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Non-invasive iontophoretic delivery of enzymatically active ribonuclease A (13.6 kDa) across intact porcine and human skins

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ABSTRACT

The purpose of the study was to demonstrate the feasibility of using transdermal iontophoresis to deliver a functional protein, ribonuclease A (RNAse; 13.6 kDa), non-invasively across the skin. Iontophoretic transport experiments were conducted using porcine skin in vitro and established the effect of current density and protein concentration on delivery kinetics. A methylene blue-based assay was used to quantify RNAse transport and to simultaneously demonstrate that protein functionality was retained post-iontophoresis. The results confirmed that intact functional RNAse was indeed delivered across the skin; cumulative permeation and steady state flux after 8 h iontophoresis at 0.3 mA/cm^2 were 224.37 ± 72.34 μg/cm^2 and 68.28 ± 23.87 μg/cm^2 h, respectively. Significant amounts of protein were also deposited within the membrane (e.g., 1425.13 ± 312.09 μg/cm^2 at 0.3 mA/cm^2). In addition to the evidence provided by the enzymatic assay with regards to RNAse integrity and functionality, SDS-PAGE gels and MALDI-TOF spectra were also used to characterize RNAse present in the receiver phase (MALDI-TOF spectra: RNAse control, 13.690 kDa cf. RNAse from permeation samples, 13.692 kDa). Co-iontophoresis of acetaminophen showed that, despite its molecular weight, electromigration was the predominant electrotransport mechanism, accounting for >80% of RNAse total flux. Increasing RNAse concentration from 0.35 to 0.7 mM in the formulation did not result in a statistically significant increase in delivery. Iontophoretic transport of RNAse across human skin was statistically equivalent to that seen with porcine skin under the same conditions; cumulative permeation across human and porcine skin was 241.48 ± 60.01 and 170.71 ± 92.13 μg/cm^2, respectively. Laser scanning confocal microscopy was used to visualize the distribution of rhodamine B-labelled RNAse in the epidermis and dermis as a function of depth following 8 h iontophoresis (results were compared to control experiments involving passive administration of the same formulation for 8 h). Although fluorescence was localized at the skin surface following passive administration, it was visible throughout the membrane after current application. In conclusion, the results demonstrate that non-invasive transdermal iontophoresis can be used to deliver significant amounts of a structurally intact, functional protein across skin.

1. Introduction

Non-invasive transdermal iontophoresis is an established technique that has demonstrated its utility for the delivery of both low molecular weight and peptide therapeutics [1,2]. The transport kinetics and the delivery mechanism depend on the physicochemical properties of the molecule [1,3]. For example, the molecular weight and charge influence the electric mobility whereas the spatial distribution of the hydrophilicity/lipophilicity can result in intermolecular interactions that lead to aggregation or binding to structures along the transport pathway [3]. Electromigration (EM) is considered to be the dominant transport mechanism for the majority of low molecular weight ions; however, the driving force for larger molecules, including peptides and proteins is not well established. In the case of cation delivery, it was originally thought that as molecular weight increased the contribution of electromigration decreased and electroosmosis (EO) determined the rate of delivery; it was putatively suggested that EO would be the dominant transport mechanism for molecules approaching ~1000 Da [4]. However, identification of the relative contributions of EM and EO to the iontophoretic delivery of triptorelin (MW 1311 Da) demonstrated that EM was the principal transport mechanism accounting for ~80% of electrotransport [5]. Furthermore, a recent study suggested that iontophoretic transport of Cytochrome *c*, a 12.4 kDa protein (0.7 mM, 0.5 mA/cm^2), was also controlled by EM (~90%; the contributions of EM and EO to flux were 249 and 16 μg/cm^2 h, respectively) [6]. Thus, it is not the molecular weight that is the limiting factor but more likely the electric mobility of the permeant and its relationship to those of the other ions in the system [7]; for proteins, with defined tertiary and quaternary structures, the fold may also influence transport rates [6]. Although there are several reports on the iontophoretic transport kinetics of peptides [2,8–17] few confirm the biological activity of the

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molecule post-delivery [10,16]. Furthermore, to the best of our knowledge, there are no studies that confirm the biological activity of an iontophoretically delivered protein.

Ribonuclease A (RNAse) was chosen as a model protein because it bears certain physicochemical and structural similarities to Cytochrome *c*; it has a molecular weight of 13.6 kDa with an isoelectric point (pI) of 8.64 (cf. MW 12.4 kDa and pI 10.2 for Cytochrome *c*) and its 124 amino acids are folded into a globular structure (pdb: 2AAS, Fig. 1) [18].

However, it has a net charge of 4 at physiological pH, which is lower than that of Cytochrome *c*; the total number of positively charged residues (Arg+Lys) is 14, and the number of negatively charged residues (Asp+Glu) is 10. RNAse also contains four His residues and at pH 6, the protein is estimated to carry a net positive charge of 7.2 (http://www.scripps.edu/~cdputnam/protcalc.html). A key difference is that since RNAse is an enzyme, the use of an activity-based assay enables simultaneous quantification of transport and confirmation of functionality post-iontophoresis.

The specific aims of this study were (i) to demonstrate the feasibility of using iontophoresis to deliver a moderately-sized protein (larger than Cytochrome *c*) across porcine and human skin in vitro, (ii) to confirm that RNAse was intact and retained its activity post-iontophoresis, (iii) to investigate the effect of experimental variables (applied current densities and protein concentration) on RNAse transport, (iv) to identify whether electromigration or electroosmosis was the dominant transport mechanism and (v) to use laser scanning confocal microscopy in conjunction with rhodamine-tagged RNAse to visualize protein distribution in the skin following iontophoresis.

2. Materials and methods

2.1. Chemicals and reagents

Ribonuclease A and ribonucleic acid (RNA) were purchased from Sisco Research Laboratory PVT. LTD. (Mumbai, India). Acetaminophen (ACM), methylene blue, sodium citrate, rhodamine isothiocyanate, silver wire and silver chloride were purchased from Sigma-Aldrich (Buchs, Switzerland). Sodium chloride, sodium bicarbonate and sodium carbonate were purchased from Fluka (Buchs, Switzerland). Citric acid and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) were purchased from Acros Organics (Chemie Brunschwig; Basel, Switzerland). Silicon tubing (3.2 mm ID, 6 mm OD, 1 mm wall) for collecting samples and PVC tubing (3 mm ID, 5 mm OD, 1 mm wall) used to prepare salt bridge assemblies were obtained from Fisher Bioblock Scientific S.A. (Illkirch, France). All solutions were prepared using deionized reverse osmosis filtered water (resistivity ≥ 18 MΩ cm). All other chemicals were at least of analytical grade.

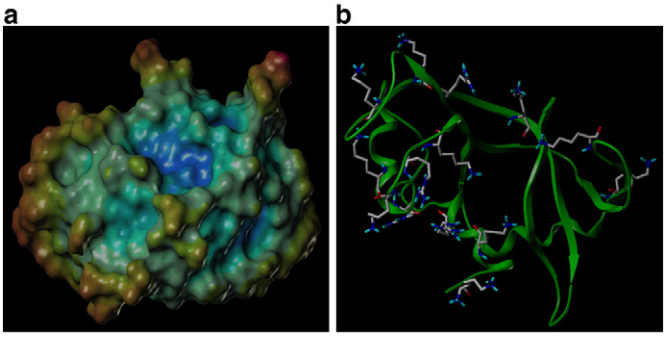

Fig. 1. Three-dimensional structure of ribonuclease (RNAse; 2AAS [18]); (a) the solvent accessible (Connolly) surface showing the electrostatic potential distribution across the molecular surface (red and blue colours represent regions of high positive and negative charge density, respectively) and (b) the protein backbone, tertiary structure and the distribution of positively charged amino acid residues (Arg and Lys). The charged amino acids are predominantly located on the protein surface.

2.2. Skin source

Porcine ears were obtained from a local abattoir (CARRE; Rolle, Switzerland), the skin was excised (thickness 750 µm) with an air-dermatome (Zimmer; Etupes, France), wrapped in Parafilm™ and stored at −20 °C for a maximum period of 2 months. Human skin samples were collected immediately after surgery from the Department of Plastic, Aesthetic and Reconstructive Surgery, Geneva University Hospital (Geneva, Switzerland), fatty tissue was removed and the skin was wrapped in Parafilm™ before storage at −20 °C for a maximum period of 3 days. The study was approved by the Central Committee for Ethics in Research (CER: 08-150 (NAC08-051); Geneva University Hospital).

2.3. Stability studies

2.3.1. RNAse stability in the presence of skin

Epidermal and dermal stabilities of the protein were determined by placing 1 ml of 0.7 mM RNAse solution (25 mM HEPES buffer; pH 6) and 1 ml 0.07 mM RNAse solution (25 mM HEPES, 133 mM NaCl; pH 7.4) in contact with epidermis (~0.8 cm^2) and dermis (~0.8 cm^2), respectively. Samples were collected hourly for 8 h and were analyzed by using an activity assay (described below). The experiments were performed in triplicate.

2.3.2. RNAse stability in the presence of current

2 ml of 0.7 mM RNAse solution (25 mM HEPES buffer; pH 6) was subjected to a current density of 0.5 mA/cm^2 for 8 h (using salt bridges to ensure connectivity between the electrodes and the formulation). Samples were collected hourly and were analyzed by using an activity assay (described below). The experiment was performed in triplicate.

2.4. Iontophoretic setup and protocol

The experimental setup used was similar to that described in earlier studies [6,17]. Dermatomed skin was clamped in three compartment vertical flow through diffusion cells (area 0.8 cm^2). The anode (containing 25 mM HEPES, 133 mM NaCl; pH 7.4) was isolated from the donor via a salt bridge assembly (3% agarose in 0.1 M NaCl) [17]. The skin was equilibrated for 40 min with 25 mM HEPES, 133 mM NaCl (pH 7.4); then, this solution was removed and replaced by 1 ml of protein solution (0.7 mM RNAse in 25 mM HEPES pH 6 with 15 mM ACM). Acetaminophen (ACM) was used to monitor electroosmotic solvent flow. The cathodal and receptor compartments were filled with 1 and 6 ml of 25 mM HEPES, 133 mM NaCl (pH 7.4) solution, respectively. A syringe pump (SP220IZ, WPI, Sarasota, FL) was used to obtain a continuous flow of buffer (1 ml/h) through the receiver compartment and samples were collected hourly. Constant current densities were applied using Ag/AgCl electrodes connected to a power supply (Kepco® APH 1000 M, Flushing, NY). At the end of the permeation experiment (duration 8 h), the amount of RNAse retained in the skin was determined by cutting the skin into small pieces and stirring them in 10 ml of 25 mM HEPES, 133 mM NaCl (pH 7.4) buffer for 18 h. The resultant extract was filtered through 0.45 µm membrane filters and RNAse quantified by enzymatic assay (see below) after appropriate dilution.

2.5. Transport studies

2.5.1. Effect of current density

In order to investigate the effect of current on RNAse electrotransport kinetics, three different current densities (0.1, 0.3 and 0.5 mA/cm^2) were applied for 8 h using the above mentioned formulation (0.7 mM RNAse in 25 mM HEPES (pH 6) with 15 mM ACM) and protocol ($n \geq 5$ in all experiments).