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Research Article

Camera-less Terahertz Imaging Investigation of the Interaction of Factor XII Protein with the Human Stratum Corneum

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ABSTRACT

This paper reports the use of camera-less terahertz imaging technique to measure zymogen factor XII (FXII) conformational changes in solution and upon binding to stratum corneum in the absence of its known endogenous potentiating complex, high molecular weight kininogen (HK)-Prekallikrein (PK) in real-time. FXII is synthesized in the liver and secreted into the circulation to serve as a blood coagulation factor. FXII has diverse biological functions. First, it serves as an accessary molecule to extend the generation of thrombin-induced platelet adhesion to the initially formed platelet monolayer. Secondly, FXII provides a robust stimulus for Bradykinin (BK)-induced hyper-permeability by disrupting vascular barriers. Using *in vitro* assays, studies have shown that FXII activation can occur on negatively charged surfaces. This activation is accelerated in the presence of kallikrein or activated Prekallikrein (PK). We demonstrate our method by measuring the conformational changes that occur upon FXII binding on stratum corneum. The subject of FXII activation remains a work in progress. Previously, Terahertz Scanning Reflectometry (TSR) and Terahertz Spectrometry (TS) was used to demonstrate that different spectral density deviations of the signal are due to the structural alterations and activation of FXII induced by the hydrated stratum corneum. The present work uses Terahertz (THz) imaging to demonstrate that a subset of zymogen FXII bond to the stratum corneum. FXII was not significantly accumulated on the hydrated stratum corneum. However, formed activated FXII (FXIIa) was observed on the stratum corneum. This finding suggests that negatively charged surface is not a necessary condition.

Keywords: Camera-less terahertz imaging; Stratum corneum; Factor XII; Protein interaction; Activated FXII; Terahertz scanning reflectometry

Abbreviations: Receptor B1: Bradykinin Receptor 1; Receptor B2: Bradykinin 2 Receptor; BK: Bradykinin; FXII: Factor XII; HK: High Molecular Weight Kininogen; KKS: Plasma Kallikrein-Kinin-System; PK: Prekallikrein; PKa: Plasma Kallikrein; PHBP: Hyaluronic Acid Binding Protein; TS: Terahertz Spectrometry; TSR: Terahertz Scanning Reflectometry

INTRODUCTION

Although dispensable for normal hemostasis, "factor XII" (FXII) is a highly desirable target for drug manufacturing for key therapeutics for a host of medical anomalies, consistent with plaque corroded arteries [1,2], thrombosis [3], and hereditary angioedema [4]. The plasma level of activated FXII is elevated in chronic spontaneous urticaria [5], angioedema [6], and bullous pemphigoid [7]. FXII knockout mouse is protected against heparin-induced skin edema [8]. Edema and inflammation are attributed to plasma kallikrein (PKa), a major activator of FXII, mediated by cleavage of high molecular weight kininogen (HK) to generate bradykinin (BK), a potent inflammatory mediator (Figure 1). BK, in turn, activates the constitutive bradykinin B2 (B_2) and inducible bradykinin B1 (B_1) receptors. Most work on the study of FXII activation has been done with *in vitro* exposure of FXII to negatively charged surfaces using the cell-free system. This autoactivation can significantly be enhanced by Pka. However, evidence shows that the zymogen FXII is stable at pH 5.3 and it's binding to dextran sulfate, a negative chemical activator of FXII, is also unaffected [9]. These *in vitro* assays do not necessarily reflect the *in vivo* FXII environment. Our understanding of the intrinsic pathway has evolved over the last decade in various fields of medicine, however, the precise mechanisms by which the activation of FXII occurs has been, and still is, unclear in humans.

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The Stratum Corneum (SC) is the topmost layer of the skin that serves as a water-impermeable barrier and has the potential to act as a buffer [10]. The dermis and epidermis provide an overlying immunological barrier of the skin [11]. The surface of SC has a net negative charge [12]. Skin changes can occur due to environmental factors, leading to itching and edema. Skin changes are associated with aging related disease including permeability barrier dysfunction and chronic non-healing wounds [13]. Moreover, type 2 Diabetes Mellitus (type 2 DM), a common chronic condition, induces many dermatological conditions such as increased susceptibility to skin infections and dry skin [14].

The expression of the serine-protease Plasma Hyaluronan-Binding Protein (PHBP) is elevated in type 2 DM-induced dry skin [15]. PHBP shares significant similarity in nucleotides with FXII [16]. Both FXII and PHBP are inhibited by C1-inhibitor [17]. Skin wound healing is compromised upon stimulation of B_2 receptor in the diabetic skin. The B_2 receptor blockade improves skin wound healing in diabetic mice [18]. Moreover, the B_2 receptor agonist impairs not only wound healing in both non-diabetic and diabetic mice but also causes skin changes and epidermis thickening [19]. Since the balance of the FXII-mediated-BK generation appears to tilt towards thrombosis and inflammation in skin, we attempt to understand physiologic basis of FXII activation in order to provide detailed insight into key step in the early stage of the intrinsic coagulation activation. This paper attempts to determine whether the autoactivation of FXII occurs in the absence of its potentiator, Pka, and is enhanced by negatively charged surfaces of the SC.

Terahertz Scanning Reflectometry (TSR) and Terahertz Spectrometry (TS) were used to study the surface mediated FXII activation, as well as penetration of the FXII, its retardant property, diffusion kinetics, and fragmentation profiles into human SC [20]. The terahertz spectral analysis via absorbance spectra indicates the adhesion of FXII onto the SC [20]. Though this process corroborates the binding of FXII to the cell membrane, as reported in the *in vitro* findings, it does not appear to be activated and degraded. It was also found that there are a number of characteristic absorbance peaks for each molecule and these peaks are uniquely shifted relative to each other when compared with the SC alone [20]. Thus, these absorbance peaks offer a means for identifying features of the protein and peptides that could be further investigated for conclusive assignment of the absorbance peaks to the specific proteins and their resonances.

The question of if and how FXII is activated on cell surface is still un-answered. Therefore, the aim of this study was to improve upon prior models of FXII binding to cells by using TSR-TS data that captures absorbance peaks. First, we describe the background of the SC-FXII interactions; second, we outline some details of the camera-less terahertz multispectral imaging followed by the materials; and third we present the imaging results followed by discussions and conclusions.

BACKGROUND

The central dogma suggests that for activated FXII formation to occur, the zymogen FXII must undergo adsorption to negatively charged surface, activation, and amplification (Figure 1). Once the activated FXII (FXIIa) is formed, it triggers the activation of plasma prekallikrein (PK) to Pka in the presence of HK. Blood circulating PK is about 75% bound noncovalently to HK and a few more plasma proteins that are not known for sure. Pka cleaves FXII and enhances the formation of two-chain FXIIa also called alpha-FXIIa (αFXIIa) that activates factor XI to produce activated factor XI (FXIa), which serves as an accessory molecule to increase thrombin-induced clot formation (Figure 1). Both kallikrein and plasmin are capable of cleaving αFXIIa to βFXIIa in blood. While βFXIIa can activate PK to kallikrein, βFXIIa loses much of its proteolytic activity toward FXI mainly due to loss of the heavy chain, the FXI binding site on FXII. HK not only functions as the principal PK transport protein, but also serves as a major plasma BK precursor. Pka also cleaves HK and releases BK, a major component of the inflammatory phase following an injury. The pro-thrombotic and pro-inflammatory roles of FXII appear to be a nonspecific response to promote wound healing and combat the establishment of infection.

FXII is not constitutively bound to cells in the intravascular compartment, but it has been hypothesized that FXII can be bound to the interstitial basal membrane when injured endothelial cells (EC) retract and/or detach. Evidence shows that FXII binds to cultured human endothelial cells, which are major contributors in the processes controlling coagulation, inflammation and the immune response [21]. Under normal conditions, FXII-dependent

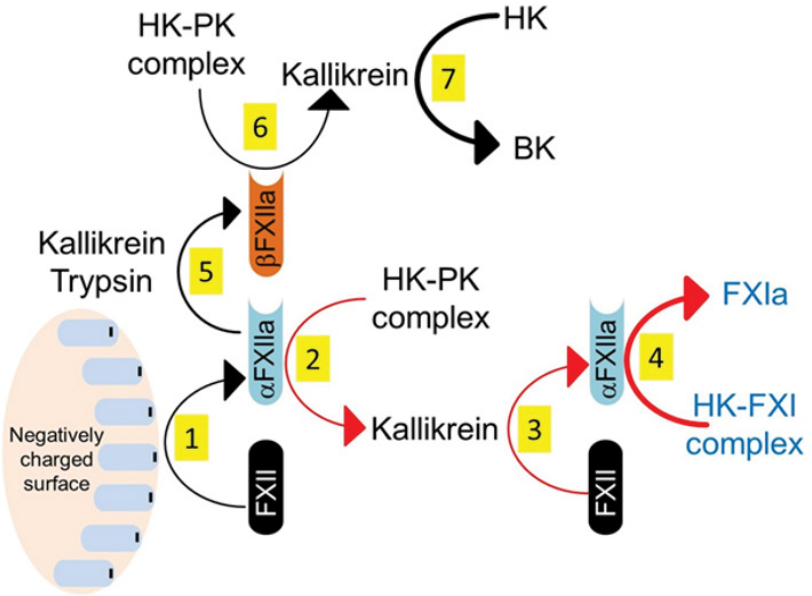

Figure 1: Schematic representing the activation of the plasma kallikrein-kinin system.

clot formation does not occur on the EC. The binding of FXII to the activated EC may tips the balance toward activation of FXII acting on coagulation, fibrinolysis [22] and angiogenesis [23]. Evidence shows that among several extracellular matrix proteins of the endothelial basal membrane, collagen contributes to activation of FXII [24]. Other surfaces such as misfolded protein aggregates, nucleic acids, and platelet and microbial polyphosphate are found to activate FXII [25]. In the context of arterial thrombosis, Von Willebrand Factor (VWF) fibers contribute to sequestration of both FXIIa and FXIa and subsequent transformation of fibrinogen to fibrin. Therefore, the mechanism by which blood FXII activation locally in response to tissue and microvascular leak remains elusive.

The view that FXIIa formation occurs on negatively charged surfaces has recently been revised. Figure 1 shows a revised version of the contact activation model in light of the present author's understanding of the work in the area of FXII. A recent evidence suggests that activation of FXII can take place on non-anionic surface [26]. Twenty-two different cancer cells of different organs are known to have negatively charged surfaces [27] compared to normal cells. However, the activation and expression pattern of FXII as a potential component of the global procoagulant profile of tumors in humans remains notably uncharacterized at present.

According to the dogma, the initial event *in vitro* is the adsorption of FXII to a negatively charged surface (vertical ovoid) where it is activated to form FXIIa (αFXIIa, step 1). High molecular weight kininogen (HK) and prekallikrein (PK) circulate as a complex (HK-PK) and αFXIIa activates PK to kallikrein (PKa) (step 2), which in turn amplifies the process through reciprocal activation of FXII (step 3). αFXIIa activates factor XI (FXI) in the FXI-HK complex to FXIa (step 4). Both kallikrein and trypsin can cleave αFXIIa to βFXIIa, the short molecule that preserves the active site but lacks the surface-binding properties (step 5). The soluble βFXIIa in turn activates PK to PKa, however, lose much of the ability of activating FXI to FXIa (step 6). Formed PKa is also responsible for the liberation of BK, a mediator of inflammation, from HK (step 7), linking coagulation and inflammation. Arrow thickness indicates magnitude of substrate activation.

In this report, this possibility is explored with FXII, placed on stratum corneum with molecular morphology analysis of FXII binding, its potential overall conformational readjustment, and its level of cell membrane penetration via integrated terahertz nano-scanning spectrometer, a non-ionizing and non-destructive detection imaging scanner. The findings indicate that the interaction between FXII and cationic and anionic charged surface cannot be thought to be the main physiological initiator of the activation of the intrinsic pathway of the blood coagulation.

Principle of camera-less terahertz multispectral imaging

The concept and the procedure for camera-less 3D image formation is described in detail elsewhere [28]. It was recently demonstrated that higher resolution images may be formed by terahertz multispectral imaging, overcoming the Abbe diffraction limit [28]. Since biological tissues are transparent to the T-rays, terahertz imaging technique offers an important opportunity to probe under the surfaces and visualize in a non-destructive, non-contact fashion. Terahertz multispectral imaging was used for early detection of skin cancer [29]. It was further demonstrated that the combination of terahertz imaging and terahertz time-domain spectrometry can be used as a powerful tool for investigating semiconductor wafers, epitaxial layers, quantum dots, and nanomaterials [30,31]. This technique also offers another advantage for user definable pixel size (or voxel size in 3D) by a hardware and software combination. In contrast, a digital camera displays and records the processed signal of an object with fixed pixel size. In a camera, an object is focused on a CCD or a focal plane array by means of a focusing lens, the output of the CCD is processed by a built-in processor that displays the image on a screen, and saves the image in a file. In contrast, the terahertz technique eliminates the CCD and lens system by a nanoscanner and a suitable computer algorithm for image generation and processing. Here, the object to be imaged is scanned (digitized) along the 3 orthogonal axes for 3D imaging; or digitized on a plane for surface imaging. A matrix containing the digitized reflected signal (or, equivalently, the transmitted signal) is recorded in a file and then processed by a suitable algorithm [29].

MATERIALS AND METHOD

Stratum Corneum (SC) samples were obtained from the New York Firefighter's skin bank from an unknown donor. The samples were kept frozen until prior to the experiments. The proteins used were obtained as follows: FXII (Enzyme Research Laboratory, South Bend, IN) and UM 8190 (an in-house compound) [32]. Other laboratory instruments used during the experiments are ovation micropipette and micro-tips, laboratory microbalance, 4 mL glass bottles, Terahertz Nanoscanning Spectrometer and 3D Imager (TNS3DI) (Applied Research and Photonics, Harrisburg, PA), software application suit for the TNS3DI, a nano-scanner built in the TSR, digital calipers, Plexiglas mount, ruler, spatula, tape, etc. and other customary lab supplies.

The FXII protein was prepared using a 16.125 μM sample that was diluted with 200 μl of deionized water. The UM 8190 was prepared with approximately ~1.18 mg with 200 μL of deionized water. These solutions were applied on the untreated SC and the solvent allowed to dry before imaging.

SC samples were mounted on a Plexiglas plate with a hole such that the terahertz beam was incident on the SC without any interference from the mounting mechanism. The measurement protocol is the same as described previously [20]. That is, samples were scanned one at a time and the reflected intensity matrix was saved in a data file that was used for image generation via the aforementioned technique [29].

RESULTS

Terahertz imaging results. Our results on terahertz images are presented in Figure 2 to all the way to Figure 8. Figure 1 exhibits terahertz images of bare SC over areas of 1 mm^2 and the same image zoomed to over 100 μm^2 area. Figure 3 shows the bare SC saturated with DI water over the same areas as in Figure 2, respectively. The comparison between Figures 2 and 3 revealed how the stratum corneum texture is modified by DI water. While the 1 mm^2 area in Figure 2 shows a relatively smooth texture, the 1 mm^2 area in Figure 3 exhibits an accentuated texture due to saturation with water. The zoomed in image over 100 μm^2 area in Figure 2 exhibited a texture that is likely to arise from the influence of collagen, but this texture is smoothed out due to water absorption as seen from zoomed in image of Figure 3. This zoomed in features from Figures 2 and 3 were compared side by side in Figure 4 in greyscale rendering, revealing the same observation is valid, that is, the texture influenced by collagen has been smoothed out due to water saturation.

Figure 5 exhibits an image of SC saturated with FXII over 1 mm^2

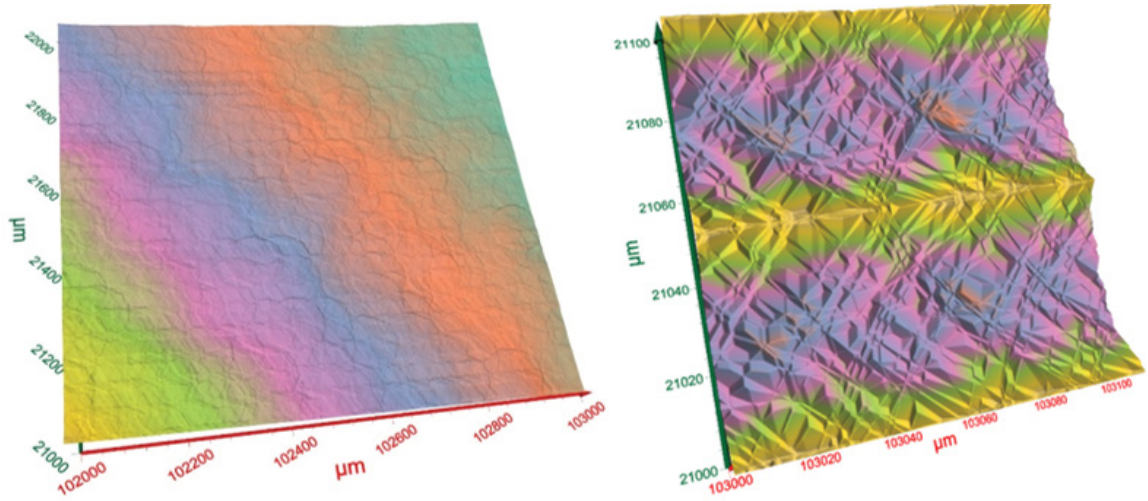

Figure 2: Left: Untreated SC, 1 mm^2; Right: Untreated SC zoomed to (100 µm)^2.

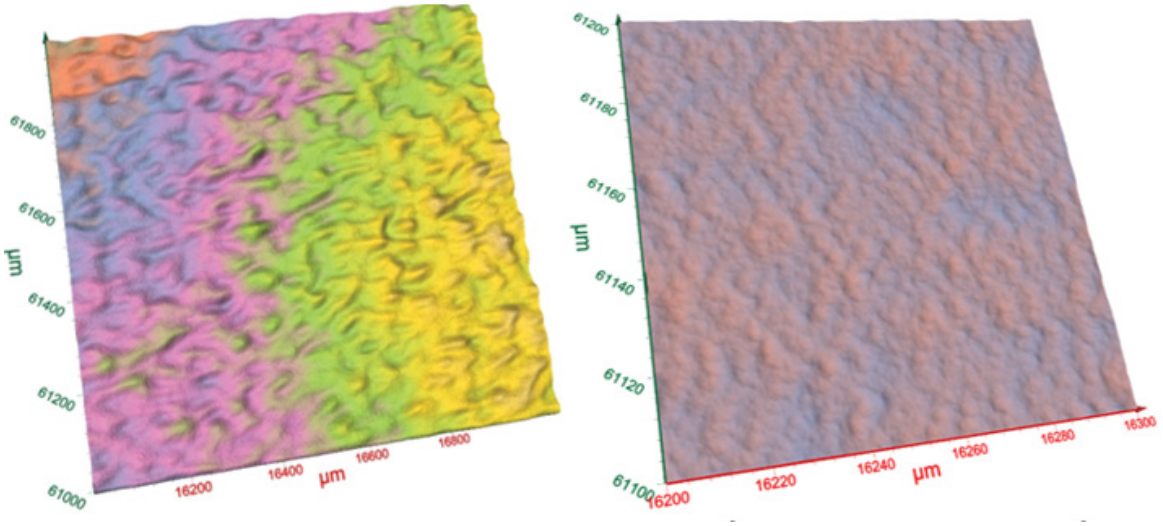

Figure 3: Left: SC saturated with DI Water, (1 mm)^2; Right: Zoomed to (100 µm)^2.

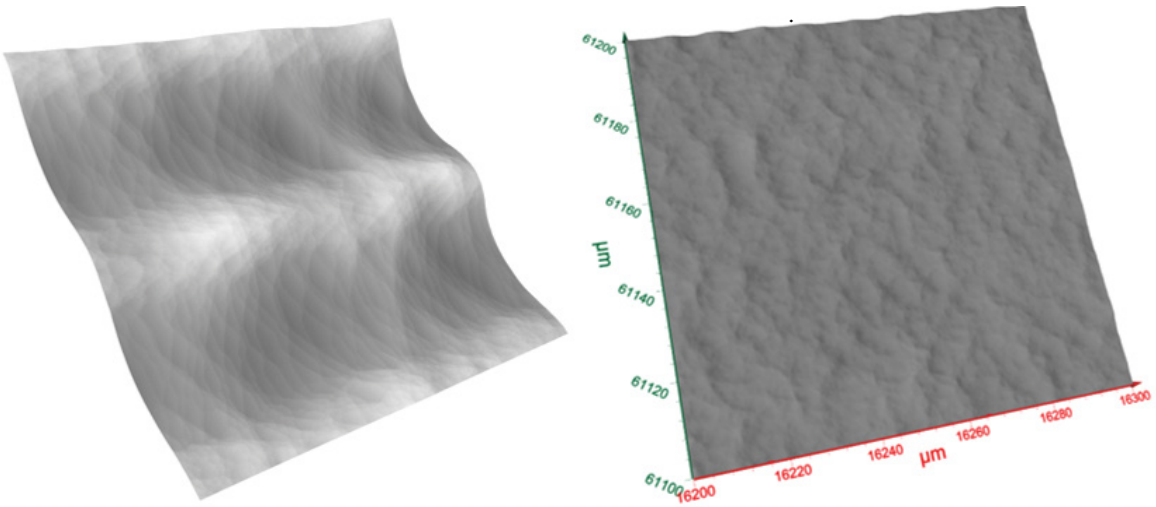

Figure 4: Left: Another view of untreated SC (100 µm)^2; Right: Another view of SC saturated with DI Water, (100 µm)^2.

and Figure 6 shows an image of SC saturated with UM-8190 also over 1 mm^2 area. UM-8190 is used as control; it has two features. First, it is a short peptide containing dodecylamides [32]. Second, it can penetrate cells. However, whether the cellular uptake of UM-8190 may occur through energy-dependent or energy-independent mechanisms is not known. Both Figures 7 and 8 compare the same situations as Figures 5 and 6, respectively, but both images are zoomed over 100 µm × 100 µm areas. It is seen from Figure 7

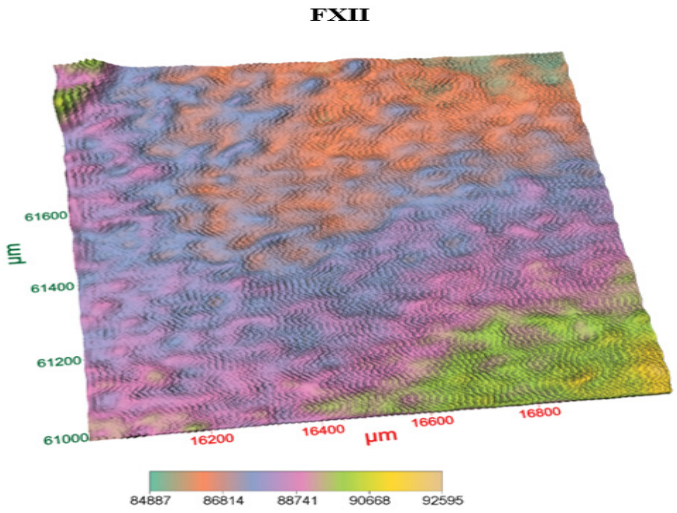

Figure 5: Stratum Corneum saturated with FXII (1 mm)^2.

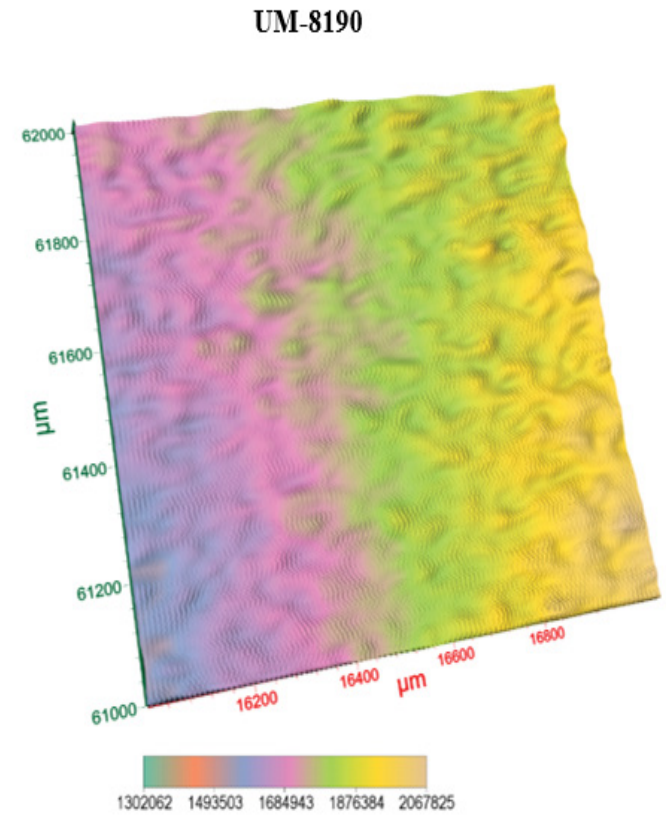

Figure 6: Stratum Corneum saturated with UM-8190 (1 mm)^2.

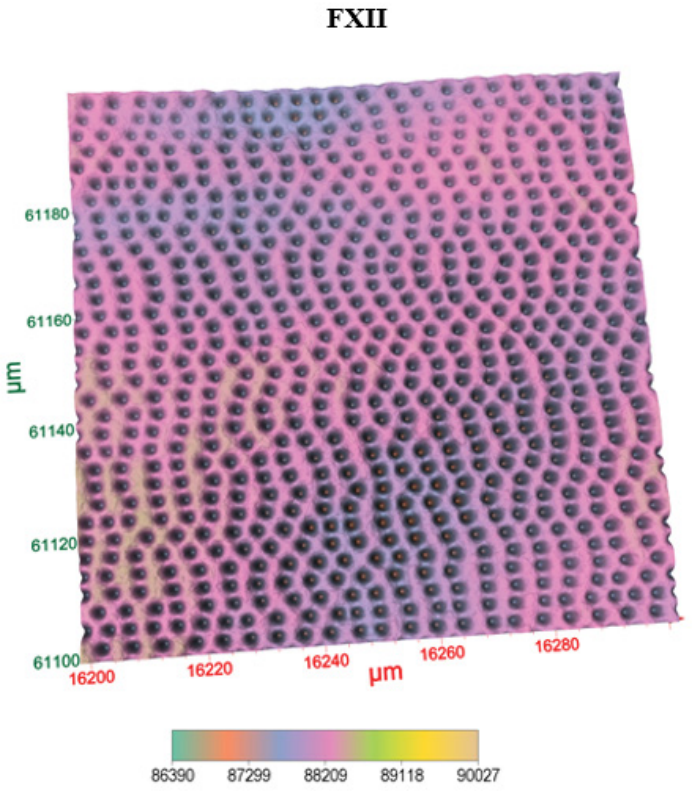

Figure 7: Close-up of SC saturated with FXII image over (100 µm × 100 µm)^2 area.

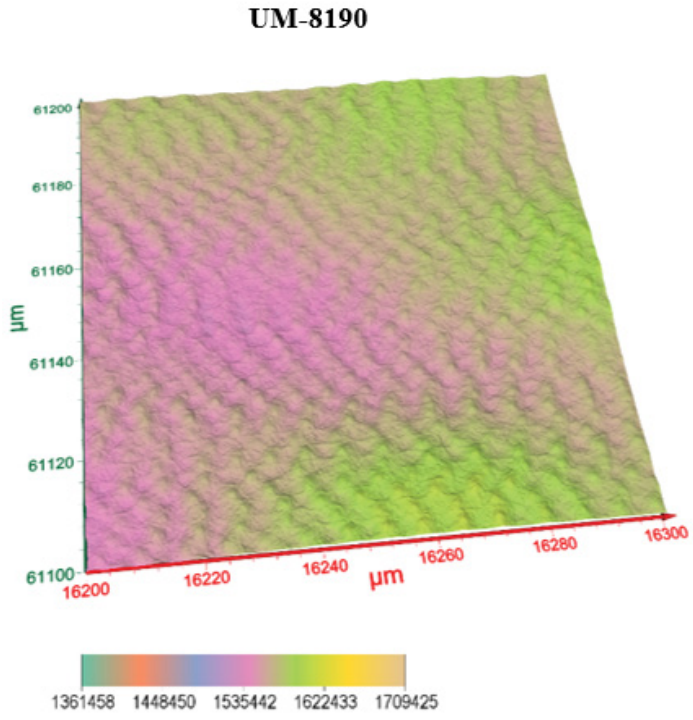

Figure 8: Close-up of SC saturated with UM-8193 over (100 µm × 100 µm)^2 area.

that the cellular network of SC was clearly visible, indicating not enough accumulation of FXII. In contrast, it is seen from Figure 8 that the cellular network of SC is obscured due to accumulation of UM-8193 over an identical area as in Figure 7.

Thus, the present results were consistent with our previous findings via terahertz scanning reflectometry that the protein FXII does not penetrate the SC while the peptide UM8190 exhibits some penetration into the SC [20].

DISCUSSION

Terahertz spectroscopy has been effectively used to detect protein

and identifying small variations in protein conformational changes and molecular interaction [33]. The present study uses camera-less terahertz imaging technique to investigate the interaction of FXII with the hydrated stratum corneum and determine whether the images can be used as a potential screening technique to distinguish the zymogen FXII from that of the UM-8193. Generated evidence suggests that surface-binding of FXII leads to a conformational shift [34], and surface-bound FXII becomes activated in a step-wise mechanism [35]. The influence of the negative surface charge on the adhesion and autoactivation properties of FXII was determined. Previously, terahertz spectral analysis via Lomb periodogram absorbance spectra indicated the adhesion of both the FXII and UM-8190 on the SC [20]. Terahertz imaging of the present work revealed that FXII was not accumulated on stratum corneum. FXII binding to collagen results in activation of FXII *in vitro* [36]. While only a subset of FXII bond to collagen, they did not appear to undergo autoactivation to alpha-FXIIa or beta-FXIIa fragments after binding to surfaces. The present results show that UM8190, a reference material, was accumulated on stratum corneum with a similar water content to that of FXII-treated stratum corneum. Previous results showed that while UM8190 penetrated SC, FXII did not [20]. Thus, both the terahertz absorbance spectral analysis [20] and the present investigation stipulate each other.

The epidermal differentiation is very dynamic and the stratum corneum is its final step, which can be considered the depositary of the epidermal transformation, considering traces of tissue activity during this process. In viable epidermis several proteoglycans such as syndecans, epican, perlecan and glypican may be found in extracellular sites. The sugar moieties may play an important functional role in the SC formation, cohesion, and desquamation during epidermal differentiation. Evidence shows that Heparin Sulfate Proteoglycans (HSPGs) are involved in contact activation [37]. Heparin and heparin sulfate, members of the glycosaminoglycan (GAG) family, are complex, linear, acidic polysaccharides [38]. The cell surface HSPGs (syndecans, glypican, epican) and matrix HSPG (perlecan) appeared to be ineffective in activating FXII.

The SC is the final interface between our body and the external world. The corneocytes work on the structure of the SC and the desquamation is the orderly process of loss of corneocytes from the surface of the skin. Proteases control some of the final steps of desquamation through the degradation of corneodesmosomes. Tissue kallikreins are very active serine proteases in skin and at least eight members of the kallikrein family are present in human SC. The serine proteases urokinase (uPA) and plasmin are also secreted as zymogens and a cross-talk among all these proteases play an important role in the pathophysiology of the skin [39]. Our data suggest that neither tissue kallikrein nor plasmin was effective in activating FXII. One potential explanation for the inability of these enzymes to activate FXII could be due to inaccessibility to their cleavage site on FXII. Alternatively, both kallikrein and plasmin are apparently inaccessible to the cell surface.

The participation of FXII in the blood coagulation, with respect to its activation state, has a number of implications for processes such as thrombosis and inflammation at the site of tissue injury. The intracellular delivery of the negatively charged formulation is found to be higher than the positively charged nanoemulsion containing prednicarbate in SC [12]. This suggests that the positively charged nanoemulsion, an impermeable molecule per se, can also alter the SC barrier property enough to penetrate at a slower rate. Thus, SC may not be considered as an ideal impermeable membrane. The epidermal barrier, when it is impaired, allows reactions such as inflammation, as a consequence of the enhanced penetration of external antigens leading to systemic immune responses. The barrier function of the skin is largely dependent on the stratum corneum, the outermost layer of the epidermis [40]. Numerous drugs cause a rash or skin changes, suggesting that drug targeted therapy alters both water-impermeable barrier and the immunological barrier [41]. FXII is the primary initiator of the intrinsic pathway of the blood coagulation on non-endothelial surfaces, which is capable of triggering coagulation by activating factor XI. Our data do not establish a positive relationship linking procoagulant surface properties of SC and FXII activation. Further studies are needed to confirm these results.

To understand the factor(s) that influence FXII's adhesive ability it is important to understand its autoactivation properties. Factor XII attachment to tissues is a complex process, affected by plasma, the activating state of the plasma circulating cells, chemical properties of the basement membrane, and the microenvironmental factors. Terahertz waves can penetrate cells, it is considered to be harmless for tissues, proteins and have no ionizing radiation to activate cells or altering the structure of proteins. Taken together, our study indicates the practical potential of TNS3DI to monitor the FXII's cell binding ability in real-time.

CONCLUSION

This paper demonstrates our technique of camera-less terahertz imaging by measuring the conformational changes that occurred upon binding of special proteins, FXII and UM-8190 on human stratum corneum. Much of the work on FXII activation remains a work in progress. Previously, terahertz scanning reflectometry and terahertz spectrometry was used to demonstrate that different spectral density deviations of the signal are due to the structural alterations and activation of FXII induced by the hydrated stratum corneum and UM-8190. The present terahertz imaging demonstrated that a subset of zymogen FXII bond to the stratum corneum. FXII was not significantly accumulated on the hydrated stratum corneum. However, formed activated FXII (FXIIa) was observed on the stratum corneum. These findings suggest that negatively charged surface is not a necessary condition for FXII to bind on the stratum corneum. Further, the present work also establish that the camera-less terahertz imaging technique is effectively useful for soft-tissue imaging both on the surface and under the surface.

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DECLARATION OF INTEREST

The authors declare that there is no conflict interest that could be perceived as prejudicing the impartiality of the research reported.

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