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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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42 Abstract

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

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

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70 Background:

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

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

87 Due to its histological structure oral mucosa shows a permeability 20 times higher
 88 to water¹³ and 4 up to 4.000 times higher to different drugs compared to skin,¹⁴ but
 89 very little is known about its behavior towards NPs penetration. It has been
 90 demonstrated that the main penetration barrier for drugs is the top third region of
 91 the epithelium, because the cells size grows, and the cells shape becomes flatter
 92 from the basal to the superficial layers.¹⁵

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

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

107 Since NPs penetration through oral mucosa is not fully known, we performed
 108 experiments to investigate AgNPs permeation. We chose to test AgNPs due to their
 109 common use as antimicrobial agents in many devices that come in contact with oral
 110 cavity. We used porcine lining mucosa because it is the most similar to the human
 111 one¹³ and is the oral region which is expected to contribute most to oromucosal
 112 absorption. In this study, experiments were performed using the Franz cell method,
 113 adapting the experience and the protocols employed during the European project
 114 EDETOX (Evaluations and predictions of Dermal absorption of TOXic chemicals)²⁴, a
 115 three-year research program (2001-2004) funded by European Union (EDETOX,
 116 2000) and already used to testing skin permeation of other metal nanoparticles
 117 such as silver, gold and cobalt.^{25, 26, 27}

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119

120 Materials and methods

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1221 Chemicals:

123 All chemicals used were of analytical grade. Sodium chloride, sodium hydrogen
 124 phosphate, potassium dihydrogen phosphate, glutaraldehyde (50% v/v), nitric acid
 125 (69% v/v), hydrochloric acid (36.5-38% v/v) were purchased from Sigma Aldrich
 126 (Milan, Italy), ammonium hydroxide (25%) from J.T. Baker (Milan Italy). Water
 127 reagent grade was produced with a Millipore purification pack system (milliQ
 128 water).

129 The physiological solution used as receptor fluid was prepared by dissolving 2.38 g
 130 of Na_2HPO_4 , 0.19 g of KH_2PO_4 and 9 g of NaCl into 1 L of milliQ water (final pH =
 131 7.35)

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133 2 Silver nanoparticles characterization

134 2.2.1 Donor phases preparation

135 AgNPs, stabilized with polyvinylpyrrolidone (content of silver: 25% w/w, polymer
 136 75%), were supplied by NanoAmor Materials Inc, (Houston, Texas, U.S.A.).

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

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

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

146 The second donor phase was prepared by the ultrafiltration of the first one to obtain
 147 only the water-soluble silver species present in the first donor phase at the moment
 148 of the experiment. Four ml of the AgNPs solution were ultrafiltered in centrifuge at
 149 5000 rpm for 30 min by means of Amicon Ultra-4 centrifugal filters (10 KDa MWCO)
 150 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

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

181

182.3 Preparation of mucosal membranes

183 Due to its morphological and enzymatic similarities with the human mucosa¹³
184 porcine oral mucosa was used for the in vitro experiments. The membranes were
185 obtained immediately after pig's slaughter (age 1 year). During the transport to
186 laboratory the tissue was stored at 4°C and then in freezer at -80°C for a period of
187 time up to, but not exceeding, 1 week. On the day of the experiment, the tissue
188 was removed from the freezer and thawed in physiological solution, at room
189 temperature, for approximately 30 min before the permeation experiment. It has
190 been shown that this method of storage does not affect the mucous barrier
191 properties, since no change in the permeability has been described.²⁸The underlying
192 connective tissue was manually removed with a scalpel blade, and uniform
193 thickness of approximately 0,6 mm was achieved with surgical scissors. Mucous
194 membranes integrity was tested as suggested by Lestari.²⁹

195

196 In vitro diffusion system

197 Mucosal permeation studies were performed using static Franz diffusion cells. The
198 receiver compartments have a mean volume of 14.0 mL and were maintained at
199 37°C by means of circulation of thermostated water in the jacket surrounding the
200 cells throughout the experiment. This temperature value has been chosen in order
201 to reproduce physiological conditions. The concentration of the salt in the receiver
202 fluids was approximately the same that can be found in the blood. The solution in
203 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 cm².

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 NH₄OH 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.

2315 Mucosa digestion after the experiment

232 All the mucosal exposed samples were collected and stored individually in freezer at
 233 -25° C for the following digestion and analysis. At the time of the analyzes, the skin
 234 membranes were dried for 2 hours at room temperature, weight, and then cut into
 235 sections and put into glass tubes with 10 mL of HNO₃ 69% v/v for digestion. The
 236 obtained solutions were heated at 80°C for 8 hours and then diluted to a final
 237 volume of 10 ml with milliQ water for the ICP-AES analysis.

238

239 Quantitative analysis

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

243 A seven-point standard curve was used for ICP-MS measurements (0.01, 0.05, 0.1,
 244 0.5, 1, 5 and 10 µg/L, ion mass 107 u.m.a.). The limit of detection of silver was
 245 0.005 µg/L for ICP-MS and the precision of the measurements expressed as
 246 repeatability (RSD %) was always lower than 5%.

247 The total silver concentration in the donor phases and in the solutions resulting
 248 from the skin sample mineralization were performed by Inductively Coupled
 249 Plasma-Atomic Emission Spectrometry (ICP-AES) using a Spectroflame Modula E
 250 optical plasma interface (OPI) instrument (by SPECTRO, Germany). The analysis
 251 were conducted using a calibration curve obtained by dilution (range: 0–10 mg/L)
 252 of Silver ICP standard solution for ICP-AES analysis (Sigma-Aldrich, Italy). The limit
 253 of detection (LOD) at the operative wavelength of 328.068 was 0.010 mg/L. The
 254 precision of the measurements expressed as repeatability (RSD %) was always
 255 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.

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.

2.8 Data 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 $3.8 \mu\text{g cm}^{-2}$ 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 polydispersity 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 reported³⁰. 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 $12.2 \pm 7.4 \mu\text{g/cm}^2$ and $11.8 \pm 11.1 \mu\text{g/cm}^2$ in cells exposed to AgNPs and to Ag ions (ultrafiltered solution), respectively. Flux permeation after 4 hours of application showed similar final values ($6.8 \pm 4.5 \text{ ng cm}^{-2} \text{ h}^{-1}$ and $5.2 \pm 4.3 \text{ ng cm}^{-2} \text{ h}^{-1}$) 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 $\mu\text{g}/\text{cm}^2$ and 1.4 $\mu\text{g}/\text{cm}^2$, 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).

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 gingiva, which contain predominantly neutral lipids.³¹

The first filter to external substances is the mucous layer³² (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

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

344 AgNPs can come in contact with human mucosa because are present in many
345 products such as toothpaste, alcohol free mouthwash³⁵, nasal sprays, endotracheal
346 tubes^{36,37} and urinary catheters,^{38,39} to prevent infections. Since the antimicrobial
347 effect of silver depends on superficial contact, the high surface area to volume ratio
348 offered by NPs allows a broader interaction with bacterial membrane and a wider
349 contact with microorganisms.

350 Few studies have been conducted to investigate AgNPs behavior towards the
351 mucosa of the digestive tract. Shahare and colleagues⁴⁰ showed that after an oral
352 administration of 3-20 nm AgNPs to albino mice for 3 weeks, at a dose of 5, 10, 15
353 and 20 mg/kg body weight, all groups treated had a significant decrease in the
354 body weight, confirming a toxic effect of the metal. Histological changes of the
355 mucosa have been reported, such as a damage of the epithelial cell micovilli and
356 the intestinal glands, which the authors hypothesized as the reason for the
357 absorptive capacity reduction of intestinal epithelium and hence for the weight loss.
358 Walczak and colleagues⁴¹ investigated the behavior of 60 nm AgNPs and of AgNO₃
359 ions in an in vitro human digestion model. They found that after gastric digestion
360 and in presence of proteins, the number of particles dropped significantly, due to
361 the formation of clusters, and subsequently disintegrated back to single 60 nm
362 AgNPs during intestinal digestion. Therefore results showed that under physiological
363 conditions AgNPs can reach the intestinal wall in their initial size.

364 No other studies investigated AgNPs mucosal absorption but 2 studies
365 demonstrated that polystyrene Nps can cross the pig mucosa: Holpuch and
366 coworkers⁴² showed that 210 nm polystyrene NPs can cross intact human
367 epithelium, derived from oral explants, and can be found in the underlying
368 connective tissue. Teubl and colleagues¹⁸ investigated more systematically NPs
369 behavior through oral mucous membrane, by performing experiments with different
370 size and superficial charge of the NPs, and at different mucosal temperatures. They
371 demonstrated that neutral 25 nm, 50 nm and 200 nm polystyrene nanoparticles
372 (PP) can all cross the mucus layer and penetrate the buccal mucosa in a size
373 dependent manner, surprisingly higher for those with bigger size. This is in contrast
374 to the generally accepted assumption that decreasing the particle diameter
375 increases the absorption^{43, 44}.

376 Our study investigated for the first time the behavior of silver NPs and its
377 ultrafiltered solution towards oral mucosa, using 19 nm AgNPs applied in vitro on
378 porcine oral explants. The aim was to distinguish the percentage of permeation, if
379 any, due to NPs themselves from the percentage due to the ions issued. The
380 findings suggest that an absorption through passive diffusion takes place, and it is
381 mainly due to silver ions. This result is consistent with the ones obtained by other
382 authors,⁴⁵ whom demonstrated that the dose-dependent toxic effects of AgNPs on
383 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 through 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.

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407

408. 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 represents 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, 25th and 75th quartiles, minimum and maximum values, outlier value of $5.97 \mu\text{g}/\text{cm}^2$ 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=500 μ m, C: bar=80 μ m, D: bar=70 μ m; E, F: bar=10 μ m).

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FIGURES

Fig.1

Fig. 2

Fig.3

Fig.4

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623 Fig.5 A

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625 Fig.5 B

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627 Fig.5 C

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629 Fig.5 D

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631 Fig.5 E

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633 Fig.5 F

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