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Evaluation of the performance of a wireless magnetoelastic pH-sensor in rumen environment

Mohammad Oli Ullah

Examensarbete / SLU, Institutionen för husdjurens utfodring och vård, **406**

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Handledare:
Supervisor: Bengt-Ove Rustas
Bitr. handledare:
Assistant supervisor:
Examinator:
Examiner: Peter Udén
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Abstract

Sub-acute rumen acidosis (SARA) is a problem with major economic importance in dairy production. The definition of SARA is a low pH (< 5.3-5.5) in the rumen during several hours in the day. Due to lack of other confirmatory clinical sign, SARA is diagnosed by measuring pH of the ruminal fluid. According to the definition continuous measurement of rumen pH is needed to diagnose SARA but until now methods for that purpose only exist for fistulated animals. In recent years, diagnostic tools in the biological field based on wireless telemetric technology have been developed. A magnetoelastic sensor which can swell and shrink in response to different pH levels was evaluated in this project. The sensor was tested in water, phosphate buffer, VOS buffer, McDougall's buffer, rumen liquid as well and in vivo in a cannulated cow. This sensor was prepared by casting layers of polymer on a magnetic ribbon, set up this ribbon in a plastic frame and then wrap ping it with magnetic paper. A box like wooden structure was used in case of in vitro which can conserve heat and provide a continuous rumen temperature. The prepared sensor upon application of a magnetic field produced mechanical vibration and thus production of a magnetic flux which can be received by a pickup coil. The measurement system consisted of the magnetoelastic sensor, a coil for excitation and pick up of the sensors resonance frequency, a control unit and a computer for signal processing and evaluation. The signals received from the sensor seemed to be affected by the use of an electric stirrer and also by gas bubbles produced during mixing of acids in buffer. However, this sensor was able to provide specific and repeatable signals in different ionic concentration. The changes in pH level of the rumen liquid were detected within a wide range of pH (from 3 to 10) whereas for the cow a more narrow range in pH (5 to 7) is required. Due to the too wide range of pH the change in frequency was difficult to calibrate against pH. Future work should be carried out with the aim to find a more appropriate polymer (e.g. peptide based) to increase the signal within the rumen pH range.

Keywords: SARA, pH, Rumen, Fistulated animal, Magnetoelastic sensor.
Author's address: Mohammad Oli Ullah, SLU, Department of Animal Nutrition and Management, Faculty of Veterinary medicine and Animal Science. E mail address: olishagor@yahoo.com.

Contents

1 Introduction

Rumen pH may be considered a reflection of the dietary management of the cow. Rumen pH varies in accordance with time, quality and quantity of diet provided and the physiological condition of the cow. An alteration of pH level outside the normal pH range can disturb normal metabolic activity of rumen microorganisms (AlZahal et al., 2007a). This condition may enhance the risk for the occurrence of SARA (sub-acute rumen acidosis). A cow affected by SARA may show feed intake depression and low fat percentages in the milk. Beside, SARA is one of the predisposing factors for cow to be affected by laminitis. The economic loss per cow per day has been calculated by North American Dairy industry for a SARA affected cow to US$1.12 (Donovan et.al., 1997; Stone et. al., 1999). Considering the economic importance and welfare of the animal, SARA should be diagnosed as soon as possible.

The existing methods practiced to diagnose SARA involves measurement of ruminal pH, milk fat testing, evaluation of odd and branched chain fatty acids in milk (Craninx et al., 2008) and measurement of the concentration of the bacterial toxin lipopolysaccharide (LPS) in fecal material (Plaizier et al., 2009b).

The limitations of manual methods to collect ruminal fluid for pH measurement have encouraged researchers to develop telemetric methods to measure rumen pH. Existing methods are based on battery driven glass electrode. Their drawbacks are limited operational time in the rumen and relatively high cost. To implement telemetric technique in practice, a low cost sensor is needed.

A functional sensor could be implemented in a sensor gate (Figure 1), which should be crossed by every cow when going to the milking or feeding areas. This gate could be implemented in a hard management tool which could be designed in such a way that if the pH reading of any cow passing through the gate deviated

from the normal pH range, it would send off an alarm making it easy to identify individuals with problems.

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Figure1. Future plan - Cows are passing through the sensor gate to go to the milking area. Cow which is not within a normal pH range will alarm us while passing the sensor gate.

2 Aims of the thesis

The general aim of this thesis was to evaluate the performance of a wireless magnetoelastic sensor based on a pH sensitive polymer, both in vitro and in vivo.

More specifically the aims of this study were:

1. To evaluate the effect of different salt concentration on sensors performance.
2. Study the ability of the sensor to detect changes in pH in rumen liquid at relevant levels.
3. To study the durability in rumen fluid in vitro
4. Evaluate the performance of the sensor in vivo.

3 Literature review

3.1 Normal pH profile in rumen

Usually pH of a dairy cow fed a high grain ration remains within the range of 5.6 to 6.2 (Oetzel et al., 2003). But this is not a constant figure as pH fluctuates over the day and a cow may experience a depression in pH at least during one period of the day. The fluctuation of rumen pH can be from 0.5 to 1 unit in a 24 hour period of a day (Oetzel et al., 2007). This variation of ruminal pH during the course of the day depends on the amount and degradation rate of fermentable carbohydrate provided in the diet. Besides this the feeding regime can affect pH in the rumen.

In early morning pH is generally relatively high due to low feed intake and long time for rumination during night (Schwertzkopf-Genswein et al., 2003). After feeding, pH drops due to the beginning of fermentation in rumen. After the first feed intake in the morning it takes around 10-13 hours to go down to the lowest pH value in a 24-hour period (Schwertzkopf- Genswein et al, 2003).

3.1.1 Nutritional factors affecting rumen pH

3.1.1.1 Chewing activity

Chewing during eating and ruminating stimulates secretion of saliva. As saliva supplies considerable amounts of buffers to the rumen fluid chewing is an important activity for counteracting pH depression in ruminants. A dairy cow typically

spends 3 to 8 hours eating and 6 to 9 hours ruminating (Beauchemin et al., 2005). The time spent on chewing depends on the source and chemical structure of the fiber and the particle size of the diet. A cow increase eating time if it consumes long dry hay compared to high moisture forage (eg pasture or silage) (Beauchemin et al., 2005). On the other hand, a fine forage particle size may provide little stimulation for chewing activity and increasing the particle size of alfalfa silage have shown to increase time spend ruminating by 4.3 hours per day in a dairy cow (Krause et al.,2006).However it is difficult to determine the optimum forage particle size in practice.

3.1.1.2 Salivary secretion

The amount of salivary secretion is critical for digestion of feed particles because saliva act as a buffering agent to neutralize the organic acids produced during fermentation in the rumen. Usually a lactating cow produces 100 to 150 liters of saliva per day (Yang et al., 2006). Saliva contains sodium bi carbonate which link up with free hydrogen ions of the produced acids and thereby buffer pH changes in rumen (Yang et al., 2006). The rate of salivary secretions was determined by Maekawa (2002) to 99ml per minute during resting and 217 ml per minute during chewing. Increasing the chewing activity for one more hour in a day has shown to increase secretion of saliva by 7 liters in a dairy cow (Maekawa et al., 2002).

3.1.1.3 Adaptation strategies

During the onset of parturition the cow needs to increase DM intake meet the physiological demands for nutrients due to increasing milk production. However, intake doesn't increase fast enough to match the increasing demand after calving. To minimize the gap between nutrient intakes and demand the cow is provided a diet with high energy density. The density of dietary energy can be increased by adding more concentrate to the diet. Therefore cows are switched from a high fiber low concentrate diet during the dry period to a low fiber high concentrate diet after calving. This shift in diet requires adaptation of the rumen ecology. Unfortunately the rumen microbial population and the rumen papilla cannot cope with a rapid change in the energy density of the diet. The reason behind this fact is rumen papilla do not remain constant in size and density for all stages in a lactation cycle.

Sizes may decrease up to 50 % during the dry period but after the onset of parturition they increase symmetrically reaching a maximum length at 4 to 5 weeks post calving (Reynolds et al., 2004). This behavior of rumen papilla was discussed in a comparative study by Reynolds (2004) where the author found that the mass of rumen papilla was greater at 10 to 22 days post calving compared to 7 and 21 days before calving. About half of the acids produced in rumen are absorbed through the rumen wall (Allen et al., 2000). If rumen papillae don't adapt to a changed diet, VFA absorption will be insufficient leading to a depressed pH.

3.2 Rumen ecology at the time of feed intake

Bacteria that are active in the rumen can be categorized into two groups: 1. Fiber digesting bacteria like Ruminococcus, Bacteriodes, and Butyrivibrio are very sensitive to low pH. They prefer a rumen pH between 6-6.8 (Slyter et al., 1970). 2. Starch digesting bacteria like Streptococcus bovis are favored by pH 5.5 – 6 (Russell et al., 1985). Beside this the number of ruminal protozoa dramatically decreases with a pH less than 5.5 (Schwertzkopf- Genswein et al., 2003). Cows switched from forage based to a grain based diet consume more fermentable carbohydrates. Degradation of these carbohydrates results in free glucose which is a substrate for bacterial fermentation and growth. Thus activity of bacteria accelerates leading to an increased amount of VFA and depression of pH in the rumen.

As pH drops, the activities of Streptococcus bovis increase relative to fibrolytic bacteria (Russell et al., 1985). Streptococcus bovis are fast growing and can reach 109 cells/ml of ruminal fluid within 20 minutes under favorable conditions (Allison et al., 1975). Glucose which is the breakdown product of starch and other carbohydrates are converted to fructose 1-6 diphosphate during the course of metabolism. This fructose -6 P is converted to pyruvate which is the substrate for the production of formate and acetate. However, the activities of Streptococcus bovis in rumen stimulate lactate dehydrogenase which provides a positive feedback for the conversion of pyruvate to lactate. Thus, there is a shift from the production of acetate and formate to lactic acid. This generates a shift in the VFA pattern with an increase in the proportion of propionic acid and a decreased proportion of acetic acid. Normal ratio of acetic and propionic acid stands at 3.2:1 which can go down to 2.2:1 due to this altered environment (Schwertzkopf- Genswein et al., 2003). However, lactic acid is a strong acid and can depress pH (Schwertzkopf- Gens-

wein et al., 2003). Under normal physiological conditions, lactate utilizing bacteria like Megasphaera elsdenii and some protozoa can utilize this lactic acid and can minimize the depression of pH (Williams et al., 1992. When microbial populations, especially lactate producing and lactate utilizing bacteria are disrupted, a lower pH environment may exist in rumen. During feed deprivation growth rate of lactate utilizers are inhibited (Schwertzkopf- Genswein et al., 2003). This makes the rumen ecosystem more susceptible to rumen acidosis. When rumen pH drops below 5.5, most of the bacteria and protozoa cannot survive. As a result, there are few species of bacteria and protozoa present in the rumen and these are not able to maintain normal pH after sudden changes in the diet. Thus, SARA can convert to acute acidosis (Williams et al., 1992).

3.3 Sub-Acute Rumen Acidosis

There is much debate at which pH levels the normal activities of microorganisms in the rumen are interrupted (AlZahal et al., 2007b). A value below a critical pH point for a certain period of time is considered as one way to diagnose SARA. Krause (2003) suggested pH 5.8 as a definition of this critical point. Others argue for a pH less than 5.6, for >3 hours in a day to indicate the occurrence of SARA (Kleen et al., 2003; Stone et al., 2004; Gozho et al., 2005). In general, cows within a range of pH 5.5 – 5.8 or above this level are considered as normal while those with a rumen pH below this point are classed as suffering from sub-acute ruminal acidosis, or SARA (Enemark et al., 2004; Penner et el., 2006; AlZahal et al., 2007 b).

Depression of dry matter intake can be identified as a clinical sign for SARA but in group fed cows, individuals with problems are not easy to detect (Devries et al., 2009). Behavior of cows can be used to detect illness. Keen observation of behavioral modifications, rumination behavior and feeding behavior, can help to identify illness of cows (Devries et al., 2009). However, it is difficult to inspect individual cows regularly (Devries et al., 2009) and therefore other ways to diagnose SARA are needed.

3.3.1 Consequences of SARA

When pH is low in the rumen, osmolarity in the ruminal fluid increase (Allen et al., 2000). A high osmolarity can limit voluntary feed consumption (Allen et al., 2000). On the other hand, cows fed grain has been shown to generate acute phase protein in blood which may cause rumen inflammation (Gozho et al., 2005; Gozho et al., 2006 and Gozho et al., 2007). This inflammation is also responsible for feed intake depression. In association with low feed intake, depressed fiber digestion is a common feature of a cow affected with SARA. This may be due to that fibrolytic bacteria which are responsible for digestion of the fiber portion of the diet can't grow and multiply at a pH less than 6 and their activities decrease in response to decreased pH (Shi et al., 2002).

A field study in New York state (USA) revealed that SARA reduced milk yield, milk fat concentration and increased milk protein concentration by 2.7 kg/day, 0.3 and 0.12% units, respectively (Stone et al., 1999). Grain pellets when added in the diet by replacing the fiber portion, usually produce higher amounts of propionic acid while the production of acetate remains the same (Bauman et al., 2003). This results in a reduction of the acetate to propionate ratio. This decreased proportion of acetate to propionate play a role in milk fat depression (Bauman et al., 2003). On the other hand, insulin which is responsible for lipolysis is usually released in higher amount in cows with positive energy balances as compared to negative energy balances (Plaizier et al., 2009a). Thus, increased amount of insulin in SARA affected cows may also be responsible for lower fat percentage in milk. It has also been established that low ruminal pH results in incomplete biohydrogination of fatty acids (Griinari et al., 1998) and the production of trans octadenic acids, especially the trans 10 isomer of trans octadenic acid which is also responsible for milk fat depression (Griinari et al., 1998).

Cow affected by SARA may have diarrhea (Nocek et al, 1997; Kleen et al., 2003 and Oetzel et al, 2003) due to low fiber content of the diet (Nocek et al, 1997). Due to more hindgut fermentation in SARA affected cows, gas and microbial protein result in feces with a foamy appearance (Nordlund et al., 2004). Fermentation in the hindgut results in increased acidity which may slough epithelium from the large intestine. The body has a self-defense mechanism in which fibrin and mucus are secreted to protect injured areas (Hall et al., 2002). As the consistency of the

fecal material becomes hypertonic, it may contribute to the development of diarrhea.

Some pathological lesions such as ruminitis and ruminal parakeratosis, erosion and ulceration of the rumen epithelium can be seen in animals with low rumen pH (Plaizier et al., 2009a; Krause et al., 2006). These lesions are caused by chemical damages of rumen epithelial cells and mucus membranes due to the increased production of acids. Inflammation of the rumen epithelium may create a suitable environment for rumen bacteria to colonize rumen wall papilla. Bacteria are then transported to the liver by the systemic circulation causing liver abscesses (Nocek et al., 1997; Kleen et al., 2003 and Oetzel et al., 2003). Bacteria in the liver eventually leave and enter the systemic circulation. They might colonize lungs, heart valves, kidneys and joints causing endocarditis, pneumonia, pyelonephritis, and arthritis, etc. (Nordlund et al., 1995; Nocek et al., 1997; Kleen et al., 2003 and Oetzel et al., 2003).

The activity of rumen microbes are disrupted at low rumen pH and this interruption causes the release of some vaso active substances like endotoxin, lactate and, possibly, histamine (Nocek, et al., 1997). These vasoactive substances give rise to a cascade of event in the circulatory system leading to lowered blood supply in the corium (Shearer et al., 2005). As a result, there may be edema, hemorrhage and necrosis of corium tissue and this promote functional disturbance of the corium. Beside this, the horn layer of the hoof capsule is also affected due to interruption of nutrient diffusion from the corium layer to the horn layer or epidermis. As a result, keratinization of horn cells in stratum sponsium is disturbed. Keratinization is responsible for the horn cell structural rigidity and strength. Due to deprivation of nutrient and oxygen, the keratinization layer may be injured or inflamed leading to the condition laminitis (Shearer et al., 2005).

3.4 Existing methods used to measure rumen pH

Different methods have been used to measure rumen fluid pH. Among them, rumenocentesis and oral stomach tubing have been practiced at farm level to sample rumen fluid from intact animal. Rumenocentesis is performed by aspiration of a

needle in the caudoventral region of the rumen to extract rumen fluid. At stomach tubing, a probe is passed through the oesophagus into the rumen from where rumen fluid can be collected. However, for research purposes, rumen cannulated animals are commonly used and samples are generally collected manually. Each of these methods has some merits and demerits. For example, samples collected with an oro-ruminal probe may be susceptible to saliva contamination (Nordland et al., 1994) and as a result, show higher pH values and higher bicarbonate concentrations compared with other collection methods (Duffield et al., 2004). Beside this, the method causes discomfort to the animal and can interfere with the normal activity of the gut. Rumenocentesis require physical and chemical (local anesthesia) restraint of the animal and it needs surgical preparation of the centesis site (Duffield et al., 2004; Nordlund et al., 1994). At rumenocentesis, there is a risk for localized abscesses or peritonitis (Duffield et al., 2004). Another drawback with these techniques is the limited number of animals that can be sampled at a time and that each animal, at least in the case of rumenocentesis, can be sampled only a few times. Furthermore, sampling could hardly be performed by a farmer. Rumen cannulation, suitable for research purpose, could hardly be practiced by the farmer due to ethical considerations, cost and hazards associated with the surgery. Beside this, it is more labor intensive and limited to only a few animals. Moreover, with growing animals like calves or lambs it is difficult to maintain the cannula. Thus, a less invasive technique which can be place inside the rumen to measure pH continuously, without interfering with the normal rumen behavior, is desirable.

Various attempts have been made to measure pH of ruminal fluid continuously. Smith (1941) was the first who described the in vivo measurement of pH in the rumen and tried to measure pH with the help of a Beckman pH meter assembled in a glass electrode. Later on Matcher (1957) and Lampila (1955) made attempts to measure pH continuously in sheep and cattle respectively with the help of a glass electrode. The glass electrode, placed in a cannulated was connected by a wire to a receiver located outside the rumen. Dado and Allen (1993) also tried to set up a system to provide continuous measurement of rumen pH with the help of indwelling pH electrodes. Their system didn't work well due to the difficulties in maintaining calibration. Measurements with the above mentioned systems were limited to tethered animal as the indwelling pH probes were connected by cables and hence restricted animal mobility. In recent years, wireless systems, able to monitor rumen pH in loose housed animals, have been developed. The accuracy of wireless measurement systems for continuous monitoring of pH over time has flourished

through the work of several researchers with different types of device, e.g. Keunen (2002), Maekawa (2002), Cottee (2004), Beauchemin and Yang (2005), Al Zahal (2007a). Wireless stand-alone systems for measuring rumen pH in grazing and unrestrained animal have been developed by Enemark (2003) and Graf (2005). These systems are in limited use and are lacking validation (Penner et el., 2006). Penner (2006) proposed a wireless measurement system named Lethbridge Research Centre ruminal pH measurement system for cannulated cows. The limitation of this system was the need for daily recalibrations. Recently, a wireless rumen probe has been promoted commercially by Kahne Limited (New Zealand) to monitor rumen pH, temperature and pressure in cattle (Kaur et al., 2010). The bulb of this probe was made of an ion sensitive field effect transistor (ISFET) sensor. The probe has been evaluated by Kaur (2010) in four rumen fistulated male sheep and the results were compared with the most common method (glass electrode) of monitoring pH. The probe was light sensitive, had a time dependent pH drift and performed poorly in comparison with a glass electrode (Kaur et al., 2010). Thus, its accuracy and usefulness in research is still a question.

Telemetric techniques with boluses that can be placed in intact animals and transmit recorded pH values are available on the market (E Cow, Dascor, Smaxtec). These systems rely on battery driven sensors, predominantly based on glass electrodes (E cow, Dascor, Smaxtec). The functional lifetime of these systems are limited to between 40 to 100 days (Gasteiner et al., 2009; Phillipps et al., 2010). Although marketed for commercial herds, the relatively high price and limited functional life time makes these systems more suited for scientific applications. They could be used to follow pH in a cow during early lactation, but if not fistulated, the cow needs to be slaughtered in order to restore the bolus.

3.5 Magnetoelastic sensors

Ruan (2003) suggested the use of a passive wireless sensor for measuring pH in the stomach of human subjects. The sensor was based on a magneto elastic film coated by a pH sensitive polymer. The cost of this technique is low, making it interesting for commercial applications.

Magneto elastic sensors are made from amorphous ferromagnetic metal film ribbons (Grimes et al., 2002). These ribbons are usually iron rich alloy which have a high mechanical tensile strength and high magneto elastic coupling co efficient (Hernando et al., 1988; O'Handley et al., 2000). The interaction between the magnetization and the strain of material can be determined by this magneto elastic coupling co efficient and a high magneto elastic coupling permits the exchange of magnetic energies to elastic energies and vice versa (Grimes. et. al., 2002). Hence, the ribbons are magnetostrictive, they can change the shape upon application of a magnetic field. A magneto elastic sensor is activated by a spatially variable magnetic field which makes the film elongate and shrink along its length axis. The magnetic field can be created by driving periodical current through a coil.

Upon activation, the magnetic metal film will generate a magnetic flux that can be detected by a pickup coil, either the same that activated the sensor (Figure 2) or a separate (Figure 3), and converted to resonance frequency (Grimes, et al., 2011). If the same coil is used for activation and detection, the response from the sensor is monitored during the periods when the current is switched off.

In the early development of magneto elastic sensors two separate coils, one to generate magnetic field and another to receive the signal generated from the sensor, were used (Figure 3). Pucket (2002) used this type of arrangement to monitor blood coagulation. With modern microcontroller based units only one coil is needed (Figure 2) to receive the signal. This type of arrangement was described more by Zeng (2006).

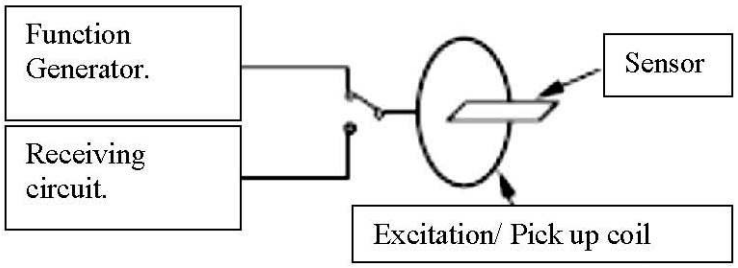

Figure 2. System for excitation and signal reception from a magneto elastic resonance sensor. A switch is used to separate the excitation circuit and the receiving circuit enabling both functions to be carried out by one single coil. Modified after Grimes et al., 2002.

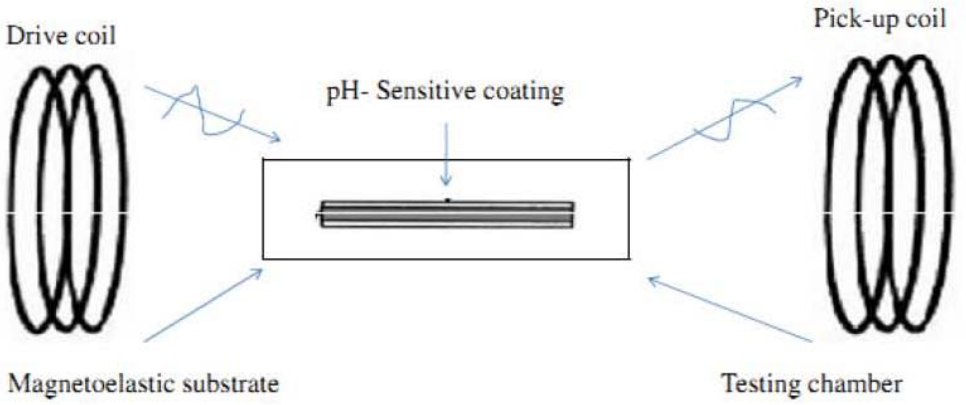

Figure 3. The organogram used in the early developmental period of magneto elastic sensor. Drive coil to provide AC/ DC magnetic field and the pick – up coil to receive the signal. (Adopted from Cai and Grimes et al., 2001)

To understand the sensor operation, an acoustic bell can be used as an analogy. The energy from a clapper that hits a bell deforms the bell and makes it vibrate. The bell vibrates with its characteristic frequency causing a specific tone. The tone is "deeper" when the bell is heavier. The magnetic field that activates the sensor is analogous to the clapper and the tone of the bell corresponds to the oscillation frequency of the magnetic flux produced by the activated sensor.

Any material deposited onto the sensor will change its resonance frequency, roughly in proportion to the deposited mass (Cai and Grimes et al., 2000). By coating the metal film with a polymer that swells and shrinks upon pH changes, shifts in the films resonance frequency can be used to detect pH changes (Ruan et al., 2003). The swelling and shrinking of pH sensitive polymers is due to functional groups that become ionized relative to pH. The functional groups might be acidic (eg -COOH) or basic (e.g. $-NH_2$). With increasing ionisation, repulsion of charged groups causes the polymer to expand, and hence swell, which increase the mass load of the sensor. Decreased ionization will make the polymer collapse and, consequently, the mass load of the sensor to decrease.

By introducing different functional groups, a polymer can be modified to react on different chemical constituents in its environment. The swelling and shrinkage of pH sensitive polymers is due to the presence of functional groups in their carbon chain. The functional groups may either be acidic (-COOH, $-SO_3H$) which will swell in alkaline solution (for example polyacrylic acid) or may have a basic group ($-NH_2$) in their carbon chain and will swell in acid pH (for example chitosan). Several attempts have been made to apply different polymers in order get an accurate pH measurement. Grimes (2001) introduced a pH sensitive polymer synthesized from 20 mol% of acrylic acid and 80 mol% of iso-octyl acrylate which eventually swell and collapse in alkaline and acidic pH respectively. But in general variation in thickness of the polymer has shown to be affected much to get signal.

Ruan (2003) tried with a new polymer structure by using (polyvinylbenzylchloride- co 2,4,5-trichlorophenyl acrylate) (VBC-TCPA) as a pH sensitive material. The authors were able to get a linear response from pH 3 to pH 9 (Ruan et al., 2003). More recently, Pang (2007) used a polymer synthesized from co-polymer of acrylic acid and iso-octyl acrylate to measure body fluid pH. The functions of these polymers rely mainly on the acrylic acid that dissociates in alkaline solution. Meanwhile, iso-octylacrylate helps the microsphere not to be dissolve in the test solutions.

4 Materials and Methods

In this project we used the mass changes of a pH sensitive polymer to measure pH in buffers and rumen environment with magneto elastic resonance (MER) technique.

The study consists of a series of experiments in vitro and in vivo to investigate if the delivered frequency from the sensor can be used to detect changes in pH and if it is repeatable. The experiments were conducted over a period of one year where different aspects that might affect the delivered signal from sensor were evaluated and its interpretation was concluded.

4.1 Polymer preparation

The polymer was poly vinylbenzyl chloride-co-2, 4, 5- trichlorophenyl acrylate (VBC-TCPA). The polymer was expected to respond linearly to changes in pH between pH-4 to pH-10 and should not be affected by the ionic strength of the test solution. The polymer which forms microspheres, was prepared at Lund University in accordance with the instruction of Seitz (1999) and Ruan (2003). This polymer (VBC--TCPA) was synthesized by using the chemicals polyvinyl pyrrolidone (PVP), acryloyl chloride (96%), 2,4,5-trichlorophenol (98%), diethanolamine, ethanol (99.%), aceton (99.5%), tetrahydrofuran(99.9%, anhydrous), triethylamine(99.9%, anhydrous), magnesiumsulfate, 4-VBC (96%) and divinylbenzene (DVB, 80%) . TCPA (trichlorophenyl acrylate) was added to the polymer formulation in order to introduce porosity and hydrophilicity to the microspheres. To introduce pH sensitive polymer groups (Figure 4), the polymer spheres were aminated with dimethylamine, diethylamine, dibutylamine and dioctyleamine follow-

ing the protocol of Ruan (2003). The aim of this amination procedure was to shorten the response time of the membrane from hours to few seconds. The diethanolamine-aminated polymer spheres were dispersed in ethanol (7.5 mg solid polymer: 1 ml ethanol). The recommendation on the protocol described by Ruan (2003) was to dissolve this polymer in dimethyl sulfoxide (DMSO). But here we tried ethanol as a solvent instead of DMSO. The reason to choosing ethanol was its low boiling point and which would help to reduce evaporation of this solvent under normal atmospheric pressure.

A solution of polymer was prepared by adding 22.5 mg solid polymer to 3 ml ethanol in a 5 ml plastic tube. The tube was sealed with a rubber lid and placed in an ultrasonic shaker at 40^0 C. It was shaken for 99 minutes, taken out for 30 minutes and then shaken again for 99 minutes to dissolve the polymer as much as possible. However, the polymer was not dissolved fully. This procedure was done at the end of the day and the tubes were kept overnight for sedimentation. On the next day the clear supernatant was carefully collected from the tube. The collected polymer solution was then ready for preparation of the sensors.

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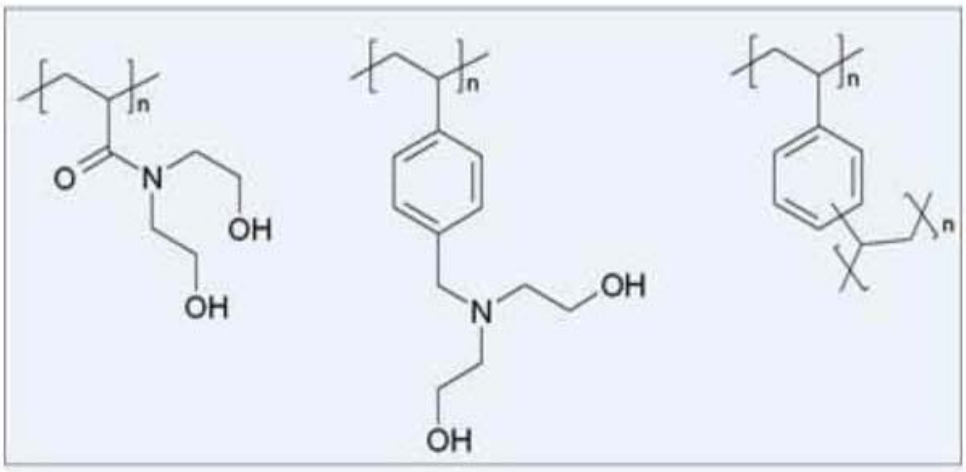

Figure 4. Functional group of the polymer used in this sensor preparation. This diethanolamine group was introduced into the polymer with the aim to shortened response time from hours to few seconds and to respond in corresponds to pH change.

4.2 Sensor preparation

Ribbons of amorphous ferromagnetic film of a high mechanical tensile strength and the composition $Fe_{40}Ni_{38}Mo_4B_{18}$ (Metglas^{TM}, New Jersey, USA) were used for these sensors. The film (Figure 5) was expected to have the capacity to convert magnetic energy into elastic energy upon application of a magnetic field and this elastic wave would then generate a signal as a frequency. The ribbons had the size 30 mm x 4 mm x 29 µm. The reasons to choose this magnetic alloy was that it takes longer time to reach a state where an increase in external magnetic field cannot increase the magnetization of the material compared to others available on the market (Scherer et al., 1987; Thomas et al., 2010). The film should have a coating by parylene to avoid rusting. A thickness of 5 µm was used in this project as a thinner coating would not be able to prevent rusting and more than 5 µm may have interfered with the response of the film because its volume and surface resistivity decreases with thickness.

To estimate the required number of polymer layers needed to cover the surface of the metal foil, we first prepared a smooth glass slide. The advantage of using a glass slide was that we could easily determine the surface coverage by the polymer under a light microscope. First a 10 µl pipette was used to pour 3-4 drops (10 µl) of a mussel adhesive protein (MAP) solution on the slide. MAP is secreted by mussel and other marine organisms and was used to increase the adherence capacity of the surface to the polymers. The presence of L-3,4-Dihydroxyphenylalanine is believed to be responsible for adherence characteristics of MAP solution. The slide with MAP solution dried for 10 minutes. Then it was rinsed with deionized water to remove surplus glue from the slide and dried for 10 minutes. Then, 10 µl of polymer solution (3-4 drops equivalent to 10 µl corresponding to one layer) was placed on the glass slide. After drying for 20 minutes, the slide was placed under a light microscope to determine the coverage of the polymer. If there were uncovered spaces, we continued to add layers of polymer until the surface was covered. For each layer of polymer, one more layer of MAP added. The observations under light microscope revealed empty space on the glass slide. To fill the empty spaces, we continued adding layers according to the described procedure and ended up with at least 5 layers to fully cover the glass slide with polymer.

To prepare the sensor, a magnetoelastic ribbon was immersed in MAP solution overnight. On the following day, the ribbon was washed in distilled water. Layers of polymer solution were added according to the procedure described for the glass slide above (Figure 5). Three sensors were prepared, one with 6 layers, another with 8 layers and one with 10 layers. There were two ears of each metal ribbon. One of these was cut and the other ear was fixed in a small plastic frame with screws (Figure 6 and 7) and the frame was wrapped in a magnetic cover made of rubber (Figure 8). A cross-section of the sensor can be seen in Figure 9.

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Figure 5. A drop of MAP (mussel adhesive protein) solutions on the magnetoelastic ribbon after one layer of polymer on it.

Figure 6. All the material used for sensor preparation (ribbon, plastic frame with screw and magnetic cover).

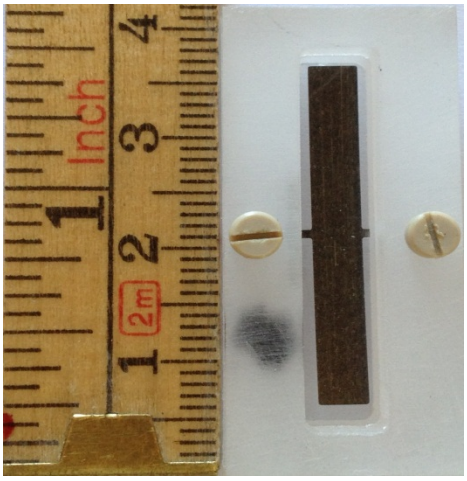

Figure 7. Prepared sensor without magnetic covering (After polymerization the ribbon was fixed inside the plastic frame with screws).

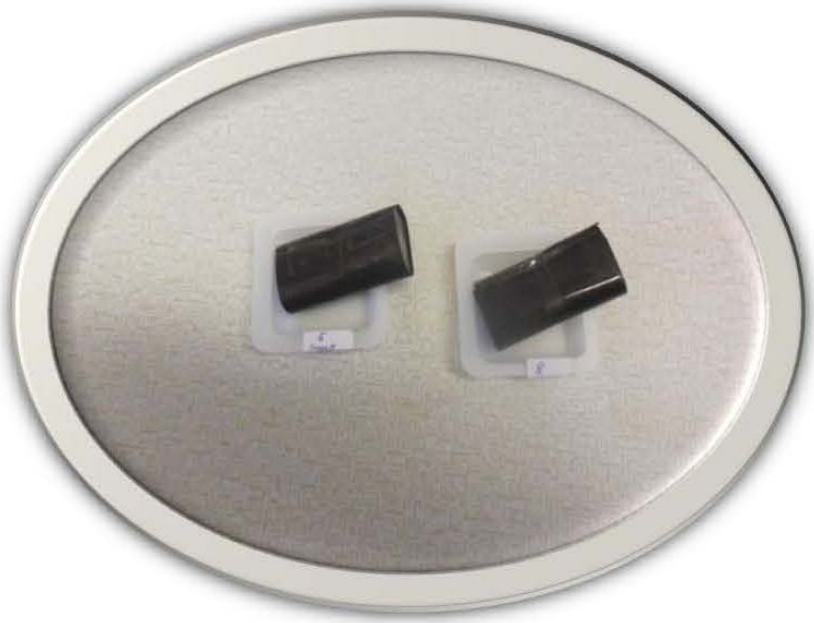

Figure 8. Two prepared sensors ready for use, One with 6 layers of polymer and the other one with 8 layers. The polymerized ribbons have been fixed in plastic frames and wrapped with magnetic cover.

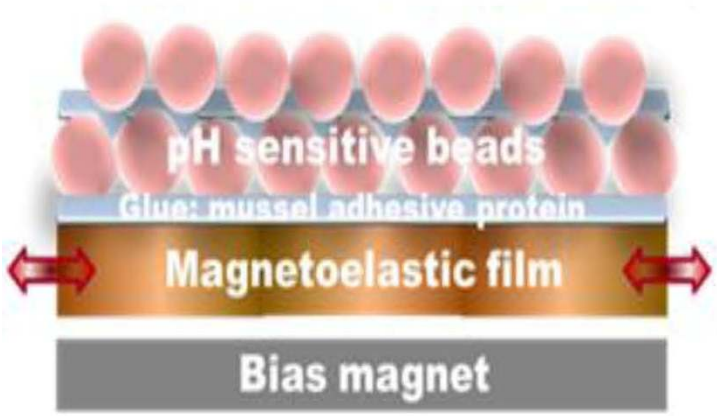

Figure 9. Cross section of a magnetoelastic sensor covered with beads of pH sensitive polymer spheres attached by a glue of mussel adhesive protein and wrapped in a magnetic cover.

4.3 Test solution preparation

The sensors were tested in different buffer solutions and rumen liquid.

4.3.1 Phosphate buffer solution

The intention was to evaluate the performance of the sensor in an isotonic solution and for that purpose, a phosphate buffer was used. For the preparation of phosphate buffer 8.01 g of NaCl, 0.20 g of KCl, 1.78 g of $Na_2HPO_4 \cdot 2H_2O$, 0.27 g of KH_2PO_4 were dissolved in distilled water and diluted up to one liter volume.

4.3.2 McDougall's buffer solution:

Secretion from saliva acts as buffering agent on the digestion of feed particles in the rumen. McDougall's buffer was used as one test solution as it resembles saliva in composition. For the preparation of McDougall's buffer 9.8 g of $NaHCO_3$, 9.25 g of $Na_2HPO_4 \cdot 12H_2O$, 0.57 g of KCl, 0.47 g of NaCl, 0.13 g of $MgCl_2 \cdot 6H_2O$ and 0.0455 g of $CaCl_2$ were dissolved in sufficient volume of distilled water to get one liter solution. It was mixed vigorously until dissolved and then provide CO_2 inside the beaker for 2 hours, kept in glass bottle in room temperature.

4.3.3 VOS buffer solution

To compare the performance of the sensor in solutions with different ionic composition an alternative buffer solution named VOS buffer was used. It is normally used together with rumen fluid in an in vitro organic matter digestibility analysis. For the preparation of one liter of solution 5.80 g of K_2HPO_4,0.50 g of $(NH_2)HPO_4$, 1.0 g of NaCl, 0.5 g of $MgSO_4 \cdot 7H_2O$,0.01 g of $FeSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2$, 8.50 g of $NaHCO_3$ and was dissolved and diluted with distilled water.

4.3.4 VFA preparation

Digestions of feed particles inside the rumen produce volatile fatty acids (VFA) which alter pH in the rumen. A mix of VFAs was used to alter pH in the test solutions. The concentration of the VFA stock solution was 1.57 M and was composed

of 65 % acetic acid, 20 % propionic acid and 15 % butyric acid based on normal molar proportion of individual acids in the rumen.

4.3.5 Rumen liquid collection

Rumen samples were collected from a rumen fistulated cow in the barn at Kungsängen Research Center. The cow was not lactating and on a diet of 4 kg of hay, 2 kg of concentrate and 2 kg of straw per day. Whole rumen content was collected manually and immediately transferred to a thermos. Within one hour after collection the rumen content was strained through a 1 mm metal sieve to remove large particles. The fluid was kept in water bath (38^0C), gassed with CO_2 and kept covered to preserve anaerobic conditions.

4.4 pH measurements

A Mettler Toledo SG2 pH meter (SevenGo ^{TM}) combined with a glass electrode was used in all experiments to measure pH. This portable pH meter had a built in memory and had automatic function to detect changes in pH in buffer solution and in rumen liquid. The pH meter was calibrated at pH 4 and pH 7 before use.

4.5 Measurement system to collect resonance frequency

A measurement system consisting of a coil, a microcontroller unit (MCU) and a computer with adapted software was used to collect information from the sensors. The coil was made of 20 turns of thin copper wire and acted as a magnetic antenna. A larger number of turns and a larger area of the coil enabled detection of weaker signals from the sensor. The microcontroller unit was connected to the coil and the computer. The coil sent the driving signal, picked up the resonance frequency and transmitted them to the computer for interpretation. The system layout is presented in Figure 10.

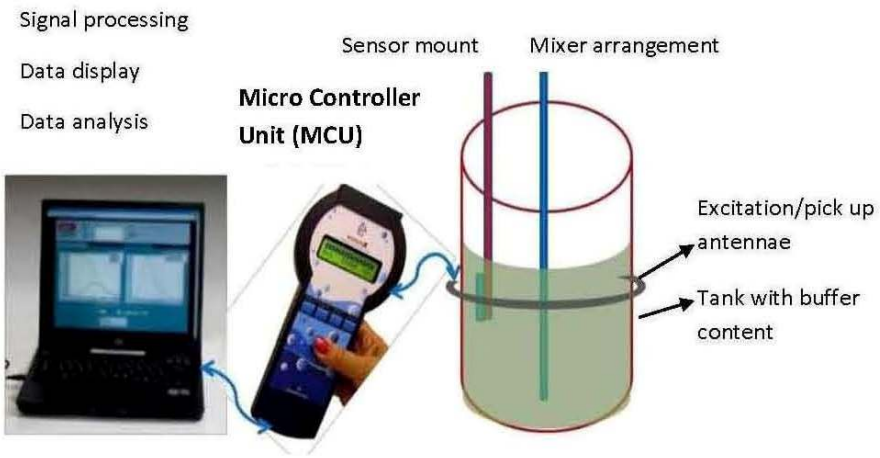

Figure 10. The measurement system used in most of the experiments.

The software used in this project was a MATLAB compiler 7.14 (Figure 11). We used 'the single measurement' option in this software at initial measurement. The single measurement revealed the amplitude of the sensor signal. After that we turned to the option 'Time sweep'. The signal was then displayed as 'Resonance frequency vs Time'. Alternative options of the software were to visualize the 'Signal to noise ratio (SNR) vs Time' or 'Q value (ability of the system to produce a large output at the resonance frequency) vs Time'. The SNR value described the strength of the signal in relation to noise and hence the quality of the measurement. The software settings could be customized, for example to restrict measurements to certain frequency intervals to reduce interfering signals from other electromagnetic sources.

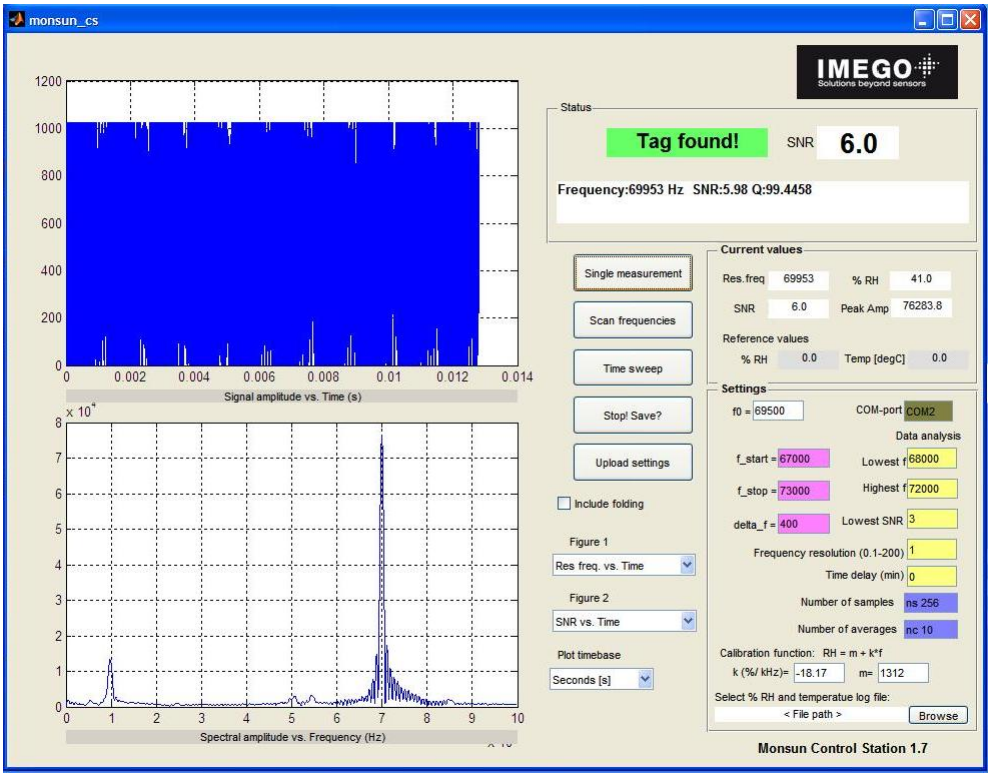

Figure 11. Snapshot of MATLAB compiler 7.14 used to analyze the delivered signal from the sensors.

4.6 Arrangement for the experiment

A wooden box (Figure 12) with the aim to hold a constant temperature of 39^0C was used in this project to attain a constant rumen temperature during the in vitro experiment. An aluminum pipe was attached at one end of the box to deliver hot air to the wooden box. The other end of the pipe was attached to a 400- W heater and a fan to circulate hot air along the direction of the heater. The wooden box was covered with plastic and on the top cover there were 4 holes for beakers inside the box. The box had mini magnetic stirrer under each beaker to mix the solution properly. The stirrer we used in this project was a color squid (Carlroth^{TM}). This stirrer had a glass platform and a plastic base made of TPC- ET (thermoplastic polyester elastomer).

The beaker was closed with a lid which has 3 openings for the sensor, pH meter and to pass the gas which was produced in the beaker. MER film coated with a bias magnet was tied with a non-metal stick to dip in buffer. The reason to use the holder was to avoid contact of MER film with any surface and to insure a stable position in relation to the antenna and the bias magnet. The opening of the beaker was closed. The coil was placed vertically around the beaker and other end of the coil was attached to the monitor (Figure- 13). A peristaltic pump (Watson – Morlow, 502s) was used to add HCl or NaOH to the beaker continuously with a speed of 9 drops of acid/minute (Figure 13). A stirrer was used to mix the added acid or alkali in the buffer solution.

Figure 12. The wooden box used in this experiment to keep the temperature constant. The wooden box was covered with plastic and on the top of cover there were 4 holes to place the beaker inside the box.

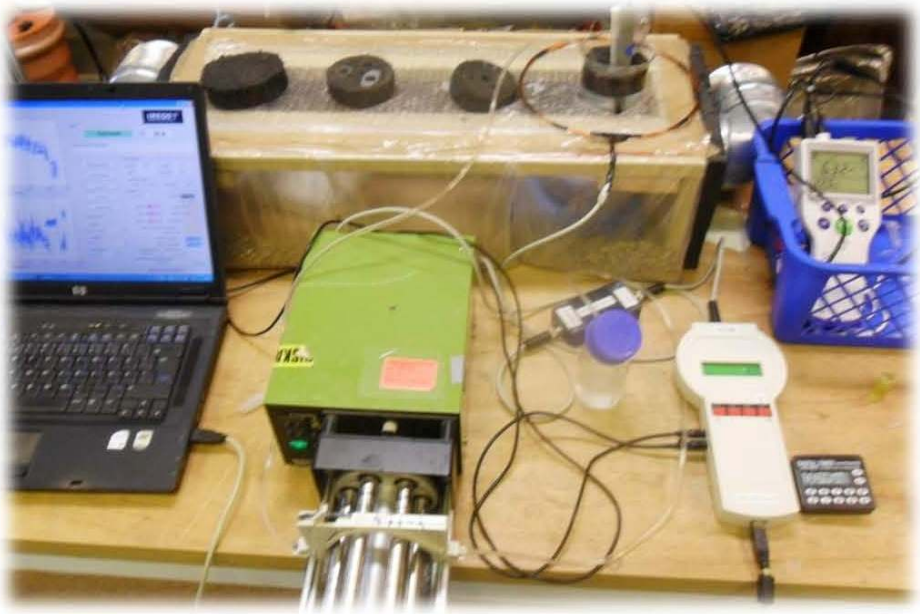

Figure 13. Complete arrangements to run the experiment, e.g coil around the beaker, micro controller unit (MCU) computer and peristaltic pump for continuous infusion of acid. The coil attached to the MCU and the MCU is connected to the computer.

5 Results

5.1 Coverage of polymer and polymer erosion

5.1.1 Observation of polymer coverage of glass slides and magneto elastic ribbons

The aim of this experiment was to evaluate the effect of increased number of polymer layers on the coverage of the prepared surface. Another aim was to evaluate the effect of exposure to the test solutions on erosion of the sensor polymer beads.

Increasing the number of layers of polymer decreased the empty space on the glass slides (Figure 14, Figure 15). The cluster of polymers didn't show much erosion after exposure to McDougall's buffer or rumen liquid (Figure 16, Figure 17). The adhesion capability of MAP seemed satisfactory as rinsing the slide with ionized water, buffer or rumen liquid didn't decrease polymer coverage.

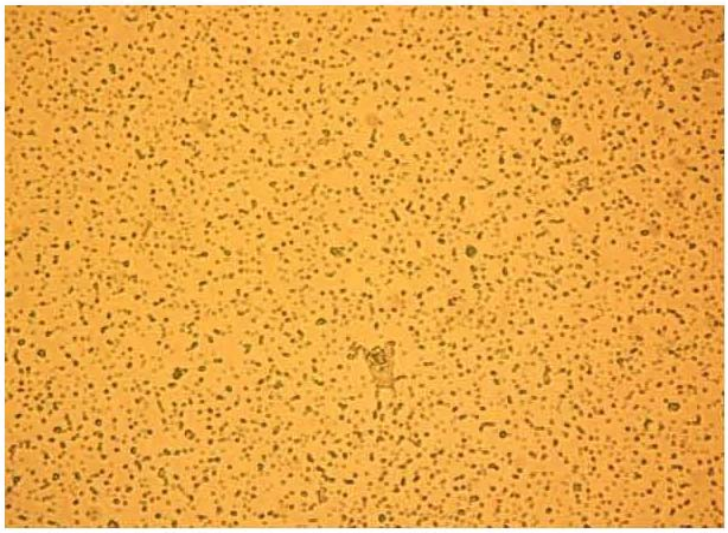

Figure 14. One layer of polymer including one layer of mussel adhesive protein (MAP) solution added to a glass slide. light microscope with 40 x magnification and light-3.

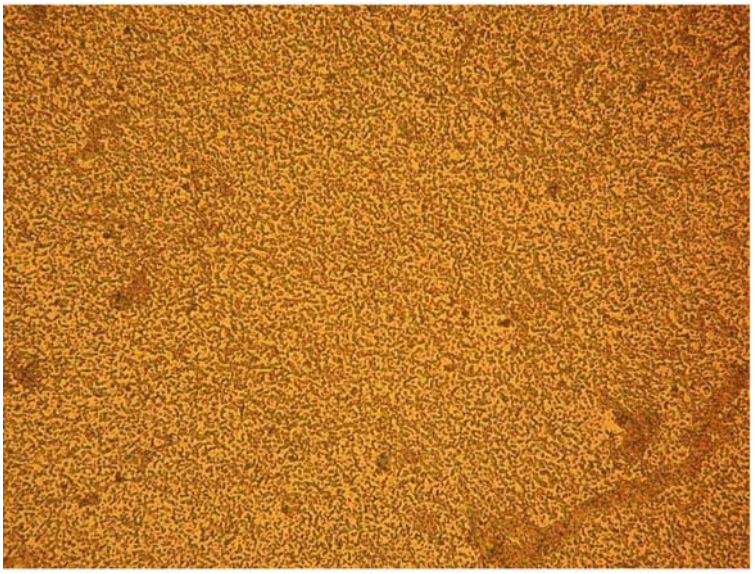

Figure 15. Six layers of polymer including six layers of MAP added to a glass slide. light microscope with 40 x magnification and light-3.

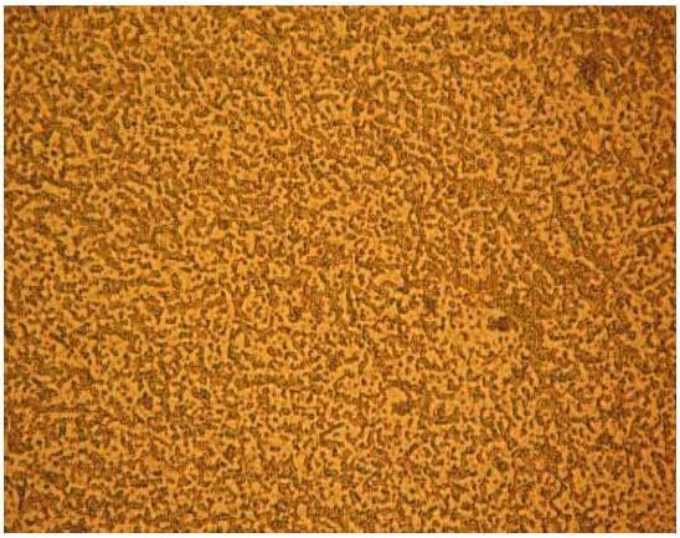

Figure 16. The glass slide after exposure to McDougall's buffer solution for 1hour (six layers of polymer including six layers of MAP added to a glass slide, light microscope with 40 x magnification and light-3.

Figure 17. After exposure to rumen liquid for one hour and rinsing in water the slide was placed under microscope (six layers of polymer including (six layers of MAP added to a glass slide, light microscope with 40 x magnification and light-3).

Glass slides were also examined by scanning electron microscopy (SEM- Olympus BX41, Tokyo, Japan) by another student from the IMEGO institute (Gothenburg, Sweden) to calculate the size of the polymer spheres. This analysis showed

an average diameter of each particle of about 250 nm (Schanzenbach et al., 2012, unpublished data). They formed a loose network which indicates weak intermolecular attractions. These particles form clusters of more than 700 nm in diameter (microscopy ruler) in the light microscope (Olympus BX41, Tokyo, Japan). Furthermore the same clusters were visible on MAP coated MER foil in the stereo microscope (Olympus SZX12, Tokyo, Japan) indicating the same binding behavior on these two surfaces (not shown). Surface profilometry measurements (Tencor P15, KLA-Tencor, US-Ca) corroborate the size of the clusters and the diameter of the spheres. Figure 18 and Figure 19 show particles with a size of 200 nm to 250 nm which form an incomplete layer on a glass slide.

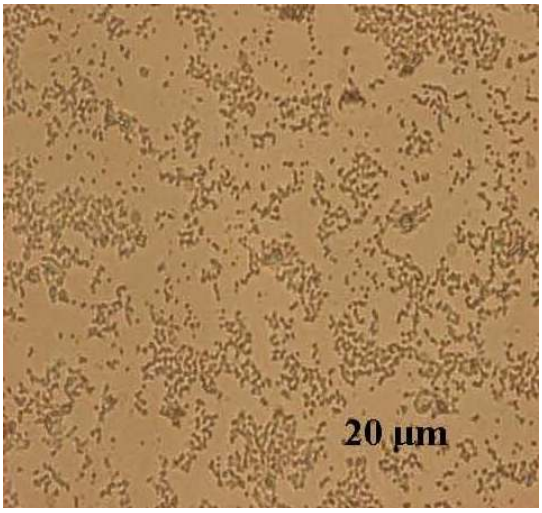

Figure 18. Light microscope photograph (40x magnification) of pH polymer spheres on a glass slide, low coverage (1 layer),

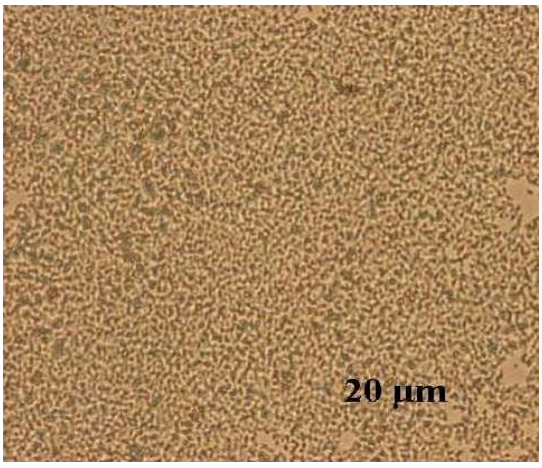

Figure 19. Light microscopic photograph (40x magnification) of pH polymer spheres on a glass slide, (5 layers).

5.1.2 Effect of the number of polymer layers on sensor performance

This was done to determine the effect of increasing number of polymer layers on to the delivered frequency from the sensor. Three sensors prepared with 6, 8 and 10 layers of polymer on a magneto elastic ribbon were used in this experiment. McDougall's buffer was used and pH was 6.15 during the experiment. All the arrangements were the same for the three different sensors during the measurement.

The resonance frequency from the sensors increased with number of layers added to the sensor (Figure 20). The SNR also increased with increased number of layers of polymer (Figure 21).

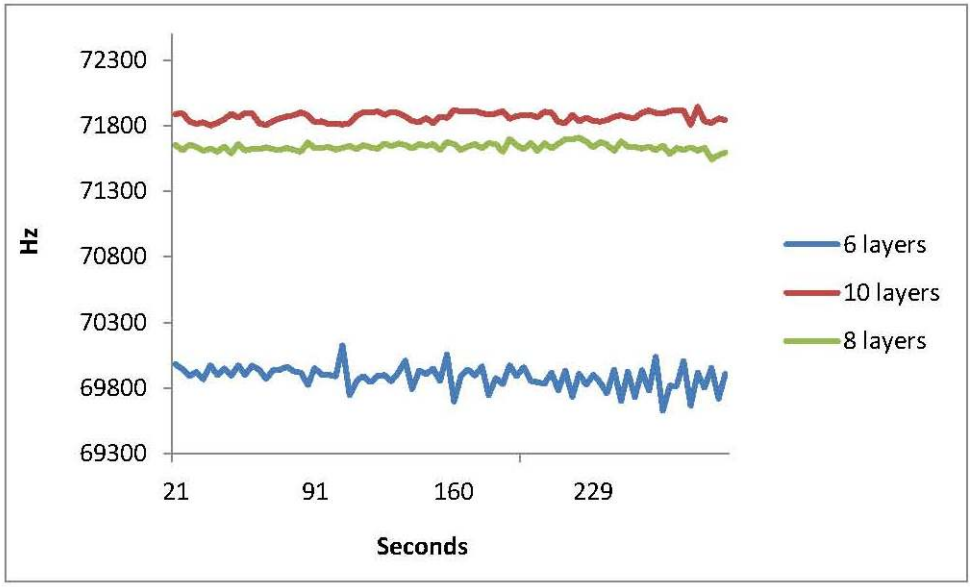

Figure 20. Comparison of delivered frequency from the sensor prepared by different layers of polymer. Sensor with a coverage of 8 layered, 6 layered and 10 layered polymers were used in McDougall's buffer solution. pH was 6.15 during measurement period.

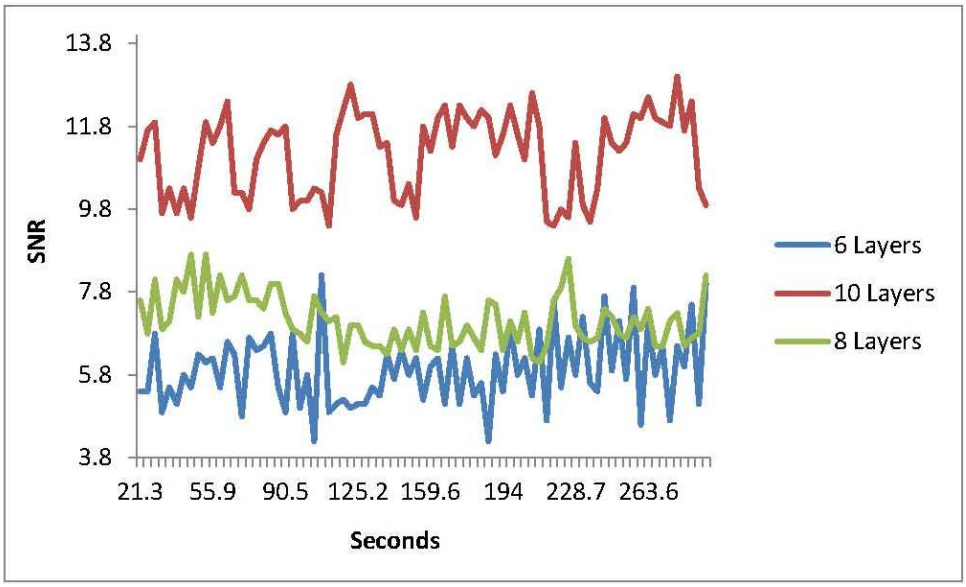

Figure 21. Comparison of SNR (signal to noise ratio) from the sensor prepared by different layers of polymer. Sensor with 8 layers, 6 layers and10 layers of polymer were used in McDougall's buffer solution. pH was 6.15 during measurement period.

5.2 Sensor environment and measurement condition

5.2.1 Effect of the environment

This experiment was done to investigate whether different environment (air, water, and buffer) would affect the response frequency from the sensor.

The sensor was placed 1) on a table; the coil was placed around the sensor, 2) the sensor was placed in a beaker with deionized water, 3) The sensor was placed in a beaker with phosphate buffer solution. The coil was placed around the beaker to capture the delivered frequency. Measurements were made for 17 minutes with the sensor in air, for 24 minutes with the sensor in water and after that the sensor was placed in a beaker with phosphate buffer. The pH of the buffer solution was measured by portable pH meter.

The sensor responded differently due to environment. This film showed a clear demarcation among these three different environments (Figure 22). The lower frequency in liquid compared with air was due to the dissipative shear force created by the liquid (Grimes et al., 2002). The higher frequency in buffer compared with water is probably due to the difference in pH (the pH of water was 7, and the pH of the buffer was 8.24).

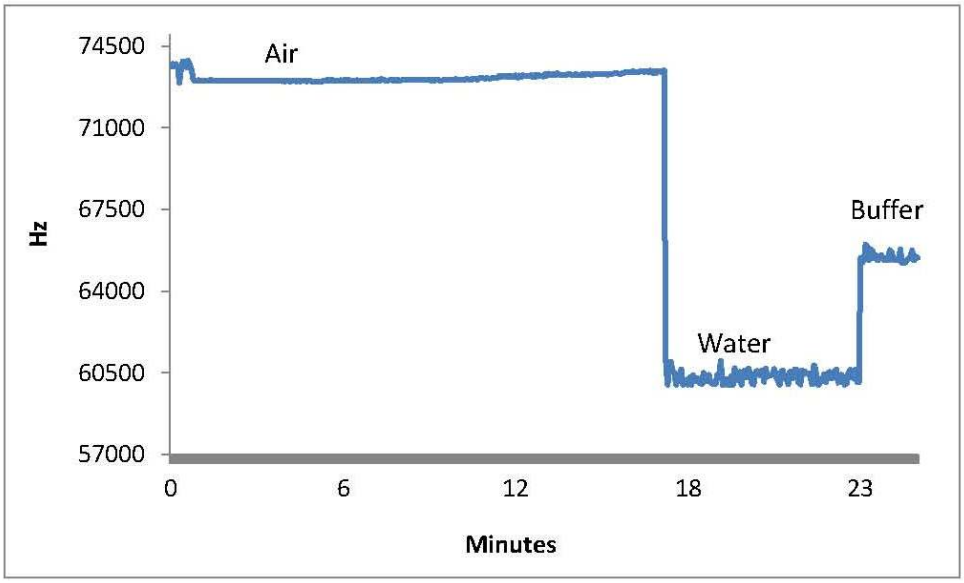

Figure 22. Performance of a sensor in air, water and buffer. Alterations of the environment enhance the sensor to generate different frequencies.

5.2.2 Effect of temperature:

As the temperature inside the rumen is around 40^0C and the polymer used previously has only been evaluated at lower temperatures we wanted to find out if the sensor was affected by temperature before using it in vivo.

To evaluate the effect of temperature, the sensor was placed in a beaker with phosphate buffer in a box like structure made of wooden frame described under 4.6 (Figure 12). The beaker was placed in the box over night with hot air flowing. The temperature slowly rose from 25^0 C to 39^0C.Temperature measured after 45, 200, 500 and 1200 minutes were 29^0C, 33^0C, 39^0C and 39^0C respectively.

At the very beginning and until 170 minutes there was some disturbance due to alteration of the position of the sensor. That's why there was a shift in frequency from 71500 Hz to 70800 Hz (Figure 23) at 170 minutes. After 170 minutes the resonance frequency was stable until the end of the measurement period. The result indicates the prepared sensor not being sensitive to temperature changes within the temperature range 25to39 ^{0}C.

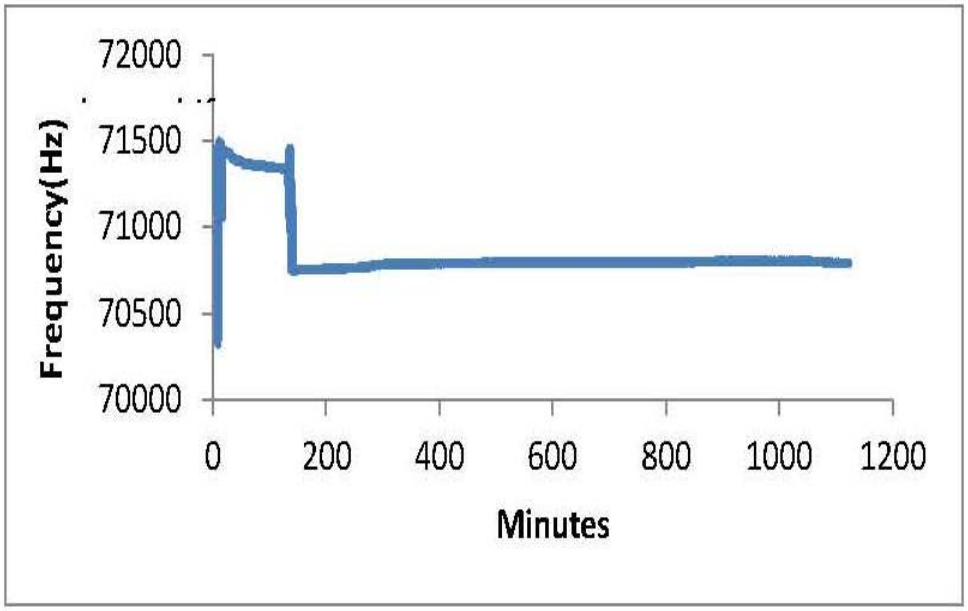

Figure 23. Effect of temperature on resonance frequency. The sensor was placed in phosphate buffer solution and temperature was increased from 25 to 39 ^{0} C.

5.2.3 Effect of buffer composition on sensor performance

This was done to compare the signal delivered by the sensor in buffers of different composition and the same pH level. The same sensor was placed into 1) a 500 ml beaker with VOS buffer and 2) another beaker with McDougall's buffer (both buffers are described under 4.3). pH was adjusted to 6.54 on both of those solutions.

The signal generated from the sensor in the two different environments is presented in Figure 24. The difference in frequency collected was comparably small (10-30 Hz) which indicates little effect of buffer composition on sensor performance.

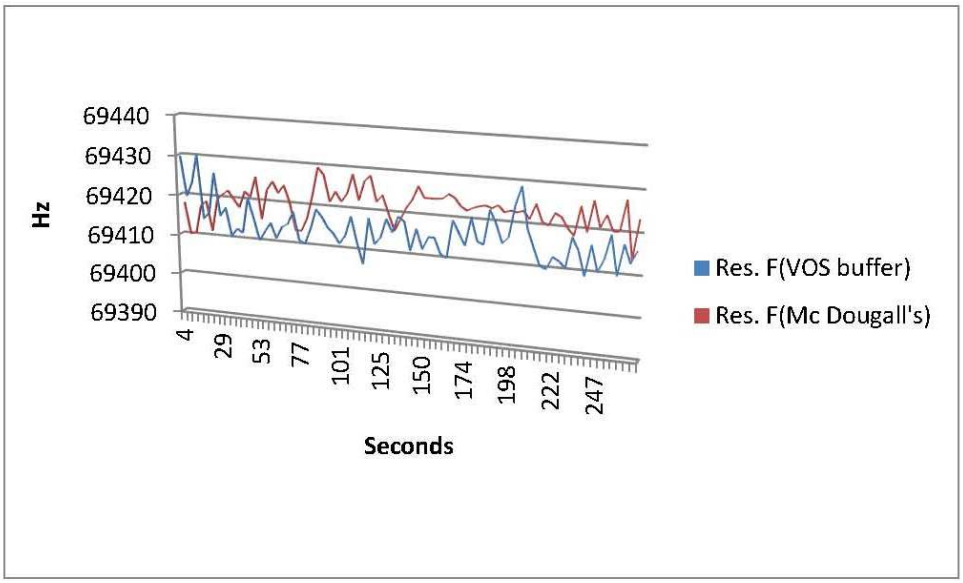

Figure 24. Frequency response in two different buffer solutions, VOS buffer and McDougall's buffer, at same pH level.

5.2.4 Effect of the distance between sensor and coil on resonance frequency

This experiment was made to evaluate the effect of the distance of coil from the sensor on the delivered signal.

The sensor was placed in a beaker with VOS buffer at constant pH of 7.2.The coil was placed around the beaker and 1) 15 cm above the sensor and 2) 15 cm below the sensor. The response is represented in Figure 25. The mean frequency when the coil was placed above the sensor was 68284Hz (SD 127) and below the sensor was 68440 (SD 49).

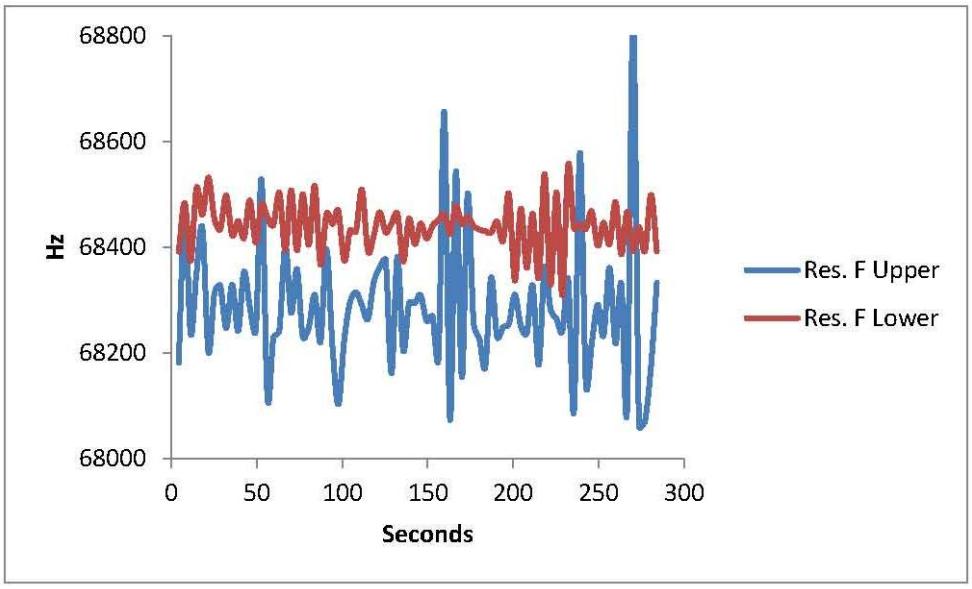

Figure 25. Sensor in VOS buffer with pH 7.2. Comparison between coil positioned above (Res. F Upper) and below (Res. F Lower) the sensor.

5.3 Detection of pH changes and influence from an electric stirrer on frequency readings

5.3.1 Detection of pH changes during continuous stirring

A 500 ml beaker with VOS buffer was placed on a magnetic stirrer kept in a box like arrangement (Figure 12). Temperature of this buffer was adjusted to 39^0C. Alteration of pH was done by addition of 0.5 M HCl continuously through a peristaltic pump (Watson –Morlow, 502s) (Figure 13). To mix HCl in the buffer solution a stirrer was run continuously. The changed chemical pattern of the buffer was measured by the manual pH meter with an interval of 10 minutes.

This unstable response may have been due to the magnetic stirrer (Figure 26). The coil probably picked up signals from the magnetic field created by the stirrer which interfered with the received signal from the sensor. Initial pH level of this buffer was 8.02 taken by the portable pH meter. pH reading by the portable pH meter were 7.69, 7.37, 7.05, 6.76, 6.41, 6.23, 6.0 at 5, 15, 25, 35, 44, 55, 65 minutes respectively (Figure 26).

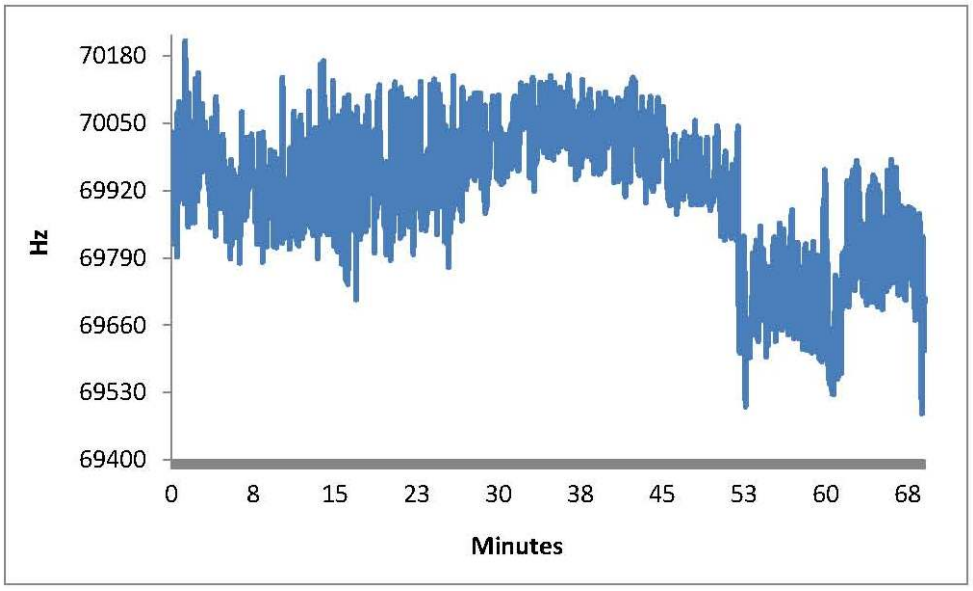

Figure 26. Frequency obtained by continuous stirring at different pH. Sensor with 6 layers of polymer was used in a VOS buffer solution within a range of pH 8.02 to 6.0. pH was changed with the help of HCl. Effect of the stirrer reveals a variable response.

5.3.2 Effect of the electric stirrer on measured response frequency

As the stirrer seemed to cause a large variation in the measured response frequency, an experiment to evaluate the magnitude of the influence was made. The sensor was positioned in a 500 ml beaker with VOS buffer and the beaker was placed on a mini magnetic stirrer. The stirrer was set at 250 rpm and was turned on and off in intervals of approximately 8 minutes. The pH of the buffer was unchanged during the measurement period.

The delivered frequency seen in Figure 27 showed a clear demarcation between the two conditions. The mean frequency with the stirrer on was 67849 (SD 5) and with the stirrer off 67873 (SD 4). The fluctuation in frequency seemed less with the stirrer off compared with the stirrer on.

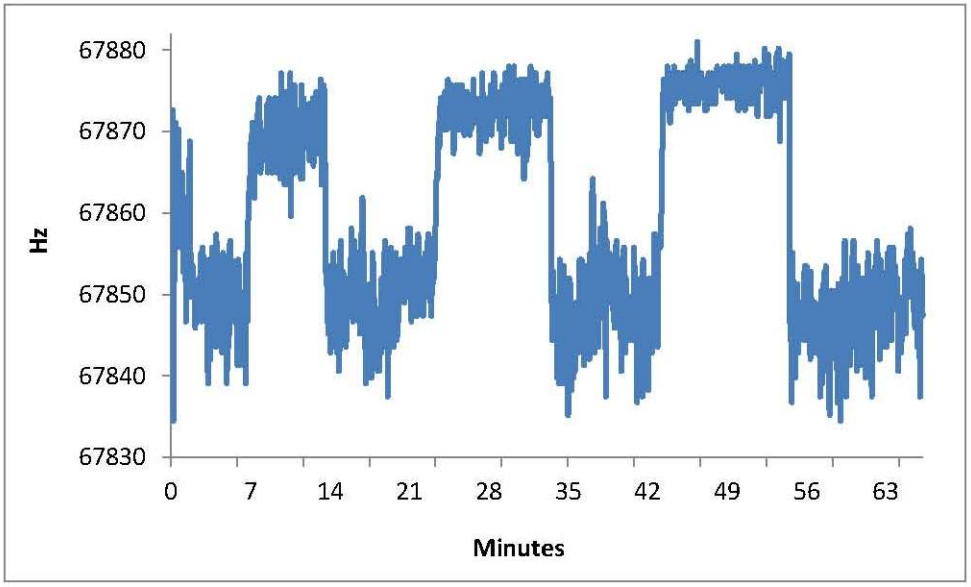

Figure 27. Effect of the magnetic stirrer on the frequency reading. A sensor with 6 layers of polymer was used in VOS buffer solutions. The stirrer was turned on at 0 to 7, 14 to 22, 30 to 42 and 54 to 67 minutes. Rest of the time stirrer was turned off.

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5.3.3 Detection of pH changes by stirring and addition of acids at intervals

5.3.3.1 HCl used to alter pH

To avoid effects of the stirrer a strategy with stirring in intervals was applied. The sensor response to decreased and then increased pH was evaluated in VOS buffer where HCl (0.5 M) and NaOH (0.1 M) was used to alter pH. Addition of HCl by pipette to the buffer was followed by stirring for 30 seconds. The stirrer was then turned off and after a couple of minutes pH was measured. This was followed by another addition of acid, 30 s of stirring and so forth. When pH reached close to 5 addition of NaOH started with the same procedure as that for HCl.

The results (Figure 28) indicate a clear frequency response in relation to the changed pH level. As for example, pH measured after 81 minutes and 141 minutes was 5.75 and the difference in frequency between 81 minutes and 141 minutes were only 15 Hz.

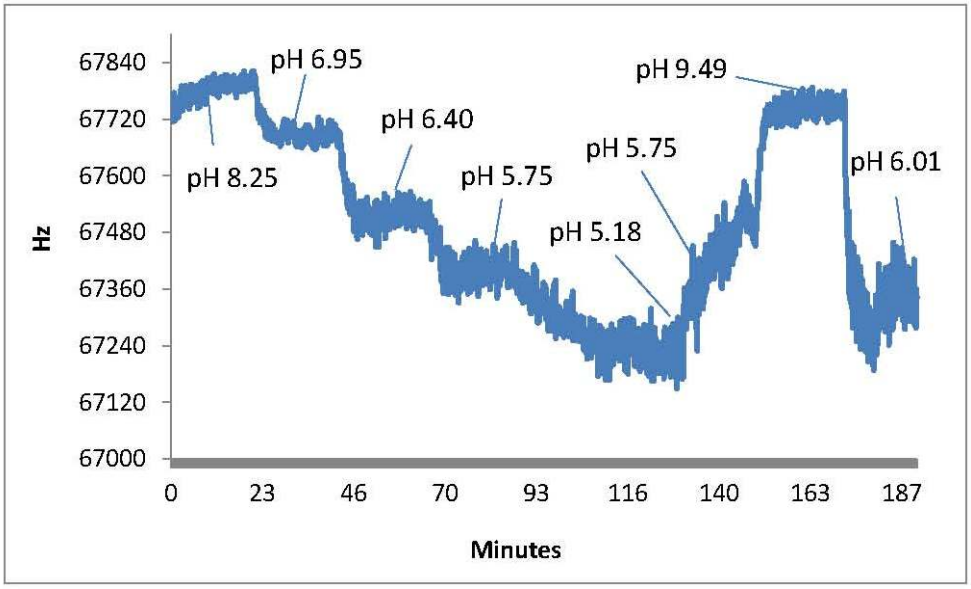

Figure 28. Continuous recording of response frequency at different pH levels in VOS buffer. HCl and NaOH were used to decrease and increase pH respectively.

5.3.2.2 VFA used to alter pH

In another experiment with VOS buffer pH was adjusted to 6.17 by the addition of the VFA mixture described under Materials and Methods. From 6.17 pH was raised to 8.04 by the addition of buffer and then pH was decreased by the addition of VFA. A pipette was used to add buffer and VFA and after each addition the stirrer was run for 30 seconds.

The frequency response was proportional to the changed pH level (Figure 29). During the early period from pH 6.17 to 6.39 the frequency increased with 114 Hz per pH unit and from pH 5.77 to 5.57, there was a 120 Hz/pH unit change.

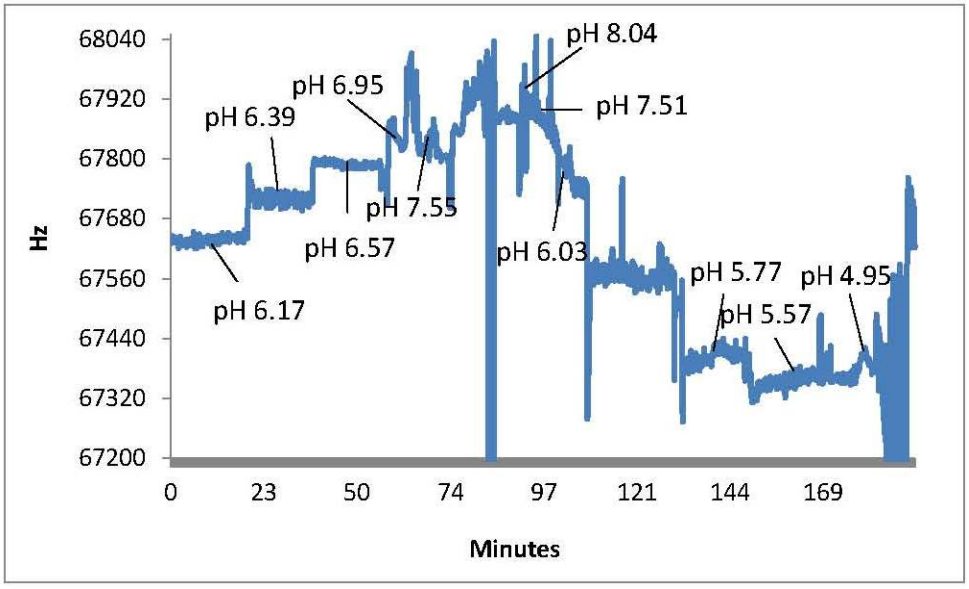

Figure 29. Continuous recording of the frequency after addition of VFA in intervals. pH was increased by adding VOS buffer and decreased by adding VFA.

5.3.4 Detection of pH changes in McDougall's buffer

In this experiment a VFA mixture was used to alter pH. McDougall's buffer was heated in a water bath to reach 38^0C and then placed in a 500 ml beaker in the temperate box described earlier. VFA was added with a pipette. A stirrer was used for mixing. All other arrangements were as mentioned above. A nylon bag was used to cover the sensor to reduce external pressure of the testing solution.

The gas production in the buffer had an influence on the delivered signal from the sensor. The $NaHCO_3$ portion of McDougall's buffer reacts with the acid resulting in production of CO_2 emerging as bubbles. After some addition of acid, bubbles accumulated on the nylon bag surrounding the coated film (Figure 30) and at the same time an increased variation in response frequency (Figure 31) was observed.

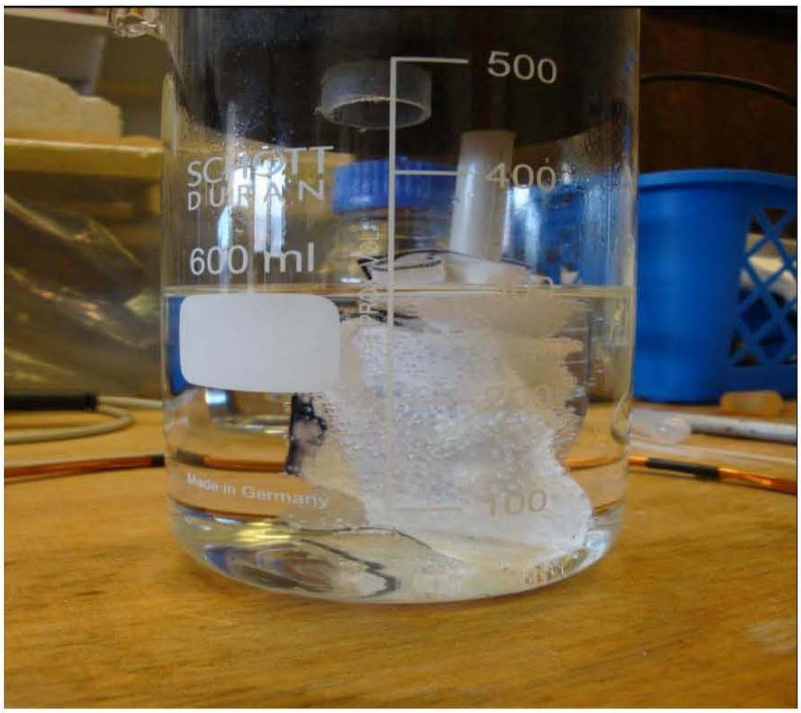

Figure 30. Bubble formation inside the beaker after addition of acid.

The frequency response showed a linear relation to pH change until bubble formation occurred (Figure 31). Noise increased markedly with bubble formation impairing sensor performance. By stirring the visible bubbles could temporarily be eradicated but without improved senor performance. This was probably due to that sensor performance was affected by bubbles on the magneto elastic film which could not be eradicated as they were protected inside the sensor cover.

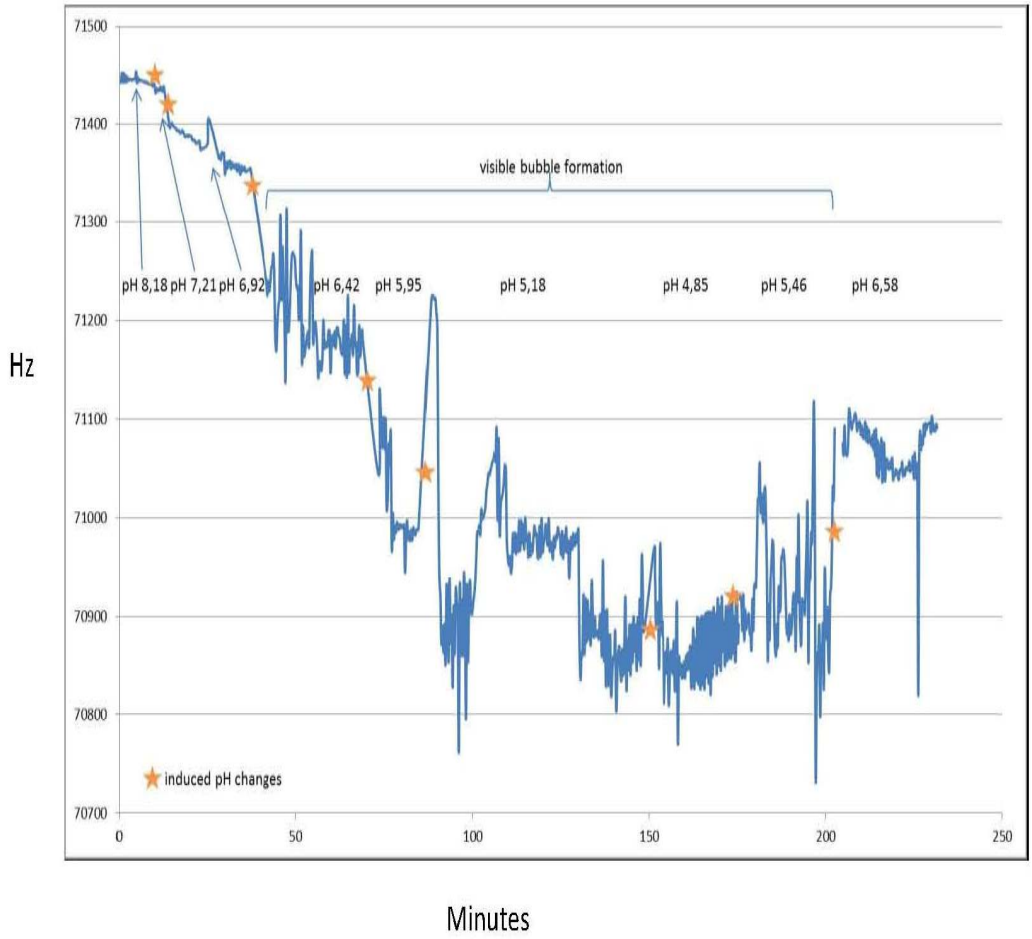

Figure 31. Resonance frequency from the magneto elastic sensor in relation changed pH level. Measurement in McDougall's buffer solution where pH was lowered by the addition of VFA. Visible bubble formation in the beaker followed enhanced resonance frequency variation.

5.3.5 Dilution of the buffer and added acid to reduce bubble formation.

This experiment was done to determine if dilution of the buffer and the addition of acid could limit bubble formation. As the stirrer seemed to have a significant effect on senor response, we used an electric shaker (Figure 32) in this experiment, hoping the shaker would affect the sensor less than the stirrer.

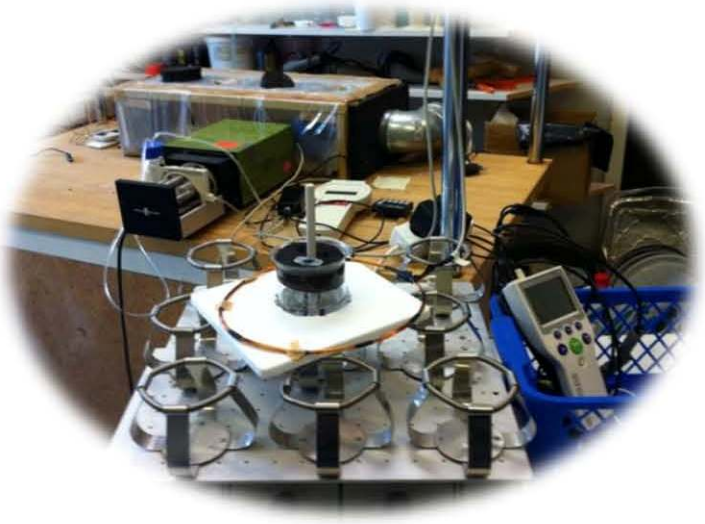

Figure 32. Electric shaker used in this experiment.

A diluted buffer (McDougall's buffer 50 ml and distilled water 150 ml) and diluted acid (20 ml VFA, 1.57 M and 100 ml distilled water) were used in this experiment. The shaker was used in every 6 minutes for 40 seconds. VFA was added continuously with a peristaltic pump.

Initial pH of the buffer was 7.45. pH was 7.13 after 6.5 minutes, 6.92 after 18.5 minutes, 6.69 after 23.5 minutes and 5.37 after 50 minutes (Figure 33). There resonance frequency changed continuously in relation to the addition of acid. The result (Figure 33) indicates that the polymer needs at least 1-3 minutes to adapt to a new environment to perform swelling and shrinkage behavior after which the resonance frequency becomes linear. Bubbles were formed during acid infusion but that did not seem to have a significant influence on the resonance frequency

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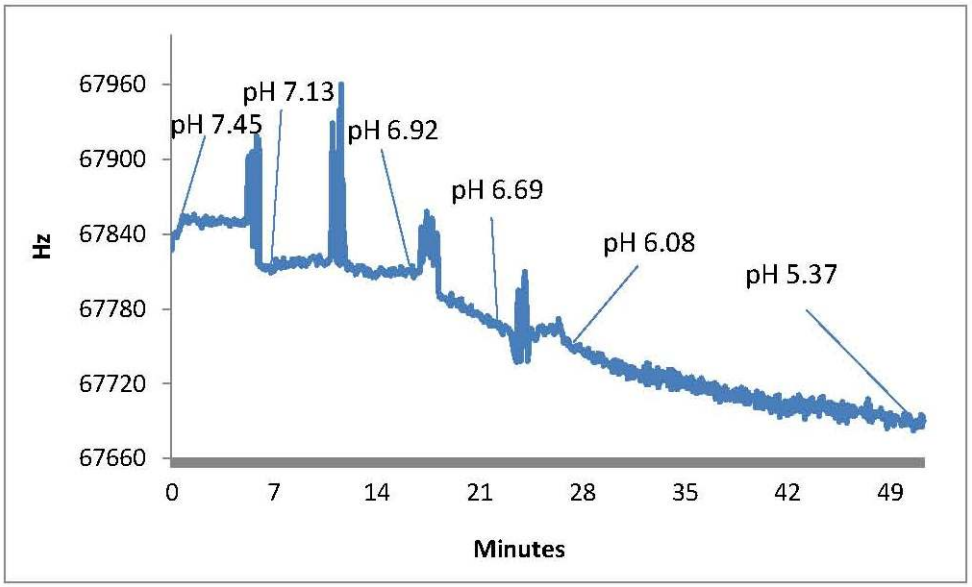

Figure 33. Frequency recordings during continuous addition of VFA to a diluted McDougall's buffer (1:3).

5.3.6 Further dilutions of the buffer

To avoid bubble formation buffer was diluted even more to get rid of the effect of the bubbles upon the delivered signal. Diluted buffer (50 ml McDougall's buffer,350 ml distilled water) was used but VFA was kept unchanged. To mix the added acid homogenously the previously described shaker was used.

A change in frequency of 48 Hz/pH unit was observed between pH 6.75 and 6.48. Similar values with 52 Hz/pH unit was seen from pH 6.48 to pH 6.27 (Figure 34).

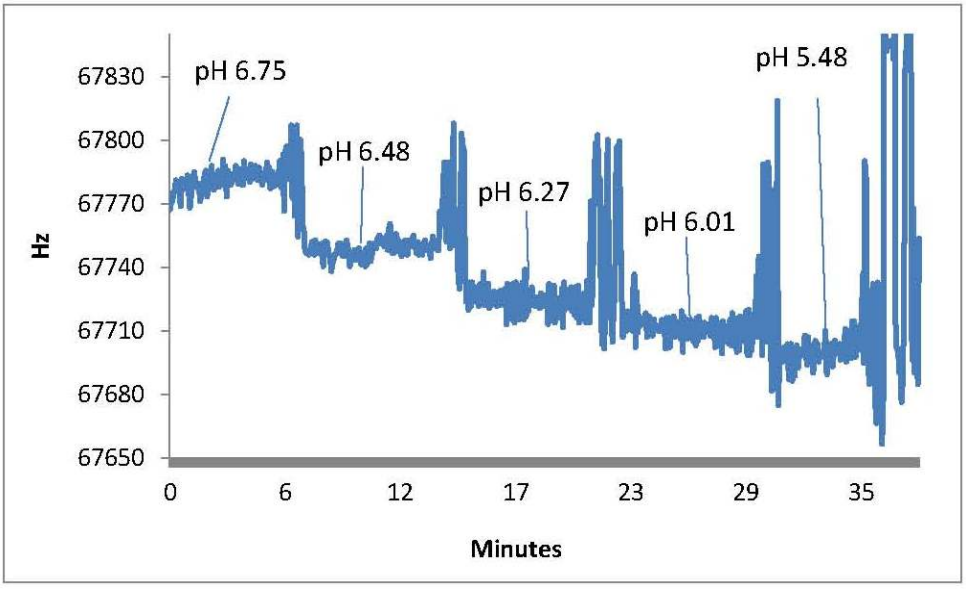

Figure 34. Continuous measurement of pH by the use of shaker (sensor with 6 layers of polymer). Diluted McDougall's buffer (1:7) was used. Shift of frequency at 5-6 minutes was due to the activity by shaker.

According to the results from Figure 33 and Figure 34, a good relation between pH and resonance frequency was observed. Frequency decreased with decreasing pH and the trend was similar in solutions of different ionic strength (Figure 35).

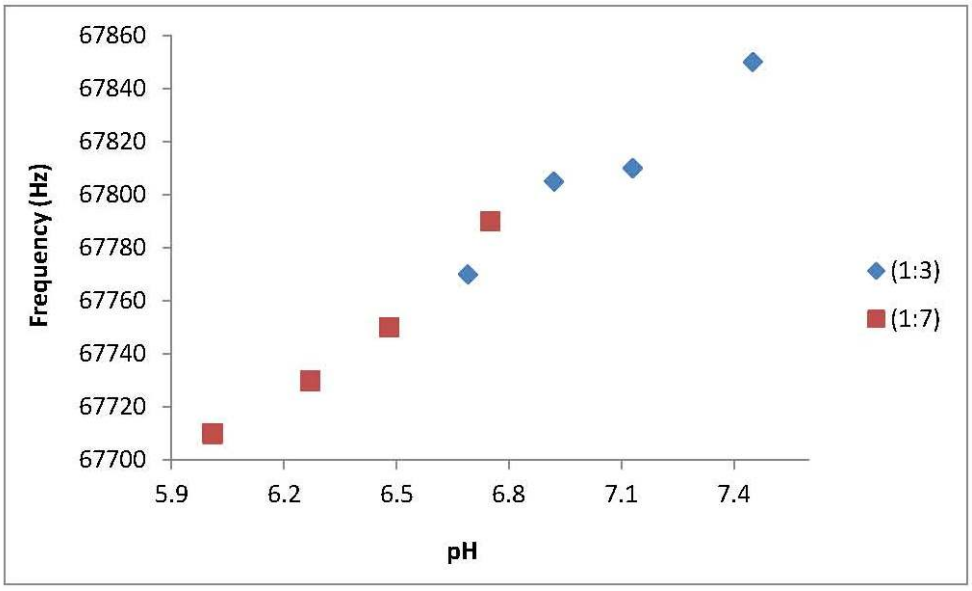

Figure 35. Comparison of the delivered frequency from diluted buffer (1:3 – A and 1:7- B) at different pH levels. A: Diluted buffer (1:3) and diluted acid, B: Diluted buffer (1:7) based on the results from Figure 33 and Figure 34.

5.4 Experiment with rumen fluid

In this experiment we used rumen fluid and sugar to enhance fermentation and VFA production. After collection, rumen liquid in a 500 ml beaker was heated to 38^0C in a water bath. After 15 minutes of heating the beaker was placed on a table to take measurement. Resonance frequency was measured continuously and change of pH was measured by a portable pH meter at intervals. To enhance the fermentation 25 g sugar was added at 32 minutes and stirred for 2 minutes to dissolve the sugar quickly. As this experiment was not performed in the temperate box described before, the beaker was heated again for 10 minutes to reach a temperature of 38^0C. pH was found 6.23, at 50 minutes. After 65 minutes we added 10 g more sugar and then heated and stirred and then got a pH of 5.91 at 80 minutes.

Frequency in response to the changed pH is shown in Figure 36. The shift of frequency followed the trend in response to the decreasing pH. The blank feature from 31 minutes to 43 minutes and 58 minutes to 63 minutes are because of the time spent in heating and stirring the beaker.

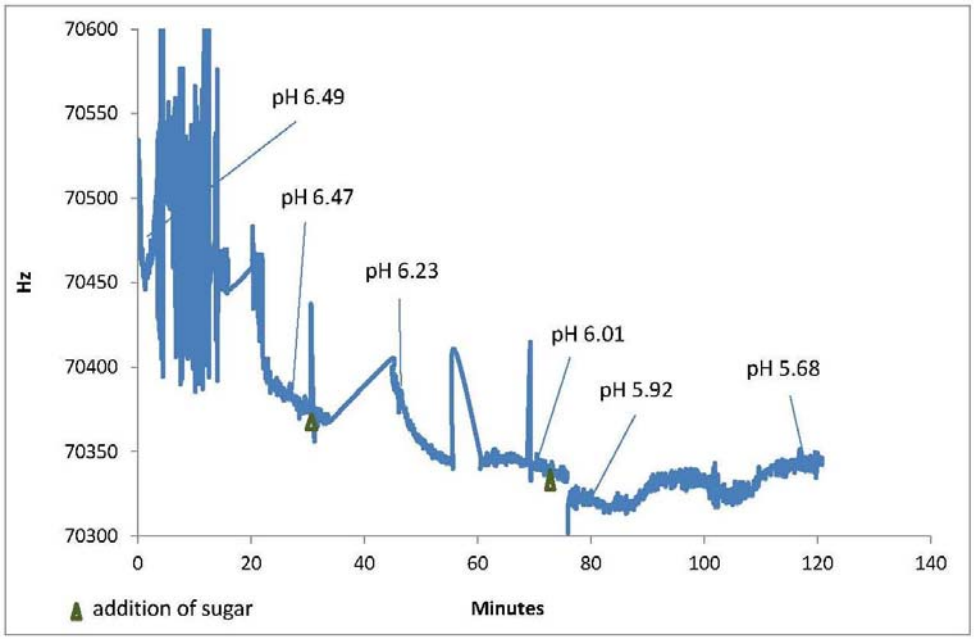

Figure 36. Continuous measurement of pH in rumen liquid in vitro (sensor with 6 layers of polymer). Change of pH was due to fermentation and the production of VFA.

5.5 Experiment in a novel macro in vitro system

This experiment was done to evaluate the sensor's capability to detect change in pH by microbial fermentation in a novel in vitro model system (Figure 37). A thermally insulated box with a heater to keep the temperature at 39^0C was used. Six polyethylene drainage pipes with water tight bottom stoppers could be placed in the box. Each of these pipes was 100 cm in height and had a capacity to accommodate 8 liters of liquid. The pipe may be closed on the upper side by a 6 cm rubber lids which has four holes for stirring mechanism, infusions, continuous gassing with CO_2 and for sampling. A 50 cm ice bore was placed inside the pipe for the purpose of mixing liquid and rumen content.

For this experiment one pipe was used. The in vitro vessels were pre heated before the pipe was filled. Five liters of McDougall's buffer solution and 3 liters of whole rumen content were poured into the poly-ethylene pipe. The pipe was filled with CO_2 to create an anaerobic environment. The content of the pipe was mixed every

3 minutes. The sensor was attached to a wooden stick which was placed in one of the holes of the rubber lid. A coil was placed around the pipe to receive signal from sensor. Sugar was added to the pipe to enhance fermentation and thereby decrease pH. As pH drops rapidly and microbial fermentation will appear to be ceased within a pH less than 5, we added some more buffers to increase pH and continue to have frequency from the sensor. After three portions of sugar were added, the buffer solution was poured into the vessel to increase pH.

Initial pH was 6.46. Addition of sugar only caused slight drops in pH which corresponded well with the stable resonance frequency (Figure 38). After the first addition of sugar there was a frequency shift of 20 Hz with a pH change of 0.03 (pH reading was 6.43 measured at 13.4 minutes). After the second addition of sugar at 24.5 min there was a shift in frequency of 12 Hz and a change in pH of 0.02 (Figure 38). A shift of 30 Hz was seen after the third addition of sugar with a change in pH of 0.04. Addition of buffer raised pH to 6.62 measured at 55 minutes with a frequency shift of 280 Hz/ unit of pH and pH 6.78 measured at 70 minutes with a shift in frequency of 230 Hz/unit of pH.

Figure 37. Arrangement of the macro in vitro system. Adapted from Uden et al, (2010).

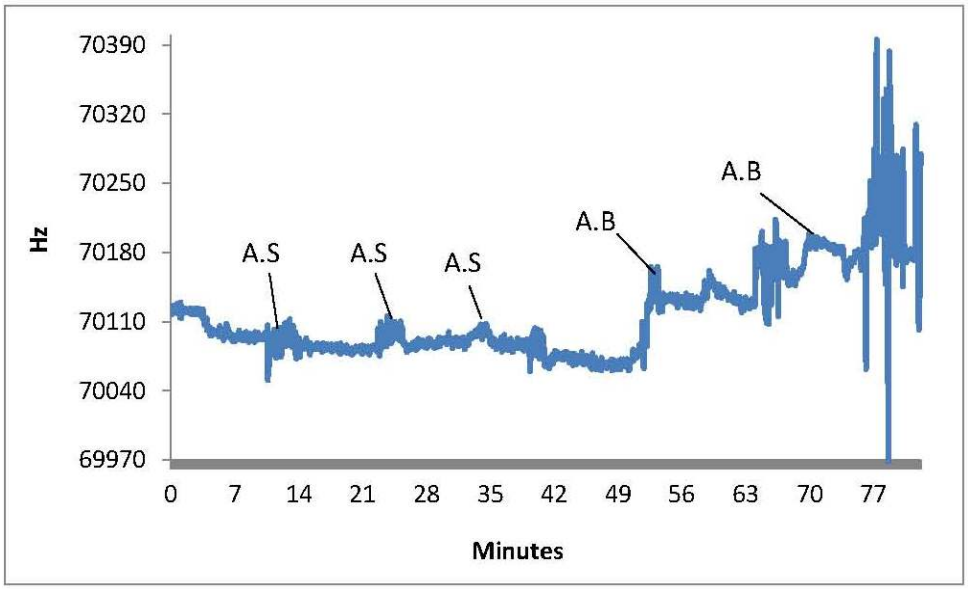

Figure 38. Performance of the sensor in the macro in vitro system. Sugar and buffer were added to the solution to decrease and increase pH level. (A.S- Addition of sugar, A.B- Addition of buffer).

5.6 Experiment in vivo

This experiment was done to evaluate the performance of the sensor in the rumen and the ability of the measurement system to catch the signal from the sensor in vivo.

The sensor was protected by a nylon bag and attached to a 0.5 kg weight of rubber (Figure 39). The purpose of the weight was to enable location and reduce movements of the sensor in the rumen. The sensor was introduced through the rumen fistula and placed in the ventral sac. The coil (60 cm, 20 turns) was placed vertically on the left side of the cow (Figure 40). A sensor with 6 layers of polymer was used to measure resonance frequency continuously.

Figure 39. Sensor attached with the weight to place it in the appropriate location of a fistulated cow.

Figure 40. Placement of the coil to get signal from the sensor.

In Figure 41, the measurement with a 6 layer sensor is seen. Signals from the in vivo measurements showed a frequency ranging from 70.5 to 70.6 KHz. The sig-

nal was lost about 11 minutes after it was placed in the rumen. After cleaning the sensor, it gave a satisfying signal again. The pH of the rumen fluid of the cow was 6.64. The value corresponds well with previous measurement in vitro with 70.46 KHz at pH 6.5.

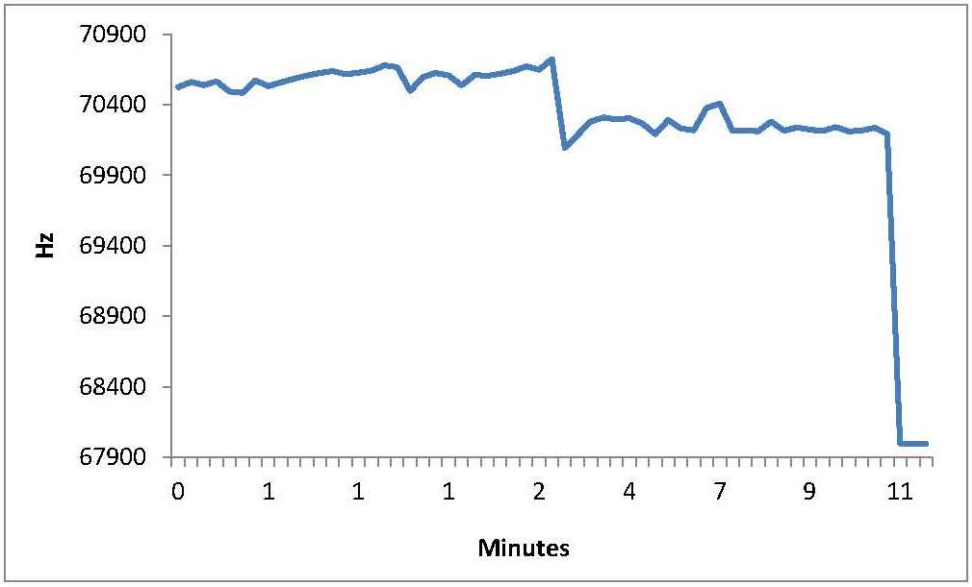

Figure 41. . Measurement of the resonance frequency from a six layered sensor in vivo. The sensor was placed in the rumen of a fistulated cow.

6 Discussion

The results of the experiments have indicated that the pH sensitive polymer beads used were consistent static and the pH response repeatable between test solutions. The polymer structure was designed in such a way that swelling and shrinkage rate was fast in order to get a quick response to pH changes. This was accomplished by amination with diethanol amine as described by Ruan (2003) to make it hydrophilic and hence sensitive to pH changes. The resonance frequency decreased when pH of the test solution was switched from high to low (e.g. Figure 28) indicating the aminated spheres swell and increase in mass in accordance with Ruan (2003).

The sensor seems to respond linearly to pH changes in buffer solutions. The sensor response to pH changes in rumen liquid were not established as well as pH changes in buffer. The variation of the frequency for different test solution in our experiments may have been due to the alteration of the placement of the coil (Figure 25) or due to difference in thickness of the polymer on the metal ribbon (Figure 20). Using the same coverage of polymer layers without changing the placement of coil showed a small frequency difference of 10-30 Hz in our experiment (Figure 24). Beside this, a thinner layer of polymer provided a lower SNR (signal to noise ratio) than the thicker one in a constant pH solution in our experiment (Figure 21) which indicate a less reliable response. This observation followed the principle described by Ruan (2003) where the authors suggested the use of a multilayer polymer bead to increase the sensitivity of a sensor.

Different ionic concentration of the test solutions didn't show much fluctuation on the delivered frequency in our experiment (Figure 24). Effect of swelling of polymer due to different salt concentration depends on the difference in functional

group of the polymer used. As for example, Ruan (2003) using VBC-TCPA polymers found a pH response almost independent of background of KCl concentration whereas Cai and Grimes (2001) working with poly (3-sulfopropyl methacrylate-co-isooctylacrylate) polymer found a response that was salt dependent but pH independent.

The production of gas bubbles upon addition of acid to buffer solutions caused a disturbance of the sensors delivered signal (Figure 31). This was caused by CO_2 formation from bicarbonate buffering. By diluting the buffer and the added acid the problem was decreased. In the experiments with buffers it was easy to visually detect bubble formation which was seen on the senor and, when used, the nylon bag protecting it. During the in vitro experiment with rumen liquid (Figure 36) it was not possible to see any bubble formation as the sensor was not detectable through the rumen fluid. However, there were no noises of the magnitude seen during bubble formation with undiluted buffer. This may have been due to the slower change of pH by fermentation and hence lower gas production rate, which was probably the reason why bubble formation was not a problem when using diluted buffers and acid. However, the lost signal and large variation in frequency response with time in vivo might indicate that bubble formation may have been a problem. Any interruption of the response of the sensor by bubbles has indicated lower SNR during the in vitro experiment with McDougall's buffer.

A longer response time from a thicker layer of polymer on the MER film compared to the thinner one were observed in our experiment (not shown) in accordance with Ruan (2003). This may be because a thicker layer needs more time for diffusion into the polymer and hence longer time to reach steady state. However, Ruan (2003) compared the response time for the polymer poly (acrylic acid co isooctylacrylate) reported by Cai and Grimes (2000) with VBC-TCPA used by Ruan (2003) and also used in our experiment. The response time has proved to be faster with polymer VBC-TCPA than poly (acrylic acid co isooctylacrylate) (Ruan et al., 2003).

From the results of our experiment it is evident that there are some factors (eg, parylene coating, thickness of polymer coverage) which can influence the response from the sensor. The magneto elastic sensor consists of iron which may be oxidized if it comes in contact with water. That's why a ribbon with a coating of parylene layer is needed. Variation in thickness of the parylene coating can be respon-

sible for a variation of the resonance frequency (Thomas et al., 2010). Beside this, how many layers of polymer we exactly need to cover the ribbon is still a question. The differences between the delivered frequency from 6 layers and 8 layers polymer preparation in McDougall's buffer solution were 1753 Hz, between 8 layers and 10 layers polymer preparation were 229 Hz (Figure 20). The difference between the thinner coatings was higher than the difference between the thicker ones. But the higher the layer thickness of polymer will allow more analyte to be absorbed in the layer and there will be a large variation of frequency between them (Cai and Grimes et al., 2001). These indicate the difference between the thinner coatings should be significantly smaller than the difference between the thicker ones. This was one of the reasons why it wasn't possible to create a calibration curve covering all sensors.

The polymer we used in this experiment responds to changes in pH on a broad scale (pH 3- pH 10) but has a rather small change in frequency in response to pH changes (< 30Hz/pH unit in rumen liquid - Figure 36, < 120 Hz/ pH unit in buffer solution - Figure 29). Ruan (2003) was also able to get a linear response within a pH range from 3 to 9 and a change of approximately 78 Hz per pH unit for a 1.5 µm thick polymer. Whereas, Grimes (2001) got a shift of 360 Hz/pH unit within a range of pH 5.4 to 7.2. But in case of in vivo a narrow range of pH (pH 5 to 7.2) is required. An appropriate polymer possibly based on peptide can help to increase the volume changes within useful pH range (Shihui et al., 2006; Dijk et al., 2008), and thus it will be easy to measure a change in pH in vivo.

Malfunction by the measurement systems were negligible during the in vitro experiment. Data acquisition system showed a good trend of resonance frequency in response to pH change. Disturbance during measurements in vivo was mainly due to the malfunction of the sensor. To improve the function of the sensor an increased number of layers were added. By standardizing measurement conditions, e.g. same positions of the coil in response to the sensor, avoiding bubble formation etc. it was possible to get a repeatable response in the same environment.

Using a sensor to measure pH in the rumen has some practical limitations which need to be overcome before proper application. The methods of using sensors to measure pH continuously inside the rumen rely on a fistulated animal or a healthy animal which has already swallowed the bolus made of this sensor. But practically it is quite impossible to recover the sensor for the purpose of recalibration or ser-

vice until the animal is slaughtered. Moreover the position of the bolus inside the rumen may change over time and the bolus may even migrate to the reticulum within a few hours after insertion. The pH environment of rumen and reticulum differs a lot because of the presence of microbes in the rumen (Enemark et al., 2003). Then, there is a probability of misleading result due to the unstable positioning of the bolus.

7 Conclusion

The interest for a device to measure pH continuously inside the rumen has increased lately. The performance of this magneto elastic sensor prepared with aminated poly (Vinylbenzychloride-co-2, 4, 5-trichlorophenyl acrylate) microspheres has been evaluated in vitro. The functional group of this polymer has shown to influence the microspheres to swell and shrink in response to different salt concentration. The resonance frequency delivered from the sensor can perform within a range of pH 3 to10. Present results from the experiment indicate that this system can be used to measure pH continuously in vitro and also responded in vivo. Different layers of polymer were used to cover the ribbon to get a stronger response and to reduce variation of delivered frequency in different salt concentration. The response from the data acquisition system used is fast and flexible and it doesn't require specific expertise to operate the system. Although we got some repeatable response from the film, it needs more work to minimize the limitation (effects of the gas bubbles, wide range of pH scale, effect of the placement and movement of the sensor etc.) and the future aim of the experiment should be lowering the drift of frequency which will help to convert the time domain frequency into pH value.

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