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Fast Surface Acoustic Wave-Matrix-Assisted Laser Desorption Ionization Mass Spectrometry of Cell Response from Islets of Langerhans

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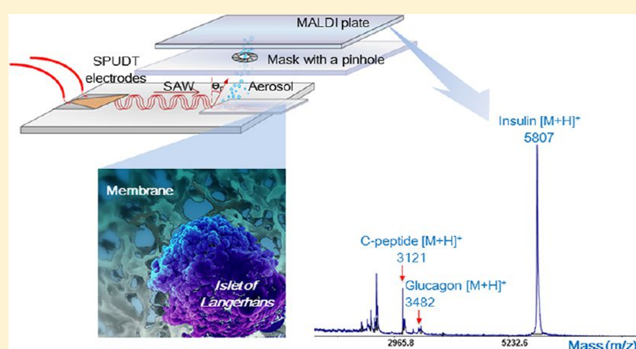
Supporting Information

ABSTRACT: A desire for higher speed and performance in molecular profiling analysis at a reduced cost is driving a trend in miniaturization and simplification of procedures. Here we report the use of a surface acoustic wave (SAW) atomizer for fast sample handling in matrix-assisted laser desorption ionization mass spectrometry (MALDI MS) peptide and protein profiling of Islets of Langerhans, for future type 2 diabetes (T2D) studies. Here the SAW atomizer was used for ultrasound (acoustic) extraction of insulin and other peptide hormones released from freshly prepared islets, stimulated directly on a membrane. A high energy propagating SAW atomizes the membrane-bound liquid into approximately 2 μm diameter droplets, rich in cell-released molecules. Besides acting as a sample carrier, the membrane provides a purification step by entrapping cell clusters and other impurities within its fibers. A new SAW-based sample-matrix deposition method for MALDI MS was developed and characterized by a strong insulin signal, and a limit of detection (LOD) lower than 100 amol was achieved. Our results support previous work reporting the SAW atomizer as a fast and inexpensive tool for ultrasound, membrane-based sample extraction. When interfaced with MALDI MS, the SAW atomizer constitutes a valuable tool for rapid cell studies. Other biomedical applications of SAW-MALDI MS are currently being developed, aiming at fast profiling of biofluids. The membrane sampling is a simplistic and noninvasive collection method of limited volume biofluids such as the gingival fluid and the tearfilm.



Over the years, bioanalysis has continually benefited at various levels from miniaturization, smaller sampling volumes,[1] and easier handling to dramatically shorten processing time. Matrix-assisted laser desorption ionization mass spectrometry (MALDI MS), an indispensable tool in protein analysis,[2−4] is usually preceded by multiple steps of sample pretreatment and purification which not only are time-consuming but might also lead to MALDI artifacts.[5] Here we introduce the surface acoustic wave (SAW) atomizer, a tool for fast ultrasound, e.g., "mechanical" sample extraction[6] from living cell samples in MALDI MS analysis.

The atomizer consists of a low-loss piezoelectric substrate where the sample droplet is placed (Figure 1A) with two single-phase unidirectional transducers single phase unidirectional transducer (SPUDT) electrodes (Figure 1B) driven at 30 MHz in this study by an electrical supply.[6−8] When the Rayleigh SAW propagates under the membrane-bound sample, SAW energy is transferred into the membrane (Figure 1C) and capillary waves[7] are generated in the fluid on the sample's surface. Because of the tremendous accelerations induced by the 30 MHz frequency SAW atomizer, the capillary waves destabilize and eject droplets from their crests; the aerosol generated possesses a nearly monodisperse diameter distribution centered at a value in the range of 2 μm that depends on the fluid's viscosity and surface tension.[7] Cavitation is absent due to the fact that the propensity of cavitation nucleation is inversely dependent on the frequency squared,[6−8] and at the frequencies used in the SAW, the power required is at least 3 orders of magnitude greater than that used for atomization. The effect of fluid shear is minimized due to the short exposure time of only a few microseconds and the fact that the time scale of SAW-driven oscillation is far away from the typical resonant time scales of molecules that might incur shear-induced damage. An insignificant degradation of less than 1% was reported for SAW atomizer-extracted ovalbumin and bovine serum albumin (BSA) for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.[6] The open format of the SAW atomizer[9] circumvents the use of sample transferring

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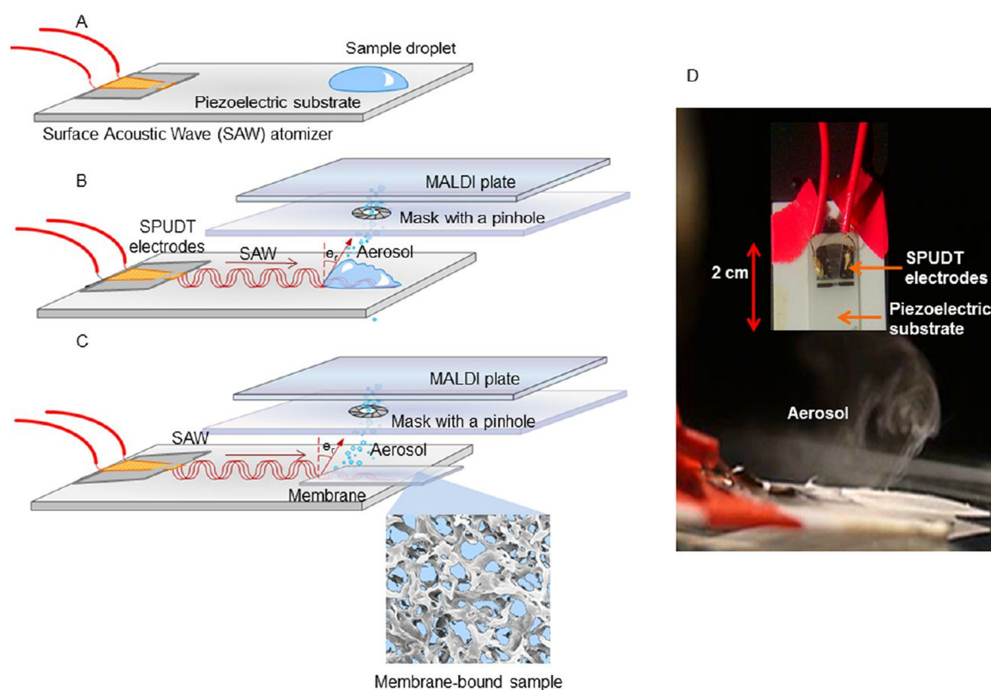

Figure 1. SAW atomizer's working principle and experimental setup. The atomizer consists of a piezoelectric material with two SPUDT electrodes which generate unidirectional Rayleigh-wave SAW that propagate toward and underneath the sample. (A) Resting droplet on the SAW atomizer, on which no SAW excitation is present. (B) The SAW has reached the droplet, destabilized, and generated a liquid aerosol with a centered monodisperse diameter of 2 μm. (C) High energy SAW drive atomization even when the liquid is previously sampled on a membrane. The resulting aerosol (B,C) are collected on a MALDI plate via a pinhole for MS analysis. (D) Frontal image of the SAW atomizer (above) and lateral image of the working atomizer (below).

microchannels[10,11] and related clogging risk. Finally, SAW atomization is simple; is essentially instant-on, instant-off technology; economical with power consumption of less than 3 W; and requires minimal personnel training. It is but one example of the burgeoning field of fluid and particle manipulation using acoustics that is solving a broad array of problems in microfluidics.[6,8]

In combination with simple filter paper or with a purpose-selected membrane, upon application of the living cell sample, the SAW atomizer offers a purification and extraction system; impurities such as cell debris or cell clusters are likely to get entrapped within the membrane fibers. Successful SAW membrane-extraction of low molecular weight analytes from blood and water have already been demonstrated by interfacing SAW with ESI-MS.[8,12] MALDI MS is better suited for direct analysis of complex biological samples due to the relatively high tolerance of impurities and simplified spectra mostly consisting of single protonated species.[13] Nevertheless, multiple experimental factors determine the spectral quality, of which the sample and matrix deposition on the MALDI plate is essential.[14,15] Ideally, the sample-matrix layer for analysis contains small, homogeneous, and equally distributed matrix crystals embedding the sample analytes. Several deposition methods to date suffer from uneven analyte distribution within the MALDI spot;[16] therefore, manually searching for "hot" spots, regions of the preparation that yield higher signal intensities, is required. Thin matrix-sample layers are reported to ionize easily and lead to high signal intensities.[17−19] The SAW-membrane platform is here used in fast screening of islet hormone secretion for future type 2 diabetes (T2D) studies in parallel to its use as a tool for thin layer MALDI sample preparation.

The disease arises from the interplay of several factors of which imbalance of pancreatic hormones, responsible for carbohydrate, fat, and protein metabolism regulation is a major contributor. Pancreatic hormones are produced from cell clusters named islets of Langerhans (Figure 2). Every islet harbors 1 000−3 000

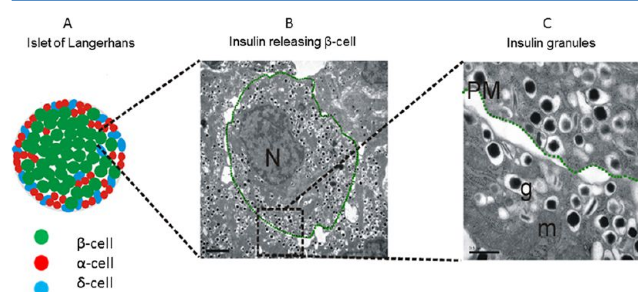

Figure 2. Illustration of an islet of Langerhans in mice. The β-cells (A), depicted in green, dominate the core of the islet, mostly surrounded by glucagon releasing α-cells (red). The electron microscopy (EM) picture (B) shows one insulin-releasing β-cell. Higher magnification of two adjacent β-cells shows numerous insulin-granules anchored in the plasma membrane. N, nucleus; g, granule; PM, plasma membrane; m, mitochondria. The green line indicates the plasma membrane. Scale bar 2 μm (B) and 0.5 μm (C).

cells of five different cell types named α, β, δ, PPi, and ε-cells. In humans, insulin-releasing β-cells constitute the main population (48−59%) followed by the glucagon-releasing α-cells (33−46%).[20,21] Glucose is the main regulator of both insulin and glucagon release, while hormones[22−24] or neurotransmitters like acetylcholine[25] modulate the response from the islet's cells. While impaired insulin release is an important factor for the disease onset, T2D patients are characterized by elevated levels

of circulating glucagon,[22] suggesting that α-cells might also play a key role in the T2D pathophysiology. The contribution of other cell types, (δ, PPi, and ε-cells) constituting a minority of the islet mass, in the disease onset is even more obscure and requires further research.

Comparative proteomics of healthy versus diseased islets as endocrine units might help identify T2D biomarkers and highlight T2D's pathogenesis. In our forthcoming studies, we intend to use the SAW atomizer as a tool for quick sample handling in MALDI MS. The chip performs an instant ultrasound-based, membrane purification/extraction of the sample, reducing the use of vials, containers, and related analysis cost.

■ EXPERIMENTAL SECTION

Reagents. Acetylcholine chloride, ammonium citrate, and bovine transferrin were purchased from Sigma-Aldrich (Buchs SG, Switzerland), human insulin from Novo Nordisk, and α-cyano-4-hydroxycinnamic acid (CHCA) from Bruker Daltonics. TFA was purchased from Merck. Islets were freshly prepared and supplied from the Clinical Research Center (CRC), Malmö. Quantitative, medium-wide pores (5−6 μm pore size) filter papers were provided by Munktell Filter AB (Falun, Sweden). Acetonitrile and Milli-Q water were of LC gradient grade. Roswell Park Memorial Institute (RPMI)-1640 media was purchased from Sigma Aldrich, while *N*-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammoniumsalt (Fluorescein-DHPE) was from Invitrogen UK.

Biological Sample Preparation. Mice used in the experiments were female NMRI (Crl:NMRI(Han) (outbred), 8 weeks old. Approximately 100 islets were isolated from each mouse, allowing for 15−20 performed experiments. After isolation, islets were suspended in RPMI-1640 medium . Prior to the analysis, they were transferred in HEPES-buffered medium, pH 7.4 (25 mM HEPES, 125 mM NaCl, 5.9 mM KCl, 12.8 mM $CaCl_2$, 1.2 mM $MgCl_2$) with various concentrations of stimulating glucose content varying from 10 to 30 mM. For the control experiments, the buffer was supplied with 3 mM glucose, a concentration below the insulin-release threshold (7 mM),[26] and acetylcholine varying in concentration (0, 1, and 100 μM). Bovine transferrin, used as a matrix additive in the insulin limit of detection (LOD) evaluation, was prepared at concentrations of 10 μM and 5 μM.

Stimulation of Islets on the Atomizer. A volume of 2 μL of HEPES buffer, containing typically 2−6 islets, was pipetted on a dry, unpretreated membrane placed on the atomizer. An equivalent volume of acetylcholine(aq) was subsequently applied on the islets located onto the membrane. Although not being the main grounds of this work, stimulation of a single islet was performed on the bare chip without employing the membrane. In this case, 1 μL of HEPES buffer containing one islet was placed on the chip followed by the addition of 1 μL of acetylcholine(aq). Stimulation time with acetylcholine was 5 min in all experiments. Stimulated islets, either on the membrane or directly on the chip, were supplied every 2 min with 0.5 μL of water to compensate for the evaporation.

Electron Micrscopy. The samples in the electron micrographs were fixed in 2.5% glutaraldehyde and 1% osmiumtetroxide prior to embedding in AGAR100 as previously described by Andersson et al.[27] Images were taken using a JEM 1230 electron microscope.

Fluorescent Nanoparticles. Lipid-based nanoparticles were prepared as described by Nilsson[28] with supplementary fluorescein DHPE.

Surface Acoustic Wave Atomizer. The SAW atomizer consists of a low-loss piezoelectric, single crystal lithium niobate 127.68° Y-rotated cut, X-propagating material sputtered with two chromium−aluminum SPUDT. When a sinusoidal electrical signal is applied between the electrodes, Rayleigh surface acoustic waves of a few nanometers in amplitude are produced and propagate unidirectionally along the *X*-axis of the substrate, toward and underneath the membrane-bound sample. The transverse component of the SAW is progressively absorbed into the fluid, generating a simple sound wave that propagates at the so-called Rayleigh angle that is only dependent upon the relative difference in the sound speed between the SAW in the lithium niobate and the sound wave in the fluid.[8] The sound wave propagating in the fluid drives the formation of a capillary wave along the fluid present atop the membrane and due to the extreme accelerations of over 108 m/s^2 present at the fluid interface, and droplets are driven from the crests of the destabilized capillary wave without cavitation to form a micrometer-order aerosol.[6] The SAW atomizer was fabricated in house by the Melbourne Centre for Nanofabrication.

Matrix Preparation. In our experiments, 2−5 mg/mL CHCA was dissolved in 50: 50:0.1% water, acetonitrile, and trifluoroacetic acid (TFA) with supplementary 2 mM ammonium citrate. In the LOD evaluation experiments, transferrin (5 μM and 10 μM) was used as a matrix additive[29] for insulin signal enhancement (Figure 7, pink trace).

Stainless Steel Plate Mask. A plate mask, set atop a plastic surface possessing a 10 mm diameter opening corresponding to 10 neighbor spots on the MALDI plate, was used to prevent the

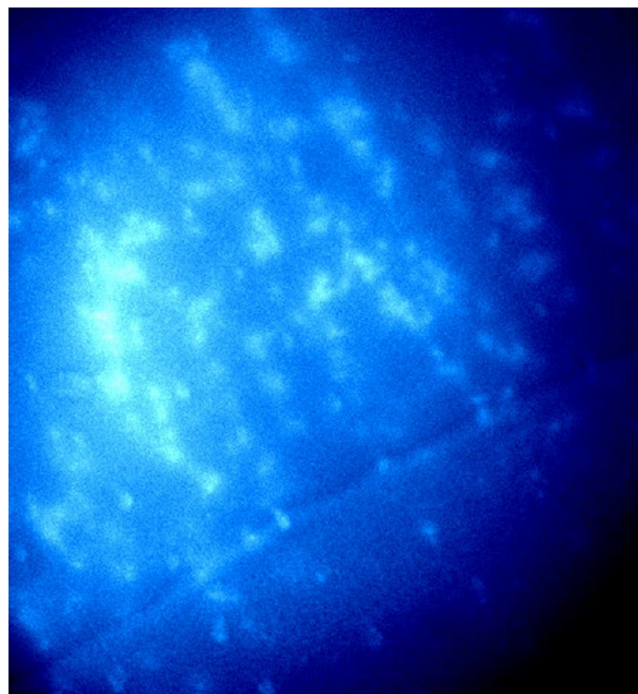

Figure 3. Visualization of SAW-extracted fluorescent nanoparticles atop a collection membrane. Nanoparticles embedded in a membrane placed on the atomizer are extracted via SAW atomization and collected on a second membrane positioned parallel to and above the atomizer, in a fashion similar to the MALDI plate. The collection membrane shows numerous SAW-extracted fluorescent nanoparticles; wherein the brighter spots represent aggregates of the extracted nanoparticles.

aerosol's deposition throughout the plate's area. The membrane size was 5 mm × 8 mm.

MALDI MS Instrument. MALDI-MS analyses of the crystallized samples were performed on a 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Framingham, MA). MS data acquisition (3000 laser shots per spot) was performed in positive linear mode. Stainless steel plates with 192 positions were used for the trapping of SAW-generated aerosol.

Sample-Matrix Deposition Method. Sample and matrix were consecutively SAW-extracted from the membrane and deposited onto a controlled area of the MALDI plate. Every SAW extraction/deposition event takes less than 15 s; therefore, sample and matrix SAW extraction/deposition takes approximately 30 s. The sample was prepared as previously described (see Stimulation of Islets on the Atomizer). A volume of 4 μL of CHCA was used for matrix-membrane saturation, following the first event of SAW extraction/deposition of the sample. When a single islet was stimulated on the chip (no membrane used), a volume of 2 μL of CHCA was applied on the cleaned atomizer.

SAW Atomizer Cleaning. After each experiment, the piezoelectric substrate was cleaned with acetone and isopropanol and rinsed with Milli-Q water as described by Ho et al.[8]

■ RESULTS AND DISCUSSION

Fluorescent Microscopy Visualization of SAW Extracted Fluorescent Nanoparticles. First, a simple approach to demonstrate the SAW operation was carried out through the extraction of fluorescent nanoparticles.[28] Nanoparticles extracted from the membrane via SAW atomization were collected on a second membrane that was subjected to conventional fluorescent microscopy (FM, Figure 3). The increases in nanoparticle concentration lead to enhanced FM signaling suggesting the role of the SAW in the extraction process.

SAW-MALDI MS Spectra of Healthy Islet Secretion. Fast MALDI MS spectra of stimulated, intact islets from healthy mice are shown in Figure 4. Islet's chemical stimulation (30 mM glucose, 100 μM acetylcholine) has selectively triggered the healthy β-cells to release insulin (Figure 4); therefore, the acquired spectra are characterized by intense peaks of insulin (relative intensity). Other cosecreted molecules such as C-peptide and amylin are also detected. Acetylcholine can evoke insulin release under nonstimulatory glucose (3 mM) at high concentrations (100 μM) only. However, in this case, insulin release is considerably reduced (spectra not shown).

The second largest cell community of the islet, α-cells, respond completely opposite to β-cells toward glucose concentrations. While high glucose above 7 mM triggers mouse β-cells to release insulin, glucagon-secretion at these higher concentrations is suppressed.[30] Low levels of glucagon in healthy individuals keep the glycemic index in control by suppressing hepatic glucose output. However T2D is characterized by reduced glucagon suppression,[22] a subject which has gained high interest in recent years. Other α-cells released peptides like glicentin-related polypeptide (GRPP), the incretin glucagon-like 1 (GL-1), and δ-cell's somatostatin-14 peptide (SMS-14) are also indicated in the spectra (Figure 4).

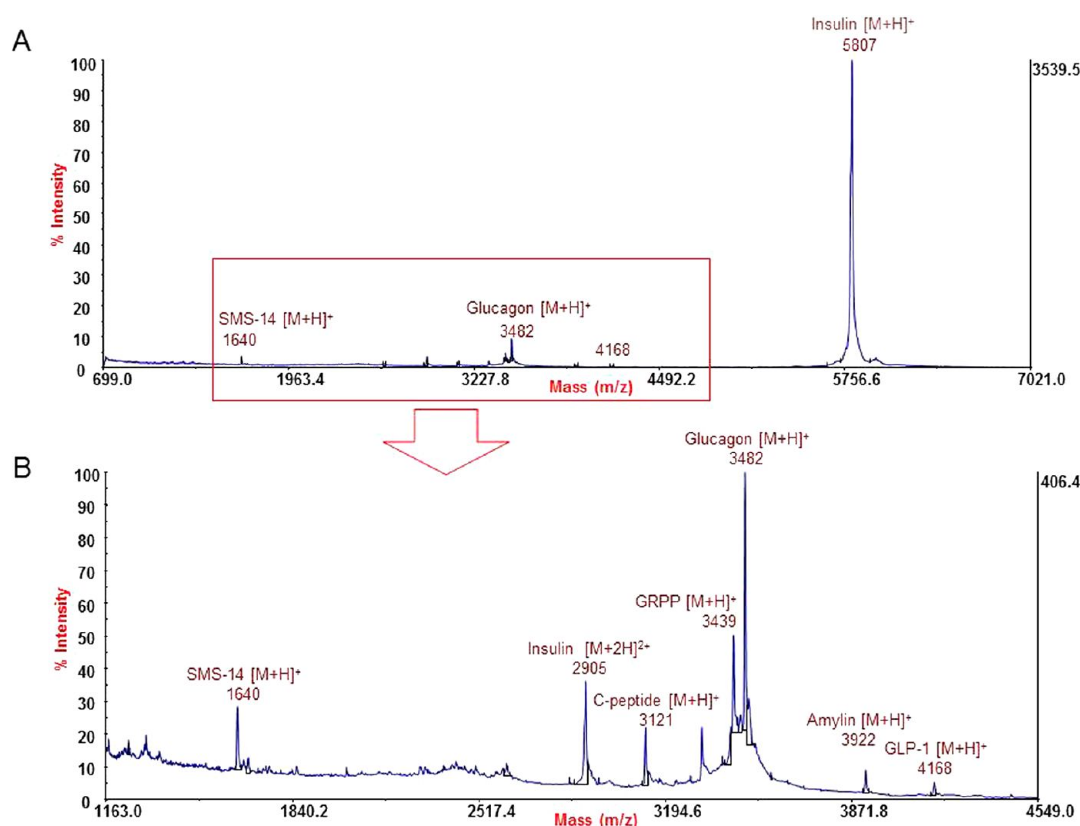

Figure 4. SAW-MALDI MS spectra of stimulated islets of healthy mice. (A) Acquired spectra from three islets in 30 mM glucose HEPES using the SAW-spotting. Single-charged insulin (5807 Da), glucagon (3482 Da), and SMS-14 (1640 Da) peptides are labeled. (B) A magnification of the preceding spectra reveals several peaks, corresponding to single-charged peptides released from α-cells: GRPP (3439 Da), glucagon-like peptide 1 (GLP-1; 4168 Da), β-cell released C-peptide (3121 Da), amylin (3922 Da), and somatostatin (SMS-14, 1640 Da) released from δ-cells. The double charged insulin is also detected (2905 Da).

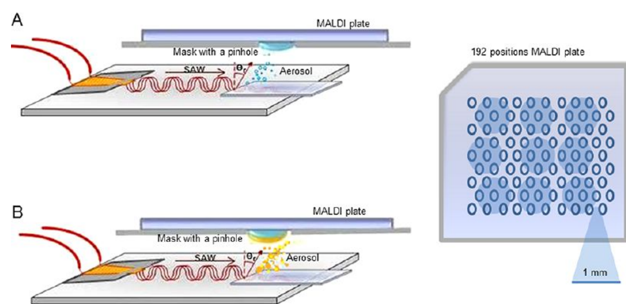

Figure 5. Illustration of the SAW-deposition method. (A) The membrane-bound sample is first extracted with SAW atomization and deposited on a controlled area (by a pinhole in the plastic mask) of the MALDI plate. (B) Following sample extraction, the matrix solution is applied on the same membrane and subsequently deposited on the plate using the SAW. (C) Depiction of the area where the thin film of SAW-extracted sample and matrix are deposited on the MALDI plate.

Sample-Matrix Deposition Method. In MALDI MS, informative and qualitative spectra acquisition is greatly dependent on the procedure of how the sample and matrix is deposited on the MALDI plate.[14,31] Additives to the matrix solution are also important; it was shown previously that the addition of phosphoric acid[32] or transferrin[29] to the matrix solution improves the detectability of intact protein. Our acquired spectra, characterized by high signal intensities and "run to run" reproducibility, have been acquired with the "SAW-sampling/SAW-matrixing" on the plate.

According to our developed method, the membrane-bound sample is SAW-extracted and deposited on the MALDI plate (Figure 5). In a second step, the same volume of matrix is applied on the membrane, subsequently, SAW-extracted, and deposited atop the sample film on the MALDI plate. The overall time of SAW sample-matrix deposition takes approximately 30 s, where every SAW extraction event takes less than 15 s.

The SAW-deposition method resulted in thin layer formation of the sample and matrix that ionizes easily and is characterized by high signal intensities.[17−19] Spraying sample and matrix on the MALDI plate with an electrospray needle and piezoelectric microdispenser[33] has also been reported to form homogeneous matrix-sample crystals characterized by high signal enhancement and reproducibility.[34−37] Besides its obvious advantages, electrospraying the matrix-sample mixture nevertheless has its downsides, including the necessity of extra equipment (e.g., transformers, etc.) and dangerously high voltages (3−5 kV). Thus, another advantage of the SAW chip emerges in our study as an easy, fast, and safe tool for thin matrix-sample crystal layer deposition for MALDI MS.

Initially we tested the "Quick & Dirty", the "Matrix-precoated layers", and the "Fast evaporation" methods of sample-matrix deposition.[15] In any method, the matrix−analyte volume ratio[38] (under matrix molar excess conditions) is of particular importance. However, small matrix pipetted volumes (0.5 μL) inundated the thin film of the SAW-extracted sample leading to loss of signal.

Reproducibility and LOD. The SAW-deposition strategy favors thin sample layer formation, associated with high signal intensities and reproducibility (Figure 6). The acquired MALDI spectra of stimulated intact islets are characterized by high S/N values of single charged insulin.

The lower insulin LOD, estimated by serial dilutions of insulin stock solutions, using the SAW-deposition method was less than 100 amol, with 2 mg/mL CHCA as a matrix (Figure 7). The signal was significantly enhanced, by at least 1 order of magnitude, when the matrix was premixed with 5 μM transferrin in a 5 to 1 ratio. Our results are in good agreement with previous

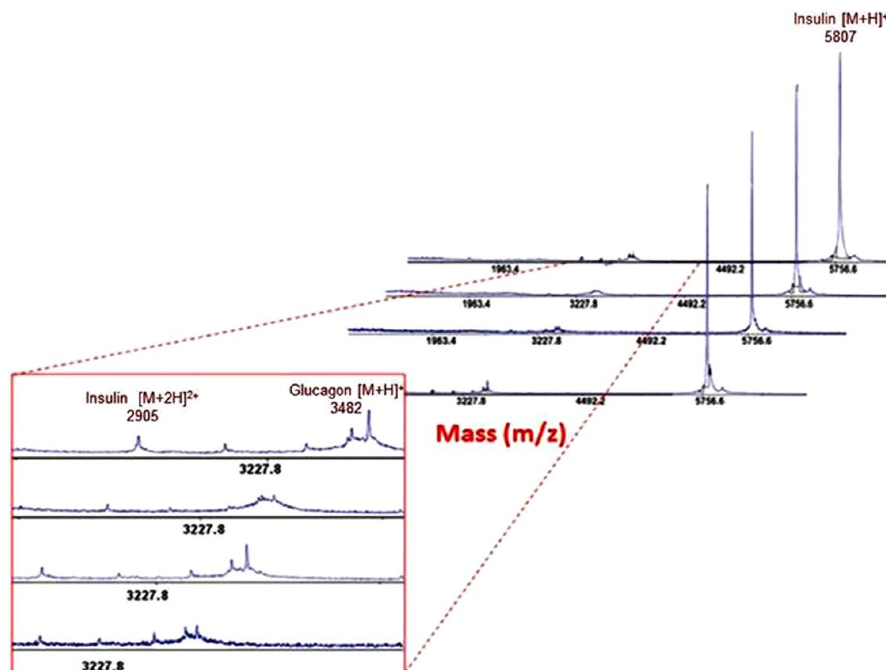

Figure 6. The MALDI MS spectra acquired and shown in this figure, were acquired by stimulation of four islets. Each MALDI spectra is characterized by a large peak corresponding to the single-charged mouse insulin (MW 5807 Da) and a small peak of single charged glucagon (MW 3482 Da). The double-charged insulin (2905 Da) is also detected. In the inset, a magnified view of the islet's response spectra is shown. Thorough investigation of low abundance peptide and proteins is needed to find new T2D markers resulting in a better understanding of the molecular mechanisms underlying the T2D disease.

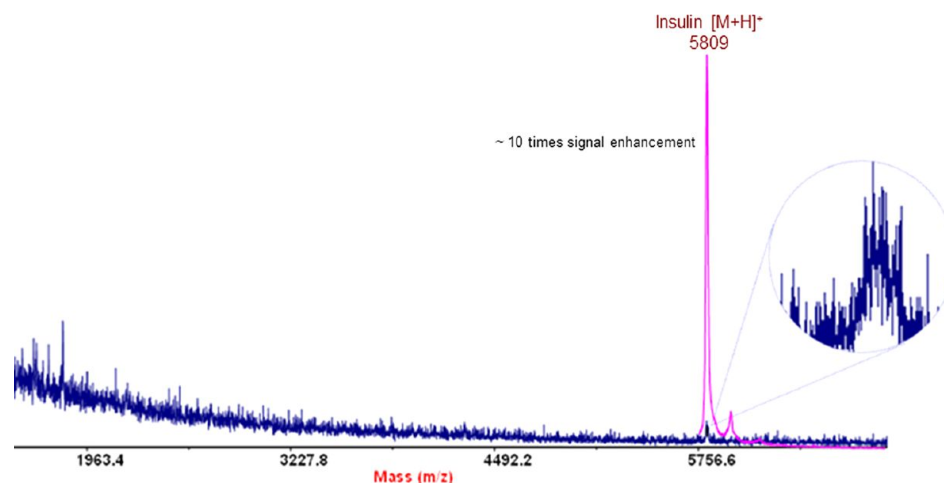

Figure 7. MALDI spectra obtained from SAW-spotting of 100 amol of human insulin (MW 5808 Da) without (blue trace) and with transferrin (red trace) in the matrix (2 mg/mL) premixed in a 5 to 1 ratio with 5 μM transferrin.

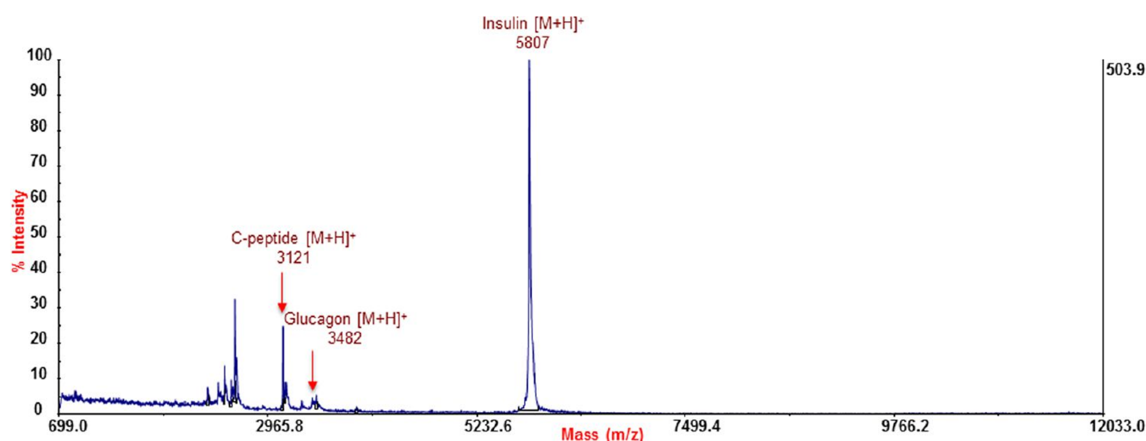

Figure 8. Single islet MALDI spectrum obtained by stimulating (100 μM acetylcholine, 30 mM glucose, HEPES buffer) the islet present on the naked chip, e.g., without membrane purification.

studies suggesting enhanced properties of transferrin when premixed with matrixes.[29] The enhancing properties of transferrin-premixed matrixes on insulin and other protein/peptides released by stimulated islets will require further study.

Islet Response Sampled on the Bare Chip. The membrane, as mentioned earlier, aids sample prepurification, "desalting", and thin crystal layer formation on the MALDI plate leading to qualitative spectra. However the atomization could be carried out without the membrane when "pure sample" is used and thus decreasing the risk of membrane sample retention that could be critical for detecting scarce analytes. Figure 8 shows the MALDI spectra from a freshly prepared and thoroughly washed single islet stimulated directly on the bare chip. The acquired spectra are characterized by a good baseline; signals corresponding to insulin, C-peptide, glucagon, and other small peptides are also detected.

■ CONCLUSIONS

The SAW atomizer can be used as a fast, ultrasound extraction tool for small volume, complex samples from a membrane. We have successfully for the first time (to our knowledge) hyphenated the SAW open chip with MALDI MS. In our experiments it was used for the extraction of islet hormones released upon stimulation with glucose and acetylcholine for future T2D studies. Since the sample here is minimally chemically pretreated, the risk of MALDI artifacts is considerably reduced. Furthermore, we have developed a SAW-based sample-matrix deposition method that generates MALDI spectra characterized by high signal intensities of the analytes with high "run to run" reproducibility. The LOD for insulin was 100 amol; however, when transferrin was premixed with the matrix, the signal was enhanced to 10 amol. Reduced sample handling analysis times, qualitative MALDI spectra acquisition, and membrane sample preservation reduces the overall cost of biomolecular screening such as comparative MS protein profiling for T2D biomarkers. Future use of SAW-MALDI MS in single cell studies can be foreseen. We are currently investigating other applications of SAW-MALDI, such as relative quantification and rapid peptide and protein profiling of membrane preserved, small volumes (less than 0.5 μL) of biofluid.

■ ASSOCIATED CONTENT

Supporting Information

Additional SAW-MALDI MS spectra and table of relative intensities of labeled peptides in the spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.
Restricted parts of this work have been presented in MS-Öresund (Sweden, 2011) and Analysdagarna (Sweden, 2012).

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