Development of Crosslinked Chitosan-Gelatin Hydrogel Based pH Sensor and Hemostatic Sponge Bandage for Real Time Wound Management

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بسم الله الرحمن الرحيم

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Development of Crosslinked Chitosan-Gelatin Hydrogel Based pH Sensor in Hemostatic Sponge Bandage for Real Time Wound Monitoring.

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Dedicated to

Pakistan Army, My Beloved Family & My best friend Nasir Malick

Who were the motivation behind successful completion of this study…

ACKNOWLEDGEMENTS

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Notation	Explanation
TCCC	Tactical Combat Casualty Care
DDA	Degree of Deacetylation
kDa	Kilo Daltons
Mw	Molecular Weight
ZOI	Zone of Inhibitions
NATO	North Atlantic Treaty Organizations
Ag	Silver
HA	Hyaluronic Acid

ABSTRACT

Real time wound monitoring through pH, temperature and moisture content in the wound bed has been extensively investigated during the past few decades. The pH milieu of wound is the most critical parameter as it indicates healing stage of injured tissues as well as the incidence of any impairment in normal healing process. A persistent alkaline pH exists in chronic wounds and bacterial infection however it progresses from acidic to alkaline and then revert back to acidic value in normal healing process. This study reports chitosan-gelatin hydrogel based pH sensor that can be used for real time wound management. The prepared chitosan-gelatin hydrogel is pH responsive and in combination with voltammetry method, it was tested for pH change in physiological range from 4 to 9.8. The size of hydrogel was 10 mm x 10 mm and it was fabricated by lyophilization process to sublimate the acidic solvent used during fabrication process. The hydrogel was casted on silicone mold and silver wires were embedded inside it. A highly linear response of 0.01 Volt/ unit change in pH was obtained. The source voltage is 5 Volt which upon change in sensor resistance varies and a minor change in pH is detectable through it. The pH response of the hydrogel with blood and simulated wound exudate have also been recorded and compared with standard pH measurement method. This sensor can be integrated in the commercial wound healing dressings where upon building interface with injured tissues, it will facilitate real time monitoring of the wound healing process. Chitosan composite sponge bandages with different polymers have been introduced to achieve effective hemostasis that aimed to reduce mortality rate of hemorrhage leading preventable deaths. In the second phase of our study, we investigated hemostasis potential of chitosan-gelatin sponge crosslinked with 5% glutaraldehyde and results were compared with uncrosslinked chitosan-gelatin sponge and other already reported chitosan

composite bandages. FTIR results showed successful crosslinking with glutaraldehyde which resulted in improved tensile (increase from 0.1MPa to 0.61MPa in crosslinked chitosan-gelatin sponge) and compressive properties (57.1% more compressible compared to uncrosslinked chitosan-gelatin sponge which was 50.1% compressible). The crosslinking affected hemostasis potential of chitosan-gelatin sponge and uncrosslinked sponge showed least absorbance (0.2) while 0.1ml crosslinked showed highest absorbance (0.974) after 30 minutes. Blood absorption capacity was highest (33.5 g/cm^2) in 0.05ml crosslinked chitosan-gelatin sponge compared to 9.4 g/cm^2 and 26.7 g/cm^2 with 0.1ml crosslinked and uncrosslinked chitosan-gelatin sponges respectively. The high blood sorption capacity of 0.05 ml crosslinked chitosan-gelatin sponge can be related to its porosity. It exhibits roughly ellipsoid shaped pores which were less dense and has 75 µm average pore size. Pore morphology with increased size and less density is found to be a favorable factor for blood sorption however addition of glutaraldehyde and its increasing concentration inhibited blood clotting efficiency of chitosan-gelatin sponges. The developed pH sensor integrated inside the chitosan-gelatin bandage can be a potential tool for real time wound management following clinical evaluation of it.

OBJECTIVES OF THIS RESEARCH STUDY

The main objectives of this research study were to develop a hydrogel based pH sensor which produce minimum hysteresis while detecting small fluctuations in pH and evaluation of hemostatic potential of chitosan-gelatin based sponge bandage.

Development of pH Sensor: Chitosan based hydrogels are known to produce minimal hysteresis in pH response (Mao et al. 2006). Our aim was to prepare chitosan-gelatin hydrogel crosslinked with 5% glutaraldehyde which can be used to measure pH change in physiological range (4-9.8) using voltammetry method. It will facilitate real-time wound monitoring when integrated inside a bandage. The pH of progressing wound differs with healing stage and help determine bacterial infection or any other biochemical impairment in normal process.

Hemostasis evaluation of Chitosan-gelatin Sponge bandage. The second phase of study involves the development of hemostatic sponge bandage using same composition of hydrogel with slight variation in experimental conditions and investigating its blood sorption capacity, mechanical and hemostatic properties.

Chapter 1

1 INTRODUCTION

1.1 Background

Real time monitoring of wound or injured tissues is critical for speedy recovery and onset of biochemical reactions cascade provide potential biomarkers which facilitate the process of wound monitoring e.g. pH, temperature, moisture level, bacterial load, cytokine and interleukins etc. Among all the biomarkers, pH has been known to have profound impact on wound healing process and it helps determine the incidence of bacterial infection in the wound (persistent elevated alkaline pH), take-rate in skin grafting, preparation of wound debridement, proteolytic activity and wound healing stage at the site of injury,. A variety of integrated sensor systems have been developed to monitor changes in pH for the determination of healing status of wound. This chapter highlights the significance of pH in determination of clinical parameters and selection of appropriate treatment regime and it presents in depth analysis of the designs and fabrication methods of integrated pH sensors which have been reported till to date for the real time monitoring of wound healing.

LITERATURE REVIEW

1.2 The Relevance of pH to the Wound Healing Process.

The wound healing process includes four phases which are overlapping; hemostasis, inflammation, proliferation and remodeling. Chronic wounds involve impairment in any of these phases and the pH milieu prevalent in it ranges from 7.15 to 8.9 (Wilson et al, 1979; Tsukada et al, 1992; Romanelli et al, 1997). It is an indicative of healing progress and status. Elevated pH in both acute and chronic wounds is an indicator of slow rate of healing compared to wounds that show approximately neutral pH (Leveen et al, 1973; Roberts et al, 1997; Gethin and Cowman, 2006).

Skin surface exhibits acidic milieu during normal conditions (Schade and Marchionini 1928) and this acidic pH varies with age and anatomical location in the range of 4-6 and act as body's first line of defense. Injuries or microbial infections in wounds disrupt the normal acidic milieu of skin and a shift towards alkaline milieu indicate bacterial colonization. High pH has been reported to support overgrowth of *Candida albicans*, a pathogenic yeast that grows on skin surface (Runeman et al., 2000). *Staphylococcus aureus* is another common pathogen which contaminates wound and thus resulting in delayed healing in presence of alkaline pH (Halbert et al. 1992). Topical applications with acidic ointments has been proved to be significant in reducing bacterial load on the skin surface of many diabetic and stroked affected patients (Kurabayashi et al. 2002). Therapeutic interventions can be devised by monitoring changes in pH during wound healing process and it will assist in elimination of bacterial contamination which is a potential co-factor that retards the wound healing process.

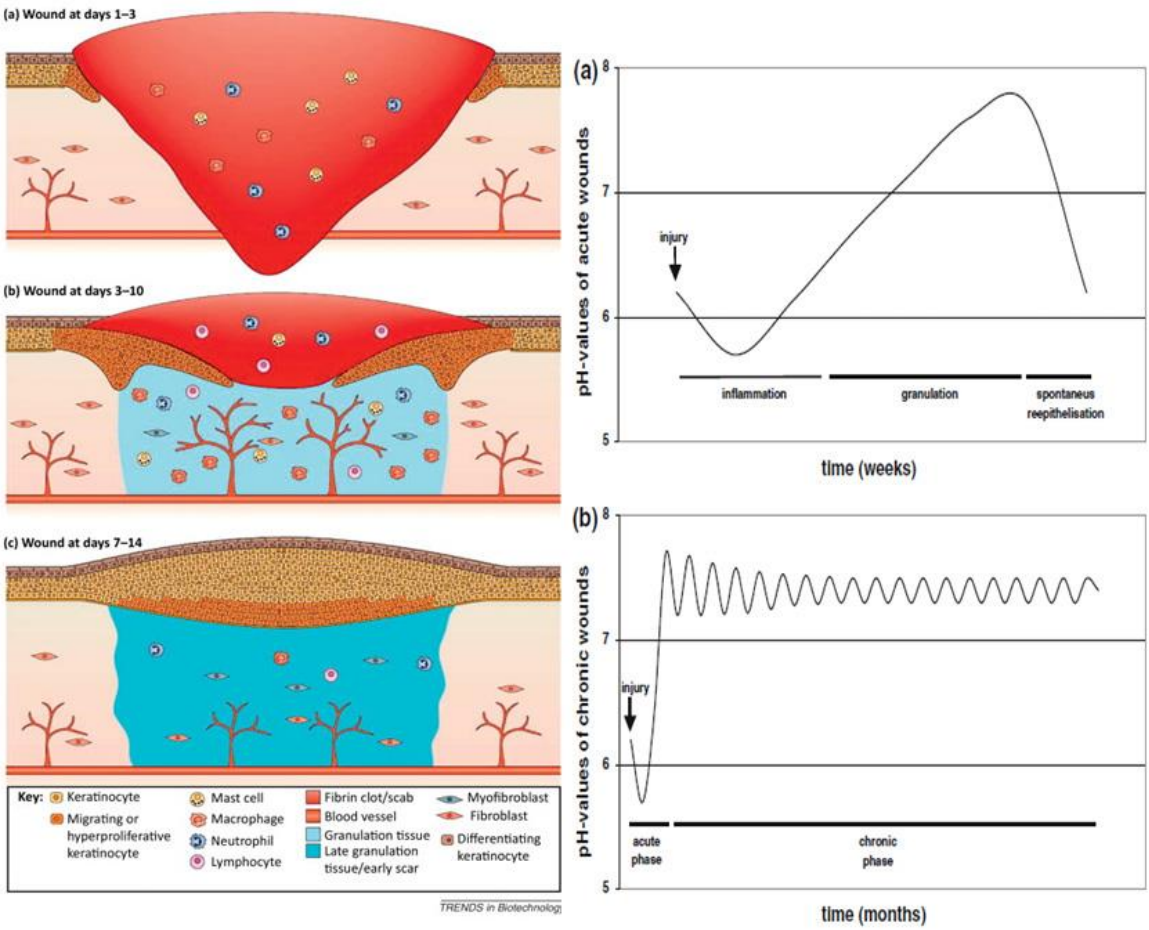

Figure 1: Wound Healing Stages and Change in pH

In chronic wounds, there exists an imbalance between tissue degradation and its assembly resulting in augmentation of catabolic activities that involves different proteases including elastase, cathepsin-G, plasmin and matrix metalloproteases also known as MMPs. The inflammatory phase prolongs and catabolic enzymes remain active. In a study, pH dependence of these proteases in chronic wounds were investigated and pH 8.0 was found to be the optimum pH for plasmin, elastase and MMP-2 activity (Greener, Hughes, and Bannister 2005; Greener et al. 2005). In the similar study, 19 samples from chronic wound patients were collected and tested. The pH range was reported to be in range of 7.5 to 8.9 indicating increased proteolytic activity (Greener, Hughes, and Bannister 2005; Greener et al. 2005). It has been suggested that shifting pH to 6.0 would assist

40-90% decrement in proteolytic activity and it will ultimately accelerate the healing process (Greener, Hughes, and Bannister 2005; Greener et al. 2005).

The ambient pH of skin changes with time course and wound healing stage. Acute wounds undergo initial temporary acidosis during which lactic is produced, oxygen demand increases and tissue perfusion stasis (Hunt et al. 1967; Kurabayashi et al. 2002; Roberts et al. 2002). During healing process high production of collagen requires increased glycolytic activity that result in augmented lactic acid concentration (Kirsti and E. 1967; Kurabayashi et al. 2002). According to a study, restoration of acidic milieu increases amount of available oxygen through Bohr-effect (Hunt et al. 1967; Leveen et al. 1973). In Bohr-effect, hemoglobin binding capacity is reduced when the ambient pH is low and thus result in increased oxygen concentration at wound edge and it accelerates the healing process (Mani 2001). On the contrary, alkaline milieu persists in chronic wounds e.g. ulcer and pressure sores except during epithelisation phase where it drops again (Glibbery and Mani 1992; Sayegh et al. 1988). The range of pH reported in patients with chronic leg ulcers was between 7.3 to 8.9 (Wilson et al. 1979) and another study reported high pH at the skin surrounding ulcer 10 cm away thus a zone with disturbed acidic milieu was observed to be formed around the ulcer and the pH value measured were within 6.2-6.6 range (Glibbery and Mani 1992). In another clinical investigation, variation of wound pH with stages was studied and it was reported that stage I has comparatively low pH (5.7 ± 0.5 SD) than stage II and III which exhibited pH values 6.9 ± 1 SD and 7.6 ± 0.2 SD (Glibbery and Mani 1992). The value of pH also differs from wound center (pH 7.6 ± 0.6 SD) to epitheliated wound borders (5.9 ± 0.4 SD) (Tsukada et al. 1992).

Table 1: pH relevance to the Wound Healing Stage

Wound healing Stage	Events	pH
Hemostasis	Fibrin Platelets Proteoglycans (Robert F. Diefgelmann & Evans, 2004)	**Acidic 4-5**
Inflammatory	Neutrophils Macrophages Lymphocytes (Robert F. Diefgelmann & Evans, 2004)	**7.2** **acidic** **6.7**
Proliferation	Fibroblasts produced Collagen Epithelial cells (epithelisation) Endothelial cells Angiogenesis (Robert F. Diefgelmann & Evans, 2004)	**pH 5** - Low pH
Remodeling	Collagen fibril cross linking Scar Maturation MMP (Percival, McCarty, Hunt, & Woods, 2014) (Robert F. Diefgelmann & Evans, 2004)	**Collagenase** **6-8** **8**

There is a great significance of pH milieu of wound on wound-bed-preparation (Mani 2001) and pus, necrotic tissues and serum crust indicate low pH i.e. 6.1 (± 0.6 SD). Ambient pH of chronic wound increases following surgical debridement of necrotic tissues (Tsukada et al. 1992) and removal by hydrocolloid dressings. Determination of pH milieu of wounds is crucial in case of preparation of proteolytic debridement as enzymes involved have their specific optimum pH range at which they function and prevalent pH defines the efficiency of suggested wound-bed-preparation involving proteolysis,

Modern regimes for wound care have considerable influence on pH prevalent in wounded regions e.g. chronic wounds when covered with non-permeable dressings show acidic pH compared to the ones covered permeable dressings. Treatment of chronic wounds releasing acidic secretions with occlusive synthetic dressing have found to retard bacterial growth and stimulate fibroblast formation (Varghese et al. 1986). In a clinical study, 36 patients were trialed to determine potential advantages of acidic milieu achieved with special dressings and patients were divided into two groups; group I received un-buffered emulsion with pH 7.3 whereas group II underwent treatment by phosphate buffered solution with pH 6.0 (Wilson et al. 1979). Group 2 with buffered solution treatment showed epithelium spread 22.6 mm/day (± 15.2 SD) while only 3.3 mm/day (± 7.4 SD) was observed in group I (Wilson et al. 1979). The results of this study suggests that targeted titration of wounded regions to acidic pH will stimulate self-healing process.

In certain types of ulcers and burns, self-healing doesn't occur and they require skin graft transplantation where take-rate of skin grafts is influenced by pH milieu. A study conducted on rat model showed relationship between the pH range at wound and skin grafts take rates. He mentioned 50-100% take rates for grafts in pH milieu 6.8 to 7.0 (Kirsti and E. 1967) then a comparative analysis of animal data with human skin transplantations of 25 patients with 2nd and 3rd degree burn was made (Kirsti and E. 1967). In humans, 90% take rate for skin grafts were achieved with pH value 7.2 (Kirsti and E. 1967). In another study, patients with both acute and chronic wounds were examined for take rate of skin grafts with variation in pH milieu and 99% skin grafts were reported to be successfully taken in wounds where pH was above 7.4 (YE 1957).

Results of these clinical investigation make it explicit that pH higher than 7.4 facilitate successful healing by transplant in chronic wounds.

1.3 Hydrogel Based pH Sensors

The discussion in section 1.2 shows that pH is crucial parameter in determining bacterial colonization, status of proteolysis activity, wound stage, healing time, take-rate of skin graft and effectiveness of modern care therapy. It also has great significance in wound debridement process. Several approaches have been adapted for fabrication of sensor that measures biological pH with precision and accuracy to facilitate the process of wound healing. In this section, we present few of the reported designs and their fabrication process which have proven to be successful in measuring biological pH for effective treatment of wounds and injuries and propose suggestions for further improvement.

Sridhar and Takahata reported a paasive wureless sensor for real time monitoring of wound pH and different types of hydrogel can be empolyed in this inductive trnasducer system. Transducer is designed by folding a coplanar dual spiral coil with dimension 5/10 mm and it was microfabricated on 50 μm thick copper clad polyimide film (Sridhar and Takahata 2009). The distance at which two aligned coils were aparat influenced the mutual inductance and for gap modulation a hydrogel gel based substance was placed in between them which changed its dimensions with ambient pH alterations (Sridhar and Takahata 2009). The inductive response to displacement was reported to be linear and its value was 0.40 nH/m whereas frequency sensitivity was 71-110 ppm/m as indicated by resonant device coupled with inductive tranducer in a wirelss measurement set-up (Sridhar and Takahata 2009). There is a variety of materials which swell in respons to changes in ambient pH, temperature, slainity and glucose concentration (Liu and Beebe

2002) and in different studies investigating MEMs based sensors which are combinations of inductor-capacitor resonant tanks, stimuli responsive hydrogels have been empolyed for radiofrequency detection (Baldi et al. 2004; Gerlach et al. 2005). In the pH sensor mentioned above, combination of poly(vinyl alcohol) and poly(acrylic acid) was used as hydrogel based pH sensitive system (Sridhar and Takahata 2009). The adoption of passive configuration is significant in designing because it facilitates development of cost effect and disposable sensor system. Variable inductors were used in passive wireless pH sensor because it obivate the requirement of diaphragm and cavity structers and hydrogel was used as biomedical chemical sensing unit. Mointrong of wound healing process is done by keeping track of changes in pH by time and it depicts progression towards recovery (Schneider et al. 2007). In the past, many macro-devices have been introduced for the insepection of injued tissue or area e.g. video imaging (Delode et al. 2001), wound depth analysis through laser (D. Nixon, A. Philps, L. Jonsson 2009) and determination of wound condition through electric resistance measurement (Spence and Pomeranz 1996).

Passive L-C circuitry consisted of a vairable inductor connected to pH responsive hydrogel and a fixed capacitor and the inductor itself included two spiral square coils having same diemnsion and pattern and there was a gap between them which influenced their mutual inductance (Sridhar and Takahata 2009). A dual coil structure similar to this was emloyed in a capacitive pressure sensing system (R. Puers, G. Vandevoorde 2000). Dimensional changes in the hydrogel was detected by using mutual inductance and its dependence on gap between the two coils . A fixed parallel plate capacitor was connected to a variable inductor to show frequency based inductance readouts. Wireless communication was developed by coupling the deivce with an external coil to detect resonant frequency of passive circuit (Sridhar and Takahata 2009). They created a flexural hinge

that allowed folding of two coplanar spiral coils and perforation were introduced in the substrate holding coils so that liquid analyte seeped into the hydrogel which was placed in between the folded coils.

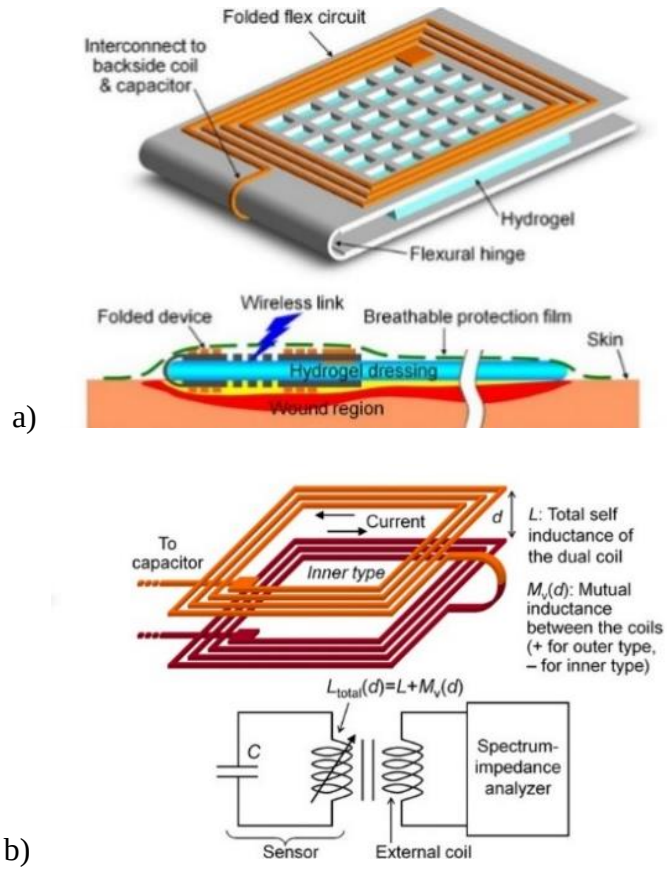

Figure 2: a) Upper Figure: Simulated version of hydrogel based passive wireless sensor a) Bottom: Sensor working in biological environment b) Upper Right: Variable dual-coil inductor c) Bottom Left: Circuit diagram of wireless sensor (Sridhar and Takahata 2009).

Polyimide film with a copper clad at one side of it was used in fabrication process and its thickness was 50m (Sridhar and Takahata 2009). First wet etching was done on the copper clad part of film and 50m thick photoresist was used to generate capacitive electrodes and contact pads. On the other side of polyimide, wet etching was done in KOH solution following lamination and patterning by 38m thick photoresist. This resulted in the formation of

perforations on the polyimide side which built liquid analyte contact with the device. Evaporation of titanium copper thin film was done prior to copper electroplating on the polyimide side and processing with oxygen and plasma was done before it to strengthen the bond between titanium and polyimide. Planar dual coil was developed following copper electroplating on polyimide side after lamination with PM240 and it also produced capacitor with 30-35m thickness (Sridhar and Takahata 2009). Electric isolation of electroplated structures was performed via wet etching of copper seed layer over titanium layer and flexural hinges were created to fold the coils by wet etching of polyimide giving it a thickness of 10m (Sridhar and Takahata 2009). Parylene CTM coating was done on the devices to make it biocompatible and insulated. Hydrogel which was subsequently sandwiched between tow coils were made of PVA/PAA as mentioned earlier and synthesis protocol was adopted from (Gerlach et al. 2005). This combination of hydrogels give improved dimensional changes in response to pH change and has been reported to shrink 170% when pH dropped from 7 to 2 (Sridhar and Takahata 2009).

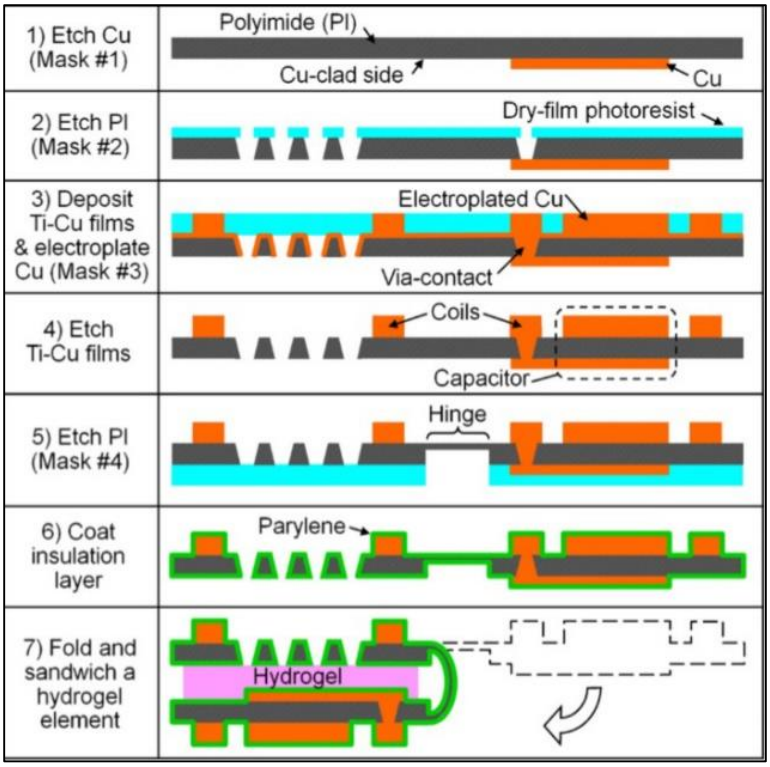

Figure 3: Sensor Fabrication steps (Sridhar and Takahata 2009)

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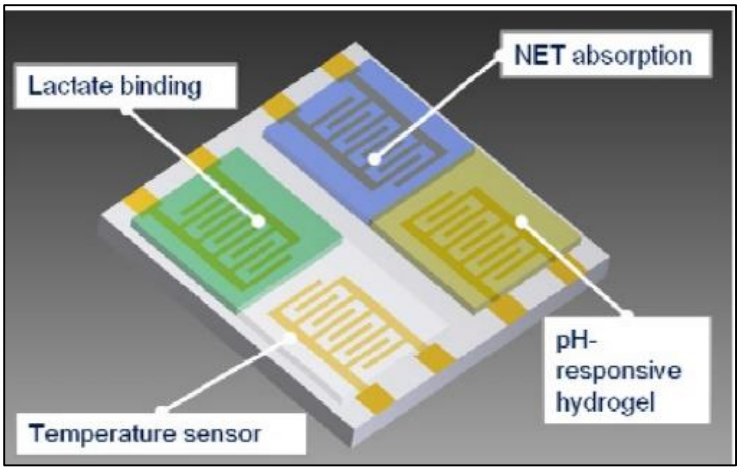
Experimental results of the study conducted by Sridhar and Takahata showed that magnetic coupling underwent degradation when device was placed in pH buffer and it was due to the development of capacitive link between coil and 1m thick dielectric layer (Parylene) and conductive liquid (Sridhar and Takahata 2009). This issue was resolved by introducing dielectric coat over the coils. They further mentioned that a biocompatible pH sensitive material such as chitosan based hydrogels (Mao et al. 2006; Sun et al. 2007) will be effective in fabrication of wireless monitoring devices for wound management (Sridhar and Takahata 2009). The change in pH that occurs during tissue injury is normally 2-3 units. Normal skin has acidic pH which increases to 7.4 if injury occurs and as the healing progress it drops down to maintain normal acidic milieu of the skin. There is a need to fabricate sensing unit which will detect smaller pH range to

bring improvement in measurement accuracy as the hysteresis behavior of hydrogel will be suppressed (Richter et al. 2008). The study suggested the use of chitosan based hydrogels in the device as it has been reported to produce minimal hysteresis (Mao et al. 2006).

A multiparametric sensor was developed by Schröter et al. to determine the bacterial infection via formation of neutrophil extracellular traps that are indicative of bacterial infection (Schröter et al. 2012). An electrode array was assembled with different functional Inh that attaches the neutrophils that comes in contact with them (Schröter et al. 2012). Electrode arrays were designed using techniques like physical vapor deposition process, lithography and spin coating and interdigitated electrode configuration prevented artifacts via production of high field strength at the surface of sensor and field distribution was maintained. Transduction system consisted of impedance analyzer and adapter and change in impedance and phase angle in response to spectra of cell cultures when they were stimulated and the resulting signals helped determine the incidence of bacterial infections at injured site (Schröter et al. 2012). Excitation power of impedimetric measurement was kept low enough to avoid disruptions in processes occurring biological unit but it was sufficiently high to generate readable signal and measurement points at specific frequencies were reduced to improve energy efficiency and thus sensor integration feature was also enhanced (Schröter et al. 2012). Electrode that measure changes in ambient pH through dimensional changes in pH responsive hydrogel act as an assistive parameter that further confirm incidence of bacterial infection when it goes beyond neutral value i.e. alkaline milieu that promote bacterial growth.

A)



B)

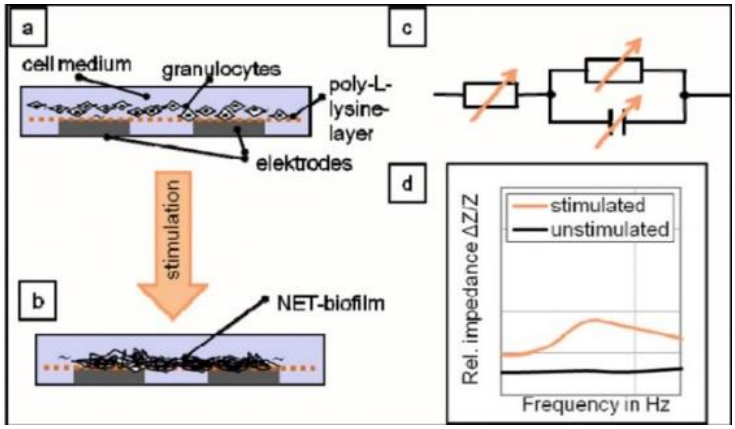


Figure 4: A) Simulation of multi-array impedance sensor with parameter selective coatings B) Schematic representation of impedance measurement Sensor working, a) unstimulated cells immobilized on interdigitated electrodes, b) NET-biofilm generation on the electrodes surface after stimulation, c) Diagram of equivalent circuit with variations after stimulation, d) Changes in impedance observed following stimulation step (Schröter et al. 2012).

Nocke et al. reported a miniaturized and textile based multilayer sensor with features like integratability, flexibility, long term stability and electric readouts (Nocke et al. 2012). Electrode one threaded integration for measurement of impedance provided required stability and it also prevented issues related with lateral displacements of electrodes. Sensor ability to detect pH changes was achieved through pH responsive hydrogel placement between electrodes which also acted as insulation layer between them (Nocke et al. 2012). Electrodes were made of gold wires with 50µm diameter and the hydrogel used for pH sensation was composed of poly(vinyl

alcohol)/poly(acrylic acid) and the range of pH that can be detected with this combination is 5 to 11 pH (V. Schulz, G. Gerlach, M. Günther, J. Magda 2010) and the method adopted for pregel solution preparation was taken from (Richter et al. 2004).

Fabrication of sensor system involved first fixation of the electrodes made of gold wire of defined dimension on a tension frame and then it was dip coated in pregel solution and then thermal treatment was given to it in an oven at temperature of 130 ^{o}C to promote crosslinking in PVA/PAA molecular structure (Nocke et al. 2012). Subsequently, outer electrode was manually wound on the formed structure and it was also made of gold wire with 50 µm diameter. Higher sensitivity and reproducibility can be attained through defined geometry and increased windings of gold wires on the hydrogel structure and automatic fabrication via twine machine will facilitate this process (Nocke et al. 2012). To improve mechanical stabilization and immobilization of electrode and gel interface, they added another coat of PVA/PAA polymer and then electrodes' connection with an impedance analyzer was developed which detected impedance in the defined range i.e. 1 Hz to 1 MHz Sensor calibration was done at pH 6 and pH value was determined immediately after every impedance measurement (Nocke et al. 2012). It was observed that output signal improved with addition of an adhesion promoter and reduction in hydrogel thickness (Nocke et al. 2012).

Results of this study showed that pH sensitivity was obtained at 323 kHz as indicated by impedance spectra and relative impedance change of PVA/PAA absolute impedance was determined over pH at 323 kHz and they reported 14% increment in absolute impedance when the pH changed from 6 to 9.1. Impedance is dependent on the water dipole elongation and distance between the electrodes which alters with hydrogel dimensional changes in response to ambient pH. Complications in field distribution and geometric configuration prevented physical modelling of this process. They further added that due to ambiguity in hydrogel swelling behavior there is a possibility that

hydrogel matrix will disrupt mechanically under extreme conditions and it requires detailed research on hydrodynamics of this subject in defined conditions.

In a study, potentiometric cell was embedded in a bandage to measure pH changes in the wounded area of the body offering dynamic response with minimum hysteresis and mechanical stress resistance feature (Guinovart et al. 2014). Flexible screen printed electrodes of silver/silver chloride along with electropolymerized polyaniline (PANi) (pH sensing unit) were used for the development of pH sensitive sensor system. A reference and a working electrode made of polyvinyl butyral polymer and PANi respectively were used; the latter composed of material which respond to pH due to transition of emeraldine salt into emeraldine base (Kaempgen and Roth 2006; Lindfors, Ervelä, and Ivaska 2003; X. Zhang, Ogorevc, and Wang 2002). They reported sensor sensitivity for pH from 4.35 to 8.00 which is in physiological range for normal wound healing.

Fabrication of sensor was performed on a substrate which was a commercially available adhesive bandage of dimensions 8 cm^2 and 0.2 cm. Screen printing of electrodes were done it using stencil patterns created on AutoCAD. Stainless steel of 75 mm thickness was used as stencil for fabrication and stencil patterning was done separately for Ag/AgCl, carbon and insulator (Guinovart et al. 2014). Tensile properties were augmented by dispersion and homogenization of 0.04% carbon fibers which were finely chopped (Windmiller et al. 2012). Ink curing was done under specified conditions and it is followed by printing of dielectric insulator on cellulose pad which shielded the area where potentiometric electrodes printing was to be done. Ag/AglCl conductive ink was printed and then a carbon layer was laid to develop working electrode. Electro conductive pathways were shielded by an insulating layer rendering only contact pads and electro-active regions exposed (Guinovart et al. 2014). Electropolymerization of aniline over carbon layer

gave working electrode functional viability and reference electrode was developed via drop casting reference cocktail membrane followed by its desiccation (Guinovart et al. 2014).

The developed sensor was reported to be tested *in vitro* using human serum which has chemical environment similar to wound healing tissues (Trengove, Langton, and Stacey 1996) having pH 7.4 however a range of pH for testing was provided by preparing dilution in Mcllvaynes buffer and sensitivity of sensor was checked against it (Guinovart et al. 2014). Testing results indicated Nernstian behavior of the sensor and the sensor was found to detect minor fluctuations in pH introduced with the passage of time. Further testing with PEGylated hydrogel emulating the healing tissue indicated that pH sensitive bandage can detect pH fluctuation in the injured tissue for an extended time period i.e. up to 100 minutes however the wound healing process takes days to weeks (Gc 2008). They concluded their study by suggesting further research in behavior of sensor over prolonged use.

Uric acid has been reported as potential biomarker for the determination wound pH and Phair et al. reported a point of care/POC based sensor system using SPE technology to measure pH (Phair et al. 2011). It enables *in situ* analysis of biofluids. They designed a capillary fill system for POC application which is disposable and it consists of multilayers of laminate sheets with 120µm thickness which underwent pre-patterning by laser etching (Phair et al. 2011). During fabrication of sensor system through cold lamination, a pressure sensitive adhesion layer was introduced and an epoxy layer which was thermally active was coated via hot lamination technique. Capillary wick of 5×5 with 120 micron thickness made of cellulose fiber was placed in the sample chamber to promote even distribution of sample (blood in this case) so that it can seep into electrodes placed beneath. A small hole of 2 mm diameter was made on the topmost laminate as sample entry zone and sensor is simple supposed to be placed on injured tissue where pH has to be determined, the

liquid will seep inside through this hole, reaching the capillary making its way to electrode where through voltametric method its pH will be readout.

Electrodes used in this sensor system was composite of polymeric (ink) binder and graphite particles and plasma pre-treatment was applied on its surface to clean it off from any residual unwanted particles plus it improves oxygen functionality and facilitate electron transfer on the carbon surface (Phair et al. 2011). Screen-printed electrodes used in this study consisted of three electrode configuration; a carbon electrode, a counter electrode and a silver/silver chloride reference electrode. Working electrode was anodized by placing it in 0.1M NaOH for 5 minutes so that it would fix at +2.0 volts (Phair et al. 2011). They report degradation in the voltammetric response at SPEs while placing laminate layers during assembly process and it was resolved by employing laminates having adhesive layers and they were pre-patterned (Phair et al. 2011). The problem was mainly due to the thermal treatment of SPEs at early lamination steps which resulted in partial dissolution of particles in binder which further altered its surface properties (Phair et al. 2011).

1.4 Conclusion

For expedited wound healing process, significance of pH mandated the need of integrated sensor system which facilitates the real time monitoring of healing wounds and obviates the requirement of redressing or complicated testing procedures which are labor intensive and also painful for patient. Different types of sensor systems have been discussed in this review which were developed using hydrogel as pH responsive system coupled with voltammetry, potentiometry, impedimetric and flex-circuit inductive transducer systems. All of the mentioned devices have considerable

potential for clinical applications and there is need of *in vivo* testing to validate their efficiency and sensitivity under practical scenarios.

Chapter 2

2 Development of Chitosan-Gelatin Hydrogel based pH Sensor

Abstract

This chapter explains fabrication of chitosan-gelatin hydrogel based pH sensor that can be integrated in bandage for real time wound management. The prepared chitosan-gelatin hydrogel is pH responsive and in combination with voltammetry method, it was tested for pH change in physiological range from 4 to 9.8. The size of hydrogel was 10 x 10 mm and it was fabricated by lyophilization process to sublimate the acidic solvent used during fabrication process. The hydrogel was casted on silicone mold and silver wires were embedded inside it. A highly linear response of 0.01 Volt/ unit change in pH was obtained. The source voltage is 5 Volt which upon change in sensor resistance varies and a minor change in pH is detectable through it. The pH response of the hydrogel with blood and simulated wound exudate have also been recorded and compared with standard pH measurement method. This sensor can be integrated in the commercially available dressings where upon building interface with injured tissues, it will facilitate real time management of the wound healing process.

2.1 Introduction

The pH milieu of wound is a critical parameter which determines the healing status of it (Percival et al. 2014). For the real time monitoring of wound, different sensors have been reported which utilized hydrogel as sensing unit (Nocke et al. 2012; Sridhar and Takahata 2009). Hydrogel is a three-dimensional polymeric structure which exhibits different swelling kinetics at varying pH (Hoffman 2012). Combination of Poly Vinyl Alcohol and Poly Acrylic acid/PVA-PAA has been extensively investigated and its response against pH was recorded using impedance and inductance based transducers (Nocke et al. 2012; Sridhar and Takahata 2009). Sridhar et al. reported hysteresis in the pH response of PVA-PAA based hydrogel when it was used at an extended period of time for measuring abrupt changes in pH (Sridhar and Takahata 2009). The swelling kinetics in response to varying physicochemical ambiance is a gradual process and past inputs cause discrepancy in signal response when the chemical environment is changed suddenly. The range of pH change in wound healing process is very small and a higher sensitivity of the sensor is crucial because a slight shift in alkaline pH from 7-7.4 or above indicates bacterial infection or impairment in normal wound healing process (Schneider et al. 2007) Chitosan based hydrogel is a material of choice in pharmaceutical and biomedical industries particularly for drug delivery applications (Hu, Sun, and Wu 2013). Chitosan possesses positive charge on its molecular structure in acidic environment and solubilizes while it retains negative charge in alkaline pH. This property makes it an ideal drug delivery vehicle (Hu, Sun, and Wu 2013). Chitosan-gelatin based hydrogel was studied for pH responsiveness using MEMs based approach. Chitosan-gelatin polyionic complexation was crosslinked with Tripolyphosphate and coated over a microcantilever (Mao et al. 2006). Variation in pH of tested solutions produced deflection

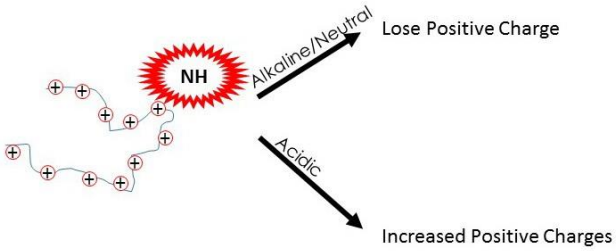


Figure 5: Chitosan Response to pH

in microcantilever which was measured in dB and calibrated against the corresponding pH (Mao et al. 2006). The hydrogel when coupled with MEMs based sensor showed delayed response to change in pH and cyclic exposure was required to generate a consistent response against pH (Mao et al. 2006). This puts a limitation on the MEMs based approach in measuring pH through hydrogel.

In this study Chitosan-gelatin hydrogel cross-linked with 5% glutaraldehyde was used for measuring pH in physiological range through voltammetry method. Voltammetry method will overcome the limitation of hysteresis that appear in the hydrogel based pH sensors. It is proposed that potential differences will arise as the acidic or alkaline pH induces changes in the electrostatic nature of polyionic complexation formed from chitosan and gelatin, see figure 5.

In the section 2, hydrogel preparation and sensor fabrication have been described while section 3 shows experimental results of this study and detailed discussion over it. Section 4 concludes the empirical analysis on the acquired data.

2.2 Experimentation

2.2.1.1 Materials

Chitosan, molecular weight 48109 Dalton was kindly prvided by Dr. M. Mujahid, Principal of School of Chemical and Materials Engineering, National University of Sciences and Technology. Glutaraldehyde (25% aqueous solution) and Gelatin Type B, Bloom 225 were purchased from DaeJung Chemicals and Metals, Korea. Glacial Acetic Acid was obtained from Scharlau Chemicals, Spain.

2.2.1.2 Methods

2.2.1.3 Preparation of Cross-linked Chitosan-Gelatin Hydrogel

Chitosan-gelatin hydrogel was prepared by first dissolving 2.5% chitosan in 1% acetic solution and it was stirred on magnetic stirrer (MS300HS, Tech Jam Instruments, China) for an hour till the solution became clear. 7% Gelatin solution was prepared by constant stirring at magnetic stirrer till clear solution was obtained. Chitosan and Gelatin solutions were mixed by the ratio of 1:5 while constantly kept on stirring till a homogeneous mixture is obtained. 5% of glutaraldehyde solution was prepared and it was added in chitosan-gelatin mixture (Foda, El-laithy, and Tadros 2004). It was poured in silicone cell and the cross-linked Chitosan-Gelatin hydrogel was air-dried and then lyophilized (EYELA, FDU 2100, Tokyo Rikakikai Co., LTD, Japan). Lyophilized samples have no residual acetic acid which can give acidic input in the testing samples. During lyophilization the solvent is sublimated and extracted thus reducing the chances of error in signal outputs at the data acquisition steps. Uncross-linked samples of hydrogel were also prepared.

2.2.1.4 Sensor Fabrication

A small square shaped cell made of silicone rubber was used to contain the gel and connect it with wires. Cell was made by casting silicone on an acrylic mold (10 mm x 10 mm). Copper wires connections were made at the bottom of cell. The liquid solution of hydrogel was poured in it through 3cc syringe and it was left for air drying. Hydrogel adhered on the copper wires inserted inside as it dried. Wires were then connected to the outer circuitry for pH measurement.

2.2.1.5 Sensor Calibration and pH measurement

Figure 6 shows the circuit configuration used in this study. A wired connection of hydrogel contained in silicone cell was made with a microprocessor that provided it 5 volts. A volt- age divider circuit was used and change in sensor resistance in response to the ambient pH was recorded through MATLAB interfaced with microprocessor. Calibration was performed by immersing sensor unit in solution with different ionic strength. The voltage change was measured and calibrated against the corresponding pH. Different samples of cross-linked and uncross-linked Chitosan-gelatin hydrogels were used to check consistency in the response. Sensitivity of the sensor was 0.01 volts/ms as calculated from the graph slope. At day 2, pH was measured in the range 10.8 to 6 and reproducible results were obtained.

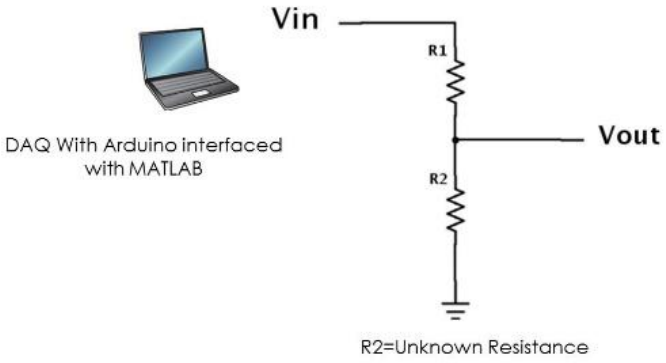


Figure 6: Voltammetry Method for pH sensing through Chitosan-gelatin Hydrogel

Initially, a slight increment in the voltage value occurs till it stabilizes and upon changing pH it increases or decreases accordingly. Day 2 reading was also taken in such a manner that voltage hysteresis effect can be determined. It was found to be minimal as the pH changes from initial 7.4 value to 10.8 and then it was dropped till 6. The time period was 30 seconds and it clearly shows that abrupt change in pH was well-responded by uncross-linked Chitosan-Gelatin hydrogel.

2.3 Results and Discussion

2.3.1.1 Crosslinked Chitosan-Gelatin pH Response

Figure 7 shows the reproducible pH response of cross-linked chitosan-gelatin hydrogel. The normal saline solution was used for the initial testing of hydrogel and its pH was decreased gradually to acidic value at day1 and day2 acquisition steps. A clear linear response of voltage change to pH can be seen in the graph. Figure 8 shows the chemistry of chitosan-gelatin polyionic complexation. Dried gel keeps the circuit open as long as it is hydrated by the surrounding moist environment and then voltage begins to change. Chitosan exhibits positively charged amine groups on its polymeric structure while gelatin contains the negative charge. They both form a complex held together by electrostatic interaction, see figure 8. Figure 5, illustrates that as the ambient pH rises, chitosan loses it positive charges and cause considerable increase in potential difference as indicated by voltage drops in this study. The positive charge increases in acidic environment and there is a significant drop in voltage as shown in figure 7.

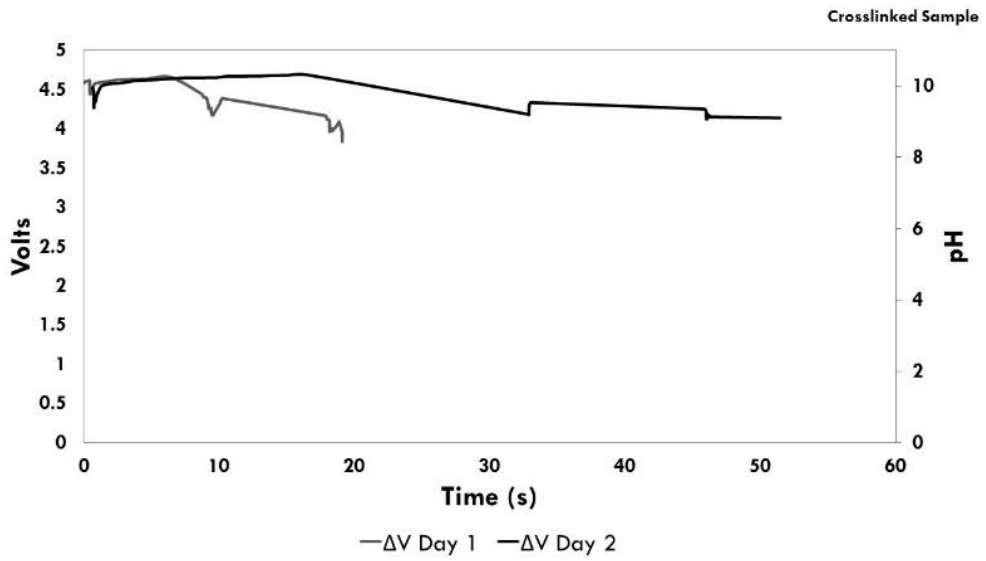


Figure 7: pH response of cross-linked Chitosan-gelatin hydrogel

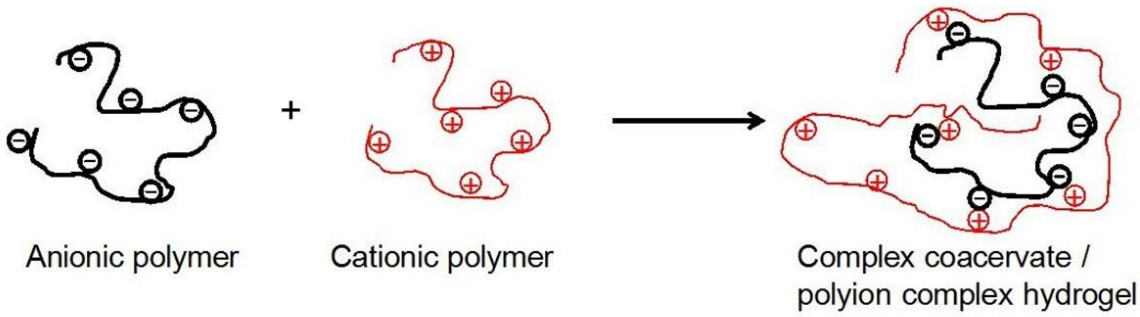


Figure 8: Chitosan Gelatin Chemistry

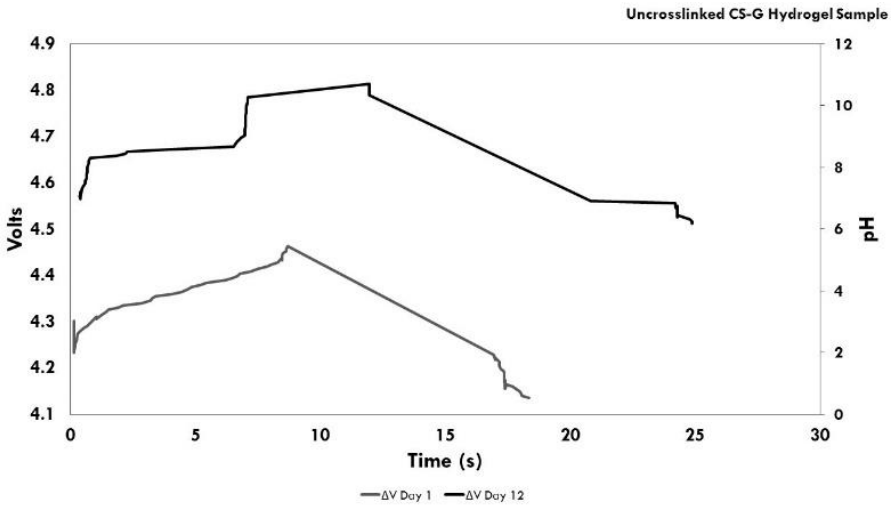


Figure 9: Uncrosslinked chitosan-gelatin hydrogel response to pH at day1 (grey)

2.3.1.2 Uncrosslinked Chitosan-Gelatin pH Response

Figure 9 shows the results of chitosan-gelatin hydrogel which was not cross-linked with 5% glutaraldehyde solution. The pH of solution was changed from 5 to 1 and the data was acquired for 0.5 minutes. The response time is considerably less because the voltage drop observed was in milliseconds. As the pH turned acidic, the voltage dropped from initial 4.3 to 4.1 values.

2.3.1.3 Measurement of Simulated Wound pH

Physiological salt solution was prepared to simulate the ionic concentration of wound exudate and it was used for measuring pH with chitosan-gelatin based pH sensor. The pH of simulated wound exudate was changed from alkaline to acidic, see figure 10. Voltage change is plotted against time and pH. A clear close to linear response was observed. As the pH turned acidic, the voltage drops drastically from 5 to 3 volts. It indicates that ionic strength variation induced only electrostatic changes in polyionic complexation structure of chitosan and gelatin. It didn't interact chemically with hydrogel which might have prevented the application of this gel for pH measurement in physiological environment.

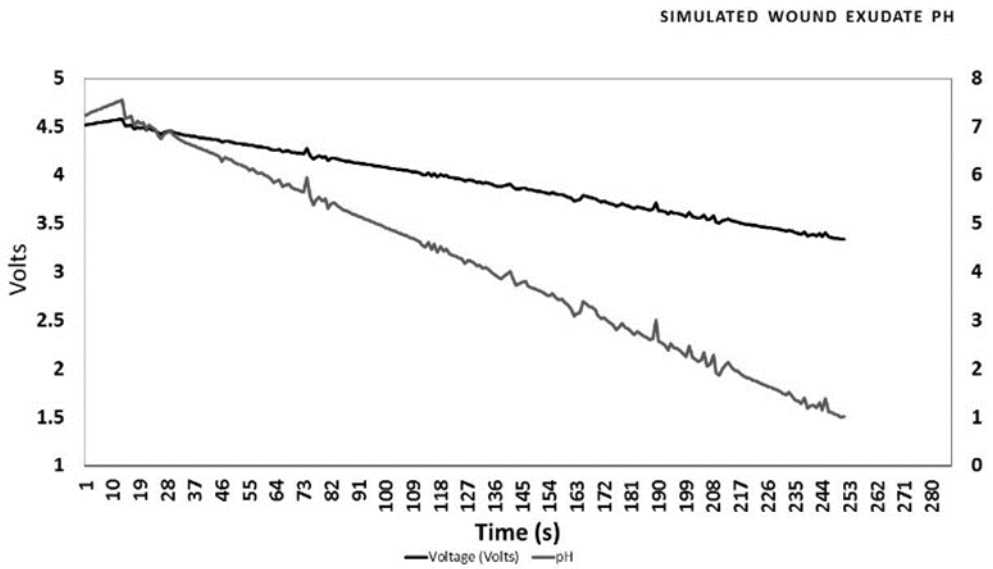


Figure 10: pH measurement with simulated wound exudate

2.3.1.4 Measurement of Blood pH

Figure 11 shows the results obtained with blood sample (kindly donated by one of the contributing authors). The sensor shows a linear increment in pH till it reaches 6.4 value which was close to the value observed with standard glass electrode pH meter (EcoMet). It was expected that blood samples will dry out and will form clumps on the hydrogel thus restricting the surface area to be in contact with liquid interface for pH measurement. Practically, it wasn't observed during the time gel was exposed to blood sample. The recorded pH was close to the one measured with standard pH meter.

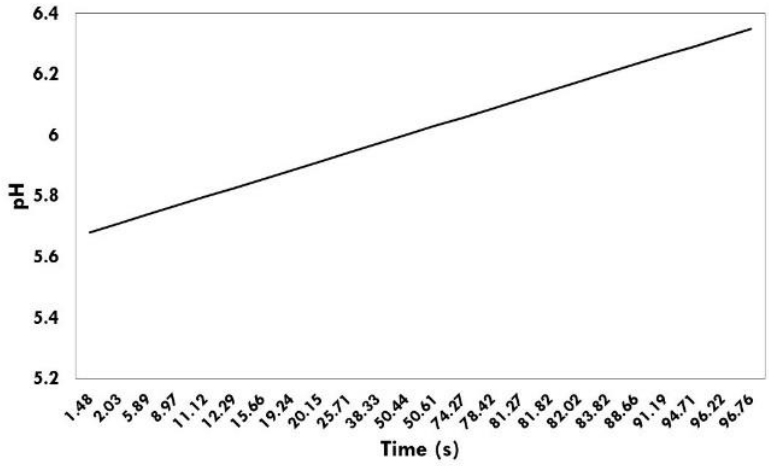


Figure 11: Measurement of Blood pH using Chitosan-Gelatin (cross-linked with 5% Glutaraldehyde)

2.4 Conclusion

Chitosan-gelatin hydrogel for the pH sensing has been studied with inductance and impedance based transduction methods. These methods require dimensional change in the hydrogel to produce change in electrical properties. An abrupt change in pH puts limit on these approaches. The sensor developed in this study utilizes electrical properties of chitosan-gelatin hydrogel which changes in response to the ambient pH. Voltammetry method has found to be more efficient in detecting pH change and it is independent of hysteresis problem which cause error in output signal and generally appears in MEMs based and other approaches which require dimensional changes in hydrogel. It is due to the reason that hydrogel changes its structure gradually in response to increasing or decreasing ionic strength. There are higher chances of error due to past input signals. No significant difference was observed in pH measurement with cross-linked and uncross-linked hydrogel samples and their electrical properties did not change. Crosslinked hydrogels were mechanically improved and can be used for extended period of time compared to uncross-linked hydrogels

of chitosan and gelatin which were fragile and broke after a certain time. At higher pH, the voltage drop is low indicating neutralization of chitosan positive charges while at lower pH, voltage drops significantly due to the increasing positive charge on its surface. The results of this study suggest that chitosan-gelatin hydrogel cross-linked with 5% glutaraldehyde can be used for pH sensing applications in real time wound monitoring sensors.

Chapter 3

3 Recent Advances in Chitosan Based Hemostatic Bandages for Hemorrhage Control in Trauma Patients

Abstract

High mortality rate of potentially survivable casualties due to severe hemorrhage is a major challenge in the advancing battlefield environment of today's world where technology has evolved and complicated the wound grades. Quality of pre-hospital care prior to patient evacuation is crucial in determining the survival rates. Considerable improvements in the hemostatic dressings have been introduced and pre-hospital care has been upgraded in many tactical combat casually care guidelines. Combat Gauze has been widely used bandage which is now been substituted by different chitosan based hemostatic dressings which not only exhibit anti-bacterial activity but also induce hemostasis via direct interaction with erythrocytes and platelets. Different generations of chitosan bandages have been developed to overcome the drawbacks of previous ones. This review provides performance analysis of chitosan bandage generations and advancement in its fabrication methods.

3.1 Introduction

In the tactical combat casualty care/TCCC, a major challenge is hemorrhage control to reduce the rate of combat death and to increase survival of injured soldiers. Hemorrhage in major arteries has been reported to cause 50% battlefield casualties while it accounts for 31% lives in civilian's clinical settings (Arnaud et al. 2011a; Hoggarth, Hardy, and Lyon 2013; Mueller et al. 2012; Schwartz et al. 2011). Nature of combat injury is entirely different from the ones seen

in civilian medical facility due to its advancing battlefield ambiance, intense and diverse nature of injury in pathophysiology, healing process and epidemiology (Champion et al. 2003).

A comparative study on combat bandages explains failure to control excessive bleeding as the main cause of high mortality in 1993 Somalian war (Rall et al. 2013; Xie et al. 2010). Normal blood circulation is impeded following hemorrhage which ultimately affects micro-circulation and induce hypoxia in brain as well as in other parts of body. The patient condition can be severed if it's not controlled on timely basis and it might cause hemorrhagic shock. Metabolic acidosis and hemorrhagic hypothermia are further other complications which can be manifested and make hemostasis process more difficult by disrupting coagulation system (Kunio et al. 2012). Blood transfusion after significant blood loss due to injury increases chances of organ rejection which complicates follow up procedure (Bijan Shams Kheirabadi et al. 2010; Satterly et al. 2013). An immediate access to proper hospital care is limited in battlefield environment due to complex geography and urgency which cause significant delay and excessive bleeding. According to a study, through proper pre-hospital care and effective hemorrhagic control mechanism, 9% of Vietnam War casualties could have been prevented (Xie et al. 2010). Conventional mode of hemorrhage control are tourniquet application and combat gauze. Tourniquet based methods fail to control bleeding when a patient receives injuries at extremities therefore hemostatic based dressings and agents are viable hemorrhage control tools in such instances. Use of combat gauze in TCCC is in practice for the last 8 years however recent advancements in hemostatic dressings introduced chitosan based hemorrhage control dressings as potential alternative for it. It has been incorporated in the TCCC guidelines of many countries including NATO militaries, U.S military Special Operation Forces and other Emergency Medical Services are already benefitting from it due to the reported clinically significant outcomes and field experience (Bennett et al. 2014). The commercially available

chitosan based hemostatic dressings are Chitogauze, Celox Gauze, Mini-sponge dressing, Hemcon, Trauma Gauze and ChitoFlex (Bennett et al. 2014; Littlejohn et al. 2011).

Chitosan based hemostatic bandages received approval from FDA for battlefield applications after being proved equally effective as combat gauze from different animal studies where extremity arterial hemorrhage wound healing was investigated using Celox trauma gauze, ChitoGauze, Mini-sponge, Celox, TraumaStat, Celox Gauze, Celox Rapid and Celox Trauma Gauze (Arnaud et al. 2011a; Hoggarth, Hardy, and Lyon 2013; Kunio et al. 2012; Mueller et al. 2012; Rall et al. 2013; Satterly et al. 2013; Schwartz et al. 2011; Xie et al. 2010). Combat gauze is commonly used to overcome hemorrhage and it has been reported to be effective in non-coagulopathic animal models while it becomes less effective in coagulopathic animal models which limits its application (Floyd, Rothwell, Risdahl, et al. 2012; Bijan Shams Kheirabadi et al. 2010). A study was conducted to compare the hemostatic efficiency of combat gauze with other dressings and in sample size of n=19, seven patients underwent combat gauze pre-hospital care and coagulopathy was manifested in two of them and failure to bleeding control was reported with combat gauze (King and Schreiber 2011). Combat gauze formulations have been modified afterwards to improve its efficacy in coagulopathic conditions (Causey et al. 2012; Sena et al. 2013). All the commercially available dressing still didn't entail the required characteristics for battlefield trauma applications (B. Kheirabadi; Mani 2001; A. H. Smith et al. 2013). Niles et al. reported that 38% military casualties related with blood transfusion has coagulopathy (Niles et al. 2008) and there is dire need for improvement in hemostatic gauze which induce hemostasis irrespective of the host coagulation system for instance chitosan or fibrin made dressings (Bijan S Kheirabadi et al. 2011). Fibrin bandages however are not an economical alternative as first responders in battlefield wound management and they are more suitable for surgical applications (King and Schreiber 2011; King 2011).

Chitosan based dressings are more economical and they have proved to be effective hemostatic agents even in coagulopathic conditions as indicated by several animal model studies (Klokkevold, P R, H Fukayama, E C Sung, and C N Bertolami. 1999; Koksal et al. 2011; R. Millner, Lockhart, and Marr 2010; Mirzadeh, Hamid, Nakisa Yaghobi, Saeed Amanpour, and Hossein Ahmadi 2002; Pozza and Millner 2011). This chapter discusses the significance of chitosan molecular weight and degree of deacetylation in hemostasis, bactericidal effect, different generations of chitosan bandages and fabrication methods.

3.1.1.1 Significance of Chitosan Molecular weight and Degree of Deacetylation in Hemostasis

Molecular weight and degree of deacetylation/DDA achieved during the purification process effect significantly on the hemostatic ability of chitosan (Hattori & Ishihara, 2015). Higher degree of deacetylation/DDA improves erythrocyte and platelets aggregation significantly which is a desirable property of hemostatic bandage (Hattori and Ishihara 2015). Chitosan belongs to polysaccharide family and its molecular structure contains 2-acetamido-2-deoxy-D-glucose and D-glucosamine which are connected with random β (1-4) linkages. Chitosan acquires positive charge as the D-glucosamine is deacetylated to form N-acetyl-D-glucosamine giving free amino groups on its molecular structure (Hattori and Ishihara 2015). The positively charged chitosan is exploited in inducing hemostasis as surfaces of platelets and erythrocytes exhibit negative charges due to the presence of phosphatidylcholine, phosphatidylethanolamine and sialic acid groups respectively (Briedé et al. 1999; Lupu and Calb 1988; vd Winkel et al. 1987). The negative charges on platelets and erythrocytes cause electrostatic repulsion between them thus hindering the aggregation process which is crucial for the onset of hemostasis process, see figure 12. The amino groups present on chitosan (poly-N-acetyl glucosamine) is involved in facilitating erythrocyte aggregation via electrostatic interaction with its surface charges and hemostasis is induced once it activates platelet (Shen

et al. 2011).

Hattori et al. reported that chitosan with 75-88% degree of deacetylation and high molecular weights (50-247 kDa) promoted protein aggregation (Hattori and Ishihara 2015). In this study, chitosan with 100% DDA failed to aggregate erythrocyte and platelets indicating that low molecular weight inhibits aggregation (Hattori and Ishihara 2015). In another study, chitosan with low degree of deacetylation was found to be more effective in aggregating erythrocytes and platelets compared to chitosan with high DDA or intermediate DDA (Yang et al. 2008). It can be postulated from the facts aforementioned that a meshwork is produced by chitosan where blood cells and platelets are entrapped and this activity is highly dependent on the DDA and molecular weight of chitosan. Hattori et al. reports erythrocyte washing out at higher rate in high molecular weight and DDA chitosan (Hattori and Ishihara 2015). The highest whole blood aggregation was seen in chitosan with 50-247 kDa Mw and 75-88% DDA (Hattori and Ishihara 2015). The author further suggests use of mixed molecular weight (8.6-247kDa) and DDA (75%-88%) to achieve best hemostatic activity (Hattori and Ishihara 2015).

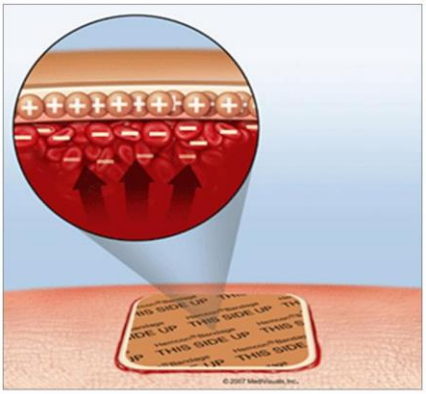


Figure 12: Chitosan based bandage hemostatic mechanism (HemCon)

3.1.1.2 Bactericidal Nature of Chitosan

Another hallmark of chitosan that makes it a suitable material for preparation of wound dressing is, its remarkable antimicrobial activity. This property is an additional advantage

which is useful in preventing bacterial infections at wounds. Free amino groups present on the chitosan give antimicrobial characteristics as they bind to bacterial cell wall causing cell lysis; it is postulated that electrostatic attraction between bacterial surface negative charge and chitosan free positive charged amino groups solubilize bacterial cell wall and membrane (Sahariah et al. 2014). However, chitosan insolubility in aqueous medium limits its antimicrobial efficacy in acidic environment and it fails to produce effective antibacterial activity at alkaline pH. One possible explanation to this limitation is positive charge neutralization at higher pH (Aiedeh and Taha 2001; Sudarshan, Hoover, and Knorr 1992). Chitosan chemical properties modified with quaternary moieties are useful in increasing its solubility in aqueous medium and induce permanent positive charges over it which augments its antibacterial properties (Jayakumar et al. 2011). Figure 13 shows a schematic illustration of antibacterial activity of chitosan.

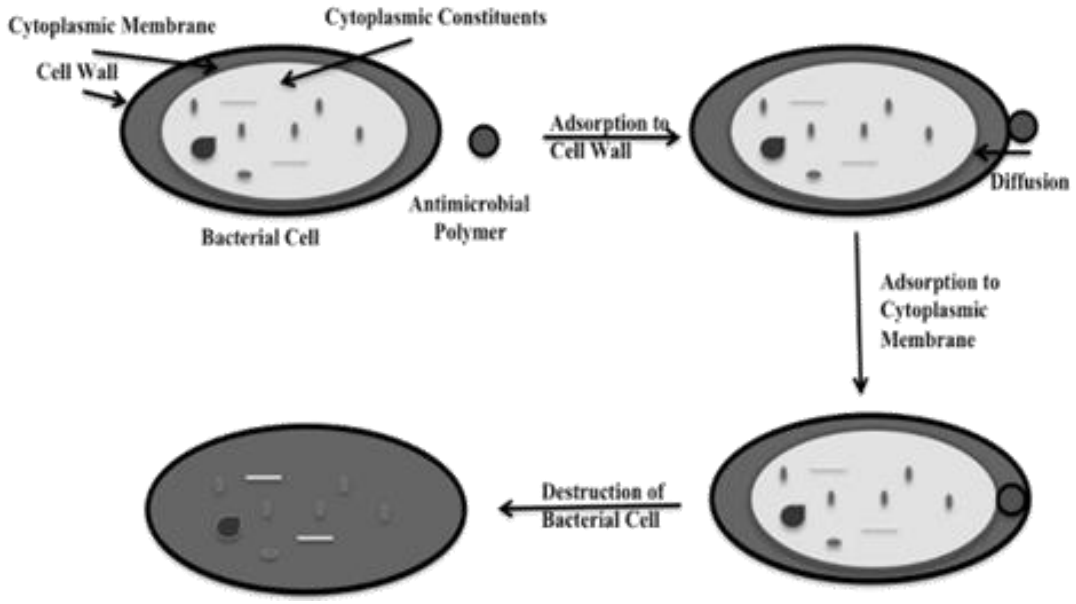


Figure 13: Schematic of chitosan antimicrobial activity

Sahariah et al. prepared chitosan derivatives by substituting amino groups with quaternary

moieties to improve its antimicrobial efficacy at alkaline pH (Sahariah et al. 2014). Chitosan with different degree of deacetylation and molecular weight were used in this study to investigate its impact on antibacterial activity and to determine relationship between cationic amino group distances from the main polymeric structure (Sahariah et al. 2014). Trimethylammonium and pyridinum groups were successfully grafted in chitosan with molecular weight ranging from 7 kDa to 23 kDa and significant improvement in antimicrobial activity was seen in Trimethylammonium grafted chitosan with complete quaternization. The author mentioned that increasing the spacer length decreased the hemolytic activity of Trimethylammonium chitosan derivative while other derivatives remained non-hemolytic. In contrast, increase in degree of deacetylation and decrease in in molecular weight of chitosan showed improved associated cytoxicity (Sahariah et al. 2014).

Clinical studies with chitosan based bandages have shown to be successful in wound management when compared with Celox Gauze in both civilian hospital care (Mueller et al. 2012; Muzzi et al. 2012; Schmid et al. 2012) and prehospital care in battlefield (Arul, Bowley, and DiRusso 2012; Tan 2011). In all the studies, safety concerns with chitosan based dressings were addressed and no complications were observed with patients undergoing treatment regime based on it (HemCon bandages and Celox particles) in military (Pozza and Millner 2011; Wedmore et al. 2006) and civilian prehospital care set ups (Brown, Daya, and Worley 2009).

Bennett et al. emphasized in his study on the inclusion of chitosan-based dressings as a potential alternate in first responder along with other medics options such as airway devices and pain medications so that hemorrhage based mortality can be prevented (Bennett et al. 2014). However it has already been adopted in TCCC guidelines of U.S. Special Operations Forces (Bennett et al. 2014). United Kingdom Ministry of Defense along with eight other NATO militaries has also approved the use of Celox Rapid and Celox Gauze (Bennett et al.

2014). ChitoGauze is now used by emergency department of Georgia medical college while other elite tactical teams in US have included Celox Gauze, Celox Rapid and ChitoGauze in first responders or prehospital care (Bennett et al. 2014). US Army 75th Ranger Regiment insists further advancement in hemorrhage control device and bandages to reduce the potentially survivable causalities up to 3% in operational forces where mortality rate due to excessive bleeding is approximately 24% (Kotwal et al. 2011).

3.1.1.3 Effective hemostasis in Coagulopathic patients

Coagulopathy is a common problem that normally appears in severe injuries and it was reported to be common in 38% of the combat casualties which were evacuated in Combat Support Hospital (Niles et al. 2008). In contrast. Coagulopathy was manifested in 25% of the trauma patients admitted in Level I trauma center (Rall et al. 2013). Chitosan hemostatic ability doesn't depend on host coagulation pathway and this is an added advantage with chitosan dressing as compared to Combat Gauze. The efficacy of chitosan dressing in coagulopathic conditions have been tested in six different animal studies (Bochicchio et al. 2009; Klokkevold et al. 1999; Koksal et al. 2011; R. W. J. Millner et al. 2011; Millner, Russell, Alan S Lockhart, and Rebecca Marr n.d.; Mirzadeh, Hamid, Nakisa Yaghobi, Saeed Amanpour, and Hossein Ahmadi 2002). However, its effectiveness in coagulopathic patients have also been verified by a cardiothoracic surgical report by civilian healthcare facility 42 and two different combat casualty (Arul, Bowley, and DiRusso 2012; Tan 2011). In contrast, Combat Gauze has not been considered as viable treatment method for hemorrhage in coagulopathic combat casualties (Floyd, Rothwell, Risdahl, et al. 2012; B. Kheirabadi). A comparative analysis of Combat Gauze and third generation chitosan-based gauzes in controlling hemorrhage should be conducted to determine the efficacy of both in coagulopathic patients. Hemostatic agents are classified as Class II (510K) medical devices and got market approval as they have proved to be substantial equivalent of already existing hemostatic methods (Bennett et al. 2014). A

localized hemostasis was induced by chitosan dressings in broken blood vessels and Celox Gauze facilitated hemostasis in multiple combat injuries received from IED blasts and sharpnel (Bennett et al. 2014). Chitosan dressings have approved to be safe and results of histological examination of exposed tissues showed clot formation in organized manner near injured tissues while it was absent inside the damaged blood vessels (Arnaud et al. 2011a; Hoggarth, Hardy, and Lyon 2013; Schwartz et al. 2011). Chitosan gauzes in many studies have been reported to be a valuable hemostatic agent where evacuation time was prolonged (from 12 to 72 hours) (Tan 2011). In world-wide remote military operational areas, the prehospital care is critical which might extend to 72 hours prior to medical evacuation for Role 2 or 3 medical care facilities and chitosan dressing has found to be successful hemostatic agent during the entire patient holding process (Inaba et al. 2011, 2013). The results of these studies suggest safety associated with long term use of chitosan dressings however the studies reporting its safety are few and requires further extensive investigations (Bennett et al. 2014).

3.2 Generations of Chitosan based bandages

Different generations of chitosan based bandages were introduced to meet the standards of ideal hemostatic bandage defined by Pusateri (Pusateri et al. 2006). Ideally, a hemostat should stop large arterial or venous hemorrhage in two minutes upon application without any pre-application procedure involved. The other standard requirements of hemostat agents exclusively for military applications apart from long shelf life are simplicity, durability and light weightiness (Pusateri et al. 2006). A committee was organized for the evaluation of first generation hemostatic bandages and it performed evaluation of HemCon and Quick-Clot efficacy on the basis of reported research studies to take decision on its selection in TCCC guidelines (Butler et al. 2007). Second generation hemostatic bandages were introduced to overcome the deficiencies reported in first generation dressings and they were evaluated by US

Army Institute of Surgical Research and Naval Medical Research Centre and they approved Combat Gauze, Celox and WoundStat compared to first generation hemostats (Arnaud et al. 2011a; B. Kheirabadi; KHEIRABADI et al. 1995). Later on, the committee set to evaluate chitosan based bandages for TCCC applications agreed on the basis of previously mentioned outcomes that combat gauze should be kept as first line treatment for severe hemorrhage which doesn't stop through tourniquet (Bennett et al. 2014).

Figure 14: Evolution of Chitosan based Hemostatic agents

Naval Medical Research conducted further investigational studies (Rall et al. 2013) and used DDA standardized Hemorrhage model (B. Kheirabadi) to evaluate four different gauze dressings and made comparative analysis with widely used Combat Gauze. This study included three chitosan dressings Celox Trauma Gauze, ChitoGauze, Celox Gauze and the fourth one was a double Combat Gauze XL, which contained higher content of kaolin than the usual one. All the bandages were tested on animal models while survival, blood loss and hemostasis were used as measurands of dressing efficacy. The study outcomes showed that FDA approved chitosan based dressing were equivalent in efficacy when compared with standard combat gauze except that hemostasis was achieved in initial 10 minutes with standard ones while the survival rate with chitosan based ChitoGauze and Celox Gauze was higher and (90 to 70%)

then combat gauze (60%) (Bennett et al. 2014).

Later on third generation hemostatic dressings were marketed which were approved by United Kingdom Defense Ministry for battlefield wound management (E. Smith and Dent 2005) and now it's under practice by Medical emergency Response Team (MERT) and air evacuation teams (Arul, Bowley, and DiRusso 2012; Wedmore et al. 2006). Tan et al. mentions the effectivity of Celox Gauze in surviving battlefield injuries in two NATO serving members without inducing any complications (Tan 2011). Figure 14 shows the different variations and development in chitosan bandages introduced by the time.

3.3 Chitosan Composite bandages with improved wound healing properties

Improvements in mechanical and drug delivery properties of chitosan bandages for wound healing applications have been extensively investigated during the past few years. Its composites with other compounds such as alginate, cellulose and gelatin etc. were developed to achieve enhanced structural integrity, porosity for oxygen permeability and antimicrobial activity. Figure 15 shows the photographs of chitosan sponges made of different composites tested for wound healing applications.

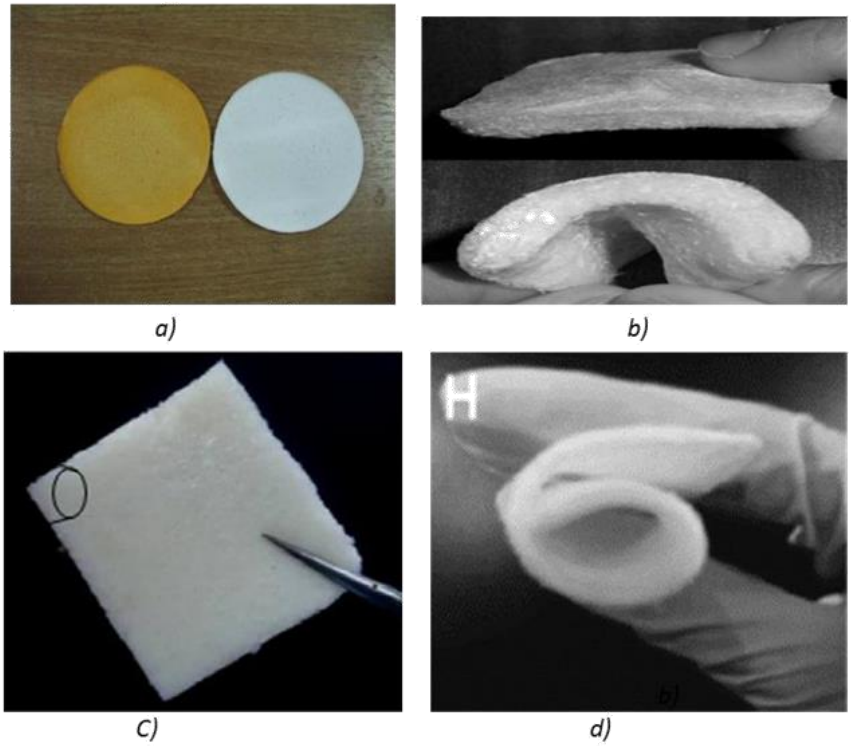


Figure 15: Chitosan Composites for wound healing a. Chitosan-Gelatin Sponge (left curcumin loaded) b. Chitosan-alginate sponge c. chitosan-hyaluronic acid sponge d. Chitosan-Nano ZnO composite bandage.

Lai et al. reported chitosan-alginate sponges with improved drug release profile and good mechanical properties, see figure 15b (H. L. Lai, Abu'Khalil, and Craig 2003a). He used sodium alginate and chitosan solution dissolved in 1% acetic acid and their mixture was freeze dried to obtain sponge like texture which was flexible and strong (H. L. Lai, Abu'Khalil, and Craig 2003b). The mixture sponge had randomly organized microstructure and it showed less resistance to compression when compared with only chitosan made sponge. Chitosan alginate sponge sustained 4N compression force compared to chitosan alone (5N) and it showed 1% elongation prior to break in tensile testing. Tensile and compressive strength of mixed sponge was found to be less than the only chitosan made sponge however it was comparatively higher than the alginate counterparts. Drug release profile was analyzed using paracetamol as a model drug and sponge made of chitosan alone had the slowest release profile (H. L. Lai, Abu'Khalil, and Craig 2003b). The author suggested in his study that manipulating the polysaccharide

composition will allow the formation of sponges with desirable mechanical and drug release properties which will be a suitable for chitosan based dressing for wound care. Figure 16 shows a comparison of tensile strength of chitosan bandages prepared with alginate (Kucharska et al. 2008), Nano-ZnO (P T Sudheesh Kumar et al. 2012) and cellulose (Wu et al. 2004). Chitosan cellulose composite sponge shows the highest tensile strength compared to others while alginate based dressings exhibit least strength. The high stiffness of chitosan-cellulose can be attributed to the strong chemical linking between OH- of cellulose and NH^{+3} of chitosan which gives it a more rigid and integrated structure. Chitosan and Nano ZnO showed intermediate tensile strengths which are sufficient for wound dressings (P T Sudheesh Kumar et al. 2012).

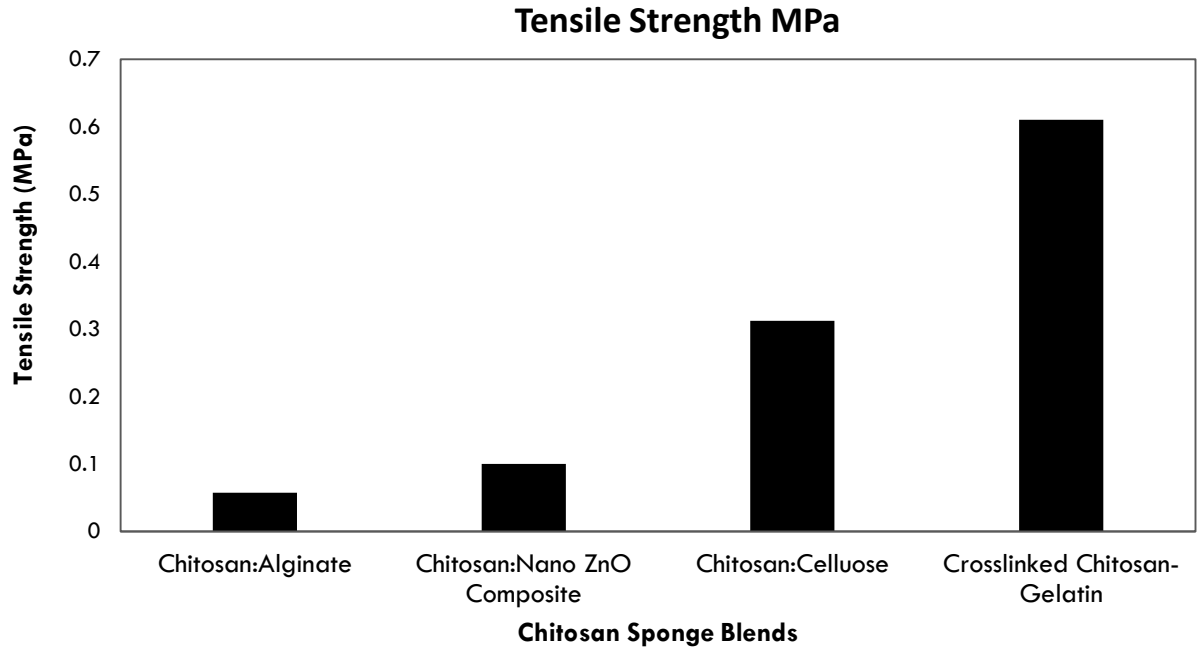


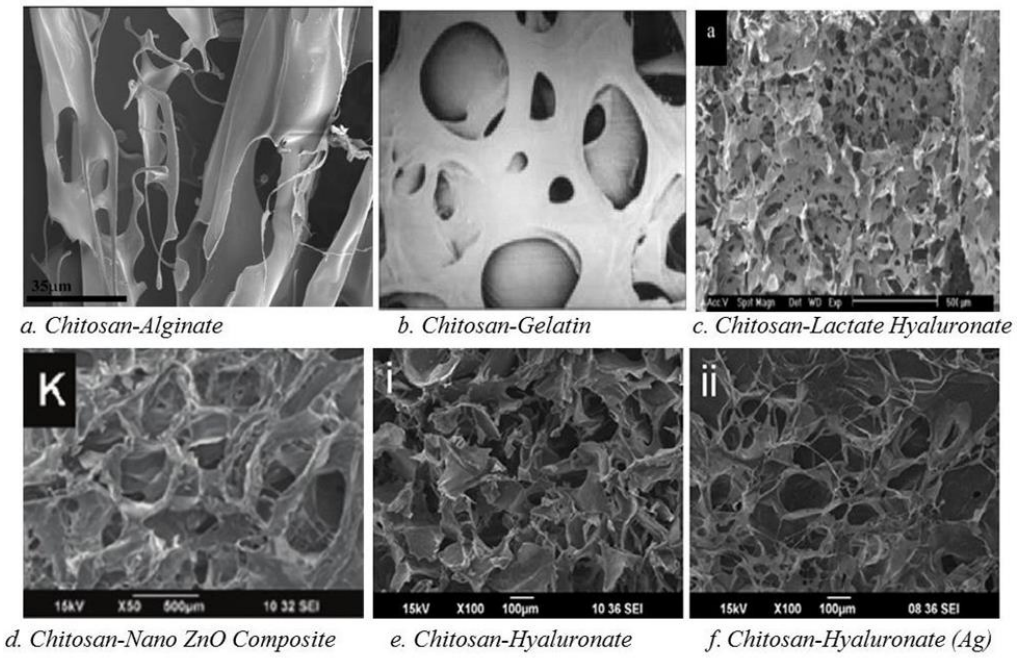
Figure 16: Tensile strength of different chitosan composites sponges for wound healing

Chitosan sponge grafted with lactic acid and hyaluronate has been developed recently in a temperature controlled aluminum vessel for as a potential wound healing dressing (C. Lai 2014b).The results of the study showed that microtubule structure in sponge can be produced

through treatment of composite mixture in a vertical temperature controlled device that contained tertiary butyl alcohol (C. Lai 2014b). Due to the presence of TBA in vessel, parallel orientation of pores was obtained resulting in high porosity and water permeability (C. Lai 2014b). Presence of lactic acid in the sponge reduced the crystallinity of chitosan thereby increasing its water solubility which eased lysozymatic degradation of chitosan-lactate-HA sponge (C. Lai 2014b). Figure 17c shows the dense porous structure of chitosan-lactate hyaluronate sponge reported in this study (C. Lai 2014b). Pores are randomly organized all across the surface while 17b indicate elliptical regularly arranged pores in chitosan-gelatin sponge (Foda, El-laithy, and Tadros 2007). This is of considerable importance in drug delivery perspective where drug is impregnated in sponge dressing for controlled and sustained release. In contrast, chitosan-alginate sponge exhibits fibrillar pore morphology that result in burst release of drugs (H. L. Lai, Abu'Khalil, and Craig 2003b). Chitosan-hyaluronate with and without embedded silver nanoparticles (Anisha et al. 2013) have surface morphology similar to the one shown by chitosan-Nano ZnO blend that contained randomly organized dense porous structures (P T Sudheesh Kumar et al. 2012). Results of these studies show that presence of gelatin in combination with chitosan give better control over drug delivery properties of the dressing sponge.

Chitosan-gelatin sponge was tested by Foda et al. for controlled drug release and improved mechanical characteristics in implantable sponges (Foda, El-laithy, and Tadros 2007). He mentioned in his study that polyionic complexation between chitosan and gelatin facilitates prolonged drug release for 8 hours (Foda, El-laithy, and Tadros 2007). He used Tramadol HCl analgesic to generate drug release profile through Higuchis diffusion mechanism and $t_{50\%}$ value in dissolution profile of chitosan-gelatin sponge was 4.73 hours which retained analgesic effect on Wister rat (testing model) for 8 hours and he explained that it was due to the combined

effect of the polyionic complexation as well as crosslinking between chitosan and gelatin (Foda, El-laithy, and Tadros 2007). Mechanical properties of chitosan was also improved due to the addition of gelatin and the sponges produced were comparatively soft, flexible and compressible with augmented moisture sorption ability (Foda, El-laithy, and Tadros 2007). Chitosan and gelatin sponges were prepared by him through freeze drying method where the factor affecting stability of foam were chitosan-gelatin ratio, whipping speed, duration and temperature as well as pH of gelatin solution. He further indicated that storing these sponges at 43-65% relative humidity retains its pliability while the best compressibility is achieved with chitosan sponges stored at 65% relative humidity (Foda, El-laithy, and Tadros 2007). Results of this study imply that the reported formulation of chitosan-gelatin sponge can be well-adapted for wound healing purpose and it requires further investigation.



a. Chitosan-Alginate *b. Chitosan-Gelatin* *c. Chitosan-Lactate Hyaluronate*

d. Chitosan-Nano ZnO Composite *e. Chitosan-Hyaluronate* *f. Chitosan-Hyaluronate (Ag)*

Figure 17: SEM images showing surface morphology of chitosan composites sponges

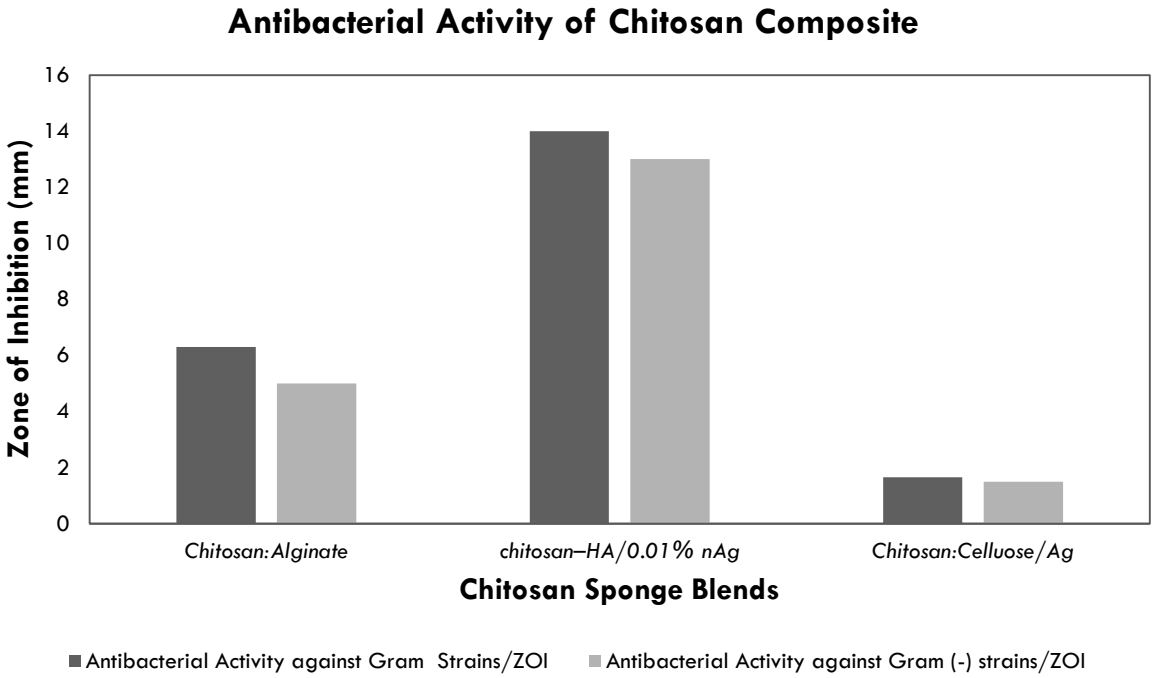


Figure 18: Antibacterial activity of chitosan composites sponges tested for wound

Chitosan exhibits broad spectrum bactericidal activity and its amino group reacts with bacterial cell wall leading to its detachment and then disruption of cell membrane which cause lysis of bacterial cells (Chung and Chen 2008). In a recent study, zinc oxide nanoparticles were incorporated in the chitosan hydrogel to bring further improvements in its antibacterial activity (P T Sudheesh Kumar et al. 2012). Incorporation of ZnO in chitosan hydrogel improved its flexibility, antibacterial activity and tensile strength (0.15MPa) (P T Sudheesh Kumar et al. 2012). Chitosan composite prepared with cellulose and 100% lyophilized have found to exhibit antimicrobial activity against gram positive as well as gram negative bacteria (Harkins et al. 2014). It was non-toxic to growing fibroblasts and their proliferation was not found to be disrupted by cellulose composite presence (Harkins et al. 2014). It also had increased blood sorption capacity that shows its potential for effective wound healing dressing however it requires animal model study for the validation of reported results of this study. However, antibacterial activity of chitosan-cellulose impregnated with silver nanoparticles was found to be less when compared with other blends reported for wound healing (Anisha et al. 2013;

Gopinath et al. 2004; Yu et al. 2005). Antibacterial activity of different chitosan sponge blends against gram positive and gram negative strains have been compared in figure 18 based on the size of zone of inhibition/ZOI. The highest area of zone of inhibition was reported in chitosan-Hyaluronate with 0.01% Ag particles composite sponge compared to blends made with alginate and cellulose. They show comparably low areas of inhibition zone, see figure 18.

In a recent study, Nguyen et al. prepared curcumin loaded sponges of chitosan and gelatin to improve wound healing process which involves higher oxidative stress resulting in inhibition of tissue remodeling process (Nguyen, Nguyen, and Hsieh 2013a). Curcumin is natural polyphenol which acts as antioxidant and prevents oxidative damage in the healing tissues when applied topically (Martin 1996). Gopinath et al. studied curcumin for wound healing applications and he incorporated it into collagen matrix for gradual release compared to burst release of it which becomes toxic to the patient (Gopinath et al. 2004). Nguyen et al. reported that curcumin loaded chitosan-gelatin sponge exhibited improved water uptake capacity and antibacterial activity (Nguyen, Nguyen, and Hsieh 2013a). He further explained in his study that increasing the gelatin concentration in composite sponge resulted in sustained release of curcumin for 240 minutes and it was observed to augment collagen production and wound closure thereby expediting the healing process (Nguyen, Nguyen, and Hsieh 2013a).

Choi et al. conducted a comparative study in which composites of gelatin and chitosan were analyzed for wound healing (Choi et al. 2001). He prepared six composites; chitosan-hyaluronate, gelatin hyaluronate and gelatin-alginate sponges and three of them were impregnated with silver sulfadiazine/AgSD to enhance antimicrobial activity (Choi et al. 2001). Significant improvements in wound healing was achieved with sponges that contained AgSD and epidermal morphometric analysis and histological examinations indicated that among all composites Gelatin-Hyaluronate produced fastest healing response in Wistar rat model used in

this study (Choi et al. 2001). The reported pore size in all the sponges was 90-160 µm except chitosan-hyaluronate sponge which showed compact structure with comparatively smaller pore size (Choi et al. 2001). The water absorption capacity was also good and the sponges were able to hold water capacity 10-40 times of their original weight (Choi et al. 2001). The author concludes that gelatin composite with hyaluronate gave best wound healing response when impregnated with AgSD. It implies that gelatin incorporation gives improved porosity, gradual release of impregnated drug and high water uptake capacity which in case of wound healing is mandatory. Pore morphology is critical in preparation of bandages that contain antimicrobial agent and controlled and sustained release of it is a major challenge which can be overcome by improving porosity of the sponge blend.

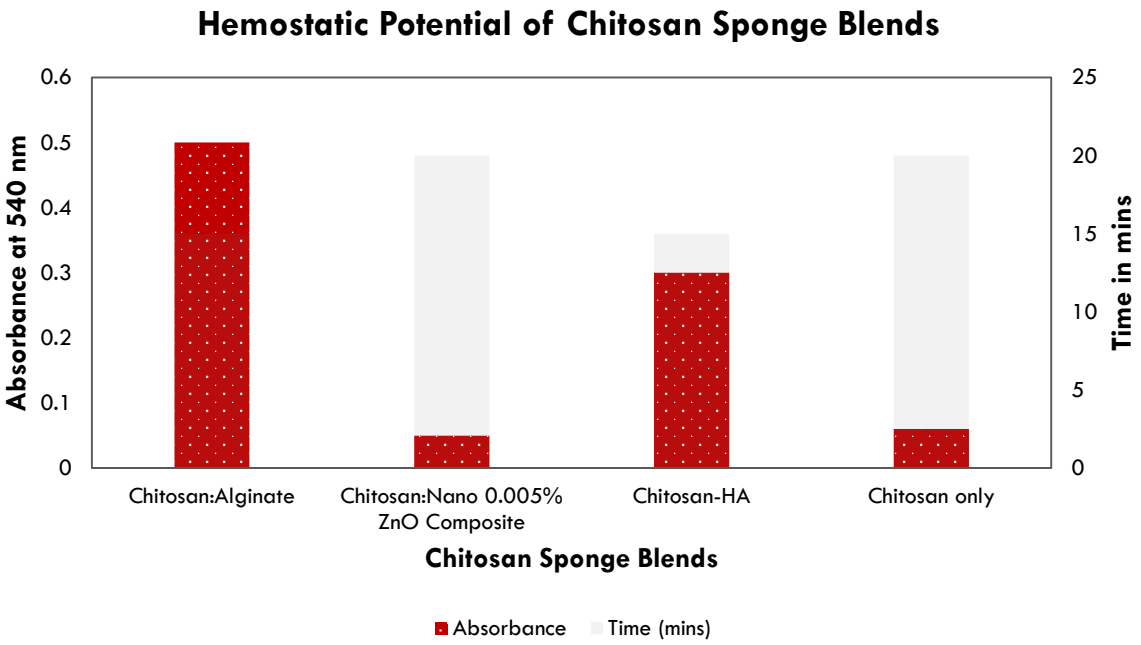


Figure 19: Comparison of hemostatic potential of different chitosan composite sponges studied for wound healing

Time to achieve hemostasis is another important property of hemorrhage control dressings and ideally a bandage should take less than 5 minutes time to stop heavy bleeding. A comparative analysis of hemostatic potential of different chitosan sponge blends have been shown in figure 19. The blends were tested for hemostasis by measuring the absorbance of

hemoglobin released by unclotted RBCs remained in solutions containing sponge dressings. The higher absorbance indicate less hemostatic activity as the concentration of RBCs that remained unclotted when exposed to bandage, is higher. Chitosan-Nano ZnO and Chitosan only bandages showed least absorbance values in less than 5 minutes which implies that higher amount of clot was formed and it suggest their good hemostasis properties compared to others.

3.4 Conclusion

Development of effective pre-hospital care following trauma requires a wound management system that has quicker hemostatic ability and inhibits infections or any other impairment that impedes the healing process. It also improves the survivability of injured patient. Variety of hemostatic agents have been developed to prevent excessive hemorrhage and the most popular in TCCC are Combat Gauze, Celox Rapid, ChitoGauze and ChitoFlex. Improvements in the fabrication methods of chitosan based dressings have been made to augment its hemostatic potential and anti-bacterial activity. Mechanical properties, gas permeability, drug delivery and wound healing characteristics of chitosan bandages have found to be augmented when some antioxidant (curcumin) or polysaccharide is integrated (cellulose/gelatin) or chemically coupled with chitosan. Further research in the implementation of integrated sensors in hemostatic dressings will facilitate real-time monitoring of wound healing process. It will also obviate repetitive dressing change which becomes uneconomical and caused hindrances in the progression towards healing.

Chapter 4

4 Development of Chitosan-Gelatin crosslinked with Glutaraldehyde Sponge as Potential Hemostatic Bandage for Trauma Patients

ABSTRACT

Chitosan composite sponge bandages with different polymers have been introduced to achieve effective hemostasis that aimed to reduce mortality rate of hemorrhage leading preventable deaths. In this study, we investigated hemostasis potential of chitosan-gelatin sponge crosslinked with 5% glutaraldehyde and results were compared with uncrosslinked chitosan-gelatin sponge and other already reported chitosan composite bandages. FTIR results showed successful crosslinking with glutaraldehyde which resulted in improved tensile (increase from 0.1MPa to 0.61MPa in crosslinked chitosan-gelatin sponge) and compressive properties (57.1% more compressible compared to uncrosslinked chitosan-gelatin sponge which was 50.1% compressible). The crosslinking affected hemostasis potential of chitosan-gelatin sponge and uncrosslinked sponge showed least absorbance (0.2) while 0.1ml crosslinked showed highest absorbance (0.974) after 30 minutes. Blood absorption capacity was highest (33.5 g/cm^2) in 0.05ml crosslinked chitosan-gelatin sponge compared to 9.4 g/cm^2 and 26.7 g/cm^2 with 0.1ml crosslinked and uncrosslinked chitosan-gelatin sponges respectively. The high blood sorption capacity of 0.05 ml crosslinked chitosan-gelatin sponge can be related to its porosity. It exhibits roughly ellipsoid shaped pores which were less dense and has 75 µm average pore size. Pore morphology with increased size and less density is found to be a

favorable factor for blood sorption however addition of glutaraldehyde and its increasing concentration inhibited blood clotting efficiency of chitosan-gelatin sponges.

4.1 INTRODUCTION

Hemorrhage is the leading cause of preventable death in trauma patients and according to a study, 50% battlefield casualties and 31% civilian casualties are due to hemorrhage (Arnaud et al. 2011b; Hoggarth, Hardy, and Lyon 2013; Mueller et al. 2012; Schwartz et al. 2011). Different blends of chitosan have been made to develop hemostatic bandages that give control over hemorrhage in major arteries (C. Lai 2014a, 2014b; Nguyen, Nguyen, and Hsieh 2013b; Yu et al. 2005; H. Zhang et al. 2015). Chitosan based bandages have already received approval from FDA for clinical and surgical applications and it has been reported to be efficacious in coagulopathic patients for stopping excessive bleeding (Floyd, Rothwell, Martin, et al. 2012; Bijan Shams Kheirabadi et al. 2010). Chitosan composite with alginate was reported earliest (H. L. Lai, Abu'Khalil, and Craig 2003b) and later on it was blended with hyaluronate (Sawada 2001), lactate hyaluronate (C. Lai 2014a), cellulose (Harkins et al. 2014) and Nano Zinc Oxide (P T Sudheesh Kumar et al. 2012) to achieve effective antibacterial activity, drug release profile, hemostatic and blood sorption abilities. Chitosan-cellulose composite bandage has good tensile strength (0.3MPa) and good blood sorption capacity (Harkins et al. 2014) however it doesn't give better control over porosity. Chitosan-alginate sponge bandage exhibits fibrillar porous structures but its blood coagulation ability is compromised (Kucharska et al. 2008). In contrast, chitosan blended with lactate and lactate-hyaluronate provided control over porosity which is important from drug release perspective (C. Lai 2014a; Sawada 2001). Chitosan-gelatin based sponges for wound healing application was reported (Nguyen, Nguyen, and

Hsieh 2013b) by Nguyen et al. and he loaded sponges with curcumin to improve antibacterial activity and to prevent oxidative damage in injured tissues. Burst release of curcumin is toxic and its sustained release is crucial which can be attained with effective control over pore size of chitosan-gelatin sponge. Chitosan-gelatin composites were also investigated by Foda et al. for dental implantable sponge applications (Foda, El-laithy, and Tadros 2004, 2007). They coated sponge with Tramadol Hydrochloride and checked the drug release profile. Chitosan-gelatin sponge which was crosslinked with 5% glutaraldehyde and prepared with 1:5 chitosan: gelatin ratio gave best results. The pores were elliptical and uniformly distributed all over the sponge (Foda, El-laithy, and Tadros 2004, 2007). In the present study, we tested chitosan-gelatin sponge crosslinked with 5% glutaraldehyde for hemostasis applications and compared the results with uncrosslinked chitosan-gelatin sponge and the composites which have already been reported.

4.2 EXPERIMENTAL

4.2.1 Materials

Chitosan, molecular weight 48109 Dalton. Glutaraldehyde (25% aqueous solution) and Gelatin Type B, Bloom 225 were purchased from DaeJung Chemicals and Metals, Korea. Glacial Acetic Acid was obtained from Scharlau Chemicals, Spain.

4.2.2 Methods

4.2.2.1 Chitosan and Gelatin preparation

First 2.5% chitosan solution in distilled water was prepared and it was stirred on magnetic stirrer (MS300HS, Tech Jam Instruments, China) for 4 hours till a clear

homogenous solution was obtained. 7% Gelatin aqueous solution was obtained by dissolving gelatin in distilled water and kept on stirring at 50^{o}C for 20-30 minutes. Gelatin and Chitosan solutions were mixed in 1:5 ratio and chitosan solution was added in gelatin solution already kept on magnetic stirring (Foda, El-laithy, and Tadros 2007). The solution was stirred till a clear homogenous mixture was obtained.

4.2.2.2 Glutaraldehyde crosslinking and Sponge formation

To make a crosslinked chitosan-gelatin solution, 5% glutaraldehyde solution prepared in distilled water was added in chitosan-gelatin solution and it was poured inside the beaker with constant stirring so that it get dissolved properly. The pH of solution was maintained at 5.5 and it was whipped with an electronic whipper at 1000rpm for 15 minutes at 25^{0}C. The whipped mixture was poured in the mold and it was kept in freezer at -20^{0}C. Glutaraldehyde (5%) was added in two different volumes in sponge; one mixture contained 100 µl in 250 ml total volume while the second one contained 50 µl of it in 250 ml of mixture.

4.2.2.3 Lyophilization conditions

Uncrosslinked Chitosan-Gelatin was frozen at -20^{o}C for 24 hours and then it was lyophilized at 15 Pascal pressure at -40 ^{o}C. The amount of solvent evaporated was 40 ml. Cross-linked chitosan gelatin with 0.05 ml and 0.1 ml glutaraldehyde were kept in freeze dryer (EYELA, FDU 2100, Tokyo Rikakikai Co., LTD, Japan) for four hours at 13.35 Pascal -40 ^{o}C. The eluent was 40 ml. Defrosting was performed at room temperature and the samples were stored at 4^{o}C in refrigerator wrapped in aluminum foil.

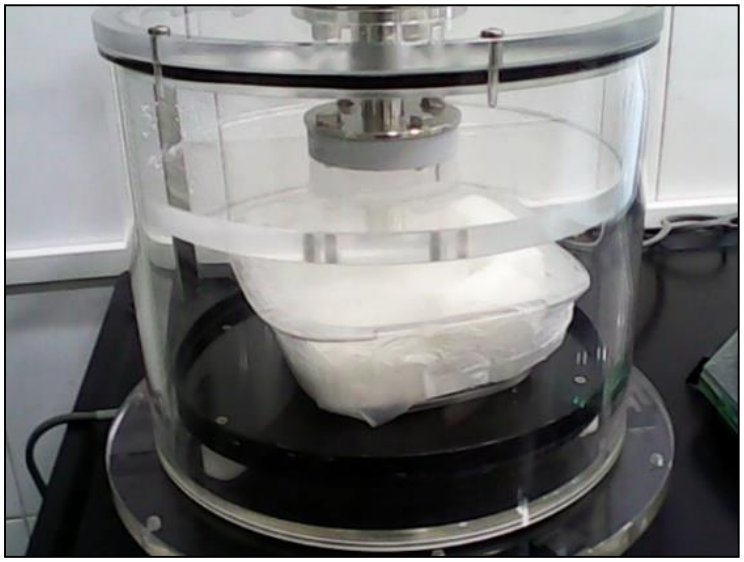


Figure 20: Chitosan-Gelatin sponge mixture kept in freeze dryer

4.2.2.4 Mechanical Testing

Compressibility testing of sponges were performed according to ASTM D57059T (Foda, El-laithy, and Tadros 2007). Cross-linked sponge's original thickness before compression was 7mm. After 25% compression by applying 20 kN for an hour in Schimadzu-UTM, the change was 3mm. It was measured after 30 mins of the release time. The measured thickness was 4mm showing 57.1 % compressibility. Uncrosslinked sponge had original thickness 5.2mm which after compression changed to 2.6mm indicating 50% compressibility. Given below is the formula used for measurement of compressibility%. (Foda, El-laithy, and Tadros 2007). The number of samples tested were 3.

$$Compressibility\ \% = \left(\frac{t_1}{t_0}\right) \times 100$$

The tensile strengths of uncrosslinked (8cm × 2cm × 0.4 cm) and crosslinked (8 cm × 2 cm × 0.4 cm) sponges were determined using Schimadzu, Universal Testing

Machine, 20kN. The crosshead speed was 25 mm/min (P. T. Sudheesh Kumar et al. 2012) and the number of samples tested was 3.

4.2.2.5 Scanning Electron Microscopy Analysis

To determine the porosity, optical microscopy in transmission mode was conducted using Optika 600, Italy at 5x. Pore Size was determined using 1mm calibration bar. Chitosan-gelatin sponge with size of 5×5mm was mounted on sample holder and adhered with tapes on both sizes. Carbon coating was performed for 2 minutes (Sputter Coater, Quorum, Q150TES, Quorum Technologies, UK) under vacuum. The thickness of coating was 87nm/mention in Angstrom. The Samples were then analyzed through Scanning Electron Microscope, MIRA3, Tescan, Czech Republic.

4.2.2.6 FT-IR Spectroscopy

To determine the extent of crosslinking, FTIR analysis was conducted (using Perkin Elmer, Spectrum 100, FTIR Spectrometer) for both chitosan gelatin sponge which are cross-linked and uncrosslinked and spectra were read from 4000 cm^{-1} to 500 cm^{-1}. Spectra were obtained from KBr Disc method. Smoothing and baseline correlation procedure were applied on it.

4.2.2.7 Hemostasis ability

The hemostasis ability of the chitosan-gelatin sponge bandage was determined by measuring the absorbance of hemolyzed erythrocytes at 540nm (Singhal and Ray 2002). Bandage samples of 1×1 cm were kept in glass vials and 0.25 ml of blood sample was added in it. It was left in incubator (WiseCube, Wisd Laboratory

Instruments, Germany) at 37 ^{0}C for 30 minutes. 20 ml of distilled water was added afterwards and its absorbance at 540nm was measured using UV-Vis Spectrophotometer, BMS UV2800. This procedures was repeated after 60 minutes incubation. Three samples of each sponge type were tested. The erythrocytes left unclotted were hemolyzed and their released hemoglobin concentration was obtained at 540 nm. It is indirectly related to the amount of erythrocyte clotted by Chitosan-gelatin sponge. Higher absorbance of hemolyzed solution at a given time interval corresponds to low hemostasis efficiency of sponge bandage.

4.2.2.8 Blood sorption capacity

Sponge of 1×1 cm of each type were cut and weighed before addition of blood sample collected in sodium citrate tube (Harkins et al. 2014). The samples were kept in petri-dishes containing 20ml of whole blood (kindly donated by a volunteer) and it was kept in incubator for 30 minutes. It was weighed again to obtain amount of blood absorbed by it (Terrill, Sussman, and Bailey 2003). The unabsorbed blood was removed by keeping sponge suspended in air for 30 seconds and then it was weighed. The procedure was repeated following 2, 5 and 6 hours of blood absorption. Three samples of each sponge type was tested for blood absorption capacity test.

4.3 RESULT AND DISCUSSION

The sponge formed following lyophilization of chitosan-gelatin mixture were very soft and pliable. Figure 2 shows uncrosslinked chitosan-gelatin sponge which is yellowish white in color while figure 3 shows crosslinked chitosan-gelatin sponges at different volumes of 5% glutaraldehyde.

Figure 21: Uncrosslinked Chitosan-Gelatin sponge

Figure 22: Left) Chitosan-Gelatin sponge crosslinked with 5% Glutaraldehyde 0.1 ml/100µl dissolved whipped mixture (Right) Chitosan Gelatin sponge crosslinked with 0.05ml/50µl of Glutaraldehyde dissolved in whipped mixture.

The uncrosslinked sponges have pale yellow color and the thickness of the sponge is different due to the difference in mold size. The crosslinked sponges had improved physical properties and they are more elastic compared to uncrosslinked chitosan-gelatin sponges.

4.3.1 Compressibility and Tensile Strength

The mechanical testing according to ASTM standards mentioned in section 2.2.4 was conducted to observe effect of glutaraldehyde crosslinking on the mechanical

characteristics of sponge. The results of tensile strength showed maximum 6.88 strain% for crosslinked sponges while the tensile strength was 0.61 MPa compared to uncrosslinked sponge which had 3.61 maximum strain % and 0.105 MPa tensile strength. The commercially available bandages have tensile strength of 0.1 MPa (P T Sudheesh Kumar et al. 2012). The crosslinked as well as uncrosslinked chitosan-gelatin sponge both exhibited the tensile strength in desirable range while crosslinked sponge had improved value due to the chemical linkage by glutaraldehyde. Figure 4 shows comparative analysis of tensile strength of chitosan composite sponges. Crosslinked chitosan and gelatin highest strength and next to it is chitosan-cellulose which has improved strength due to its inherent structural properties (Harkins et al. 2014). In contrast, tensile strengths of uncrosslinked chitosan-gelatin sponge and chitosan-Nano ZnO composite (P T Sudheesh Kumar et al. 2012) are comparable and less than the former two composites.

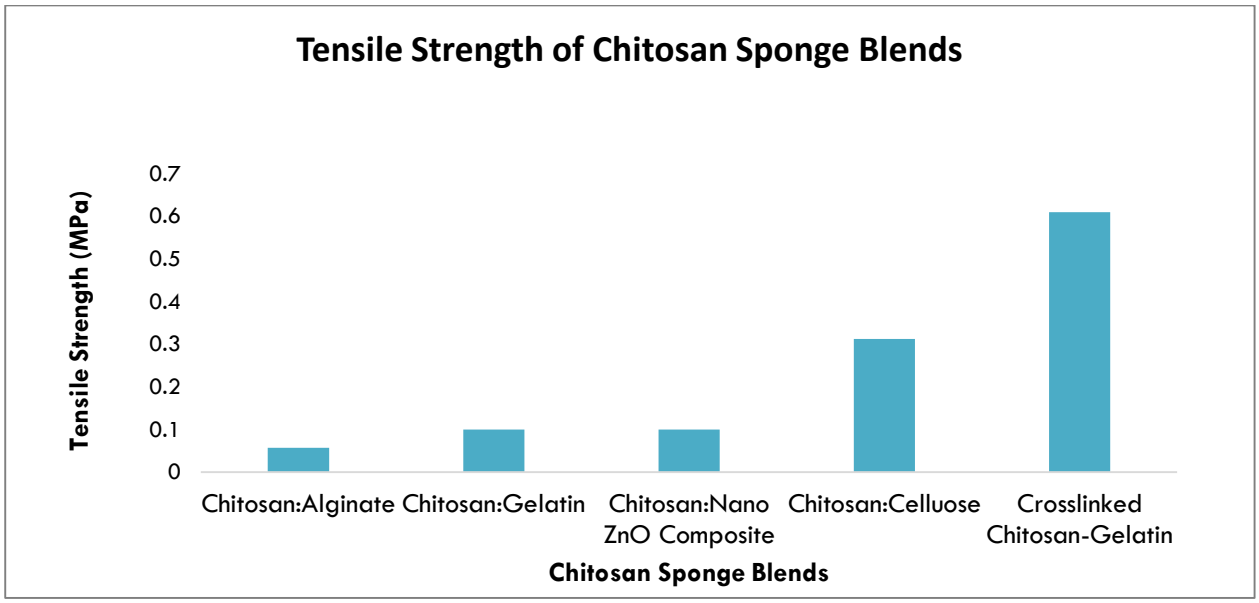
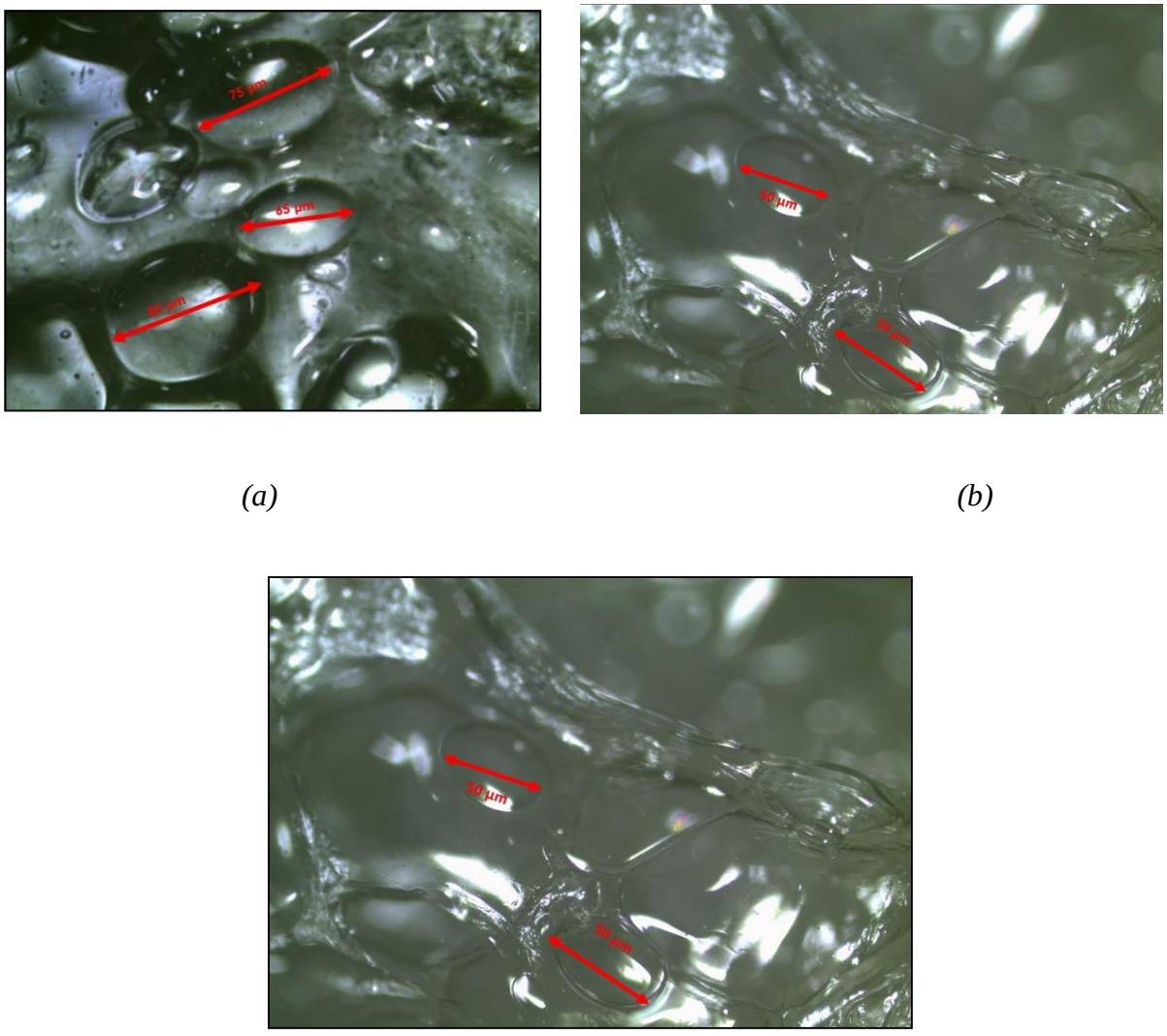


Figure 23: Comparative analysis of tensile strength of chitosan composite sponges.

Compressibility testing shows that crosslinked chitosan-gelatin sponge was 57.1% compressible to its original thickness (7 mm) while uncrosslinked chitosan-gelatin sponge showed resistance to compression and it was 50% compressible to its original thickness (5.2 mm). The elasticity achieved in crosslinked sponge was due to the presence of glutaraldehyde chemical linkage which made the polymeric structure more flexible and pliable.

4.3.2 Surface Morphology and Porosity analyzed through Optical Microscopy and SEM

Pore morphology of the prepared chitosan-gelatin sponge bandages were analyzed through optical microscopy in transmission mode and scanning electron microscopy. Figure 5 shows the pore size measured through calibration bar of 1mm. In figure 5a, the pores obtained and have average 75 µm pore size (calculated from different fields). Chitosan-gelatin sponge crosslinked with 5% glutaraldehyde (100 µl in 250 ml) shows large interconnected pores with irregular geometric mixed with small ellipsoid pores (average 50µm size), see figure 5b. In contrast, uncrosslinked chitosan-gelatin sponge have similar pore morphology with pore size (50µm).



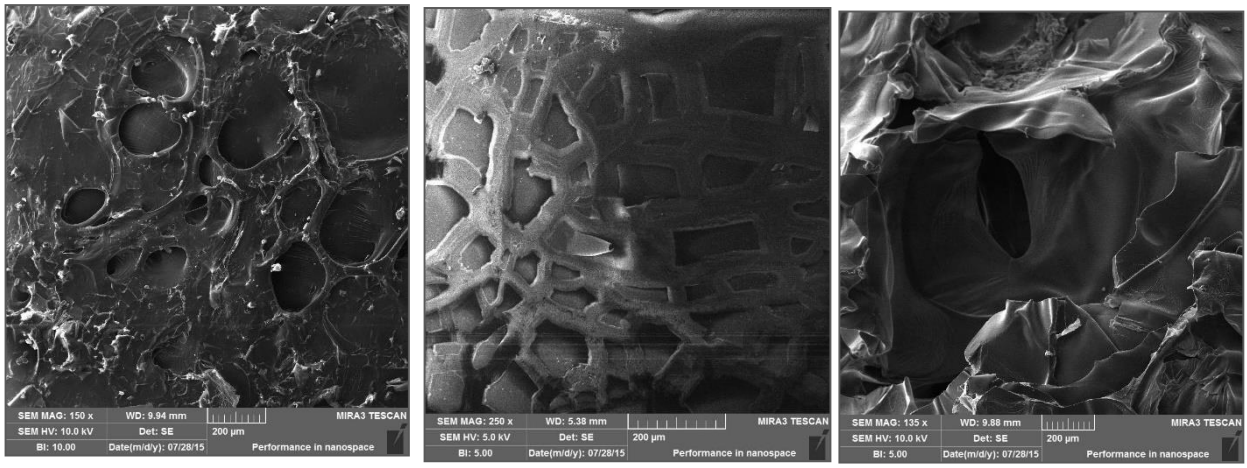
(a) *(b)*

(c)

Figure 24: Surface morphology analyzed through optical microscopy. a.Crosslinked Sponge with 5% Glutaraldehyde (50 µl) b.Crosslinked Sponge 5% Glutaraldehyde (100µl) c. Uncrosslinked Sponge.

SEM results of these sponges have been shown in figure 6. Uncrosslinked chitosan-gelatin sponge with roughly elliptical pores are randomly organized in the given field, see figure 6a. Figure 6a and 6b and confirms high pore density in chitosan-gelatin uncrosslinked and crosslinked with 100 µl of 5% Glutaraldehyde. On the contrary, chitosan-gelatin crosslinked with less volume of glutaraldehyde (50 µl of 5%) showed less dense porous structure, see figure 6a. Results of SEM and optical microscopy were in consistent with

ones reported by Foda et al., (Foda, El-laithy, and Tadros 2007) and it further confirms that pore size of sponge composite is tunable through glutaraldehyde concentration added in mixture solution and the optimum concentration for obtaining average 75 µm pore size with less density is 50 µl of 5% aqueous solution of it.



(a) *(b)* *(c)*

Figure 25: SEM analysis of a. Chitosan-Gelatin uncrosslinked sponge b. Chitosan-Gelatin crosslinked 100µl Glutaraldehyde c. Chitosan-Gelatin crosslinked with 50µl Glutaraldehyde.

4.3.3 FTIR Spectroscopy Analysis

Confirmation of crosslinking by glutaraldehyde in chitosan-gelatin composite was performed through FT-IR spectroscopy analyses. Figure 7 shows stretches at 1658 and 3467 which corresponds to chitosan- NH_3 stretching (symmetric deformation) and Gelatin-OH groups indicating complex formation between chitosan and gelatin (Silva and Andrade 2009). Stretching at 1416 shows gelatin –COO bonds. These peaks disappear in the second spectrum and a new stretch at 1617 shows formation of Schiff's base N=C bond indicating crosslinking by glutaraldehyde (Li et al. 2013).

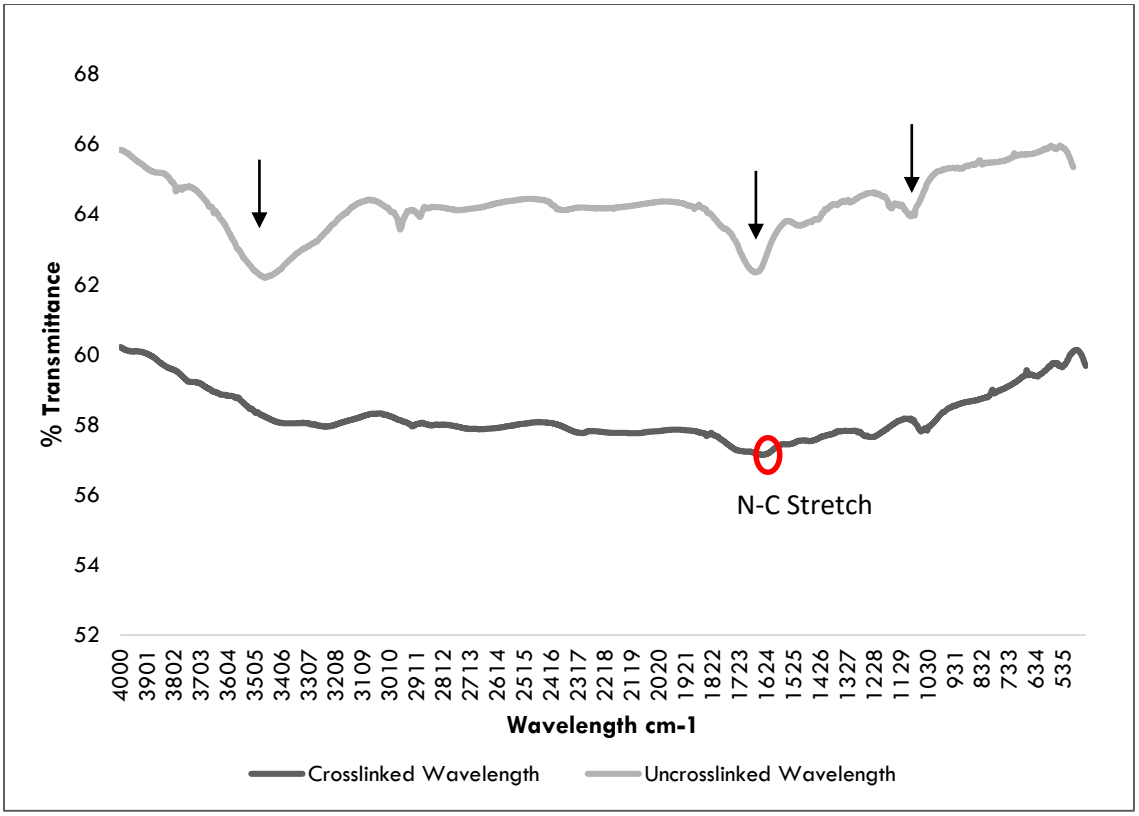


Figure 26: FT-IR spectra of crosslinked and uncrosslinked chitosan-gelatin sponge

4.3.4 Hemostasis Ability

Chitosan-Gelatin sponges were tested for hemostasis and the absorbance recorded at 30 minutes and 60 minutes interval corresponds to hemoglobin released by lysed erythrocytes and used as a measure of clotting efficiency which indicates chitosan composite sponge ability to achieve hemostasis. The absorbance was measured at 540 nm and figure 8 shows that uncrosslinked chitosan-gelatin sponge has minimum absorbance after 30 minutes while the crosslinked sponges have higher absorbance relative to uncrosslinked sponge, implying less hemostasis in initial 30 minutes by them. The graphical analysis further suggests that crosslinking by glutaraldehyde has affected the hemostatic efficiency of chitosan-gelatin sponge and in case of crosslinked Chitosan-gelatin sponge with 0.1ml/100µl volume of glutaraldehyde, the absorbance decreased after 60 minutes. It

indicates delayed hemostasis while in 0.05ml crosslinked sponge the absorbance continued to increase after 60 minutes.

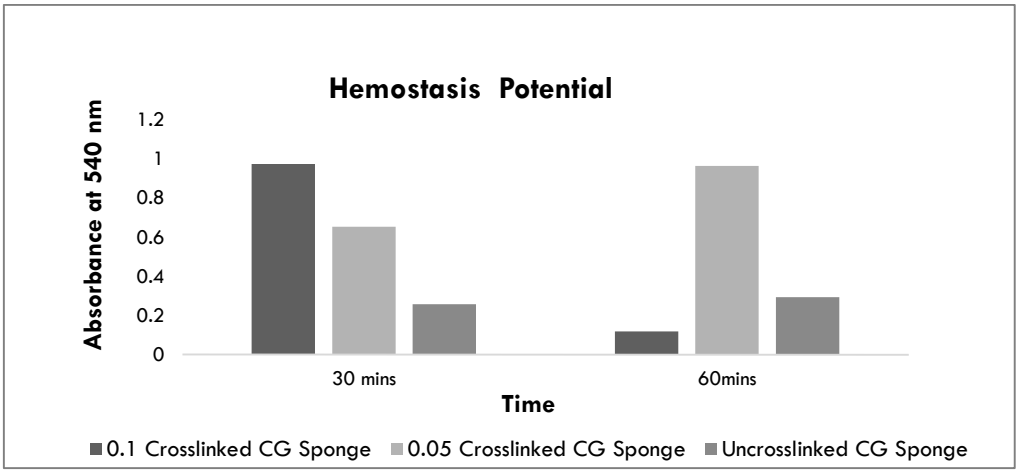


Figure 27: Hemostatic efficiency of chitosan sponges when measured at 30 minutes interval and 60 minutes time interval.

It can be concluded that crosslinking with glutaraldehyde causes delayed hemostasis in chitosan-gelatin bandages.

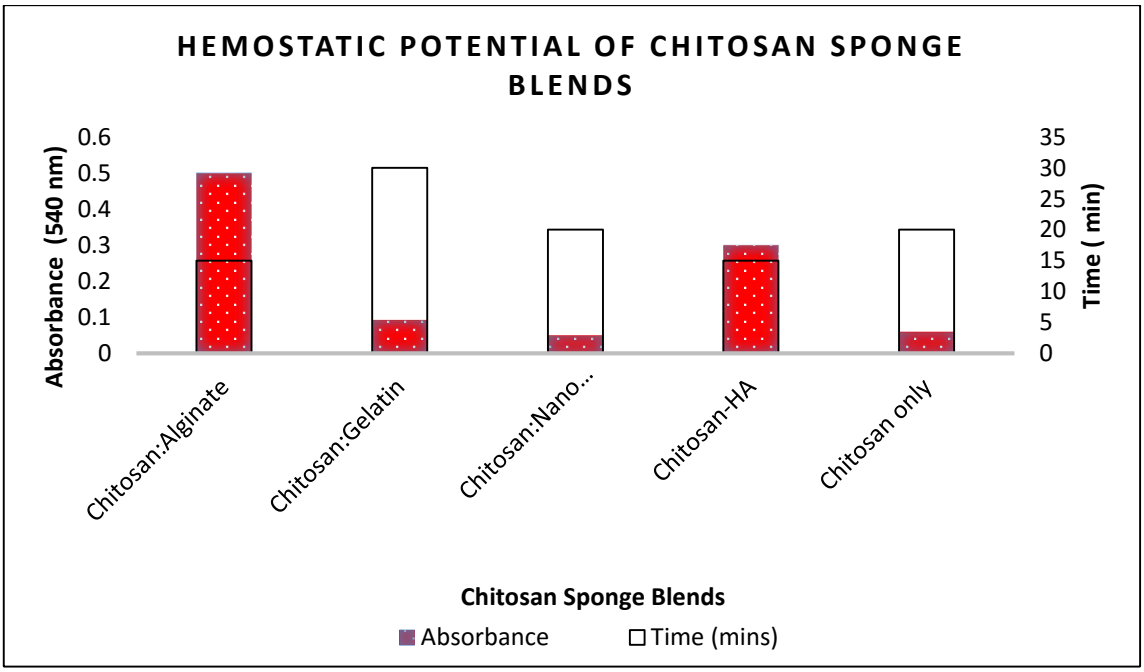


Figure 28: Chitosan-gelatin uncrosslinked sponge hemostasis potential compared with the ones reported with other chitosan composite sponges (alginate, Nano ZnO, Hyaluronate and only Chitosan).

Figure 9 shows a comparative analysis graph of chitosan-gelatin uncrosslinked sponge hemostasis ability with the ones obtained with blends of chitosan sponges (Anisha et al. 2013; H. L. Lai, Abu'Khalil, and Craig 2003a; P. T. Sudheesh Kumar et al. 2012). Chitosan-Gelatin hemostasis potential was comparable with one reported in Chitosan-Nano ZnO composite and chitosan only. Composites made with alginate and hyaluronic acid (Anisha et al. 2013) exhibits higher absorbance i.e. 0.5 and 0.3 after 15 minutes and 20 minutes respectively. It suggests that their hemostatic potential was comparatively less than the one shown by chitosan only, chitosan-gelatin and chitosan-Nano ZnO composites.

4.3.5 Blood Sorption Capacity

The blood absorption capacity for all three types of sponges is shown in figure 10 at time 0 minute, 30 minutes, 2 hours, 5 hours and 6 hours. At time 0, there was no blood added in the sample and it simply shows the weight of sample sponge 1× 1 cm^2.

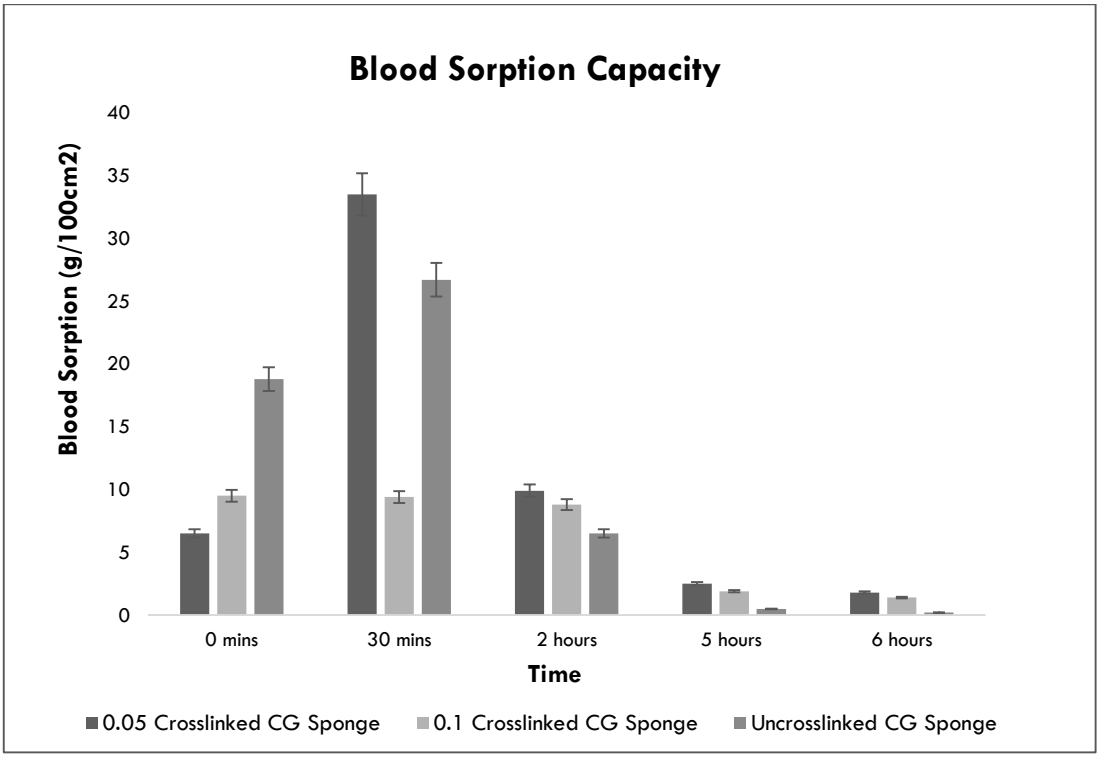


Figure 29: Chitosan-gelatin sponges blood sorption capacity measured in g/100cm2 at 0 min (no blood added), 30 minutes, 2 hours, 5 hours and 6 hours.

Increase in values at 30 minutes show the change in weight of sponges following blood absorption and 0.05 ml crosslinked chitosan-gelatin sponge has the highest absorption capacity. The value is 33.5 g/cm^2 which is close to the values reported by Harkins et al. for gauze sponge bandages (Harkins et al. 2014). The weight of sponges started decreasing as they dried and the blood absorption capacity in uncrosslinked and 0.1ml crosslinked sponges were comparatively less than the ones obtained with crosslinked 0.05ml sponge.

4.4 CONCLUSION

High mortality associated with excessive bleeding in trauma patients can be significantly reduced by effective pre-hospital care. Different composites with chitosan were prepared to improve it hemostasis ability that would help patient sustain its injury before hospital care and stops bleeding. Chitosan was blended with alginate, hyaluronic acid, lactate hyaluronate, cellulose, Nano ZnO, and gelatin and addition of polymeric structure produce variation in its blood sorption and hemostasis characteristics. In this study, we investigated crosslinked chitosan-gelatin for hemostasis and results of these studies suggest that addition of glutaraldehyde has considerable impact on pore morphology of resulting sponge which ultimately influence its blood sorption capacity and hemostasis property. However, tensile strength and compressibility increases with glutaraldehyde crosslinking. Optimum pore size is attained with 0.05 ml crosslinked chitosan-gelatin sponge which exhibits 33.5 g/cm^2 blood sorption capacity however its hemostasis ability is compromised and it can be improved with use of chitosan with molecular weight above 50 kDa.

Chapter 5

5 CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

5.1 Conclusions

- Prepared chitosan hydrogel respond very well to the change in pH
- Voltage value is higher in alkaline pH while it decreases as the pH turns acidic
- Data discrepancies (unit change of ± 0.6) observed due to the calibration difference in pH meter as highest and lowest values are based on it.
- Cross-linked lyophilized gel has improved mechanical properties and structural integrity which allows its use at an extended period compared to the previous one.
- Wired sensor configuration
- pH sensing requires moist interface – Gel upon complete drying opens up the circuit.
- The abovementioned limitation prevents pH measurement of dry skin.
- Coagulation assay show 0.016 OD at 5 min indicating effective Hemostasis by this time

5.1 Future Recommendations

- ❖ Integration with RFICs: Incorporation of RFIC will permit wireless monitoring
- ❖ Healing Status: On the basis of pH, healing stage of a wound can be determined

- Recovery Time: Along with other healing markers, recovery time can be calculated and included in the sensor design to let the user know - when it's the appropriate time to remove the bandage.

- **50% battlefield casualties** and **31% casualties in civilian's** trauma centers - reported due to Hemorrhage in major arteries (Mueller et al., 2015) –
- can be prevented by developing effective wound care regime

- Integration of pH sensor in a Hemostat bandage will not only facilitate real time wound monitoring but it will also accelerate the healing process via controlled release of impregnated drugs

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