TITLE:

Nano-thermal imaging of the stratum corneum and its potential use for understanding of the mechanism of skin penetration enhancer

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ABSTRACT:

Nano-thermal analysis (nano-TA) is a localised thermal technique which maps a surface in terms of thermal transitions by combining atomic force microscopy with the use of thermal probes, allowing a spatial resolution of sub-100nm. In this communication, we describe the application of a localised nano-TA approach, transition temperature microscopy (TTM), to investigate the thermotropic properties of porcine SC (PSC) as a function of depth and the influence of penetration enhancer on the nano-thermal properties of PSC. The investigations were conducted on PSC removed using tape strips. The transition temperature of PSC recorded at ~220ºC was ascribed to protein denaturation/degradation. A decrease in the transition temperature was observed with an increase of skin depth. 'Transition depression' was observed when PSC was treated with propylene glycol, suggesting its water extraction effect on SC protein and a drop in the biomechanical properties of the SC. TTM has the potential to be extended to on *in situ* investigations of various penetration enhancers.

KEYWORDS:

Nano-TA; stratum corneum; protein denaturation/degradation; porcine skin; skin penetration enhancer

ABBREVIATIONS:

AFM: Atomic force microscopy

HSC: Human stratum corneum

LTA: Localised thermal analysis

Nano-TA**:** Nano-thermal analysis

PG: Propylene glycol

PSC: Porcine stratum corneum

SC: Stratum corneum

TTM: Transition temperature microscopy

MANUSCRIPT BODY:

Advances in the field of nanotechnology and instrumentation in recent years have opened up new opportunities to probe drug delivery at the molecular level. As an extension of localised thermal analysis (LTA), nano-thermal analysis (nano-TA) has gained popularity in pharmaceutical research as a physical characterisation tool for solid dosage forms. By employing a thermal probe in a scanning probe microscope, spatially resolved localised measurements on the surface of a sample is achieved using nano-TA (1). The nano-machined probes measure a thermal event via penetration of the probe into the sample because of surface softening upon heating. Unlike bulk thermal analysis, nano-TA provides spatially resolved information about the surface properties of materials. The technique has been used to differentiate various materials including amorphous and crystalline forms, to map samples with high spatial resolution and to provide 3D information (2). Transition temperature microscopy (TTM) applies the same principles of nano-TA but measurements are carried out in a grid pattern (1). This

creates a TTM image with each pixel referring to a transition temperature assigned using a colour palette. TTM was first used to map the surface of paracetamol/hydroxylpropyl methylcellulose (HPMC) compacts and to determine distribution of materials (1). TTM provided insight into the phase separation in nilvadipine/HPMC spray dried particles at different nitrogen flow rates (3), fentanyl-poly(vinylpyrrolidone) (PVP) solid dispersion thin films under high humidity (4) and cyclosporine A-Eudragit E PO hot melt extruded dispersions prepared at different mixing temperatures (5). It has also been used to characterise PVP nanofibers to identify the materials (6). The application of TTM in characterising the solid drug products has been shown to be a very promising approach to understand the distribution of different materials on the surface. This technique shows advantages over basic atomic force microscopy (AFM) by providing thermal information of the sample apart from the topographical images. The identification of different materials present in a sample renders difficult in AFM images.

To date, the thermal behaviour of the stratum corneum (SC) has been studied using bulk thermal analysis, namely differential scanning calorimetry. This method, however, provides only global information on the overall thermotropic properties of the SC. The advent of nano-characterisation methods such as TTM is the major motivation for the present investigation in order to understand the thermal properties of the SC at the nano-scale with high spatial resolution.

Penetration enhancers have been extensively used in topical and transdermal drug delivery to accelerate the transport of drug molecules into the skin (7). However, the underlying mechanisms of most penetration enhancers have not been fully understood.

Therefore, it is also of interest to explore the potential of TTM to provide more information regarding to the possible enhancement mechanism of penetration enhancer in improving transport of drug molecules into the skin.

TTM is a highly sensitive tool which works best for samples with a consistent thickness or a flat surface. The normal skin surface, however, is rough and uneven and is not suitable for direct contact with TTM probes as it may damage thermal probes. Therefore, the SC was collected by tape stripping for this work.

Porcine ear skin was used as it is an accepted surrogate model for human skin. Fresh porcine ears were obtained from a local abattoir and washed carefully with deionised water. The outer skin membrane was carefully isolated from the underlying tissues and the hair was trimmed carefully using scissors. The prepared skin was stored at -20ºC and thawed at room temperature before use. The skin was mounted in Franz-type diffusion cells (diffusional area = ~1 cm^2) at 32 ± 0.5ºC with and without application of PG (2 µl/cm^2) for 24 h. The receptor phase was filled with phosphate buffer saline (pH 7.3 ± 0.2). After 24 h, *in vitro* tape stripping with porcine ear skin was carried out as reported by Klang, Schwarz (8) (9). Excess PG was removed gently using dry tissues before tape stripping. A total of 20 sequential D-Squame® tapes were obtained for the tape stripping procedure. This *in vitro* tape stripping procedure was standardised in terms of intensity with a pressure applicator and duration of pressure application (5 s) as well as the speed of tape stripping to ensure reproducibility of the measurements. The amount of SC protein collected on each tape strip was quantified with an infrared (IR) densitometer SquameScan® 850A at a wavelength of 850 nm (Heiland Electronic GmbH, Wetzlar, Germany) based on the absorption values obtained from IR

densitometry – absorption (%) = 0.41 x mass of protein (μg/cm^2) (8). From the measured area that was tape stripped area, the SC thickness, which reflects the depth of the SC barrier may be calculated using a SC density of 1 g/cm^3. (8,10)

TTM measurements were conducted using a VESTA® Nano Thermal Analyser (Anasys Instruments Corp., Santa Barbara, CA, US) with a nano-TA probe (Bruker AXS S.A.S, Marne la Vallee Cedex 2, France) as described elsewhere (5). The device was connected to a nanoTA2 controller (Anasys Instruments Corp., Santa Barbara, CA, US) for voltage adjustment to the tip. The cantilever deflection is monitored by the sensor signal (V). Temperature calibration was carried out for the probe using the manufacturer-supplied melting point standards – polycaprolactone (55ºC), polyethylene (116ºC) and polyethylene terephthalate (235ºC). The softening of the materials caused by the penetration of the probe into the surface with heat was determined as a thermal event. Samples were firmly attached to a magnetic stud using double-sided tape before mounting on an X-Y translation microscope stage. TTM imaging was performed based on thermal transition temperatures in LTA. An underlying heating rate of 10ºC/s was applied in LTA from room temperature (25ºC) to 250ºC, with a cooling rate of 100ºC/s and a data rate of 20 point/s. An area of interest (30 × 30 μm or 10 × 10 μm) on a sample surface was identified and an optical microscope was used to capture the image of the sample surface. The TTM image was constructed based on a particular colour palette where a colour was assigned to each transition temperature detected. The resolution of the TTM image was set at 1 × 1 μm and the distance between locations for measurement was fixed at 1 μm.

The mass of protein removed by sequential tape stripping is shown Figure 1. The amount of protein removed progressively decreased with the number of tape strips. Similar observations have been reported by Klang, Schwarz (8) using the same tapes (D-Squame®). A study comparing *in vitro* and *in vivo* tape stripping on human skin also confirmed a similar pattern using the same tapes (10). The cumulative thickness of the SC removed increases with the number of tape strip. The calculated SC thickness was 6.6 ± 0.8 µm but this does not represent the actual thickness of the SC because the 20 sequential tape strips does not remove the entire SC.

TTM images of the first and second tape strips are illustrated in Figure 2. Two domains (red/yellow and purple colours) with transition temperatures of ~220ºC and 25ºC were identified in both images. The transition temperature of ~220ºC within the red domain refers to the SC. The first two tape strips were scanned with an area of 30 × 30 µm in order to elucidate the differences between the SC (red domain) and the tape adhesive (purple domain). The detection of the transition temperature around 220ºC for the SC is largely related to protein denaturation and degradation. Bulk thermal analysis of PSC using differential scanning calorimetry (DSC) has confirmed that at about 90ºC there is generally an irreversible SC protein denaturation (11-16). Bulgon and Vinson (17) suggested that denaturation of keratin in human stratum corneum (HSC) may contribute to the transition temperature above 180ºC. The transition temperature of PSC reported in this study is also similar to the highest transition temperature determined using thermomechanical analysis of HSC (18,19). This is generally correlated with the thermal decomposition of skin tissue and was confirmed by DSC and visual observation (20). Recent work studying the effects of heat on human skin indicated that the SC started to

155 decompose when it was heated from 150 – 200ºC (21). Epidermal tissue changes under
156 the influence of heat are complex. The increase of tension of the epidermis upon
157 heating may be attributed to the disruption of the α-helix and the build-up of β keratin
158 (22). When heat is applied to the SC, α-keratin is converted to the β form because of
159 fractures in cross-linkages of keratin filaments (15).

160 The anisotropic structure of SC is greatly influenced by the organisation of keratin.
161 Because of the heterogeneous distribution of keratin filaments, the effect of temperature
162 on viscoelastic properties of SC will vary from one tape strip to the next. Comparisons of
163 all 20 tape strips were carried out using a scanning area of 10 × 10 μm on the SC and
164 excluding the tape adhesive (Figure 3).

165 Figure 4A shows the correlation of the transition temperature and protein mass
166 collected on each strip. Protein mass extracted indicates the thickness of the SC
167 sample. The transition temperature of PSC decreases gradually with the number of tape
168 strips removed. The mean transition temperature between each layer of tape strips are
169 statistically significant differences (ANOVA, $p < 0.05$). In addition, it is interesting to note
170 that the higher amount of SC protein/keratin (or the thicker of the SC extracted) resulted
171 in a higher transition temperature (Figure 4A). The relationship between the mean
172 transition temperature and protein mass is shown clearly in Figure 4B. Heat is required
173 to break the cross linking of keratin filaments in the SC, converting the α-keratin to the β
174 form (15). Substantial energy is required for transforming α-keratin to the β form in tape
175 strips containing more keratin. An increase in the β/α ratio with heating was observed by
176 Lin and co-workers indicating a continuous conversion of these structures (15). It may
177 happen similarly in this work where a high degree of keratin denaturation in a thick SC

sample (high protein mass) may result in a late penetration of the nano-TA probe into the SC. Thus, this causes a higher transition temperature.

The correlation between the transition temperature and protein mass may be further understood using the variations in the colour assigned (red to yellow) for PSC for distribution of keratin content. In this case, the red colour refers to a higher level of keratin filaments, resulting in a higher transition temperature. This may reflect a late penetration of the probe into the SC (penetration at a higher transition temperature) because more energy in the form of heat is needed to break the cross linking of the keratin filaments. The yellow region is assigned to an area with less keratin filaments, showing an early penetration temperature of the probe. A recent approach using AFM with a tunable IR laser source (AFM-IR) showed that the variation of the intensities of absorption peak at 3290 cm^{-1} (mostly due to the N–H stretching vibration of amide A in the keratin) in the HSC sample is related to the total protein content at the point of measurement (23). A weaker band at 3290 cm^{-1} was linked to a lower total protein content relative to the hydrophobic lipid-like compounds. This observation supports the appearance of two different colours (red/yellow) within the same domain assigned to the PSC in this study. The colour variation in the PSC, therefore, reflects different amounts of protein (keratin) or different thickness of the SC sample at the scanning point as explained above.

PG is a commonly used glycol in topical formulations especially for improving the transport of poorly soluble materials into the skin. Thermal analysis of the effect of PG on skin has demonstrated the possibility of a dehydrating effect of PG on the SC

protein(24). Therefore, PG is shown as a good candidate in this work as TTM data reported the transition temperature of SC protein instead of the lipids.

The SC thickness removed (a total of 20 tape strips) from the skin treated with PG was 6.6 ± 0.7 µm and there is no statistically significant difference reported in the SC thickness removed compared with the control study (Mann-Whitney test, $p > 0.05$). The amount of the removed protein from individual tape strip also showed no statistical differences (Mann-Whitney test, $p > 0.05$) compared with the control with the exception of the first tape ($p < 0.05$) (Figure 1). The SC in this first tape has the highest exposure time to PG before PG evaporates or permeates through the skin. PG has no large impact on the removal of the SC in the deeper layers. Although PG does not have a major influence on the protein content removed by tape stripping, it shows a pronounced effect on the thermal properties of PSC. From the TTM images in Figure 5, a homogenous yellow/green region was obtained consistently for all selected tapes. Transition temperatures for this yellow/green zone ranged from 175ºC to 190ºC as listed in Table 1, suggesting a significant drop in the transition temperature of PSC as compared to the same tape reported in the untreated samples (Student's *t* test, $p < 0.05$). This phenomenon is termed here as 'transition depression'.

Previous DSC studies which investigated the change of thermal behaviour after treatment with PG revealed the absence of the protein denaturation endotherm (24). The loss of the protein peak may reflect water extraction from the protein by PG (24,25). Ostrenga, Steinmetz (26) previously reported that skin pliability was reduced due to the dehydration effect of PG. The water extraction effect on the SC protein could be related to the transition depression observed for the SC in the presence of PG. Dehydration of

the SC protein could change the biomechanical properties of the SC (27). The SC plasticised with water has a high stretching capacity which requires more energy to weaken the biomechanical properties of the SC (27). In less hydrated SC, biomechanical strength withstanding the tension applied without fracture is far weaker than that of hydrated SC. It may be hypothesised that water extraction from the protein content by PG has weakened the biomechanical properties of PSC in the same way. Loss of water has impaired the integrity of SC to resist force applied by the heated probe. Consequently, the cantilever of the probe deflected to a smaller extent, giving rise to an early penetration of the probe into the SC. The greatest reduction of transition temperature was reported in the first few layers (Table 1). This may be due to the highest contact time of these SC layers with PG before PG depleted from the skin. Under these circumstances, water extraction by PG is more substantial in the upper layer of the SC, further weakening the mechanical strength of SC. This modification of the biomechanical properties of SC associated with the use of PG has not been reported previously using other thermal analyses.

In summary, we demonstrated that TTM is a powerful nano-thermal technique in describing the thermal properties of PSC *in situ* at a high spatial resolution. TTM is able to characterise the thermal properties of the SC as a function of depth which is impossible to be achieved by using a conventional thermal analysis such as DSC. Also, the thermal measurement using TTM can be conducted at ambient environment which mimics the actual condition. This technique is convenient to use and does not require any other accessories such as pan and purging gas. By having these advantages, we managed to show that the influence of PG on the local thermal and biomechanical

properties of the SC can be reflected by the changes of the transition temperature. Therefore, we believe that this technique could be adapted to further evaluate the underlying mechanism of various skin penetration enhancers which have been previously reported to have interaction with SC protein and ability to modify the protein conformation such as dimethyl sulphoxide (7). However, the application of TTM is limited to the changes occurred to solid objects such as protein. The observation involving liquid such as SC lipid is not suitable by using TTM. Techniques such as DSC and ATR-FTIR spectroscopy may be used to support the action of skin penetration enhancers on the SC lipid.

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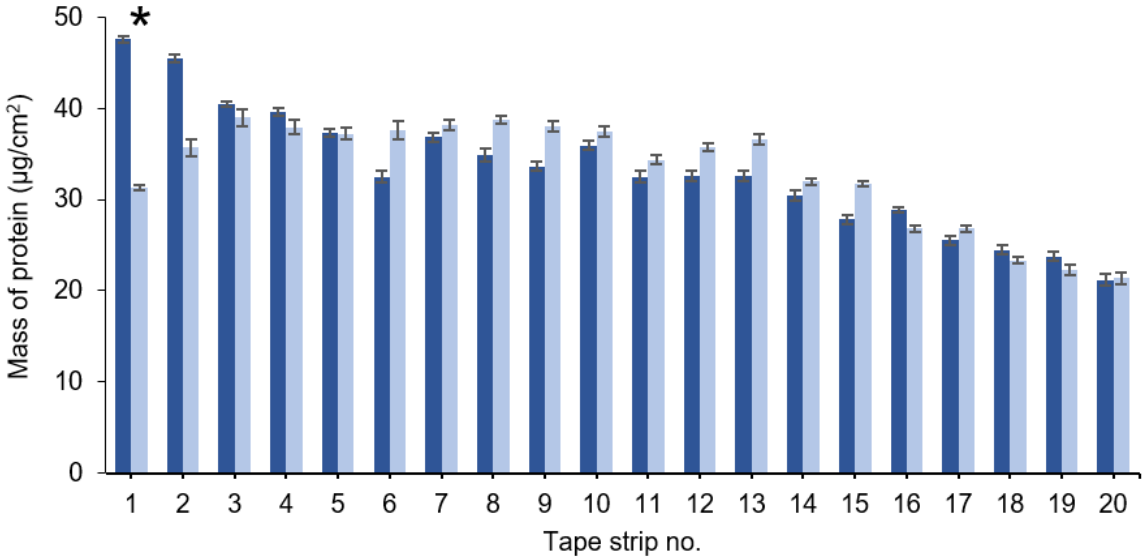
353 **FIGURES:**



354

355 Figure 1 Mass of SC protein removed across the porcine ear skin with 20 tape strips
356 with (light blue bars) and without (dark blue bars) the application of PG (n = 5, mean ±
357 SD). * indicates statistically significant difference (Mann-Whitney test, $p < 0.05$).

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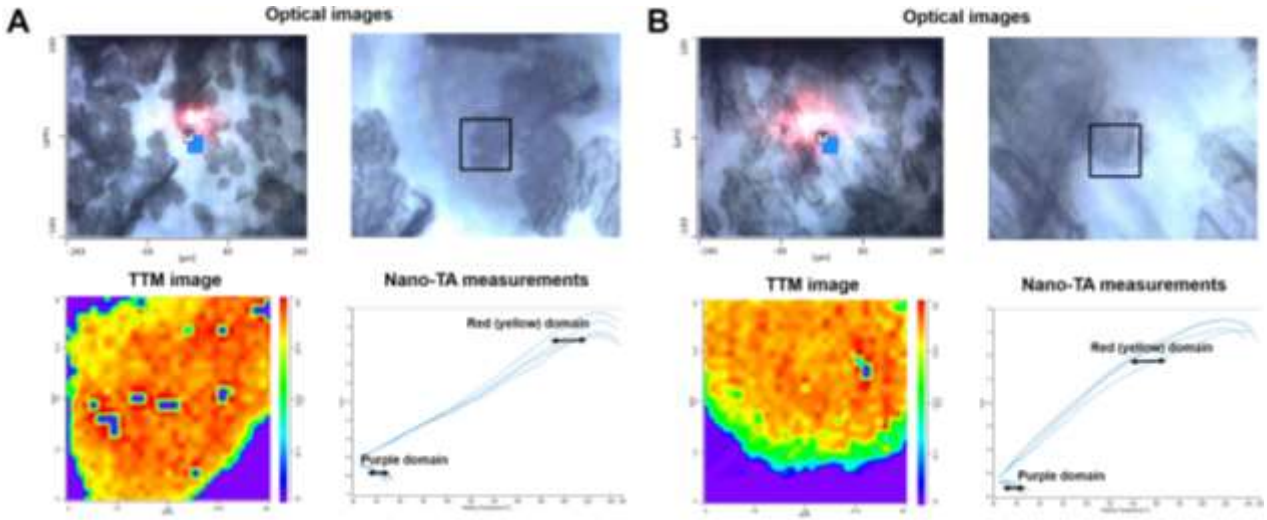

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360 Figure 2 TTM data for the (A) first and (B) second tape strips of PSC in the control study

361 (area: 30 × 30 μm; resolution: 1 × 1 μm)

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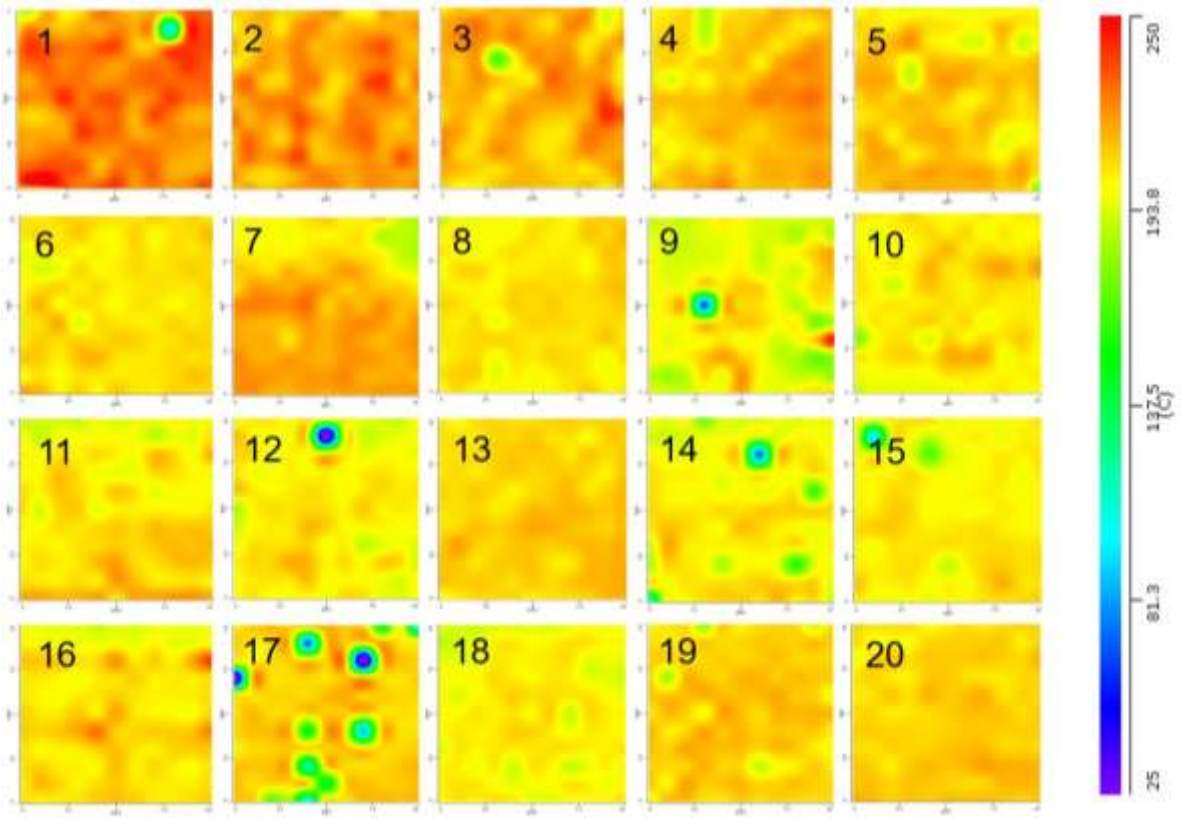

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365 Figure 3 TTM images for all 20 tape strips of PSC in the control study (Area: 10 × 10
366 μm; Resolution: 1 × 1 μm). The number in the TTM images refers to the tape strip
367 number.

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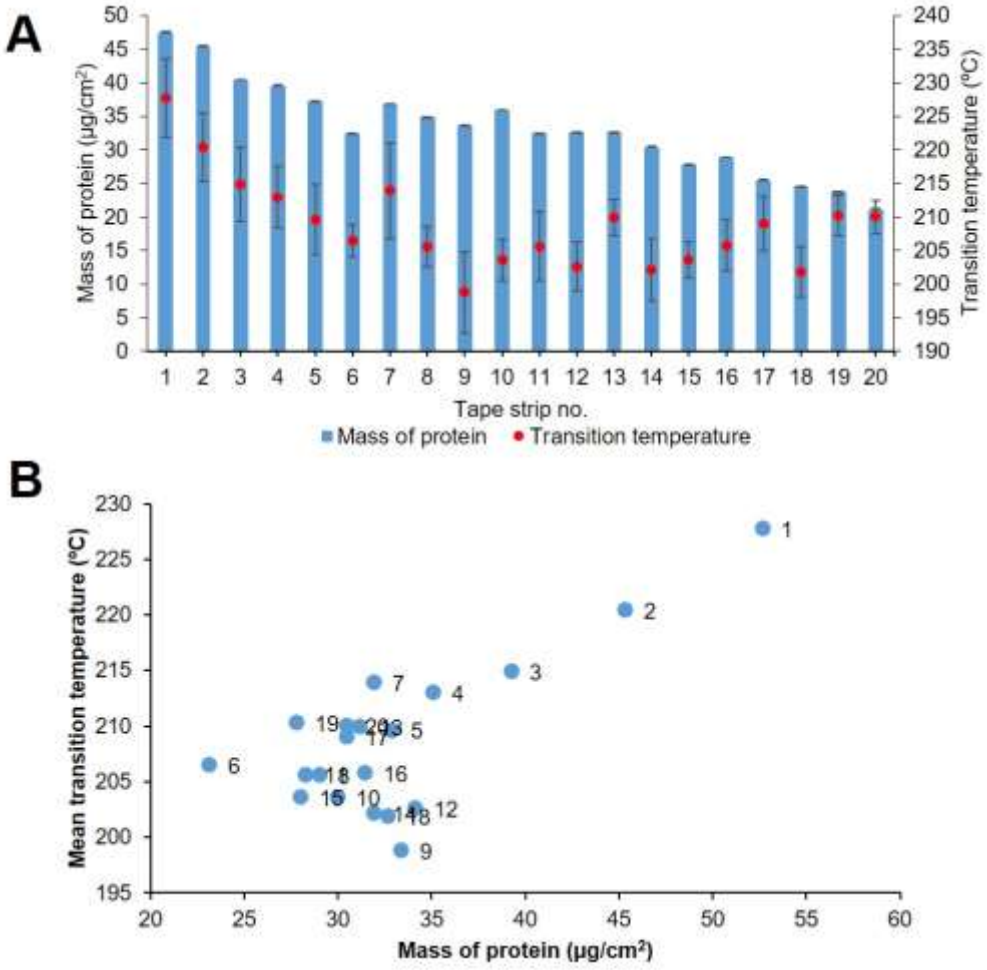

370

371 Figure 4 Transition temperature (n = 100, mean ± SD) and SC protein mass (n = 5,
372 mean ± SD) profiles (A) and their correlation (B) for all 20 tape strips (control study).
373 The number in the plot B represents the tape strip number.

374

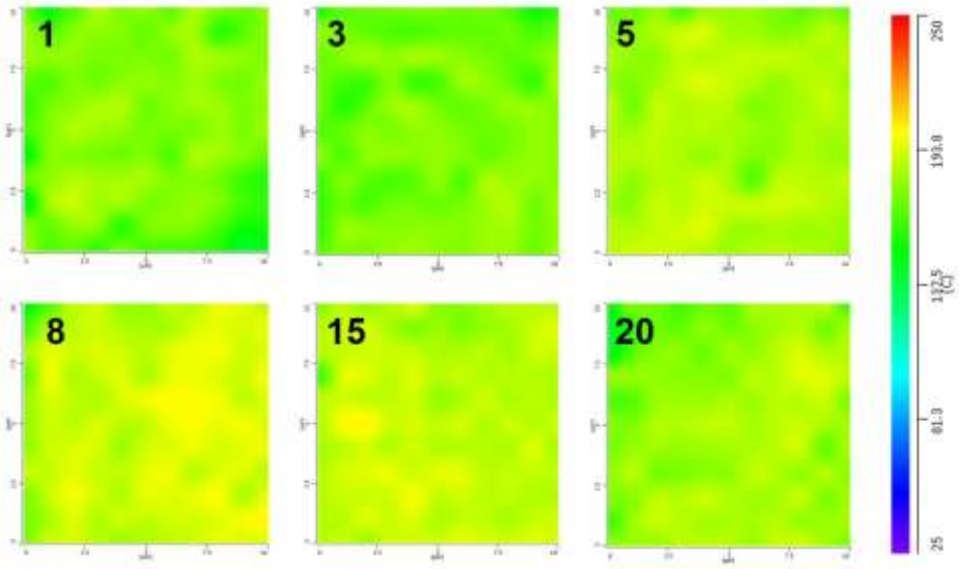

Figure 5 TTM images of the PSC removed from the selected tapes after application of PG only (area: 10 × 10 μm; resolution: 1 × 1 μm). The number in the TTM images refers to the tape strip number.

TABLE:

Table 1 Comparison of transition temperatures for the selected tapes with and without the application of propylene glycol (PG) (n = 100, mean ± SD)

Tape strip no.	Transition temperature (ºC)	
	PG	Control
1	175.29 ± 5.90*	227.76 ± 5.90
3	174.85 ± 4.72*	214.89 ± 5.55
5	184.74 ± 3.68*	209.60 ± 5.29
8	191.38 ± 4.16*	205.55 ± 2.98
15	188.19 ± 3.91*	203.54 ± 2.71
20	181.90 ± 5.18*	209.98 ± 2.47

* indicates statistically significant difference (Student's *t* test, $p < 0.05$)