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Nanoscale Mapping of the Physical Surface Properties of Human Buccal Cells and Changes Induced by Saliva

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Nanoscale mapping of the physical surface properties of human buccal cells and changes induced by saliva

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7 **Nanoscale mapping of the physical surface**
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11 **properties of human buccal cells and changes**
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15 **induced by saliva**
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51 microscopy, oral epithelial cells, mucosal pellicle, mucins,
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53 cell hydrophobicity, salivary proteins.
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3 ABSTRACT
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7 The mucosal pellicle, also called salivary pellicle, is a thin
8 biological layer made of salivary and epithelial constituents,
9 lining oral mucosae. It contributes to their protection against
10 microbiological, chemical or mechanical insults. Pellicle
11 formation depends on the cells' surface properties, and in turn
12 the pellicle deeply modifies such properties. It has been reported
13 that the expression of the transmembrane mucin MUC1 in oral
14 epithelial cells improves the formation of the mucosal pellicle.
15 Here, we describe an approach combining classical and
16 functionalized tip atomic force microscopy and scanning microwave
17 microscopy to characterize how MUC1 induces changes in buccal
18 cells' morphology, hydrophobicity and electric properties to
19 elucidate the physicochemical mechanisms involved in the
20 enhancement of the anchoring of salivary proteins. We show that
21 MUC1 expression did not modify drastically the morphology of the
22 epithelial cells' surface. MUC1 expression, however, resulted in
23 the presence of more hydrophobic and more charged areas at the
24 cell surface. The presence of salivary proteins decreased the
25 highest attractive and repulsive forces recorded between the cell
26 surface and a functionalized hydrophobic AFM tip, suggesting that
27 the most hydrophobic and charged areas participate in the binding
28 of salivary proteins. The cells' dielectric properties were
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3 altered by both MUC1 expression and the presence of a mucosal
4 pellicle. We finally show that in absence of MUC1, the pellicle
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6 appeared as a distinct layer poorly interacting with the cells'
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8 surface. This integrative AFM/SMM approach may usefully describe
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10 the surface properties of various cell types, with relevance to
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12 the bioadhesion or biomimetics fields.
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TEXT

Introduction

Mucosal surfaces, which line various body cavities (eye, mouth, nose, vagina...) or internal organs (lungs, stomach, gut...), represent the first line of defense against invading pathogens and xenobiotics. They also ensure protection against mechanical wear and dehydration through their lubricating properties. These functions are partly fulfilled by a mucous layer covering the often mucin-secreting epithelial cells. The situation differs slightly for the mucosal surfaces of the oral cavity, where epithelial cells are lined by a thin biological layer, up to 100 nm high, made of both epithelial and salivary constituents. This biological structure termed the mucosal or salivary pellicle is constituted through selective adsorption of salivary proteins (MUC5B, MUC7, sIgA etc.) onto the cell's surface^[1]. Among those, the heavily glycosylated mucins MUC5B are prominently involved in the lubricating and hydrating properties of the pellicle^[2]. Adsorption of MUC5B and other salivary proteins to the cells' surface is suggested to occur mainly through non-covalent (in particular hydrophobic) interactions^[3]. We have also showed that the presence of the transmembrane mucin MUC1 enhances anchoring of MUC5B onto the cell's surface ^[4], suggesting that protein-protein interactions between MUC1 and salivary proteins are also involved

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3 in the mucosal pellicle formation. Cross-linking between salivary
4 proteins mediated by transglutaminase further consolidates the
5 architecture of the pellicle^[1a].
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10 Oral mucosal surfaces play a role in the perception of food
11 sensory attributes, in particular astringency or aroma
12 persistence. For both sensations, the suggested mechanism by which
13 the oral surface intervenes relies on the ability of the cell
14 surface and mucosal pellicle constituents to interact non-
15 covalently with tannins^[5] or aroma compounds^[6]. Noncovalent
16 interactions can be of different natures (hydrophobic effect,
17 electrostatic interactions, Van der Waals forces or π -effects),
18 depending on the structure and physico-chemical properties of the
19 partners.
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34 Therefore, determining the physicochemical characteristics of
35 epithelial cells and mucosal surfaces may help understanding
36 further the nature of non-covalent interactions involved both in
37 the anchoring of salivary proteins forming the mucosal pellicle
38 and in flavor perception. More generally, such information would
39 be of great importance to gain deeper knowledge on the phenomenon
40 of bioadhesion on oral soft surfaces.
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51 To study *in vitro* biological events involving the mucosal
52 surface, we have recently developed a cellular model of oral mucosa
53 surmounted by a mucosal pellicle. The model is based on the use of
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3 TR146 cells which have been stably transfected to express the
4 transmembrane mucin MUC1, incubated with human saliva. We have
5 demonstrated that MUC1 expression increases by 30% the anchoring
6 of salivary MUC5B at its surface^[4]. This model proved useful to
7 study the structural alterations of the mucosal pellicle induced
8 by tannins^[5b].
9

10
11 Atomic Force Microscopy (AFM) is a very high-resolution type of
12 scanning probe microscopy which allows investigating with
13 subnanometer resolution capacity the morphology and
14 physicochemical properties of biological samples in their
15 physiological environment. The AFM technique consists of scanning
16 the surface of a sample by a sharp tip and recording the inter-
17 atomic forces between the sample and the tip. AFM can be applied
18 to a variety of biological samples such as protein assemblies^[7],
19 viruses^[8] or live eukaryotic cells^[9]. Functionalization of AFM
20 tips with a ligand enables probing specific interactions such as
21 antibody-antigen or DNA-protein interactions^[10]. Another AFM
22 technique, termed scanning microwave microscopy (SMM), combines
23 the electromagnetic measurement capabilities of a microwave vector
24 network analyzer with the resolution power of AFM. Our laboratory
25 has developed a network analyzer giving access to several
26 frequencies in the range 0.1-16 GHz ^[11]. The signal characterizes
27 the local conductance and dielectric properties of the sample,
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3 enabling to perform three-dimensional tomographic investigations
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5 on the electric properties of a sample. While the dielectric
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7 properties of a medium determines the strength of interactions
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9 between charged species, the dielectric properties of a surface
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11 provide indications on its ability to establish electrostatic
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13 forces. Moreover, as the dielectric micro-environments are complex
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15 and variable, SMM will provide an overview on its spatial variation
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17 both horizontally and vertically.
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22 Here, we report an integrative approach combining classical AFM,
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24 force spectroscopy and SMM to identify the physicochemical
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26 mechanisms involved in the formation of the mucosal pellicle and
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28 characterize the influence of biological supramolecular structures
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30 (the transmembrane mucins and /or the mucosal pellicle) present at
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32 the cell surface on its physico-chemical properties. While
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34 classical AFM discriminated the structures present at the cell
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36 surface, functionalization of the probe's tip allowed evaluating
37
38 the hydrophobicity of cell's surface. In parallel, the electric
39
40 properties of the cell's surface were explored at resonance
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42 frequencies of 2, 4.3 and 8.6 GHz by the SMM device. The thorough
43
44 description of physico-chemical properties of the cell surface and
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46 how they are affected by the presence of mucin and salivary
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48 proteins will provide new insights into the mechanisms at the
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50 origin of bioadhesion on soft surfaces of the oral cavity.
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3 Bioadhesion is crucial to maintain a healthy and comfortable
4 hydrated mouth feeling. Any new knowledge in this field may lead
5 to innovations in, for example, the development of oral hygiene
6 products or saliva substitutes for patients suffering from dry
7 mouth.
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19 **Experimental Section**

20 **Saliva collection**

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25 The study was performed in agreement with the guidelines of the
26 declaration of Helsinki. Informed consent was obtained from the
27 subjects who donated saliva. Unstimulated saliva was collected
28 from seventeen volunteers who declared to be in good oral health.
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30 The volunteers were instructed to refrain from smoking, eating and
31 drinking for at least two hours before the collection sessions.
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33 The collection sessions lasted 30 minutes during which volunteers
34 spat out the saliva accumulating naturally in their mouth into
35 plastic vessels kept on ice. All samples were pooled and
36 centrifuged at 14 000 g for 20 min at 4 °C. The clarified saliva
37 was aliquoted and frozen at -80 °C.
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51 **Cell culture**

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3 The native (TR146) and transfected (TR146/MUC1) epithelial cells
4 were routinely grown according to the protocol described
5 previously ^[4]. The cells were seeded at 4×10^4 cells/ml density
6 on Cell-Tak™ coated glass cover slips (Agar Scientific). The cells
7 were incubated at 37 °C in a humidified atmosphere containing 7.5
8 % CO₂ for two days in order to reach confluence. The confluent
9 cultures were incubated with clarified saliva for two hours and
10 then washed twice with phosphate-buffered saline (PBS, pH 7.0 -
11 7.3, Gibco®) to eliminate unbound saliva. Fixation of cells was
12 carried out with 2.5% glutaraldehyde (Sigma-Aldrich) in PBS (pH
13 7.8 ± 0.2 , Bio-RAD) during 30 min, followed by rinsing with PBS.
14 AFM imaging (topography and tip-sample force measurements) was
15 performed in liquid PBS medium. SMM analysis was performed on
16 samples processed as above and further dehydrated through graded
17 baths of ethanol (from 30 to 100%). Drying was performed by the
18 critical point drying method using a Leica CPD 030.

40 **AFM imaging**

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43 AFM characterizations were performed using Multimode 8 AFM
44 microscope (Bruker, Santa Barbara, CA, USA). The topographic
45 features of cells without and with a mucosal pellicle were
46 investigated using silicon nitride probes (ScanAsyst Air HR, $k =$
47 0.4 N/m and $f = 70 \text{ kHz}$, Bruker). The topographic images were
48 acquired at high resolution ($512 \times 512 \text{ pixel}^2$) using Peak force

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3 mode. In Peak force mode, the AFM tip oscillates at a frequency of
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5 2 kHz with amplitude of 20 nanometers. This mode is well adapted
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7 for imaging soft and delicate samples such as cells at high
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9 resolution.
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11 12 13 **Tip - sample force measurements** 14

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16 Gold-coated AFM probes were functionalized with hydrophobic self-
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18 assembled monolayers (SAMs) using alkanethiols. Forming a SAMs
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20 assembly on gold surfaces is a well-controlled process having many
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22 practical advantages^[12]. The SH-groups of alkanethiols has a high
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24 affinity on gold substrates^[13] via chemical binding and it can
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26 remain stable during days to weeks after its formation^[12]. Gold-
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28 coated cantilevers, with the nominal spring constant of 0.12 N/m
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30 and the nominal resonance frequency of 23 kHz, were purchased from
31
32 Bruker (NPG-10, Santa Barbara, CA, USA). The self-assembled
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34 monolayers were formed on the gold-coated cantilevers according to
35
36 a previous procedure^[14]. Gold-coated cantilevers were immersed in
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38 1 mM solutions of alkanethiols terminated with CH₃ (1-
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40 octadecanethiol, Sigma-Aldrich) during 18 hours. Firstly, a teflon
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42 substrate (Sigma Aldrich) was scanned to verify the AFM tip
43
44 functionalization. Secondly, the adhesion forces between the
45
46 functionalized tips and cells were measured using contact imaging
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48 mode. AFM enables to probe the forces between the tip and sample
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50 by monitoring the deflection of the cantilever when it approaches,
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3 contacts and retracts from a surface. Contact imaging mode records
4 a force curve for each contact point of tip in function of the
5 approach - retract curves providing information about the
6 elasticity and adhesion. The tip - sample adhesion forces were
7 measured on 5 x 5 μm^2 of area on the cell surface by taking in
8 account 256 x 256 contact points. Images were acquired from 4
9 different glass slides per condition (N, NS, T, TS).

20 **Statistical analysis for tip - sample force measurements**

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23 To determine the characteristic adhesion forces between each
24 sample and the hydrophobic tip, a total of 94208 force curves were
25 recorded per sample. For each image, cumulated attractive and
26 repulsive forces were plotted against the force intensity. The
27 D25, D50 and D75, which correspond to the force intensity at 25,
28 50 and 75 percent of the cumulated force, were extracted. The
29 average attractive and repulsive forces were also extracted. The
30 impact of the sample type on such data was tested using one-way
31 analysis of variance (ANOVA). When a significant difference was
32 observed (P value < 0.05), means were compared using a Tukey
33 pairwise comparison test (significance level set at 5%).

49 **SMM imaging**

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52 Scanning microwave microscopy combines the use of atomic force
53 microscopy with a vector network analyzer (VNA). This

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3 configuration offers simultaneous measurement of the topography
4 and complex reflection coefficient of a sample with high
5 sensitivity. The VNA can generate microwaves in the high frequency
6 range. The incident microwave signal is transmitted through a
7 resonant circuit to a conductive probe. The conductive probe
8 enables to transmit and receive microwaves from the contact point
9 on sample. The applied microwave penetrates into the sample and a
10 part of the signal is reflected back through the cantilever. The
11 VNA detects the reflected signal (alternatively transmitted
12 signal) in the reflection mode and thereby measures the complex
13 reflection coefficient (S_{11}) of the contact point. The measured S_{11}
14 value represents the local impedance resulting from the tip-sample
15 interactions. The amplitude and phase of S_{11} signal are recorded
16 for each contact point simultaneously with topographic data of the
17 sample.

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38 We have performed SMM analysis using a commercial AFM (Keysight
39 5600LS, Agilent Technologies) equipped with a vector network
40 analyzer (VNA N5230A, Agilent Technologies). The VNA can generate
41 microwaves ranging from 800 MHz to 13 GHz and detects the reflected
42 signal from sample. Topography, VNA phase and VNA amplitude images
43 of the samples were recorded using contact mode in ambient
44 conditions. The surfaces of samples were scanned via a platinum-
45 iridium coated tip with nominal radius of 20 nm (SCM-PIT, $k = 2.8$
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3 N/m and $f_0 = 75$ kHz, Bruker) at 50 μm scan size with 1 Hz scan
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5 rate. Each sample was analyzed at three frequencies (2 GHz, 4.3
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7 GHz and 8.6 GHz). Images were acquired from 4 different glass
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9 slides per condition ((N, NS, T, TS).

13 **Statistical analysis of SMM images**

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16 A total of 174 SMM images were used for statistical analysis: the
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18 number of images at each frequency (2, 4.3 and 8.6 GHz) was
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20 respectively 12, 12 and 10 for native cells (N), 9, 10 and 9 for
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22 native cells with a salivary (mucosal) pellicle (NS), 17, 20 and
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24 16 for transfected TR146/MUC1 cells (T) and 19, 19 and 21 for
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26 transfected cells with a salivary pellicle (TS). The mean values
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28 of each amplitude and phase image were obtained using AFM software
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30 [15]. One-way ANOVA was performed to evaluate at each frequency the
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32 impact of MUC1 and the mucosal pellicle on mean amplitude and
33
34 phase SMM values.
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40 **Impedance images**

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43 The complex reflection coefficient was converted into complex
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45 impedance data using a Matlab script. The reflection coefficient
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47 S_{11} was measured in reflection mode. In this way, a ratio of the
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49 incident / reflected electromagnetic waves was recorded by the
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51 VNA. The microwave images (amplitude and phase signals) are induced
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53 by interaction variations between the tip and the sample at every
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3 contact point, traducing local changes of the constituted
4 equivalent electrical circuit. These local changes give access to
5 nanoscale electrical impedance values of the sample, called DUT
6 (Device Under Test).
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13 Impedance is a complex number with strong frequency dependence.
14 A simple method to measure impedance at microwave frequencies is
15 to connect the DUT (here biological sample) of unknown impedance
16 to the end of a coaxial line (here the AFM tip). The other end of
17 the coaxial line is linked to the VNA, the microwave source. Thus
18 the SMM measurement allows to calculate the corresponding real and
19 imaginary impedance components by first expressing the real and
20 imaginary parts of the reflection coefficient as follows:
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$$\Gamma_r = |\Gamma_L| \cos\left(\phi \frac{\pi}{180}\right) \quad (1)$$

$$\Gamma_i = |\Gamma_L| \sin\left(\phi \frac{\pi}{180}\right) \quad (2)$$

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40 With ϕ the measured phase and $|\Gamma_L|$ the modulus of the measured
41 amplitude variation of the reflection coefficient expressed as:
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$$|\Gamma_L| = 10^{(S_{11}/20)} \quad (3)$$

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50 Unknown impedance reflects a part of the microwave signal. Thus,
51 the amount of reflected signal from the DUT, Γ_L , is directly
52 dependent on the degree of mismatch between the microwave source
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characteristic impedance, Z_0 , (a real industry normalized value of 50 Ω) and the DUT impedance. Its expression can be noted as follows:

$$\Gamma_L = \frac{V_{refl}}{V_{inc}} = \frac{Z_L - Z_0}{Z_L + Z_0} = \Gamma_r + j\Gamma_i \quad (4)$$

Where Z_L is the unknown impedance of the DUT.

By fixing Z_0 to 50 Ω , we can then define normalized load impedance by

$$Z = \frac{Z_L}{Z_0} = \frac{R + jX}{Z_0} = r + jx \quad (5)$$

With this simplification, we can rewrite the reflection coefficient formula as:

$$\Gamma_L = \Gamma_r + j\Gamma_i = \frac{Z_L - Z_0}{Z_L + Z_0} = \frac{(Z_L - Z_0)/Z_0}{(Z_L + Z_0)/Z_0} = \frac{Z - 1}{Z + 1} = \frac{r + jx - 1}{r + jx + 1} \quad (6)$$

and express the normalized load impedance as:

$$Z = r + jx = \frac{1 + \Gamma_L}{1 - \Gamma_L} = \frac{1 + \Gamma_r + j\Gamma_i}{1 - \Gamma_r + j\Gamma_i} \quad (7)$$

By setting the real and the imaginary parts of Z , two independent relationships are obtained:

$$r = \frac{1 - \Gamma_r^2 - \Gamma_i^2}{(1 - \Gamma_r)^2 + \Gamma_i^2} \quad (8)$$

$$x = \frac{2\Gamma_i}{(1 - \Gamma_r)^2 + \Gamma_i^2} \quad (9)$$

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3 r , the real part of the impedance, translates a resistance (Ω)
4 of the DUT and x , the imaginary part, is the reactance of the DUT
5 (Ω).
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11 As the sensitivity of the reflection coefficient Γ_L to impedance
12 r rapidly decreases for very high impedances ($r > 10 \text{ k}\Omega$ for $|\Gamma_L| >$
13 0.99) and for very low impedances ($r < 250 \text{ m}\Omega$ for $|\Gamma_L| < -0.99$),
14 the imaging frequencies are chosen according to their matching
15 degree with the microwave source to avoid losses in reflection
16 coefficient sensitivity.
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25 In this article, images representing the variation of the real
26 part of the impedance of the DUT are presented as qualitative
27 measurements (one representative image per condition at 8.6 GHz).
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33 **Data availability**

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36 The data supporting the findings of this study are available from
37 the corresponding author upon reasonable request.
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45 **Results and discussion**

46 **Morphology of human oral epithelial cells**

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49 Fig. 1 presents the retention of the salivary mucins MUC5B on
50 human buccal TR146 native cells (Fig. 1b) and on TR146/MUC1
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3 transfected cells (Fig. 1c) and the topography of native and
4 transfected cells without or with a mucosal pellicle imaged by AFM
5 (Fig. 1a and 1d to 1h). As previously reported^[4], the expression
6 of MUC1 in transfected cells enhanced the retention of salivary
7 mucins MUC5B. MUC5B immunostaining showed larger between-cells
8 variability in transfected cells. Cells had a rather flat shape
9 (Fig. 1a) with an apex in height in the range of 1-2 μm . There was
10 no substantial difference in the morphological features of native
11 and transfected cells (Fig. 1d and 1e). Membrane folds
12 (microplicae), a few hundreds of nanometer-high, were visible on
13 both types of cells. They varied in size and density as previously
14 observed by scanning electron microscopy^[4]. Microplicae are
15 typical of squamous epithelial surfaces^[16] where they increase the
16 exchange surface between cells and the external medium, saliva
17 here, with consequences on drug absorption for example. They may
18 also enhance mucus retention thereby preventing infections^[16].

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40 The overall appearance of the cell surface was not drastically
41 modified by the presence of a mucosal pellicle on native cells
42 (Fig. 1f). It was not possible to determinate precisely its
43 thickness because of the uneven cells' topography. However,
44 microplicae remained distinguishable under the pellicle as
45 previously found^[5b], translating that only a very thin layer was
46 deposited on the cell surface. This is also consistent with former
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3 measurements of the pellicle's thickness (0 to 100 nm) on human
4 buccal cells^[17]. On transfected cells, the same conclusions applied
5 to most acquired images (Fig. 1g) although a fine filamentous
6 network was also occasionally observed (Fig. 1h). AFM imaging
7 provided a detailed topographic picture of this fragile structure
8 in liquid medium. This network resembles the filamentous structure
9 observed by cryoscanning electron microscopy in
10 submandibular/sublingual saliva, which was interpreted as a mucin
11 network^[18]. It is also comparable to a MUC5B network formed on mica
12 observed by AFM^[5a]. This distinctive appearance of some TR146/MUC1
13 cells can be related to the heterogeneous retention of MUC5B (Fig.
14 1c): it is likely that such a salivary network would form
15 specifically on cells retaining most strongly MUC5B mucins.
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37 **Impact of a mucosal pellicle on the hydrophobicity of epithelial** 38 **cells** 39

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42 Chemical force microscopy (CFM) with hydrophobic tip has been
43 previously successfully used to performed direct measurement of
44 hydrophobic forces at the cell surfaces ^[19]. To investigate the
45 impact of a mucosal pellicle on cells' hydrophobicity, we applied
46 CFM with hydrophobic functionalized gold AFM tips to monitor
47 interactions in liquid medium at the cell surface (Fig. 2). Tips
48 were functionalized with alkanethiols terminated with CH₃ as
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3 previously reported^[14] and checked on hydrophobic teflon
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5 (Supplementary Fig. S1): a greater adhesion force was observed
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7 with the functionalized tip. Then the cell surface was scanned
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9 leading to adhesion force mapping. Fig. 2a shows a representative
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11 map of adhesion force for the four tested conditions. It reveals
12
13 that there were both attractive (negative values) and repulsive
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15 (positive values) forces between the functionalized tip and the
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17 cell surface, with large differences among conditions. Pictures of
18
19 native TR146 cells without (N) or with a mucosal pellicle (NS)
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21 were very similar, with attractive and repulsive forces at low
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23 intensities. Conversely, much higher intensities (both for
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25 positive and negative forces) were recorded on images of TR146/MUC1
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27 transfected cells without (T) or with a mucosal pellicle (TS). In
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29 addition, the presence of the mucosal pellicle seemed to decrease
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31 overall the number of areas with the highest repulsive and
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33 attractive forces.
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40 To compare maps, we plotted for each image the cumulative force
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42 as a function of the adhesion forces' intensity, both for
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44 attractive (negative values) and repulsive (positive values)
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46 forces. Fig. 2b provides the resulting curves for the particular
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48 images presented in Fig. 2a. This confirmed that attractive and
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50 repulsive forces recorded at the surface of native cells without
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52 or with a mucosal pellicle were weak since their absolute values
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3 never exceeded 10 nN. The curves were very close for the two
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5 conditions N/NS: the presence of saliva hardly modified attractive
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7 and repulsive forces at the cell surface. In contrast, the curves
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9 obtained for the transfected cells showed higher attractive (down
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11 to -80 nN) and repulsive (up to 130 nN) forces. On these cells,
12
13 the presence of a mucosal pellicle induced a rather large
14
15 difference in adhesion forces, particularly with a decrease of the
16
17 maximum intensity of attractive and repulsive forces.
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22 From these curves were extracted the values D25, D50 and D75,
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24 which represent respectively the attractive or repulsive forces at
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26 25, 50 and 75 percent of the total attractive or repulsive forces.
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28 These were determined for all the acquired images on different
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30 cells (n=18, 16, 18 and 24 for the conditions N, T, NS and TS
31
32 respectively). Fig. 2c represents the repulsive and attractive
33
34 D25, D50 and D75 values (mean \pm STD) while Fig. 2d presents the
35
36 average repulsive and attractive forces at the cell surface (mean
37
38 \pm STD). ANOVA testing evidenced statistical differences between
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40 conditions for the D25 (repulsive forces: $F=7.92002$, $p=0.00014$;
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42 attractive forces: $F=10.3863$, $p=1.09 \times 10^{-5}$), D50 (repulsive forces:
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44 $F=7.69584$, $p=0.00017$; attractive forces: $F=11.0893$, $p=5.52 \times 10^{-6}$),
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46 D75 (repulsive forces: $F=8.99411$, $p=4.4724 \times 10^{-5}$; attractive
47
48 forces: $F=11.564$, $P=3.49 \times 10^{-6}$) and average (repulsive forces:
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50 $F=8.29782$, $p=9.22 \times 10^{-5}$; attractive forces: $F=10.0733$, $p=1.50 \times 10^{-5}$)
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3 values. Tukey pairwise comparison tests were applied. For native
4 cells, the presence of salivary proteins at the surface (N vs NS)
5 did not modify significantly the repulsive and attractive forces.
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7 In contrast, the presence of MUC1 alone (N vs T) significantly
8 increased the adhesive and repulsive forces for all extracted
9 values: D25, D50, D75 and average. The anchoring of salivary
10 proteins on TR146/MUC1 transfected cells (T vs TS) slightly reduced
11 adhesion forces but not significantly, except for the highest
12 attractive and repulsive forces (D75).
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24 Attractive and repulsive forces suggest the occurrence of
25 noncovalent interactions between the AFM tip and the surface of
26 the cells. Attractive forces could be due to hydrophobic effect,
27 indicating the presence of hydrophobic surfaces, or London
28 dispersion, which are not expected to play an important role due
29 to the weakness of the CH \cdots HC interaction and their too small
30 number, while repulsive forces suggest the presence of hydrophilic
31 surfaces, presenting either formal or partial charges. Therefore,
32 expression of MUC1 increased the intensity of both hydrophobic and
33 charged areas. The protein encoded by the gene used to transfect
34 TR146 cells (MUC1-Y-LSP) presents a shorter extracellular domain
35 than the full-length MUC1. This 167 amino-acids extracellular
36 domain contains 11 basic amino-acids (Arg, Lys) and 15 acidic
37 amino-acids (Asp, Glu), with a theoretical isoelectric point of
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3 5.08. At the pH of the cell culture medium (6.8–7.2) or PBS (7.0–
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5 7.8), it tends to be negatively charged, consistently with the
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7 increase in repulsive forces. In addition, although the structure
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9 of this extracellular domain is not described, its composition in
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11 amino-acids suggests that it would comprise both hydrophobic and
12
13 charged areas, which would explain the present observations. The
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15 great variability (indicated by large standard deviations
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17 associated to average values) both for attractive and repulsive
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19 forces is probably related first to the heterogeneous between- and
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21 within-cells expression of MUC1, and second to heterogeneity in
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23 the surface properties of MUC1-Y-LSP. This isoform of MUC1 could
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25 bear 5 N-glycosylations but in contrast to other isoforms of MUC1
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27 it does not include the variable number tandem repeat (VNTR)
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29 module, which is abundantly glycosylated. Thus, this isoform does
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31 not fully represent the properties of other isoforms of MUC1, such
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33 as MUC1/1. The numerous glycosylations of the VNTR module are
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35 expected to be negatively charged increasing the hydrophilicity at
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37 the cell surface and should decrease the access to hydrophobic
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39 domain of the peptidic chain through steric hindrance.
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47 The presence of salivary proteins decreased the highest
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49 attractive and repulsive forces (Fig. 2c and 2d), suggesting that
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51 the most hydrophobic and charged areas participate in the binding
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53 of salivary proteins. MUC5B in particular have high affinity for
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3 hydrophobic substrates^[20]. Therefore, the highly hydrophobic areas
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5 observed upon MUC1 expression probably participate to the higher
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7 retention of MUC5B on transfected cells compared to native cells
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9 (Fig. 2a and 2b). More generally, our results support the previous
10
11 suggestion that hydrophobic effects and electrostatic interactions
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13 intervene in salivary protein binding during pellicle formation^[21].
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21 **A mucosal pellicle modifies the dielectric properties of** 22 **epithelial cells** 23 24

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26 After documenting cells' hydrophobicity, we investigated the
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28 cells' dielectric properties by scanning microwave microscopy
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30 (SMM). This technique has been previously applied to cells ^[22]
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32 where the amplitude of the microwave signal yields information
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34 about the sample's conductivity determined for example by the ionic
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36 strength, while the phase of the microwave signal reflects the
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38 dielectric losses arising from the fluid density/water content
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40 characteristic of the sample. An advantage of microwave near field
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42 investigation over infrared, optical waves or AFM force field is
43
44 the penetration ability of microwaves, allowing subsurface imaging
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46 even in poorly conductive biological materials. Since penetration
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48 decreases with increasing frequency, it is possible to estimate
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50 whether differences in dielectric properties occur at the sample
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52 surface or deeper.
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3 The samples were analysed with a scanning microwave microscope
4 set up as shown in Fig. 3. In the imaging mode where a fixed
5 frequency is chosen, SMM allows to simultaneously obtain the
6 topography of the surface by means of its AFM tip and the
7 reflection coefficient of the microwave signal acquired by the
8 vector network analyzer at each point of measurement. The microwave
9 amplitude and phase images are dependent on the frequency as well
10 as the topography of the cells. In our case, the topography was
11 overall comparable from one sample to another as shown by AFM
12 images (Fig. 1 and Fig. 4a). Any significant difference in the
13 amplitude and phase SMM images relates therefore mainly to
14 differences in dielectric properties among samples.
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31 Each sample was examined at resonance frequencies of 2, 4.3 and
32 8.6 GHz. A typical topography image of each type of sample is
33 displayed with the corresponding SMM amplitude and phase images
34 obtained at a resonance frequency of 8.6 GHz (Fig. 4). SMM images
35 obtained at 2 and 4.3 GHz are presented in Supplementary Fig. S2.
36 Overall, the contrast of amplitude and phase images between samples
37 increased with resonance frequency (Fig. S2). The difference in
38 dielectric properties between cells was therefore much more
39 visible at high frequency (near the sample's surface) than at low
40 frequency (deeper down). This is expected since cells differ by
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3 the presence or not of a transmembrane mucin, and the presence or
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5 not of a mucosal pellicle on their surface.
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9 The impact of MUC1 expression on electric properties was
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11 evaluated by comparing native and transfected cells at 4.3 GHz and
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13 8.6 GHz (Fig. 4e). At 4.3GHz, the amplitude microwave signal was
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15 significantly higher for native cells (DF=30, F=12.70, p= 0.0012).
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17 At 8.6 GHz, amplitude and phase signals were significantly higher
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19 for transfected cells (amplitude: DF= 25, F=6.29, p= 0.0190; phase:
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21 DF= 24, F= 10.63, p= 0.0033). The large differences in values at
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23 8.6 GHz (e.g. phase of ~0.060 deg vs ~ 0.50 deg for N and T,
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25 respectively) confirm that the microwave-sample interactions
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27 differed mainly near the sample's surface, most likely as a result
28
29 of the presence of the transmembrane protein. MUC1-Y-LSP contains
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31 a short cytoplasmic tail, a membrane-spanning domain and an
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33 extracellular domain overall negatively charged. Charge variations
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35 induced by the presence of MUC1 possibly influenced the complex
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37 conductivity, thereby the SMM amplitude and phase signals.
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44 The impact of a mucosal pellicle on electric properties was
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46 evaluated by comparing both native and transfected cells without
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48 or with a mucosal pellicle at 4.3 and 8.6 GHz (Fig. 4f). For both
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50 cell types, the presence of the pellicle had no significant impact
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52 on the SMM amplitude signal. In contrast, the SMM phase signal was
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3 significantly increased (DF=17, F= 6.38, p= 0.0217) after pellicle
4 formation for native cells at 8.6 GHz.
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8 The difference in phase signals of N and NS cells indicated a
9 change in the dielectric environment of the material due to the
10 mucosal pellicle. This change was larger in absolute value at high
11 frequency (8.6 GHz) i.e. near the surface. At microwave
12 frequencies, the dielectric properties of biological tissues are
13 highly influenced by the presence of water. For example, a SMM
14 study on Chinese hamster ovary cells reported that increasing the
15 relative humidity raised their conductance^[23] since water adsorbed
16 onto the cells' surface is responsible for the dissipation of
17 microwave energy. With an increase in humidity content, the
18 dielectric loss values (translated by the phase of the microwave
19 signal) increase. Our results are in line with this: the mucosal
20 pellicle most likely increased residual humidity at the cells'
21 surface.
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41 Results of phase signal for transfected cells were rather
42 unexpected: the presence of MUC1 promotes binding of MUC5B on the
43 cells' surface and enhances mucosal pellicle formation^[4], which
44 was expected to result in higher dielectric losses. Nevertheless,
45 a slight increase in microwave phase signal was observed in TS in
46 comparison to NS (0.30 deg vs 0.23 deg) which may relate to a
47 higher wettability of the cell surface. Thus, a direct correlation
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3 between mucins concentrations and wettability of saliva was
4 reported: saliva containing more MUC5B presented lower contact
5 angles^[18]. Assuming there is an increase in surface wettability in
6 transfected vs native cell, this would explain the higher microwave
7 phase signal. The only representation of microwave raw signals
8 (phase and amplitude), however, is not sufficient to fully
9 understand the impact of saliva on dielectric properties of native
10 and transfected cells.
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22 Biological media are defined by their complex conductivity and
23 permittivity, these properties varying with the frequency of the
24 electric field passing through the medium. In our case, the complex
25 reflection coefficient can be converted into complex impedance in
26 order to obtain electrical and dielectric properties, i.e.
27 capacitance and conductance properties of the sample ^[24]. Fig. 5
28 shows impedance images deduced from phase and amplitude values for
29 one representative image per condition N, T, NS and TS at 8.6 GHz
30 (Fig 5a to 5d). After mucosal pellicle formation, the maximum
31 impedance increased. Saliva, containing 99% of water, can be
32 considered as a polar dielectric system. When a dielectric material
33 is excited by an electric field at microwave frequencies, the
34 rotational movement is no longer synchronized with the variations
35 of the electric field and a resonance phenomenon occurs. There is
36 thus a phase difference between the electric field and the
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3 rotational movement, thereby producing dielectric losses,
4 translated into the impedance increase which was observed. Even
5 more remarkably, the 3D impedance images show that this effect is
6 different between the two cell types. For native cells specifically
7 (Fig. 5c), the effect is characterized by the dissociation of the
8 impedance of the native cells (low level) from that induced by the
9 mucosal pellicle formation (high level) which can be interpreted
10 as a low affinity of native cells for saliva, in accordance with
11 the hydrophobic functionalized tip results. In contrast for
12 transfected cells, the impedance response is not dissociated. With
13 the presence of an electric field, the effects on the impedance
14 are complex: they combine first a dielectric loss effect due to,
15 as previously mentioned, increasing binding between transmembrane
16 MUC1 and salivary MUC5B and thereby formation of the mucosal
17 pellicle and second a conductivity effect induced by the
18 displacement of the charges in dense areas. The present impedance
19 results indicate a strong binding between the transfected cells
20 and the mucosal pellicle.
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48 **Conclusion**

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52 The present study innovatively combines different techniques
53 and methodologies of atomic force microscopy, in order to
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3 characterize the surface properties of a model of mucosa. Classical
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5 AFM technique brought information on the topography of the cells
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7 and the structure of the mucosal pellicle. Chemical force
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9 microscopy (CFM) investigation, with CH₃ functionalized tip
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11 allowed to characterize the hydrophobicity at the cell surface and
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13 revealed that the expression of MUC1 leads to the presence at the
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15 cells' surface of more repulsive and attractive forces suggesting
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17 an increase of both hydrophobic and hydrophilic areas. It indicates
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19 also that the adhesion of the salivary proteins involves probably
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21 the most hydrophobic and charged areas of MUC1. The SMM approach
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23 gave complementary information on the electric properties of the
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25 cell surface and the main results obtained indicate that salivary
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27 proteins and MUC1 are strongly interacting together. In absence of
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29 MUC1, salivary proteins appeared as a distinct layer poorly
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31 interacting with the cells' surface.
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38 These findings open perspectives about the use of AFM to
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40 characterize biological structures and their formation. Regarding
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42 the oral mucosa and the formation of the mucosal pellicle, these
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44 results call for research aiming at gaining a deeper understanding
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46 of the mechanisms involved in the formation of the mucosal pellicle
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48 and the regions or domains of MUC1 which are involved in the
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50 binding of salivary proteins. Future research is also required to
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3 identify the salivary proteins which specifically bind MUC1 and
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5 the noncovalent interactions involved.
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FIGURES

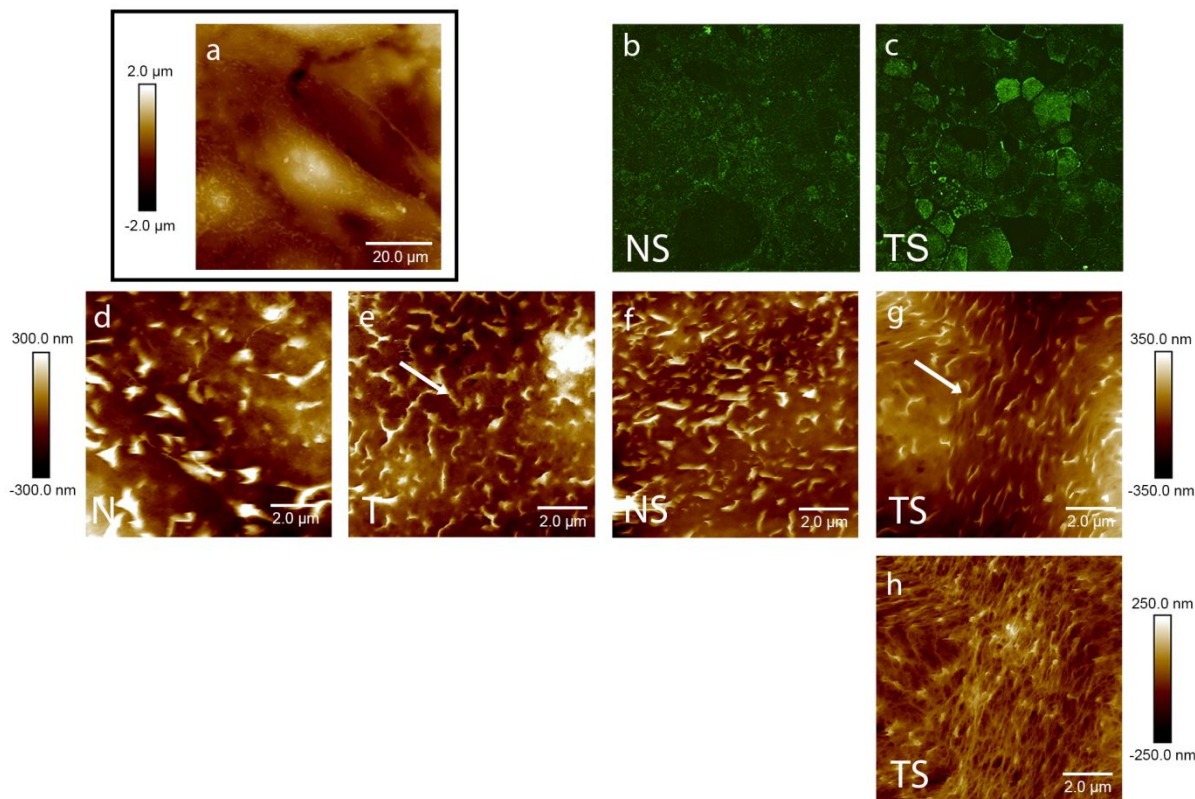


Figure 1. Retention of salivary mucins and topography of human epithelial buccal cells. Immuno-staining of MUC5B bound at the cell surface of native TR146 cells (b) and transfected TR146/MUC1 cells (c). Typical topographical image of epithelial cells (here are presented TR146/MUC1 cells) (a). Topography of TR146 native cells (d), TR146/MUC1 transfected cells (e), native cells with a mucosal pellicle (f) and transfected cells with a mucosal pellicle (g, h).

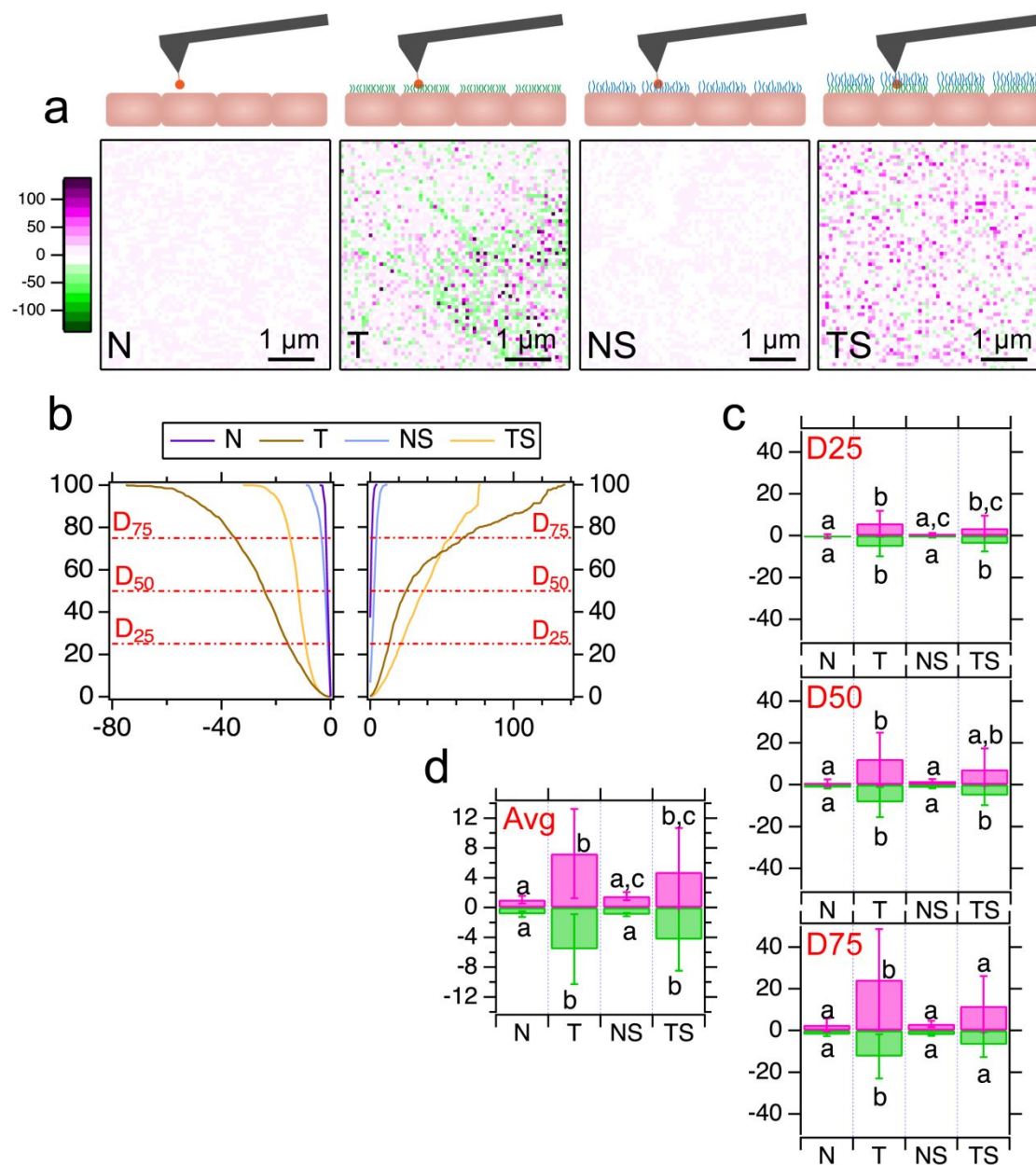


Figure 2. Hydrophobicity of human epithelial buccal cells. **a.** Schematic illustration of sample-tip interactions and corresponding adhesion force maps are given for TR146 native cells (N), TR146/MUC1 transfected cells (T), native cells with a mucosal pellicle (NS) and transfected cells with a mucosal pellicle (TS), respectively. **b.** Cumulated attractive (left) and repulsive (right) force curves as a function of force intensity. **c.** Means of the D75, D50 and D25 positive and negative value. **d.** Means of the average values of repulsive and attractive forces. All forces are expressed in nN.

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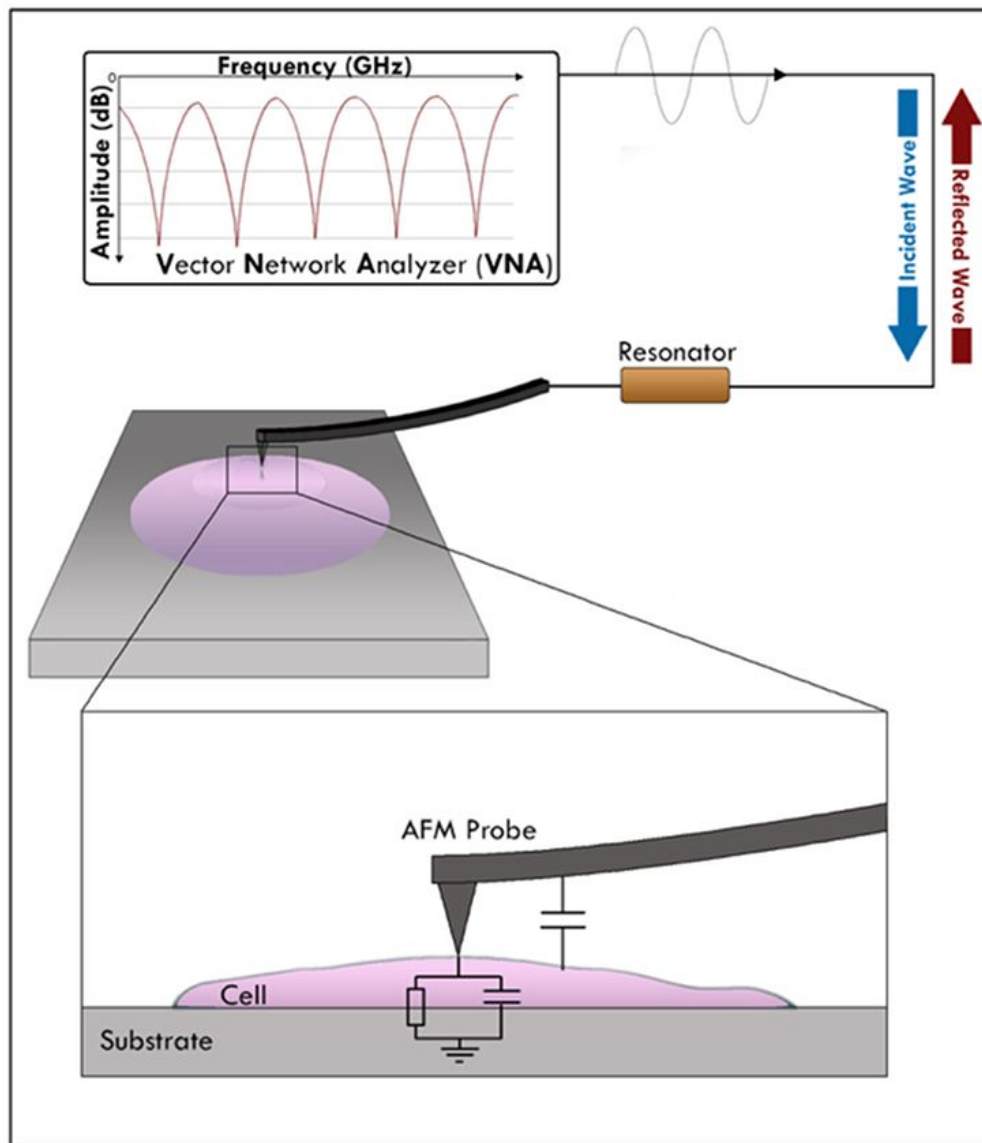


Figure 3. Schematic illustration of cell analysis by scanning microwave microscopy.

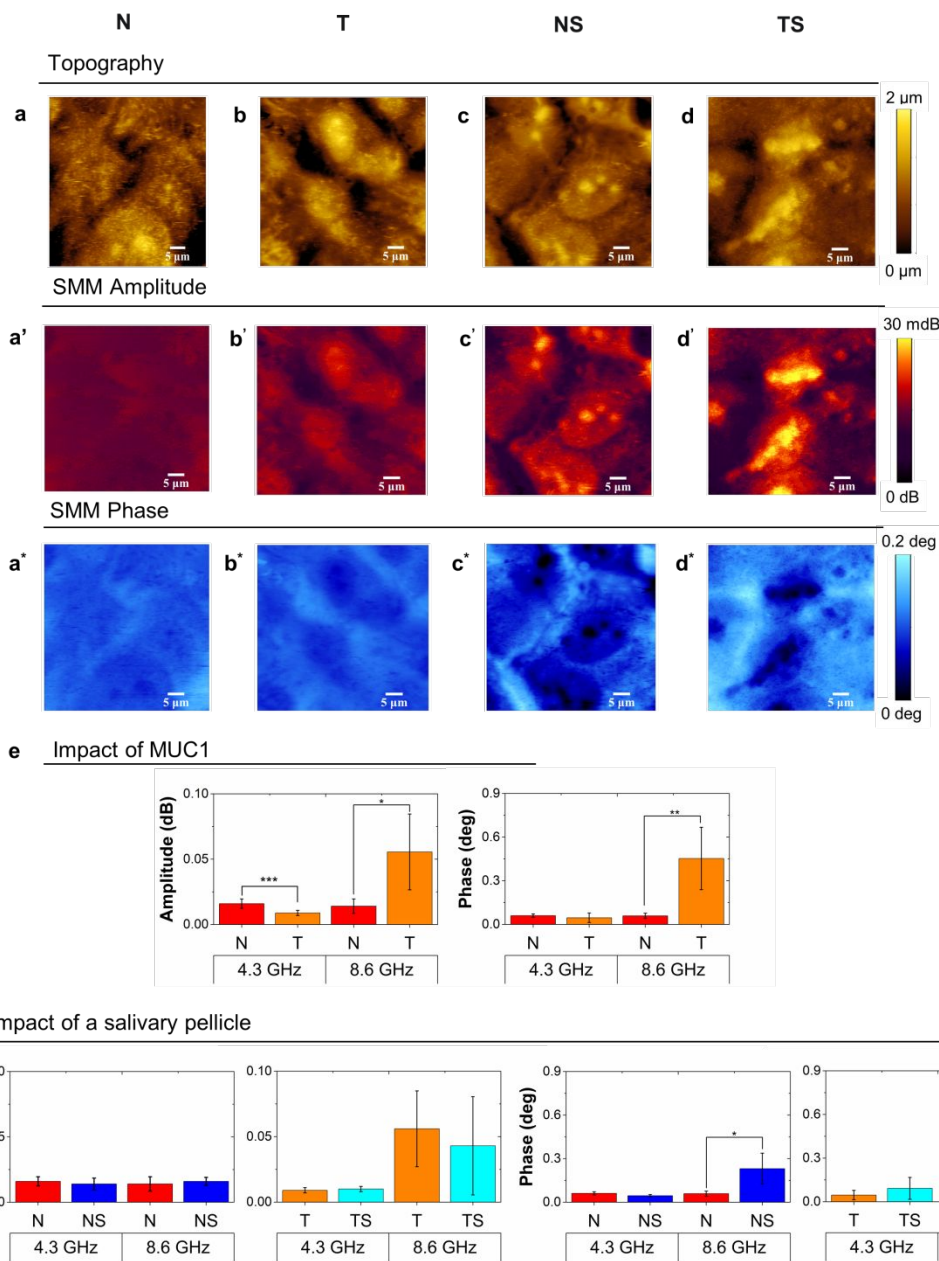


Figure 4. Scanning microwave microscopy analysis of human epithelial buccal cells. Topography (**a-d**), SMM amplitude (**a'-d'**) and SMM phase (**a*-d***) images for TR146 native cells (N), TR146/MUC1 transfected cells (T), native cells with a mucosal pellicle (NS) and transfected cells with a mucosal pellicle (TS). SMM amplitude and phase images were measured at a resonance frequency of 8.6 GHz. All images are at $50 \times 50 \mu\text{m}^2$ scanning size.

e, f: SMM amplitude and phase values (mean \pm STD) recorded at 4.3 and 8.6 GHz for native (red) and transfected (orange) cells, native cells with a mucosal pellicle (dark blue) and transfected cells with a mucosal pellicle (light blue). ANOVA tests were

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3 performed to evaluate the statistical impact of the presence of
4 MUC1 protein (**e**) or a mucosal pellicle (**f**) on amplitude and phase
5 values (*****p < 0.05, ******p < 0.005 and *******p < 0.001).
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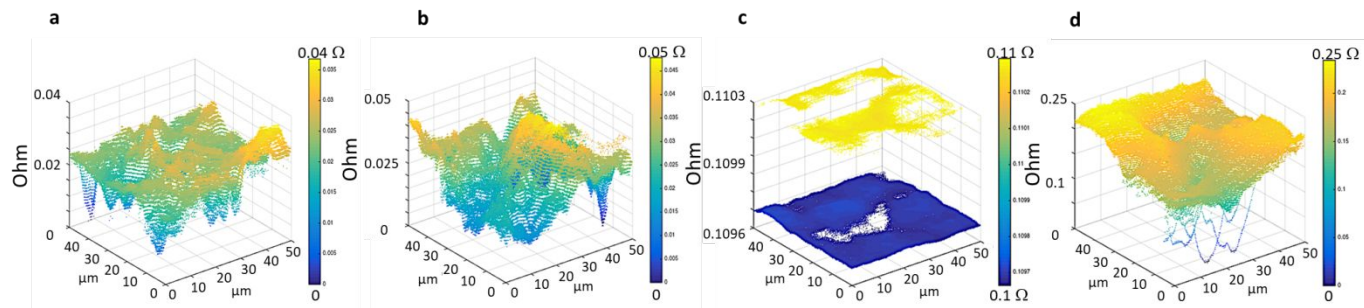


Figure 5. Impedance images of TR146 epithelial buccal cells.

Impedance images were constructed for one representative image per condition from amplitude and phase values obtained at 8.6GHz. **a:** TR146 native cells (**N**), **b:** TR146/MUC1 transfected cells (**T**), **c:** native cells with a mucosal pellicle (**NS**) and **d:** transfected cells with a mucosal pellicle (**TS**).

ASSOCIATED CONTENT

Supporting Information.

Supporting Figure S1. Verification of AFM tips functionalization.

Supporting Figure S2. Scanning microwave microscopy analysis of human epithelial buccal cells.

AUTHOR INFORMATION

Corresponding Author

Francis Canon

Author Contributions

E.N.A., E.B., E.L and F.C. conceived and designed the work.

E.N.A. performed all the AFM experiments. S.P. and M.B. produced the TR146/MUC1 cells, maintained the cell lines and performed immunohistochemistry. B.D.F. calculated and plotted impedance images. E.N.A., E.B., M.M., E.L and F.C analyzed and interpreted the data. E.N.A. drafted the manuscript with input from all authors. E.B., B.D.F., M.M., E.L. and F.C. revised the manuscript. All authors have approved its submitted version.

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11 PIA2/iSite-BFC, ANR-15-CE09-0002-02, ANR-17-EURE-0002 EIPHI
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13 Graduate School).

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24 (Grant ANR-14-CE20-0001-01 MUFFIN). The French National Research
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26 Agency also provided support for establishing and maintaining
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29
30 PIA2/iSite-BFC, ANR-15-CE09-0002-02, ANR-17-EURE-0002 EIPHI
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32 Graduate School).

33 34 35 36 37 38 39 40 ABBREVIATIONS

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42 AFM, atomic force microscopy; MUC1, Mucine-1; MUC5B, Mucine-5B;
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44 N, native cells; NS, native cells with a mucosal pellicle; PBS,
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46 phosphate-buffered saline; SMM, scanning microwave microscopy;
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48 T, TR146/MUC1 transfected cells; TS, transfected cells with a
49
50 mucosal pellicle; VNA, vector network analyzer.

51 52 53 54 REFERENCES

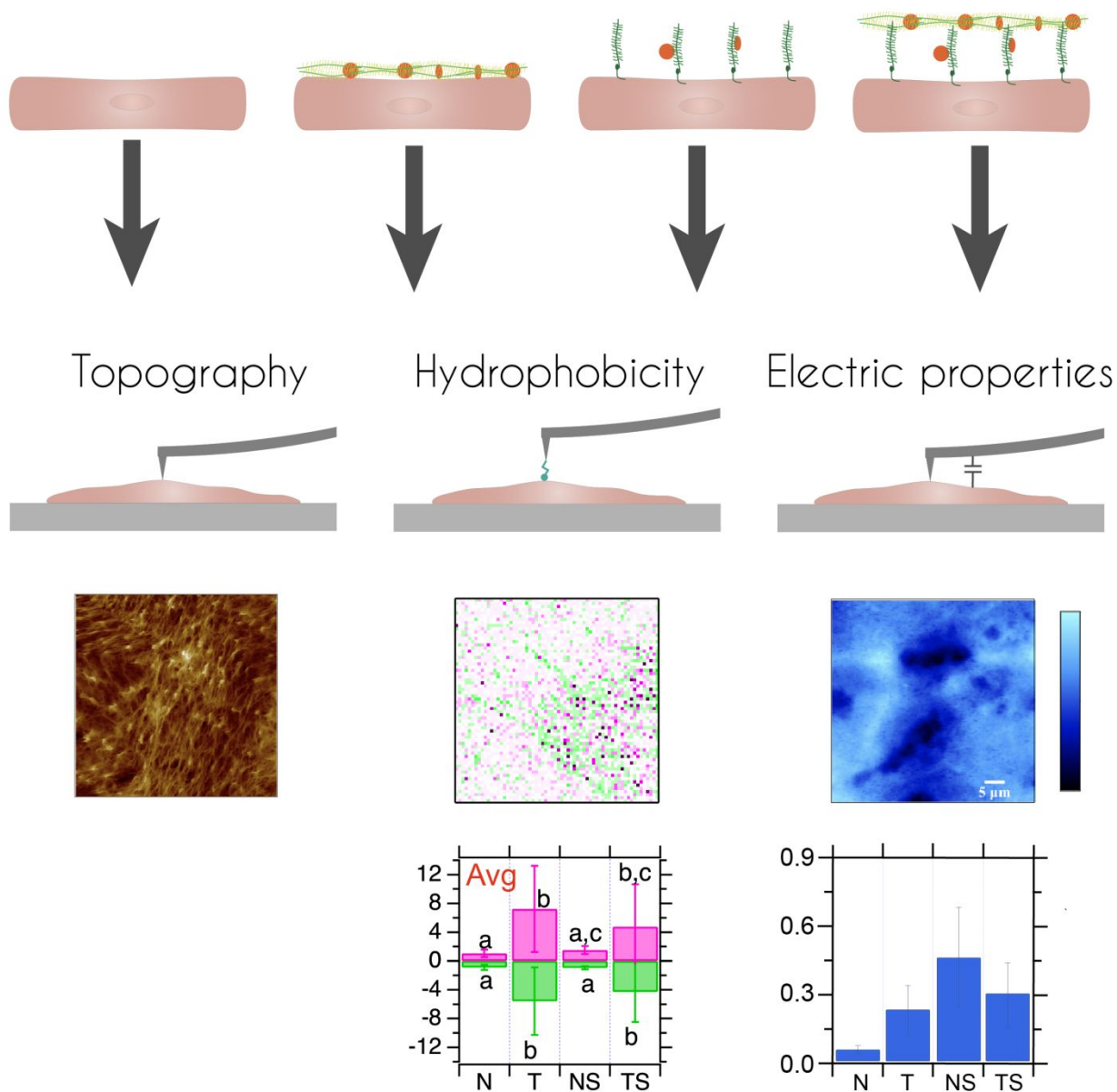
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SYNOPSIS

Mucosal pellicle



Comment citer ce document :

Aybeke, E. N., Ployon, S., Brulé, M., de Fonseca, B., Bouilliot, E., Morzel, M., Lesniewska, E., Canon, F. (2019). Nanoscale mapping of the physical surface properties of human buccal cells and changes induced by saliva. *Langmuir*, 35 (39), 12647-12655. , DOI : 10.1021/acs.langmuir.9b01979

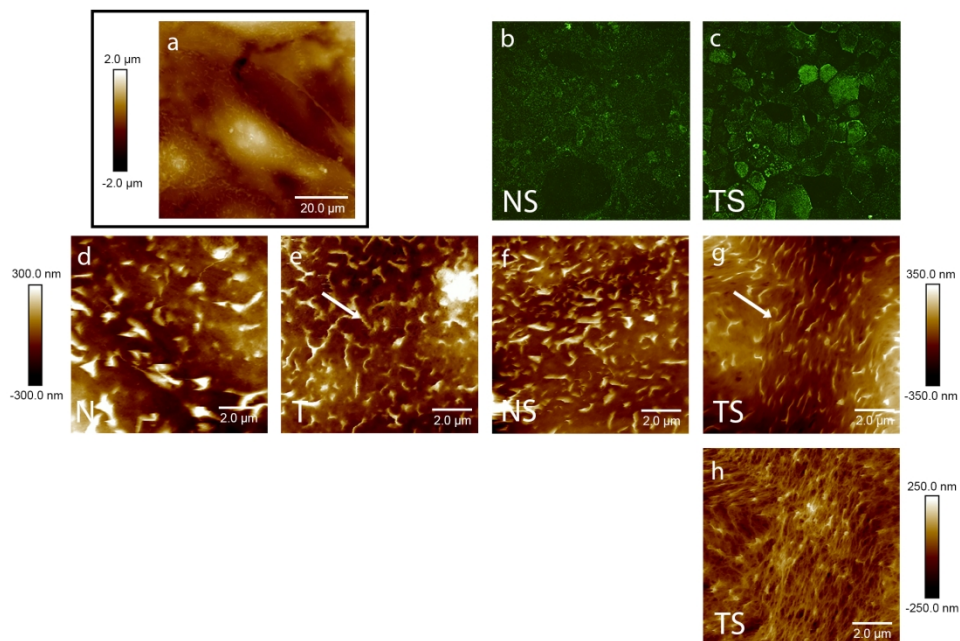


Figure 1. Retention of salivary mucins and topography of human epithelial buccal cells. Immuno-staining of MUC5B bound at the cell surface of native TR146 cells (b) and transfected TR146/MUC1 cells (c). Typical topographical image of epithelial cells (here are presented TR146/MUC1 cells) (a). Topography of TR146 native cells (d), TR146/MUC1 transfected cells (e), native cells with a mucosal pellicle (f) and transfected cells with a mucosal pellicle (g, h).

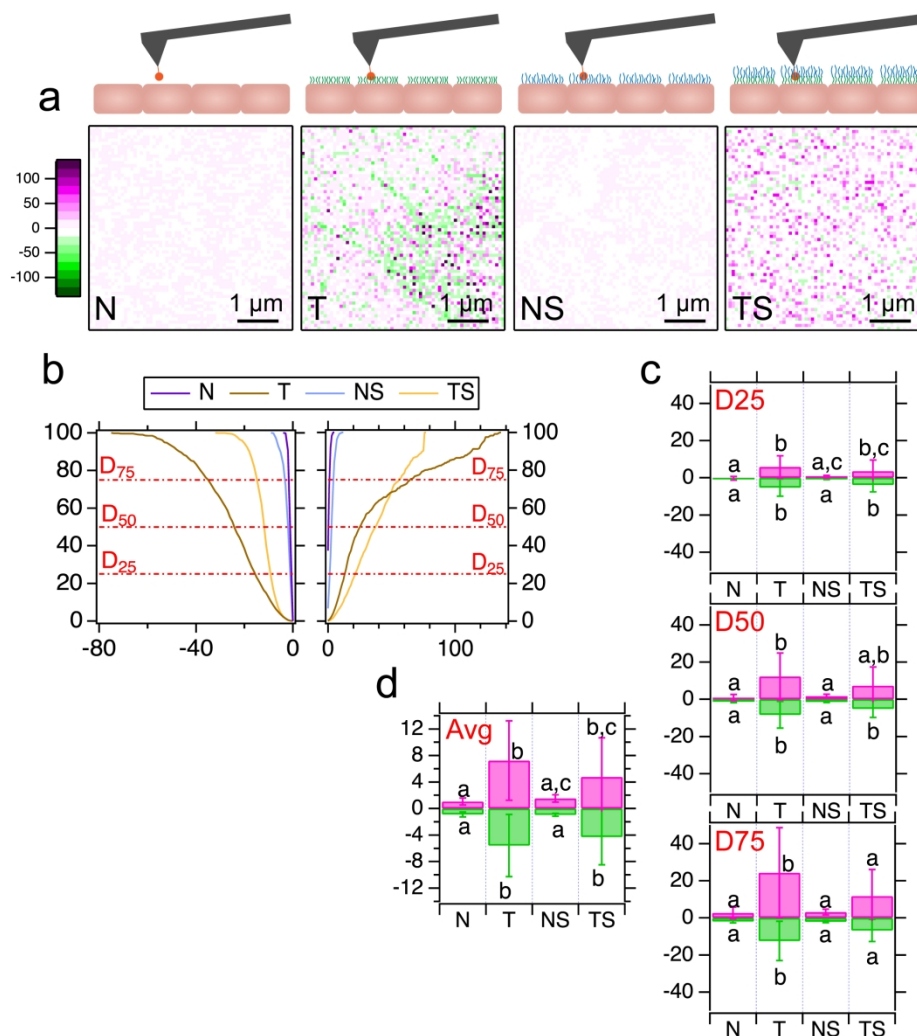


Figure 2. Hydrophobicity of human epithelial buccal cells. a. Schematic illustration of sample-tip interactions and corresponding adhesion force maps are given for TR146 native cells (N), TR146/MUC1 transfected cells (T), native cells with a salivary pellicle (NS) and transfected cells with a salivary pellicle (TS), respectively. b. Cumulated attractive (left) and repulsive (right) force curves as a function of force intensity. c. Means of the D75, D50 and D25 positive and negative value. d. Means of the average values of repulsive and attractive forces. All forces are expressed in nN.

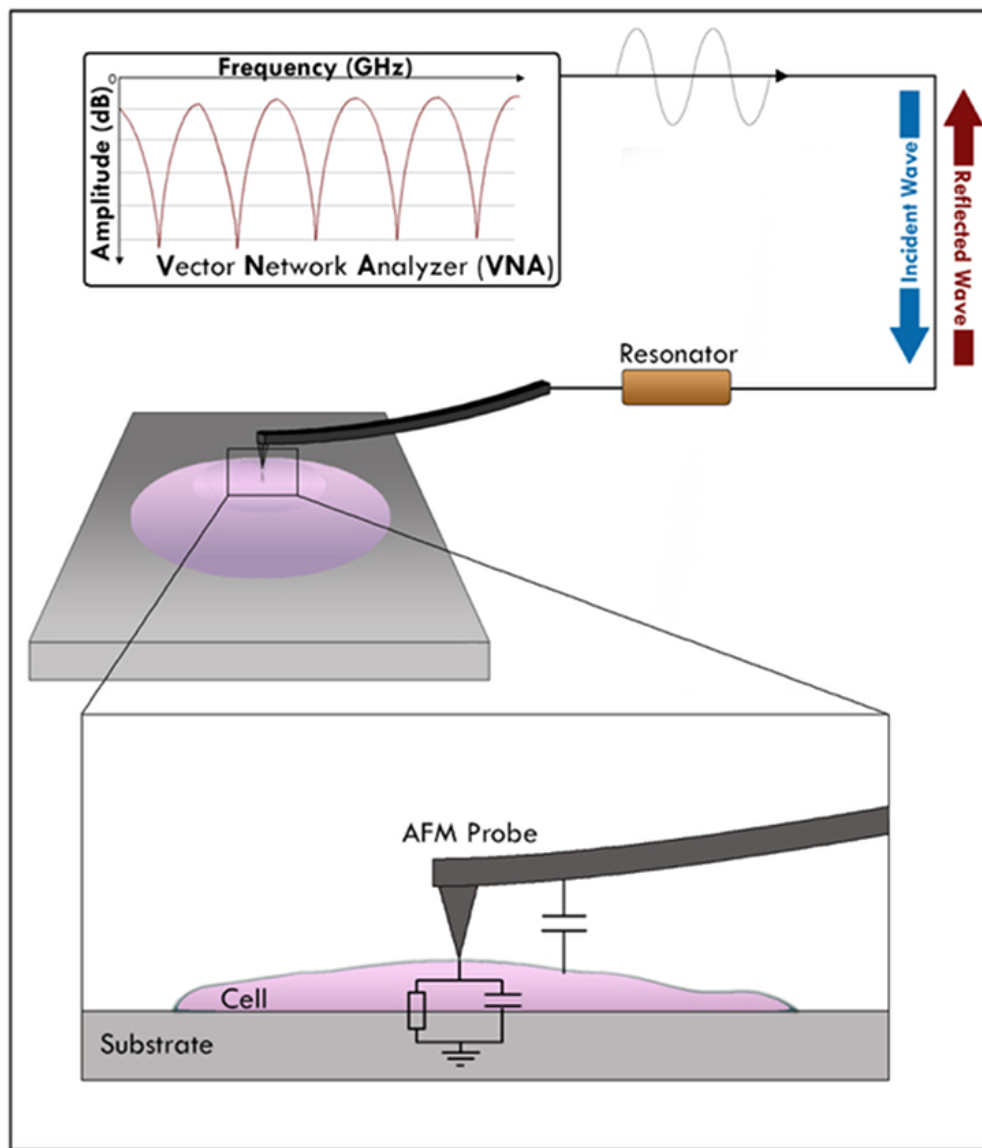
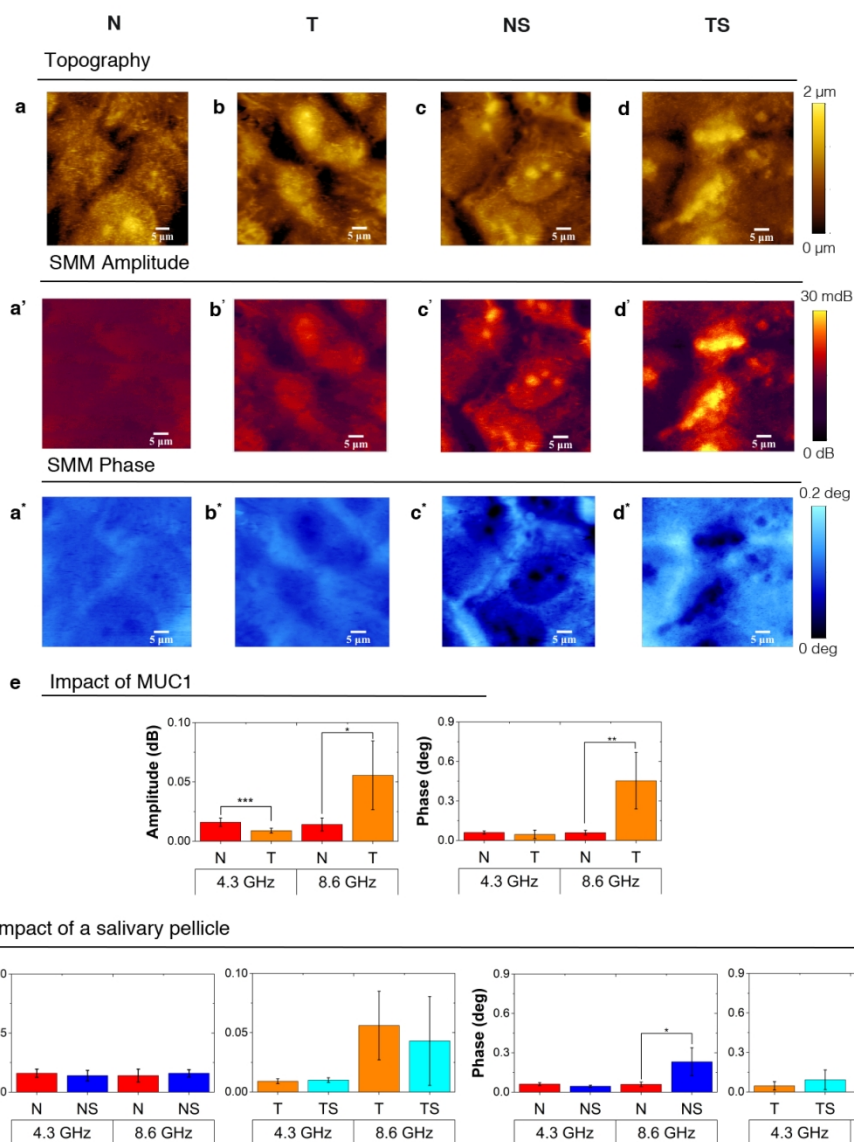


Figure 3. Schematic illustration of cell analysis by scanning microwave microscopy.

61x71mm (300 x 300 DPI)



43 Figure 4. Scanning microwave microscopy analysis of human epithelial buccal cells. Topography (a-d), SMM
44 amplitude (a'-d') and SMM phase (a''-d'') images for TR146 native cells (N), TR146/MUC1 transfected cells
45 (T), native cells with a salivary pellicle (NS) and transfected cells with a salivary pellicle (TS). SMM
46 amplitude and phase images were measured at a resonance frequency of 8.6 GHz. All images are at 50 x 50
47 μm^2 scanning size.

48 e, f: SMM amplitude and phase values (mean \pm STD) recorded at 4.3 and 8.6 GHz for native (red) and
49 transfected (orange) cells, native cells with a salivary pellicle (dark blue) and transfected cells with a
50 salivary pellicle (light blue). ANOVA tests were performed to evaluate the statistical impact of the presence
51 of MUC1 protein (e) or a salivary pellicle (f) on amplitude and phase values (* $p < 0.05$, ** $p < 0.005$ and
52 *** $p < 0.001$).

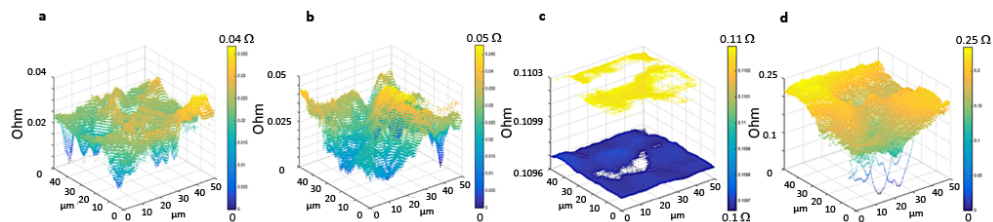


Figure 5. Impedance images of human epithelial buccal cells. Impedance images were constructed for one representative image per condition from amplitude and phase values obtained at 8.6GHz. a: TR146 native cells (N), b: TR146/MUC1 transfected cells (T), c: native cells with a salivary pellicle (NS) and d: transfected cells with a salivary pellicle (TS).