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IDENTIFICATION OF NITRIC-OXIDE DEGRADATION PRODUCTS OF ASCORBIC ACID

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By

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ABSTRACT

Nitric oxide (NO) has been shown to possess many Anti-inflammatory effects. Ascorbic acid which is naturally present in human body is known to react with S-nitrosothiols (RSNO) directly to reduce RSNO to NO and produce free thiol groups, if present in more than millimolar concentration. Higher concentration of ascorbic acid is required because the reduction of RSNO is not caused by ascorbate instead, but instead a decomposed product of ascorbic acid. S-Nitroso-N-Acetylpenicillamine is used as an NO donor. An HPLC method is used for determination of the degradation product of ascorbic acid and simultaneously identifying the compound responsible for NO production from RSNO by running the compound through Nitric Oxide Analyzer. Once the compound is identified it has a potential medical use for NO production. For example, it could provide a means that initiates RSNO decomposition in polymeric biomaterials. Thus, increasing the functionality of the medical device coated with such materials and preventing thrombus formation

1. INTRODUCTION

With the increasing size of the population and the increasing multitude of illnesses such as diabetic, cardiovascular and neurological disorders which cannot be mitigated alone with medications, there is an increasing demand for implantable devices

[1]. Over the last 60 year's implantable devices have undergone a significant transformation becoming a valuable tool for monitoring and measuring physiological conditions. Success of these devices has heavily relied on the development of electronic technologies capable of interfacing with living tissues and organs at the micro and nanoscale level [2]. With increased in vivo stability, miniaturization along with some other parameters has led to the development of implantable electronic devices, such as sensors, cardiac pacemakers, implantable cardioverter defibrillators, cochlear implant, and bone stimulator, to name a few. These devices encompass a wide range of duration of intended use, level of invasiveness and device complexity. For example, needle-type glucose sensors are placed in subcutaneous tissue and intended to monitor glucose levels in interstitial fluid that is related to blood glucose levels. Another example are prosthetic knee implants which are intended to be imbedded in body within the patient and restore physiological function of the knee joint for 10 years.

Halperin et al. report that over 25 million US citizens are reliant on implantable medical devices for life-critical functions [3]. Despite of substantial innovations in the fabrication and application of implantable biomedical systems since the first implantable heart pacemaker of 1958, the modern implants patient is still faced with several challenges. Long-term reliability of the device is extremely important considering the high cost and time associated with surgical implantation along with patient recovery. The location of the implant space is a chemically harsh environment, where the surface of the implant will be continuously attacked by the highly conductive and corrosive physiological medium which also carries a variety of biochemically reactive organic molecules and metabolically active cells. The drive towards small, light, and flexible devices may affect the mechanical robustness of implants. The ensuing in vivo degradation and loss of integrity may be detrimental to the performance of the device, potentially leading to the device failure. Studies to improve the quality and design of both intravascular and subcutaneous sensors has been carried out since early1960s [4]. Only a handful of sensors have succeeded in the in vivo environment, with most failures due to the unpredictable performance resulting from the robust and dynamic biological response mounted by the body in response to the foreign device within the physiological system.

Achieving biocompatibility is a complex matter due to the dynamic host response to the synthetic and organic material used for device production. The host response must be balanced with the intended life time of a device and what is required of the device for appropriate functionality within the body. Focusing on the specific case of implanted sensors, one of the solutions under investigation is using a biodegradable material for fabrication of device which will allow controlled dissociation over time. Degradation

products will cause minimal toxic response and are removed from the implantation site by normal metabolic activity [5].

One such example of biodegradable materials is using Magnesium based alloys. Stent used in cardiovascular application can be made from magnesium alloy. It has many advantages i.e. high biocompatibility (Mg is present in the body and will not be recognized as foreign material) , Alloy's element are dissolved in the human body (no toxic waste), not visible be X-ray or CT scan (no artifacts) . However, they do have some limitations like low corrosion resistant and suboptimal biocompatibility. Coating technology is one of the leading approach used to overcome these problems. Coatings on Mg-based stents can vary from metal and inorganic coatings to biodegradable coatings. Among these, biodegradable polymer coatings with drug-eluting features might be a better choice because of their advanced biocompatibility and capability to reduce late restenosis compared with other coatings. Developing implantable electronic device with biodegradable, non-toxic electronic materials on a such a small scale is difficult. A combination of robust and reliable electronics with a bio resorbable polymer platform offers both the flexibility of the device and sufficient bulk degradation that the immune response to the remaining material is minimal. For the technology to be clinically implanted certain challenges such as controlling degradation kinetics and biocompatibility of the device should be addressed. A review of the major issues that are involved in biological response to implanted devices follows, beginning with the risk of infection associated with implanted devices, the tissue response toward implanted devices, and coagulation inspired by implanted devices. By examining these separate processes, potential solutions to overcoming the host response to implanted devices can be developed.

1.1. IMPLANT ASSOCIATED INFECTIONS

Implantable devices have undergone a significant development due to the advancement in packaging designed to protect the indwelling device from hash physiological environment. Nonetheless, the risk of infections resulting from bacteria adhering to the implant is significant in terms of both morbidity and mortality of the patient and high costs associated with treating these device-associated infections. The highly invasive nature of the surgical procedure along with the health condition and inadequate immune response from the patient contribute to the causes of implant associated infection. Depending upon the severity of the infection treatment may involve localized antibiotic therapy to complete removal of infected implant. The incidence of infections associated with cardiac pacemaker implant ranges from 1%–19%, with 7%– 8% attributed to contamination during laboratory handling or the event of implantation [7]. Typically, the pre- and post-operative pathways are the most common pathways of biomaterial associated infection. If a patient is suffering from diabetic disease or using anti-inflammatory medication like corticosteroids and other immunosuppressive drugs may slow down surgical site healing and patient recovery, making the host more susceptible to developing an infection [8]. In addition, pathogens,

can originate elsewhere in the body and spreading to the implant site via blood, this route of infection is particularly important for devices that are in contact with the blood.

The physiochemical properties of the implanted surface render them susceptible for colonization by bacteria. The non-living nature of the implant surface means that it does not respond to the presence of bacteria nor does it produce chemical signals to notify the surrounding tissues of the imminent danger. Certain combination of surface properties can be employed to mitigate the host response to a certain level, but these modifications are helpless against bacterial cells that manage to adhere to implant surfaces. Furthermore, bacterial cells that adhere to implant surfaces release a significant amount of extracellular matrix to form a biofilm around the implant. In this biofilm state, they are protected from the host response and are not susceptible to systemic antibiotics. Hence, there has been more emphasis on developing surfaces capable of both preventing the bacterial adhesion and replication, and eliminating attached bacteria by releasing antibacterial drugs. The drug eluting property can be imparted onto the implanted module via encapsulation or surface modification. In addition to traditional antibiotics, a range of alternative antimicrobial agents have been considered, including silver ions, nitric oxide, bioactive antibodies, and other bactericidal compounds [9,10].

1.2. HOST RESPONSE TO FOREIGN MATERIALS INFLAMMATION

Inflammation and Coagulation are two major factors that significantly impact the function of the implanted device. When the properties of the surface materials are not properly matched with the characteristics of the surrounding tissues and cells the longterm utilization of the material may not be successful [11]. Surfaces of the implants may be contaminated by bacteria or bacterial cells which induces an inflammatory response. The physical presence of the implant, e.g., pressure it exudes onto the surrounding tissues and organs, can also trigger inflammatory mechanisms where affected cells release a host of chemical signalling molecules. Surgical intervention for introducing the implant triggers a pro-inflammatory response which delays the tissue healing. After the implant is exposed to chemically harsh environment, to the implant may begin to degrade as a result of this cell-mediated inflammatory response. Furthermore, the loosening of the implant has been linked to the increased incidence of inflammations, complications, and less successful functional performance of the implant.

Device implantation and associated tissue injury triggers a cascade of inflammatory and wound healing responses. The inflammatory response is comprised of two stages: acute and chronic phases. The initial acute phase lasts from hours to days. The acute phase is mostly responsible for cleaning the wounded site and provisional matrix formation. Vessels dilate and excess blood flows into the injury site. Numerous blood and tissue proteins such as cytokines and growth factors are released, and leukocytes adhere to the endothelium of the blood vessels and infiltrate the injury site.

Monocytes are then called into the site and these differentiate into macrophages [12]. Persistent inflammatory stimuli without resolution, such as the continual presence of the biomaterial/medical device, lead to chronic inflammation. Chronic inflammation is histologically less uniform when compared to acute inflammation, and the wound healing response is generally dependent on the size and/or degree of injury. This phase is generally characterized by the presence of monocytes, macrophages, and lymphocytes, as well as the proliferation of blood vessels and connective tissue to restructure the affected area. The formation of blood vessels is essential to wound healing, supplying necessary nutrients. Eventually, the granulation tissue is replaced by an extracellular matrix (ECM). The ECM acts not only as a physical scaffold but also as a crucial modulator of the biological processes, including differentiation, development regeneration, repair, as well tumor progression [13]. The end stage of the foreign body response involves walling off the implant by a vascular and collagenous fibrous capsule that is typically 50–200 μm in thickness. This fibrous wall confines the implant and consequently prevents it from interacting with the surrounding tissue.

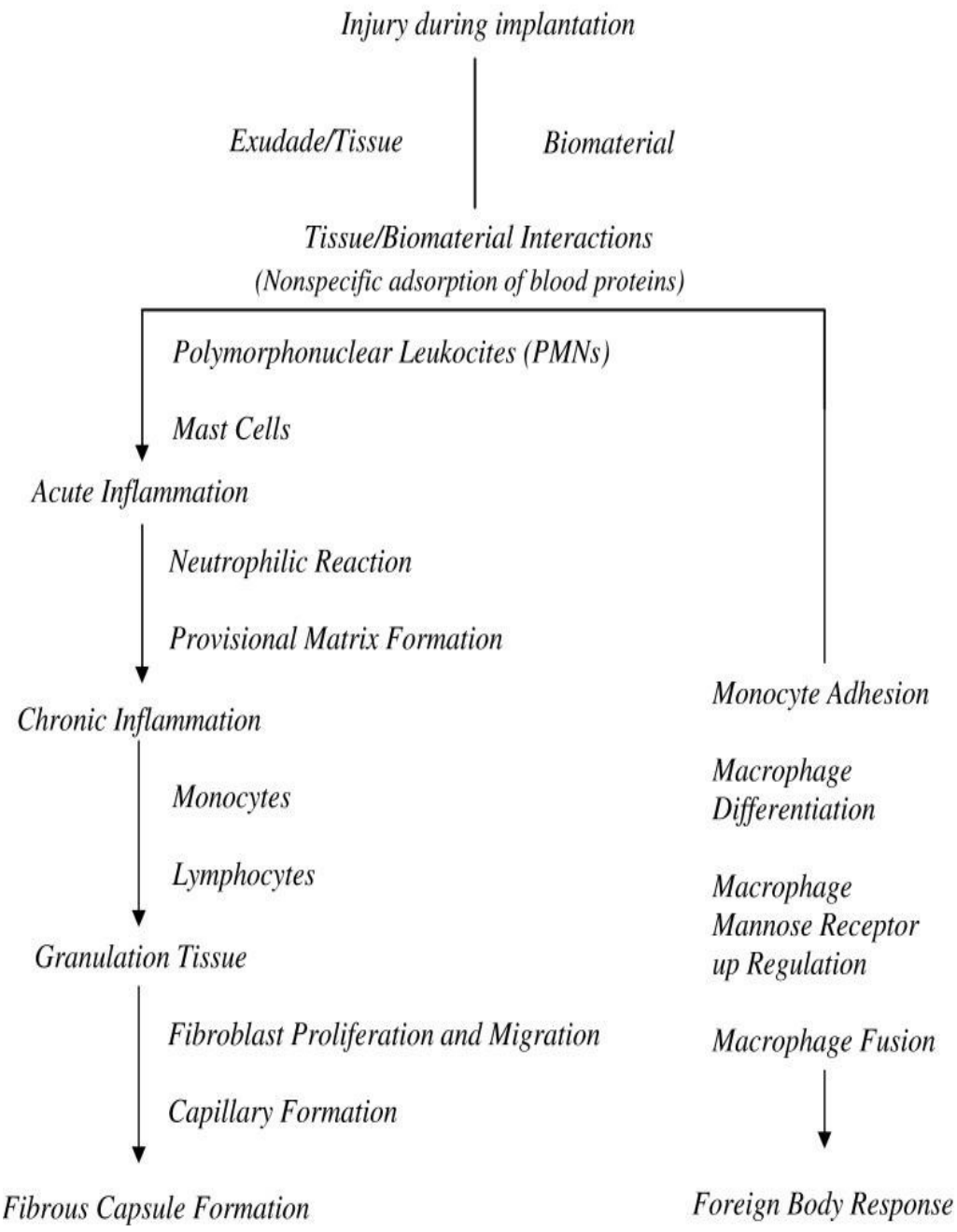

Figure 1.1 Sequence of events involved in the foreign body response to an implantable device.

1.2.1 COAGULATION RESPONSE TO FOREIGN MATERIALS

Intravascular implants offer more advantage for patience care and improving the life style of the individual. However, they do face some major issues like size limitations and biocompatibility. Sensors not only have to be smaller in size but also must function fully in the presence of blood and the coagulation cascade.

Implantable devices as they come in contact with the blood, this will activate the coagulation cascade [14]. The end result of this response is a device coated with the provisional matrix that is a blood clot which contains metabolically active cells. This encapsulation can deeply affect the functionality of the sensor and resulting embolism risks for the patient of the blood clot breaks apart and travels to other locations within the vasculature [15].

Most common example of implant being encapsulated with calcium deposits are heart valves. Calcific deposits are seen on the heart valves within 3 years of postimplantation. It occurs due to chemical interaction within the aldehyde groups phospholipids and circulating calcium ions, immunological response. Calcification often starts at the commissure and the basal area of the cusp. Calcification can lead to stenosis due to cusp stiffening and regurgitation or incompetence, due to associated cusp tears. Occasionally, calcific deposits on the surface of the cusps may embolize to distant sites.

The blood coagulation cascade is can be initiated by either the intrinsic pathway or the extrinsic pathway. Details of the intrinsic cascade are shown in Figure 1 Briefly, Initially, proteins adsorb on the surface of the sensor immediately upon implantation. The composition of the blood bound proteins varies and change over the course. Composition of proteins adsorbed on to the surface depends on the Vroman effect and the nature of the implant (i.e. type of surface, curves, smooth etc.). Next platelet bound to these surface proteins via expression of adhesion receptors (e.g. glycoprotein IIb/IIIa which binds to the plasma ligand fibrinogen), contractile protein within the platelets tighten and the platelet flattens to form “false-legs” or pseudopodia [16]. This enables them to cover more surface, at the same time contraction causes degranulation of platelets. Degranulation releases a number of chemicals including ADP and thromboxane A_2. These chemicals are potent platelet activators, thus attracting nearby platelets to the surface increasing the platelet adhesion and aggregation. In the end, the implant surface will be coated with stabilized activated platelet that binds with factor V to activate local thrombin. Thrombin once formed interacts with fibrinogen to form a mesh of fibrin around the implant. Fibrin interacts with platelets to Breaking of the thrombi and freely entering the circulating blood causes poses a serious threat. Only presence of a monolayer of thrombus will not affect the sensor or make it less responsive to the analyte.

However, thrombus formed with the metabolically active cells creates a different local environment which is different compared to the bulk blood. Thus, misleading the sensor

from analyzing the actual condition of the analyte (i.e. bulk blood). Additionally, there will reduced blood flow to the implanted area i.e. vasoconstriction which will change the local form a consolidated structure around the implant which can entrap circulating cells. Thus, aggregated platelets, cells and fibrin thread forms a thrombus known as blood clot. Shown in Figure 1.3

Breaking of the thrombus and fragments freely entering the circulating blood poses a serious threat. Thrombus once circulating freely into the blood stream can block any artery or vein leading to a blood clot, hence it is as serious threat. Only presence of a monolayer of thrombus will not affect the sensor or make it less responsive to the analyte. However, thrombus formed with the metabolically active cells creates a different local environment which is different compared to the bulk blood. Thus, misleading the sensor to from analyzing the actual condition of the analyte (i.e. bulk blood). Additionally, there will reduced blood flow to the implanted area i.e. vasoconstriction which will change the local metabolic activity, thus affecting the sensor data even further by misinterpreting the condition. It is clear that understating this coagulation cascade and stopping it in its pathway is extremely important for full functionality of the sensor

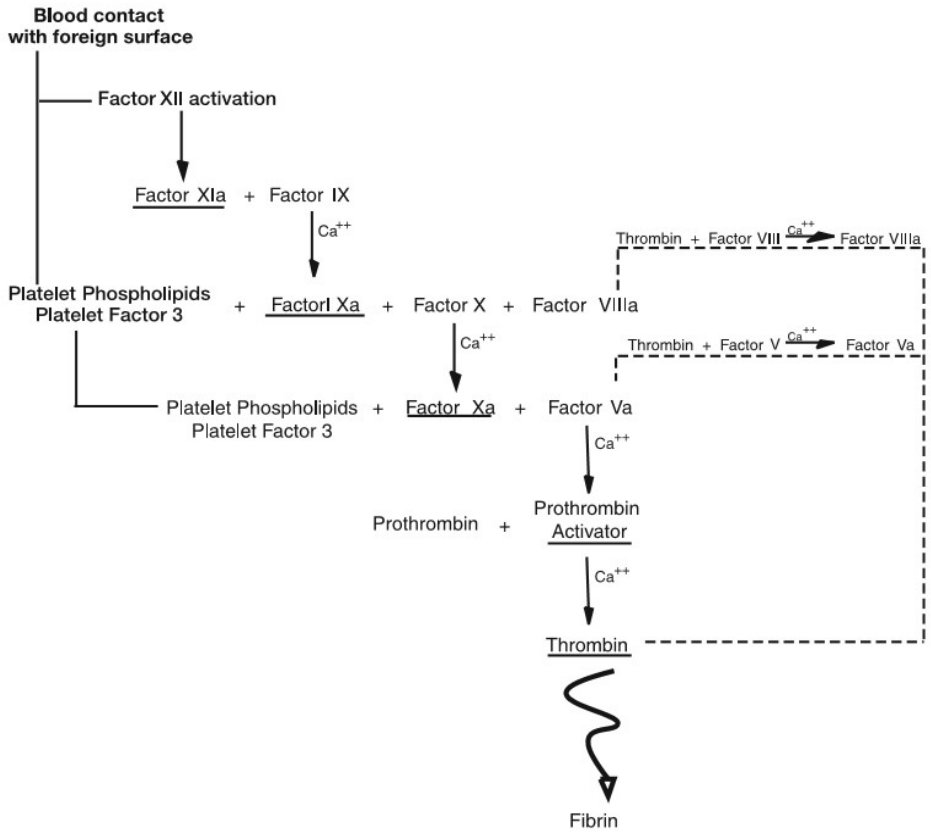

Figure 1.2 Intrinsic Coagulation cascade initiated by blood contact with foreign materials implanted within the vasculature. Dotted lines show potential pathways of positive

feedback mechanism. Typical end product of cascade is the formation of a thrombus on the exposed surface of the implant. Adapted from Dee [17]

Form a consolidated structure around the implant which can entrap circulating cells.
Thus, aggregated platelets, cells and fibrin thread forms a thrombus known as blood clot. Shown in Figure 1.3

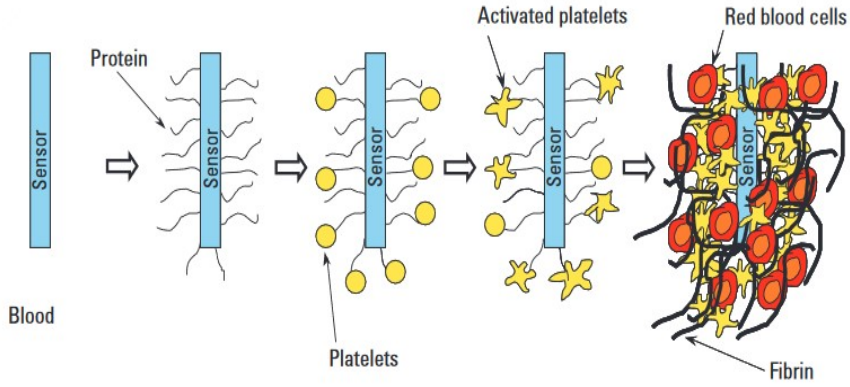

Figure 1.3 Depiction of exposed surface of an implanted sensor in contact with the blood. Protein adhesion facilitates platelet adhesion and activation which initiates intrinsic coagulation cascade leading to the thrombus formation. Adapted from Frost [5]

CONTROL POINTS IN COAGULATION CASCADE

- Coagulation cascade is a complex system with both feedback and feedforward loop. Controlling this complex process can eventually yield us control over implantable sensor functionality and improving the lifetime of the sensor. Steps within the cascade which are vital to the process are listed below (Adapted from Tissue-Biomaterials Interactions. 2002: John Wiley &Sons [17])
- Calcium: - Calcium is required at each step of the coagulation cascade, except for the factor XII and XI activation. Without calcium blood with not clot.
- Thrombin: - Thrombin is major component of the feedback loop, controlling the availability of thrombin and its reactivity a major portion of the cascade can be controlled
- Clotting factors: - Clotting factors are the key to the coagulation cascade. Controlling few clotting factors like Factor X can yield a potential control point because it is a common link between the pathways.

- Platelets contribution: - Platelets can be viewed as a surface catalyst for coagulation process. Platelets are the first thing to adhere to the foreign body, initiating platelet aggregation.

1.2.2 EXTRINIC COAGULATION CASCADE

Activated factor Xa is the site at which the intrinsic and extrinsic coagulation cascades converge. The extrinsic pathway is initiated at the site of injury in response to the release of tissue factor (factor III) and thus, is also known as the tissue factor pathway. Tissue factor is a cofactor in the factor VIIa-catalyzed activation of factor X. Factor VIIa, a gla residue containing serine protease, cleaves factor X to factor Xa in a manner identical to that of factor IXa of the intrinsic pathway. The activation of factor VII occurs through the action of thrombin or factor Xa. The ability of factor Xa to activate factor VII creates a link between the intrinsic and extrinsic pathways. An additional link between the two pathways exists through the ability of tissue factor and factor VIIa to activate factor IX. The formation of complex between factor VIIa and tissue factor is believed to be a principal step in the overall clotting cascade. Evidence for this stems from the fact that persons with hereditary deficiencies in the components of the contact phase of the intrinsic pathway do not exhibit clotting problems. A major mechanism for the inhibition of the extrinsic pathway occurs at the tissue factor-factor VIIa-Ca2+-Xa complex. The protein, lipoprotein-associated coagulation inhibitor, LACI specifically binds to this complex. LACI is also referred to as extrinsic pathway inhibitor, EPI or tissue factor pathway inhibitor, TFPI and was formerly named anticonvertin. LACI is composed of 3 tandem protease inhibitor domains. Domain 1 binds to factor Xa and domain 2 binds to factor VIIa only in the presence of factor Xa.

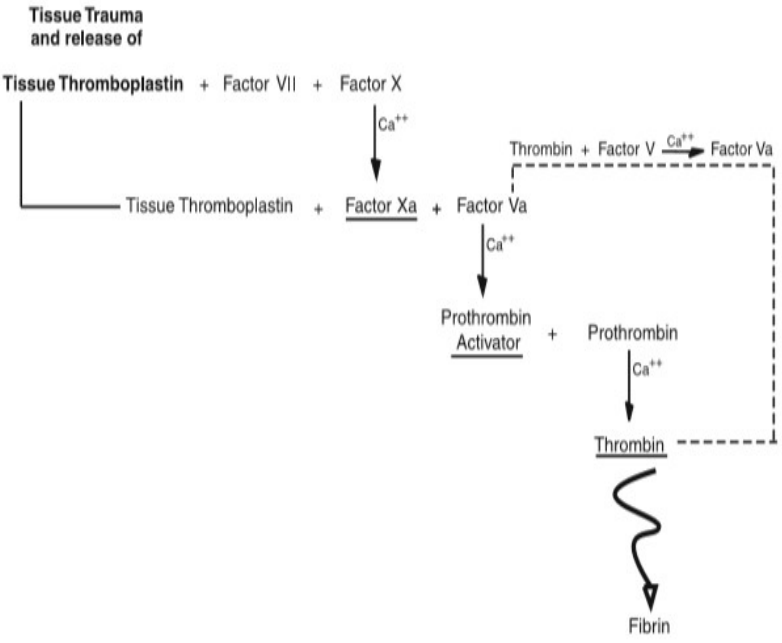

Figure 1.4 the extrinsic coagulation pathway. Reaction products are underlined; the dashed lines denote potential feedback loops. After the production of thrombin has been achieved, the reactions outlined in

1.3 COPING UP WITH HOST COAGULATION RESPONSE

The most common method to prevent coagulation in patients with implanted devices is the use of anticoagulants. The most widely used anticoagulant is heparin. Heparin inactivates thrombi in the clotting process, this stops the formation of fibrin hence prevent blood clot. Heparin is also used to treat blood clots formed abnormally inside the blood vessel. Heparin is administrated both orally and intravascularly. Heparin interacts with antithrombin III and enhances its ability to inactive coagulation enzyme thrombin, which in turn prevents blood clot formations. It also affects other clotting factors like Factor Xa and IXa [18]. However, heparin may have several negative effects. Heparin can cause you to have bleeding episodes while you are using it and even several weeks after you stop using it. It also interacts with a number of plasma proteins which are not even involved in the coagulation cascade.

Studies have shown that plasma protein available for interaction with heparin may vary from person to person [19], thus heparin is only partially effective at inhibiting the thrombus formation on the implanted device. Moreover, prolonged used of heparin can cause bleeding issues, for few weeks after the use. Extreme effects of heparin administration are bone loss by decreasing bone formation i.e. osteoporosis and reducing the platelet count [20] i.e. thrombocytopenia [21]. Many patients who have implanted

sensors in their body already have hemostatically compromised condition, using heparin may worsen their condition. Hence. the use of heparin to improve the biocompatibility is still under question.

It is clear that we cannot rely on the efficiency of the anticoagulants, there is a need for development of new techniques for passivating the host response. Techniques like development of polymer coating or outer membrane that could potentially passivate the implanted device to some extent of the host response. Number of approaches like naturally derived materials, diamond like carbon, hydrogels, topology treatments etc. were used [22]. Many of these materials showed good progress during in vitro study of cell or protein adhesion, but in vivo study some resistance to host response. Ultimately, body's defense will overcome the static surface and initiate a host response which will affect the sensor performance.

1.4 SOLUTION TO OVERCOME FOREIGN BODY RESPONSE

Properties of biomaterial surface influences how it will react with proteins once it comes in contact with the blood. Blood comprises of about 150 different type of proteins. Surface properties is usually grouped into three categories: geometric, chemical, and electrical. Thumb rule being greater the surface area more possible sites for protein interaction. For example, surface with grooves and pores will have greater surface are compared to smooth surfaces. So, grooves and pores should be avoided unless they are a part of the design. Also, surface features like the machine marks also provide additional site for protein interaction. So, the key is keeping the implant surface as simple as possible.

Chemical composition of the material will determine which functional species are available for interaction with biomolecules. The oxidized (passivated) surface of a metallic biomaterial exposes metal and oxygen ions. Similarly, ceramic, and some glass, surfaces comprise metal and nonmetal ions. A variety of functional species, such as amino, carbonyl, carboxyl, and aromatic groups, can be present on the surface of polymeric biomaterials. Depending on which species are exposed, biomolecules (or even particular regions of the molecule) may have different affinities for various surfaces. For example, hydrophobic surfaces tend to bind more protein as well as binding it more tenaciously.

Biomaterial surfaces are often in homogeneous. Patches, or domains, of different functionality can exist on biomaterial surfaces, and these patches can interact differently with biomolecules. For example, many metallic biomaterials contain at least two different phases, such as the a- and b-phases in Ti-6Al-4V. Not only can the different phases behave differently when interacting with biomolecules, but grain boundaries behave differently than do grain interiors. In polymers, segregation resulting from folding of macromolecular chains can give microstructural domains. Depending on the chemical species present within the various domains, proteins will have different affinities for the patches.

Controlling this initial protein interaction on the surface of biomaterial can help dictate the entire foreign body response.

To overcome the limited in vivo functionality and longevity of implantable devices, some important approaches have been reported

1.4.1 BIOCOMPATIBLE MATERIAL COATING

The use of biocompatible materials for coating implantable devices is based on their ability to mask the underlying surface. Masking is achieved by producing a hydrophilic interface between the device surface and the tissue fluids, thereby minimizing tissue reactions induced by device implantation [23]. The formation of these biocompatible layers improves implantable device/host tissue interactions and consequently improves device functionality and life span

Various natural, synthetic, and semisynthetic materials are currently utilized in the fabrication of implantable device coatings. Naturally occurring materials include (a) alginate, (b) chitosan, (c) collagen, (d) dextran, and (e) hyaluronan. These methods offer the advantage of being very similar to macromolecular substances that the biological environment is prepared to recognize and to deal with metabolically. On the other hand, serious disadvantages are (a) natural polymers are frequently immunogenic, (b) these polymers typically decompose or undergo pyrolytic modification at temperatures below their melting point, thereby precluding the convenience of high-temperature thermoplastic processing methods (such as melt extrusion) during the manufacturing of the implant, and (c) since they are derived from animal or plant sources, natural variability in macromolecular structure are expected. Numerous synthetic polymeric materials have been employed as coating materials, e.g., poly(lactic-acid) and poly (lactic co-glycolic acid) (PLGA) , poly(ethylene-glycol), 2-hydroxyethyl methacrylate , poly(ethylene glycol) (PEG) , and poly(vinyl-alcohol) (PVA). Knowledge of the physical and chemical properties of the polymer is a useful tool to rationalize the choice of the coating material.

Quinin and et al developed [24] Photo-crosslinked copolymers of 2-hydroxyethyl methacrylate, poly (ethylene glycol) tetra-acrylate and ethylene dimethacrylate for improving biocompatibility of biosensors. They coated the copolymer created on the electrode of a glucose sensor and compared with a control electrode which did not have any copolymer coating. The explanted samples were evaluated using scanning electron microscopy. The control electrodes were highly encapsulated with fibrous material, while the copolymer-treated electrodes induced much less encapsulation. The results show this copolymer to be a candidate as a biocompatible coating for electrically wired oxidoreductase-based subcutaneous biosensors.

1.4.2 ANGIOGENIC DRUGS

Biosensor functionality and longevity can be compromised by a biofouling response and the formation of an avascular fibrous capsule around the device that greatly decreases both the transport of analyte from the tissue to the sensor and the diffusion of reaction products from the sensor to surrounding. Therefore, controlling fibrotic encapsulation at the implant site would appear to be critical to achieve a functional and extended life-time biosensor in vivo. One approach to improve the analyte transport around

the implant is the promotion of angiogenesis. This can be achieved by inducing new blood vessel formation in the vicinity of the sensor using growth factors such as the vascular endothelial growth factor (VEGF) [25]. It is noteworthy that well-vascularized tissue at the implant site is also critical for healing the trauma caused during implantation. Another issue associated with the use of corticosteroid drugs to treat the inflammation process is that these drugs also downregulate endogenous VEGF, thereby inhibiting angiogenesis. Accordingly, a two-pronged approach (control of inflammation and induction of angiogenesis) may be necessary.

Patil SD and et al developed [26] Dexamethasone-loaded poly(lactic-co-glycolic) acid microspheres/poly (vinyl alcohol) hydrogel composite coatings for inflammation control. Dexamethasone-loaded PLGA microspheres were prepared using a solvent evaporation technique. Composites were fabricated by dispersing microspheres in PVA solution and performing freeze-thaw cycling. Composites were implanted into subcutaneous tissue of rats. PLGA microsphere/PVA hydrogel composites eluting dexamethasone were successful in controlling negative tissue reactions at the sensortissue interface by reducing the level of inflammation-mediation cells to those observed in normal tissue.

1.4.3 IMPLANTED POLYMERS

Recent studies showed that active approach is much more effective than coated materials developed in the past. Worked done earlier on surface bound heparin [27] has been extended to materials which release heparin in vivo. Gutowaska et al. [28]. Developed thermosensitive polymer blends using Biomer/poly (Nisopropylacrylamide)/[poly(NiPAAm)]. This technique was based on temperature dependent swelling of the material. At room temperature, i.e., below the lower critical solution temperature (LCST) of poly (NiPAAm), the Biomer/(poly(NiPAAm) coatings are highly swollen. Swelling enables fast loading of hydrophilic macromolecules (e.g., heparin) into the coating by a solution sorption technique. At a body temperature, i.e., above the LCST of poly (NiPAAm) the coatings are in a de-swollen state and the absorbed macromolecules may be slowly released from a dense coating via a diffusion controlled mechanism. The results show that the heparin release from

Biomer/poly(NiPAAm)-coated surfaces resulted in a significant reduction of thrombus formation on test surfaces in contact with venous blood as compared to control surfaces in vitro studies. However, considering the complexity of the in vivo environment, there is need for further study to access the efficacy of heparin releasing materials in improving the biocompatibility of the implanted sensors.

Nitric Oxide (NO) is another potentially viable option to help control the coagulation cascade.NO has a diverse array of functions in vivo including help controlling the blood vessel tone and adhesion molecule expression. Nitric Oxide is also a potent inhibitor of platelet aggregation and activation [22,23], due to its role in stimulation calcium sequestration and inhibiting the synthesis of thromboxane A_2 in platelet. NO is also involved in endothelium cell and smooth muscle cell proliferation; it has a direct role in

destruction of bacteria during phagocytosis by macrophages [24]. Vascular endothelium cells generate NO flux of $1.0\text{-}4.0*10^{-10}$ mol/cm^2 min continuously [25]) has been their primary basis for the non-thrombogenic properties of the vascular endothelium, surface which elicits no host response despite constant contact with precursor molecules to the intrinsic coagulation cascade. Endogenous NO supply diffuses into the lumen of the blood vessels and smooth muscles cells that surrounds all the arteries where it plays an important role in preventing platelet activation in healthy blood vessels.

The innate physiological function of NO makes it a strong candidate for improving the biocompatibility of the implanted sensors. NO releasing sensors will be single handed capable of controlling the both platelet adhesion and activation along with minimizing vasocontraction. NO releasing subcutaneous implanted sensors will ensure increased blood flow to the sensor surface, increasing transfer of analyte and reducing bacterial infection. Half-life of NO in blood is 0.5-1.8ms [23], thus the NO generated will no create any systemic effects.

1.5 NO RELEASING MATERIALS

The encouraging work in early NO studies led to serious efforts for creating NO releasing materials which has been ongoing for 20 years. Organic nitrites, metal complexes, nitrosamines, nitrosothiols and diazeniumdiolates are some of the potential candidates arising from the work. The diverse structures used for NO donors causes varied chemical reactions and NO-release kinetics [26], which can be used for controlling the dose of NO delivered.

There are two mechanisms from which NO can be generated from their donors, 1) exogenous NO release and 2) those which generate NO by decomposing endogenous NO donors.

1.6 EXOGENOUS NO DONORS

Recently a class of NO donor's diazeniumdiolates has getting much attention known as N-diazeniumdiolates, or "NONOates" as an exogenous NO donor. They produce predictable amount of NO and ease to prepare. NONOates generate NO spontaneously under physiological condition and stable at ambient temperature and can be covalently attached to polymer films [28,29]. Release of NO from NONOates is influenced by biological factors so no tolerance can be developed against it. The stability of N-diazeniumdiolates depends on the concentration of the donor in material as well as pH temperature and/or structure of substrate. Study by Frost et al. [30] studied the performance of a Clarke-style amperometric oxygen-sensing catheter coated with a ~ 100μm layer of polymer covalently bonded to diazeniumdiolates. Results suggested that NO release through this technique greatly reduces thrombus formation.

RSNOs also known as S-nitrosothilos emerged as a potential candidate for exogenous NO release. There are three known mechanisms for NO generation from RSNOs: -

- Metal ion i.e. copper mediated decomposition

- Ascorbate mediated decomposition
- Photo-initiated decomposition [31]

Light can be used as an ON/OFF switch to generate NO if decomposition via metal ions. Ascorbate mechanism is independent of light. Frost et al. developed a tri-layer silicon rubber (SR) film wherein one of the layer is blended with S-Nitroso-N-acetyl-DLpenicillamine derivatized fumed silica was sandwiched between two layers of pure SR.

Film showed photolytic NO generation without being sensitive to copper and ascorbate. NO generated was dependent on the intensity and duration of light it was exposed to. This points out that RSNOs with the help of a controllable light source could be used to anchor the NO generation on the surface of the implantable sensor.

1.7 ENDOGENEOUS NO DONORS

NO is generated naturally in mammalian body, NO precursors in the form of nitrites and RSNOs are present in the body in micro-molar concentration. Cu^{2+} metal ions along with other metals are known to cause reduce RSNOs to generate NO in aqueous solution [32]. Wu et al. developed polymeric coating with Cu^{0} particles which will slowly corrode to produce reactive ions to decompose RSNOs to produce NO [34]. These coatings were applied to catheters and tested in vivo showed positive results. However, few incidences showed that control and NO generating fiber showed thrombus formation, because of the low concentration of RSNOs in those animals.

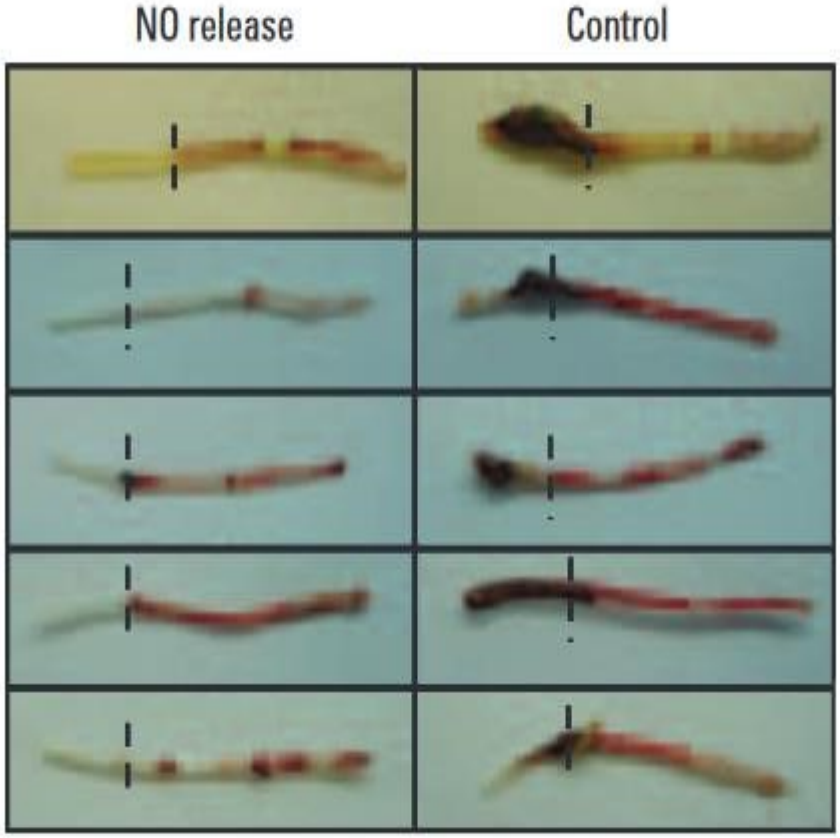

Figure 1.5 Clarke type amperometry intravascular sensor post-explanation. The region to the left with dotted lines shows the portion of the sensor which was placed within the vasculature. The sensor on the left incorporated NO releasing material sensor on the right was without modification

1.8 BACKGROUND OF ASCORBIC ACID

Ascorbic acid is an isolated nutrient which is part of vitamin C. Ascorbic acid can be normally found in human body. Recently studies on ascorbic acid has been increasing because of its ability to react with RSNOs and produce NO. Holmes et al. reported that ascorbic acid can interact directly with RSNOs if present in more than milli-molar concentration to produce NO. Preliminary evidence suggested that it is a decomposition product of ascorbate responsible foR the NO from nitrosotiols rather than high concentration of ascorbate. Higher concentration is required because the species responsible for the decomposition is not actually ascorbate. A partial decomposition pathway can be seen in Figure 4.

Frost and et.al. used S-Nitroso-N-acetyl-DL-penicillamine (SNAP) as NO donor and treated with both fresh ascorbate and decomposed ascorbate. Nitric oxide measured using via chemiluminescence using nitric oxide analyzer. Ascorbate was allowed to decompose naturally under ambient condition for 8 days. It showed that oxidized ascorbate releases 10 times more NO than the fresh ascorbate. Seen in Figure 5. This shows clear evidence that it is actually a decomposition product of ascorbate than the ascorbate itself.

Depending on the decomposition pathway (Figure 4) they suspected the compound to be a four-carbon sugar Threose. They treated threose with SNAP to measure the amount of NO release. Results showed that only 50% of the threose was used, which states that clearly there is another decomposition product present in threose syrup which is responsible for NO release. Clearly a lot of work has to be done in identifying the actual product that causing it to release NO.

1.9 STATEMENT OF PURPOSE

The extent to which host response can be controlled is directly proportional to the functionality of the implanted device. Controlled NO release from the implanted device is the key. Given that it is now know how different fluxes of NO generated will impact the host response at the tissue -material interface in the acute and chronic response, it cannot be known the best way to mitigate the host response using NO. Determining an ideal therapeutic flux of NO and then being able to deliver the needed amount will help fight against the host response tremendously.

Natural source of reducing agent along with naturally available NO donors could be instrumental in developing a new technique for NO release. Ascorbic acid is naturally present in human body and traces of S-nitrosoglutathione is been found in the form of proteins. The goal of this work is to identification of NO generating decomposition product of ascorbic acid. High performance Liquid Chromatography (HPLC) along with chemiluminescence (NO Analyzer) can be used for separating compound from decomposed ascorbate and measuring NO generated from it. Once the compounds are separated Mass Spectroscopy can be used to identify the compound.

To collect real time NO data from the HPLC separated compounds we need a device which is capable of providing a medium for the compounds to react and generate NO. A new 3-D printed device needed to be created for integrating HPLC with NO analyzer (Discussed in detail in chapter 2). Thus a 3-D printed device is able to serve as a medium for HPLC separated compound to react with RSNOs to generate NO.

If the active compound identified is non-toxic and can be stabilized, it could prove as a novel method too initial RSNOs decomposition in polymeric material or endogenously from RSNOs present in the body. E.g. Medical grade polyurethane doped with the reducing agent can be formulated in such a way that when it comes in contact with the blood the active agent leaches from the material decomposing circulating nitrosothiols.

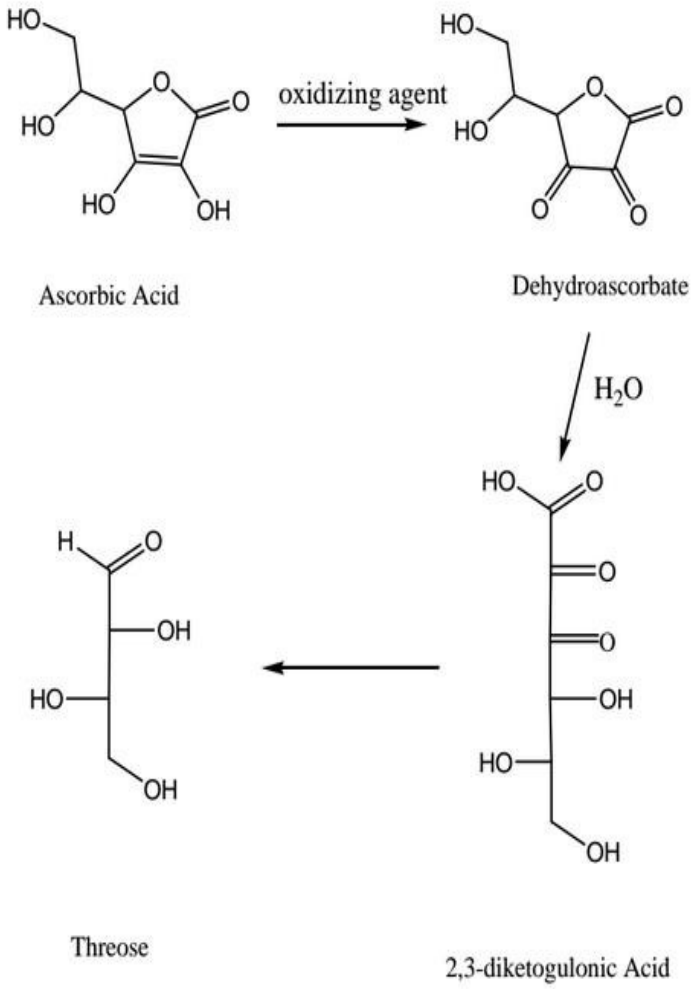

Figure 1.6 Decomposition pathway of ascorbic acid to the reducing sugar threose in neutral pH

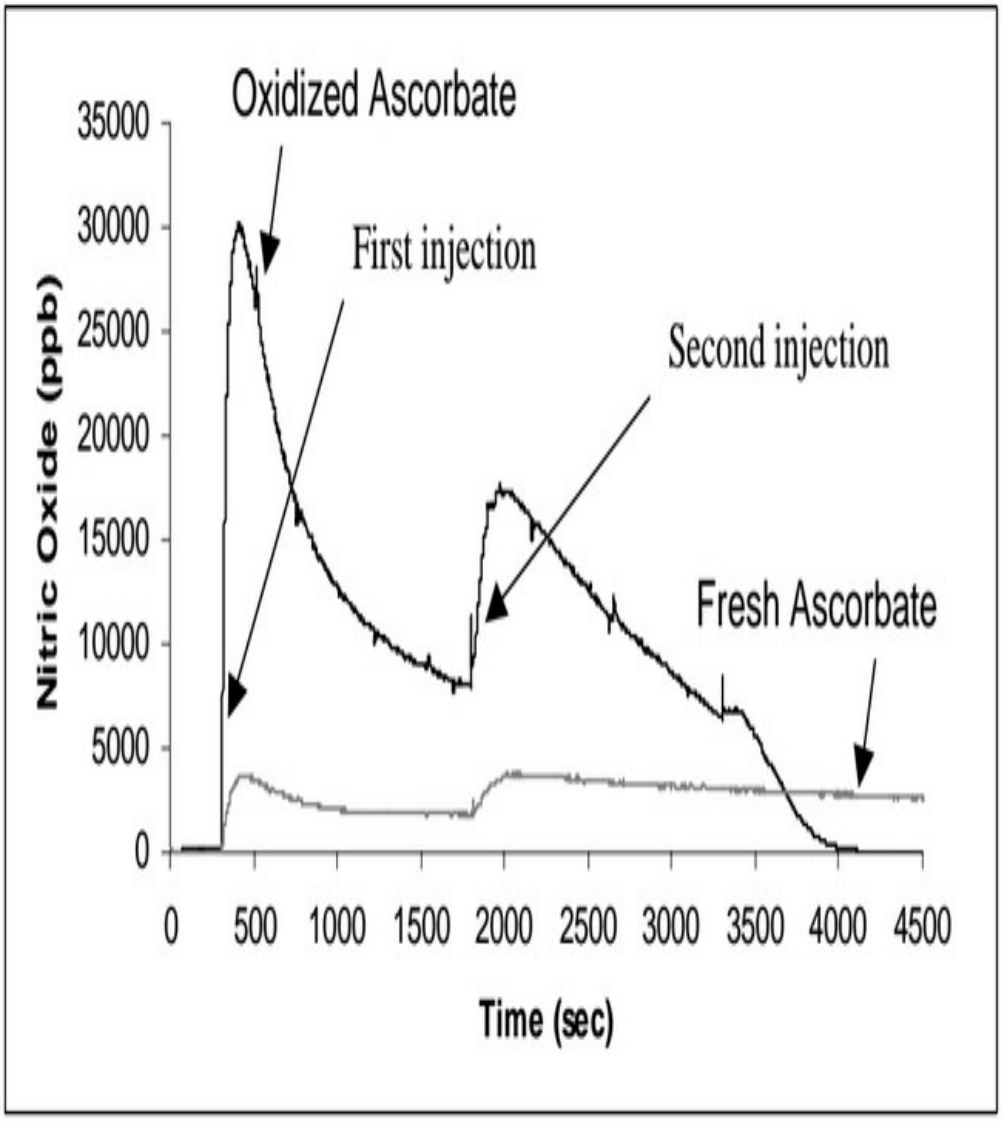

Figure 1.7 Final concentration of oxidized ascorbate and fresh ascorbate were 2.5 mM after the first inject and 5.0 mM after the second injection.

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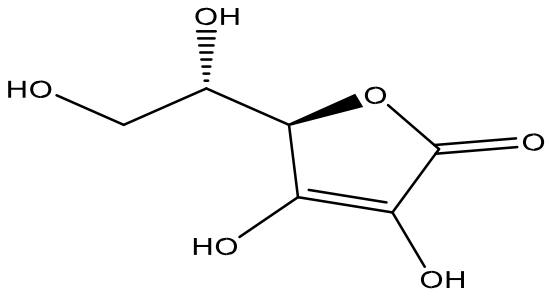
2. ASCORBIC ACID

Ascorbic acid or vitamin C is a small molecule nutrient that is essential for normal functioning in the human body (see Figure 2.1). Among its many functions, it acts as an antiscorbutic agent (curing scurvy) and is a cofactor in a variety of enzymatic process [1]. Ascorbic acid is also known for its antioxidant property in both *in vitro* and *in vivo*. The first metabolic decomposition product of ascorbic acid is its conversion to dehydroascorbic acid (DHA); this process is reversible which is suspected to be responsible for antioxidant properties of ascorbic acid. DHA will irreversibly degraded to 2,3-diketogulonic acid and then to over 50 species containing five or fewer carbons [2]. The complete pathway of degradation is yet unknown. Ascorbic acid is also known to react with RSNOs to produce NO [3]. Much work has been done on studying degradation products of ascorbic acid and their functions in the body, but this complex pathway is far from being understood. Gaston Vernin, et al. studied thermal degradation of ascorbic acid at 300^0 C yielded furan derivatives and $\propto, \beta$-unsaturated cyclic ketones with a fivemembered ring [4]. Since ascorbic acid is a part of our daily food cycle it makes it all the more important to identify its decomposition product triggering NO release, since it is naturally present in our body.



Ascorbic acid

Figure 2.1 The structure of ascorbic acid.

There are three known mechanics of NO generation from S-nitrosothiols (RSNOs) [5]. Copper mediated decomposition in which Cu^{2+} is first reduced to Cu^{+} and then Cu^{+} reduces RSNOs to NO and produces disulfide bonds. Common species used to reduce Cu^{2+} to Cu^{+} are free thiolate ions present and ascorbic acid. Ascorbic acid itself has been reported

to directly interact with RSNOs when it is present at greater than millimolar concentrations to reduce RSNO to NO and produce free thiol groups. The mechanism of this reaction is not known [6]. The third known mechanism of NO generation from RSNOs is by means of photoinitiation.

Dasgupta and Smith published a study entitled "Kinetics and Mechanism of the Decomposition of S-Nitrosoglutathione (Type of RSNOs) by Ascorbic Acid and Copper Ions" [6]. They state that S-nitrosothiols severs as a good source of NO mainly due to the ease of cleavage of S-N bond which consequently produces NO. The reductive decomposition of the specific RSNO, S-nitrosoglutathione (GSNO) by ascorbic acid produced NO which was monitored both electrochemically and spectrophotometrically. The rate of reaction and NO release occurs in both an ascorbate concentration and a pH dependent manner. Where ascorbate is required in greater than millimolar levels and little to no decomposition at low pH (below 5.5). Reductive decomposition of GSNO by ascorbate in an aqueous solution was carried out in the presence of EDTA to suppress any Cu^{2+} present.

Dasgupta and Smith proved that GSNO decomposed with a liner dependence on the concentration of ascorbate from 10-55mM. In their analysis. They could only account for the 90% of ascorbate used initially when combining total ascorbate and DHA present initially, claiming that the lost 10% was probably due to "analytical error". This suggests that ascorbic acid decomposes into different or additional products and it is possible that one of these products is an active agent that is responsible for the release of NO from RSNOs. (i.e., more favorable redox potential and/or kinetics of electron transfer to react directly with RSNOs).

Figure 2.2 shows NO generated from 0.227 mM SNAP solutions in PBS at pH 7.4 upon the addition of fresh ascorbate and the addition of the same amount of oxidized ascorbate from the same solution after 8 d of decomposition under ambient conditions using chemiluminescenc detection [7]. After the first injection of ascorbate or oxidized ascorbate, the final concentration of ascorbate was 2.5 mM and 5 mM after the second

injections. Clearly there is more NO generated from the oxidized ascorbate than from the fresh ascorbate, by nearly a factor of 10

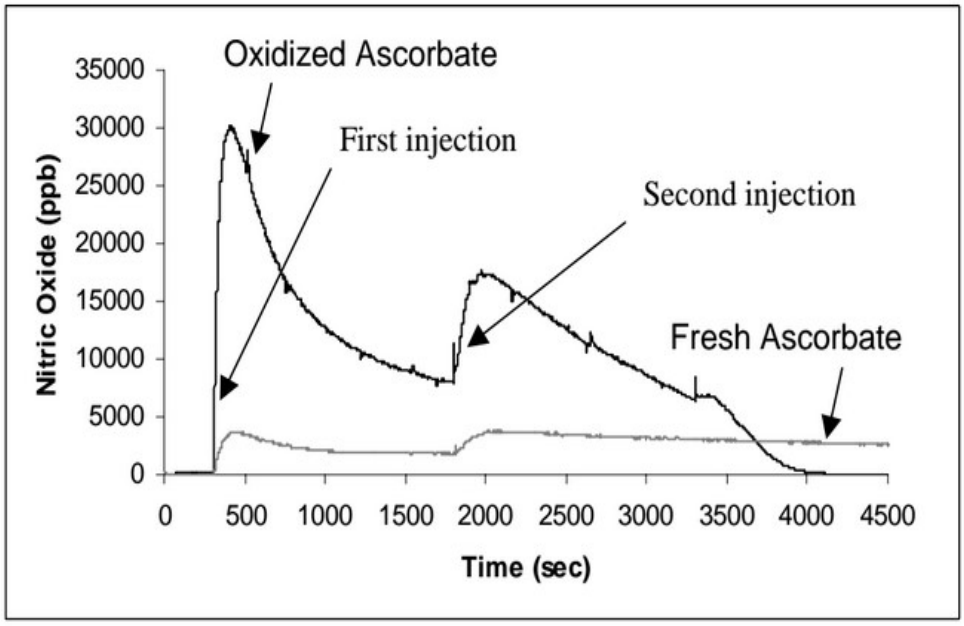


Figure 2.2 Comparison of NO generated from 0.227 mM SNAP solution in PBS at pH 7.4 [7].

None of the work published to date has been able to identify the mechanism of ascorbic acid to directly decompose RSNOs nor have decomposition products of ascorbate been investigated as potential species that release NO from them. Correctly identifying the product or products that may be responsible for RSNO decomposition may provide us with new candidate material(s) for coatings on the implanted device and may also improve their performance *in vivo* and help mitigate host response. This study has developed a new experimental method to help separate the decomposition products which are responsible for NO release from the well-known RSNO, S-nitroso-Nacetylpenicillamine (SNAP).

As previously discussed in chapter 1 the amount of NO generated from decomposed ascorbic acid is more than that produced by fresh ascorbic acid. This clearly indicates that it is not actually ascorbic acid but one its decomposition product which leads to increased production of NO.

2.1 WORKFLOW OF THE EXPERIMENTS

The general idea is to provide clear evidence that one or more of the products in decomposed ascorbic acid is capable of generating NO from a standard RSNO solution. Fresh ascorbate was allowed to decompose and then the products were separated with reversed-phase HPLC. UV-VIS spectroscopy of known standards of both ascorbic acid and dehydroascorbic acid were used for determining peak absorption wavelength of these compounds A diode array detector that recorded full UV-Vis spectra was used with the HPLC system to achieve detection of the components present in the decomposed ascorbate solution. Once peaks of the decomposed ascorbate were obtained along with the retention time, spectral analysis was carried to track how the absorbance changes over time. This helped further confirm the presence of more than one compound in decomposed ascorbic acid. Once the separation was successful, the HPLC eluent was merged with a stream of S-nitroso-N-actylpenicillamine (SNAP) and the headspace was sampled to detect the production of NO. The compounds separated via HPLC that generated NO were then analyzed with mass spectrometry to identify candidate compounds that are responsible for NO generation.

2.2 MATERIALS AND METHODS

L- (+)-Ascorbic Acid used for experiments was purchased from J.T BAKER. 20 ml disposable scintillation vials were used for storage of ascorbic acid samples; Deionized water was used as base solvent for preparation of ascorbic acid. Ascorbic acid of varying concentration was prepared ranging from 1 □M to 100 mM depending upon the needs of the experiments. Ascorbic acid was allowed to decompose naturally for 8 days under ambient conditions in the presence of normal sunlight at pH 5.5, decomposed ascorbic acid pH was measured to be 4.5. A PerkinEmler Lambda 35 UV/VIS Spectrometer was used to read solution absorbances. Disposable spectrophotometer cuvettes purchased from VWR International (Randor, PA) were used. A PerkinELmer HPLC systems consisted of Series 200 Vacuum Degasser, Series 200 pump, Series 200

EP Diode Array Detector and a 20 □L injection loopwas used. The column used for HPLC separation was C_{18} (5 μm, 10 cm x 3.2 mm) purchased from ES Industries. C_{18} is combination of hydrophobic and silanophilic mechanisms. C_{18} packings are highly retentive for polar and non-polar compounds. Two mobile phase solvents were used: Mobile Phase A – HPLC grade Methanol + 0.1% Trifluoroacetic acid, Mobile phase B – Deionized water + 0.1% Trifluoroacetic acid.. Sievers 280i Nitric Oxide Analyzer was used for the direct detection of NO via chemiluminecnce detection. The syringe pump used for controlled release of RSNOs was purchased from Kent Scientific (Farmingdale, NY).

2.2.1 SNAP SYNTHESIS

N-Acetyl-DL-penicillamine, sodium nitrite ($NaNO_2$) and methanol were obtained from Sigma (St.Louis, MO). Sulfuric acid (H_2SO_4) and hydrochloric acid (HCl) were obtained from Fisher Scientific (Pittsburgh, PA). SNAP crystals were made using the method of Field,136 et. al [7]. One-hundred mg of N-acetyl-DL-penicillamine, 5 mL of 1 M NaNO2, 10 mL of methanol, 10 mL of 1 M HCl and 0.5 mL of H_2SO_4 were combined.

A very dark green solution developed nearly instantly. The solution was stirred for ~40 min. The volume of the solution was reduced by blowing nitrogen gas over the solution and the flask was ice cooled. Green crystals were collected via suction filtration and washed with water. Crystals were re-crystalized from methanol and allowed to air dry.

2.2.2 SNAP-PDMS SYNTHESIS

Detailed synthesis of this materials has been described elsewhere [7], briefly 1.6 g of hydroxy-terminated PDMS was mixed with 0.3 g of 3-aminopropyl trimethoxysilane, 2.4 mg of dibutyltindilaurate and 8 mL of toluene. The solution was stirred for 24 h to crosslink the PDMS. A self-protected thiolactone was synthesized according to the method by Moynihan [8]. Fifty milligrams of this thiolactone was then mixed with 1 mL toluene followed by 2 mL other crosslinked PDMS. The solution was stirred for an additional 24 h to allow the thiol-containing compound to react with the primary amine groups present on the cross-linking agent. The resultant free thiol was then converted to the corresponding

RSNO by reaction with t-butylnitrite, producing the characteristic emerald green SNAP-PDMS.

All the experiments were carried out in two sets, first it was performed on fresh ascorbic acid and then repeated on decomposed ascorbic acid. For verifying the consistency of the results experiments was repeated at least three times. In-order to separate the compounds using HPLC there was need to identify the wavelength window of fresh and decomposed ascorbic acid. This wavelength window will help setup the detection limit for both compounds while the separation is being performed. So the first setup is to identify the absorption wavelength of both the compounds using UV-VIS spectroscopy.

2.2.3 UV-VIS SPECTROSCOPY

To establish the detection limit of the HPLC system toward ascorbic acid, UVVIS Spectroscopy was used. UV-VIS Spectroscopy works on Beer-Lambert law. Ascorbic acid and decomposed ascorbic acid sample was prepared and diluted to 1 micro-molar concentration for accuracy. Since de-ionized water was used for preparation of both the samples, it was used as a base sample for the experiment. Since the concentration of the samples are known UV-VIS Spectroscopy helps to identify the absorption wavelength of the samples.

2.2.4 NO ANALYZER

To integrate the complex system and get a real-time analysis of the amount of NO released a medium was required. 3-D Printed device was developed to act as a medium to integrate multiple system together details of the device are describe in detail in the latter half of the chapter. Initially NO analyzer was used to track the amount of NO released when fresh ascorbic acid and decomposed ascorbic acid when treated with SNAP using the 3-D Printed device. This data clearly indicates that decomposed ascorbic acid produces more NO than fresh ascorbic acid. (Figure 2.11 provides sufficient evidence). Left and the right opening of the 3-D printed device receives input from syringe pump i.e. SNAP and Fresh/Decomposed ascorbic acid. Central opening is connected to the NO analyzer which collected the NO reading data across the gas permeable membrane. Graph below represents NO analyzer data.

Once HPLC is used for separation of decomposed ascorbic acid, NO analyzer is used again to repeat the same procedure as above to track the amount of NO released. 3D printed device receives one input from syringe pump i.e. SNAP and the other input from HPLC i.e. separated compounds. This provides real time data and a direct comparison which compound produces more NO.

2.3 RESULTS AND DISCUSSION

2.3.1 UV/VIS DATA

UV/VIS spectroscopy was performed on both fresh and decomposed ascorbic acid. Ascorbic acid (1mM) and decomposed ascorbic acid (1mM) were diluted to 1 □M concentration for higher resolution and more accurate data. Since deionized water was used as a base solvent for preparing both compounds, hence it was also used a standard reference for both the experiments. Detection window for UV-VIS is between 200-700 nm

Figure 2.3 shows a representative UV/VIS spectrum for fresh ascorbic acid and decomposed ascorbic acid. Data suggests that peak absorption wavelength of ascorbic acid is 263nm which is consistent with the literature. Several peaks with consistent change of absorbance can be seen in the figure. Additionally, the absorbance of the ascorbic acid peak at 263nm is reduced in intensity from 0.56 to 0.25, indicating a loss of compound. This confirms that the ascorbic acid did indeed decompose and other compounds are present in the solution. These peaks range from 200 – 400 nm. This information is vital because it will help setup up the detection range for HPLC while physically separating these compounds.

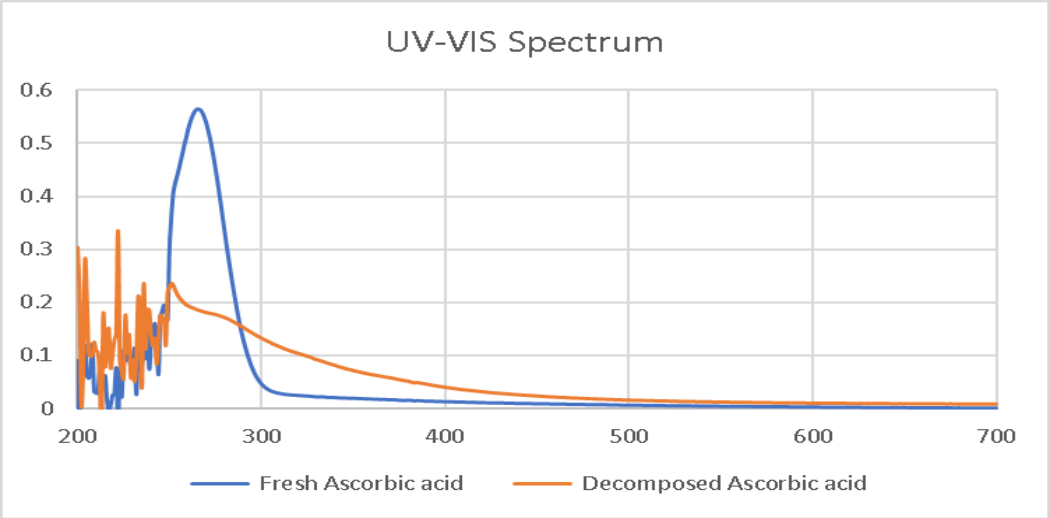


Figure 2.3 UV/VIS data for fresh ascorbic acid sample and Decomposed ascorbic was allowed to decompose in natural light and ambient temperature for 8 days, acid with 1 micro-molar concentration.

2.3.2 HPLC DATA

Because Figures 2.5 and 2.6 clearly indicate that some product in the decomposed ascorbate solution is initiating release of NO, reversed phase HPLC was used to separation the decomposed ascorbic acid in an effort to allow the identification of which component(s) were responsible for this NO release. In this technique, the binary mobile phase (i.e. mobile phase was polar and the C_{18} stationary phase was non-polar was used).

Elution conditions were optimized for both fresh and decomposed ascorbic acid. Compounds once eluted and separated are detected by the Series 200 EP Diode Array Detector. Optimized separation conditions are listed in table 2.1

Table 2.1. Represents working condition for HPLC. Mobile phase A consists of Methanol + 0.001% trifluoracetic acid and mobile phase B consists of de-ionized water + 0.001% trifluoracetic acid.

Parameters	Fresh ascorbic acid	Decomposed ascorbic acid
Solvent condition	Mobile Phase A 90% and Mobile Phase B 10%	Mobile Phase A 75% and Mobile Phase B 25%
Flow Rate	1 ml/min	1 ml/min
Detection Range	200-300 nm	200-450 nm
Pressure	1000-1200 psi	1000-1200 psi

To make sure that mobile phase solvents are not interfering with the separation of compound we also ran a blank compound i.e. Mobile phase A (Methanol + TFA) solvent as a compound to be injected. Figure 6 represents HPLC data for blank sample.

Figure 2.4 shows that ascorbic acid is retained at time 1.407 minutes and no other compound being eluted from fresh ascorbic acid sample. Figure 2.5 shows two peaks at 0.691 minutes and 1.198 minutes which surely suggests presence of more than one compound in decomposed ascorbic acid sample. A UV-Spectrum analysis was carried out on the two peaks obtained (seen in Figure 2.6) shows how the absorbance for the peaks changes over time.

Figure 2.6 shows that for the first peak absorbance is 0.05 and gradually changes as the peak descend and when the second peak ascends, the absorbance is 0.16 indicating a different compound and it reaches to 0.2 when the peak descend; Once again providing a clear evidence of presence of more than one compound.

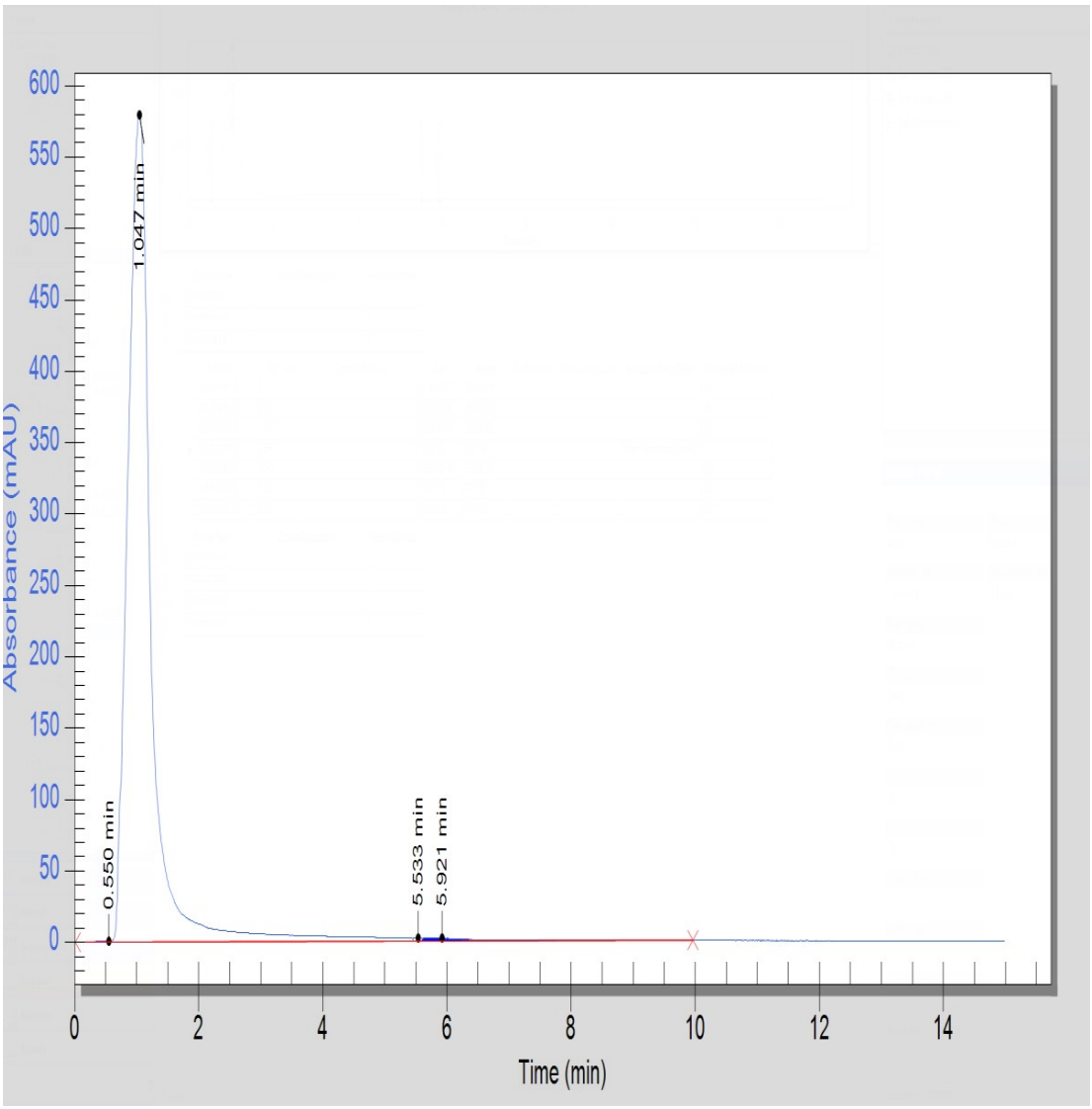


Figure 2.4. HPLC for fresh ascorbic acid sample represents the elution time of the sample along with the absorbance. Concentration of fresh ascorbate used was 100 mm. 50µL of the sample was injected.

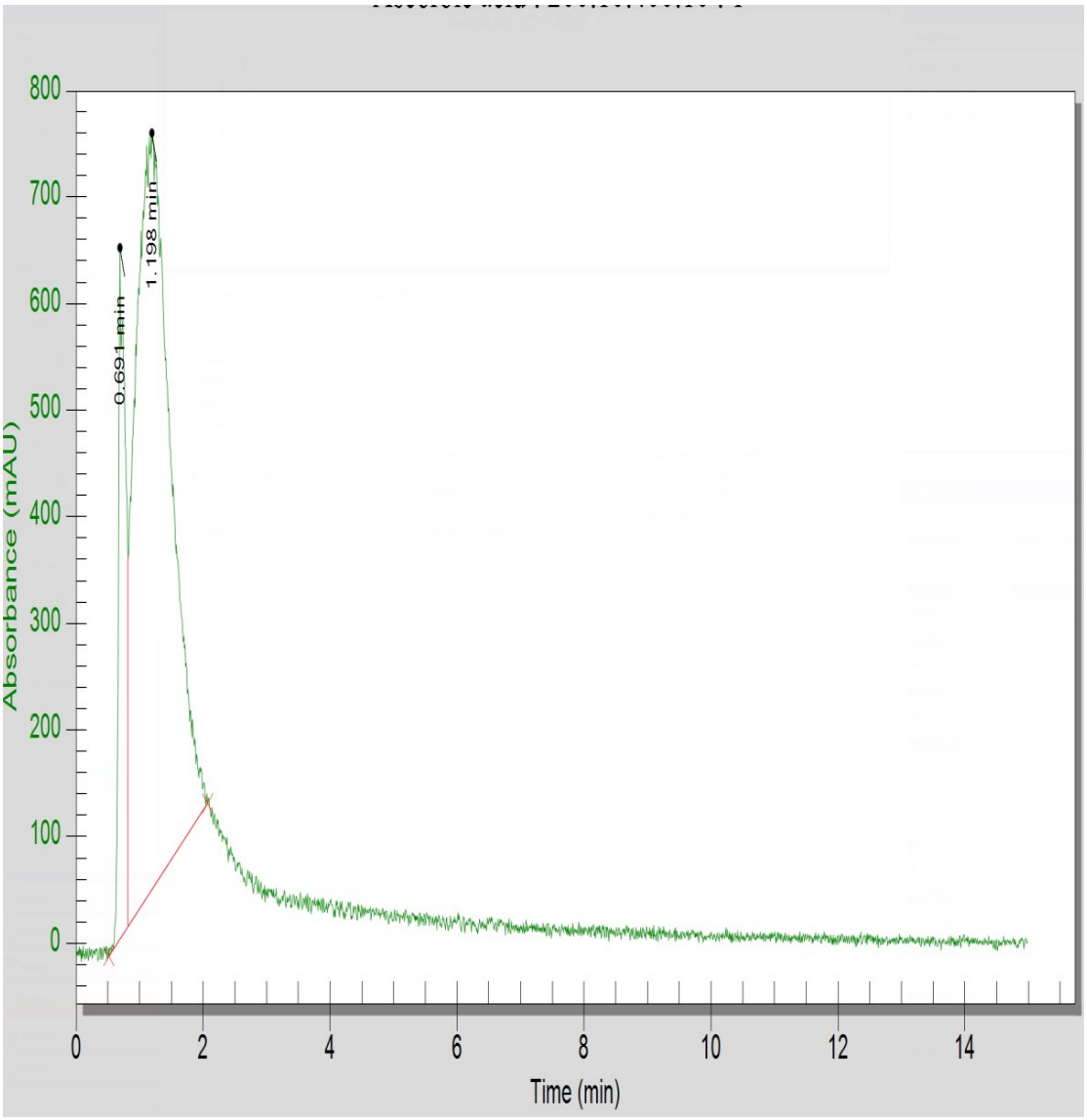


Figure 2.5. HPLC for decomposed ascorbic acid sample. Represents the elution time of the sample along with the absorbance. Concentration of decomposed ascorbate used was 100 mm. 50µL of the sample was injected.

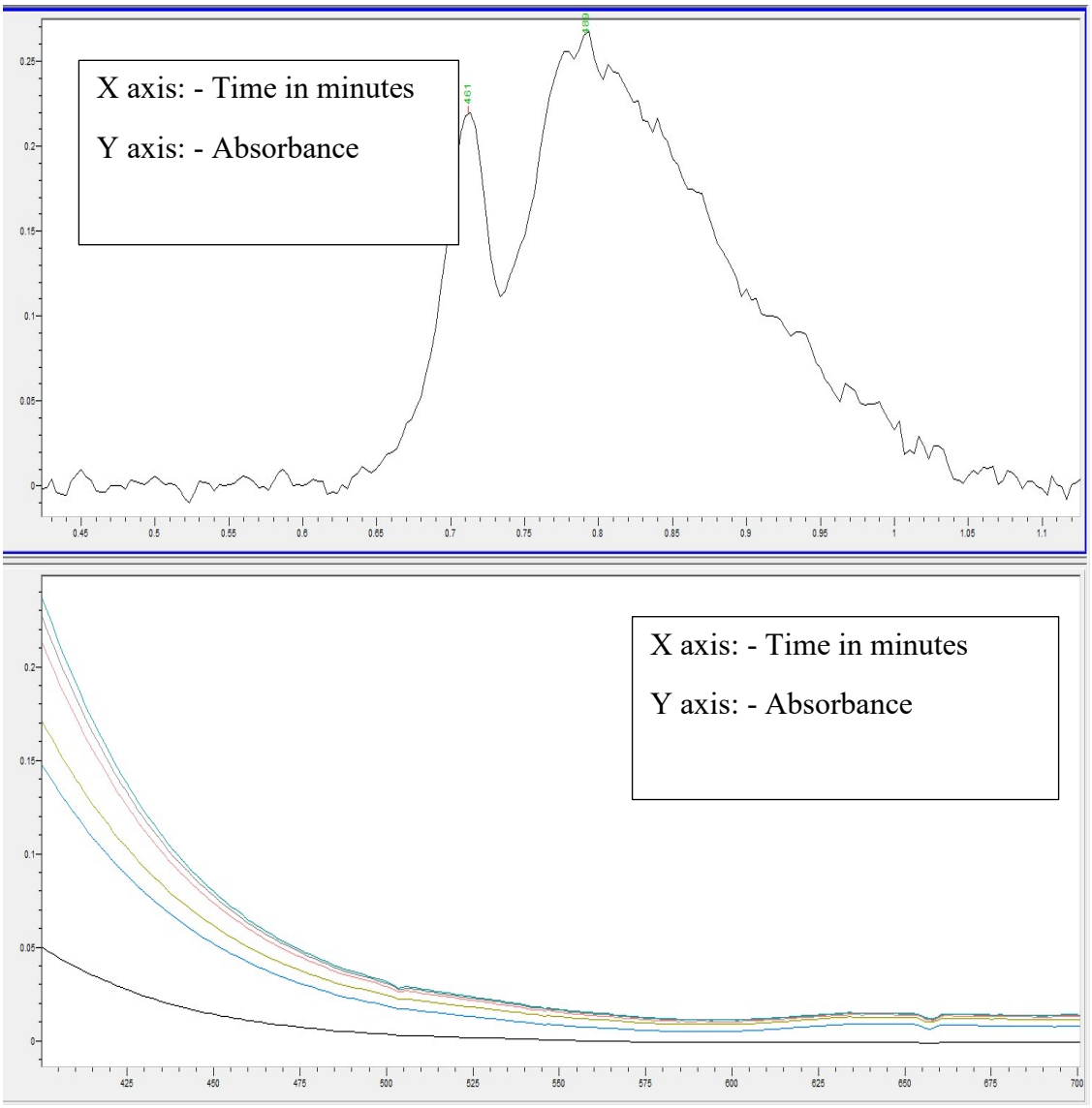


Figure 2.6. UV-SPECTRUM of the peaks obtained from decomposed ascorbic acid. Absorbance changes for the two peaks. From 0.06 for the first peak to 0.2 for the second peak. Indicating two different compounds.

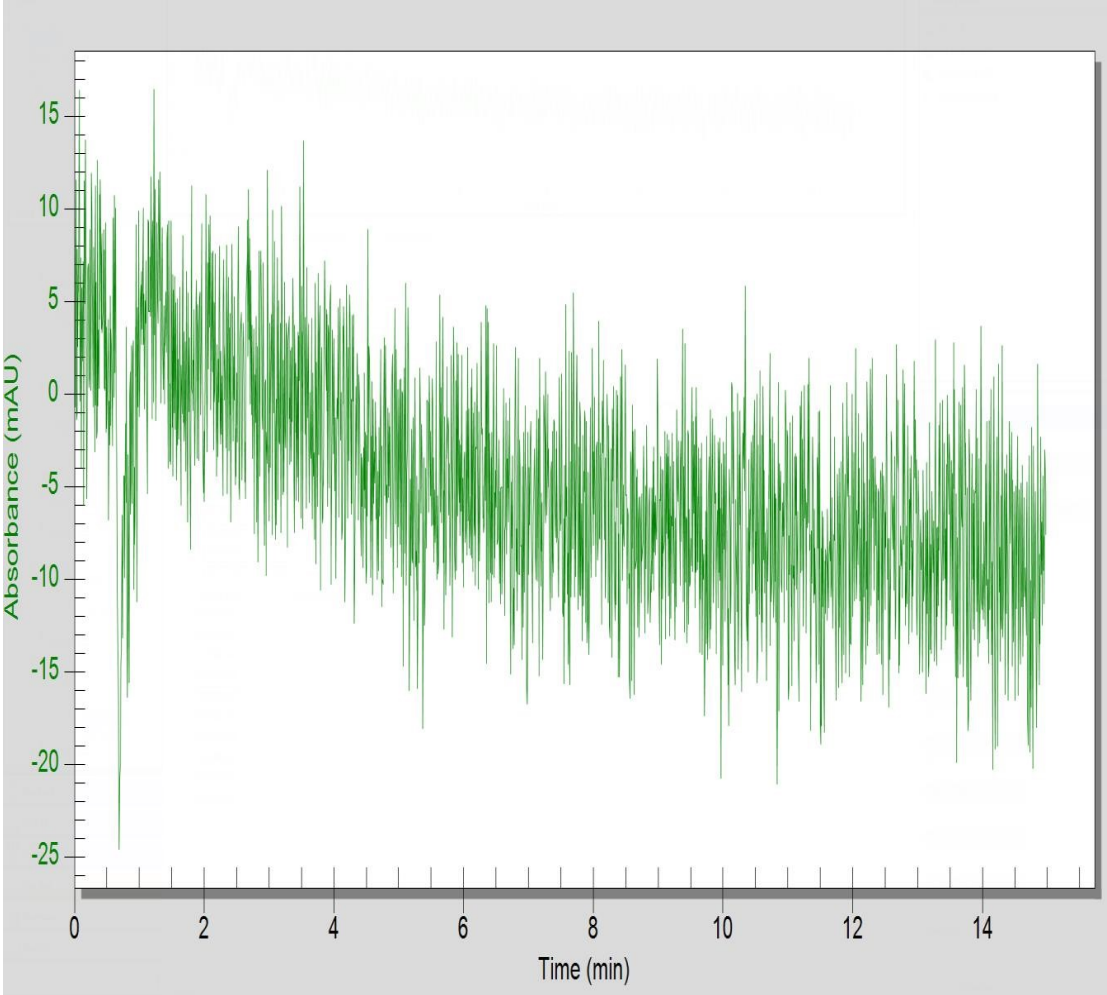


Figure 2.7. HPLC for blank sample. Shows no compound coming out of the HPLC system. Indicating Mobile phase solvents do not interfere with the separation of decomposed ascorbic acid.

2.4 3-D PRINTED DEVICE

2.4.1 NEED FOR THE DEVICE

In order to identify which component(s) in these 2 solutions (fresh and decomposed ascorbic acid) was responsible for initiating NO release from SNAP, a novel device was designed and used to couple the separation capacity of the HPLC with the direct NO measurement capability of chemiluminescence. Much of the earlier work done in the literature are just techniques used to separate compounds from decomposed ascorbic acid

and identify them. None of the work done to this point directly studied which of these compounds are responsible for NO release when in contact with RSNOs.

2.4.2 SPECIFICATION OF THE DEVICE

Device was printed by Dr. Joshua Pearce using an open source method of scientific hardware design [Pearce 2012; Pearce 2014; Baden 2015] [8] The main apparatus was augmented with a 3-D Printed Complex-Carbohydrate-Mixer, first developed by Brian Swanson and made available on Github [Swanson, 2014]. [9,10]. This device facilitates NO detection by allowing the compounds to mix and react with the small mixing chamber located at the center of the device. Released gases are detected by allowing them to pass through a filter which is a gas permeable membrane. The membrane is placed between the device and the 3-D printed cover, allowing the sampling of any NO generated when the eluent from the HPLC mixes with the stream of RSNO via chemiluminescence. Figure 2.10 represents the 3-D printed device.

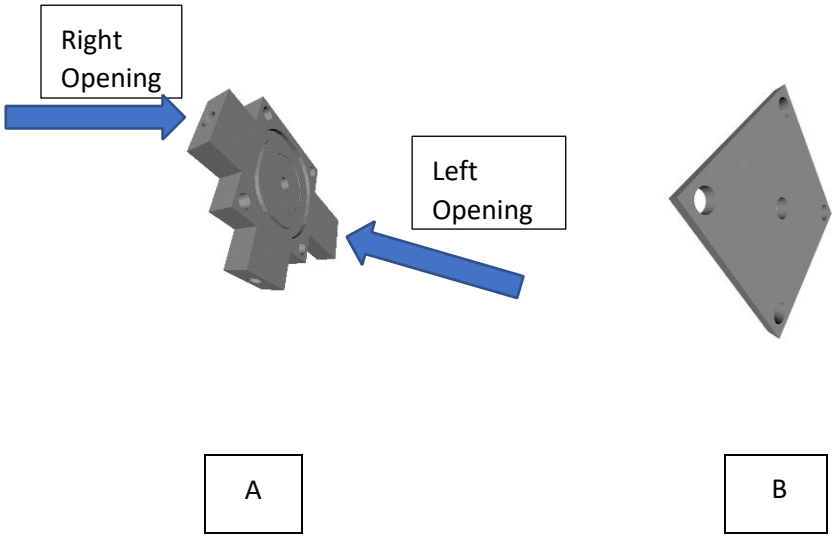


Figure 2.8 CAD Design of the 3-D printed device.

A: - Represents the actual device, left opening takes input from the syringe pump and right opening takes input from HPLC.

B: - Represents the cover of the Meshlab screen shot of STL of 3-D printed cover with mounting holes

The 3-D printed parts can be manufactured in any fused filament fabrication (FFF) based 3-D printer such as the open source RepRaps (self-replicating rapid prototypers) [Bowyer; Sells; Jones] [11,12,13]. The custom parts are 3-D printed in polylactic acid (PLA) or similar hard thermoplastic, which is chemically stable with the experiment. In this case, natural PLA was printed at 100% infill with 100 micron layers. Previous work that investigated the mechanical properties of PLA has found that these materials are optimal for strength [Tymrak; Wittbrodt] while also minimizing contamination from unknown pigments in commercial 3-D printing filament.

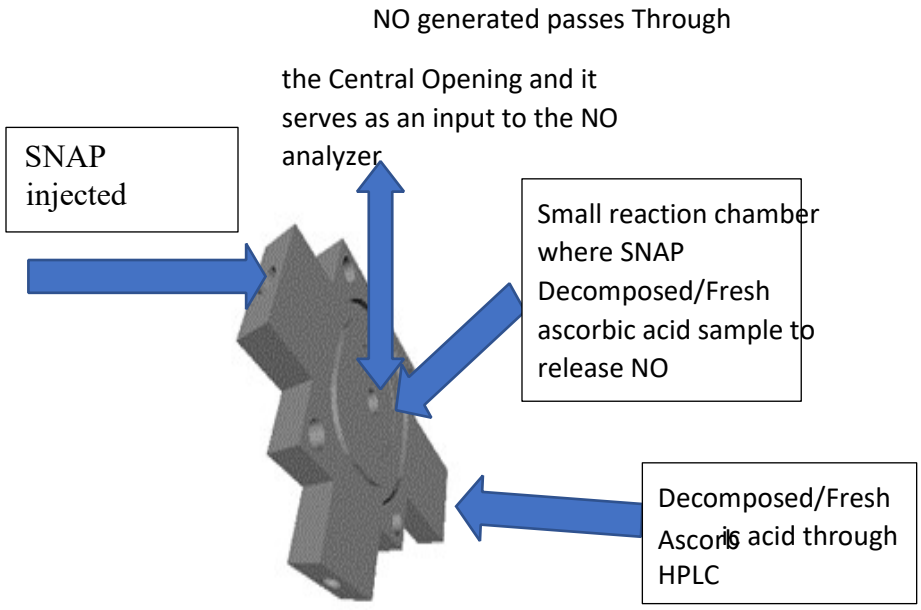


Figure 2.9 represents schematic flow of compounds for 3-D printed device.

2.4.3 VALIDATING THE DEVICE

To validate proper functionality of the device, experiments were performed that directly injected solutions of a known RSNO and known samples of agents that would

initiate NO release from the RSNO solution. Fresh 100mM ascorbic sample and SNAP were injected through the two openings on the left and the right side of the 3-D printed device using a syringe pump. Flow rate was set at 1ml/min. NO Analyzer input was connected to the Central opening on the 3-D printed plate placed above the gas permeable membrane. Figure 2.10 shows NO Release Profile of the fresh ascorbic acid sample. To setup a detection limit for the system this experiment was repeated 3 times with varying concentration of SNAP starting from 1mg and going down to 0.2mg (i.e. 1mg/50ml of de-ionized water). Table 2.2 represents the compound of NO released by each sample.

Table 2.2 Total amount of NO released by each sample.

Samples	Concentration	Amount of NO released
S1	1mg SNAP + 100mm Ascorbic acid	4.07×10^{-9} moles
S2	0.5mg SNAP + 100mm Ascorbic acid	2.33×10^{-9} moles
S3	0.2mg SNAP + 100mm Ascorbic acid	6.62×10^{-11} moles

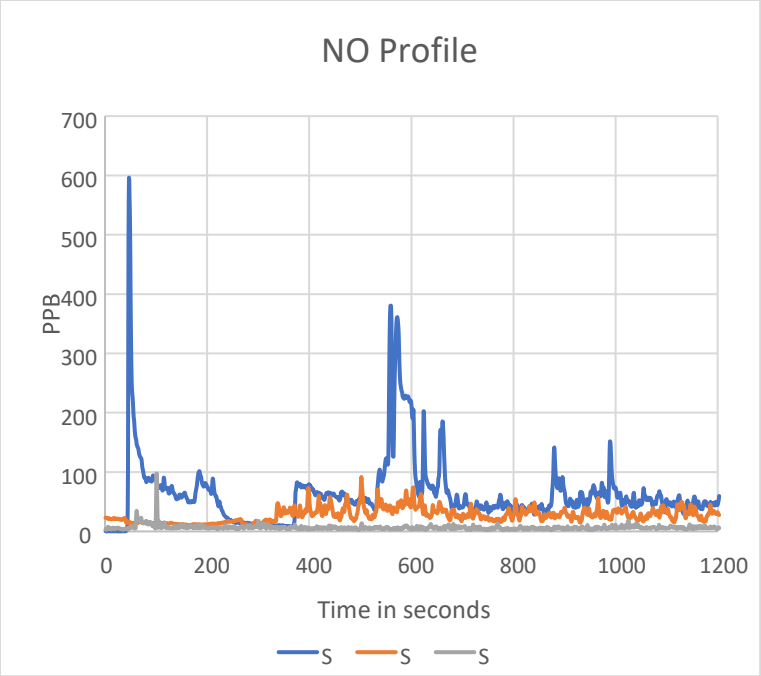


Figure 2.10 Represents NO release profile of Samples S1, S2 & S3

The second part of the experiment, samples of 100mM decomposed ascorbic acid and SNAP (0.5mg/50ml of di-ionized water) were injected through the two openings. Decomposed ascorbic acid was obtained by allowing fresh ascorbic acid sample to decompose in ambient conditions for eight days. The NO analyzer input was connected to the central opening on the 3-D printed plate placed above the gas permeable membrane.

Figure 2.11 shows the NO release profile of the decomposed ascorbic acid sample.

It can be clearly seen from the data that decomposed ascorbic acid releases twice the amount of NO compared to fresh ascorbic acid sample. Thus, validating that device can be used to integrate HPLC system directly with the NO analyzer. The difference will be that one of the inputs is from the HPLC system and the other using Syringe pump, instead of both coming from syringe pump as in case of the above experiments.

2.5 HPLC INTEGRATED WITH NO ANALYZER

Once the compounds had been separated via HPLC, the next task was to integrate it with the chemiluminecscne detector for NO with the system to prove that decomposed ascorbic acid generates more NO than fresh ascorbic acid. To integrate the two systems, we use the device 3-D Printed Device. Figure 2.11 shows a schematic representation of the integrated system.

Workflow of the setup is simple: waste coming out of the HPLC acts as one of the inputs for the bridge and the second input is SNAP which acts as an NO donor. The two compounds are allowed to react at the center of the "3-DPrinted Device". A gas permeable membrane is placed over the central opening which is directly connected to the NO Analyzer. Waste from the reaction is collected into an empty vial. There is a Lag time between the compounds reaching the BRIDGE and the NO Generated.

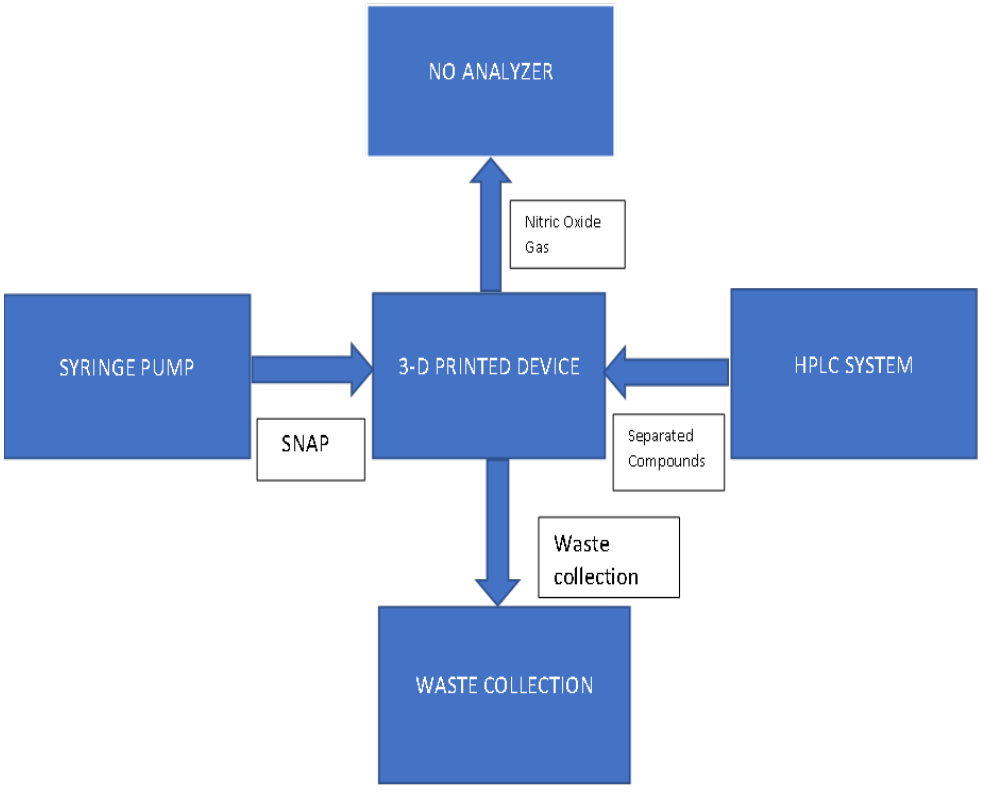


Figure 2.11 Schematic flow of the integrated system. Two inputs to the 3-D Printed device are from HPLC and syringe pump. NO Analyzer data is collected through an opening across the gas permeable membrane

Figures 2.12 Represent the amount of NO Released from the blank sample, Fresh and decomposed ascorbic acid. Spike in the NO profile for blank sample is due to the SNAP alone and lag time in HPLC compound reaching the 3-D Printed Device. The amount of NO released from the decomposed ascorbic acid is more than twice the amount released from fresh ascorbic acid. This proves that when ascorbic acid decomposes it release more NO than fresh ascorbic acid, which concludes that a new compound is formed when ascorbic acid decomposes.

Table 2.3 Represents Total NO released by blank sample, fresh and decomposed ascorbic acid

Compounds	Total NO released ±(SD)
Fresh ascorbic acid	$170x10^{-12}$ ($1.07x10^{-10}$) moles
Decomposed ascorbic acid	$350x10^{-12}$ ($3.50x10^{-10}$) moles
Blank sample	$6.29x10^{-12}$ ($6.29x10^{-12}$) moles

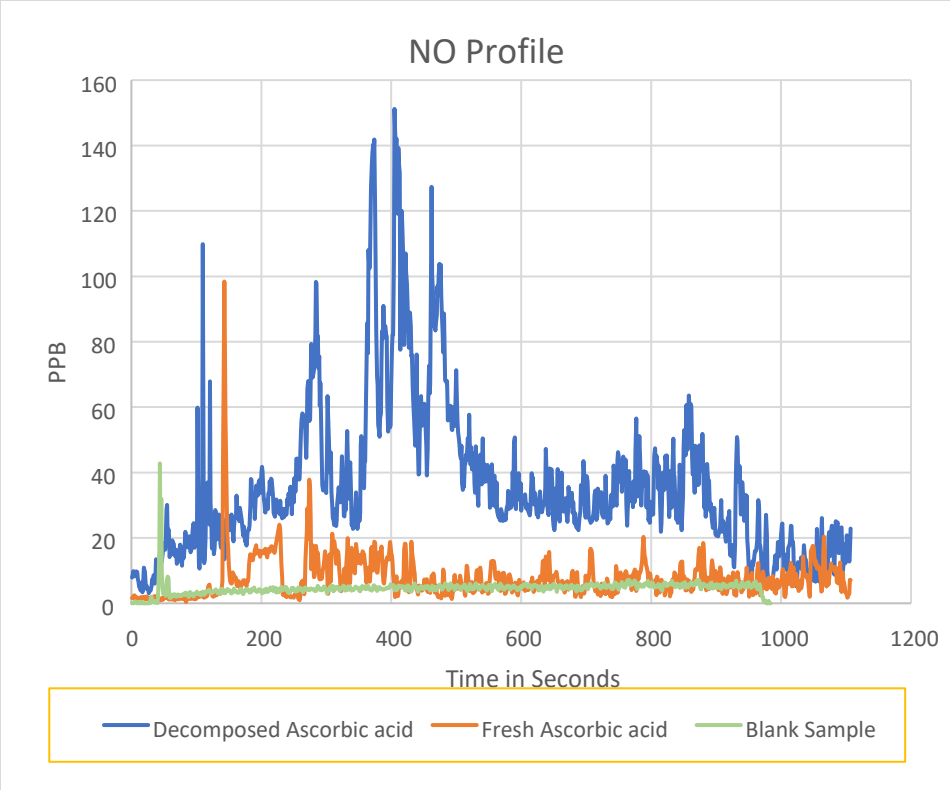


Figure 2.12 NO release profile for blank sample, fresh and decomposed ascorbic acid.

2.6 CONCLUSION

UV-VIS Spectroscopy was used to identify the absorption wavelength of fresh and decomposed ascorbic acid. Once the detection wavelength was established it was used to setup a wavelength window for separation of decomposed ascorbic acid. HPLC separation technique helped to separate decomposed& fresh ascorbic acid.

Figure 2.5 shows the separation of decomposed ascorbic acid with retention time. Change in the absorbance from the UV-Spectra graph represents presence of more than one compound present. Once the separation was complete NO data was collected for both decomposed and fresh ascorbic acid by using 3-D Printed device as a medium to integrate HPLC with NO analyzer to obtain real time data.

Figure 2.12 represents the amount of NO released from decomposed ascorbic acid when it was separated using HPLC. Same procedure was also repeated using a blank, which

proves that it is actually decomposed ascorbic acid that causes more NO to be released as compared to Fresh ascorbic acid. The amount of NO released by decomposed ascorbic acid is almost twice the amount released by fresh ascorbic acid.

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3. IDENTIFICATION OF NO RELEASING COMPOUND

Ascorbic acid is a well-known antioxidant and vitamin which is widely distributed in nature, and also part of our daily diet. Ascorbic acid is also known for its antioxidant property in both *in vitro* and *in vivo*. The initial step in ascorbic acid metabolism is conversion into dehydroascorbic acid (DHA); this process is reversible and is suspected to be responsible for its antioxidant properties. DHA is degraded to 2,3diketogulonic acid and then to over 50 species containing five or less carbons [2]. The complete mechanism of degradation is unknown due to the highly reactive and complex pathways available for decomposition [2]. Ascorbic acid is also known to react with RSNOs to produce NO. Much work has been done on studying degradation products of ascorbic acid, but the mechanism of decomposition under physiological conditions has not been elucidated. Gaston Vernin, et al. studied thermal degradation of ascorbic acid at 3000 C yielded furan derivatives and ∝, β-unsaturated cyclic ketones with a fivemembered ring. Since ascorbic acid is a required part of our daily food cycle it makes it all the more important to identify its decomposition product(s) triggering NO release, since it is naturally present in our body.

There are three known mechanics of NO generation from S-nitrosothiols (RSNOs) [3]. Copper mediated decomposition in which Cu^{2+} is first reduced to Cu^{+} and then Cu^{+} reduces RSNOs to NO and produces disulfide bonds. Common species used to reduce Cu^{2+} to Cu^{+} are free thiolate ions and ascorbic acid. Ascorbic acid itself has been reported to directly interact with RSNOs when it is present at greater than millimolar concentrations to reduce RSNO to NO and produce free thiol groups. The mechanism of this reaction is not known [4]. The third known mechanism of NO generation from RSNOs is by means of photoinitiation.

Dasgupta and Smith published a study entitled “Kinetics and Mechanism of the Decomposition of S-Nitrosoglutathione (Type of RSNOs) by Ascorbic Acid and Copper Ions” [6]. The state that S-nitrosothiols severs as a good source of NO mainly due to the ease of cleavage of S-N bond which consequently produces NO. The reductive decomposition of the specific RSNO, S-nitrosoglutathione (GSNO) by ascorbic acid produced NO which was monitored both electrochemically and spectrophotometrically.

The rate of reaction and NO release occurs in an ascorbate concentration and pH dependent manner. There is little to no reaction at low pH (below 5.5). Reductive decomposition of GSNO by ascorbate in an aqueous solution was carried out in the presence of EDTA to suppress any copper catalysis.

Dasgupta and Smith proved that GSNO decomposition had a liner dependence on the concentration of ascorbate from 10-55mM. In their analysis. they could only account for the 90% of ascorbate used initially when combining total ascorbate and DHA present initially, claiming that the lost 10% was probably due to “analytical error”. This suggests that ascorbic acid decomposes into different or additional products and it is possible that one of these products is an active agent that is responsible for the release of NO from RSNOs. (i.e., more favorable redox potential and/or kinetics of electron transfer to react directly with RSNO).

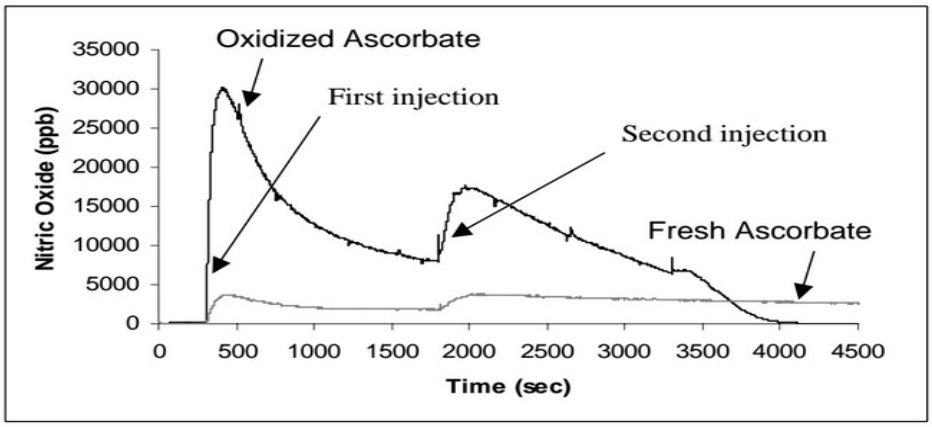


Figure 3.1 Comparison of NO generated from 0.227Mm SNAP solution in PBS at Ph 7.4 [5].

Figure 3.1 shows NO generated from 0.227 mM SNAP solutions in PBS at pH 7.4 upon the addition of fresh ascorbate and the addition of the same amount of oxidized ascorbate from the same solution after 8 d of decomposition under ambient conditions using chemiluminescenc detection. After the first injection of ascorbate or oxidized ascorbate, the final concentration of ascorbate was 2.5 mM and 5 mM after the second injections. Clearly there is more NO generated from the oxidized ascorbate than from the fresh ascorbate, by nearly a factor of 10.

None of the work published to date has been able to identify the mechanism of ascorbic acid to directly decompose RSNOs nor have decomposition products of ascorbate been investigated as potential species that release NO. Correctly identifying the product or products that may be responsible for RSNO decomposition may provide us with new candidate material(s) for coatings on the implanted device and may also improve their performance in vivo and help mitigate host response. This study has developed a new experimental method to help separate the decomposition products which are responsible for NO release.

As previously discussed in chapter 1 the amount of NO generated from decomposed ascorbic acid is more than that produced by fresh ascorbic acid. This clearly indicates that it is not actually ascorbic acid but one its decomposition product which leads to increased production of NO.

Ascorbic acid upon oxidation is converted into dehydro-L-ascorbic acid (DHA), which later is non-enzymatically converted to a variety of products [2]. These products are known as ascorbic cascade. We used high performance liquid chromatography (HPLC) with a photo diode array detector (PDA) along with Series 400 quaternary pump to separate the decomposed ascorbic acid. As concluded in Chapter 2 we did separate compounds from decomposed ascorbic acid and integrated it in real time with NO analyzer using the "3-D Printed device". We concluded from the results that it is not actually ascorbic acid but one of the decomposition products of ascorbic acid that produces NO from RSNOs. Although HPLC separated some of the components of the complex mixture that resulted from the decomposition of ascorbic acid, the component(s) that eluted at 1.98 mins (Figure 2.7, Chapter 2) that cause release of NO need to be identified. There for MS analysis of this peak was carried out using a Thermo Finnigan LCQ Advantage Ion-Trap was the device used to run the Mass spectroscopy analysis.

As stated in chapter 2, HPLC was carried out using PDA detector and series 400 pump. Separation was achieved using C_{18} column. Mobile phase was methanol and deionized water each containing 0.01% trifluoracetic acid. Eluent was pumped at the flow rate of 1ml/min. The chromatographic data i.e. retention time, wavelength, absorbance was

obtained as contour maps through multichannel PDA detector, which covered a wavelength range of 200-450 nm. Elutes were examined by absorption spectrum, further characterization and identification of the compounds were done using Electron Spray Ionization technique was used during mass spectrometry.

3.1 ASCORBIC ACID DECOMPOSITION CASCADE

Once ascorbic acid is decomposed into DHA, it can then rapidly convert into a variety of transformation products possessing absorption maximum between 240 and 350 nm. Eiji kimoto et al (2). Studied the decomposition cascade of ascorbic acid. Using electrochemical detection-HPLC analysis. Figure 3.2 shows the structures of compounds involved in this cascade. They suggested a possible decomposition pathway of DHA. DHA is easily delactonized into 2,3-diketo-L-gulonic acid(DKG). DKG undergoes isomerization to form the 6-membered R-345. DKG solution may also be subjected to decarboxylation to form L-xylosone (XLS) as reported by whiting and coggins. (1) Subsequently transformation produces various five-carbon.

XLS is converted to Compound **VI.** Compound **VI** is dehydrated to from compound **VIII**, the unsaturated α- keto acid, which is further dehydrated to produce 2 Furoic acid (2FA) (compound **XI**) and/or 3OH2P (Compound **XII**) of the 2-pyrone form. As an alternative pathway XLS is converted to enediol form, Compound **VII.** Its aldehyde group is oxidized to carboxyl group which is lactonized to form EAsA. Intramolecular redox reaction of compound **VII** leads to rearrangement from the aldehydic to the carboxylic group and from the hydroxymethyl at the

C (5) position to the methyl group, thus producing compound **X.** It undergoes ring closure to form MDT. Otherwise, the aldehydic group of compounds **VII** reamins intact. It dehydrated and reduced i.e. loss of oxygen to form compound **IX** of the structure. Compound **IX** is dehydrated to form FF of the furan form, or it is rearranged to form the bialdehydic structure as an unstable intermediate which undergoes ring closure easily to form RA.

Identification of the actual compound that initiated NO release form RSNOs is extremely important. If the identified compound is stable and non-toxic it could provide a means that initiate RSNO decomposition in polymeric materials or endogenously from RSNOs present in biological systems. For example, a polymer such as medical grade polyurethane doped with the reducing agent could be formulated such that when exposed to blood the active agent slowly leaches from the material, decomposing circulating nitrosothiols and preventing thrombus formation on the device.

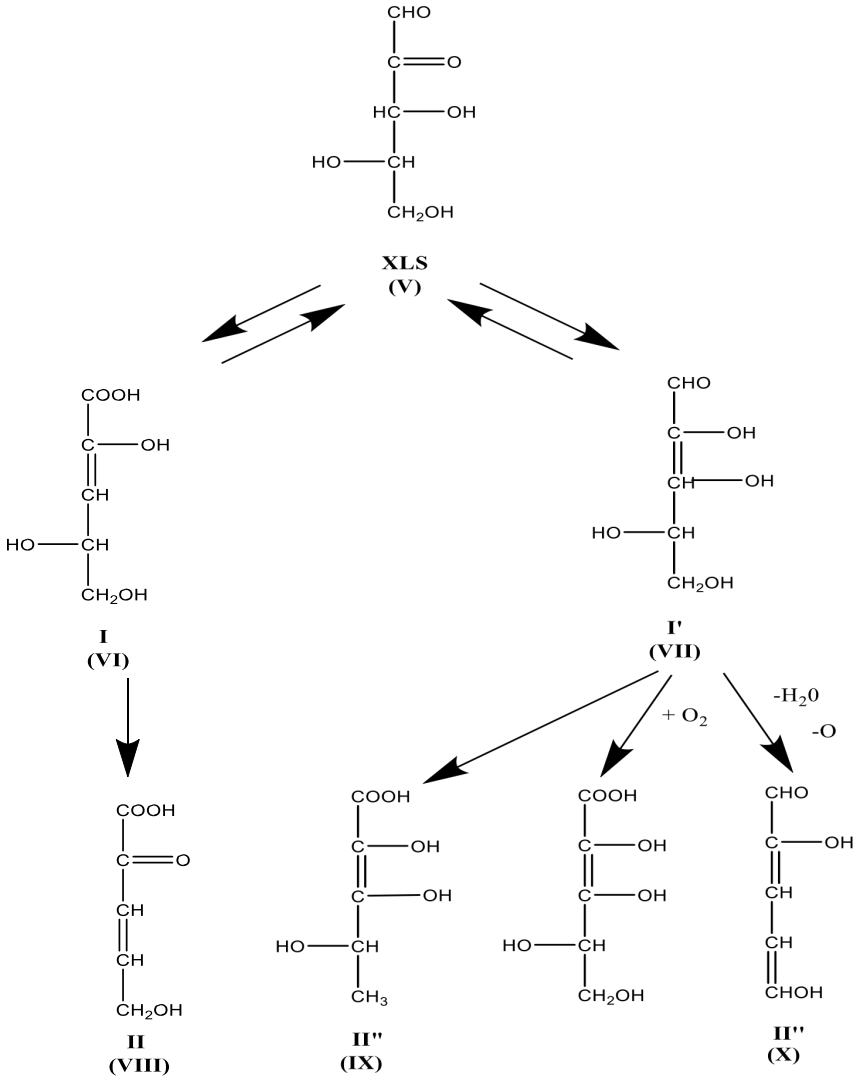
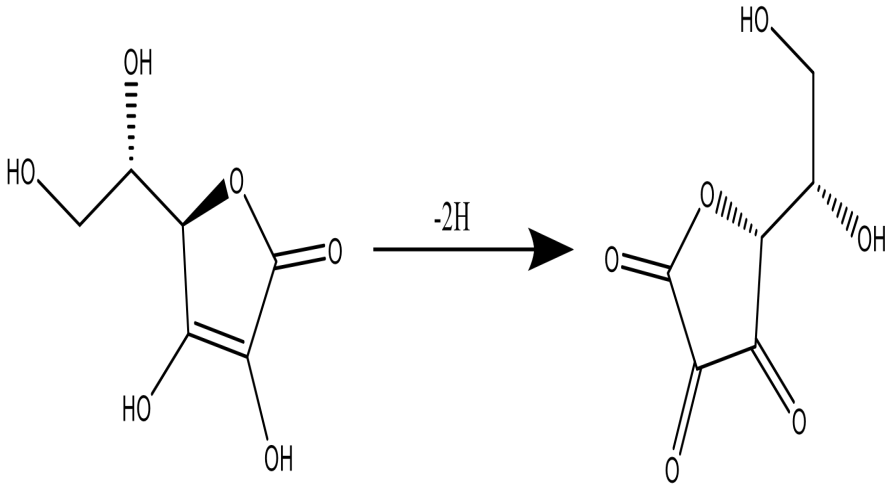
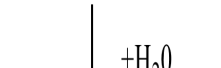


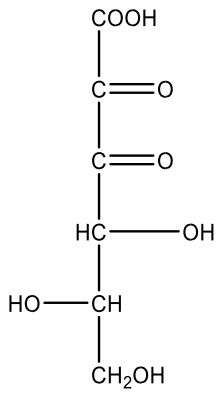
Figure 3.2 Represents structure of the 5-Carbon compounds involved in the cascade.



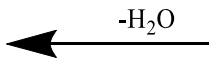
Ascorbic acid
(I)

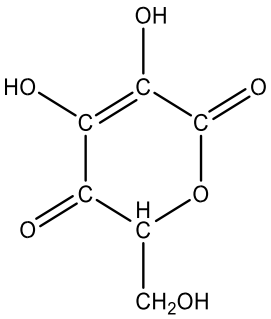
Dehydroascorbic acid
(II)



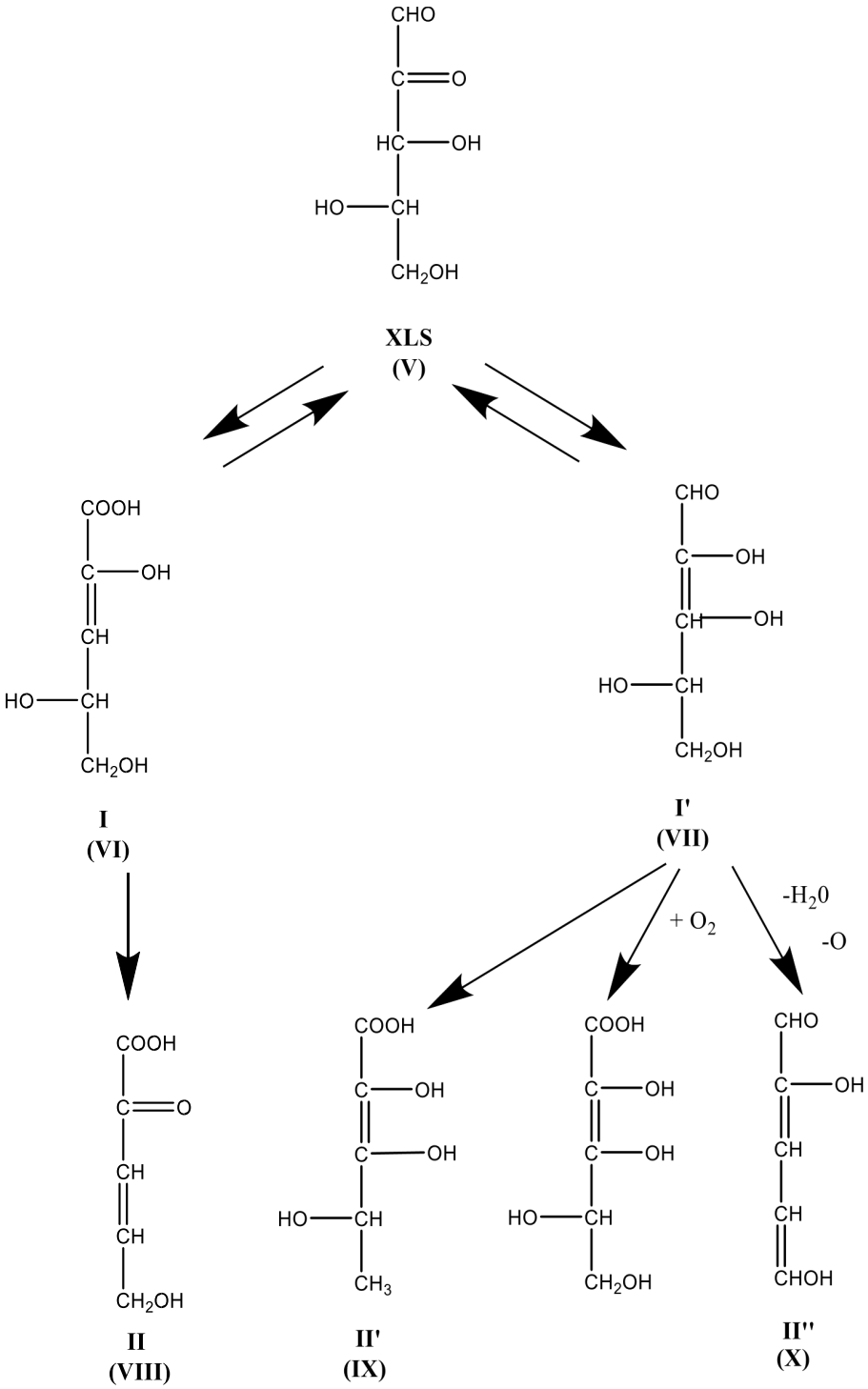


2,3 diketo-L-gluconic acid
(III)





R-35
(IV)


CHO
C=O
HC—OH
HO—CH
CH2OH
XLS
(V)
COOH
C—OH
CH
HO—CH
CH2OH
I
(VI)
CHO
C—OH
CH—OH
HO—CH
CH2OH
I'
(VII)
-H20
+ O2
-O
COOH
C=O
CH
CH
CH2OH
II
(VIII)
COOH
C—OH
C—OH
HO—CH
CH3
II'
(IX)
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CHO
C—OH
CH
CH
CHOH
II''
(X)

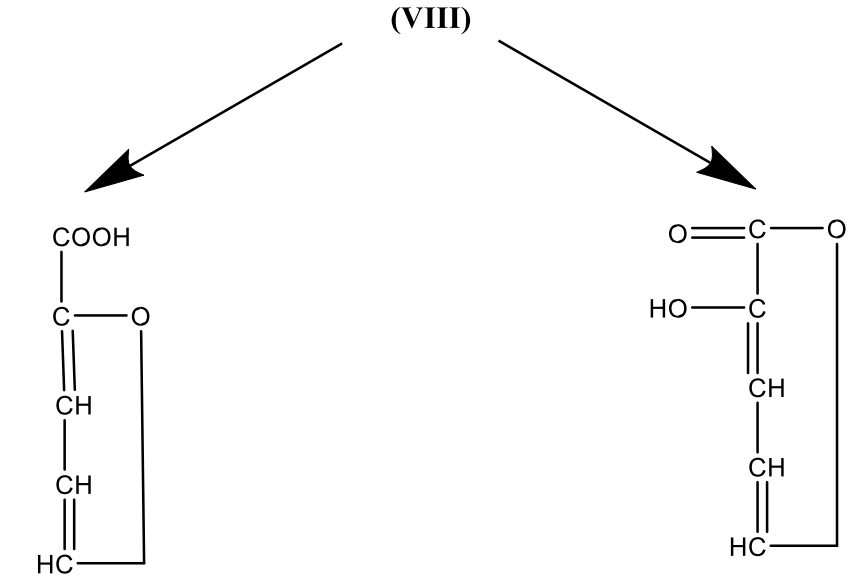

Figure 3.3 Possible reaction pathway for the transformation products of ascorbic acid [2].

3.2 EXPERIMENTAL PROCEDURE FOR MASS SPECTROSCOPY

Reagents and chemicals of highest grade was obtained from Sigma-Aldrich (St. Louis, MO, USA). Nitrogen gas was purchased from General Air Service and Supply (Denver, CO, USA). DHA and 2FA were also purchased from Sigma-Aldrich. Methanol along with di-ionized water were used as solvent for mass spectroscopy in 50-50 % ratio.

Thermo Finnigan LCQ Advantage Ion-Trap was used for mass spectroscopy.

The Finnigan LCQ Advantage is an ion trap mass spectrometer. This instrument is capable of performing both single stage LC/MS a double stage LC/MS/MS. Sample solution is introduced to the mass spectrometer via loop injection. It uses electrospray ionization (ESI) ESI results in the generation of highly charged droplets directly from the infused solution. Multiply and/or singly charged analyte molecules desorb from the sprayed droplets and are sampled through the mass spectrometer for mass analysis. Rest of the equipment consists of quadruple ion trap where the ions are trapped followed by a photodiode array (PDA) detector. Compounds collected from HPLC separation were used an input samples for mass spectroscopy. 500µl of samples is injected, data is recorded for

11 minutes. After running each sample solution of methanol and de-ionized water is used for cleaning injector module. Once it is cleaned another sample with same amount is injected for analysis.

3.3 DATA ANALYSIS FOR MASS SPECTROSCOPY

Upon introducing the eluent from the HPLC peak of interest into the MS, the components were first ionized. The ions then travel to the mass analyzer and arrive at the different part of the detector according to the mass/charge ratio. After the ions make contact with the detector useable signals are generated and displayed on the computer. Yaxis is labelled as the relative abundance, which is actually the relative intensity. This is the intensity relative to the tallest peak in the spectrum with the tallest peak set to 100%. X-axis is the mass divided by the charge ratio m/z. since the charge is always +1 with ESI, the m/z ratio always represent mass. All the data collected is in the positive ion mode since there were compounds detected in the negative ion mode.

First MS analysis was carried out on the HPLC separated compounds for decomposed ascorbic acid. Figure 3.3 represents MS data for HPLC separated decomposed ascorbic acid. Spectra show a number of peaks which suggests presence of multiple compounds. Each peak obtained from the HPLC separation was further broken down for full mass analysis. Compounds with m/z charge ratio higher than 550 m/z did not break down into smaller fragments. However, peak with 430.8 m/z ratio was further broken down to 158.9 m/z ratio. Using the ascorbic acid decomposition cascade as reference we suspected this peak to be either dimers of dehydroascorbic acid or 2-furoic acid keeping their molecular mass in account with either addition or donation of hydrogen ions. After narrowing it down to two compounds i.e. dehydro-ascorbic acid and 2-furoic acid we performed MS on these two compounds to see if we could find any overlap.

Reference material 2-Furoic acid and Dehydro-ascorbic acid of highest purity was purchased from Sigma-Aldrich (St. Louis, MO, USA) for testing NO release and perform mass spectroscopy to see if any compounds overlap with the existing data. Five different samples were prepared for both MS analysis and then as pure substances that would

directly initiate release of NO from SNAP measured in the chemiluminescence detector. Table 3.1 lists compounds selected for further testing suggested from MS analysis: -

Table 3.1 Represents the compounds selected for testing along with their respective concentration.

Compound I	2-furoic acid (2FA)	1mm
Compound II	Dehydro-ascorbic acid (DHA)	1mm
Compound III	DHA + 2-furoic acid	1mm(DHA) 50% + 1mm 2-FA (50%
Compound IV	2-furoic acid + Ascorbic acid	1mm (2FA) 50% + 1mm Ascorbic acid 50%
Compound V	Ascorbic acid	1mm

Table 3.2 Represents m/z ratio of each of the compound tested, it also includes a further breakdown of the m/z ratio which were possible.

Compound	m/z ratio	m/z ratio (Further breakdown)
HPLC separated decomposed ascorbic acid	430.8	158.9
2-Furoic acid	424.9	157
DHA + 2FA	424.9	157
Ascorbic acid	178	
Ascorbic acid + 2FA	Multiple peaks	

Mass spec analysis was performed on the HPLC separated compounds, to further identify the exact compound mass spec analysis was performed on all of the five

compounds listed in Table 3.1 to find any overlap with the HPLC separated compounds. 2-Furoic acid had compound with m/z ratio of 424.9 which was found in decomposed ascorbic acid, i.e. compound with compound with 424.9 m/z broke down into 157m/z as seen in figure 3.5(Figure 3.5 shows mass spec data for 2-FA). So, did the combination of DHA + 2-FA losing a pair of hydrogen atoms, i.e. compound with m/z 424.9 which further broke down in to 157 m/z like that found in Figure 3.1(Figure 3.4 shows Mass spec data for DHA + 2FA). Since there is an overlap of m/z ratio from both 2FA and 2FA + DHA with the originally separated decomposed ascorbic acid it was concluded that either 2FA or DHA is one of the decomposition products of the ascorbic acid responsible for the large amount of NO release. Further analysis on the compound i.e. 2FA + Ascorbic acid shows no overlap in m/z ratio with the HPLC separated decomposed ascorbic, which concludes that ascorbic acid suppresses the presence of 2-furoic acid and forms a complex mixture, that might be one of the reasons why ascorbic acid produces a lower amount of NO as compared to 2 furoic acid. (Figure 3.9 & 3.10 describes it in more detail)

Figure 3.5 represents MS data for 1mm 2-furoic acid. It also showed presence of multiple peaks. Peaks with m/z charge ratio above 550 did not show any break down fragments in full mass analysis of the spectrum, however the peak with m/z ratio 424.9 was further broken down into peak with m/z ratio 157. Which indicates similar compound as seen in HPLC separated decomposed ascorbic acid.

Similarly Figure 3.6 represents MS data for the combination of DHA and 2-FA (1mm). Data reported presence of the same compound with m/z ratio of 424.9 which could be further broken down into fragment of m/z ratio 157.

Figure 3.7 represents MS data for fresh ascorbic acid sample (1mM). Molecular mass of ascorbic acid is 176 g/mol so the m/z ratio should be 177 with the addition of one hydrogen atom, which is seen in the figure 3.5 ascorbic acid with peak of m/z ratio 177.

Figure 3.8 represents MS data for the combination of fresh ascorbic acid with 2furoic acid. Data showed no overlap with any of the peaks seen earlier, which lead us to

believe that ascorbic acid suppress the presence of 2-furoic acid to form some other compounds.

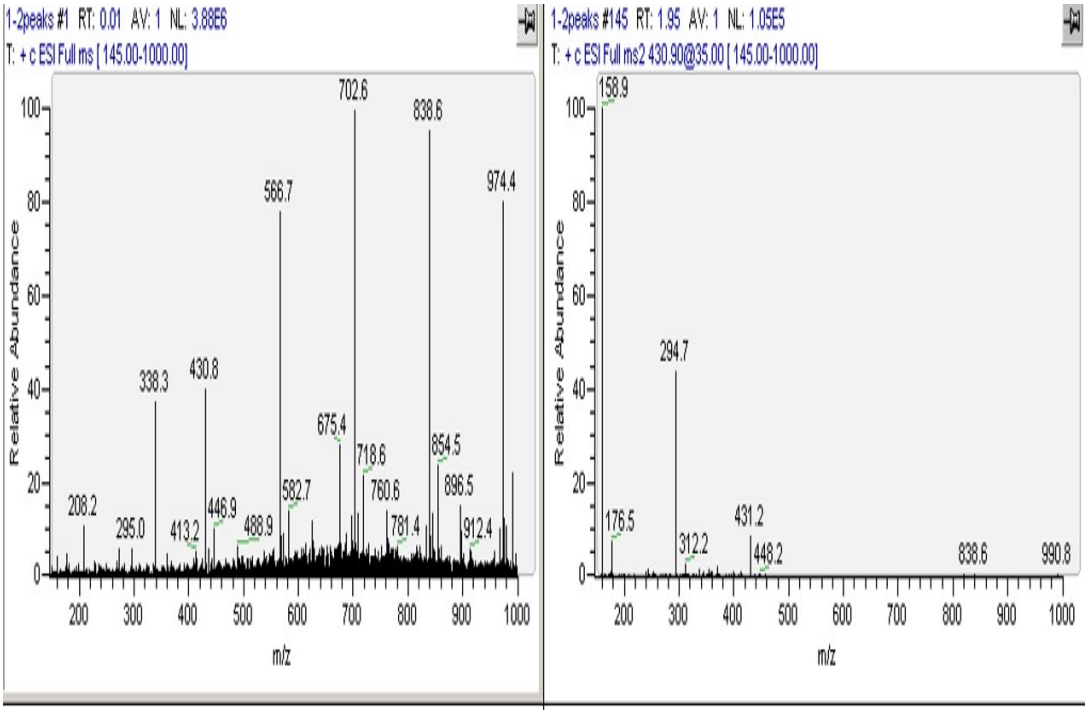

Figure 3.4 Mass Spectroscopy data after HPLC Separation for Decomposed Ascorbic acid. Left side represents spectrum view over the range of 200 – 1000 m/z ratio. Right side represents Further breakdown of 430.8 m/z ratio.

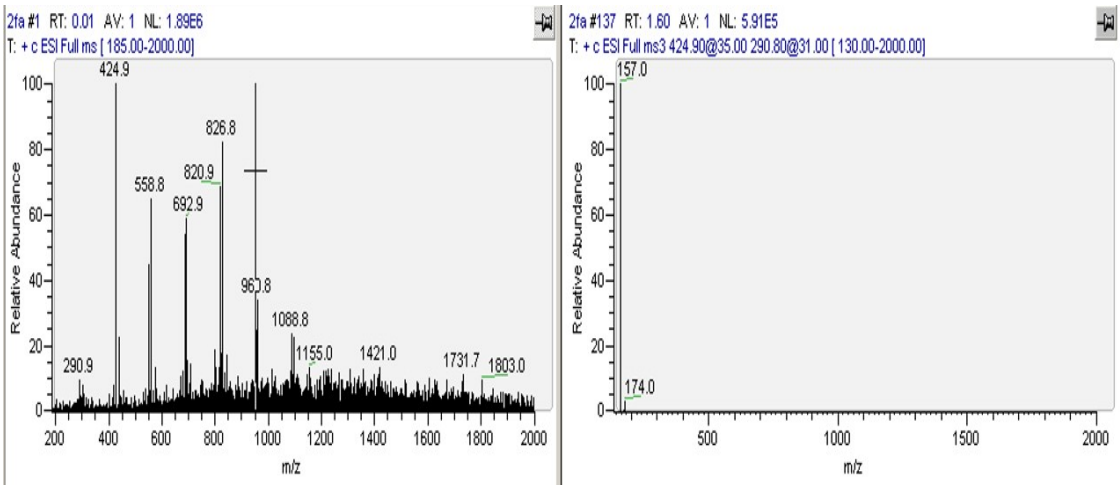

Figure 3.5 Mass Spectroscopy data for 2-Furoic acid. Left side represents spectrum view over the range of 200 – 2000 m/z ratio. Right side represents Further breakdown of 424.9 m/z ratio.

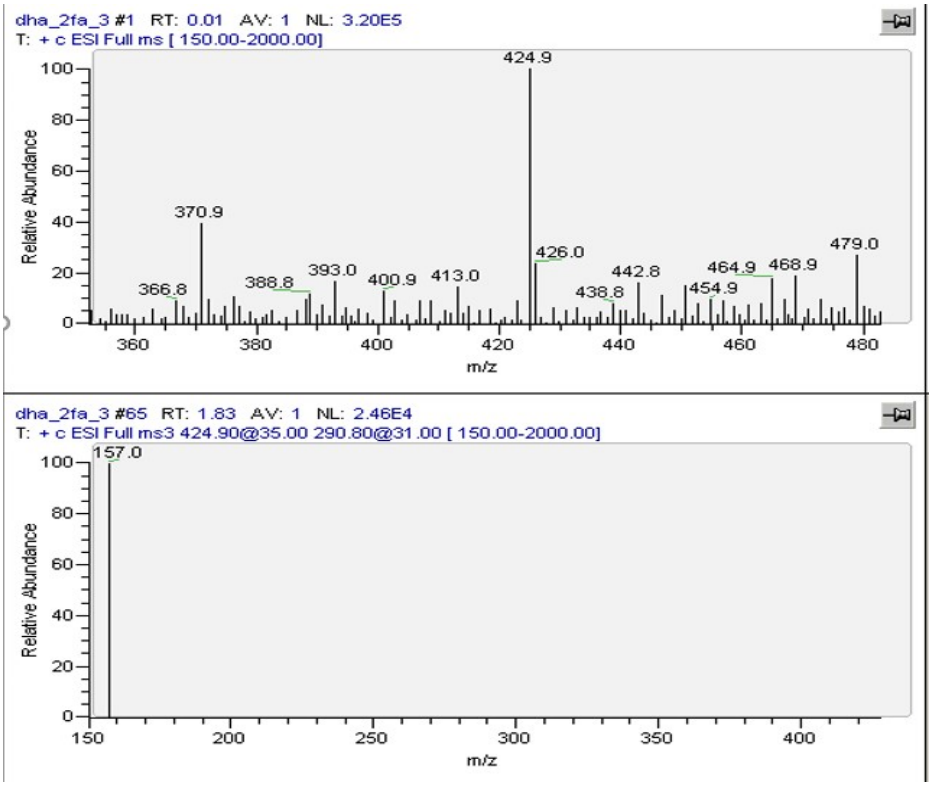

Figure 3.6 Mass Spectroscopy data for DHA + 2FA. Breakdown of 424.9 m/z ratio.

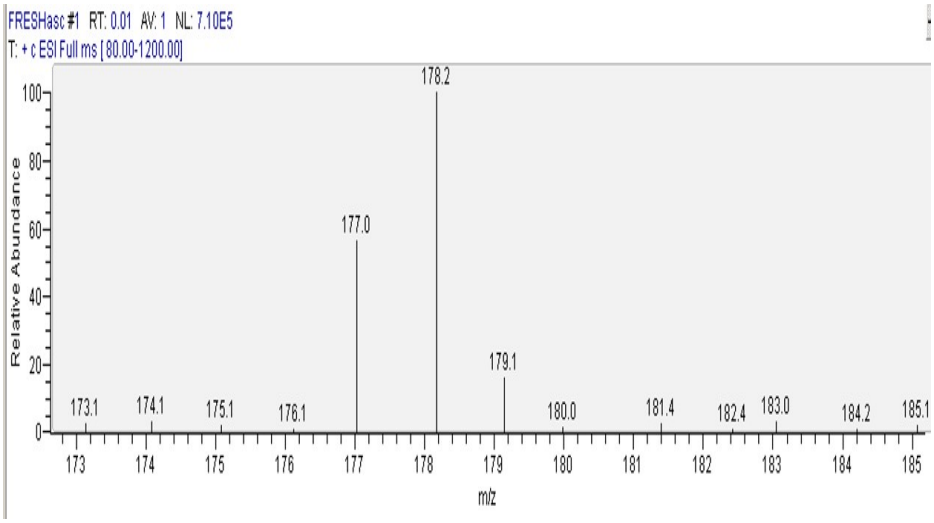

Figure 3.7 Mass Spectroscopy data for Fresh ascorbic acid sample.

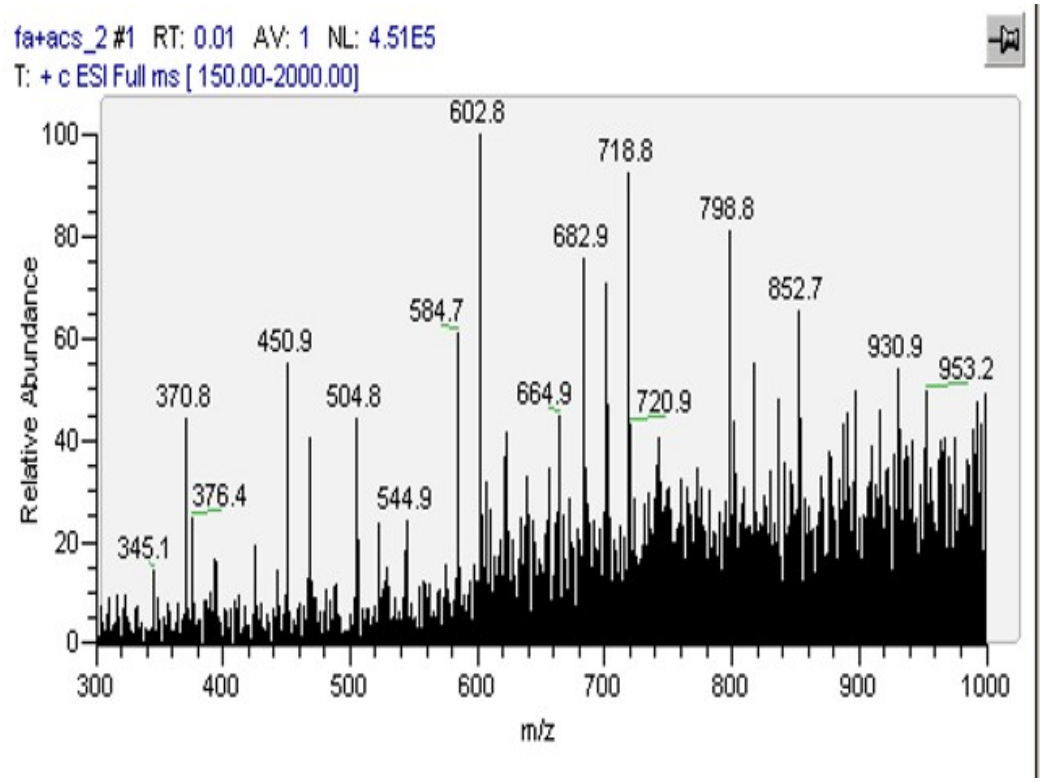

Figure 3.8 Mass Spectroscopy data for Fresh ascorbic acid + 2-furoic acid in 50-50% ratio. NO overlap of compounds for comparing with decomposed ascorbic or 2-furoic acid mass spec data.

3.4 DETERMINATION OF NO GENERATION

To calculate the amount of NO release from each compound we designed the folloing expeperiemnt. Nitrogen was used as a sweeping gas for all the four analyses. SNAP crystals of 0.8 mg were used in each experiment. SNAP was placed in a dark vial and connected to the NO analyzer with the sweeping gas. After 10 minutes, 2ml of Phosphate Buffer Saline (PBS) of concentration mixed with Chelex 100 is added to the SNAP crystals. This causes NO release because of reaction between trace metal contaminants and SNAP. After a period of 1 hour we injected the one of the four compounds listed above recording the NO profile, as the readings reaches a plateau we inject 1mm $CuCl_2$ to it in order to initiate complete decomposition of the SNAP remaining in solution.

3.5 DATA ANALYSIS FOR NO ANALYZER

Each set of experiments was performed in triplicates. Dark colored vial was used to avoid NO release through photoinitiation. Chelex 100 is used a chelating agent for purification of compounds via ion exchange. Copper chloride was added in the end just to show that system still contains nitric oxide.

Initial readings show zero nitric oxide released for first 10 mins which indicates that SNAP crystals used are stable. Upon addition of PBS it releases nitric oxide. Once the PBS readings reaches a plateau we inject the compound of interest. Data shows that 2-Furoic acid releases maximum amount of NO i.e. 900 PPB. When ascorbic acid is combined with 2-furoic acid the amount of NO released is almost 1/3 in comparison to that intuited from pure 2-furoic acid.

Table 3.3 Represents Amount of NO released from 2-furoic acid, dehydroascorbic acid and fresh ascorbic acid

Compounds	Amount of NO Released
2 Furoic acid	$101x10^{-08}$ moles
Dehydroascorbic acid	$1.01x10^{-8}$ moles
Fresh Ascorbic acid	$28.2x10^{-08}$moles

Table 3.4 Represents Amount of NO released from 2-furoic acid + ascorbic, dehydroascorbic acid + ascorbic acid and fresh ascorbic acid

Compounds	Amount of NO Released
2 Furoic acid + ascorbic acid	15.1×10^{-8} moles
Dehydroascorbic acid + ascorbic	11.4×10^{-8} moles
2 furoic acid	30.4×10^{-8} moles

Figure 3.9 shows a direct comparison between the NO released from the 2-Furoic acid, Dehydroascorbic acid and Fresh ascorbic acid. Last spike in the Figure is due to injection of Copper Chloride which reacts with SNAP to give huge amount of NO.

From this data, it is very suggestive that 2-furoic acid or dimers of this compound are responsible for initiating NO release from RSNOs.

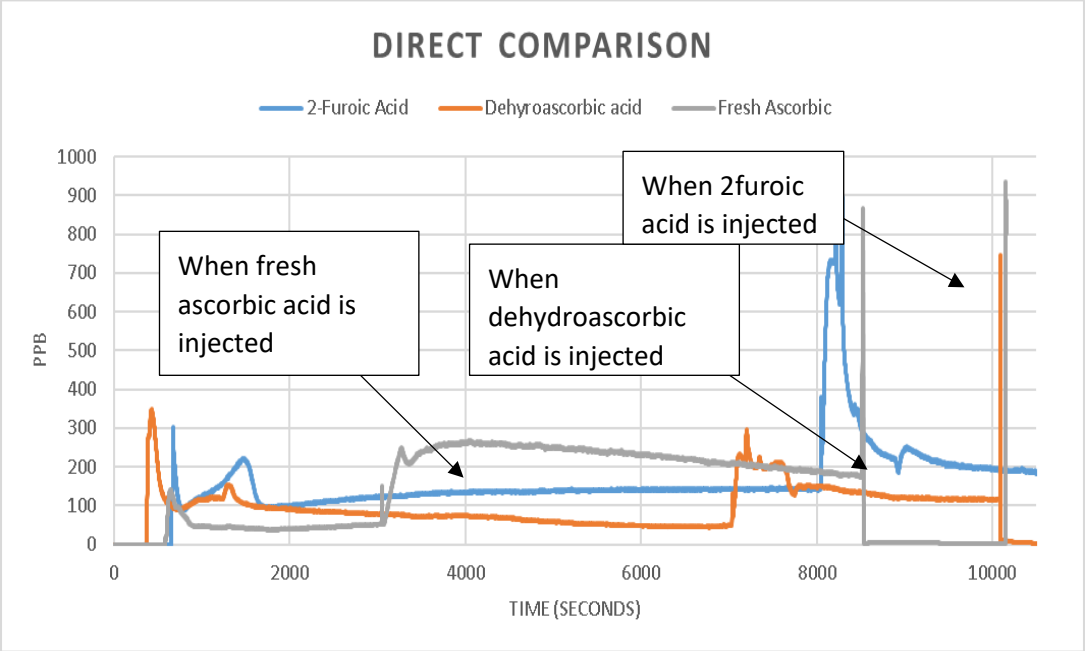

Figure 3.9 Direct comparison between 2-furoic acid, Dehydroascorbic acid and fresh ascorbic acid. 2-furoic acid releases highest amount of NO.

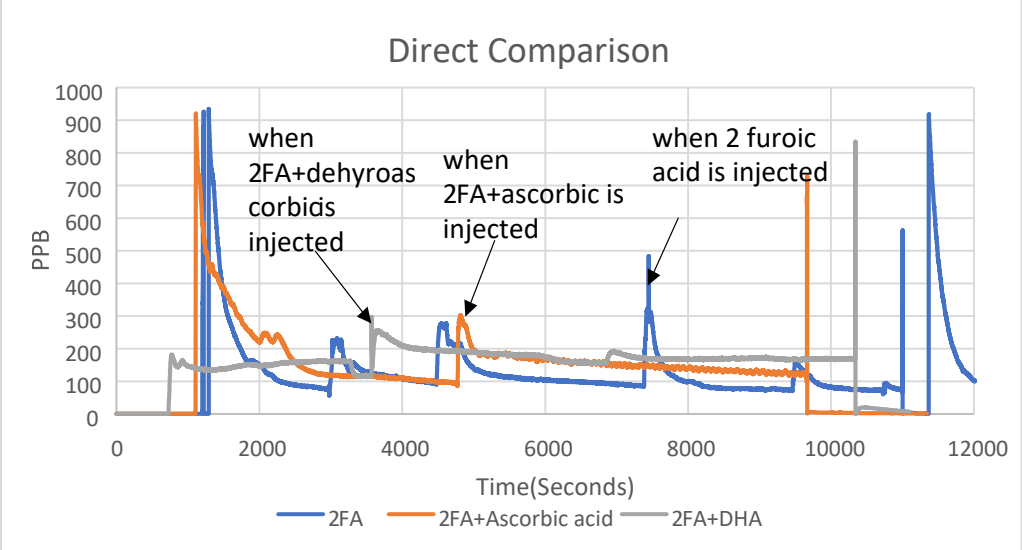

Figure 3.10 Direct comparison between 2-furoic acid, dehydroascorbic acid & fresh ascorbic acid. 2-furoic acid releases highest amount of NO.

3.6. CONCLUSION

The goal of the project was to identify the compound responsible for production of NO. In order to do that there was a need to study and identify decomposition products of ascorbic. Compound separation was done using HPLC. Once the compounds were separated real time NO analysis was carried out on both decomposed ascorbic acid and fresh ascorbic acid taken from HPLC using 3-D printed device as a medium. It was concluded that decomposed ascorbic acid produces more NO compared to fresh ascorbic acid. Next step was to identify the compounds present in the decomposed ascorbic acid and then test those compounds separately for NO generation. Identification of compounds were carried out using mass spectroscopy.

Based on the MS data and the testing of pure compounds from the ascorbic acid decomposition cascade, the potential compounds responsible for the release of NO from RSNOs was narrowed down to either DHA or 2-FA. All the compounds tested showed

some amount of NO released, but 2-FA, by itself, showed almost three times the amount of NO released compared to other compounds. Combination of both fresh ascorbic acid and 2-FA release NO similar to that of the combination of DHA and 2-FA which indicates that the ascorbic acid suppresses the presence of 2-furoic acid in the system, similar to be said for DHA since it is an immediate decomposition product of ascorbic acid.

Thus, both the MS data where peak of m/z i.e. 424.9 ratio of the 2-furoic acid coincides with the peak of the HPLC separated decomposed ascorbic acid. Furthermore, the NO data showing maximum amount of NO released is from 2-FA out of the combination of all the compounds. Therefore, it is likely that 2-furoic acid is the agent that causes NO release instead of high concentrations of ascorbic acid.

2-furoic acid is a carboxylic acid, consisting of a five-membered aromatic ring. It is an organic product most widely used in food products as a preservative and a flavoring agent. 2-furoic acid is no-toxic. Whole goal of the project was to eventually develop a polymer with 2-furoic acid in its backbone, which can be coated on a medical device upon contact with blood it leaches and reacts with biological RSNOs to produce NO.

Since 2-furoic acid is potentially used in many of the food products so the human body is not subjected to any toxic products.

3.7 REFERENCES

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4. CONCLUSION AND FUTURE DIRECTIONS

With the increasing number of illness which cannot be tackled with medication alone there is an increasing need for implantable medical devices. Studies for improving the quality and design of implantable devices has been carried out since early 1960s and tremendous progress has been made in miniaturization of reliable devices that are capable of monitoring and improving physiological function in vitro. There is still a stark crop in performance when these devices are actually placed in the complex and dynamic in vivo environment. Understanding the host response is extremely important in development of implantable devices. One of the emerging solution is nitric oxide (NO) releasing polymer coatings on implanted medical devices. The focus of the work described herein is to identify the NO generating decomposition product of ascorbic acid.

Nitric oxide is potentially viable option to help control the coagulation cascade and wound healing responses, i.e. driving pathways that determine ultimate host response. NO has a diverse array of functions in vivo including help controlling the blood vessel tone and adhesion receptor expression. NO is also a potent inhibitor of platelet aggregation and activation. NO is also involved in endothelium cell and smooth muscle cell proliferation; it has a direct role in destruction of bacteria during phagocytosis by macrophages. Vascular endothelium cells are estimated to generate NO flux of 1.0-4.0 x 10^{-10} $mol \cdot cm^{-2}$ min^{-1} continuously and is the primary basis for the non-thrombogenic properties of the vascular endothelium, surface which elicits normal host response (i.e., the coagulation cascade is not activated) despite constant contact with precursor molecules to the intrinsic coagulation cascade. The endogenous NO supply diffuses into the lumen of the blood vessels and smooth muscles cells that surrounds all the arteries where it plays an important role in preventing platelet activation in healthy blood vessels.

The innate physiological functions of NO make it a strong candidate for improving the biocompatibility of the implanted devices. NO releasing sensors will be capable of controlling both platelet adhesion and activation along with minimizing vasocontraction. NO releasing subcutaneous implanted sensors have been shown to increase blood flow to the sensor surface, increase transfer of analyte, reduce bacterial adhesion and reduce

fibrous encapuslation. Because the half-life of NO in the biological system is 0.5-1.8ms, the NO generated should not create any systemic effects.

There are three known mechanics of NO generation from S-nitrosothiols (RSNOs). Copper mediated decomposition in which Cu^{2+} is first reduced to Cu^{+} and then Cu^{+} reduces RSNOs to NO. Common species used to reduce Cu^{2+} to Cu^{+} are free thiolate ions present as impurities in RSNO solutions and ascorbic acid. Ascorbic acid itself has been reported to directly interact with RSNOs when it is present at greater than millimolar concentrations to directly reduce RSNO to NO and produce free thiol groups. The mechanism of this reaction is not known. The third known mechanism of NO generation from RSNOs is by means of photoinitiation.

The work presented here focused on elucidating the species responsible forNO generation from ascorbic acid. Literature states that ascorbic acid when reacts with RSNOs it releases NO if present in more than millimolar concentration but we have shown that it is not actually ascorbic acid that causes NO release, rather a product that results from ascorbic acid decomposition under ambient conidtions . When ascorbic acid decomposes, it produces a complex mixture, it is one of these compounds that is actually responsible for the NO generation. Main goal of this project was to study the decomposition products of ascorbic acid and identify the compound responsible for generating NO.

UV-VIS spectroscopy was used to identify the absorption wavelength for both fresh ascorbic acid and decomposed ascorbic acid. Data suggests that peak absorption wavelength of ascorbic acid is 263nm which is consistent with the literature.

Decomposed ascorbic acid showed several peaks with consistent change of absorbance. Additionally, the absorbance of the ascorbic acid peak at 263nm is reduced in intensity from 0.56 to 0.25 after 8 days of passive decomposition, indicating a significant loss of parent compound. This confirms that decomposition products of ascorbic acid are present in substantial quantities in the solution. The peaks resulting from these compounds have absorbance maximum ranging from 200 – 400 nm. This information was vital because it will help setup up the detection range for HPLC while physically separating these compounds.

HPLC system was used for separation of these decomposition compounds. Under the isocratic separation conditions utilized, ascorbic acid has a retention time 1.407 minutes and no other compound being eluted from fresh ascorbic acid sample. Decomposed ascorbic acid showed two peaks at 0.691 minutes and 1.198 minutes which surely suggests presence of more than one compound in decomposed ascorbic acid sample. A UV-Spectrum analysis was carried out on the two peaks obtained which showed how the absorbance for the peaks changes over time. Th UV-Vis spectrum obtained from the diode array detector in the HPLC system showed that for the first peak absorbance is 0.05 and gradually changes as the peak descend and when the second peak ascends, the absorbance is 0.16 indicating a different compound and it reaches to 0.2 when the peak descends; Once again providing a clear evidence of presence of more than one compound.

To identify which component(s) in these 2 solutions (fresh and decomposed ascorbic acid) was responsible for initiating NO release from SNAP, a novel device was designed and used to couple the separation capacity of the HPLC with the direct NO measurement using NO analyzer. NO analysis was performed on Fresh ascorbic acid, decomposed ascorbic acid and a blank sample. The amount of NO released from the decomposed ascorbic acid is more than twice the amount released from fresh ascorbic acid.

This proves that when ascorbic acid decomposes it release more NO than fresh ascorbic acid, which concludes that a new compound is formed when ascorbic acid decomposes.

Table 4.1 Represents Total NO released by blank sample, fresh and decomposed ascorbic acid

Compounds	Total NO released ±(SD)
Fresh ascorbic acid	$170x10^{-12}$ ($1.07x10^{-10}$) moles
Decomposed ascorbic acid	$350x10^{-12}$ ($3.50x10^{-10}$) moles
Blank sample	$6.29x10^{-12}$ ($6.29x10^{-12}$) moles

Next step was to identify the compound responsible for NO release. Mass Spectroscopy was used for identifying decomposition product of ascorbic acid. Mass spectrum of a sample is a pattern representing the distribution of ions by mass (more

correctly: mass-to-charge ratio) in a sample. First MS analysis was carried out on the HPLC separated compounds for decomposed ascorbic acid. Spectrum showed number of peaks which suggests presence of multiple compounds. Each peak was further broken down for full mass analysis.

Compounds with m/z charge ratio higher than 550 m/z (mass by charge ratio) did not break down into smaller fragments. However, peak with 430.8 m/z ratio was further broken down to 158.9 m/z ratio. Using the ascorbic acid decomposition cascade as reference we suspected this peak to be either dimers of dehydroascorbic acid or 2-furoic acid keeping their molecular mass in account with either addition or donation of hydrogen ions. After narrowing it down to two compounds i.e. dehydro-ascorbic acid and 2-furoic acid we performed MS on these two compounds to see if there was an overlap.

Reference material 2-Furoic acid and Dehydro-ascorbic acid of highest purity was purchased for testing NO release and perform mass spectroscopy to see if any compounds overlap with the existing data.

Table 4.2 lists compounds selected for further testing suggested from MS analysis:

Compound I	2-furoic acid (2FA)	1mm
Compound II	Dehydro-ascorbic acid (DHA)	1mm
Compound III	DHA + 2-furoic acid	1mm(DHA) 50% + 1mm 2-FA (50%
Compound IV	2-furoic acid + Ascorbic acid	1mm (2FA) 50% + 1mm Ascorbic acid 50%
Compound V	Ascorbic acid	1mm

Table 4.3 represents the compounds selected for testing along with their respective concentration.

Compound	m/z ratio	m/z ratio (Further breakdown)
HPLC separated decomposed ascorbic acid	430.8	158.9
2-Furoic acid	424.9	157
DHA + 2FA	424.9	157
Ascorbic acid	178	
Ascorbic acid + 2FA	Multiple peaks	

Table 3.2 Represents m/z ratio of each of the compound tested, it also includes a further breakdown of the m/z ratio which were possible.

2-Furoic acid had compound with m/z ratio i.e.424.9 which was found in decomposed ascorbic acid i.e. compound with 424.9 m/z broke down into 157m/z. So, did the combination of DHA + 2-FA losing a combination of hydrogen atoms i.e. compound with m/z 424.9 which further broke down in to 157 m/z. Since there is an overlap of m/z ratio from both 2FA and 2FA + DHA with the originally separated decomposed ascorbic acid it was concluded that either 2FA or DHA is one of the decomposition product of the ascorbic acid responsible for the large amount of NO release. Further analysis on the compound i.e. 2FA + Ascorbic acid shows no overlap in m/z ratio with the HPLC separated decomposed ascorbic, which concludes that ascorbic acid suppresses the presence of 2-furoic acid and forms a complex mixture, that might be one of the reasons why ascorbic acid produces less amount of NO as compared to 2 furoic acid.

Last step was to perform NO analysis on these compounds to provide definitive evidence that 2-furoic acid is the actual compound that causes NO release. Data showed that 2-Furoic acid releases maximum amount of NO i.e. 1.01×10^{-6} moles. When ascorbic acid is combined with 2-furoic acid the amount of NO released is almost 1/3 in comparison to that intuited from pure 2-furoic acid. From this data, it is very suggestive that 2-furoic

acid or dimers of this compound are responsible for initiating NO release from RSNOs. Therefore, it is likely that 2-furoic acid is the agent that causes NO release instead of high concentrations of ascorbic acid.

For the scope of the experiments a very basic design of a 3-D printed device was created, which functioned sufficiently under these initial conditions. For future work, the 3-D printed device should be optimized i.e. the size and shape of the reaction chamber,the angles for the incoming solutions into the reaction chamber, the dimensions of the channels into the reaction chamber. These optimizations should make the device generally more applicable to other potential HPLC separations coupled to gas reactant products.

2-furoic acid is a carboxylic acid, consisting of a five-membered aromatic ring. It is an organic product most widely used in food products as a preservative and a flavoring agent. 2-furoic acid is no-toxic. Whole goal of the project was to eventually develop a polymer with 2-furoic acid in its backbone, which can be coated on a medical device upon contact with blood it leaches and reacts with biological RSNOs to produce NO. Since 2-furoic acid is potentially used in many of the food products so the human body is not subjected to any toxic products. Because this a simple compound that is stable, there are a wide variety of different conditions in which it may be used to create NO generating materials and surfaces.