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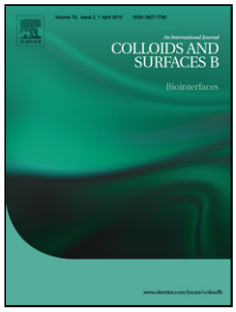

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HIGHLIGHTS

- Many devices are coated with silver NPs and come in contact with oral mucosa
- 19 nm AgNPs and only silver ions have been investigated through oral porcine mucosa
- Results showed similar flux permeation in both experiments
- It can be suggested that the permeation is mainly due to ions released from NPs
- AgNPs flux permeation through oral mucosa is higher compared to skin permeation

In vitro permeability of silver nanoparticles through porcine oromucosal membrane

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Abstract

Silver nanoparticles (AgNPs) can come in contact with human oral mucosa due to their wide use in food industry and hygiene devices. We evaluate transmucosal absorption of 19 nm AgNPs using excised porcine buccal mucosa applied on Franz diffusion cells. Two donor solutions were used: one containing AgNPs (0.5 g/L) and one derived from the ultrafiltration of the former and containing only Ag in its soluble form. Experiments were carried out separately for 4 hours. Silver flux permeation was demonstrated through oral mucosa, showing similar values for AgNPs (6.8 ± 4.5ng cm^{-2} h^{-1}) and Ag ions (5.2 ± 4.3ng cm^{-2} h^{-1}). Our study demonstrates that silver can permeate the oromucosal barrier and that absorption is substantially due to Ag ions, since no permeation difference was found using the two solutions. Mucosal absorption has to be considered in further risk assessment studies.

Keywords: Silver nanoparticles, mucosal membrane, in vitro, Franz cells, permeation.

Background:

Silver nanoparticles (AgNPs) are diffusely used in food packaging, containers, toothpaste and teeth brushes, nipples and nursing bottles, water purification devices etc. [1,2,3] These particles are therefore able to come in contact with oral mucosa, whose penetration properties are not completely known. Silver is used for its good antimicrobial properties and its safe profile,[4] but in literature silver intoxication (argyria) has been described through oral route, in people who drank it for deliberate uptake, [5, 6] or through skin route, when wound dressings containing Silver NPs are used on burns for more than 30% of the skin surface.[7] The Agency for Toxic Substances and Disease Registry (ATSDR) describes argyria as a "cosmetic problem", since it consist mostly in a not reversible bluish-gray discoloration of the skin.[8] Nevertheless there are isolated reports of more serious neurologic, renal and hepatic complications caused by the ingestion of colloidal silver.[9, 10]

Oral mucosa traditionally acts as first barrier to xenobiotics in the digestive tract, but it is also a possible drug delivery route for medical formulations, [11] since it can avoid liver metabolism if compared to the traditional intestinal route.[12]

Due to its histological structure oral mucosa shows a permeability 20 times higher to water[13] and 4 up to 4.000 times higher to different drugs compared to skin,[14] but very little is known about its behavior towards NPs penetration. It has been demonstrated that the main penetration barrier for drugs is the top third region of the epithelium, because the cells size grows, and the cells shape becomes flatter from the basal to the superficial layers. [15]

Since the spread of nanotechnologies has taken place in many fields of everyday life, there are many available products containing AgNPs but the knowledge on NPs permeation properties through mucosal membranes is still lacking.[16] Some authors demonstrated the capability of mucus layer to embed polystyrene NPs,[17] others demonstrated that them can cross this barrier and penetrate the buccal mucosa in a size dependent manner.[18] Nanosized pathogens too (Norwalk virus, 38 nm

diameter, and HPV, 55 nm diameter) can easily diffuse through the mucus layer that protect the gastric and nasal mucosa.[18, 19] On this basis mucosal vaccines have been developed in recent years and some of them are delivered through oral mucosa (as the vaccines against cholera, rotavirus and typhoid fever) while others through nasal mucosa by spray. [20, 21, 22] There is evidence that the administration of antigens at mucosal portals of entry inside lipid nanocapsules can induce a T-cellular immune responses up to 13-fold higher rather than the equivalent soluble formulation. [23]

Since NPs penetration through oral mucosa is not fully known, we performed experiments to investigate AgNPs permeation. We chose to test AgNPs due to their common use as antimicrobial agents in many devices that come in contact with oral cavity. We used porcine lining mucosa because it is the most similar to the human one[13] and is the oral region which is expected to contribute most to oromucosal absorption. In this study, experiments were performed using the Franz cell method, adapting the experience and the protocols employed during the European project EDETOX (Evaluations and predictions of DErmal absorption of TOXic chemicals)[24], a three-year research program (2001-2004) funded by European Union (EDETOX, 2000) and already used to testing skin permeation of other metal nanoparticles such as silver, gold and cobalt.[25, 26, 27]

Materials and methods

Chemicals:

All chemicals used were of analytical grade. Sodium chloride, sodium hydrogen phosphate, potassium dihydrogen phosphate, glutaraldehyde (50% v/v), nitric acid (69% v/v), hydrochloric acid (36.5-38% v/v) were purchased from Sigma Aldrich (Milan, Italy), ammonium hydroxide (25%) from J.T. Baker (Milan Italy). Water reagent grade was produced with a Millipore purification pack system (milliQ water).

The physiological solution used as receptor fluid was prepared by dissolving 2.38 g of Na_2HP0_4, 0.19 g of KH_2P0_4 and 9 g of NaCl into 1 L of milliQ water (final pH = 7.35)

2.2 Silver nanoparticles characterization

2.2.1 Donor phases preparation

AgNPs, stabilized with polyvinylpirrolidone (content of silver: 25% w/w, polymer 75%), were supplied by NanoAmor Materials Inc, (Houston, Texas, U.S.A.).

In order to better distinguish the permeation between AgNPs and silver ions, released from the NPs, two different donor phases were prepared just before the experiments.

The first donor phase, consisting of the AgNPs solution, was prepared using 200 mg (ratio metal:polymer=1:4) of AgNPs dispersed by sonication in 100 ml of physiological solution to obtain a concentration of 0.50 g/L (as metal content).

The nanoparticles suspension in water had a presence of 5% of silver in ionized form, determined using the ultrafiltration technique. The silver ions presence did not significantly change in four hours.

The second donor phase was prepared by the ultrafiltration of the first one to obtain only the water-soluble silver species present in the first donor phase at the moment of the experiment. Four ml of the AgNPs solution were ultrafiltered in centrifuge at 5000 rpm for 30 min by means of Amicon Ultra-4 centrifugal filters (10 KDa MWCO) in order to separate the AgNPs from the aqueous solution. The filtration has been

repeated on five different aliquots in order to obtain an adequate solution volume to perform silver quantification analysis and permeation experiments. The five filtered aliquots were mixed for a total of 20 ml and used during the permeation experiments.

2.2.2 Ion release from AgNPs

In order to define the percentage of silver ions inside the AgNPs solution, the donor phases have been analyzed by means of Inductively Coupled Plasma –Atomic Emission Spectroscopy (ICP-AES).

2.2.3 Transmission electron microscope characterization

AgNPs dispersed in physiological solution were characterized to obtain nanoparticles size and morphology on a transmission electron microscope (EM208; Philips, Eindhoven, The Netherlands operating at 200 kV) with an high definition acquisition system based on a side-mounted TEM camera OSIS Morada and a iTEM software platform (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

2.2.4 Dynamic light scattering measurements

The average values of the AgNPs size and polydispersity, defined as a relative width of the size distribution, were determined from dynamic light scattering (DLS) measurements, using a Zetasizer Nano Z (Malvern Instruments Ltd) analyzer applying a 633 nm laser oriented at 173° relative to the sample.

The software was optimized to report summary statistics based upon the intensity of light scattered. Four hundred µl sample volumes from nanosilver dispersion (dilution 1:5 in physiological solution) were loaded into low size disposable cuvette (supplied by manufacturer) and summary statistics were obtained using quadruplicate 3 min analysis (total analysis time=12min). Instrument performance was verified using a polymer reference standard known to be 60 nm.

2.2.5 Zeta potential measurement

Measurements were carried out using a ZetasizerNano ZS (Malvern). An aqueous suspension of silver nanoparticles was diluted 1:5 in a physiological solution. The zeta potential was calculated using Henry's equation.

2.3 Preparation of mucosal membranes

Due to its morphological and enzymatic similarities with the human mucosa[13] porcine oral mucosa was used for the in vitro experiments. The membranes were obtained immediately after pig's slaughter (age 1 year). During the transport to laboratory the tissue was stored at 4°C and then in freezer at -80°C for a period of time up to, but not exceeding, 1 week. On the day of the experiment, the tissue was removed from the freezer and thawed in physiological solution, at room temperature, for approximately 30 min before the permeation experiment. It has been shown that this method of storage does not affect the mucous barrier properties, since no change in the permeability has been described.[28]The underlying connective tissue was manually removed with a scalpel blade, and uniform thickness of approximately 0,6 mm was achieved with surgical scissors. Mucous membranes integrity was tested as suggested by Lestari. [29]

2.4 *In vitro diffusion system*

Mucosal permeation studies were performed using static Franz diffusion cells. The receiver compartments have a mean volume of 14.0 mL and were maintained at 37°C by means of circulation of thermostated water in the jacket surrounding the cells throughout the experiment. This temperature value has been chosen in order to reproduce physiological conditions. The concentration of the salt in the receiver fluids was approximately the same that can be found in the blood. The solution in each cell was continuously stirred using a Teflon coated magnetic stirrer.

Each excised sheet of mucosa was clamped between the donor and the receptor compartment in such a way that the epithelium faced the donor, and the connective tissue region faced the receiver compartment; the mean exposed area of the mucous membranes was 3.29 cm^2.

The experiments were performed as follows:

Exp. 1: At time 0, the exposure chambers of 4 Franz diffusion cells were filled with 1 mL of physiological solution and 0.5 mL of AgNPs suspension (75 μg cm^{-2}), in order to provide an infinite dose: the concentration in each cell has been confirmed at the end of the experiments by means of ICP-AES analysis.

At selected intervals (30, 60, 90, 120, 150, 180, 210, 240 min) 1 mL of the receiving bathing solution was removed and collected for the analysis, and immediately replaced with an equal volume of fresh made physiological solution. In order to avoid the precipitation of silver chloride (AgCl), 100 μl of NH_4OH 1N was added to each sample collected.

The experiment was carried out for 4 hours, as suggested in other studies.[16]At the end of the experiment the mucosa pieces were removed, washed abundantly with milliQ water, and subsequently stored in the freezer together with mucosal bathing solutions and the donor solutions for the following analysis

The experiment was repeated twice for a total of 8 cells.

Exp. 2: the exposure chambers of 4 Franz diffusion cells were filled with 1 mL of physiological solution and 0.5 mL of the Ag ultrafiltered solution. The other test conditions were the same of the experiment 1. The experiment was repeated twice for a total of 8 cells.

Blanks: for each experiment, two cells were added as blank. The blank cells were treated as the other cells with the exception that the exposure chambers were filled only with physiological solution.

Mucosa digestion after the experiment

All the mucosal exposed samples were collected and stored individually in freezer at -25° C for the following digestion and analysis. At the time of the analyzes, the skin membranes were dried for 2 hours at room temperature, weight, and then cut into sections and put into glass tubes with 10 mL of HNO_3 69% v/v for digestion. The obtained solutions were heated at 80°C for 8 hours and then diluted to a final volume of 10 ml with milliQ water for the ICP-AES analysis.

Quantitative analysis

An Inductive Coupled Plasma Mass Spectrometer (ICP-MS 7500 CE Agilent instrument with integrated autosampler) was used to determinate the total silver concentration in the receiver phases.

A seven-point standard curve was used for ICP-MS measurements (0.01, 0.05, 0.1, 0.5, 1, 5 and 10 µg/L, ion mass 107 u.m.a.). The limit of detection of silver was 0.005 µg/L for ICP-MS and the precision of the measurements expressed as repeatability (RSD %) was always lower than 5%.

The total silver concentration in the donor phases and in the solutions resulting from the skin sample mineralization were performed by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) using a Spectroflame Modula E optical plasma interface (OPI) instrument (by SPECTRO, Germany). The analysis were conducted using a calibration curve obtained by dilution (range: 0–10 mg/L) of Silver ICP standard solution for ICP-AES analysis (Sigma-Aldrich, Italy). The limit of detection (LOD) at the operative wavelength of 328.068 was 0.010 mg/L. The precision of the measurements expressed as repeatability (RSD %) was always lower than 5%.

All standard solutions used for calibration curves had been prepared using physiological solution and 10% of ammonium hydroxide 1N in order to reproduce the matrix of the samples.

2.7 SEM-EDX analysis

One mucosal sample for each experiment (one blank, one exposed to AgNPs and one to ultrafiltered soluble silver) was fixed with glutaraldehyde 10% v/v, washed with ethanol-water at increasing concentration of ethanol and stored in ethanol 98% until SEM analysis.

Analysis were performed by means of a Scanning Electron Microscope (Hitachi, TM 3000) equipped with Energy Dispersive X-ray Spectroscope (EDX SwiftEd 3000) with a magnification of 30000x and an accelerating voltage of 15 kV. With this setting silver clusters with a diameter above 50 nm were easily detected.

2.8Data analysis

Data analysis was performed with Excel for Windows, release 2007 and Stata Software, version 11.0 (StataCorp LP, College Station, TX, USA). All data were reported as mean or median as measures of central tendency and standard deviation (SD) or quartiles as measure of dispersion. The difference among independent data was assessed by means of the Mann-Whitney test. A p value of <0.05 was considered as the limit of statistical significance.

3. Results:

3.1 characterization of AgNPs colloidal dispersion

The colloidal dispersion of AgNPs in water showed Plasmon absorption at 405 nm. Transmission Electron Microscopy (TEM) measurements revealed that AgNPs used in donor solution were quite uniform in size and shape and as small as 19 ± 5 nm (number of measured nanoparticles: 100). At the concentration used in the permeation experiments no aggregates have been visualized (fig. 1).

The quantitative analysis of the filtered aliquots revealed that 5% of the donor solution was ionized and a dose of 3.8 μg cm^{-2} of silver was applied as donor phase in exp 2. Size distributions obtained by DLS are quite narrow, as presented in figure 2. The analysis revealed a z-average size (d.nm) equal to 57.1 and a polidispersity index (PdI) of 0.28 (fig. 2), while Zeta potential was equal to – 11.4 ± 0.2 mV.

The apparent mismatch between TEM and DLS sizes is the result of various facts, as elsewhere reported[30]. Firstly, the laser scattering technique measures the hydrodynamic diameter inclusive of PVP and coordinated molecules. Furthermore, polymer-protected metal NPs can form agglomerates consisting of various metal cores wrapped up in the same polymer chain. The mean hydrodynamic diameters of these agglomerates, revealed by DLS, are therefore larger than the mean sizes of the primary NPs, revealed by TEM.

3.2 Ag permeation through mucous membrane

Passive silver flux permeation was demonstrated through oral mucosa. Figure 3 shows the time-dependent increasing trend of metal concentrations in receiving phases. The final values, expressed as mean and standard deviation, were 12.2 ± 7.4 μg/cm² and 11.8 ± 11.1 μg/cm² in cells exposed to AgNPs and to Ag ions (ultrafiltered solution), respectively. Flux permeation after 4 hours of application showed similar final values (6.8 ± 4.5 ng cm^{-2} h^{-1} and 5.2 ± 4.3 ng cm^{-2} h^{-1}) and lag times (1.9 ± 0.7 h and 1.7 ± 0.7 h) using AgNPs and ultrafiltered solution, respectively (mean and standard deviation).

Silver content inside the mucosa showed similar values in both experiments too (median 0.8 μg/cm^2 and 1.4 μg/cm^2, 25th Pct 0.5 and 0.9, 75th Pct 0.1 and 0.2, in membranes exposed to AgNPs and to Ag ions (ultrafiltered solution), respectively) as showed in fig.4.

SEM-EDX investigations showed no traces of AgNPs clusters in the tissue. SEM analysis revealed the presence of electrondense zones upon the mucosal tissue exposed to Ag-NPs, but microanalysis on that points showed the absence of silver or silver chloride particles (fig. 5A-F).

4. Discussion

The oral mucosa is an attractive biological membrane, since it owns a dual role in the body: on one side it acts as the first barrier towards xenobiotics and human pathogens, and on the other it acts as the first gateway to systemic circulation towards substances which can permeate it. Many drugs have been studied in order to be absorbed through sublingual administration, but very few is known about permeation properties towards nanoparticles.

Previous studies have shown that the oral mucosa permeability depends mainly on the type of epithelium, the type and amount of intercellular lipids and the chemical nature of the substances applied. Regions coated with nonkeratinized epithelium, such as buccal mucosa and floor of mouth (lining mucosa), which we used in the study, contain glycosylceramides, and have a significantly higher permeability compared to regions with keratinized epithelium, such as hard palate and gengiva, which contain predominantly neutral lipids. [31]

The first filter to external substances is the mucous layer [32] (average thickness of 70-100 μm), which consists mostly of a high molecular weight mucin, called MG1, which is a component of the saliva that binds to the surface of the buccal epithelium.[33, 34] However it has been demonstrated that the main penetration barrier for drugs is the top third region of the epithelium, due to the growing size and shape of the cells that go up from the basal to the superficial layers.[15]

The xenobiotics that can cross the hindrance of this barrier reach the underneath connective tissue, called "lamina propria", which provides support and nourishment to the mucosa through a network of blood vessels, capillaries and smooth

muscles,[11] and from here substances can spread throughout the body via systemic circulation.

AgNPs can come in contact with human mucosa because are present in many products such as toothpaste, alcohol free mouthwash[35,] nasal sprays, endotracheal tubes [36,37] and urinary catheters,[38,39] to prevent infections. Since the antimicrobial effect of silver depends on superficial contact, the high surface area to volume ratio offered by NPs allows a broader interaction with bacterial membrane and a wider contact with microorganisms.

Few studies have been conducted to investigate AgNPs behavior towards the mucosa of the digestive tract. Shahare and colleagues [40] showed that after an oral administration of 3-20 nm AgNPs to albino mice for 3 weeks, at a dose of 5, 10, 15 and 20 mg/kg body weight, all groups treated had a significant decrease in the body weight, confirming a toxic effect of the metal. Histological changes of the mucosa have been reported, such as a damage of the epithelial cell micovilli and the intestinal glands, which the authors hypothesized as the reason for the absorptive capacity reduction of intestinal epithelium and hence for the weight loss. Walczak and colleagues[41] investigated the behavior of 60 nm AgNPs and of $AgNO_3$ ions in an in vitro human digestion model. They found that after gastric digestion and in presence of proteins, the number of particles dropped significantly, due to the formation of clusters, and subsequently disintegrated back to single 60 nm AgNPs during intestinal digestion. Therefore results showed that under physiological conditions AgNPs can reach the intestinal wall in their initial size.

No other studies investigated AgNPs mucosal absorption but 2 studies demonstrated that polystyrene Nps can cross the pig mucosa: Holpuch and coworkers[42] showed that 210 nm polystyrene NPs can cross intact human epithelium, derived from oral explants, and can be found in the underlying connective tissue. Teubl and colleagues[18] investigated more systematically NPs behavior through oral mucous membrane, by performing experiments with different size and superficial charge of the NPs, and at different mucosal temperatures. They demonstrated that neutral 25 nm, 50 nm and 200 nm polystyrene nanoparticles (PP) can all cross the mucus layer and penetrate the buccal mucosa in a size dependent manner, surprisingly higher for those with bigger size. This is in contrast to the generally accepted assumption that decreasing the particle diameter increases the absorption[43, 44].

Our study investigated for the first time the behavior of silver NPs and its ultrafiltered solution towards oral mucosa, using 19 nm AgNPs applied in vitro on porcine oral explants. The aim was to distinguish the percentage of permeation, if any, due to NPs themselves from the percentage due to the ions issued. The findings suggest that an absorption through passive diffusion takes place, and it is mainly due to silver ions. This result is consistent with the ones obtained by other authors, [45] whom demonstrated that the dose-dependent toxic effects of AgNPs on animals (death, weight loss, cardiac enlargement, altered liver enzymes levels and

immunological effects) were substantially mediated by silver ions released from AgNPs. Gaillet and coworkers support the same theory in a recent review.[46]

Indeed Silver, in whatever form, is not an essential mineral for humans, and so it can exert toxic effects. Systemic intoxication, called "argyria", is fortunately a rare event, but a more common effect in human is the uptake reduction of some drugs, such as thyroxine, penicillamine and of some antibiotics,[47].

For this reason the governmental agency Food and Drug Administration (FDA) issued numerous warning letters to e-commerce sites which promoted colloidal silver as antibiotic or drug for medical purposes.[48, 49, 50]

It could be interesting compare our results with those obtained by Bianco and coworkers,[51] where AgNPs have been applied on full thickness human skin in similar experimental conditions. Interestingly the flux through oral mucosa is about 1 order of magnitude higher compared to skin, and the time required to reach a constant flux trough the membrane is definitely lower through the mucosa. This higher permeability is attributable to a slightly different histological structure of the mucosa compared to skin.

Our study adds important information to understand how nanoparticles can enter the body but nevertheless the protocol used presented some limitations related to: 1) the in-vitro condition, which can underestimate real world scenarios, since only passive diffusion can be studied using Franz-cells and 2) the use of porcine mucosa, which is a good model to study human's mucosa but there are no data, yet, which allow to bridge interspecies results.

8. Conclusions

Our study investigated the permeation of 19 nm silver nanoparticles (AgNPs) across excised porcine oral lining mucosa, using an in vitro diffusion cell system.

We demonstrated for the first time that AgNPs, can lead to silver absorption through oral mucosa, in a similar amount when AgNPs or silver soluble form is used, suggesting that the permeation of the mucosa is related mainly to ions diffusion. A further support to this hypothesis comes from the SEM-EDX results, since no evidence of AgNPs clusters has been revealed, while the quantification of total silver on the mineralized tissue ensures the presence of the metal.

Moreover the comparison with flux permeation values through the skin barrier, when similar experimental conditions were used, suggest that the permeability of silver through oral mucosa is one order of magnitude greater compared to skin, leading to a higher uptake in in-vivo conditions.

Even if the amount of silver found should be not hazardous for human health, these data suggest that oral cavity should be part of further risk assessment studies, since it acts as the first barrier for systemic uptake and can come in contact with different types of nanoparticles. Moreover this study investigated only the intact mucosa, but in everyday life there are common circumstances which may damage the mucosal integrity, such as gastroesophageal reflux, infections or accidental abrasions, which all can lead to an increase in the oromucosal uptake.

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FIGURE LEGENDS

Fig.1. TEM images of the AgNPs dispersed in physiological solution: NPs appeared uniform in size and shape and as small as 19 ± 5 nm (A: bar=200nm; B: bar=100nm).

Fig.2. DLS analysis.The curve represent AgNPs size distribution by intensity.The summary statistics is based upon the intensity of light scattered of 6 different samples derived from nanosilver dispersion.

Fig.3. Silver permeation profile in receiving phases of 8 cells exposed to AgNPs (square) and of 6 cells exposed to Ag ions (diamonds) expressed as mean and standard deviation.

Fig.4. Silver concentration in the mucosa of 7 cells exposed to AgNPs and 5 cells exposed to Ag ions (median values, 25^{th} and 75^{th} quartiles, minimum and maximum values, outsider value of 5.97 $\mu g/cm^2$ in the mucosa exposed to AgNPs not showed in the figure). p = 0.61 (Mann-Whitney test).

603 Fig.5. SEM images at increasing magnifications of the mucosal tissue exposed to
604 AgNPs with EDX microanalysis on the yellow spots (A: the entire sample –
605 bar=2mm; B: bar=500μm, C: bar=80μm, D: bar=70μm; E, F: bar=10μm).

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607 FIGURES

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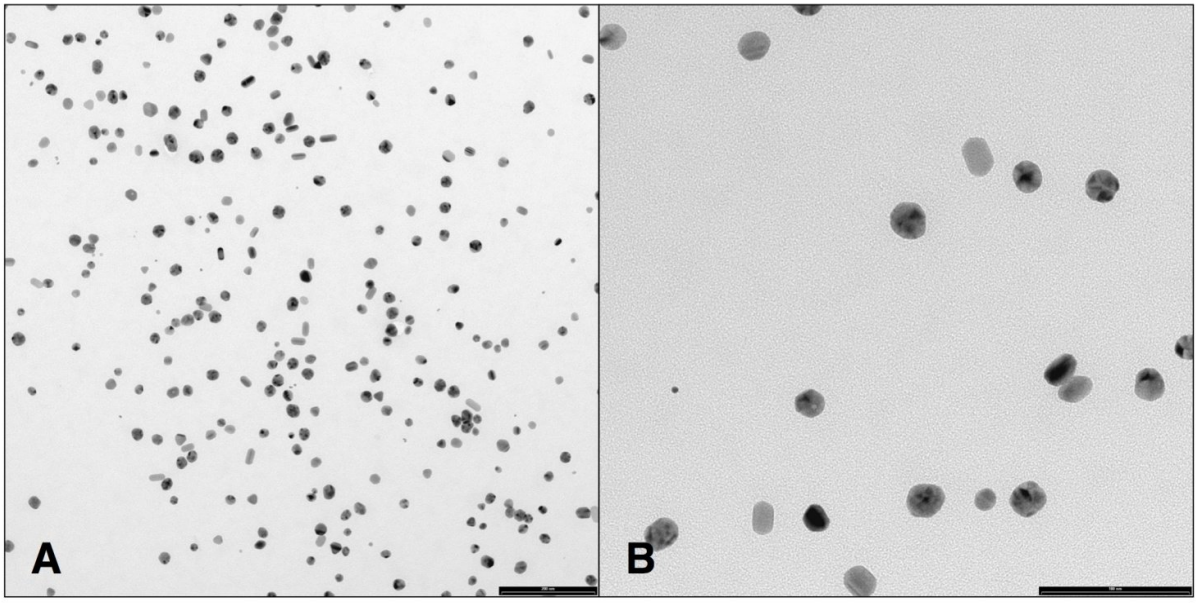

609

610 Fig.1

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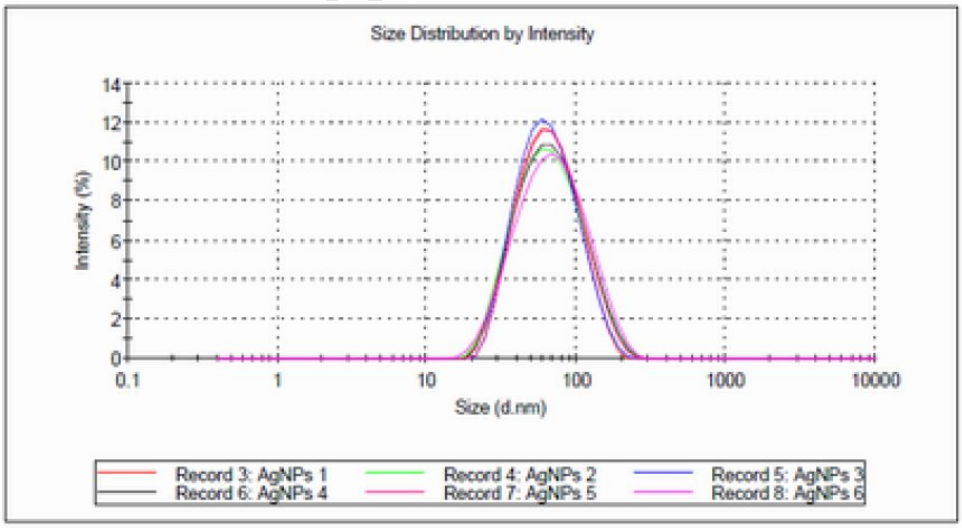

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613 Fig. 2

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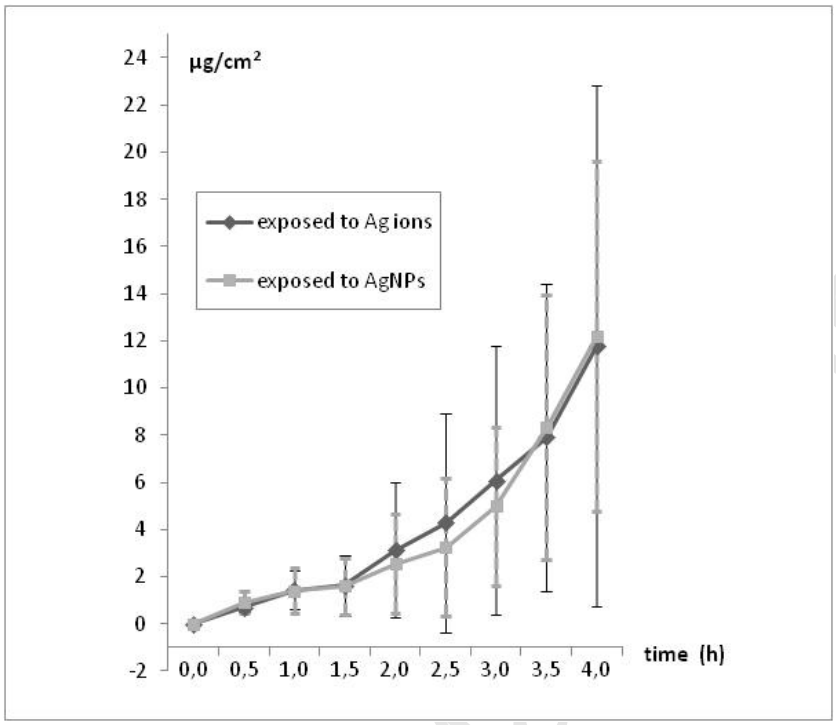

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617 Fig.3

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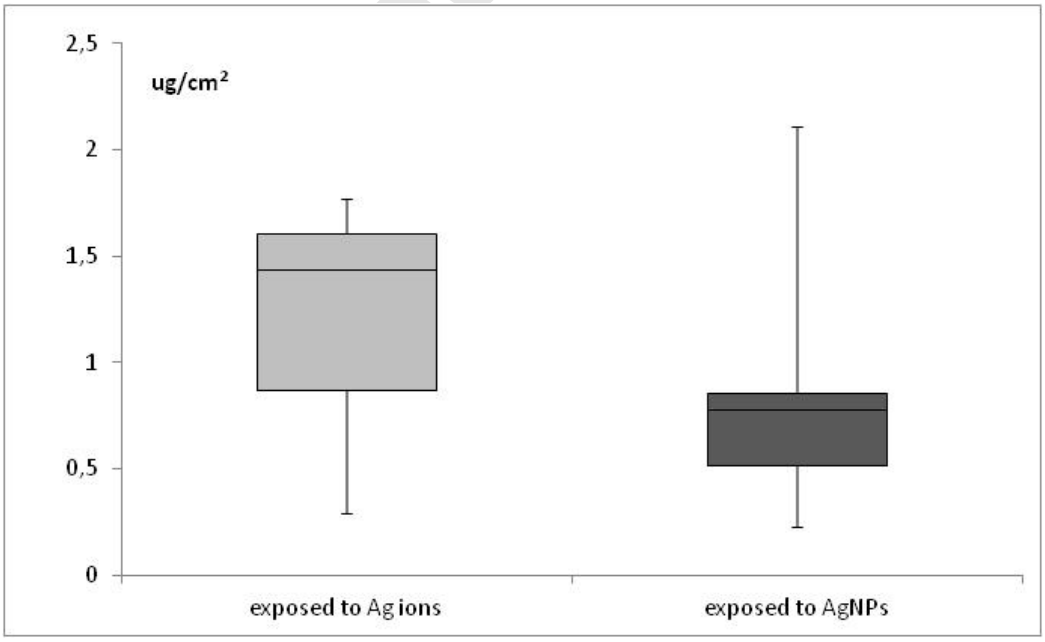

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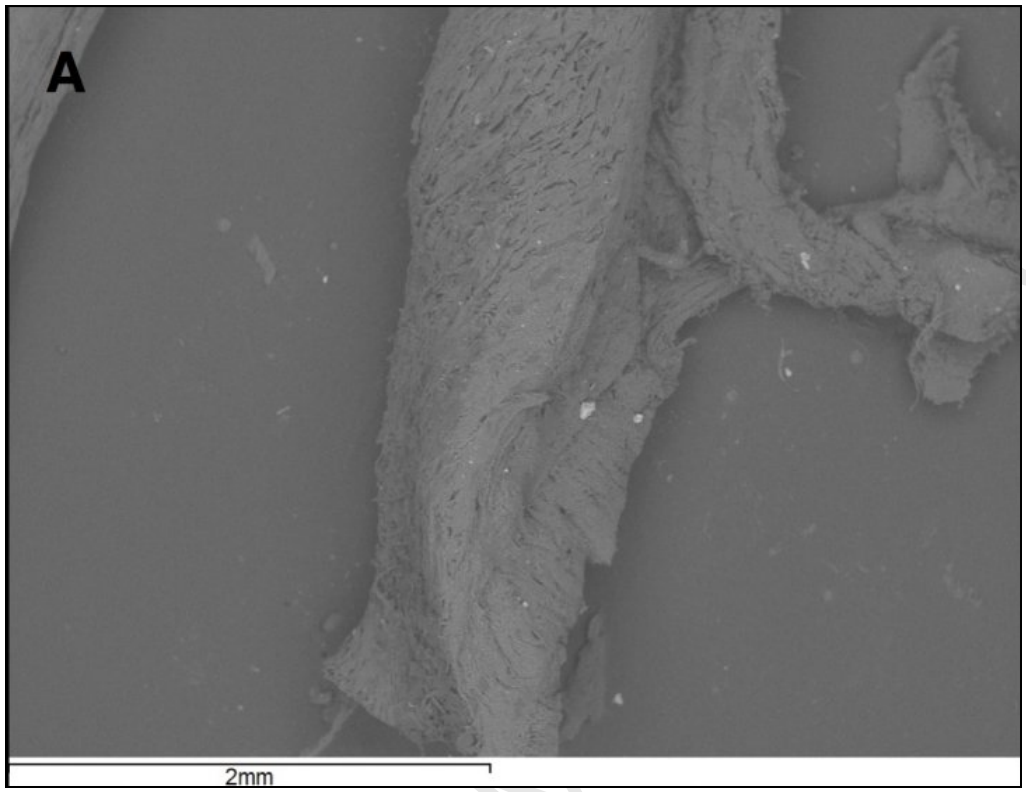

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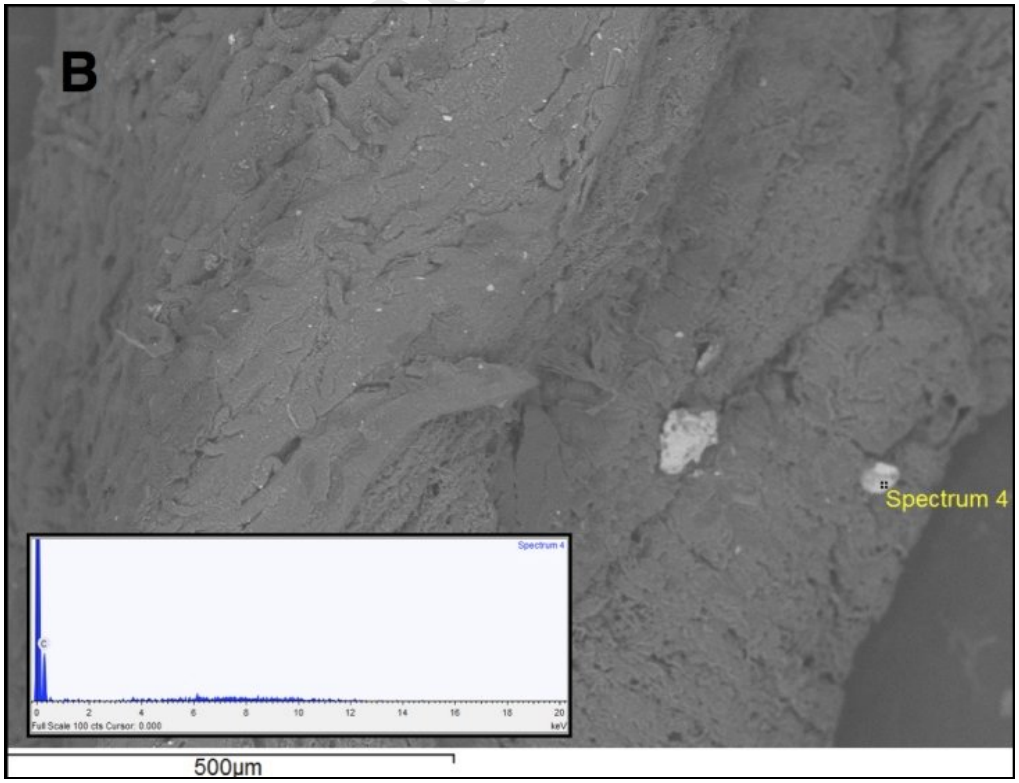

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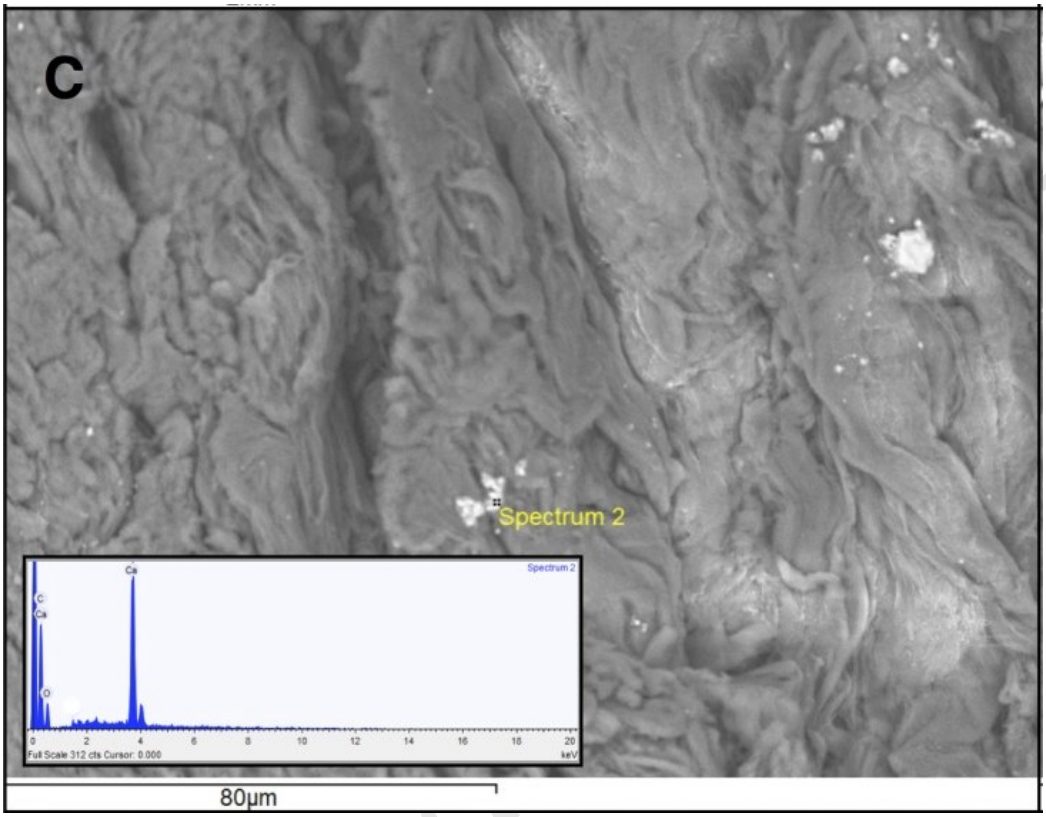

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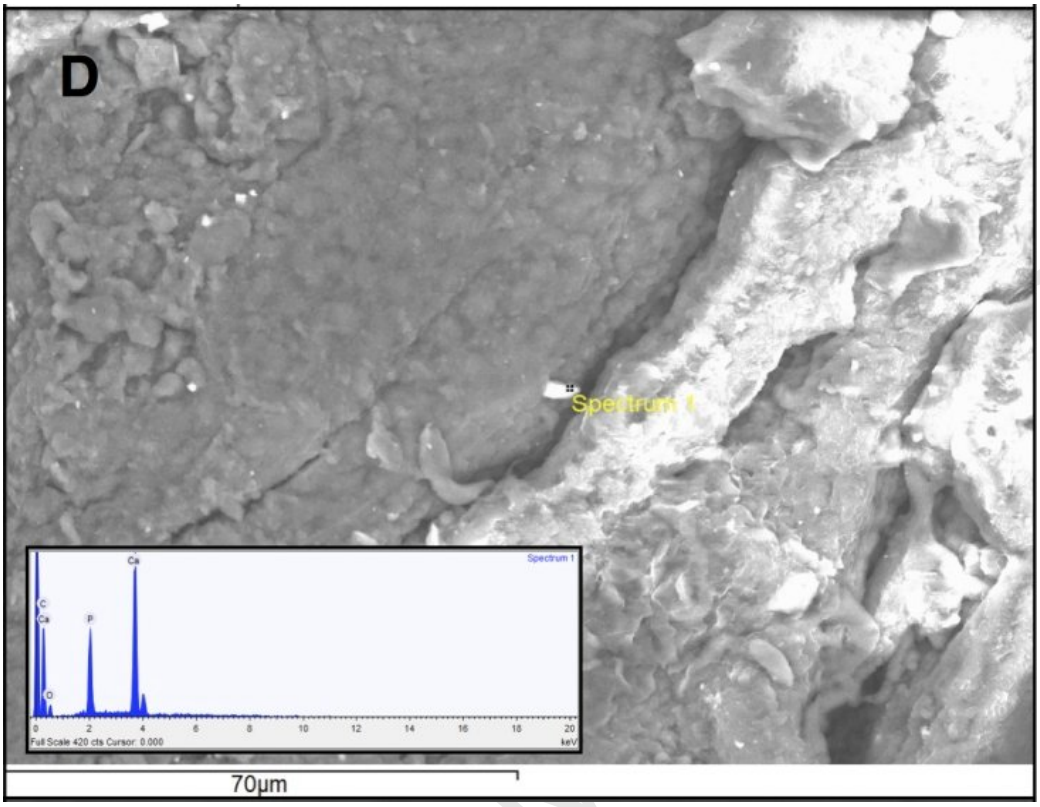

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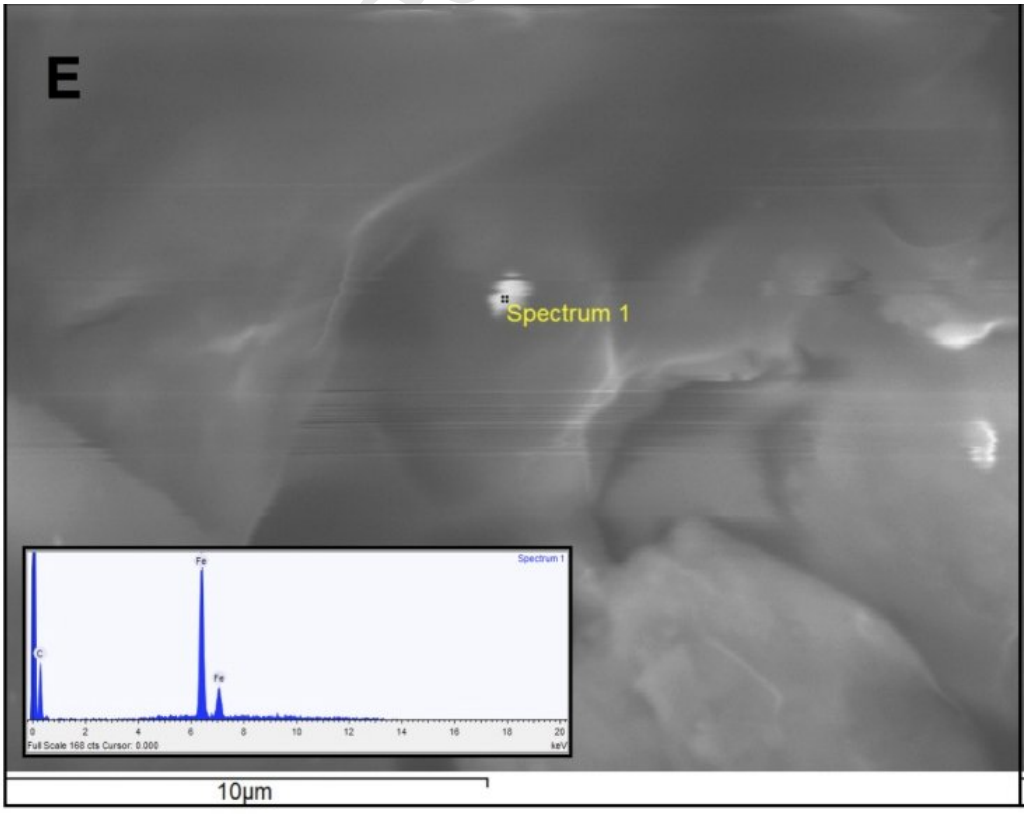
629 Fig.5 D



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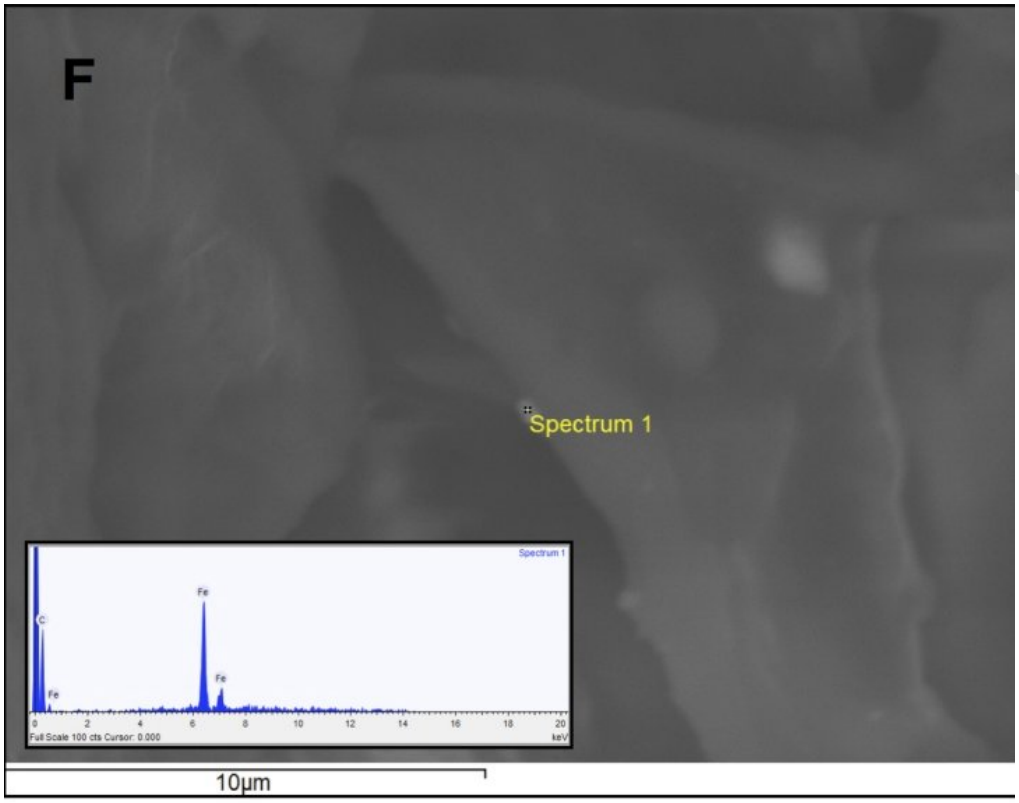

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633 Fig.5 F

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Graphical Abstract (for review)

Donor solution:

1 mL of physiological solution and 0.5 mL of AgNPs solution (75 µg cm^{-2})

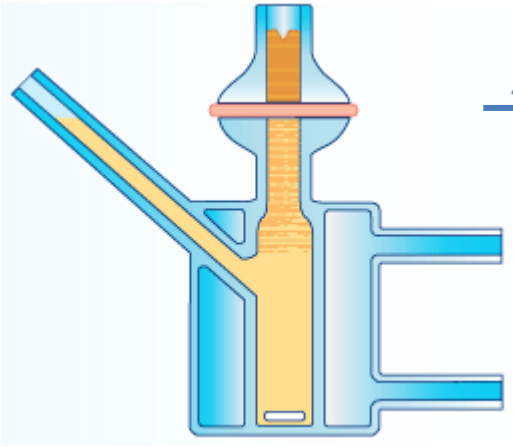
Filtered

Donor solution :

1 mL of physiological solution and 0.5 mL of the ultrafiltered AgNPs solution (silver ions)

Separate experiments

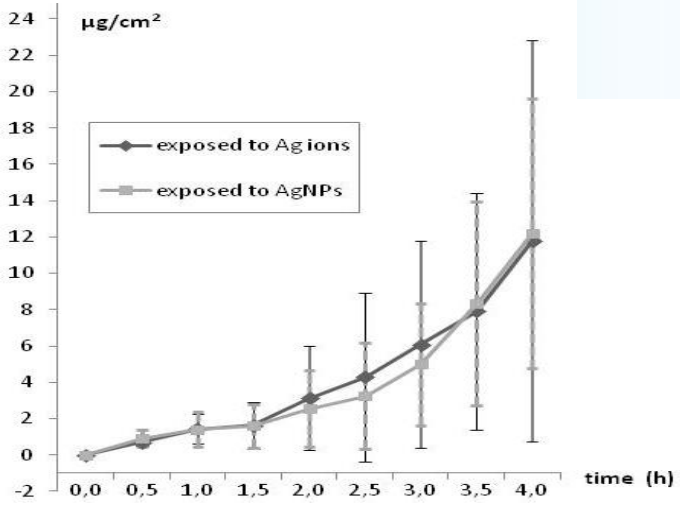
1,5 ml samples
collected at selected intervals



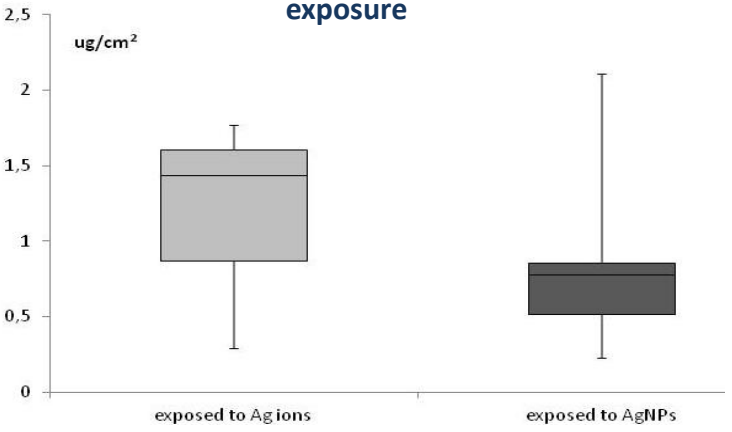
Thermostated system
37°C

Porcine oral mucosa

Silver content inside receiving solutions



Silver content inside oral mucosa after 4 h of exposure



Results expressed as mean and standard deviation

Median values, 25th and 75th quartiles, minimum and maximum values, p = 0.61 (Mann-Whitney test).