Colloids and Surfaces B: Biointerfaces 132 (2015) 10–16

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

In vitro permeability of silver nanoparticles through porcine oromucosal membrane

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ARTICLE INFO

Article history:
Received 23 January 2015
Received in revised form 26 April 2015
Accepted 27 April 2015
Available online 5 May 2015

Keywords:
Silver nanoparticles
Mucosal membrane
In vitro
Franz cells
Permeation.

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 h. Silver flux permeation was demonstrated through oral mucosa, showing similar values for AgNPs (6.8 ± 4.5 ng cm^{-2} h^{-1}) and Ag ions (5.2 ± 4.3 ng 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.

1. Background

Silver nanoparticles (AgNPs) are diffusely used in food packaging, containers, toothpaste and teeth brushes, nipples and nursing bottles, water purification devices etc. [1–3]. 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 toward 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 they 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 [20–22]. 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

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http://dx.doi.org/10.1016/j.colsurfb.2015.04.061
0927-7765/

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 [25–27].

2. Materials and methods

2.1. 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_2HPO_4, 0.19 g of KH_2PO_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, TX, USA).

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 4 h.

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 milliliter 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 microliter 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 = 12 min). 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 $\mu g/cm^2$), 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.