The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them.

Sir William Bragg (1862 - 1942)

Physicist

Chapter 7

CHANGES TO EPICUTICULAR WAX OF MANGO (*Mangifera indica* L.) DUE TO HANDLING ON A COMMERCIAL PACKLINE

7.1 ABSTRACT

All plant surfaces are covered by an epicuticular membrane that plays an important role in plant defence mechanisms. This membrane consists of the epicuticular and cuticular wax, cutin and auxiliary, superficial structures such as trichomes and stomata. Each component of the epicuticular membrane of mango fruit contributes towards protection of the developing fruit. All packline practices and physical handling during the packing process will affect these structures. A problem that is often encountered and that decreases the quality of export fruit is the discolouration of lenticels associated with mango fruit rind. This is a study into the extent of changes imposed on the epicuticular wax during normal packline operations, including an investigation into the contribution of these changes to lenticel discolouration. Fruit samples from the cultivar 'Keitt' was collected from progressive points along a packline and studied using scanning electron microscopy. It was found that packline practices inflict substantial physical changes on the epicuticular structures of the fruit surface. No correlation between lenticel discolouration and epicuticular changes could be demonstrated.

7.2 INTRODUCTION

Increasing demand for mango (*Mangifera indica* L.) fruit has lead to worldwide attempts at improving the selection of commercially popular cultivars (Knight, 1997; Yadav, 1997). The fruit, a fleshy drupe, develops on sparsely bearing thyrses, and has variable metamorphic and sensorial characteristics. Consumers get their first impressions from visual aspects like colour, shape, size and aesthetics, with taste and smell only explored if the appearance is satisfying. Judgement of quality therefore relies largely on the external condition of the fruit, with external appearance correlated to physiological condition.

Display of sensorial characteristics depend primarily on combinations of genetic traits manipulated through cultivar selection (Judd *et al*., 2002, Human & Rheeder, 2004), but are also influenced by meteorological and geographic features of the cultivating area (Mercadante & Rodriguez-Amaya, 1998; Kruger *et al*., 2003). Apart from cultivar selection, other horticultural aspects under constant revision include preharvest pest control, picking and handling practices, and postharvest treatments (Macnish *et al*., 1997; Jacobi, *et al*., 2001, Reddy *et al*., 2003). All these different factors contribute towards difficulties experienced during production and selection of export quality fruit.

Mango fruit is climacteric and harvesting is done when fruit is considered to have reached physiological (or horticultural) ripeness. The wide-ranging variability of cultivar characteristics results in subjective determination of when picking should commence (Abbott, 1999). Parameters that are monitored include shape (the development of a fully rounded shoulder), internal and external colour development, and firmness (Knight, 1997; Kruger *et al*., 2003). Internal colour intensity is judged by means of an in-house colour chart developed by the South African Mango Growers Association (SAMGA, version 1997). Considerable quantities of fruit are either rejected for export, or downgraded, due to problems based on visual assessment of the produce.

Fruit is still very firm when it is picked, creating an erroneous perception of robustness. Picking practices are therefore quite forceful, aimed towards speed and bulk handling rather than physiological considerations. By the time fruit are packed and ready to be sold, it has been subjected to an array of physical practices. All of these have an impact on the outer layers of the fruit, notably the highly hydrophobic epicuticular wax layer that is the interface between the fruit and the environment (Barthlott & Neinhuis, 1997). This layer plays a decisive role in the attachment and colonisation potential of micro-organisms, providing innate fungal resistance (Comménil *et al.*, 1997; Mariani *et al.*, 2000). The fruit achieves this resistance by presenting a physical and chemical barrier that consists of wax crystalloids on the outside of the fruit. The morphology and composition of fruit wax is dependent on the species involved and often appear as complex, pliable structures (Kolattukudy, 1996).

Physical interference with the epicuticular wax layer may bring about permanent alteration of the crystalloid structures, but the plant can often rectify mechanical removal of the epicuticular wax by deposits on or over the altered area (Jackson & Danehower, 1996). Picking, handling and packline procedures cause physical and chemical changes to the epicuticular structures of fruit. The most noticeable changes are brought about by the

application of commercial wax coatings. The effect of these changes on the cuticular layers below the epicuticular wax and the postharvest quality of mango fruit is not known. One such effect is well demonstrated by a serious postharvest problem known as 'lenticel damage' (Tamjinda *et al*., 1992).

Annually causing a significant loss of return on investment in export fruit, this condition is characterised by a discolouration of the superficial area surrounding the lenticel. Lenticel discolouration does not manifest with the same severity in all commercial cultivars. 'Tommy Atkins' (early maturing) and 'Keitt' (late maturing) are both susceptible cultivars, with 'Tommy Atkins' exhibiting less severe symptoms than 'Keitt' (Willis & Duvenhage, 2002). Symptom expression can be attributed to the differences in fruit colouration, since 'Tommy Atkins' is a red cultivar that masks the discolouration more effectively. However, while 'Kent' and 'Keitt' are both more yellow cultivars, 'Kent' is not prone to the development of lenticel discolouration. Currently, there is no consensus about the environmental or horticultural initiators or triggers for the development of the lesions (Tamjinda, *et al*., 1992; Pesis *et al*., 2000; Willis & Duvenhage, 2002). The unpredictability of this condition results in consignments dispatched to export markets sometimes being downgraded by inspectors at the destination port due to late blemish development. It is also impractical and risky to make changes to commercial scale crop management systems based on experimental simulations of conditions that seem to induce the discolouration of lenticels (Willis & Duvenhage, 2002; Fallik, 2004).

The purpose of this study was to investigate the nature of physical changes of the epicuticular wax brought about by practices during the postharvest period, and to determine how these changes affects the lenticel structures.

7.3 MATERIALS AND METHODS

During the mango seasons of 2002, 2003 and 2004 samples were collected fortnightly from Bavaria Fruit Estates (S 24° 22' E 31° 02') in the Hoedspruit area of the Limpopo Province, South Africa. Cultivar 'Keitt' was used for the study and all fruit were regarded as physiologically mature, with three fruit replicates from each predetermined sample point. All samples were collected from the orchards and packhouse of this single location, thereby excluding any influence from geographical and meteorological factors. Sampling points were selected based on the severity of mechanical impact or where a change in the line environment occurred. Eight predetermined points were used for sampling, namely:

- Orchard (control fruit to determine the natural appearance of the surface, no visible interference or alteration)
- Picking bins
- Dumping bath
- Warm water bath
- Prochloraz application
- Immediately after commercial wax application
- After commercial wax drying (air knives)
- Packed cartons

Samples were processed within 18 hours of collection. Surfaces of collected fruit were protected at the time of sampling, whereafter each fruit was transported in confinement, so that no further alteration was possible. This ensured that the true surface condition during each stage of handling was reflected. Wet fruit were packed and allowed to air dry in the boxes used for transport; they were not exposed to windy or sunny conditions to accelerate the drying process.

Fresh material was sectioned and from each fruit, ten 5 x 5 mm squares were cut from the fruit shoulder area, which had been exposed towards the outside of the tree during growth. This side showed better colour development than the shaded side of the fruit. After sectioning, samples were immediately cryofixed in liquid propane at -180 °C, vacuum dried (Custom built, Tshwane University of Technology, Pretoria, South Africa) at -80 °C and 10^{-7} mBar (10^{-5} Pa) for 72 hours, and coated in 0.5 % Ruthenium vapour to obtain proper conductance. Scanning electron microscopy (SEM) (JSM 840, JEOL, Tokyo, Japan) at 5kV and a working distance of 12 mm was used to study the sample material.

7.4 RESULTS AND DISCUSSION

The fruit surface from control sample surfaces presented an undisturbed epicuticular covering (Fig. 1A) that was highly organised into two distinctive layers of wax crystalloids (Fig. 1B & C). The top (outermost) crystalloids were sepaline (Fig. 1D) and arranged in architecturally complex, funnel shaped lattices. The innermost layer adjacent to the cutin consisted of dense and anamorphous wax. Fruit surfaces from the picking bins were subjected to physical disruption and areas where chafing and scratching had taken place,

or where pressure had been applied, can be discerned (Fig. 2A & B, white arrows). From figure 2C and D it could be seen that the more pliable, delicate sepaline layer was affected by mechanical interference, with the more robust, amorphous layer still intact. Fruit entered the packline through a dumping bath, the first sampling point. There was no discernable difference in the nature of the surface disturbance between fruit collected from storage bins and fruit from the dump bath, although the amount of surface disruption had increased (Fig. 3A - D). Despite the fact that the first bath was intended to remove all dust and foreign matter from the fruit surface (Bally *et al*., 1996), loose debris positioned on top of the depressed wax layer was visible on some fruit surfaces, indicating that it had been deposited from the water in the bath (Fig. 3A). Overall, the surface topography became smoother due to the rolling and rubbing of fruit as they travel into and through the bath (Fig. 3B). Flattened crystalloids on the fruit surface trapped debris particles present in the affected area (arrows in Fig. 3C & D). There are several sources from where debris originate, one being the blank newspaper used to line the picking bins. The paper was dumped into the dumping bath along with the fruit and, by disintegrating, eventually contaminated the line, as did dust particles blown into the packhouse and onto the packline.

Exiting the dumping bath, the line passed over sorting tables into a warm water bath. Fruit was kept submerged in water at 47 °C (± 2 °C) for five minutes, so that the temperature of the fruit epicuticular membrane reached the ambient water temperature. Figure 4A show the radical change in the architecture of fruit wax crystalloids of the fruit surface at this point. Scarring by the brushes on the packline became visible (Fig. 4B). The warm water exposure caused the soft sepaline layer to collapse on the harder wax below (Fig. 4C & D). Entering the dumping bath containing a postharvest fungicide, the external fruit temperature was still 45 °C ± 2 °C, but matched the water temperature upon exit from this bath (34 °C ± 3 °C). Most of the fruit surface showed increased mechanical flattening of the epicuticular crystalloids (Fig. 5A - D), exacerbated by the softening of the wax in the warm water bath.

Immediately after the application of a commercial wax emulsion (17 % solids), but before passing through a jet of air, known as air knives, a set of fruit samples was collected. Left to dry naturally, the commercial wax coating had an uneven and lumpy appearance (Fig. 6A & B), which accentuated the importance of the air knives. In transverse sections, areas where the commercial wax emulsion had integrated poorly with the epicuticular wax were identified (Fig. 6C). In areas where some integration did take place, the incomplete process was visible as unevenly distributed densities in the wax layers (Fig. 6D). Another

set of samples were collected after the waxed fruit had passed through the air knives. (Fig. 7A - E). These focussed, forceful air jets levelled the wax film surface (Fig. 7A - C) and contributed to integration of the two wax fractions. The increased rate of integration resulted in a film with more consistent thickness and integrity (Fig. 7D & E). Lenticels from market-ready fruit are mostly flooded by the commercial wax (Fig. 6A & B, Fig. 7A & B), but the wax film has sufficient gas permeability not to have a suffocating effect (Louw, pers. comm., 2004). While not all fruit coated with commercial wax will develop discoloured lenticels, fruit trapped on the wax rollers are always blemished within a short time. Blemishes develop towards the tapered ends of the fruit, which is where excess wax accumulates on such trapped fruit. Although assurance of the gas permeability of the commercial wax was given, it must therefore be argued that when lenticels flooded or covered by a thick layer commercial wax, gaseous exchange will be impeded. This indicates the relevance of careful control over the thickness of the applied wax coating (López *et al*., 1995; Manzano *et al*., 1997) and the uninterrupted flow of fruit on the packline.

Application of commercial wax eventually alters the fruit surface into a smooth, featureless capsule. However, the protective wax film itself was exposed to physical damage, with scratches and cracks visible on fruit from collected packed cartons (Fig. 8A). Such damage could have been inflicted anytime after waxing and drying, since the fruit rolled along the packline, was handled by packers and rubbed against each other as they were placed into boxes. Damage was presented in the form of cracking and tearing of the wax film, and the fruit surface was visible where the combined layer of plant wax/commercial wax was defaced (Fig. 8A & B). Fungal spores trapped in the wax film (Fig. 8C & D) could have originated from a number of sources. These include inefficient prior washing of the fruit surface in the various baths, contamination from the baths or packline, or introduced into the packhouse by wind. The commercial wax emulsions were stored in sealed containers up to the point of application by spray nozzles; therefore this was not a likely source of contamination.

The fruit cuticle from all the samples from the various sampling points seemed unaffected by the physical impacts. No changes in layer thickness, intracuticular wax distribution or cutin integrity were observed in any of the material. In Figure 9A, recrystallised fruit wax was observed on the exterior surface of the commercial wax coating fruit samples from the cartons packed for shipping. These crystals differed morphologically from the natural wax crystalloids (Fig. 9B) with the most likely reason the loss of wax constituents during the migration of these fractions through the integrated wax film. The origin of wax

constituents is still debated but it is accepted that cuticular waxes accumulate in the cutin (Lui & Post-Beittenmiller, 1995; Kolattukudy, 1996). Cuticular waxes play a role in cuticular transport and act as the source of precursors for epicuticular crystalloids (Merk *et al*., 1998; Riederer & Schreiber, 2001). Environmentally induced changes in the morphology and chemical properties of the epicuticular wax layers may, however, influence the functionality of these as well as successive layers (Kolattukudy, 1996; Batzli & Dawson, 1999; Haas *et al*, 2001). This will impact on the physiology of the total fruit and ultimately, its postharvest appearance (López *et al*., 1995).

Fruit sampling from the packline was done at random, and most samples had no lenticel discolouration. Despite the existence of structural variance in terms of morphology, internal lenticel structures did not alter due to the amount of physical impact as it travelled down the packline. This is illustrated by Figure 10A, which shows a lenticel from a sample taken from the dumping bath and Figure 10B, which shows a lenticel from a sample taken from the warm water bath. The external fruit wax in the vicinity of lenticels showed equal scarring to the rest of the surface at every sampling point (Fig. 10C). Using SEM, it was impossible to distinguish between the external morphology of a discoloured lenticel (Fig. 10C) and a non-discoloured lenticel (Fig. 10D).

7.5 CONCLUSION

Epicuticular wax crystalloids undergo morphological changes during the progression of fruit along the packline due to the impact of commercial postharvest practices on these structures. The protective function of the crystalloids during fruit development is indicated by the angular alignment and denseness, as this relates to light scattering abilities (Juniper & Cox, 1973; Grant *et al*., 2003). Epicuticular wax therefore has an impact on the temperature regulation and subsequent water management by the plant during fruit development (Toivonen, 2002).

Mango fruit receives rigorous postharvest treatment on commercial packlines aimed at prolongling shelf life, for postharvest disease control measure and to improve fruit appearance (Srinivasa *et al*., 2004). It was shown that debris scattered around lenticels could enter these epicuticular structures from the packline environment during postharvest handling. Such foreign objects will injure the soft internal mesophyll tissue, triggering plant defence systems (Beckman, 2000). Evidence of commercial wax flowing into lenticels confirmed that blockage by commercial wax was possible. According to López *et al*.

(1995) and Manzano *et al*. (1997) blocked lenticels would experience altered gaseous exchange, which, in turn, would affect the rate of metabolism, and influence physiological reactions in the fruit tissue. Despite the physical impact of temperature changes, mechanical packline procedures and waxing on the epicuticular structures, cutin had an unaffected appearance, confirming the resilience of the biopolymer structure.

Uneven spread of the fungicide applied immediately after the warm water bath indicated the possible removal or chemical change of heat labile fractions in the fruit wax. In a study on the effect of hot water treatments, Fallik (2004) described heat induced biochemical changes in the fruit rind involving polyamines and proteins. However, no information on the chemical interaction (or absence of such reactions) between the natural fruit wax and the chemicals applied on the packline was available. Plant pathogens and phytophagous insects have evolved chemical targeting mechanisms based on the alkanes, lipids, fatty alcohols, sterols and wax esters of the epicuticular layer (Eigenbrode, 1996; Espelie, 1996; Jackson & Danehower, 1996; Comménil *et al*, 1997). Determining the exact nature and composition of the fruit wax constituents will enable more precise tracking of the induced chemical changes in the epicuticular layer. This will give credence to toxicity studies of the mango fruit wax layers towards micro-organisms, contributing to a closer understanding of the function of the different wax layers (Schwab *et al*., 1995; Jackson, & Danehower, 1996; Comménil *et al*., 1997). Identifying biologically active compounds will also advance beneficial characteristics of commercial fruit coatings.

This study has shown that a threat posed by the application of commercial coating wax is the embedding of micro-organisms. Furthermore, damage to the integrity of the integrated wax film may serve as entry points for postharvest organisms (Schwab *et al.*, 1995, Comménil *et al.*, 1997). Mango fruit is climacteric and continuing physiological changes during further ripening will lead to cracking of the commercial wax coating. Maintenance of metabolic stasis through suitable postharvest storage is therefore imperative.

Fruit that are trapped on the packline often exhibit the worst signs of discolouration and physiological stress. The primary role of lenticels is gaseous exchange and its development is synchronised with the development and maturation of the epicuticular layers of the fruit rind. Although it is a harmless and natural phenomenon, development of excessively visible lenticels are indicative of physiological stress (Larson *et al*., 1991; Batzli & Dawson, 1999). Trapped fruit is subjected to prolonged submersion (in a dumping bath) or excessive wax application (wax rollers). The lenticels will eventually become flooded, inhibiting gaseous exchange. Gaseous exchange within the fruit rind and in the

particular vicinity of the lenticel cavity takes place in micro atmospheres. These micro atmospheres are modified by physical additions to the epicuticular layer, creating a point of physiological stress. Affected fruit tissue will perceive this induced stress as injurious, triggering an oxidative burst in localised cells, and resulting in a cascade of reactions to form self-defence phenolics (Grace & Logan, 2000).

The build-up of phenolics, combined with the layers of cutin and epicuticular wax lining the lenticel lumen, ensure that the lenticel area is delimited by chemical as well as physical measures against infection (Beckman, 2000). It has been found that a discernable relationship between lenticel discolouration, lenticel morphology and cultivar exists.

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7.7 FIGURE CAPTIONS

Figure 1 Scanning electron micrograph of mango fruit surface with pristine wax (A). Transversal section of the epicuticular membrane, showing the cutin and wax bilayer (B). Close-up of the wax bilayer, with an amorphous layer separating the cutin and the layer of sepaline crystalloids (C). Micrograph of a typical sepaline crystalloid from the outer wax layer (D).

Figure 2 Fruit from picking bins with marks where chafing and scratching had taken place, or where pressure had been applied can be discerned (A & B, white arrows). The more pliable, delicate top layer was affected by mechanical interference, with the more robust, amorphous layer still intact (C & D).

Figure 3 Fruit from the dump bath, with increased surface disruption (A & B). Debris trapped in the flattened wax is indicated by arrows in C and D.

Figure 4 The change in the architecture of the epicuticular wax crystalloids on surfaces of fruit emerging from the warm water bath (A). A scar produced by the brushes on the packline can be seen in (B). The soft, pliable top layer of epicuticular wax had collapsed on the harder wax below and debris became trapped in the flattened wax (arrows, C & D).

Figure 5 Progressively increased flattening of the epicuticular wax crystalloids from fruit surfaces after Prochloraz application (A - D), exacerbated by softening of the wax in the previous warm water bath. Crystalloids lining the grooves in between epicuticular plates (white arrows, B & C) were less exposed to both mechanical abrasion and the effect of heat than the planar surfaces (black arrows, C & D).

Figure 6 Without forced air drying, commercial wax emulsion had an uneven and lumpy appearance immediately after application (A & B). Transverse section showing poor integration of the commercial wax with epicuticular wax (C). Incomplete polymerisation of the commercial wax (black arrow), with destructured fruit wax visible as a dark layer adjacent to the cutin (white arrow, D).

Figure 7 Lenticels flooded with commercial wax emulsion (A & B). Focussed jets of forced air smooth the commercial wax after application (C). Forced air drying effects close union of commercial and epicuticular wax, resulting in good film formation (D) and more uniform integration (E).

Figure 8 Compromised surfaces from fruit in commercial sales cartons with defaced wax film and the exposed cutin underneath (A & B). Contaminating fungal spores trapped by the wax film (C & D).

Figure 9 Recrystallisation on top of the commercial wax film (A). Reconstituted crystalloids showed architectural differences in comparison to normal crystalloids (B).

Figure 10 Structural variance of lenticels were not due to impact from the various sample points (A & B). No distinguishing morphology between a discoloured lenticel (C) and non-discoloured lenticel (D) was detectable by scanning electron microscopy.

7.8 FIGURES

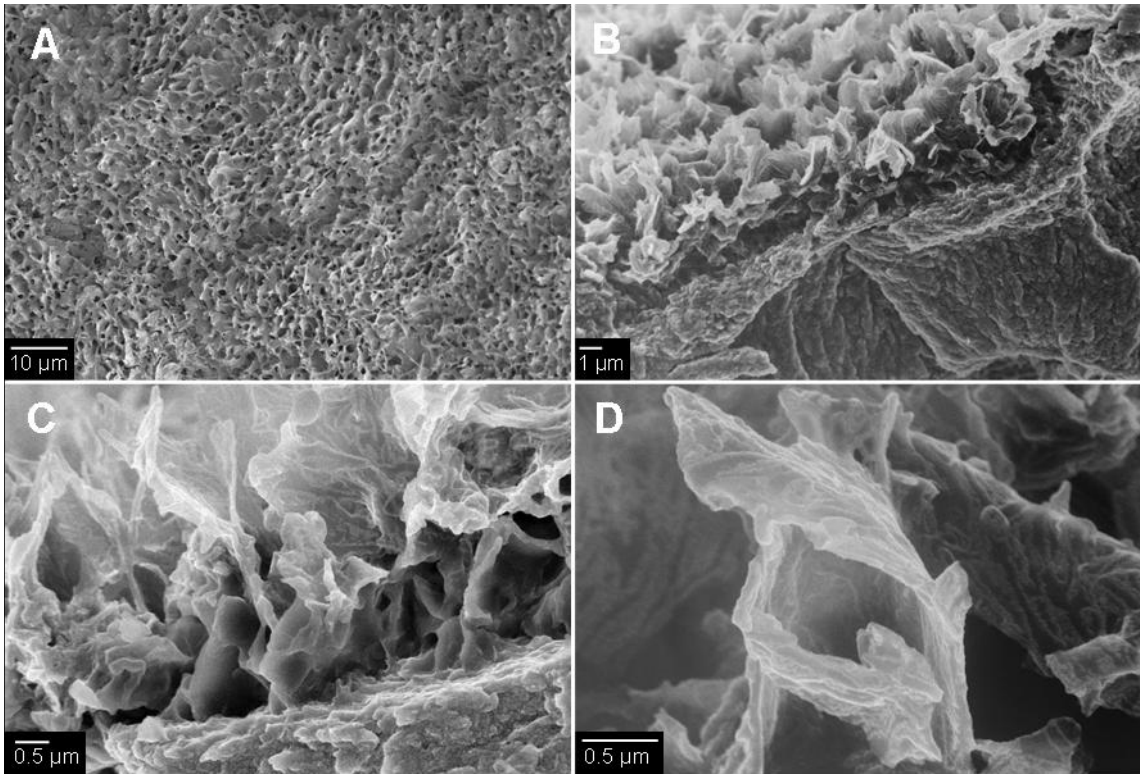

Figure 1

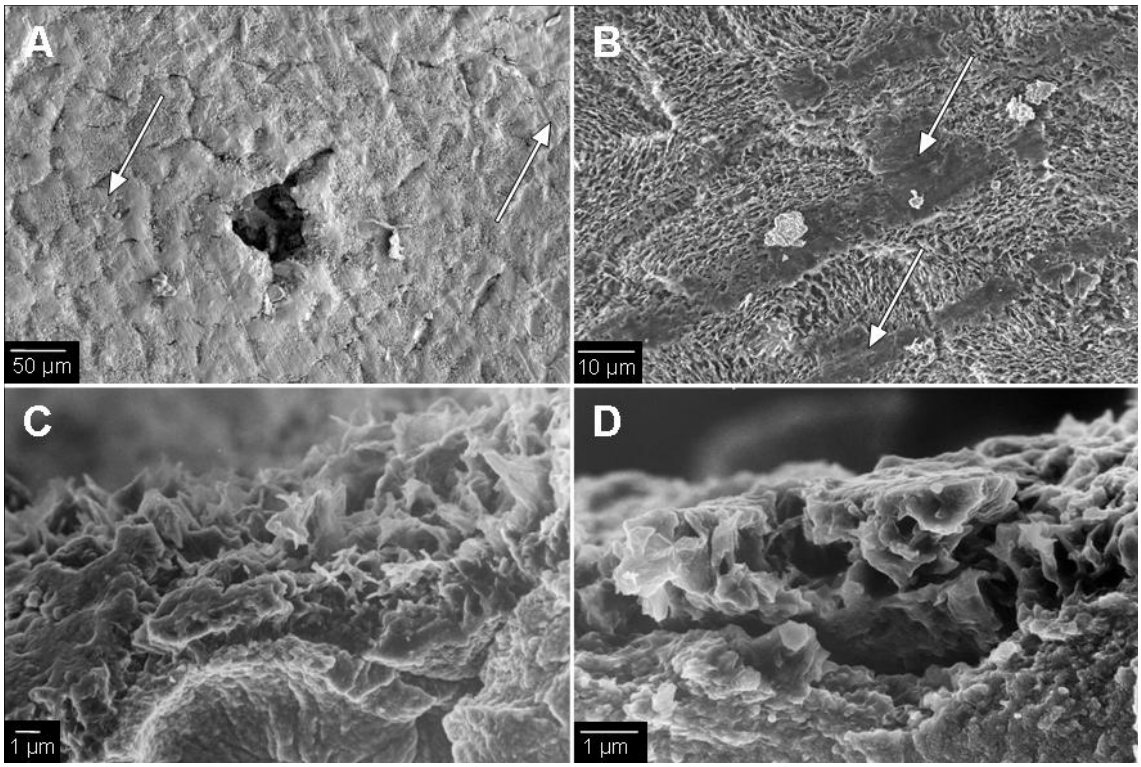

Figure 2

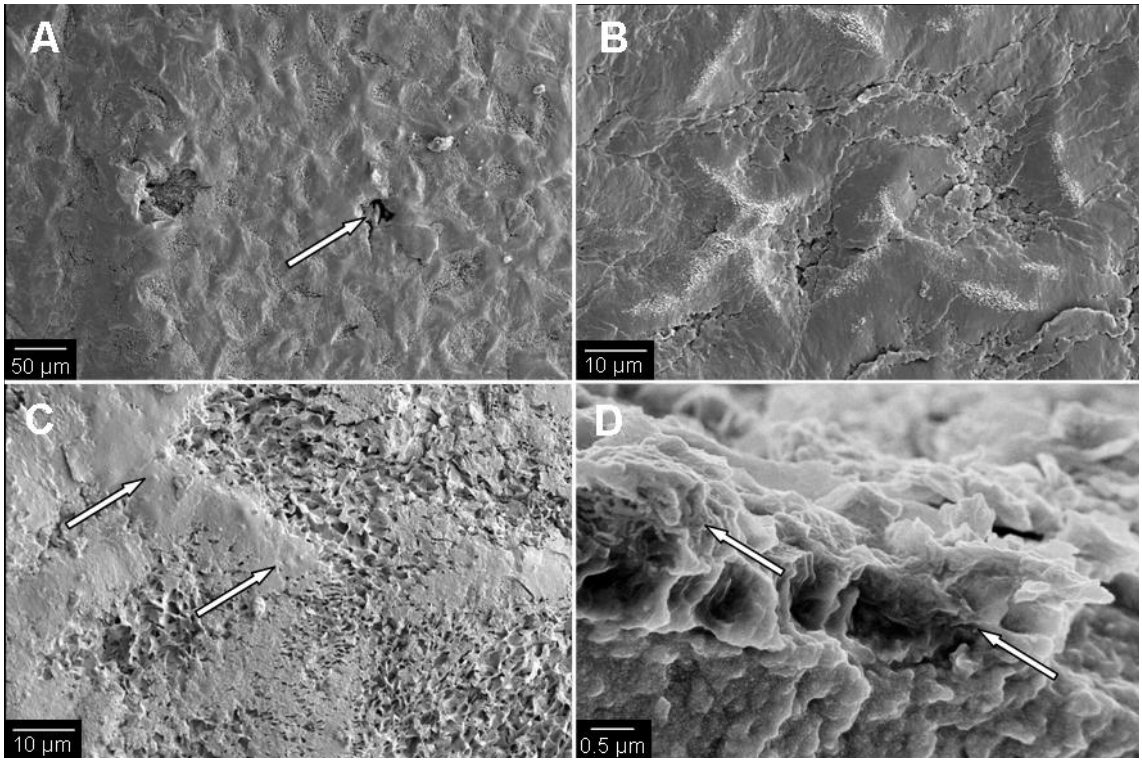

Figure 3

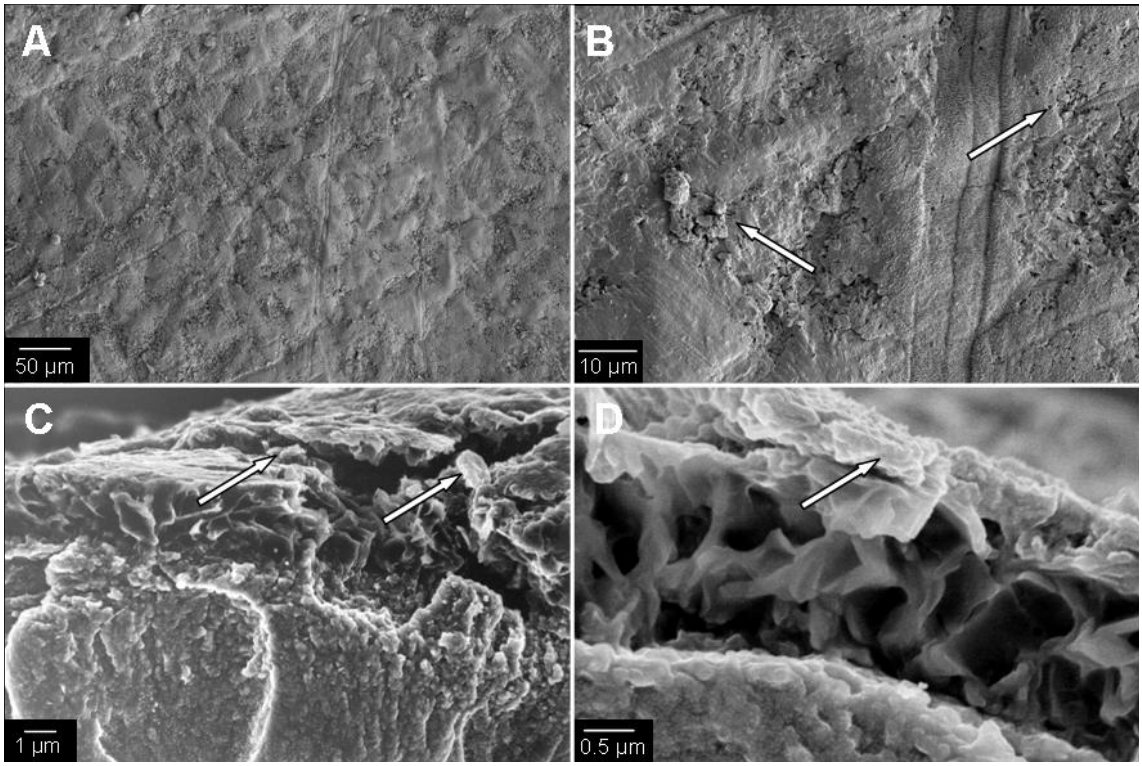

Figure 4

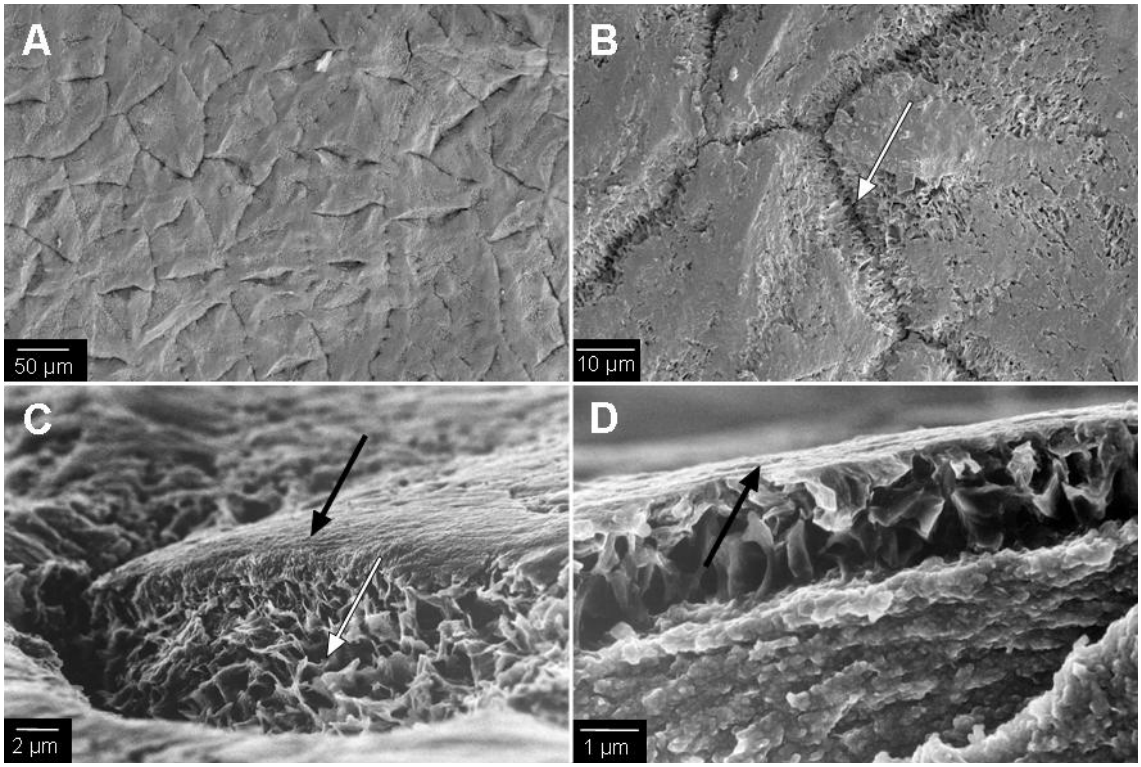

Figure 5

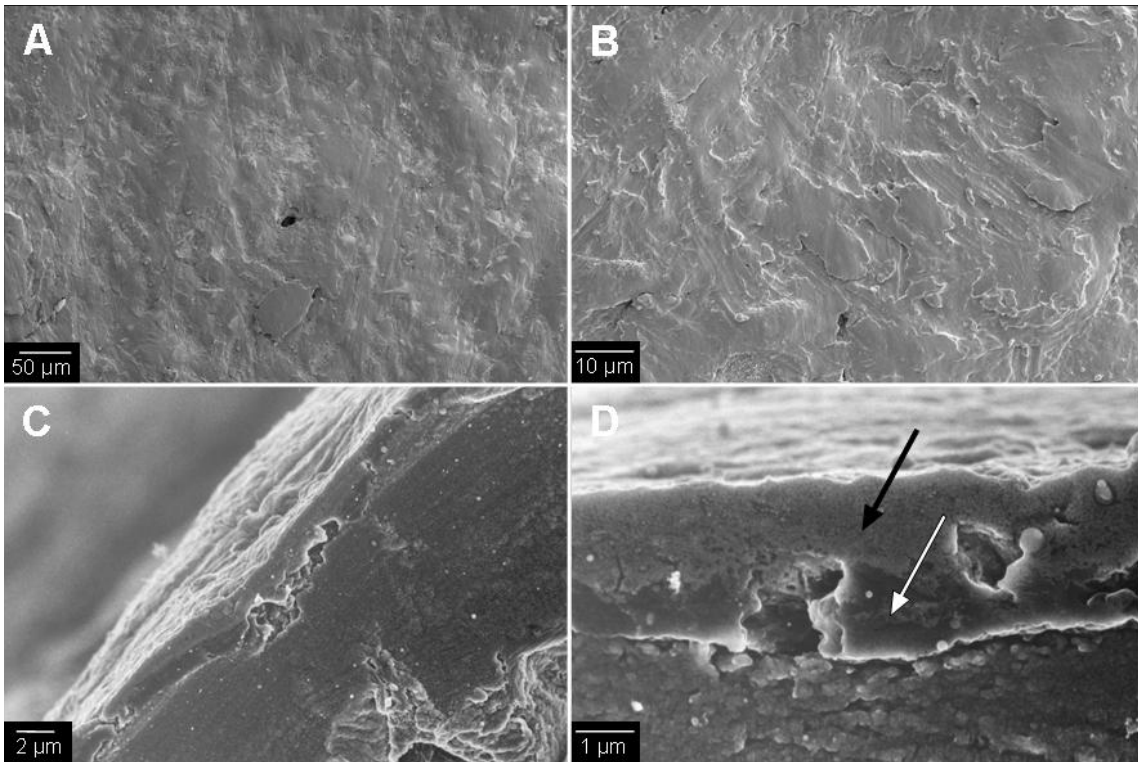

Figure 6

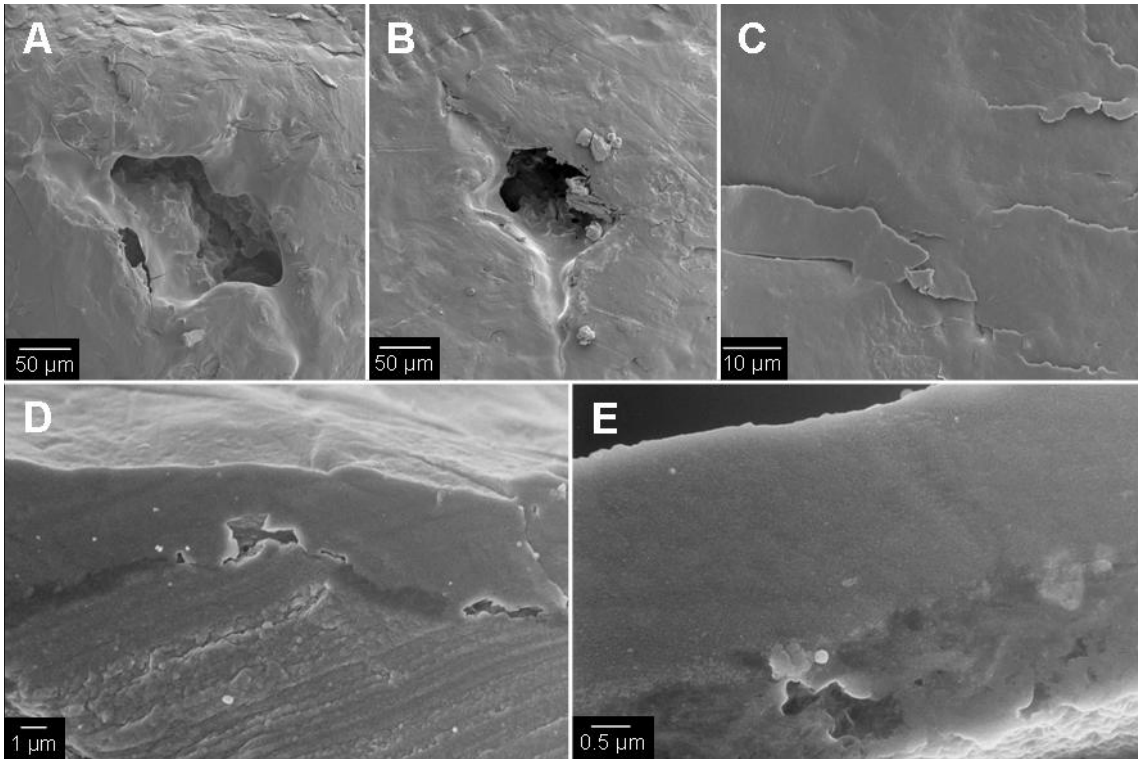

Figure 7

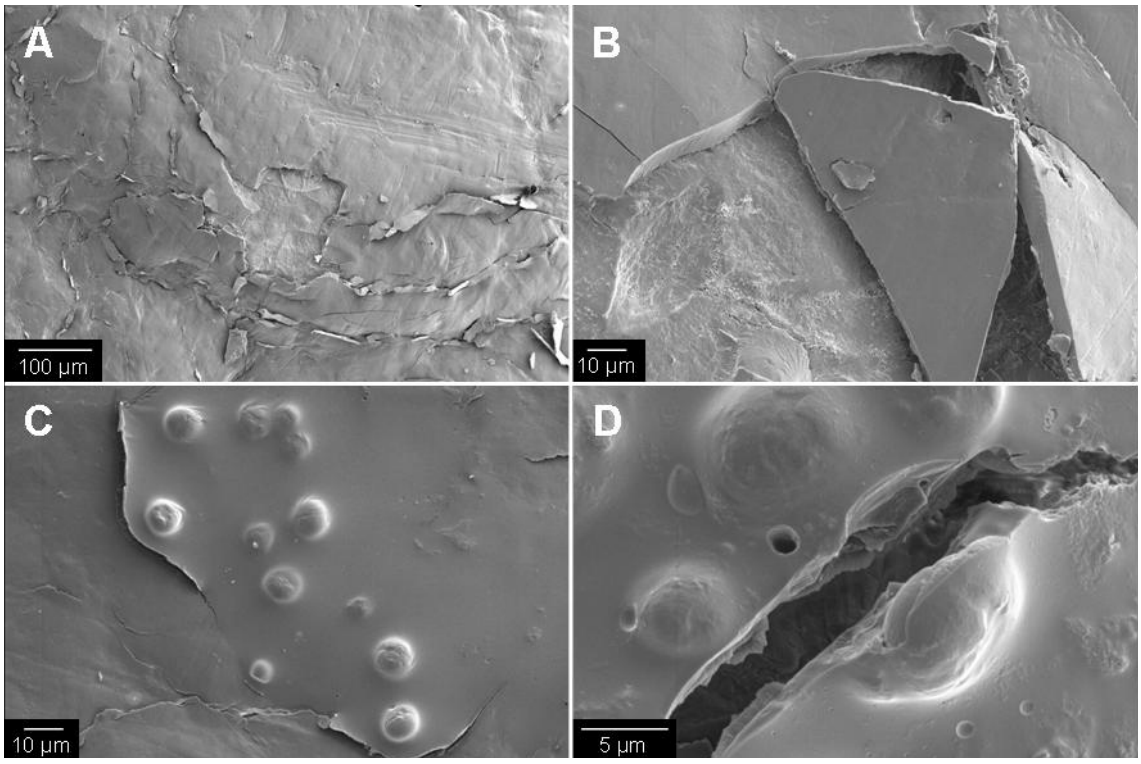

Figure 8

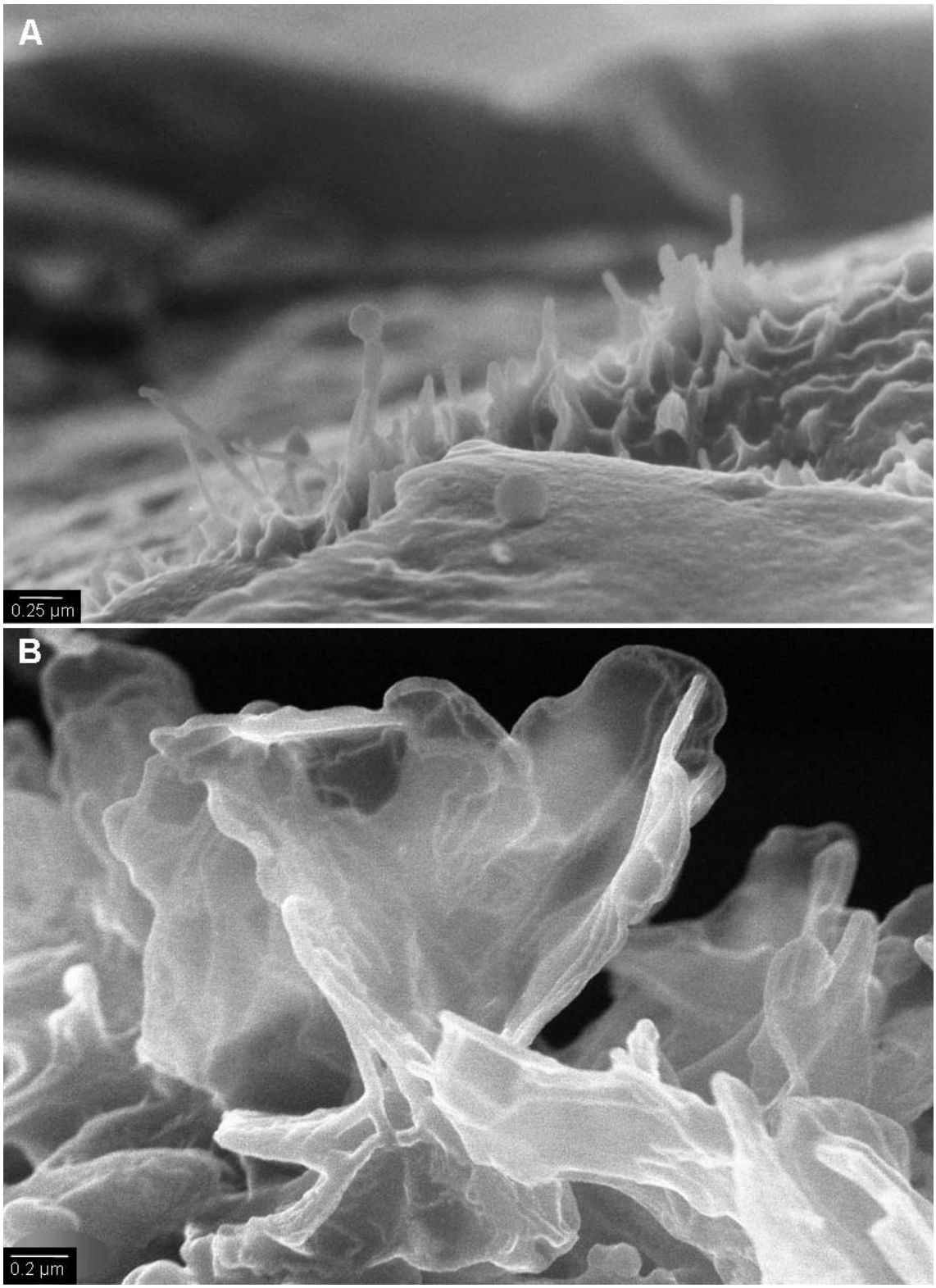

Figure 9

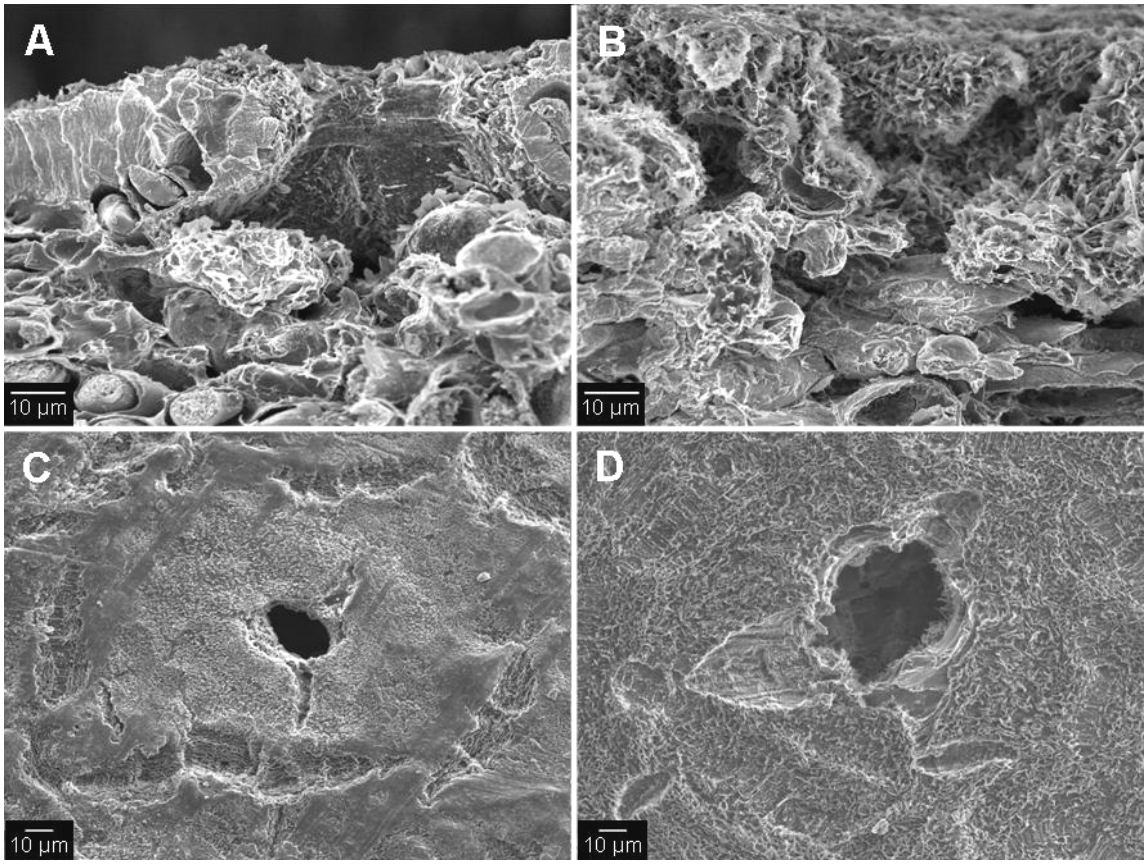

Figure 10