

# Airborne system in Type 2 Diabetes studies

Research focused on Langerhans islets

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Thesis of International Master in Chemistry program, Lund  
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## Summary:

The explosive increase in expansion of diabetes mellitus, particularly type 2 diabetes (T2D) is becoming a threat to public health which is associated with modern lifestyle, abundant nutrient supply, reduced physical activity, and obesity. It is realized that T2D only develops in insulin-resistance subjects which starts with  $\beta$ -cell dysfunction. In order to clarify the relationship between hormone release and metabolism in isolated pancreatic islets, the secretory effects of a non-fuel stimulator like acetylcholine was studied.

The miniaturized "airborne system" was combined with MALDI-MS to acquire data from single Langerhans islets and further  $\beta$ -cell metabolism. Levitation technique is a method which has been developed to study intra-and extra- cellular reactions at single or few cell levels. With this technique it is possible to handle 50 nl to 1500 nl sample volumes without contamination from solid walls. In combination with this method a CCD camera was used to check the islet in droplet during the experiments for surveillance of the droplet, for example it was possible to take pictures in different periods of time to calculate droplet size after evaporation to understand change in concentration by time.

MALDI-TOF mass spectrometry is a sensitive analytical technique with good limit of detection. In this work it was combined with airborne system (levitation technique) to analyze proteins/peptides that are released from Langerhans islets.  $\alpha$ -cyano-4-hydroxy Cinnamic acid (CHCA) was used as matrix to crystallize the sample on MALDI plate. There are three different components in the MS 1. Ionizer, 2. Mass analyzer, and 3. Detector. **An Ionizer** is used to ionize the proteins and peptides from activated single Langerhans islet, then there is a **Mass analyzer** that differentiates the Ions with respect to the Mass/Charge (M/Z) finally a **detector** to measure the Ion beam current.

In this study, a single Langerhans islet picked for each experiment and it was positioned in the levitator with a fused silica capillary. Stimulation of insulin secretion from the islets of Langerhans was accomplished by adding acetylcholine to the levitated droplet and addition of stimulators were stopped after desired concentration. A piezoelectric dispenser was used to add all the reagents to the droplet. At the end, epinephrine was added to stop the reaction and a fused silica capillary was used to transfer the droplet to MALDI plate. Sample was crystallized with CHCA matrix and it was analyzed with MALDI-MS spectrometry.

Peaks observed from mass spectrometry gave information about molecular mass, intensity and charge of the detected proteins and peptides.

The whole process is shown in Figure 1:

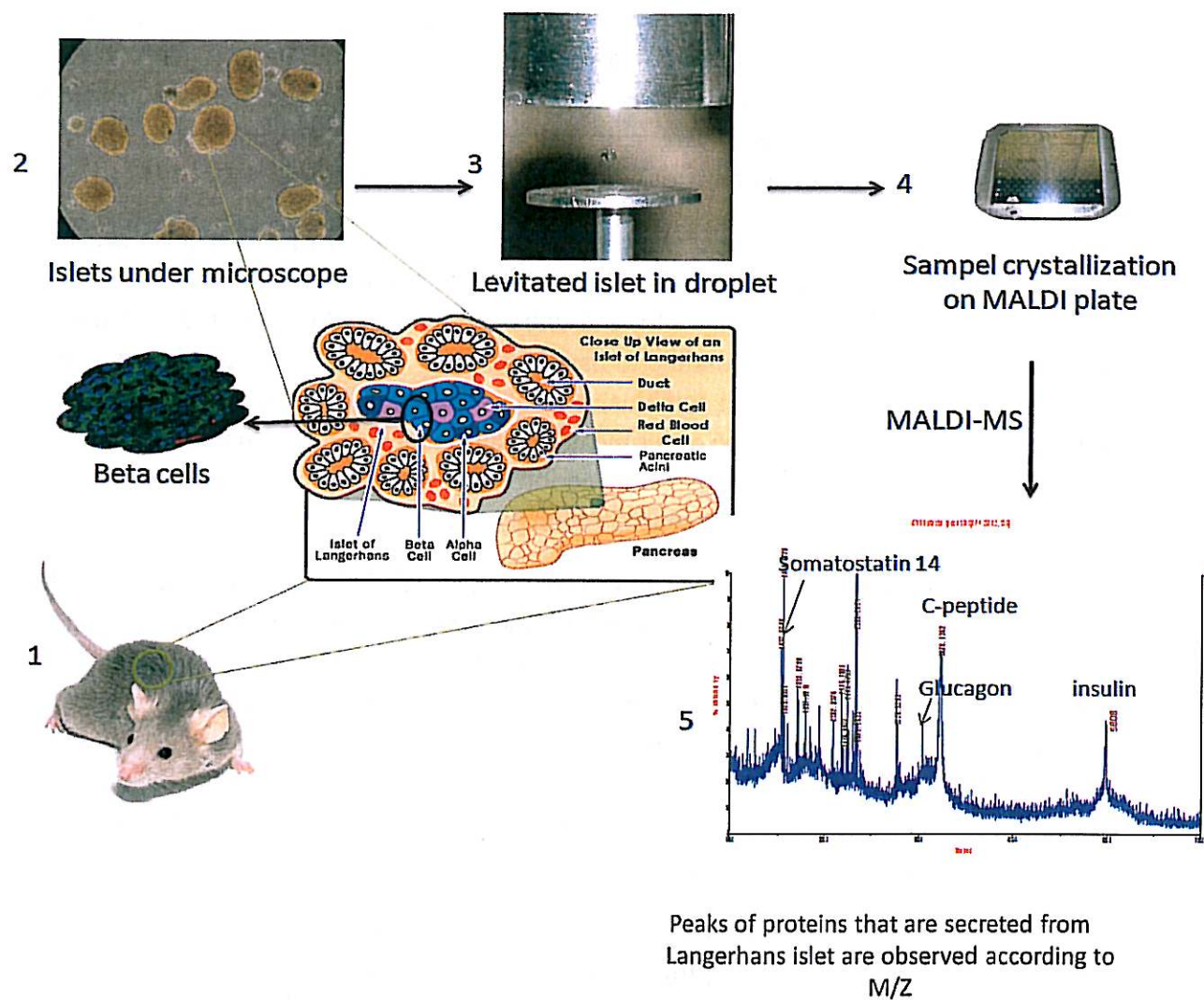


Figure 1: Schematic view of work flow, using airborne system to study Langerhans islets.

Keywords:  $\beta$  cells, Insulin, acetylcholine chloride, MALDI-TOF mass spectrometry, MALDI LTQ Orbitrap XL, Epinephrine.

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List of abbreviations:

Ach:	acetylcholine
BSA:	bovine serum albumin
CHCA:	alpha-cyano-4-hydroxy cinnamic acid
MALDI-TOF:	matrix assisted laser desorption ionization time of flight
T1D:	type 1 diabetes
T2D:	type 2 diabetes
PTM:	post translational modification
PKA:	c-AMP protein dependent kinase
VDCC:	voltage dependent calcium channel
IP3:	inositol triphosphate
GLT1:	glutamate transporter 1
GLP-1:	glucagon-like peptide 1
GLP-1R:	glucagon-like peptide 1 receptor
GIP:	glucose dependent insulinotropic polypeptide
PKC:	protein kinase C
GLUT2:	glucose transporter 2
P:	Proinsulin
I:	Insulin
HCD:	higher energy dissociation
L.O.D:	Limit of detection



## **1. Introduction:**

### **1.1 Theoretical background**

#### **1.1.1 Diabetes**

The World Health Organization estimates that 220 million people worldwide today have diabetes and WHO predicts the prevalence worldwide to reach 366 million by 2030 [1] which 90% of them suffer from type 2 diabetes [2].

Diabetes reduces life expectancy because it affects arteries that supply the heart, brain and lower extremities. It damages nerves and blood vessels. Blindness can be one of the consequences of being diabetic which is associated with poor glucose control [3]. High glucose levels lead to kidney failure and amputation.

There are 3 main kinds of diabetes:

- Type 1 diabetes: The body's immune system destroys beta cells as a pathogen. [4]
- Type 2 diabetes: A condition that  $\beta$ -cell in pancreas does not produce enough insulin or target cells cannot use insulin properly.
- Gestational diabetes: In the case of a pregnant woman who has never had diabetes before, has a high blood glucose level during pregnancy.

In this investigation type 2 diabetes and hormones related to this disease are studied.

#### **1.1.2 Type 1 diabetes**

Diabetes type 1 is a chronic disease that usually begins in childhood and adolescence [5] and is due to the antibodies destroying the cells that produce insulin [6]. Type 1 diabetes is partially hereditary, in general it is possible to get it in any age. Insulin therapy is used as a treatment for T1D that can be provided with daily injections of insulin.

With modern treatment and dosing of insulin it is possible to live a good life despite the disease.

#### **1.1.3 Type 2 diabetes**

Type 2 diabetes is the most common type of diabetes and covers about 90% of diabetics. Approximately 300,000 Swedes or four percent of the population suffer from type 2 diabetes [7]. Many have the disease without knowing it. The disease becomes increasingly common, not only in Sweden but throughout the world.

Both genetic and environmental factors contribute to insulin deficiency and insulin resistance. In T2D, beta cell dysfunction plays a major part of the disease. Under normal conditions, blood glucose concentrations are regulated within a very tight range. After a

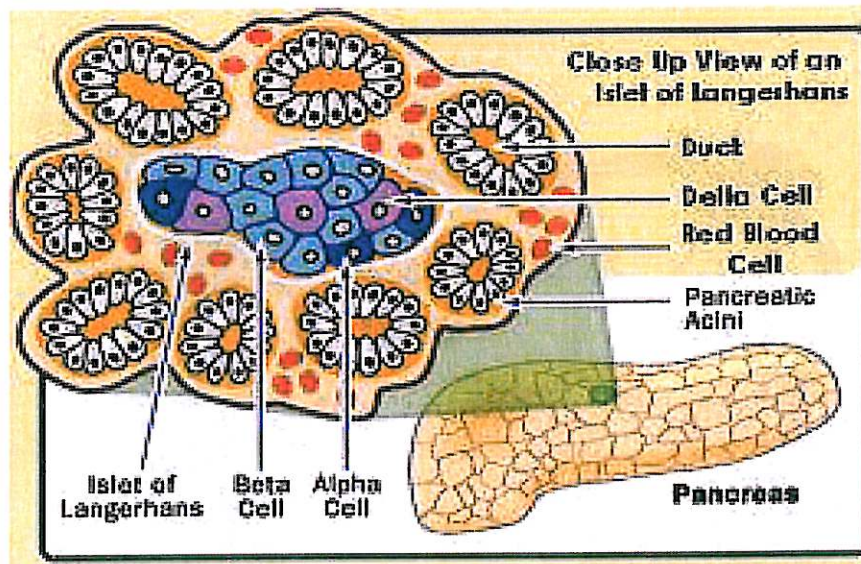
meal, insulin secretion from beta cells is stimulated, whereas glucagon secretion from the alpha cells is suppressed. Insulin facilitates glucose uptake into the cells, and glucagon stimulates glucose production in the liver by releasing glucose from glycogen. Liver cells have glucagon receptors. When glucagon binds to the glucagon receptor, the glycogen polymer is converted to the individual glucose molecules in the liver cells and they will be released into the blood stream.

The expression of the glucose transporter on skeletal muscle seems to be increased with vigorous exercise. Type 2 diabetes usually occurs in adults and, particularly often, in overweight people (over 85% of people with T2D are overweight). However, over the last few years in the U.S, the incidence of type 2 diabetes in children has grown to the point where they now account for 20% of all newly-diagnosed cases (and, like their adult counterparts, they are usually overweight). Recently, a team of researchers of University of Oxford have found that a gene linked to type 2 diabetes and cholesterol levels is in fact a 'master regulator' gene which controls the behavior of other genes found within fat (adipose tissue) in the body [8].

#### 1.1.4 Langerhans islets

Inside the pancreas there are many clusters of cells called islets of Langerhans (see figure 2). Islets of Langerhans are the region of the pancreas that contains its hormone-producing cells. Islets comprise only 1-2% of the pancreatic mass, they are in direct contact with a capillary vessel [9]. Islets receive arterial blood supply from one to three arterioles, which usually branch into capillaries in the  $\beta$ -cell core of the islets[10]. Pancreatic islet size can vary between 50 to 250  $\mu\text{m}$  in size [11] and there are about one million islets in a normal pancreas.

Histologically, the islets consist of four cell types: Alpha, Beta, Delta and PP (polypeptide) cells. Three first cells have great role in hormone release. **Beta cells**, which synthesize and secrete insulin, make up about 60-75% of typical islets. **Alpha cells** are the source of glucagon and comprise 20% of the islet tissue. Glucagon is a polypeptide of 29 amino acids, its secretion is stimulated by low levels of glucose and it is inhibited with high levels of glucose or by amylin [12]. Amylin is secreted from  $\beta$ -cells into the local islet portal circulation and might be carried to  $\alpha$ -cell on its passage to the islet cortex, and act there directly to inhibit glucagon secretion [13]. **Delta cells**, which are considerably less abundant, produce somatostatin. This hormone consist of two polypeptides, one of 14 amino acids and one of 28. Somatostatin has variety of functions including the inhibition of growth hormone secretion from the anterior pituitary gland. Somatostatin 14 has the sequence Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys, with a disulfide link between cysteines 3 and 14. Somatostatin reduce the rate at which food is absorbed from the contents of the intestine [14].



*Figure 2: Close view of different compartments in single islet of Langerhans [15]*

#### **1.1.4.1 Relationship between Alpha and Beta cells:**

**Alpha cells** can behave like adversaries (enemies) for beta cells. Glutamate toxicity is a new mechanism of beta cell destruction which was not previously known. Several studies have revealed that GLT1 (glutamate transporter 1) protein which regulates glutamate can protect beta cells [16]. This protein controls the microenvironment of beta cells with respect to glutamate concentration. GLT1 localizes to the plasma membrane of  $\beta$ -cells, modulates hormone secretion, and prevents glutamate-induced cytotoxicity as shown by the fact that its down-regulation induced  $\beta$ -cell death, whereas GLT1 up-regulation promoted  $\beta$ -cell survival [16].

**Beta cells** synthesize and release insulin, the hormone that controls the level of glucose in the blood. They occupy the central region of the islet or micro lobules within islets, whereas alpha cells occupy the outer rim [17]. Recently pioneers prove that adult pancreatic alpha-cells can be a new source of cells for beta-cell regeneration. It is also possible that regeneration of beta-cell occur by replication of pre-existing beta cells [18].

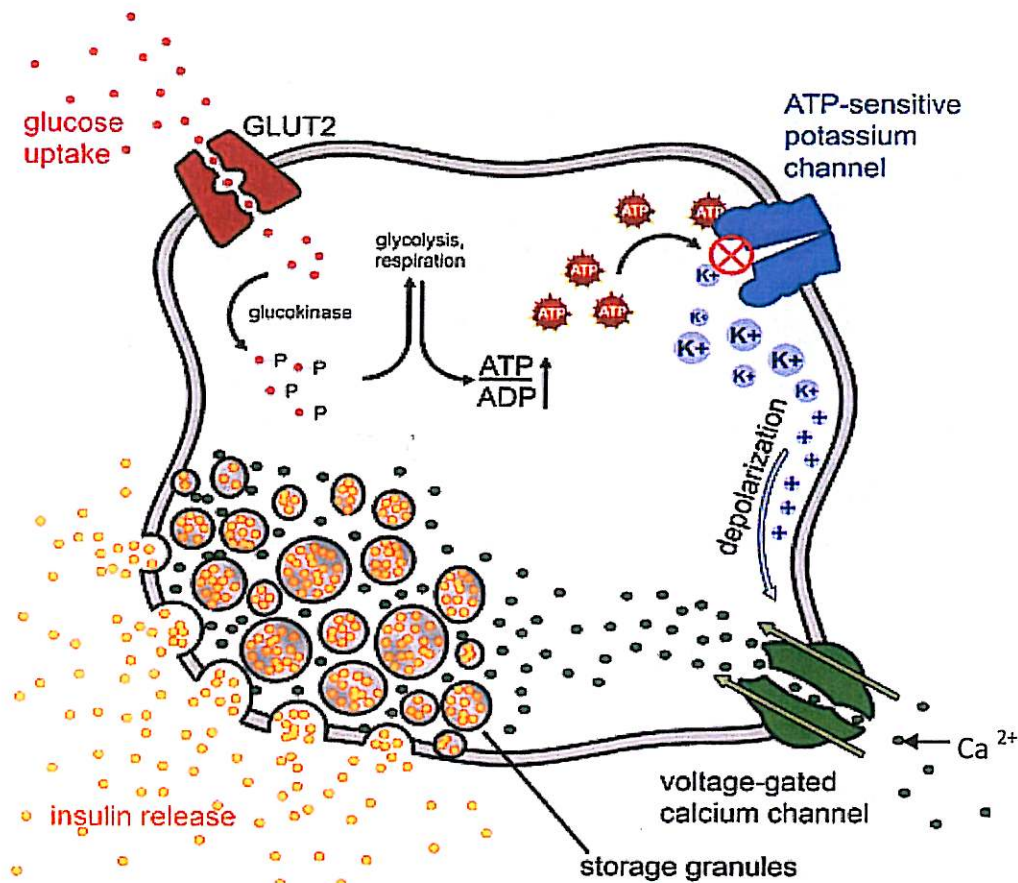


Figure 3: Pathways involved in insulin secretion and glucose uptake through GLUT2 transporter in pancreatic  $\beta$ -cells. [19]

Insulin secretion from beta cells is triggered by rising blood glucose levels. Starting with the uptake of glucose by GLUT2 transporter, the glycolytic phosphorylation of glucose causes a rise in the ATP: ADP ratio. This rise inactivates the potassium channel which depolarizes the membrane, causing the calcium channel to open up allowing calcium ions to flow inward. It causes the exocytotic release of insulin from their storage granule. (Figure 3)

Another factor which contributes to insulin release is glucagon-like peptide (GLP-1). It elevates intracellular cAMP level through the activation of GLP1 receptor (GLP-1R) in beta-cell membrane. It closure K<sup>+</sup> channel and increase Ca<sup>2+</sup> concentration, thereby stimulating insulin secretion and lowering blood glucose [20, 21]. 'Glucose-dependent insulintropic (GIP) is secreted by K cells from the upper small intestine after ingestion of carbohydrates, amino acids and fats while GLP-1 is mainly produced in the enteroendocrine L cells located in the distal intestine after each meal. Their effect is mediated through their binding with specific receptors. (See Figure 4)

### The Effect of GLP-1 and GIP

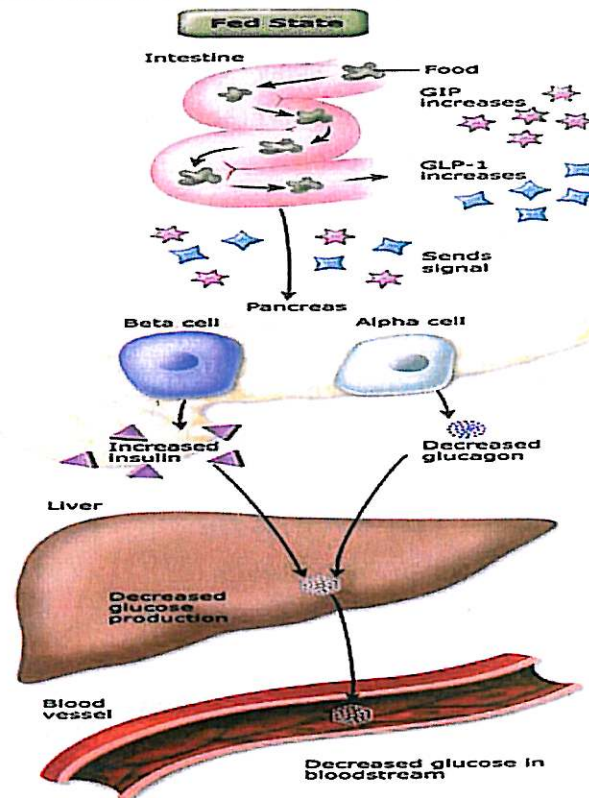


Figure 4: Summary of glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) that secret from the intestine. They secret in response to each meal which lead to stimulation of insulin secretion and lowering glucose level. [22]

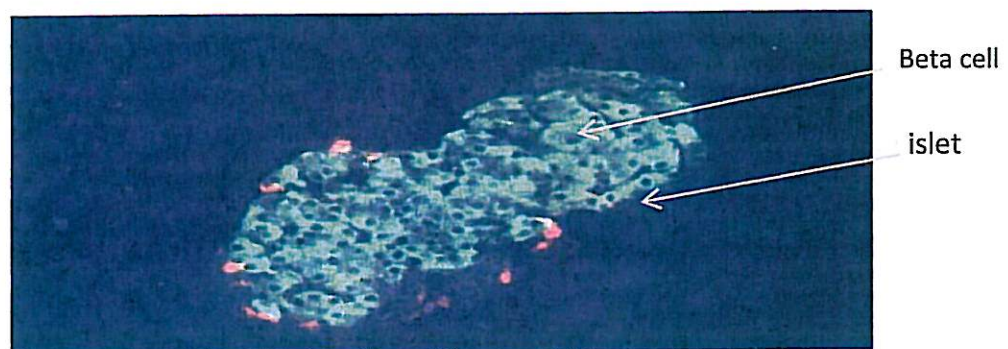


Figure 5: Fluorescent image of Beta cells (blue) and alpha cells (red) in one islet. [23]

### 1.1.5 Insulin:

**Insulin** is composed of two unbranched peptide chains joined together by two disulfide bridges (figure 6 and 7). The two chains of insulin and their disulfide cross-bridges are derived from the single chain proinsulin molecule, from which a 31-residue peptide, called the connecting peptide (C peptide). Conversion of proinsulin to insulin takes place slowly. The C peptide therefore accumulates within granules in equimolar amounts with insulin [24]

(see Figure 6). Type 2 diabetes is characterized by increased proinsulin-to-insulin ratio (p/I ratio), increased glycation and oxidative stress, and  $\beta$ -cell dysfunction [25]. It has been shown that proinsulin levels provides a highly specific predictive marker for insulin resistance in T2D subjects [26], implicating that P/I ratio using proinsulin can be expected to be a more accurate marker of  $\beta$ -cell dysfunction.

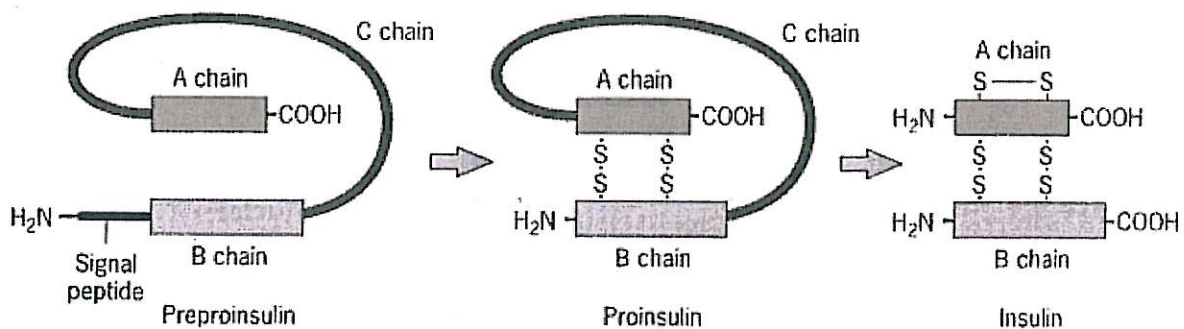
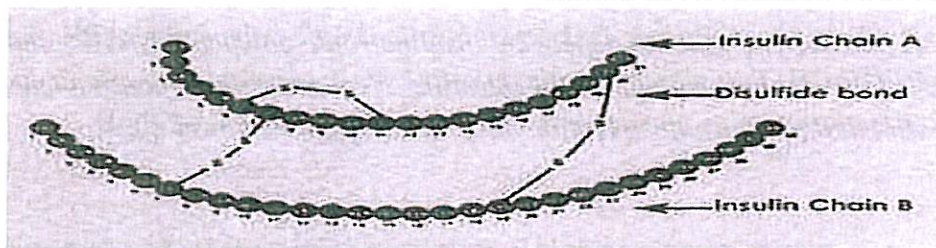


Figure 6: post transitional processing of preproinsulin. [27]

**Insulin** is a small protein consisting of an A chain of 21 amino acids linked by two disulfide (S—S) bridges to a B chain of 30 amino acids.



Beta cells have channels in their plasma membrane that serve as glucose detectors. Beta cells secrete insulin in response to a rising level of circulating glucose.

Figure 7: Structure image if two chains of insulin (A chain and B chain). [28]

Insulin affects many organs. It

1. "Stimulates skeletal muscle fibers to:  
Take up glucose and convert it into glycogen  
Take selenocysteine from the blood and convert them into the proteins
2. Acts on liver cells, stimulating them to take up glucose
3. Acts on fat (adipose) cells by inhibiting the intracellular lipase
4. Acts on cells in the hypothalamus to reduce appetite" [29]

The insulin storage granule contains a variety of proteins that are also released in to the extracellular space whenever insulin is secreted. One such protein is called *amylin*

which is a peptide hormone that may contribute to the amyloid “(insoluble fiber protein)” that accumulates in and around beta cells in state of insulin hyper secretion [30]. It also contributes to loss of  $\beta$ -cell mass in T2D [31].

Amylin is polypeptide of 37 amino acids, some of its actions are:

- “Inhibits the secretion of glucagon;
- Slows the emptying of the stomach;
- Sends a satiety signal to the brain”. [29]

All its action tends to provide insulin and reduce the level of glucose in the blood [32].

## 1.2 Experimental background:

### 1.2.1 Airborne analytical system-ultrasonic levitator and the flow through dispenser

Micro-scale analysis is now becoming more attractive in the research fields of chemistry, biochemistry, biology and pharmacy. To enable miniaturized bio analysis, the airborne analytical system was developed. This system offers some advantages: easy handling of small volumes, decreased risk of adsorption to walls and sample contamination [33, 34].

Acoustic levitation is a wall less test tube. This technique performs contact less handling of small solid or liquid samples in gaseous environment to minimize sample adsorption [35, 36].

An acoustic levitator has two parts, a transducer which is the sound source and a reflector that bounces the sound wave back. Objects will float in the area of the sound waves where it is calm node's area [37]. (Figure 8)

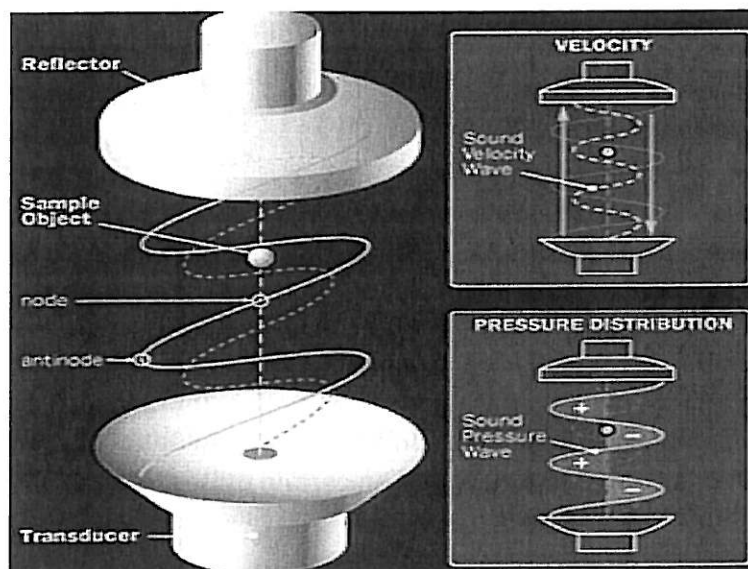


Figure 8: Acoustic levitation uses sound pressure (100Hz) to allow objects to float in calm area of sound waves.[38]

The Flow through droplet dispenser is a reliable sample delivery which can provide highly precise, reproducible liquid supply in the pL range. The dispenser droplets are ejected from a flow through channel formed by joining two micro structured silicon plates [35].

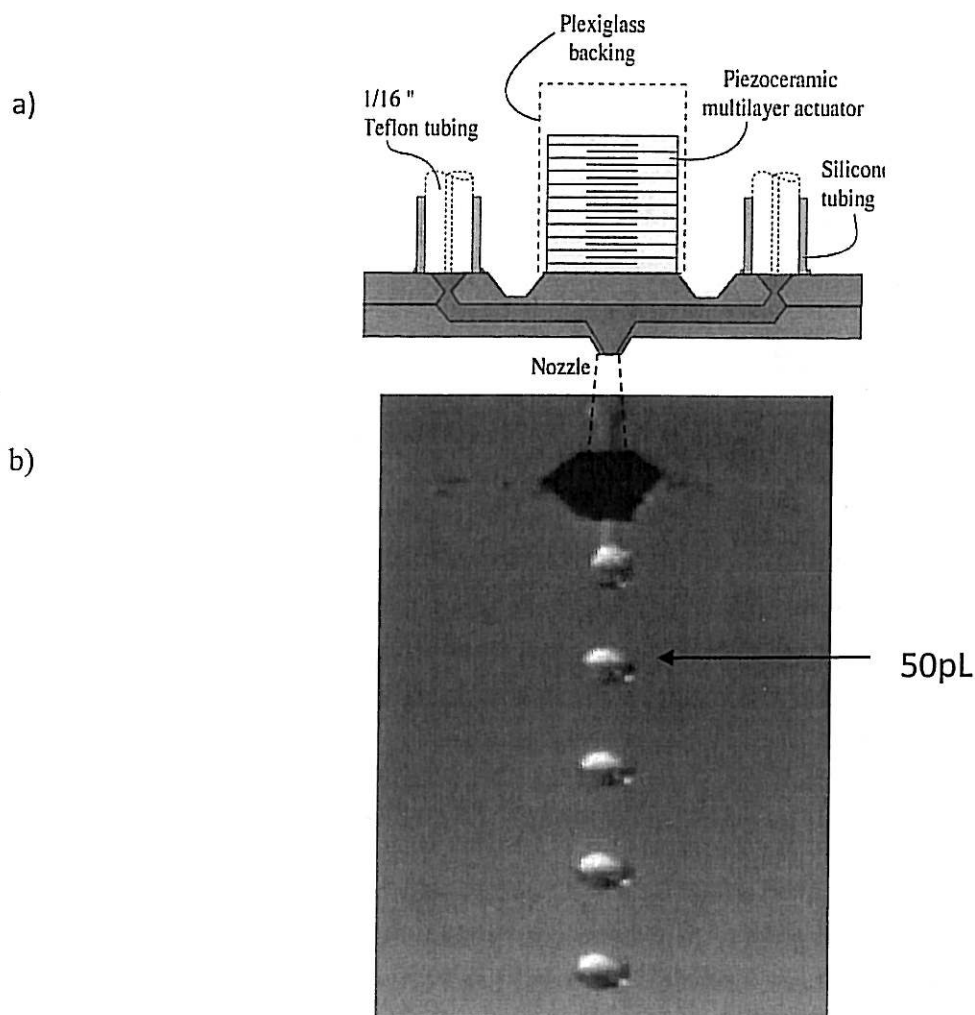


Figure 9: a) Schematic of the flow-through dispenser, b) Continuous droplet formation from the nozzle, each droplet is 50 pL in size.

In the center of the channel, a protruding pyramid shaped nozzle is formed. A multilayer piezo-electric element is connected to the channel wall opposing the nozzle. By applying a short voltage pulse across the piezo-electric element it elongates and pushes into the channel generating a pressure pulse in to the liquid (Figure 9 a). The increased pressure accelerates the liquid in the nozzle and a droplet with precise volume (50 pL) is ejected. It is also useful for controlling the size of levitated droplet and maintaining the volume constant with time. It can be done by setting the power box on certain pulses in time.

## 1.2.2. MALDI-TOF mass spectrometry - analyzing substances released from Langerhans islet

### 1.2.2.1: Mass spectrometry:

Mass spectrometry is a powerful analytical tool which is used to identify unknown compounds based on separation of molecules according to their mass-to-charge ratio.

It can also give structural information about molecules, using fragmentation of the sample by ionizing, separating, and measuring molecular ions.

Mass spectrometers are used in industry and academia for both routine and research purposes. The following list is just a brief summary of the major mass spectrometric applications:

- **“Biotechnology:** *the analysis of proteins, peptides, oligonucleotides*
- **Pharmaceutical:** *drug discovery, combinatorial chemistry, pharmacokinetics, drug metabolism*
- **Clinical:** *neonatal screening, hemoglobin analysis, drug testing*
- **Environmental:** *PAHs, PBCs, water quality, food contamination*
- **Geological:** *oil composition [39]”*

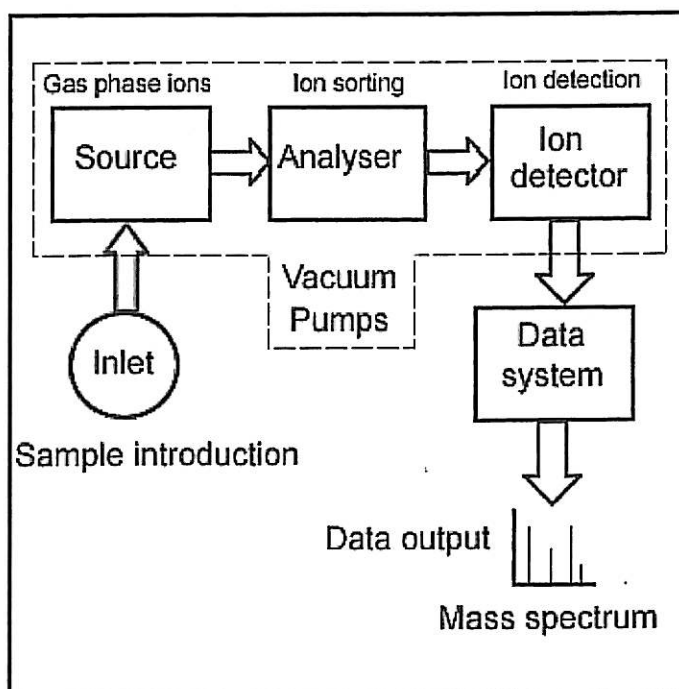


Figure 10: Basic parts of mass spectrometer[40]

The MS principle induces ionizing the sample to generate charged molecules that are subsequently accelerated in an electrical field according to their mass to charge and eventually detected in a detector as shown in figure 10 [41].

Each step in a mass spectrometer can be followed in figure 10.

3 steps are described briefly:

The best **ionization** methods which are used for the majority of biochemical analyses are so far Electro spray Ionization (ESI) and Matrix Assisted Laser Desorption Ionization (MALDI) [42].

**Mass analyzer** separate and resolve the ions which are formed in the ionizer according to  $m/z$  (mass to charge). There are many types of mass analyzers, the most well-known include Time of flight (TOF).

The **detector** monitors and amplifies the signal. Signals are transmitted to the data system where it is recorded in the form of mass spectra. The recorded time is converted by the spectrometer and is reported as an  $m/z$  ratio, where  $m$  is the mass of the ion in Daltons, and  $z$  is the ion charge "The  $m/z$  values of the ions are plotted against their intensities to show the number of components in the sample, the molecular mass of each component, and the relative abundance of the various components in the sample" [43].

#### 1.2.2.2. Matrix Assisted Laser Desorption:

MALDI, matrix assisted laser desorption ionization, is a laser-based soft ionization method that is used for analysis and investigation of large molecules. The mass accuracy depends on the type and performance of the analyser of the mass spectrometer, but most modern instruments should be capable of measuring masses to 0.01% of the molecular mass of the sample, at least up to ca. 40000 Da[43]. MALDI is based on bombardment of sample molecules with a laser light to ionize the sample. Most MALDI mass spectrometers have a pulsed nitrogen laser of wavelength 337 nm. The sample is pre-mixed with a highly absorbing matrix which transforms the laser energy to excitation energy for itself and the sample, it leads to the sputtering of analyte and matrix ions from the surface of the mixture [44].

#### 1.2.3. MALDI-TOF:

MALDI-TOF (time of flight) is used during this study. It performs well in fast and accurate determination of varieties of bio molecules. In this method, samples are mixed with a light-absorbing matrix compound on a metal plate with different spots. The matrix and sample molecules can co-crystallize on the spots during evaporation. Ionization usually occurs with nitrogen laser beam after sample plate is placed in the instrument. The matrix can absorb the laser energy and facilitating analyte ionization [45, 46] (See Figure 11). TOF is the mass analyzer which is combined with MALDI.

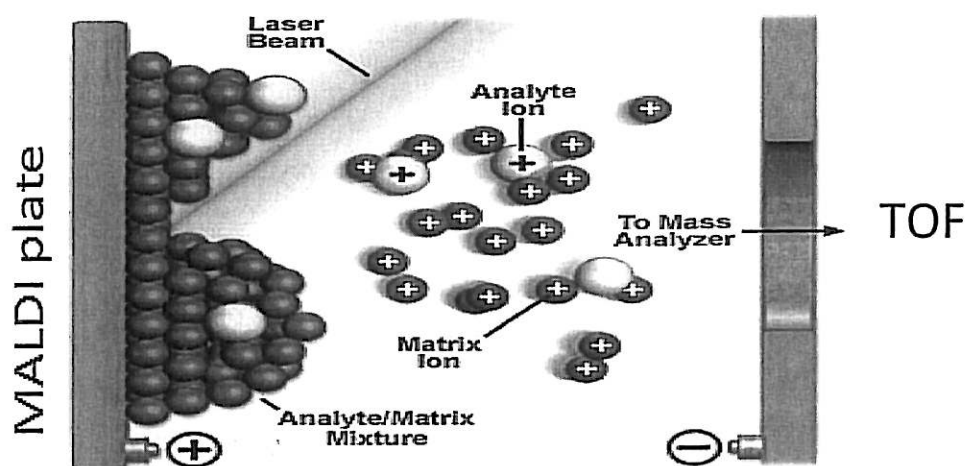


Figure 11: Matrix assisted laser desorption ionization (MALDI) [46].

A high electric field along the tube of the spectrometer causes the ionized molecules to "fly" down the length of the tube. The "time of flight" (TOF) is the time it takes the ions to reach the detector at the end of the tube and depends on its mass/charge ( $m/z$ ) [47].

The results are reported as a spectrum, a list of peaks that represent the peptides contained in the original sample.

In this study a MALDI-TOF 4700<sup>TM</sup> was used. It has two modes: 1-linear mode 2- reflectron mode. Reflectron mode can improve the mass resolution but it has 1000 times lower peak intensity. The reflectron, located at the end of the flight tube, is used to compensate for the difference in flight times of the same  $m/z$  ions of slightly different kinetic energies by means of an ion reflector (figure 12). "The more energetic ions that have slightly faster velocities will penetrate further into the ion mirror and hence be slightly delayed relative to less energetic ions thus both will tend to reach the detector at the same time" [48].

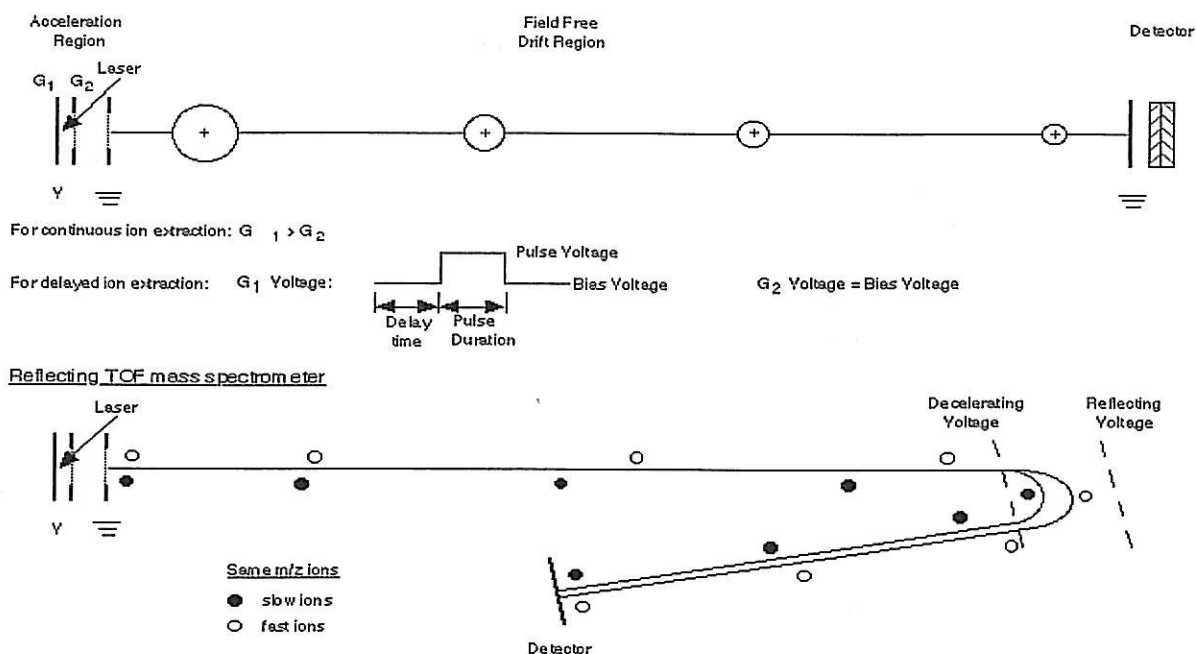


Figure 12: basic components of a linear (upper) and reflecting (lower) MALDI-TOF mass spectrometer [49].

### MALDI Orbitrap LTQ XL:

In this study MALDI Orbitrap LTQ XL was used in parallel to the MALDI TOF 4700 instrument and results were compared.

"MALDI Orbitrap LTQ XL basically consists of a MALDI ion source, a linear ion trap, a curved linear ion trap, a collision cell in elongation to the curved linear ion trap and an Orbitrap analyzer which is placed to accept extracted ions from the curved linear ion trap". Molecular ions are produced by MALDI, then they are sent to the linear ion trap or Orbitrap detector (figure 13). Chemical noise is reduced in the Orbitrap detection compared to conventional MALDI-TOF instrumentation [50].

Combining Orbitrap technology with LTQ XL linear ion trap enables faster, more sensitive and more reliable detection and identification of compounds. It is used for smaller molecules applications (size up to 4000 Da). Its great mass accuracy, mass resolution and high sensitivity performance makes it a good alternative to existing hybrid time of flight system [51]. The LTQ Orbitrap XL features the HCD (higher energy c-trap dissociation) collision cell for increased flexibility in MS/MS fragmentation application including peptide quantitation, superior mass accuracy and high resolution. HCD collision cell was not used for Langerhans islet experiment because known proteins/peptides were studied, so MS data was enough to get result.

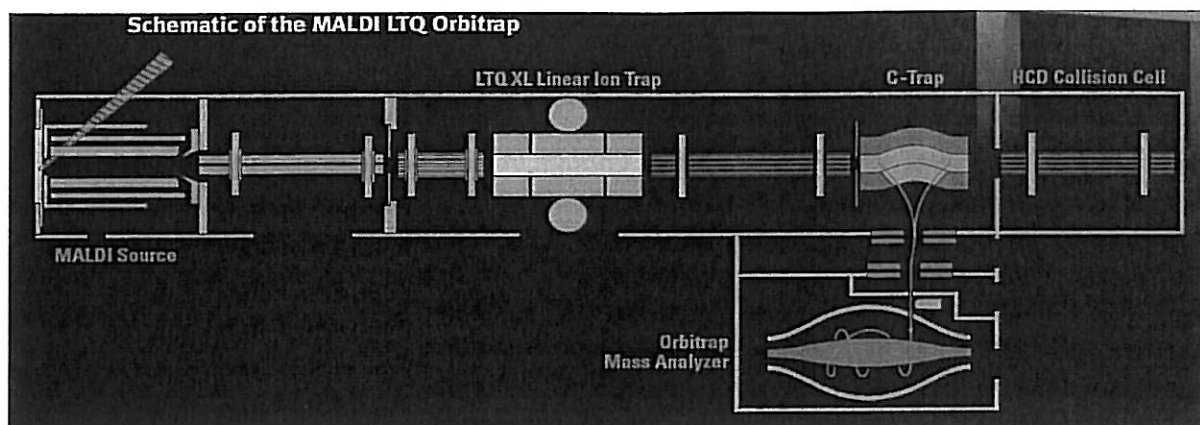


Figure 13: Schematic of MALDI LTQ Orbitrap [52]

## 2. Material and Methods:

### 2.1 Islets of Langerhans

Mouse pancreatic islets were provided from Clinical Research Center (CRC) in Malmö. It was prepared by collagenase digestion and incubated in Hepes buffer 10 mM, pH 7.4 (120 mM NaCl, 4.7 mM KCl, 2.5 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgCl}_2$ , 3.3 mM glucose). The medium was exchanged every 2 hours.

### 2.2 Reagents

Human insulin was a gift from Novo Nordisk®, Gentofte, Denmark. C-peptide was provided by Sigma-Aldrich®, Sweden. Acetylcholine chloride was obtained from Sigma-Aldrich®, Sweden. Stock solution (350mM) acetylcholine chloride in MQ water was prepared and added to the levitated droplet for stimulation of islet with final concentration of 5  $\mu\text{M}$ . Epinephrine,  $\alpha$ -cyano-4-hydroxy cinammic acid was obtained from Sigma-Aldrich, Sweden. The water used in all solutions was prepared using a Milli-Q system (Millipore, Bedford®, MA, and USA).

### 2.3 Sample handling

A 100  $\mu\text{L}$  pipet was used to pick Langerhans islets and put them in Krebs buffer solution. There is no need to use microscope because islets are big and they can be seen with the naked eye. Immediately without any incubation in Krebs buffer (Krebs buffer was only used to wash the islets from their previous environment), a 1.5 $\mu\text{L}$  islet-containing droplet was transferred to a fused silica capillary and positioned in the levitator (Figure 14). All reagents were added to the levitated droplet using a piezoelectric dispenser and additions were tuned to give the desired concentration. Stock solution of 35  $\mu\text{M}$  acetylcholine chloride was used to stimulate islet by shooting for 5 minutes while the power box of the piezoelectric dispenser was set to 10 pulses and pulse frequency of 50 Hz to get the final concentration of 5  $\mu\text{M}$  in the droplet. Then 50  $\mu\text{M}$  epinephrine was used as a stock solution to stop the

reaction to a final concentration of 10  $\mu\text{M}$ . The droplet remained in the levitator for 3 more minutes for more evaporation, because size of droplet is still 1.5  $\mu\text{L}$  and each spot in the MALDI plate can just handle 0.5  $\mu\text{L}$  of the sample. What is more, amount of sample and matrix must be almost the same to have enough matrix for certain amount of sample. Before addition of sample on the MALDI plate, 0.5  $\mu\text{L}$  matrix solution of  $\alpha$ -4-hydroxycinnamic acid (CHCA) was performed on the plate to crystallize the sample for analyzing. Matrix solution contains approximately 5 mg/ml CHCA in 50% acetonitrile, 0.1% TFA and 0.1 M citric acid, CHCA is the most common matrix that is used in MALDI-MS for crystallization of proteins and peptides with molecular mass of less than 10 KDa.

### 3. Results and discussion:

#### 3.1. Insulin release from $\beta$ -cell in a Langerhans islet

Acetylcholine was used to stimulate insulin release on isolated single Langerhans islet. Acetylcholine activates  $\text{Na}^+$  channel on  $\beta$ -cell membrane, resulting in depolarization and opening of  $\text{Ca}^{2+}$  channels (VDCC) which lead to granule translocation along the cell and exocytosis. In parallel, it mobilizes the intracellular calcium in the  $\beta$ -cell, via  $\text{IP}_3$  and activates PKC, have effect on insulin secretion[53] (Figure 14). In general stimulation of  $\beta$ -cell with Ach, increase concentration of  $\text{Ca}^{2+}$  inside the cell that contributes to insulin secretion from the  $\beta$ -cell.

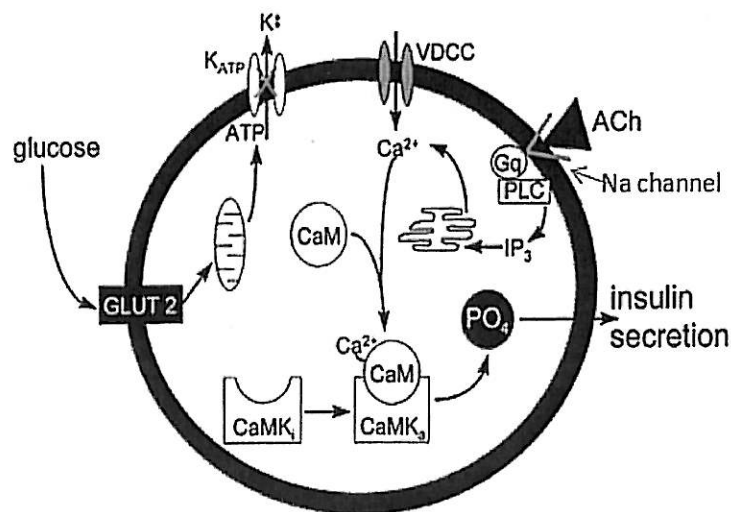
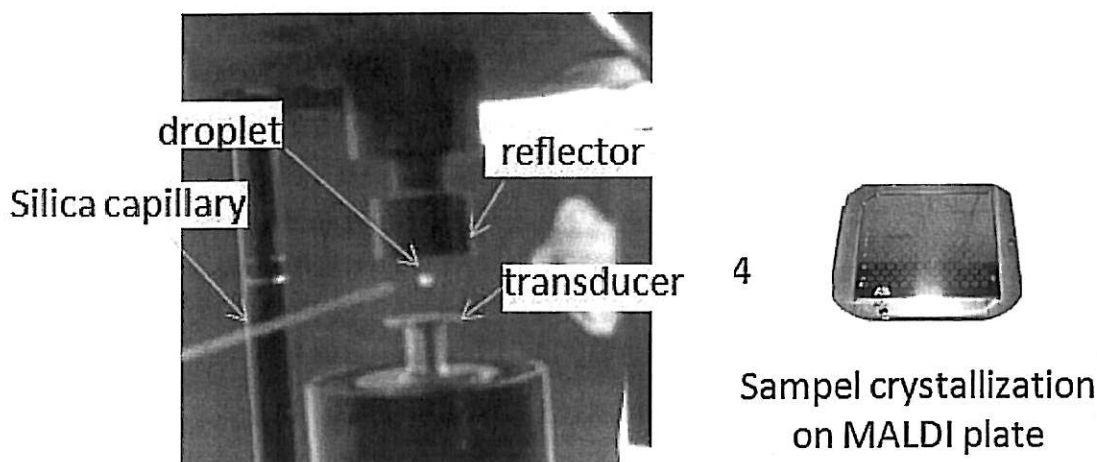


Figure 14: Regulation of insulin secretion by Acetylcholine in a  $\beta$ -cell [54].

In this experiment, single islet-containing buffer droplet was positioned in ultrasonic standing wave and stimulated during 5 minutes. A 10  $\mu\text{L}$  automatic pipet was used to pick one islet for each experiment. The islet which was soaked with Krebs buffer (1.5  $\mu\text{L}$  volume of sample) was transferred inside the Levitator with a fused silica capillary. Acetylcholine chloride was used as stimulator and it was added to the droplet using the flow-through-dispenser, to a final concentration of 5  $\mu\text{M}$  in the droplet.

After 5 minutes the droplet was picked outside of the levitator and transferred with a fused silica capillary to a MALDI plate which was previously coated with matrix. Matrix was almost dry when droplet was positioned on the plate. (See Figure 15)



*Figure 15: Manipulation of a fused silica capillary to pick the droplet from the levitator*

MALDI-TOF mass spectrometry was used to analyze the sample. The mass spectra demonstrate the proteins/peptides which are released from mouse pancreatic islet after stimulation with Acetylcholine chloride. Insulin (5808 Da), C-peptide (3672 Da), Somatostatin 14 (1637.9 Da) and Glucagon (3485 Da) are the main proteins secreted from Langerhans islet. (Figure 16)

A blank experiment (without using Ach) showed that islets themselves did not give response.

Mass spectra in Figure 16 show the reflector mode result after stimulation with Acetylcholine chloride and crystallization on MALDI plate.

### **3.2. MALDI-TOF 4700 result:**

Although both experiments were done the same way, signals in figure 16.a and b varying in intensity. Even more peaks (signals) are observed in the second mass spectra. This result could depend on the difference in Krebs buffer composition. Figure 16.b shows the better result (more peaks) because it has lower concentration of salts in Krebs buffer +  $\text{KH}_2\text{PO}_4$ . This is intended to provide better biochemical conditions for the stimulation of islets which was confirmed by more number of peaks and with increasing intensities. MALDI instrument performance can also affect the result.

To provide appropriate condition for crystallization of proteins/peptides that release from Langerhans islet, two different concentration of Acetylcholine chloride were tested. Both stock solution of 350  $\mu\text{M}$  and 35  $\mu\text{M}$  Acetylcholine chloride were used as stimulator to compare crystallization condition and protein release from the islet. Crystallization of

droplet which was stimulated with 350  $\mu\text{M}$  Ach was possible but it took longer time than crystallization with 35  $\mu\text{M}$  Ach. What is more peaks were observed when 35  $\mu\text{M}$  Ach was used. So in the next experiments the lower concentration of Ach was used as stimulator (It was added to the droplet through using piezoelectric dispenser with 10 pulses for 5 minutes) to get the final concentration of 5  $\mu\text{M}$  in the droplet.

Crystallization of the sample on the MALDI plate has great role in the final result because if proteins/peptides do not crystallize inside the matrix well they cannot be ionized. So choice of matrix is one of the crucial things that should be tested before the experiment. In this study, different conc. of CHCA was performed to compare crystallization of insulin on MALDI plate. 5mg/ml CHCA is the appropriate conc. of matrix which provides good crystals. Crystallization was checked under microscope before running the sample in MALDI mass spectrometry.

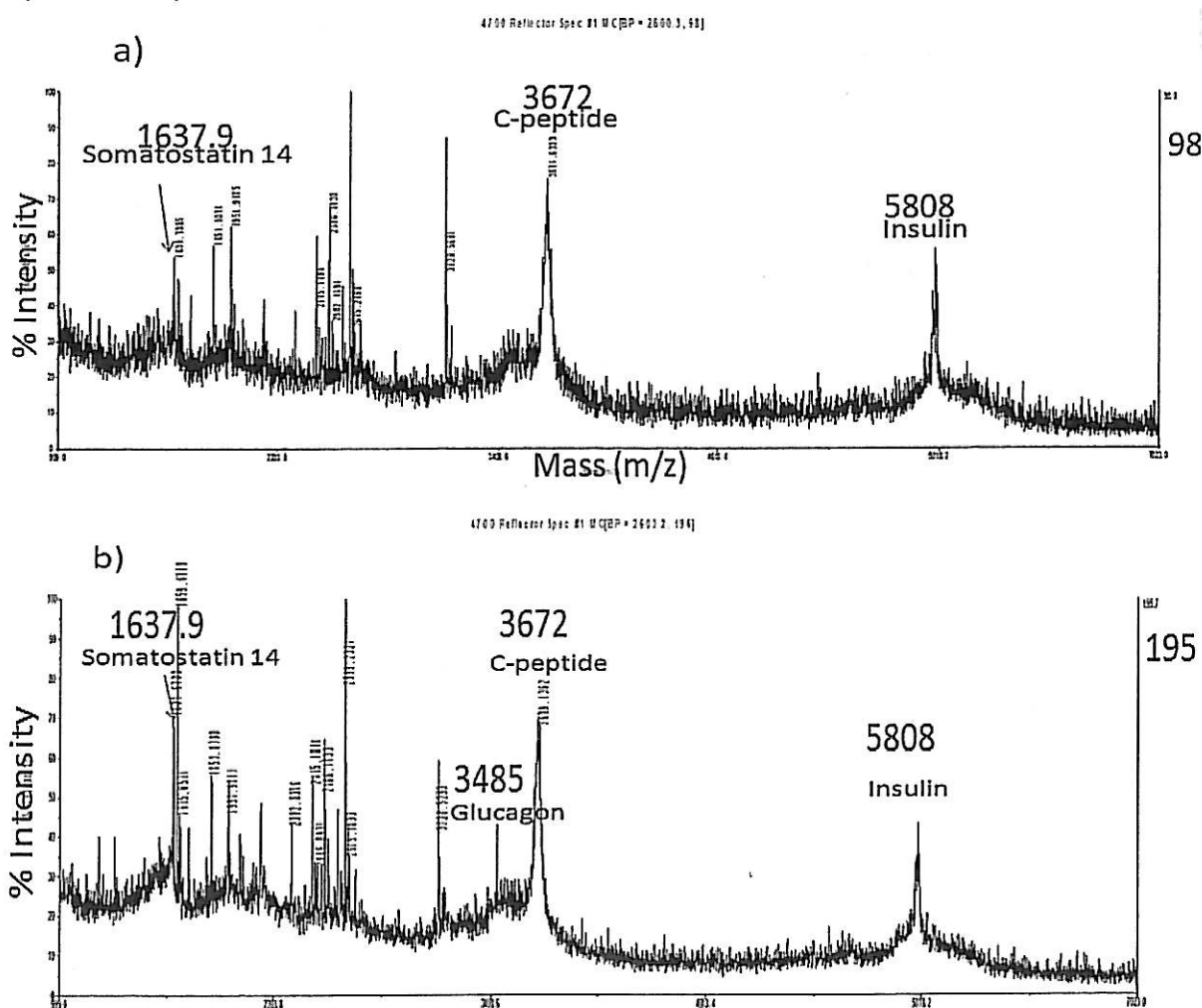


Figure 16: MALDI-TOF mass spectra in reflector mode recorded for droplets containing single Langerhans islet after stimulation with Acetylcholine chloride to final concentration of 5  $\mu\text{M}$ . Both experiments were performed in the same manner but with different concentration of ions in the Krebs buffer. a) **High** conc. (see material and method) of ions in the buffer ( $\text{CaCl}_2$  is at least 6 times more concentrated). b) **Low** conc. (see material and method) of ion in the buffer +  $\text{KH}_2\text{PO}_4$ .

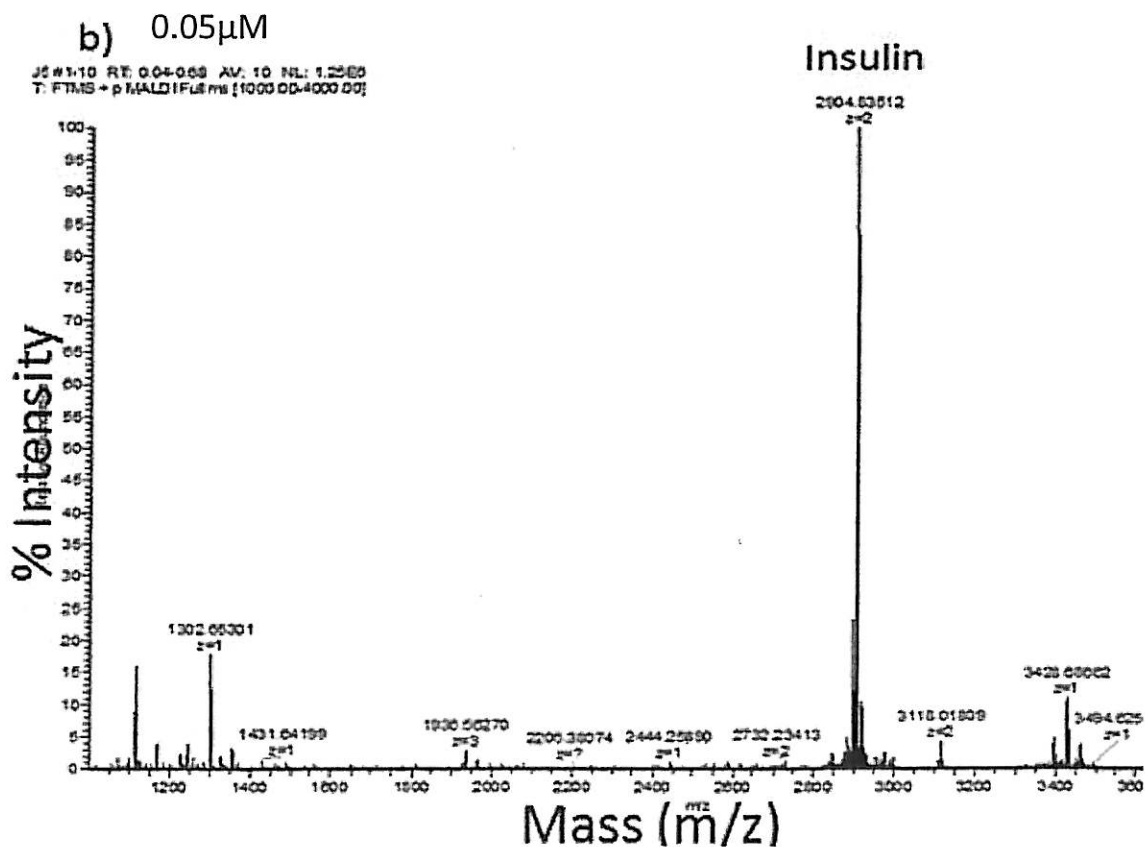
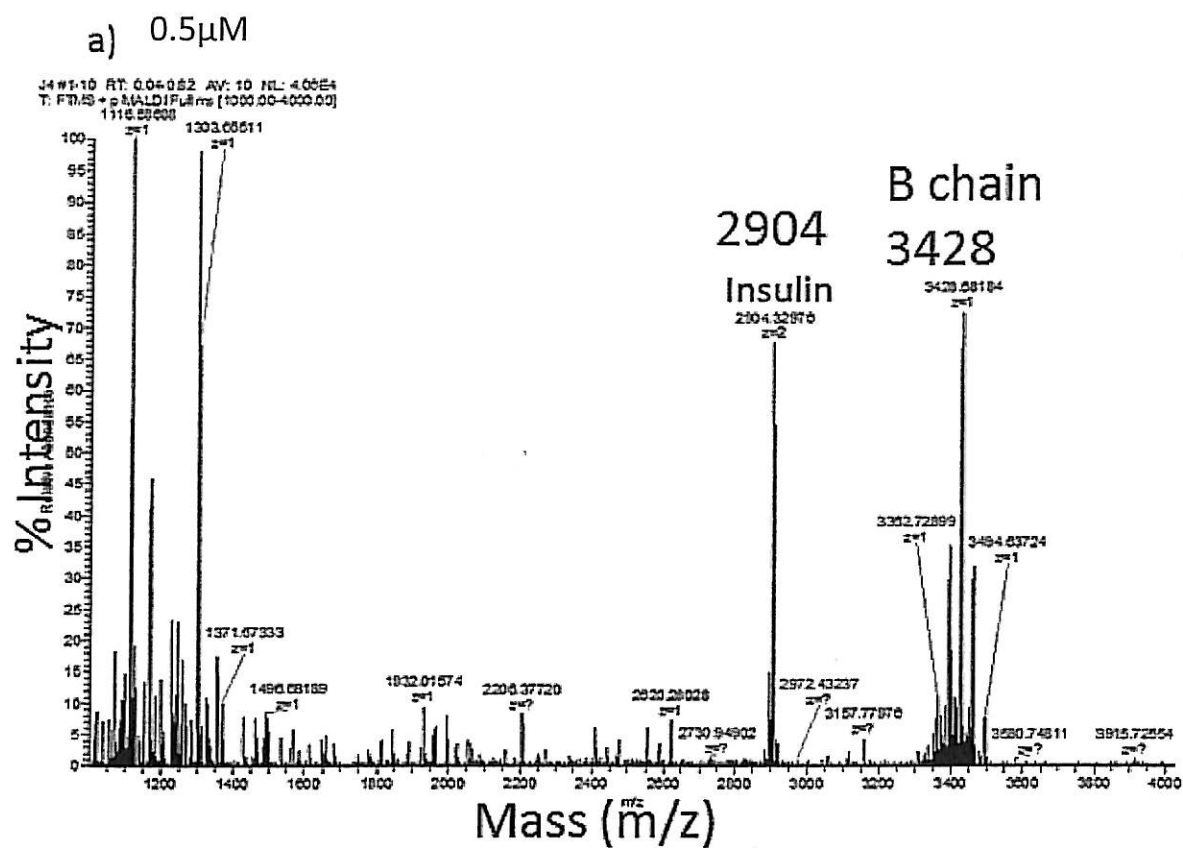
For analyzing the mass spectra, it was preferred to use linear mode since it gives peaks with higher intensity (1000 times higher than reflector mode and better L.O.D). However, in this study it seems that in reflector mode peaks (signals) are more distinct; therefore it was easier to distinguish them from each other.

Interestingly, the signal representing C-peptide is rather strong compared to insulin (signal is almost two times higher). That was unexpected since C-peptide is produced in equimolar to insulin, by splitting proinsulin into insulin and C-peptide. Half-life of C-peptide is 2 to 5 minutes more than insulin, it can be the reason for stronger signal of C-peptide. Additionally, another interesting observation was made; the absence of glucagon peak in the spectra in figure 16 a) suggests that stimulating effect and cell activity is drastically different with little change in cell environment (buffer) since Glucagon and some other signals cannot be seen. It seems that islet did not have proper condition for stimulation and releasing desired hormones because both experiments were performed in the same manner, the only difference was conc. of Krebs buffer. Another reason for this result can be MALDI performance or Langerhans islets themselves, because for each experiment one Langerhans islet was picked.

### **3.3. MALDI LTQ XL Orbitrap Result:**

Another experiment was carried out to see the possibility of using MALDI LTQ XL Orbitrap for Langerhans islet study because of its high resolution (60000 resolutions at 1Hz scan cycle) [52]. Low mass range of this instrument (until 4000 Da) was the only obstacle for this study since molecular weight of insulin is 5808, thereby  $Z^{+2}$  of insulin was checked instead of  $Z^{+1}$  because its molecular weight is 2904 which is in MALDI LTQ XL Orbitrap mass range. So it was possible to investigate insulin's signal with  $Z^{+2}$  in different dilutions.

To investigate the insulin signal with two charges and L.O.D of MALDI LTQ XL Orbitrap, different dilutions of insulin were prepared and with direct spotting they applied on the MALDI plate. Dilution shows that insulin with two charges gives higher signal (% intensity) when it is more diluted, until certain concentration (0.005 $\mu$ M). Then signal start to decrease after more dilution (figure 18). In 0.0005 $\mu$ M of insulin, no signal was observed in the spectrum (1  $\mu$ l of sample was applied on each spot of the MALDI plate in Orbitrap XL). Therefore L.O.D for insulin in MALDI LTQ XL Orbitrap was down to 5 attomole. The purity of insulin was checked to investigate the result from two charges of insulin. Insulin which was used here had some modification (zinc was used to make hexamer with insulin because insulin stores in the body as a hexamer while the active form is monomer). B chain of insulin was also observed in 0.5  $\mu$ M and 0.05  $\mu$ M insulin but its signal was decreased with dilution of insulin.



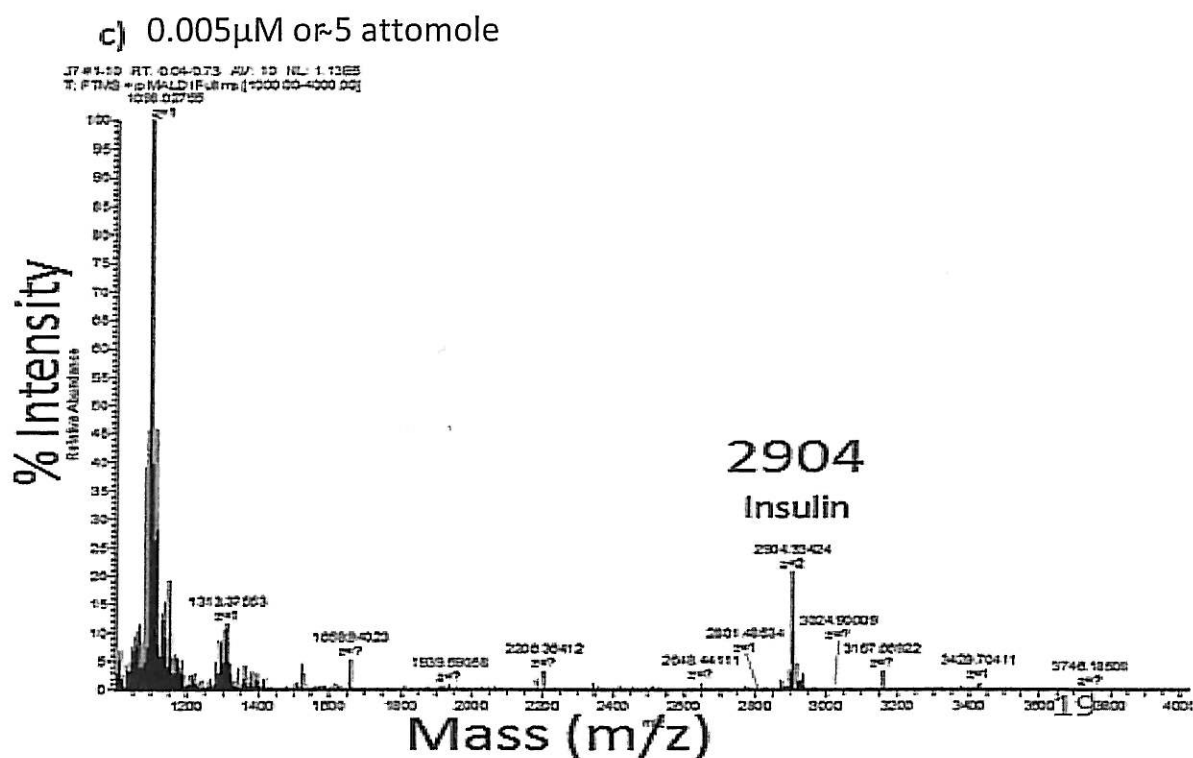


Figure 17: shows signal intensity in different concentration of a) 0.5 $\mu$ M (500 attomole) b) 0.05 $\mu$ M (50 attomole) c) 0.005 $\mu$ M (5 attomole) insulin with direct spotting of insulin on MALDI plate.

This experiment demonstrates that other peptides and ions can affect the result (Figure 17). With more diluted sample, lower signal of insulin was expected but interestingly higher signal of insulin was seen. In general it seems that suppression of other ions (which is Zn here) could cover the peak because some insulin could be still in the form of hexamer with zinc. In this form it had high molecular weight that was out of the mass range, so the hexamer was not observed in the mass spectra but it could cover the two charges of insulin. It is possible to overcome this problem and control the ion suppression, but since it is easier and more time consuming to work with MALDI-TOF 4700 in comparison with MALDI LTQ XL Orbitrap, MALDI-TOF 4700 was used as a mass spectrometer. It has ability to detect high range of molecular weight. Insulin's  $Z^{+1}$  molecular mass is higher than other hormones that secret from the islet, there for its signal cannot be covered by other ions.

In another experiment, MALDI Orbitrap LTQ XL was used to investigate ability of this instrument for Langerhans islet study. For this reason, 5mM DTT was added to levitated insulin droplet for 1 min using piezoelectric dispenser in order to cleave insulin in two chains (A chain and B chain). A chain and B chain molecular weight are 2382 Da and 3427.68 respectively and they are connected to each other by disulfide bond. So after using DTT it was possible to investigate insulin because both A and B chains have molecular weight of less than 4000 Da which is in the limited range of MALDI Orbitrap detection.

Surprisingly just B chain was observed in this experiment (Figure .18). It seems that A chain did not ionize enough or percentage of signal intensity was so low that it was not detected in the spectrum. However there are many factors that can affect this result, like suppression by other ion (zinc) which can cover the A chain peak. For this reason, it was preferred not to use this method for Langerhans islet study because DTT can cleave S-S bond in all hormones that release from the islet which have disulfide bond. So using this method could make the experiment too complicated to identify the proteins containing disulfide bond (insulin).

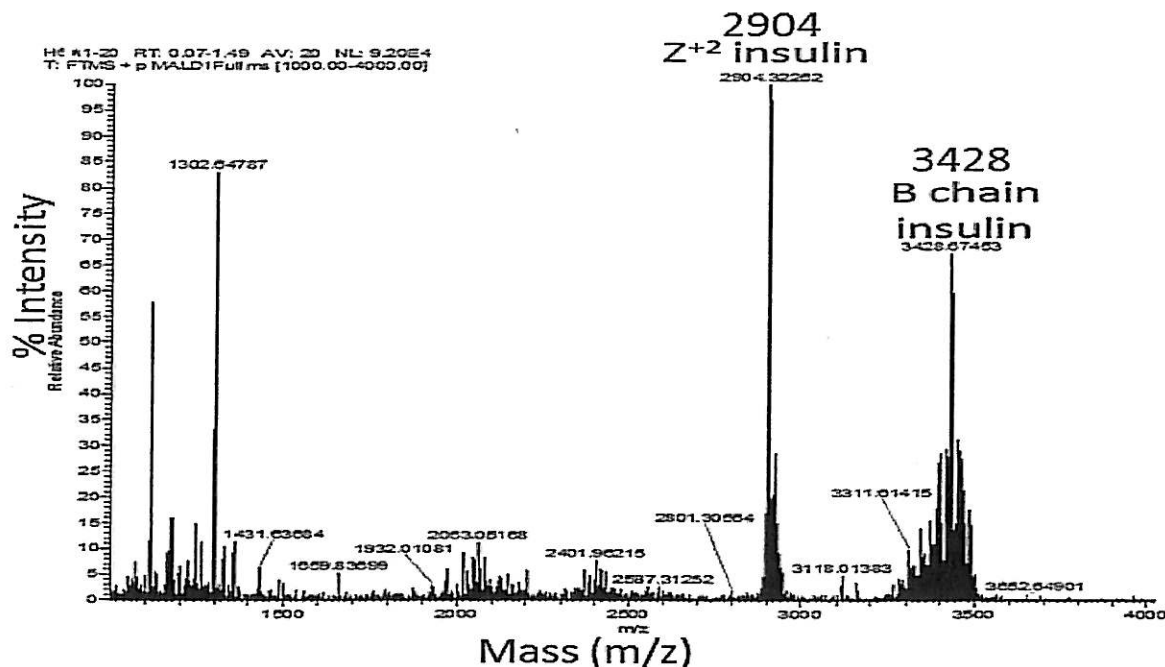


Figure 18: B chain and  $Z^{+2}$  (two charges) insulin after addition of 5mM DTT which was applied with piezoelectric dispenser for 1 min to single Langerhans islet in levitated droplet.

With MALDI-TOF instrument, it was possible to achieve limit of detection (L.O.D) for insulin and other proteins (L.O.D down to 20 femtomoles) see figure 19.

Bovine insulin (obtained from Sven Kjellström, CMPS, Center for Chemistry and Chemical Engineering, LU) was applied on MALDI plate with direct spotting and without levitation, and then matrix was added to sample to crystallize it.

MALDI-TOF was set on the linear mode to analyze the sample with high intensity.

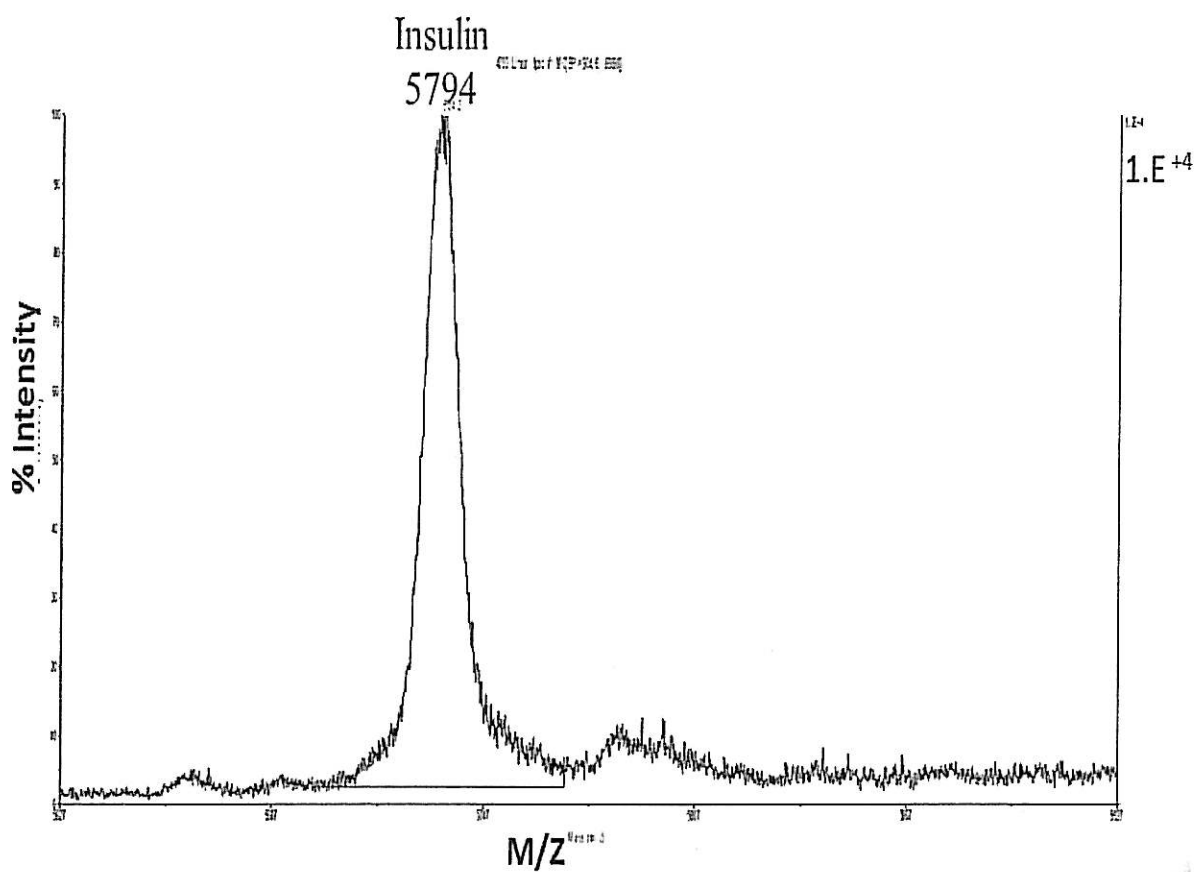


Figure 19: Spectrum of 20 femtomole of bovine insulin in linear mode run of MALDI-TOF 4700.

## Conclusion:

The combination of airborne analytical system (levitator) with two MALDI mass spectrometers for single Langerhans islet study in T2D research has been shown. The results clearly confirm the suitability of the method using MALDI-TOF mass spectrometry.

As proteins/peptides release from islet were observed, it manifests that cells are not damaged by ultrasonic field because sample levitates in the "calm" area of sound waves (the nodes). Living cells were levitated in this "wall-less" test tube, which contribute to less contamination of sample and no losses of sample to container walls.

Flow through dispenser enable addition of small volume of reagents to the droplet without any physical contact and contamination.

An interesting observation was that, a little change in cell environment (composition of Krebs buffer) affected stimulation of cell by changing amount of proteins/peptides that release from the Langerhans islet and different percentage of signal intensity. Therefore it is very important to find appropriate conditions for cell reaction to get desirable result. Crystallization of sample on MALDI plate is also important and it might be one of the reasons for this result.

According to the results, the signal representing Insulin is lower than C-peptide which was unexpected since C-peptide production should be in equimolar to insulin. C-peptide has 2 to 5 times longer half-life than insulin [55], it can be one of the reasons that percentage of insulin intensity is lower than that of c-peptide.

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## References:

1. Williamson, D. and A. Bardsley-Elliott, *Glossary of genetics/genomics terms: diabetes mellitus*. Mol Diagn Ther, 2008. **12**(6): p. 401-3.
2. van den Donk, M., et al., *Screening for Type 2 diabetes. Lessons from the ADDITION-Europe study*. Diabet Med, 2011.
3. Sep;30(3):139-44., P.R.H.S.J., et al.
4. Sep;6(5):599-605., et al.
5. Murphy, H.R., et al., *Closed-Loop Insulin Delivery During Pregnancy Complicated by Type 1 Diabetes*. Diabetes Care, 2011. **34**(2): p. 406-411.
6. Battaglia, M. and M.G. Roncarolo, *Immune intervention with T regulatory cells: Past lessons and future perspectives for type 1 diabetes*. Semin Immunol, 2011. **23**(3): p. 182-94.
7. Hemminki, K., et al., *Familial risks for type 2 diabetes in Sweden*. Diabetes Care, 2010. **33**(2): p. 293-7.
8. McCarthy, M.I., et al., *Identification of an imprinted master trans regulator at the KLF14 locus related to multiple metabolic phenotypes*. Nature Genetics, 2011. **43**(6): p. 561-U90.
9. Lin, C.C. and K.S. Anseth, *Cell-cell communication mimicry with poly(ethylene glycol) hydrogels for enhancing beta-cell function*. Proceedings of the National Academy of Sciences of the United States of America, 2011. **108**(16): p. 6380-6385.
10. Jansson, L., *The Regulation of Pancreatic-Islet Blood-Flow*. Diabetes-Metabolism Reviews, 1994. **10**(4): p. 407-416.
11. Cavallari, G., et al., *Rat pancreatic islet size standardization by the "hanging drop" technique*. Transplantation Proceedings, 2007. **39**(6): p. 2018-2020.
12. Silvestre, R.A., et al., *Selective amylin inhibition of the glucagon response to arginine is extrinsic to the pancreas*. American Journal of Physiology-Endocrinology and Metabolism, 2001. **280**(3): p. E443-E449.
13. Fan, R., et al., *Exendin-4 protects pancreatic beta cells from human islet amyloid polypeptide-induced cell damage: potential involvement of AKT and mitochondria biogenesis*. Diabetes Obes Metab, 2010. **12**(9): p. 815-24.
14. <http://diamondhead.net/ncnhc.htm>.9/23/2011
15. [http://www.daviddarling.info/encyclopedia/l/islets\\_of\\_Langerhans.html](http://www.daviddarling.info/encyclopedia/l/islets_of_Langerhans.html).9/23/2011
16. Folli, F., et al., *The Glial Glutamate Transporter 1 (GLT1) Is Expressed by Pancreatic beta-Cells and Prevents Glutamate-induced beta-Cell Death*. Journal of Biological Chemistry, 2011. **286**(16): p. 14007-14018.
17. Bosco, D., et al., *Unique arrangement of alpha- and beta-cells in human islets of Langerhans*. Diabetes, 2010. **59**(5): p. 1202-10.
18. Chung, C.H. and F. Levine, *Adult pancreatic alpha-cells: a new source of cells for beta-cell regeneration*. Rev Diabet Stud, 2010. **7**(2): p. 124-31.
19. [http://ciitn.missouri.edu/cgi-bin/pub\\_view\\_project\\_ind.cgi?g\\_num=11&c\\_id=2007009](http://ciitn.missouri.edu/cgi-bin/pub_view_project_ind.cgi?g_num=11&c_id=2007009).9/23/2011
20. Thorens, B., *Expression cloning of the pancreatic beta cell receptor for the gluco-incretin hormone glucagon-like peptide 1*. Proc Natl Acad Sci U S A, 1992. **89**(18): p. 8641-5.
21. Ahren, B., *Glucagon-like peptide-1(7-36)amide increases cyclic AMP accumulation in normal islets*. Pancreas, 1996. **12**(2): p. 211-3.
22. <http://dtc.ucsf.edu/types-of-diabetes/type1/understanding-type-1-diabetes/how-the-body-processes-sugar/blood-sugar-other-hormones/>.9/23/2011
23. <http://www.google.se/imgres?q=fluorescence+of+beta+cells+alpha+cells+in+one+islet&um=1&hl=sv&biw=1034&bih=603&tbs=ic:specific,i.b.t.i.t>.9/23/2011
24. <http://www.diabetescare.page.tl/Proinsulin-.htm>.9/23/2011

25. Hirose, H., et al., *Relationship between proinsulin-to-insulin ratio and advanced glycation endproducts in Japanese type 2 diabetic subjects*. Diabetes Research and Clinical Practice, 2007. **78**(2): p. 182-188.
26. Pfutzner, A., et al., *Fasting intact proinsulin is a highly specific predictor of insulin resistance in type 2 diabetes*. Diabetes Care, 2004. **27**(3): p. 682-687.
27. [http://www.mun.ca/biology/scarr/Insulin\\_posttranslational\\_modification.html](http://www.mun.ca/biology/scarr/Insulin_posttranslational_modification.html).9/23/2011
28. <http://faculty.smu.edu/jbuynak/images/Diabetes%20MellitusBuynak.ppt>.9/23/2011
29. <http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/P/Pancreas.html>.9/23/2011
30. fibrils, I.a.p.I.a.i.I.o.h.b.-c.b.c.b.d.n.f., et al.
31. Zraika, S., et al., *Identification of the amyloid-degrading enzyme neprilysin in mouse islets and potential role in islet amyloidogenesis (vol 56, pg 304, 2007)*. Diabetes, 2007. **56**(6): p. 1746-1746.
32. Hettiarachchi, M., et al., *Rat amylin-(8-37) enhances insulin action and alters lipid metabolism in normal and insulin-resistant rats*. American Journal of Physiology-Endocrinology and Metabolism, 1997. **273**(5): p. E859-E867.
33. Omrane, A., et al., *Laser techniques in acoustically levitated micro droplets*. Lab Chip, 2004. **4**(4): p. 287-91.
34. Santesson, S., et al., *Cell-cell communication between adipocytes and pancreatic beta-cells in acoustically levitated droplets*. Integr Biol (Camb), 2009. **1**(10): p. 595-601.
35. Santesson, S. and S. Nilsson, *Airborne chemistry: acoustic levitation in chemical analysis*. Anal Bioanal Chem, 2004. **378**(7): p. 1704-9.
36. Leiterer, J., et al., *Acoustically levitated droplets: a contactless sampling method for fluorescence studies*. Ann N Y Acad Sci, 2008. **1130**: p. 78-84.
37. Petersson, M., et al., *Sample enrichment in a single levitated droplet for capillary electrophoresis*. J Chromatogr B Biomed Sci Appl, 1998. **714**(1): p. 39-46.
38. <http://science.howstuffworks.com/acoustic-levitation1.htm>.9/23/2011
39. Spectrometry, A.I.t.M., et al.
40. <http://www.hull.ac.uk/chemistry/masspec3/principles%20of%20ms.html>.9/23/2011
41. Ho, C.S., et al., *Electrospray ionisation mass spectrometry: principles and clinical applications*. Clin Biochem Rev, 2003. **24**(1): p. 3-12.
42. Chapman, J.R., *Mass spectrometry. Ionization methods and instrumentation*. Methods Mol Biol, 1996. **61**: p. 9-28.
43. <http://www.astbury.leeds.ac.uk/facil/MStut/mstutorial.htm>.9/23/2011
44. Arakawa, R., et al., *Mass spectrometric analysis of low molecular mass polyesters by laser desorption/ionization on porous silicon*. J Mass Spectrom, 2004. **39**(8): p. 961-5.
45. Bizzini, A. and G. Greub, *Matrix-assisted laser desorption ionization time-of-flight mass spectrometry, a revolution in clinical microbial identification*. Clin Microbiol Infect, 2010. **16**(11): p. 1614-9.
46. [http://www.magnet.fsu.edu/education/tutorials/tools/ionization\\_maldi.html](http://www.magnet.fsu.edu/education/tutorials/tools/ionization_maldi.html).9/23/2011
47. Cotter, R.J.T.-o.-f.m.s.C., OH: American Chemical Society.
48. <http://keck.med.yale.edu/prochem/procmald.htm>.9/23/2011
49. Spectrometry, M.A.L.D.I.T.-o.-f.M., J.J. Lennon, and U.o. Washington.
50. Verhaert, P.D., et al., *Imaging of similar mass neuropeptides in neuronal tissue by enhanced resolution MALDI MS with an ion trap - Orbitrap hybrid instrument*. Methods Mol Biol, 2010. **656**: p. 433-49.
51. <http://www.ionsource.com/Instrumentation/ThermoFisher/OrbiTrap.htm>.9/23/2011
52. [www.thermo.com](http://www.thermo.com).9/23/2011
53. Gilon, P. and J.C. Henquin, *Mechanisms and physiological significance of the cholinergic control of pancreatic beta-cell function*. Endocrine Reviews, 2001. **22**(5): p. 565-604.
54. Jones, P.M. and S.J. Persaud, *Protein kinases, protein phosphorylation, and the regulation of insulin secretion from pancreatic beta-cells*. Endocr Rev, 1998. **19**(4): p. 429-61.

55. <http://shop.perkinelmer.com/applicationssummary/applications/insulin-Cpeptide.htm>.9/30/2011