Surface Properties of the Skin of the Pilot Whale *Globicephala melas*

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On the skin surface of delphinids small biofoulers are challenged to high shear water flow and liquid–vapor interfaces of air-bubbles during jumping. This state of self-cleaning is supported by the even, nano-rough gel-coated epidermal surface of the skin. The present study focussed on the intercellular evolution of gel formation and the chemical composition of the gel smoothing the skin surface of the pilot whale, *Globicephala melas*, using X-ray photoelectron spectroscopy (XPS) in combination with cryo-scanning electron microscopy (CSM), and transmission electron microscopy (TEM). In the superficial layer of the epidermis, the stratum corneum, intercellular material was shown by electron optical methods to assemble from smaller into larger covalently cross-linked aggregates during the transit of the corneocytes towards the skin surface. XPS measurements showed that the surface of the skin and the intercellular gel included approximately the same amounts of polar groups (especially, free amines and amides) and non-polar groups, corresponding to the presence of lipid droplets dispersed within the jelly material. It was concluded from the results that the gel-coat of the skin surface is a chemically heterogeneous skin product. The advantages of chemically heterogeneous patches contributing to the ablation of traces of the biofouling process are discussed.

Keywords: *Globicephala melas*; pilot whales; dolphin; self-cleaning; photoelectron spectroscopy; skin

INTRODUCTION

With the aim of lessening the environmental impact of antifouling paint compositions by biomimetic principles, the properties of delphinid skin, for example, the topological basis of contact reduction contributing to the self-cleaning abilities of the skin of the pilot whale, *Globicephala melas*, have been investigated (Baum *et al.*, 2001a; 2002a). The skin of delphinids displays a variety of adaptations to the aquatic environment. As in all cetaceans, the skin lacks glands and it was therefore assumed that the protective power of the epidermis was exclusively based on the biochemical adaptation of the keratinocytes which transit from the basal layer to the layer of the stratum spinosum and the superficial stratum corneum. In pilot whales, biochemical enzyme-based, topological and rheological properties of the epidermal surface are important co-factors of the high antifouling performance. These factors especially support the desorbative potential of air-bubbles and high shear flow during jumping by the dolphin (Baum *et al.*, 2001b; 2002a; 2002b). Dolphins benefit from the even gel-coated skin surface which exhibits low numbers of contact points and micro-niches where biofoulers could adhere or hide (Baum *et al.*, 2002a). The even gel on the skin surface displays the properties of a viscoelastic solid, which withstands the high shear regimes during jumping (Baum *et al.*, 2002b). The retention of hydrolytic enzymes such as peptidases and glycosidases within the gel broadens the self-cleaning abilities of the dolphin integument to some extent (Baum *et al.*, 2001b), and it was suggested that the degradation of adhesive biopolymers from the biofouling process

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particularly increases the fluidity of such adhesives and, thus, decreases their retention on the skin surface during jumping. Although the rate-dependent desquamation *vs* rate-dependent adhesion of contaminations is an unknown dynamic feature of the attachment-release process, it is assumed, that the continuous desquamation process in general, initiated by hydrolytic enzymes, subsequently contributes to the removal of micro-foulers, if smaller than the size of the desquamating cells (approximately $50 \times 80\,\mu m$) and the cell clusters ($100 \times 300\,\mu m$). In the common dolphin, *Tursiops truncatus*, the rate of desquamation can reach values of 12 cell layers a day (Brown *et al.*, 1983). With respect to the duration of the gel formation within the epidermis of *G. melas* (Baum *et al.*, 2002b) the rate of desquamation cannot exceed 2 layers a day.

In the present study new insights are provided into the chemical composition of the skin surface and the topological and chemical evolution of the intercellular gel using X-ray photoelectron spectroscopy (XPS) in combination with electron optical methods. The topological and chemical characteristics with respect to the properties previously described (Baum *et al.*, 2001b; 2002a; 2002b) are discussed with the focus on the chemical composition of the epidermal surface of the pilot whale. It is known that the chemical composition of a surface controls the dynamics of adsorption of biofoulers and biofilm precursors at solid–liquid interfaces as well as the (de-) wetting dynamics at solid–vapor interfaces of air-bubbles (Suarez-Gomez *et al.*, 1999; Suarez-Gomez, 2001) and the ablation of biofilm precursors (Pasmore *et al.*, 2002).

MATERIALS AND METHODS

Material

Small samples of the epidermis of the pilot whale, *G. melas* (approximately 0.25 cm^2), were immediately taken under seawater from animals beached in three authorised hunts by Faroese people in 1999. During these hunts the dolphins are forced to swim very fast towards a beach, producing air bubbles and waves of high shear water flow. The samples were deep-frozen and stored in liquid nitrogen (−196°C), or fixed in 2% glutaraldehyde in 0.05 mol l^{-1} Sörensen phosphate-buffer (pH 7.4). In addition, abraded flackey cell clusters of the stratum corneum were centrifuged mechanically in order to collect the intercellular fluid, which transformed from sol to gel within 2 h at room temparature (20°C) (for details, see Baum *et al.*, 2001b; 2002b). After the gel formation, samples were kept frozen in liquid nitrogen until used for XPS.

Transmission Electron Microscopy (TEM)

Aldehyde-fixed samples were subsequently dehydrated with ethanol and acetone, and then embedded in Unicryl. Ultrathin sections were stained with lead citrate and uranyl acetate. The elastic biogel of the liquid-centrifuged stratum corneum formed was fixed in 2% glutaraldehyde in 0.05 mol l^{-1}(pH 7) PIPES buffer (piperazine-N,N'-bis[2-ethanesulfonic acid]) for 45 min, and postfixed in 2% osmic acid. Samples were then embedded in EPON 815, and ultrathin sections were stained with lead citrate (see Baum *et al.*, 2002b). Sections were viewed in a Zeiss TEM CEM 902 at 80 kV.

Cryo-scanning Electron Microscopy (CSM)

The quench-frozen skin samples were glued with Tissue Tek (Miles) onto a pre-cooled sample holder (−196°C).

A batch of samples was cryo-sectioned at −139°C from the deeper to uppermost skin layers using a BalTec MCS 010 microtome. The ice crystals were removed from the sample surfaces by mild sublimation under an argon atmosphere within the microtome chamber (for 5 min, −139°C, 1.7×10^{-3} Pa). Subsequently, the surfaces of the samples were sputter-coated with gold (40 s). The samples were sputtered twice and in opposite directions (40 mA, 4–5 Pa, argon atmosphere) without leaving the vacuum system of the microtome. The coated samples were transferred *via* cryo-shuttle to a LEO 1430 VP cryo-scanning microscope (LEO Electron Microscopy Ltd), and viewed under vacuum conditions (5×10^{-5} Pa) with an accelerating voltage of 10 kV.

X-ray Photoelectron Spectroscopy (XPS)

Analyses of the chemical composition of the uppermost 5–8 nm of the skin surfaces and the intercellular jelly material were performed in an AXIS ULTRA photoelectron spectrometer (Kratos Analytical, UK). During the whole measuring procedure all samples were kept at a temperature of approximately −120°C by cooling with liquid nitrogen. Hence, the pressure in the analysis chamber was always $< 10^{-9}$ mbar. The spectometer was equipped with a monochromatized Al Ka X-ray source of 300 W at 15 kV, a hemispherical analyser with a pass-energy of 160 eV for survey spectra and 20 eV for high-resolution spectra. To compensate for the influence of excess charges, the C 1s peak of saturated hydrocarbons was used as a reference peak to adjust the binding energy axis of the spectrum. It was set on a binding energy of BE = 285.00 eV. Quantitative elemental compositions were determined from peak areas in relation to

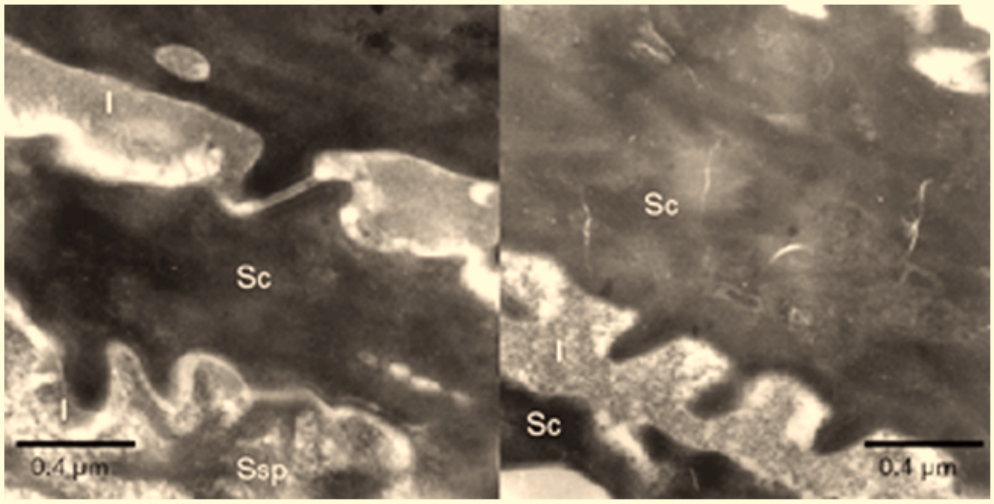

FIGURE 1 Epidermis of *G. melas* (TEM). Variability in the consistency of the intercellular space (l) between the stratum spinosum (Ssp) and the stratum corneum (Sc). a = homogeneous deposits of the aldehyde-fixed intercellular fluid; b = the uppermost intercellular space of the stratum corneum containing agglomerates of the intercellular fluid.

the sensitivity factors and the spectrometer transmission function.

RESULTS

TEM and CSM

Electron optical methods showed that the consistency of the intercellular fluid changed within the stratum corneum. In TEM, the intercellular substance of the lower layers of the stratum corneum (Figure 1a, the transition zone between the stratum spinosum and the stratum corneum) was less structured and aggregated than that of the uppermost five layers of the stratum corneum (Figure 1b).

CSM was combined with freeze drying under electron optical control (compare Baum *et al.*, 2000; 2002a). Changes in the morphology of the intercellular compartments of the deeper layer of the stratum corneum were induced (Figure 2, unfilled intercellular spaces, open arrows), when the water of the intercellular material sublimed along the gradient of water content during the freeze-drying process. In contrast, the uppermost 5 to 6 layers of the stratum corneum remained filled (Figure 2, closed arrows) under the same experimental conditions. The intercellular material from within the stratum corneum displayed the characteristics of the "seawater-side" intercellular space at the skin surface.

As known from previous reports (Baum *et al.*, 2000; 2002a; 2002b), the skin surface is covered by a gel-like medium filling the pores of the desmosomal junction and also by lipidic areas, whose morphology was always unaffected by the freeze-drying process (Figure 3a). Alternating patches of (hydrophobic)

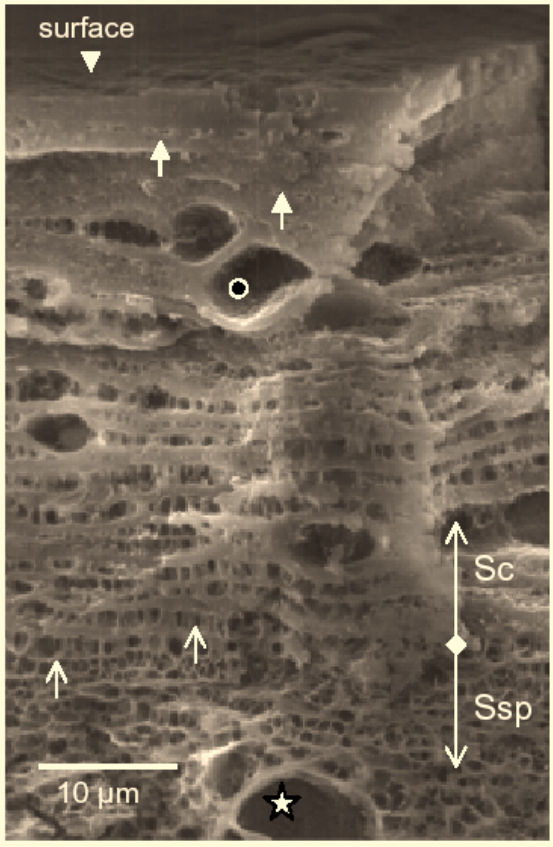

FIGURE 2 CSM micrograph of the stratum spinosum (Ssp) adjacent to the stratum corneum (Sc) of the epidermis of *G. melas*. In the deeper layers of the stratum corneum freeze-drying extracted water from the intercellular space (open arrows). The intercellular space of the upper 5 to 6 layers and the "water-side" intercellular space at the skin surface retained water-filling (closed arrows), indicating the presence of a covalently cross-linked matrix. * = empty lumen of the nucleus of a living cell of the stratum spinosum. Sectioning the skin samples induced fractures of the interdigitating squames (white circle), which were then cryo-preserved.

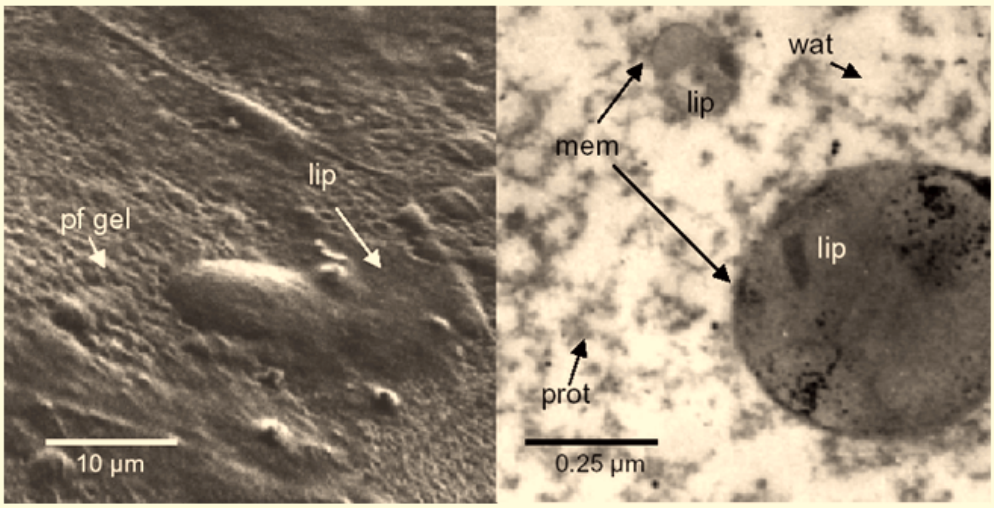

FIGURE 3 a = CSM micrograph of the skin surface of *G. melas*, displaying the alternating patches of the pore-filling gel (pf gel) and the lipids (lip) spread over the surface; b = TEM showing lipid droplets (lip) ranging from 0.1 to 0.5 µm in diameter were recovered in the centrifuged intercellular fluid transformed from sol to gel within 2 h; the lipid droplets, considered to be the primary resource of the skin-covering lipids, were coated by membranes (mem) embedded in a proteinaceous network (prot) and a watery phase (wat).

lipids and (hydrophilic) gel-like materials covered the skin surface.

After treating the gel with osmic acid and aldehydes, proteinaceous gel components and membrane-coated lipid droplets were visible (Figure 3b). The shape and the size of the droplets have been described in detail by Menon *et al.* (1986); Pfeiffer and Jones (1993), and Baum *et al.* (2000; 2002b). These lipids droplets were embedded within the proteinaceous gel-like components. The cross-linked proteinaceous components of the centrifuged biogel displayed polymorphic clustering comparable to the structured material in the uppermost intercellular space of the stratum corneum (compare Figures 1b and 3b).

XPS

XPS measurements carried out on deep-frozen fresh epidermis samples (Figure 4) and on the gel-like intercellular substances (not shown) showed the presence of polar (dark pie sections in Figure 4) and non-polar (white pie section in Figure 4) derivatives of carbon. The amounts and the species of these were comparable in both surfaces. The hydrocarbons amounted to more than 70% of all derivatives of carbon, which included conjugated unsaturated carbon C=C and sulphur–carbon C–S. The concentration of both derivatives ranged in the background level of the peak of hydrocarbons. Free amino-groups and carboxyl-groups were detected in both samples. In contrast to the skin surface, the surface of the intercellular material was more enriched in amines (11%) and esters (7%), and contained fewer amides (3%), alcohols/aldehydes/ketones (6%) and hydrocarbons (70%) compared to the values for the skin surface (Figure 4).

DISCUSSION

TEM and CEM

In line with the predictions of the macromolecular coating concept of Geraci *et al.* (1986), evidence was found for the biogel-forming process starting with the aggregation of a homogenous matrix during the transit of the corneocytes towards the skin surface. This variability is an intrinsic property of

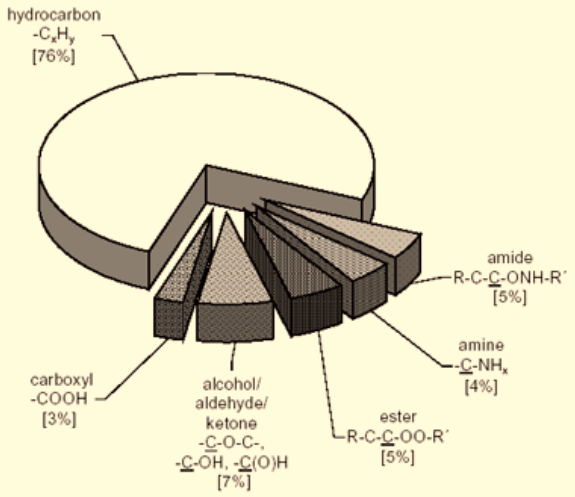

FIGURE 4 Distribution of polar (dark sections) and non-polar (white section) derivatives of carbon on the skin surface of *G. melas*, using XPS on deep-frozen skin samples.

the structuring process of the gel-like material (*cf.* Baum *et al.*, 2002b). Comparable to the chemical differentiation of the intercellular material using fixatives, the loss of water during freeze-drying applied under cryo-electron optical control induced the difference in the above-mentioned layers of the stratum corneum. The deeper intercellular spaces of the non-fixed cryo-preserved samples of the stratum corneum were demonstrated to release water during the freeze-drying process, leaving nearly empty intercellular spaces which indicates the presence of non-cross-linked material. At the same degree of dehydration, the uppermost five to six intercellular spaces and the intercellular space at the skin surface remained filled, indicating that the material was more resistant to freeze-drying than the non-cross-linked material from the deeper layers.

In conclusion, both the electron optical results showed that chemical cross-linked intercellular material assembles from smaller homogenous to larger polymorphic, proteinaceous aggregates (see Figure 3b) during the transit of corneocytes towards the epidermis surface. This increase in the size of the aggregates was also anticipated from the results of multi-angle laser-light scattering and gel kinetic experiments (Baum *et al.*, 2001b; 2002b). The uppermost intercellular spaces demonstrated here exhibited the same morphological characteristics as the intercellular space of the skin surface. The results indicate that the characteristics of the skin surface derive from the intercellular precursor material. This conclusion is supported by the results of the XPS measurements (see below) which show that the surface chemistry of the centrifuged biogel and the epidermis surface were nearly identical.

XPS

XPS measurements carried out on deep-frozen fresh skin samples revealed the presence of polar and non-polar derivatives of carbon. In this connection it seems reasonable to assume that the polar groups detected (for example, free amino-groups and amides) lead to the major chemical characteristics of the viscoelastic gel covering the skin surface, whereas the ester groups and the majority of non-polar derivatives of carbon potentially correspond to the presence of small lipid droplets and lipidic areas. It is concluded from the nearly identical chemical composition of the skin surface and the surface of the gel-like intercellular fluid, that the intercellular fluid defines the chemical composition of the skin surface of the pilot whale.

Since it is evident from the results, that the skin surface of the pilot whale exhibits patchily distributed chemical heterogeneity which would be expected to contribute to opposite wettabilities, further investigation related to the wettability of the skin of the pilot whale are needed. It has been reported by Gucinski *et al.* (1984) and Gucinski (1986), that the critical surface tension of the skin of delphinids varies between 20–30 mN m^{-1}. These values were calculated from the contact angles of non-polar fluid droplets of known surface tension placed on the skin surface of dolphins in air. However, such low energy surfaces de-wet in seawater, a phenomenon which is uncommon in jumping dolphins. It is hypothesised that like in other hydrated gels (Sedev *et al.*, 1996) the major proteinaceous components of the gel of the skin surface including the above-mentioned amines and amides lead to full hydration of the gel under natural conditions. This high degree of hydration probably causes a high wettability of the skin, a hypothesis based on preliminary wetting experiments. Employing dynamic drop shape analysis (DSA) (unpublished), the *in vitro* gel displayed advancing and receding contact angles of approximately 20.0° and low values of hysteresis (approximately 1°) using seawater to wet the surface in air. It was found that the measurements of wettability were sensitive to drying, converting hydrophilic precursor gel into hydrophobic gels, and to dilution of lipids which dissolve in hydrophobic liquids used for wetting (*cf.* Sedev *et al.*, 1996).

XPS was employed in order to detect traces of the biofouling process on the skin surface. Biofilms and adhesion molecules of biofoulers are known to contain hetero-polysaccharides or sulphated organic molecules (*e.g.* Cooksey & Wigglesworth-Cooksey 1995; Beveridge et al. 1997). It was expected that their presence would change the chemistry of the skin surface, since theses layers are thicker than 10 nm and the depth of information is approximately 8 nm in XPS measurements. The results obtained show that the skin surface exhibited no other carbon derivatives than those recovered in the intercellular space. The carbon derivatives were in the same range. It is concluded from both results that no biofilm layers were present on the samples (collected under seawater from fresh skin) indicating that the skin of the pilot whale has the potential to display a high defouling performance (see Materials). Since the skin surface areas investigated were neither trace-loaded with biofilm or contaminated with biofoulers, it is suggested that the self-cleaning mechanisms are directed and adapted against the early stages of the biofouling process, *i.e.* the establishment of the conditioning layer.

In the present study the focus was on the chemical composition of the skin surface of the pilot whale in relation to the control of the dynamics of adsorption of biofoulers and biofilm precursors at solid–liquid interfaces, as well as the (de-) wetting dynamics at solid–vapor interfaces of air-bubbles (Suarez-Gomez *et al.*, 1999; Suarez-Gomez, 2001). Generally

independent from the wettability, air-bubbles in contact with smooth surfaces can remove up to 50% of any kind of particle per contact cycle depending on the contact time, the size of the bubble and its velocity (Suarez-Gomez, 2001).

In addition, the chemical composition of a surface determines the period of ablation of epibiontic organisms. It seems that the initial steps in the adsorption of a conditioning layer and the attachment of organisms is less prominent on polar surfaces (*e.g.* Pasmore *et al.*, 2002). In contrast, the retention of an established biofilm is low on non-polar surfaces challenged to shear or air-bubbles (*e.g.* Bos *et al.*, 2000). Although their are contrary findings on the relationship between chemical composition and period of ablation, it is assumed that the chemical heterogeneity of the skin surface of the pilot whale, combined with the jumping behaviour, may have co-evolved for the prevention of both the short-term and the long-term settlement of biofoulers.

By analogy with the skin properties of the dolphin and its jumping behaviour, it therefore seems reasonable to combine both components in tandem to prevent all phases of the biofouling process (the establishment of a conditioning layer, the formation of a biofilm and the settlement of epibiontic larvae). This would provide eco-friendly prevention of biofouling on harbouring ships, replacing toxic panels. Further experiments are planned to evaluate the cleaning effect of air-bubbles on cleaning ships' hulls which are at risk of being overgrown by biofoulers when stationary.

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