A POROUS BIOCOMPATIBLE MATERIAL FOR DRUG DELIVERY

A PROJECT REPORT

Submitted by

VIJAYALAKSHMI NANDAKUMAR

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of

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ANNA UNIVERSITY: CHENNAI 600 025

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CENTRAL LEATHER RESEARCH INSTITUTE
(Council of Scientific & Industrial Research)
Adyar, Chennai 600 020 India

Dr. A. Rajaram
Scientist F
Biophysics Laboratory

Certificate

This is to certify that this dissertation entitled " **A Porus Biocompatible Material for Drug Delivery**" submitted to the Kumaraguru College of Technology, Coimbatore, in partial fulfilment of the requirements for the award of Degree of Bachelor of Technology in Industrial Biotechnology, is a record of work done by **Vijayalakshmi Nandakumar** during her period of training from January 18, 2005 to March 31, 2006 in the Biophysics Laboratory, Central Leather Research Institute, Adyar, Chennai –20, under my supervision.

Place: Chennai

Date: 1-4-06

Rajaram

Dr. A. Rajaram

(Supervisor)

BONAFIDE CERTIFICATE

Certified that this project report **"A POROUS BIOCOMPATIBLE MATERIAL FOR DRUG DELIVERY"is the bonafide work of "VIJAYALAKSHMI NANDAKUMAR"who carried out the project work under my supervision.**

SIGNATURE

Dr.S.Sadasivam
HEAD OF THE DEPARTMENT

Department of Biotechnology
Kumaraguru College Of Technology
Chinnavedampatti
Coimbatore-641006.

18/4/06
SIGNATURE

Dr.Stephen Rapheal
SUPERVISOR

Senior Lecturer

Department of Biotechnology
Kumaraguru College Of Technology
Chinnavedampatti
Coimbatore-641006.

CERTIFICATE OF EVALUATION

COLLEGE : Kumaraguru College Of Technology

BRANCH : Industrial Biotechnology

SEMESTER : Eighth Semester

NAME OF THE STUDENTS	TITLE OF THE PROJECT	NAME OF THE SUPERVISOR WITH DESIGNATION
Vijayalakshmi Nandakumar.	"A Porous Biocompatible material for Drug Delivery"	Dr.Stephen Raphael Senior Lecturer.

The Report of the project work submitted by the above students in partial fulfillment for the award of Bachelor of Technology degree in Industrial Biotechnology of Anna University were evaluated and confirmed to be report of the work done by the above students and then evaluated.

(INTERNAL EXAMINER)
29/04/06.

29/4/06.
(EXTERNAL EXAMINER)

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ABSTRACT:

Artificial implants had given rise to a lot of complications like local infections, donor site morbidity, donor shortage,immunologic response etc.To overcome these demerits to a considerable extent, a porous biocompatible material has been studied and investigated .On SEM analysis, the material seemed to be very porous with substantial interconnectivity suitable for drug delivery. The material was heated at 900°C overnight to remove the organic matter. Incorporation of Penicillin-G into the matrix was carried out by a simple vacuum infiltration method. The in vitro release characteristics of the antibiotics were studied in phosphate buffered saline (PBS) of pH 7 at 37°C by recording it's release profile and estimating the amount of Penicillin G.The material was characterized to study it's collagen content, biochemical components and fluorescence nature. The organic matrix of the shell was decalcified in 0.5M EDTA and subjected to acid hydrolysis.The collagen content of the hydrolysate was measured by the estimation of hydroxy proline in the material. The acid hydrolysate has also been investigated for the presence of other amino acids such as Tyrosine and Tryptophan since the presence of non-collagenous proteins was indicated in the remaining part after collagen estimation. The fluorescence of the

material was studied by fluorescence spectroscopy. The UV and fluorescence spectra of authentic samples of Tyrosine and Tryptophan were compared with that of acid hydrolysate to identify the presence of specific amino acids. In addition, Type I collagen from Rat- tail tendon has also been purified, which was used as a standard throughout the study.

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CHAPTER 1

INTRODUCTION

INTRODUCTION:

Tissue engineering is a field of research, which aims at regenerating tissues and organs. A major goal of tissue engineering is the preparation of a suitable scaffold for cells to proliferate, migrate and differentiate. The scaffold should assist cells to form the desired tissue. In tissues and organs,major extracellular matrix components are collagen,elastin and glycosaminoglycans.Each tissue / organ has its own unique set and content of these biomolecules.Bone like materials are needed to correct orthopedic defects, bone neoplasia and tumors,pseudoarthrosis treatment, stabilization of spinal segments as well as in maxillofacial,craniofacial,orthopedic,reconstructive,trauma and neck and head surgery.

BIOMATERIAL:

Biomaterials are materials (synthetic and natural; solid and sometimes liquid) that are used in medical devices or in contact with biological systems. Although biomaterials are primarily used for medical applications, they are also used to grow cells in culture, to assay for blood proteins in the clinical laboratory, in processing biomolecules in biotechnology, for fertility regulation implants in cattle, in diagnostic gene arrays, and in the investigational biochips. The commonality of these applications is the interaction between biological systems and synthetic or modified natural materials.

Biomaterials are rarely used on their own but are more commonly integrated into devices or implants. Thus, the subject cannot be explored without also considering biomedical devices and the biodesigning of

scaffolds to promote tissue growth has received a huge amount of focus in recent years. Scaffolds must be biodegradable and can be made from either natural or synthetic polymers. The purpose of the scaffold is to serve as a temporary support for growing cells to form new tissue around. As the scaffold degrades, the end result consists of only cells and tissue.

In general there are several approaches to using scaffolds for tissue engineering. One approach is to fabricate a porous scaffold of defined shape and architecture onto which cells are seeded. The cell scaffold is placed in a tissue culture system to support growth, called a bioreactor. Once the 3D tissue equivalent has formed, the engineered tissue is surgically implanted into the host and fixed into place. Alternatively, the scaffold containing cells can be directly implanted, allowing the body to serve as the bioreactor. A third approach is to design a scaffold that forms when placed in the body, such as an injectable liquid that gels *in situ*. The attractive feature of this process is that cells can be combined with a liquid solution and injected into the desired regions, creating a minimally invasive surgical procedure. Once injected, the liquid solidifies, encapsulating the cells within the scaffold

NATURAL BIOMATERIALS:

Natural polymers, or polymers derived from living creatures, are of great interest in the biomaterials field. In the area of tissue-engineering, for example, scientists and engineers look for scaffolds on which one may successfully grow cells to replace damaged tissue. Typically, it is desirable for these scaffolds to be:

- Biodegradable

- Non-toxic/non-inflammatory
- Mechanically similar to the tissue to be replaced
- Highly porous
- Encouraging of cell attachment and growth
- Easy and cheap to manufacture
- Capable of attachment with other molecules (to potentially increase scaffold interaction with normal tissue)

Natural polymers often easily fulfill these expectations, as they are naturally engineered to work well within the living beings from which they come. Three examples of natural polymers that have been commonly studied for use as biomaterials are: collagen, chitosan and alginate.

COLLAGEN:

Collagen is the main protein of connective tissue in animals and the most abundant protein in mammals, making up about 1/4 of the total. It is one of the long, fibrous structural proteins whose functions are quite different from those of globular proteins such as enzymes. It is tough and inextensible, with great tensile strength, and is the main component of cartilage, ligaments and tendons, and the main protein component of skin ,bone and teeth.

Being natural it offers several advantages like aiding in wound healing, low antigenicity,biocompatible,biodegradable,ability to be processed into variety of forms,resorbable and can be readily isolated and purified in large quantities. Apart from these features it has served as an excellent carrier for drug-delivery systems. TypeI collagen is a triple helix with short non-helical terminal domains called telopeptides,which is thought to be responsible for

causing an immunogenic response when introduced into Xenogenic hosts. To eliminate this problem,solubilized skin collagen can be digested with pepsin to remove telopeptides.The main sources of type-I collagen for biomedical applications are either animal skin(predominantly bovine or porcine)or Achilles tendon, which is usually of bovine or equine origin.

Structure:

Collagen comprises three polypeptide chains (α-chains), which form a unique triple-helical structure[1, 2]. For the three chains to wind into a triple helix they must have the smallest amino acid, glycine, at every third residue along each chain. Each of the three chains therefore has the repeating structure Gly-Xaa-Yaa, in which Xaa and Yaa can be any amino acid but are frequently the imino acids proline and hydroxyproline.

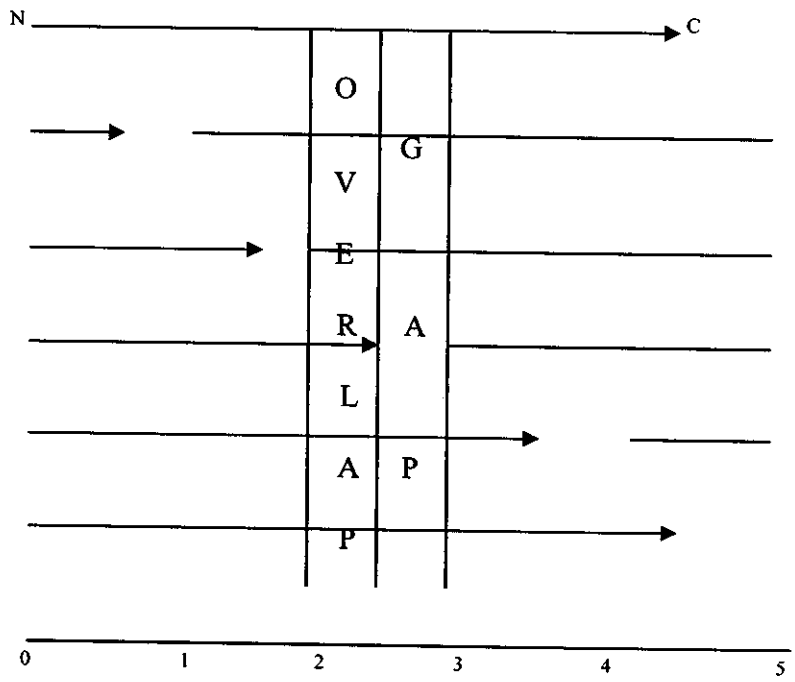
Collagen has an unusual amino acid composition and sequence. Glycine (Gly) is found at almost every third residue, and collagen contains large amounts of proline, (Pro) — as well as two uncommon derivative amino acids not directly inserted during translation of mRNA: hydroxyproline (Hypro) and hydroxylysine. Prolines and lysines at specific locations relative to glycine are modified post-translationally by different enzymes, both of which require vitamin C as a cofactor.

The *tropocollagen* subunit is a rod about 300 nm long and 1.5 nm in diameter, made up of three polypeptide strands, each of which is a left-handed helix. They are twisted together into a right-handed coiled coil, a triple helix, a cooperative quaternary structure stabilized by numerous hydrogen bonds.

COLLAGEN TRIPLE HELIX

There are non helical extensions (telopeptide) at both the N and C terminal ends. These telopeptides which comprises of 16 and 25 amino acids in the N and C terminal ends respectievely, do not have glycine at every third residue and are relatively rich in hydrophobic and charged amino acids. The lysine or hydroxylysine at positions 9(N-terminus) and 1047(C-terminus) in telopeptide region of the molecule are oxidised by the enzyme lysly oxidase to aldehydic forms allysine and hydroxyallysine which are involved in iniatiation of cross links of collagen .

The triple helical collagen molecules are arranged in a quarter staggered array giving rise to 64nm periodicity when viewed under transmission electron microscopy . The molecule is highly stable not attacked by pepsin,trypsin and other proteases. The structural rigidity of the molecule can be understood from its quarter staggered arrangement. This gives rise to overlap zones and hole zones visible in electron microscopy.



Schematic view of the axial stagger relationship of TypeI collagen molecules (arrows) in the native fibril.The molecules are about 4.5D long,whee D=67nm.The non-integral length results in overlap and gap regions with protein densities.It is believed that the 1D and 4D staggers,as shown,are important nearest –neighbour relationships,but they may not be the only ones in the fibril

In the case of rat tail's collagen, the arrangement is more organized that, when the molecule is treated with 0.5M Acetic acid, it leads to the cleavage of double bond in C=NH,leading to the dissolvation of collagen. But in the really hard core materials like Bone,cartilages,skin and turtle shell(material used in this project),the Schiff's base formed undergoes a lot of rearrangements leading to complicated cross-links which is really hard to dissolve. The material also exhibits some kind of fluorescence which may be due to fluorescence components present in the inorganic ions,non-collagenous or collagenous part of the material.

Classification of collagen :

Collagen occurs in many places throughout the body, and occurs in different forms known as types, which include:

- Type I collagen - This is the most abundant collagen of the human body. It is present in scar tissue, the end product when tissue heals by repair. It is found in tendons and the organic part of bone.
- Type II collagen - Articular cartilage
- Type III collagen - This is the collagen of granulation tissue, and is produced quickly by young fibroblasts before the tougher type I collagen is synthesized.
- Type IV collagen - basal lamina; eye lens
- Type V collagen - most interstitial tissue, assoc. with type I, associated with placenta
- Type VI collagen - most interstitial tissue, assoc. with type I
- Type VII collagen - epithelia
- Type VIII collagen - some endothelial cells
- Type IX collagen - cartilage, assoc. with type II
- Type X collagen - hypertrophic and mineralizing cartilage
- Type XI collagen - cartilage
- Type XII collagen - interacts with types I and III
- Type XIII collagen - interacts with types I and II

There are 27 types of collagen in total.

Collagen can be resorbed into the body, is non-toxic, produces only a minimal immune response (even between different species), and is excellent

for attachment and biological interaction with cells. Collagen may also be processed into a variety of formats, including porous sponges, gels, and sheets, and can be cross-linked with chemicals to make it stronger or to alter its degradation rate. Cells grown on collagen often come close to behaving as they do within the body, which is why collagen is so promising when one is trying to duplicate natural tissue function and healing.

However, some disadvantages to using collagen as a cell substrate do exist. Depending on how it is processed, collagen can potentially cause alteration of cell behavior (*e.g.*, changes in growth or movement), have inappropriate mechanical properties, or undergo contraction (shrinkage). Because cells interact so easily with collagen, cells can actually pull and reorganize collagen fibers, causing scaffolds to lose their shape if they are not properly stabilized by cross linking or mixing with another less "vulnerable" material. Fortunately, collagen can be easily combined with other biological or synthetic materials to improve its mechanical properties or change the way cells behave when grown upon it.

CHITOSAN:

Chitosan is derived from chitin, a type of polysaccharide (sugar) that is present in the hard exoskeletons of shellfish like shrimp and crab, exoskeletons of crustaceans and insects as well in the cell walls of some bacteria and fungi. Chitin, in fact, is one of the most abundant polysaccharides found in nature, making chitosan a plentiful and relatively inexpensive product. Chitosan has recently sparked interest in the tissue-engineering field due to several desirable properties:

- Minimal foreign body reaction
- Mild processing conditions (synthetic polymers often need to be dissolved in harsh chemicals; chitosan will dissolve in water based on pH)
- Controllable mechanical/biodegradation properties (such as scaffold porosity or polymer length)
- Availability of chemical side groups for attachment to other molecules

It is processed by removing the shells from shellfish such as shrimp, lobster, and crabs. The shells are then ground into powder and this powder is then deacetylated. Chitosan is extremely popular all over the world in water purification plants.

.Chitosan exhibits a variety of physiochemical and biological properties resulting in numerous applications in fields such as waste and water-treatment,agriculture,fabric textiles,cosmetics,nutritional enhancement and food processing. Due to lack of toxicity and allergenicity, biocompatibility and biodegradability it is a very attractive substance for applications as a biomaterial.

Chitosan has already been investigated for use in the engineering of cartilage, nerve, and liver tissue. Chitosan has also been studied for use in wound dressings and drug delivery devices. Current difficulties with using chitosan as a polymer scaffold in tissue engineering, however, include low strength and inconsistent behavior with seeded cells [. Fortunately, chitosan may be easily combined with other materials in order to increase its strength and cell-attachment potential. Mixtures with synthetic polymers such as poly (vinyl alcohol) and poly (ethylene glycol), or natural polymers such as

collagen, have already been produced. These combinations have displayed promise for improving the performance of the combined construct over the behaviour of either component alone.

ALGINATE:

Alginate is a polysaccharide derived from brown seaweed. Like chitosan, alginate can be processed easily in water and has been found to be fairly non-toxic and non-inflammatory, enough so that it has been approved in some countries for wound dressing and for use in food products. Alginate is biodegradable, has controllable porosity, and may be linked to other biologically active molecules. Alginate forms a solid gel under mild processing conditions, which allows it to be used for entrapping cells into beads and other shapes. Encapsulation of certain cell types into alginate beads may actually enhance cell survival and growth. In addition, alginate has been explored for use in liver, nerve, heart, and cartilage tissue engineering. Unfortunately, some drawbacks to alginate include mechanical weakness and poor cell adhesion. Again, to overcome these limitations, the strength and cell behavior of alginate have been enhanced by mixtures with other materials, including the natural polymers agarose and chitosan .

ANTIBIOTICS TO PREVENT LOCAL INFECTION:

Usually for bone infection,vancomycin,ciprofloxacin,gentamicin are used.But the initial choice of antibiotic inevitably depends on the causal pathogen and its sensitivity pattern. Antibiotic considered bactericidal against the infecting organisms are often considered necessary, although the need for this with respect to osteomyelitis has not been experimentally proven. One such antibiotic is cephalexin,semi synthetic

cephalosporin.Penicillin is a water-soluble antibiotic(water+acetone) and it has an inhibitory effect against Streptococcus,Enterococcus species in cases of meningitis,osteomyelitis ,etc.

The material used here is obtained from the carapace of turtle shell. The shell is made up of two components viz;an inorganic matrix and an organic matrix. After removal of organic matrix by ashing,porous network with high interconnectivity is observed. The large surface area of this porous matrix is exploited to hold high concentrations of the antibiotic, which is loaded by a simple vacuum infiltration method. It consists of a highly porous network, with substantial interconnectivity. The release characteristic of the antibiotic loaded matrix is studied. Investigations are carried out to check whether the organic matrix is made up of collagen.

CHAPTER 2

LITERATURE REVIEW

LITERATURE REVIEW:

The need for biomaterials and the potential role for natural tissues in various applications such as cardiac valves, bone grafts, bone injuries and vascular grafts have been recently realized. Upon implantation, unmodified natural materials are subject to chemical and enzymatic degradation, seriously decreasing the life of the prosthesis.

NEED FOR NATURAL BIOMATERIAL:

Large bone repair is always a tough problem in surgery. The natural biological material for bone defect repair including allografts and autografts, each has its own shortcomings such as donor site, morbidity and donor shortage for autografts,immunological response and endemic risks for allografts.Although numerous synthetic bone substitutes using metals, ceramics and polymers have been developed to promote bone regeneration for several decades, there exists a lack of efficiency in their biological performance.Since natural bone can induce infections and antigenic reaction, therefore ,the preparation of artificial; material with composition, structure and biological feature comparable to those of bone is a goal to be pursued Therefore, methods such as glutaraldehyde and polyepoxide cross linking treatments and dye-mediated photo oxidation have been developed to stabilize the tissue while attempting to maintain its natural mechanical properties. Also, residual cellular components in a bioprosthetic material have been associated with undesired effects, such as calcification and immunological recognition, and thus have been the motivation for various decellularization processes. The effects of these stabilization and decellularization treatments on mechanical, biological and chemical

properties of treated tissues have been investigated, specifically with regard to calcification, immunogenicity, and cytotoxicity concerns.

CERAMIC-BASED SYSTEMS:

Among all implantable delivery systems described in the pharmaceutical literature,ceramic-based systems have created great interest[1]Naturally occurring bioceramics like calcium sulphate(plaster of paris) have been widely studied because it is non-immunogenic,fully biodegradable,and the rate of resorption is dependent upon the density of the crystal.Surgical grade calcium phosphate is a relatively pure alpha hemihydrate which can be hydrolysed to solid implants.The custom-made calcium sulphate as a local antibiotic delivery implant for chronic osteomyelitis have been developed and characterized.[2].The advantages of loading hardened calcium sulphate beads with various antibiotic solutions and their release kinetics have been studied[3]It was shown that on doubling the antibiotic load of the beads gives a more prolonged elution and a two-fold increase in antibiotic release.Even though,calcium sulphate beads facilitate individualized chemotherapy,it exhibits cytotoxic effects. Hydroxyapatite (HAP), a principal calcium phosphate is another bioceramic that have been studied extensively for drug delivery.This biomaterial has been used successfully in bone injury and other biomedical applications because its chemical composition is close to that of bone mineral and it displays bipocompatibility,osteoconductivity and bioactivity.Hydroxyapatite is resistant to mechanical forces, is effective in filling cavities and defects in bone, and any antibiotic can be used with hydroxyapatite as there is no risk of thermal damage and no second surgery procedure is required for removal(because of biodegradability).Most of the impregnated antibiotic is

released over a period of time. The porosity of the block being used and the nature of the antibiotic are factors that determine release.

The disadvantages of synthetic porous calcium phosphate ceramics are that these contain numerous closed pores which prevent the invasion of bone tissue into the material and the residue of the synthetic material would be is found within the bone after more than a year following implantation. To overcome the above disadvantages some natural biomaterials both of cortical and cancellous have been developed. [4]

ANTIBIOTIC IMPREGNATED IMPLANTS:

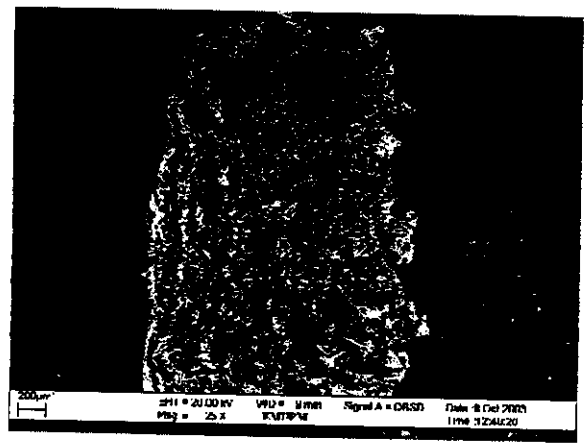
The most widely used antibiotic-impregnated implants are methlmethacrylate beads and cements. Polymethylmathacrylate (PMMA), a dense acrylic, non-biodegradable polymer loaded with gentamicin sulphate has been tested by elution studies for treating bone infection [5] PMMA beads provided localized treatment and some stability.Tobramicin,Gentamicin and Vancomycin are the antibiotics that have been combined with various types of methylmethacrylate and have been tested by elution studies[6]The release profiles of gentamicin from different PMMA bone cements were studied[7]They found a typical fast release within the first week and also showed that the most of the drug(.90%) remains within the closed pores of the non-biodegradable bone cement after the first week and being very slowly released. As PMMA beads are non-biodegradeable,non-resorbable and exhibit low biocompatibility, they may have to be surgically removed after a few months and replaced either by new beads to prolong the antibiotic therapy or by a bone substitute to accelerate bone reconstruction. This increases the chance of reinfection.Moreover PMMA does not aid in bone repair and there is a

possibility of thermal damage to the tissue and the antibiotics, as heat is evolved when the antibiotic is loaded into the polymerizing PMMA beads. Thus PMMA is not an ideal antibiotic implant.

In another case, Synthetic porous hydroxyapatite blocks containing Vancomycin powder was implanted in patients with MRSA osteomyelitis.The blocks were removed after a few months and their release kinetics were studied in vitro[8]Ciprofloxacin implants composed of hydroxyapatite ,β-tricalcium phosphate,poly lactic acid,and ciprofloxacin have been characterized for bone infection[9].Hydroxyapatite cement (HAC) as a carrier for Vancomycin in the treatment of chronic osteomyelitis has been studied[10]Hydroxyapatite cylinders with bimodal pore size distribution were vacuum infiltrated with the antibiotic solution and its release in phosphate buffer saline(PBS) was assayed.A cumulative release of 80% was achieved in 10 days[11]Interporous hydroxyapatite ceramics in the form of cubic blocks were vacuum infiltrated with isepamicin sulphate(IPS).They released the antibiotic with a mean concentration of 0.41μg/ml over of 18 days[12] A calcium phosphate cement that could be molded into any desired shape due to its plastical consistency was developed. The powder component of the cement consists of a-tricalcium phosphate and tetracalcium phosphate, which were made by the decomposition of hydroxyapatite ceramic blocks. The liquid component consists of citric acid,chitosan and glucose solution. When incubated in physiological saline, the cements were transformed to hydroxyapatite at 3,and 6 weeks, the compressive strengths were also increased. The inflammatory response developed on the bone implanted into the rats disappeared after 4 weeks and the cement had bound to bone. [13]

CHITOSAN AS A BIOMATERIAL:

There are a lot of cases where Chitosan had gained special importance as a biomaterial. Chitosan,which is extracted from crab or prawn shell, is essentially a polyglycosaminoglycan,that has been tried by many researches for use along with collagen.Chitosan exhibits good physical properties and is also biocompatible.

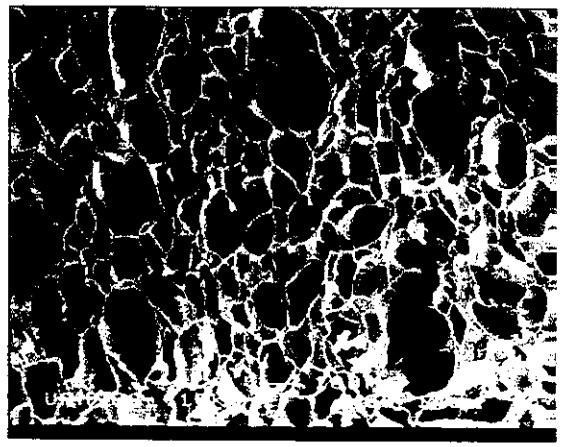


POROUS CHITOSAN SPONGE

Research efforts have been directed towards developing safe and efficient chitosan-based particulate drug d Dodane and Vilivalam reviewed new approaches on pharmaceutical applications of chitosan and discussed its mechanisms of action in various vitro and in vivo models.Recent reviews addressed the issues on biomedical, pharmaceutical and biological aspects of chitin,chitosan and their derivatives

elivery systems. [14] Chitosan is extensively used in developing drug delivery systems It is used in the preparation of mucoadhesive formulations [15] improving the dissolution rate of the poorly soluble drugs [16], drug targeting [17] and enhancement of peptide absorption. [18]

Chitosan scaffolds reinforced by ß-tricalcium phosphate (ß-TCP), Calcium phosphate invert glass and Sodium chloride were constructed and their microstructures and mechanical performance were studied



CHITOSAN SCAFFOLD FABRICATED WITH SODIUM CHLORIDE

The composite scaffolds were microporous and maintained the regularly oriented pore structure. The salient differences in microstructures of chotosan scaffolds with and without glasses suggested that not only physical incorprration occurred,but also the chemical complexation between the chitosan and the fillers might be involved[19]

The chitosan tubes derived from crab tendons form a hollow tube structure,which is useful for nerve regeneration.But there were certain problems due to the mechanical strength and spatial arrangement caused by swelling.This problem was solved when apatite was made to react with chitosan tubes to enhance the mechanical strength and was further annealed at 120?C to prevent from swelling and simultaneously forming into triangular shape to imolantation..By these treatments, the hollow tubes could keep their shape even in vivo after implantation.Animal tests using SD rats

further showed that the chitosan tubes effectively degraded and absorbed in vivo. [20]

COLLAGEN AS A BIOMATERIAL:

Lee (International Journal Of Pharmaceutics, 221,1-22(2001) and Freiss(European journal of Pharmaceutics and Biopharmaceutics 45,113-136,1998),collagen, an ubiquitous connective tissue protein has been purified and extensively used as an important biomaterial for the purpose of wound healing. Pins and Silver (Material Science and Engineering C 3,101-107, 1995) have discussed the use of a reconstituted collagen scaffold for tissue engineering applications.

Yurugi et al (Aesthetic Plastic Surgery, 26,360-364,2002) have found that there is variable wound contraction attributable to the collagen wound healing material. Also in natural tissues,collagen occurs in association with other proteins and proteoglycans to varying extents(as in cartilage)depending on the function of tissue. This feature has prompted the researches to explore possibilities to provide a composite material of collagen and other biocompatible materials.

S uzuki et al (Biomaterials 11,356-360, 1990) used a bilayer of collagen and silicone as an artificial skin when after resorption of collagen,the synthetic silicone layer peeled off. Reference may be made to Li et al (US Patent No.6303136), who have provided a matrix of general nature by coating extracellular material on a non-degradable fibre matrix. The matrix can attract cells during wound healing. But the major limitation is that it is non-degradeable,thereby running the risk of rejection at a later stage or even in growth of connective tissue that may not be desirable because of the subsequent possibility of calcification.

Collagen can be readily purified from animal tissues, such as skin and tendon, as well as human tissues, such as placenta, and reconstituted into gels by changing the pH and temperature of suspensions of the precursor components. Cell migration in these materials can proceed through mechanisms that either require the action of matrix metalloproteinases(MMPs)[21]or are independent of MMPs[22]Collagen has been widely used in the cross linked form to extend the durability of collagen. The chemical treatment influences the structural integrity of collagen molecule resulting in the loss of triple helical characteristic. The structural characteristic of collagen is importantly related to its biological function for the interaction with cell. The triple helical structure of EGCG treated collagen could be maintained at 370C in comparison with collagen, which is confirmed by spectra analysis. The structural stability of EGCG treated collagen provided a favorable support for cell function in cell adhesion. EGCG treated collagens provided a practical benefit to resist the degradation by collagenase retaining its structural characteristics and can be a suitable biomaterial for biomedical applications [23]

SKIN TISSUE ENGINEERING:

Tissue-engineering for skin replacement, have developed widely in recent years though it exists from early times.

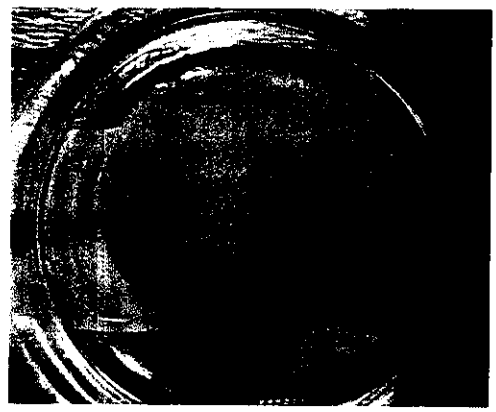
Ma et. al(Biomaterials 24,4833-4841,2003) have used porous collagen/chitosan scaffolds for tissue engineering .Collombel et al(US Patent No.5166187) prepared a mixture of collagen and chitosan by mixing of respective solutions along with glycocaminoglycans and making different layers of these mixtures .The collagen made in these preparations was not necessarily formed at a pH of around 7.0 and so were without the native periodicity of 68nm.It was not clear whether there were any chemical

crosslinks between layers or even between constituents. The major disadvantage associated with the product is that the layers not being chemically cross linked could separate under physiological conditions and would degrade without any preferred mode. This might not facilitate preferential cell growth and would delay the healing process.

Mao et al. (Biomaterials, 24,1067-74,2003) prepared a gelatin-chitosan matrix by first forming a porous membrane of chitosan (or gelatin) and then forming a membrane There are many references to show the use of collagen and chitosan in biomedical applications. For example collagenous material and chitosan in its various physical forms were used in wound dressing and in treatment of various skin defects due to its biodegradability and biocompatibility.

Within native tissues cells were held within the extracellular matrix (ECM), which had a role in maintaing homeostasis,guiding development and directing regeneration. Efforts in tissue engineering had aimed to mimic the structure and characteristics of ECM, but had also considered ways to reproduce its molecular properties including its bioadhesive character,proteolytic susceptibility and ability to bind growth factors.[24]

Porous scaffolds for skin tissue engineering were fabricated by freeze-drying the mixture of collagen and chitosan solutions. Glutaraldehyde (GA) was used to treat the scaffolds to improve their biostability.A detailed investigation found that a steady increase of the biostability of the collagen/chitosan scaffold was achieved when GA concentration was lower than 0.1%,then was less influenced at a still higher GA concentration up to 0.25%



CHITOSAN-COLLAGEN SCAFFOLD

In vitro culture of human dermal fibroblasts proved that the GA-treated scaffold couldretain the original good cytocompatibility of collagen to effectively accelerate the fibroblasts infiltration from the surrounding tissue.Immunohistochemistry analysis of the scaffold embedded for 28 days indicated that the biodegradation of the 0.25%GA-treated scaffold is a long-term process[25]

DEVELOPMENT OF SCAFFOLDS FOR ORTHOPEDIC DEFECTS:

Eventually a lot of bilayer scaffolds are being developed recently in cases of bone defects and fractures. A novel biocompatible matrix that can be used in Bone damage and fractures and which is different from presently available materials.The intention is to incorporate collagen with chitosan,a polymer of glucosamine and galactosamine which has similarities to other extracellular matrix components i.e.hyaluronic acid and

mucopolysaccharide which are associated with collagen in native tissue Of gelatin.(or chitosan)over it. There was no chemical crosslinking between layers. The porosity was adjusted by freeze-drying at different low temperatures.The major limitation of this product was that of this product was that gelatin,which was essentially a denatured collagen, does not support cell growth as effectively as collagen with its natural structure and organization.

The treatment of experimental osteomyelitis caused by methicillin-resistance *Staphylococcus aureus* (MRSA) with a low molecular weight and bioabsorbable polymer D -, L- dilactide containing pefloxacin resulted in eradication of MRSA [26] The composite materials from bio active glass powders, ploy (L- lactic acid) (PLA), biostable polymer PMMA and gentamicin have been developed, where a fast initial release during the first 10 hrs of soaking followed by a controlled release of the drug was observed [27].

Because of the long degrading time of lactic acid polymers the more, rapidly degrading co polymer mixtures of poly (lactide – co – glycolides) (PLA: PGA, 50:50) was used for the controlled delivery of antibiotics.These polymers degrade by bulk hydrolysis of ester bonds and break down into their constituent monomers,lactic and glycolic acids,which are excreted from the body or metabolized.The in vitro and in vivo elution characteristics of vancomycin,cefazolin sodium and gentamicin sulphate from the above copolymer as antibiotic beads for the long-term drug release have been studied[28]The release profile of ciprofloxacin hydrochloride from PLGA microcapsules has been characterized[29] The release period of antibiotics from the bead could be prolonged by increasing the size of the beads or by adopting multi-layered beads.To obtain the sustained release of a

hydrophilic antibiotic drug from PLGA based nanofibrous scaffolds without the loss of structural integrity and bioactivity,an amphilic poly-b-poly-lactide(PEG-b-PLA) diblock copolymer was added in the polymer solutions to encapsulate the hydrophilic drug[30]

An artificial biomaterial for knee joint replacement has been developed by building a graded structure consisting of ultra-high molecular weight polyethylene (UHMWPE) fibre reinforced high-density polyethylene combined with a surface of UHMWPE.The ingrowth behaviour of titanium implants into hard tissue can be improved by depositing a graded biopolymer coating of fibronectin,collagen types I and III with a gradation, derived from the mechanisms occurring during healing in vivo.Functionally graded porous hydroxyapatite (HAP) ceramics can be produced using alternative routes such as polylactide,poly-co-glycolide and polyglycolide,with carbonated nanocrystalline hydroxyapatite.HAP-collagen I scaffolds are an appropriate material for in vitro growth of bone. The scaffold has to be functionally graded in order to create an optimized mechanical behaviour as well as the intended improvement of the cell in growth. [31]

The effect of biomaterials on specific functions was studied, employing cells of the same type of those which will be in contact with the device in vivo.osteoblasts,either from primary culture or an established line ,are used to study the interaction with materials for bone implants. The effect of dental cements on replication cycle and S-phase inhibition, and immune response was studied. [32]

BILAYER SCAFFOLDS:

With all this studies and details,Collagen cross-linked with another biomaterial has been used in many cases. For instance,

biodegradable antibiotic implant to treat chronic osteomyelitis has been studied for antibiotic delivery including natural and synthetic polymers in ceramic.

A direct nucleation of Hydroxyapatite on self-assembled collagen fibers to set up a collagen –hydroxyapatite nanocrystals composite as a biomaterial for bone repair and reconstruction was done.X-ray diffractometric technique,thermogravimetric(TG-DTG),spectroscopic (FT-IR,ICP),microscopic(SEM,TEM) analyses have been used to highlight the likeness of the artificial biomimetic HA/Col composite with natural bone tissue.[33]

TypeI collagen fibrils,elastin fibres and chondroitin sulphate(CS) were used for the preparation of molecularly-defined collagen-elastin-glycosaminoglycan scaffolds A .Electron microscopy showed that collagen and elastin physically interacted with each other.A total of 12 different scaffolds were prepared with four different ratios of collagen and elastin(1:9,1:!,9:1 and 1:0),with and without chemical crosslinking,and with and without CS.Collagen was essential to fabricate coherent,porous scaffolds.Electron microscopy showed that collagen and elastin physically intgeracted with each other and that elastin fibres were enveloped by collagen.By carbodiimide-crosslinking,amine groups were coupled to carboxylic groups and CS could be incorporated.More CS could be bound to collagen scaffolds(10%)than to collagen –elastin scaffolds(2.4-8.5% depending on the ratio.)The attachment of CS increased the water-binding capacity to up to 65%. Scaffolds with higher collagen content had higher tensile strength whereas addition of elastin increased elasticity. Scaffolds were cytocompatible as was established using human myoblast and

fibroblast culture systems. It is concluded that molecularly-defined composite scaffolds can be composed from individual,purified,extracellular matrix components.[34]

A biomaterial composed of sintered (1100°C) and powdered hydroxyapatite and type I collagen,both of bovine origin,designed for osteoconductive and osteoinductive scaffolds.Studies and analysis showed that the sintered bone was composed essentially of Hydroxyapatite with minimum additional groups such as surface calcium hydroxide,surface and crystal water, free carbon dioxide and possibly brushite,carbonates at A and B sites of HA,and weakly bound to the structure. Human osteoblasts adhered and spread on both the HA particle surface and the collagen fibres, which seemed to guide cells between adjacent particles [35]

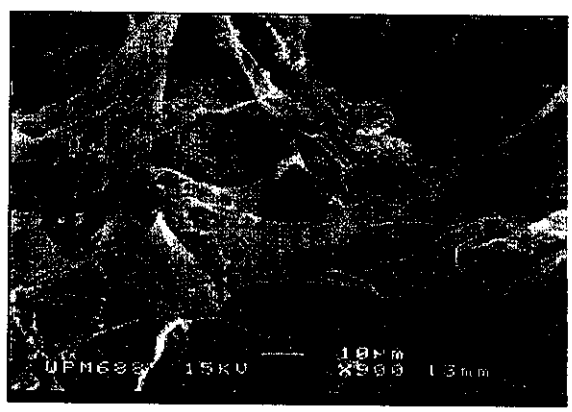
Injuries to peripheral nerves can have different causes and may lead to disorders affecting mobility, sensitivity and loss of motor function as they progress. Weiss, in 1944, introduced tubulisation to promote the regeneration of a sectioned nerve. In this study the biomaterial Chitosan was used to induce and stimulate the regeneration of the sciatic nerve in dogs. At the same time, taking advantage of the characteristics offered by Chitosan the neurosteroid, progesterone was included in its matrix, as a promoter of axonal growth.

COLLAGEN-CHITOSAN SCAFFOLD:

Matrix composed of collagen and chitosan may create an appropriate environment for the regeneration of livers .A new collagen /chitosan matrix (CCM). The CCM was made by using cross linking agent

1-ethyl-3- (3-dimethylaminopropyl)-carbodiimide(EDC) in N-hydroxysuccinimide(NHS) and a 2-morpholinoethane sulfonic acid(MES) buffer system. Studies and analysis showed that CCM has excellent blood and cell compatibility. [36]

Porous scaffolds for skin tissue engineering were fabricated by freeze-drying the mixture of collagen and chitosan solutions. A detail investigation found that a steady increase of the biostability of the collagen/chitosan scaffold was achieved when Glutaraldehyde concentration was lower than 0.1%, and then was less influenced at a still higher GA concentration up to 0.25%. In vitro culture of human dermal fibroblasts proved that the GA-treated scaffold could retain the original good biocompatibility of collagen to effectively accelerate cell infiltration and proliferation. In vivo animal tests further revealed that the scaffold could sufficiently support and accelerate cell infiltration and proliferation. In vivo animal tests further revealed that the scaffold could sufficiently support and accelerate the fibroblast infiltration from the surrounding tissue. [37]



ATTACHMENT OF FIBROBLASTS TO CHITOSAN-COLLAGEN SCAFFOLD

CRUSTACEANS AS BIOMATERIALS:

Biomaterials from natural sources such as shells of corals, carapace of turtle, skeletons of sponges, tendons of crab seemed to be very promising in recent types

The material isolated from the Margaritifera shell of fresh waters, in granule forms, were implanted into the back muscles and femurs of rats for 1, 2, 4, 8 and 16 weeks.. The interface between bone and the implants showed close fusion by scanning electron microscopy (SEM). Bonding between this natural aragonite and bone seems to occur via a phosphorous-rich intermediate layer. [38] Energy dispersive X-ray analysis (EDAX) demonstrated a phosphorous-rich zone in the interface between bone and the implants, and no electron-dense layer in the interface was found by transmission electron microscopy (TEM). We conclude that Margaritifera shells are biocompatible, biodegradable and osteoconductive materials.

Nanocrystalline Hydroxyapatite, which is similar in composition and crystal structure of natural bone, is found to be ideal bone graft substitute due to its controlled resorption in the body fluid upon implantation. This paper reports on a simple combustion technique for synthesizing nanocrystalline hydroxyapatite powder from Eggshell, Diammonium hydrogen phosphate and Citric acid. Fourier - Transform Infrared Spectroscopy and powder X-ray diffraction methods were employed to characterize the sample for phase formation. The particle size is calculated using XRD data, which shows an average diameter of 44 nm. Chemical analyses to determine the Ca:P atomic ratios in synthesized ceramics were performed and it is found to be 1.6.[39]

A natural marine sponge skeleton was selected as a potential scaffold on the basis of the hydration potential of the fiber, the presence of open interconnected channels created by the fiber network, the collagenous composition of the fiber, and the structural diversity of fiber architecture. The skeleton of an undetermined species of *Spongia* (Class Demospongiae: Order Dictyoceratida: Family Spongiidae) composed of spongin, supported growth of human osteoprogenitor cells. Cell attachment and invasion into the framework were observed within 16 h, followed by development into membranous sheets between the sponge fibers by concentric infilling. Histochemical staining for alkaline phosphatase and type I collagen indicated formation of bone matrix as confirmed by birefringence. At 9 and 14 days alkaline phosphatase-specific activity in sponge fiber-osteoprogenitor cell cultures was significantly greater than in control cultures on cell culture plastic. Adsorption with recombinant human bone morphogenetic protein 2 confirmed the potential of this sponge skeleton as a delivery scaffold for osteogenic factors. The abundance and structural diversity of natural marine sponge skeletons and their potential as multifunctional, cell conductive and inductive frameworks indicate a promising new source of scaffold for tissue regeneration. Such a scaffold can be used for seeding stem cells to generate functional three-dimensional tissues. [40]

In this project, a porous biocompatible material isolated from the carapace of the turtle shell has been studied and characterized to be used as a suitable drug delivery system to prevent infections accompanying surgical procedures during implantations and to set right the orthopedic defects.

CHAPTER 3
AIM AND SCOPE

AIM AND SCOPE:

The development of new biomaterials for medical applications is one of the challenging tasks for material science today. There is an obvious need for better implants as well as for the manufacturing of artificial tissues. A characteristic feature of this research area is the increasing acceptance of biologically inspired approaches. The tissue engineering approach to bone regeneration is founded upon the use of polymeric scaffolds that serve to support, reinforce and in some cases organize the tissue regeneration. The synthetic macro porous scaffolds fabricated from biocompatible, biodegradable polymers play an important role in the formation of new tissues and provide a temporary scaffold to guide the new tissue growth and organization.Biodegradeable materials are becoming increasingly more important in the biomaterial field, particularly for wound healing ,tissue reconstruction and controlled drug delivery.

The main advantage of biodegradable over non-biodegradable materials is the disappearance of implanted foreign materials from the body as a result of their biodegradation. This is a marked contrast with non-biodegradable biomaterials, which might elicit foreign body reactions from the host defense system during their long-term contact with a living structure.

Large bone repair is always a tough problem of surgeons in clinics. The natural biological materials for bone defect repair, including autografts and allografts, each has its own shortcomings such as donor site morbidity and donor shortage for autografts,immunologic response and endemic risk for allografts although synthetic bone substitutes using metals, ceramics and

polymers have been developed to promote bone regeneration for several decades.

The main objective of this project is to use a porous biomaterial isolated from the carapace of a turtle shell that would overcome the demerits associated with other biomaterials mentioned above. Before reaching the final product, the material has to be characterized and studied to study its biochemical nature, collagen content and fluorescence properties. To prevent local infection during implantation performed through surgical procedures in the cases of orthopedic defects, bone neoplasia and tumours,Osteomyelitis etc.the porous material has to be incorporated with antibiotics(Penicillin G) and its release profile through the porous interconnectivities of the material has to be recorded and studied. This may be attributed to the drug delivery in vivo conditions and its rate of release through the blood vessels to prevent infection.

CHAPTER 4

MATERIALS AND METHODS

MATERIALS:

REAGENTS AND INSTRUMENTS	SOURCE
Phenyl Methane Sulfonyl fluoride (PMSF)	Sigma
Tyrosine	S.D Fine –Chemicals Ltd.
Tryptophan	
Hydroxy proline	
Sodium Chloride	
di-Sodium hydrogen orthophosphate anhydrous Purified (LR)	
Ethylene diamine tetraacetic acid Disodium salt (GPR)	BDH
B-Mercaptoethanol	HIMEDIA
Citric acid	
Acetic acid (glacial)(AR)	RANBAXY
Glycine	
Acetone (AR)	RANKEM
Methanol (LR)	FISCHER
Sodium hydroxide	Sisco Research Lab
Tri Glyceride(Tris)	
Penicillin G	USB
Formalin	Qualigens
Microbalance	Mettler Toledo Model AG245
Top Pan Balance	Mettler Toledo Model PB1502
Low temperature cabinet (-20◦ C)	Cryoscientific
Cold Centrifuge	Himac CR22, Rotor R22A2
UV-Spectrophotometer	Specord 200
Fluorescence Spectrophotometer	Varian
Scenning Electron Microscope (SEM)	Fei-Quanta 200

METHODS:

PREPARATION OF HYDROXYAPATITE MATRIX

The material was isolated from the carapace of turtle's shell collected from Snake park,Chennai A highly porous hydroxyapatite matrix was obtained from the carapace of turtle's shell, after sintering at 900° C overnight to remove the organic content and other impurities.

DECALCIFICATION OF THE MATERIAL:

Reagents:

0.5 M Ethylenediamine tetra acetic acid disodium salt, Sodium hydroxide pellets, Acetone, Isopropanol.

Method:

The turtle shell pieces were suspended in 0.5 M EDTA (pH 7.4) over a period of 15-20 days. The dissolution was done with constant stirring at 4°C .The decalcified matrix was then subjected to intense washing procedures using isopropanol and acetone followed by several washes in distilled water to remove EDTA.

HYDROLYSIS OF THE MATERIAL:

Reagents:

Sample material, 6 N HCl

Procedure:

A known weight / volume of sample were hydrolyzed with 6N HCl in sealed tubes at 110°C for 16 – 18 hours. After hydrolysis, the sample was evaporated to dryness by dissolving it in water and drying it every time (2 to 3 times) to remove hydrochloric acid completely. The residue was dissolved and made up to a known volume with distilled water.

DETERMINATION OF FLUORESCENCE OF THE MATERIAL

Reagents:

Hydrolysate, Tyrosine (1 mg/ ml), Tryptophan, polished material.

Procedure:

Initially, UV-Visible spectra for the tryptophan,Tyrosine and hydrolysate was run seperately and the peak corresponding to the wavelength of maximum absorbance was noted. Then fluorescence spectroscopy was done on the following:

1. Tryptophan
2. Tyrosine
3. Hydrolysate
4. Solid material

Assays are performed in 10 mm quartz cuvettes of 1 ml capacity. The cuvettes were held at a thermostatted compartment, and preferably the spectrometer has a recording mechanism with a constant speed chart drive., eg the sample solution of specific concentration was prepared.. The speed, excitation and emission split widths were adjusted. The excitation wavelength was also set and the fluorescence spectra corresponding to the emission wavelength is noted.

ESTIMATION OF HYDROXY PROLINE:

Reagents:

- Buffer (pH 6.0): 50 g of citric acid, 12 ml of Acetic acid, 120 g of sodium acetate, 34 g of NaOH was made upto 1 litre and pH was adjusted to 6.0.
- Chloramine -7: 0.5 M chloramines-7 was prepared by dissolving 1.41 g of the same, in 20 ml of water, 30 ml of Methyl cellosolve and 50 ml of buffer (pH 6.0).

- Perchloric acid: 10.8 ml of 70% perchloric acid made upto 40 ml.
- P-Dimethylaminobenzaldehyde (PDAB): A 20% solution of PDAB in methyl celllosolve was prepared freshly before use.
- Hydroxy proline standard: a Stock solution was prepared by dissolving 10 mg of L-hydroxy praline in 100 ml of 0.001N hydrochloric acid. Standards were prepared by diluting the stock with water to obtain a concentration of 1 - 5 µg / 2 ml.

Procedure:

2 ml portion of the samples were placed in test tubes. A series of standards were prepared. 1ml of freshly prepared chloramine T was added and was allowed to stand for 20 min at room temperature. The reaction with chloramines T was stopped by adding 1 ml of perchloric acid (3.15 M), mixed well and allowed to stand for 5 min. finally, 1 ml of freshly prepaced p-dimethyleaminobenzaldehyde (20%) solution was added, heated in a water bath at 60°C for 20 min and the absorbance of the solution at 557 nm was measured after cooling to room temperature.

INCORPORATION OF PENICILLIN G INTO POROUS HYDROXYAPATITE MATRIX:

Reagents:

Penicillin solution (1 mg / ml) (water: acetone)(4:1) dried hydroxyapatite pieces.

Procedure:

The processed hydroxyapatite pieces were left in the desiccator for a period of 15 days for complete drying. The other part of it was vacuum infiltrated with antibiotic solution (Penicillin dissolved in nanopure water and acetone) of known concentration for 20 min and the pieces were dried using a vacuum desiccator for 15 min. Then the pieces were left in the desiccator for

24 hours for complete drying. The change in weight of the pieces, before and after filtration was observed. Any observed change in weight was assumed as an absolute amount of antibiotics present in the pieces

SEM ANALYSIS

The shell pieces having been ashed and infiltrated with Penicillin-G were subjected to SEM analysis.

PENICILLIN G RELEASE PROFILE EVALUATION:

a) UV VISIBLE SPECTROSCOPY FOR PREPARATION OF STANDARDS:

Reagents:

Penicillin in water [1 mg /ml], penicillin in phosphate buffer saline [1 mg /ml],,Phosphate buffered saline.

Preparation of phosphate buffer Saline:

(From buffer chart –G.Gomori, W.M. Clark and H.A Lubs, J.Bacteriol 2,1 (1917)).

A: 0.2 M solution of Monosodium phosphate (27.8 gin 1000 ml).

B: 0.2 M solution of dibasic sodium phosphate (53.05 g of $Na_2HPO_4.7H_2O$ or 71 g

Or $Na_2HPO_4.12H_2O$) in 1000 ml.19 ml of A and 81 ml of B was mixed and diluted to a total of 200 ml. the pH was adjusted to 7.4

Procedure

UV visible spectra was recorded seperately for penicillin in water, and penicillin in buffer for different concentrations of 0.005 mg / ml, 0.01 mg / ml, 0.015 mg / ml and 0.02 mg / ml from a stock of 1 mg / ml.

Assays were performed in 10 mm quart cuvette of 1 ml capacity. The cuvette was held in a thermostatted compartment and the parameters were set with a constant speed chart drive. The zero correction was performed

with water/ buffer as blank solution.Same was used as the reference solution. In, the assay cuvettte, at different concentrations the spectra of the sample was scanned.

b) RELEASE CHARACTERISTICS OF PENICILLIN G

An invitro elution method was employed to determine the release characteristics of Penicillin from the hydroxyapatite matrix. A phosphate buffered saline (PBS) of pH 7.4 was used as the medium. A known quantity of the infiltrated pieces with Penicillin-G was incubated in 2ml of PBS at 37°C. The medium was collected at suitable time interval (5-30 mins) and a UV visible spectrum was run each time until all the penicillin release into the medium.

The UV –visible spectra was run as explained above using PBS as the blank and reference solution. The unknown concentration of penicillin in the dissolution media was calculated by comparing it with the standards.

EXTRACTION OF STANDARD COLLAGEN FROM RAT-TAIL TENDON (RTT)

Collagen was extracted and purified from rat-tail tendon. The tendons were teased from the tail of 6-month-old albino rats.

Reagents:

0.9%Sodium chloride, 0.5 M Acetic acid, glacial, 2m M Phenyl Methane sulfonyl fluoride (PMSF), 0.02 M, di –Sodium hydrogen orthophosphate anhydrous.

Method

All subsequent procedures were performed at 4°C. The tendons were stirred overnight in 0.5 M acetic acid. Protease inhibitors like N-ethylmaleimide (2m M) and phenyl methane sulfoxyfluoride(PMSF)(1m M) were added to the mixture. The solution was then filtered and centrifuged at 50,000g for 40

min and the residue discarded. The solubilised collagen of 5% Nacl and left overnight. The precipitate collagen was recovered by centrifugation at 35,000g for 30 min. the residue was redissolved in 0.5 M acetic acid by stirring overnight. The solution was centrifuged at 50,000g for 40 min and supernatant collected was dialyzed extensively against 0.02 M disodiumhydrogen phosphate buffer with 2 to 3 changes of buffer each day. After dialysis, the precipitated collagen was centrifuged at 35,000g for 30 min and the precipitate was redissolved in 0.5 M Acetic acid. The dissolved collagen in 0.5 M Acetic acid was centrifuged at 50,000g for 40 min and the Supernatant was dialyzed against 0.05 M Acetic acid. After dialysis the solution was centrifuged at 50,000g for 40 min and purified collagen was stored at 4°C in acetic acid solution.

PREPARATION OF PROTEIN EXTRACT FROM TURTLE SHELL:

Reagents:

Calcium chloride, Pepsin, 0.05M Glacial Acetic acid, Phenyl Methane sulphonyl Chloride (PMSF)

Procedure:

The decalcified pieces were then cut carefully into small dimensions. The cleaned pieces were dried in a desiccator overnight with calcium chloride as the drying agent. A 150 ml solution of 0.05 M Acetic acid with three pinches of pepsin in it was prepared. The pH was adjusted to 6.8 and small quantities of phenyl methyl sulphonyl fluoride were added to it (inhibitors of protease activity). The cut pieces were added to it and kept for constant stirring at 4°C over a period of 48-hours .The solution is dialyzed against 0.02 M Disodium Hydrogen orthophosphate Buffer

SDS- POLYACRYLAMIDE GEL ELECTROPHORESIS:

SDS – PAGE is a widely used technique for the separation of protein and peptide mixtures. It was carried out by the method of laemmli (laemmli, U.K, 1970).

Reagents:

- Stock solution of the reagents of given concentration were prepared.
- Acrylamide: N, N-Methylene bisacrylamide(30% : 0.8%)
- SDS 10%.
- 1 M Tris Buffer (pH 6.8)
- 1.6 M Tris Buffer (pH 8.8)
- Ammonium persulphate(APS 10%)
- TEMED
- Tank Buffer (5X) of pH 8.3:

Tris base-7.55g (0.025 M), Glycine-36.58g (0.195 M), SDS-2.5g (0.1%).

Solutions:

a) Fixing solution: 10% Trichloroacetate acid.

b) Staining solution: 10% Methanol, 7% Acetic acid, 0.1% Coomasie Brilliant Blue dye.

c) Destaining solution: 10% Methanol, 10% Acetic acid.

d) Storing solution: 7% acetic acid.

e) Tank Buffer (5X) of (pH 8.3): From this stock 500 ml of 1X tank Buffer was prepared.

Gel composition:

REAGENTS	SEPERATINGGEL (5.5%) (10 ml)	STACKING GEL (3.8%) (5 ml)
(Acrylamide	1830 (µl)	633 (µl)
Tris Buffer	2000 (µl) pH 8.8	600(µl) pH 6.8
Distilled H_2O	5890 (µl)	3657 (µl)
SDS (10% -0.1%)	100 (µl)	50 (µl)
APS (10%-0. 1%)	80 (µl)	50 (µl)
TEMED	10 (µl)	10 (µl)

PREPARATION AND CASTING OF GEL

The gel plates were sterilized, wiped and dried before clamping it with spacers and sealing it with cello-tape [2 or 3 coating]. The gels were formed in the space between the two glass plates.1 ml of sealing gel was polymerized into the space between the gel plate. Separating gel (5.5%) was then prepared and added carefully till 3-4th of the plate was filled taking care to avoid any air bubble. Few drops of water were layered over it. The water was decanted after the polymerization of the separating gel. The surface was dried and wiped with tissue paper and the stacking gel (3.8%) was prepared and stacked. Before its polymerization a comb was inserted to provide sample wells. After complete polymerization, the comb and the cello-tapes were removed and the cast plates was clamped in the gel tank and connected to the power pack using chords. The samples were loaded into the wells [10 – 30(µl)]. The electrophoresis was performed at 50 V at room

temperature for a period of two and half hours. The gel was transferred to the fixing solution (45 min) and then put in staining solution overnight in a rocker. The gel was then destained 3 or 4 times and the bands were observed in an illuminator.

SILVER STAINING:

Silver staining was done according to the method of Nesterenko et al.

Reagents:

All solutions used for silver staining were prepared first before use.

- Milli Q water.
- Destaining solution: 40% Methanol, 10% Glacial Acetic acid
- Fixative solution: 40% Methanol 35% formalin, 25% Milli Q water.
- 0.02% sodiumthiosulphate.
- Sliver Nitrate 100 mg/200 ml (to stored in dark).
- Developer solution: 4.5g of Sodium carbonate.
  3 ml of 0.02% Sodiumthiosulphate
  75 (µl) of formaldehyde.
  150 ml of Milli Q water.
- Stopper solution: 50 ml of developer solution without formaldehyde 2 M citric acid (22.08g).

Method:

1. The gel was destained thoroughly with destaining solution followed with Milli Q water.
2. The gel was washed thoroughly with Milli Q water.

3. The gel was soaked for 10 min in fixative solution and subjected to gradual shaking.
4. The gel is then washed for 2 times for 5 minutes with Milli Q water.
5. The gel was soaked and treated with 0.02% Sodium thiosulphate for 1 min.
6. This was followed by 3 washes with Milli Q water for 45 seconds.
7. Silver nitrate solution was added to the gel and stained for 10 min.
8. The gel was washed with Milli Q water for 45 sec.
9. A developer solution was added to the gel, colour was allowed to develop till the bands were visible.
10. The reaction was stopped by the addition of stopper solution.
11. The gel was further stored in Milli Q water.

CHAPTER 5

RESULTS AND DISCUSSION

RESULTS: TABLES

TABLE 1: UV VISIBLE SPECTRA OF PENICILLIN G IN WATER

WAVELENGTH (nm)	0.005 mg/ml	0.01 mg/ml	0.015 mg/ml	0.02 mg/ml
200	0.6111	1.0948	1.5722	1.9074
210	0.2562	0.4867	0.7193	0.9599
220	0.1253	0.2342	0.3473	0.4653
230	0.0684	0.1278	0.1925	0.2591
240	0.0781	0.1463	0.2226	0.3009
250	0.1258	0.2369	0.3590	0.4835
260	0.1743	0.3291	0.4967	0.6685
270	0.1871	0.3546	0.5335	0.7171
280	0.1540	0.2916	0.4389	0.5909
290	0.0918	0.1727	0.2596	0.3497
300	0.0351	0.0658	0.0995	0.1343
310	0.0093	0.0174	0.0272	0.0368
320	0.0075	0.0000	0.0070	0.0143
330	0.0055	0.0000	0.0040	0.0112
340	0.0019	0.0000	0.0022	0.0075

TABLE 2: UV VISIBLE SPECTRA OF PENICILLIN G IN BUFFER

WAVELENGTH (nm)	0.005 mg/ml	0.01 mg/ml	0.015 mg/ml	0.02 mg/ml
200	0.3295	0.2712	0.3295	0.3060
210	0.6997	0.5662	0.6997	1.0047
220	0.3918	0.2772	0.3918	0.5260
230	0.1533	0.1144	0.1533	0.2013
240	0.0634	0.0541	0.0634	0.0796
250	0.0285	0.0308	0.0285	0.0333
260	0.0188	0.0220	0.0188	0.0200
270	0.0129	0.0139	0.0129	0.0118
280	0.0087	0.0083	0.0087	0.0052
290	0.0088	0.0066	0.0088	0.0047
300	0.0088	0.0056	0.0088	0.0047
310	0.0085	0.0058	0.0086	0.0047
320	0.0061	0.0019	0.0061	0.0043
330	0.0064	0.0077	0.0064	0.0033
340	0.0079	0.0146	0.0079	0.0047

Table 3 UV VISIBLE SPECTRA OF THE RELEASE PROFILE OF PENICILLIN-G

WAVELENGTH(nm)	5 mins	10 mins	15mins
200	0.301	0.4075	0.7085
205	0.5242	0.5816	0.8060
210	2.8402	2.9648	2.0374
215	2.0162	2.1110	2.000
220	1.2998	2.4082	1.8410
225	1.2990	1.6194	1.7701
230	1.2000	1.0248	1.6645
235	0.7972	0.6396	1.5208
240	0.4771	0.4210	0.6240
245	0.2960	0.3048	0.3940
250	0.1995		0.2722

TABLE 4: RELEASE PROFILE OF PENICILLIN-G

TIME (mins)	ABSORBANCE AT 210 nm	**CONCENTRATION OF PENICILLIN G (µg)**
0	0	0
5	1.8120	32.002
10	1.8706	33.038
15	2.0374	35.984
25	1.9022	333.595

1. CALCULATION OF ε VALUE:

$$\varepsilon^{0.001\%}_{210\ nm} = \frac{\textbf{ABSORBANCE AT 210 nm}}{\textbf{CONCENTRATION (w/v)\% X PATH LENGTH}}$$

$$= \frac{0.5662}{0.001X\ 1cm}$$

$$= 566.2\ cm^{-1}\ (conc.)^{-1}$$

Note: Stock: 1mg/ml, which is 0.1% (w/v)%; Sample:0. 01mg/ml, which is 0.001%(w/v)%

CALCULATION OF PENICILLIN G CONCENTRATION:

$$\textbf{C\%} = \frac{\textbf{ABSORBANCE}}{\varepsilon^{0.001\%}_{210\ nm}\ \textbf{X PATH LENGTH}}$$

$$= \frac{1.8120}{566.2\ X\ 1cm}$$

$$= 0.00320\%$$

$$= 3.2x\ 10^{-5}\ gm/ml$$

$$= 32\ \mu g$$

Table 5: ESTIMATION OF HYDROXY PROLINE

Name	Vol of HP (µl)	Vol of H_2O (µl)	Conc. of HP (µg)	Vol of Chl-T (µl)	20 mins At 37 °c	Vol of Pech acid (µl)	5 mins At 37 °c	Vol of pDAB	60 °c for 20 mins	OD at 557 nm
Blank	-	2000	0	1		1		1		0
1	10	1990	1	1		1		1		0.059
2	20	1980	2	1		1		1		0.150
3	30	1970	3	1		1		1		0.236
4	40	1960	4	1		1		1		0.259
5	50	1950	5	1		1		1		0.351
Test	10	1990	?	1		1		1		0.221

2.CALCULATION OF HYDROXYPROLINE CONCENTRATION:

From Graph 4,

The test sample (10µl) contains 2.8µg of Hydroxyproline

Therefore the 3000µl of the hydrolysate (containing 11 mg of the material) contains

840µg of Hydroxy proline

0.84 mg of Hydroxy proline

It's collagent content is given by 0.84 X 7.14

= 5.9976 mg of Collagen

Note: 7.14 is the conversion factor of Hydroxy proline to collagen since Collagen Contains 14% of Hydroxy proline

Amount of material in the hydrolysate = 11 mg

Therefore the percentage of collagen present in 11 mg = 54.52%

Table 6: UV VISIBLE SPECTRA OF TRYPTOPHAN

WAVELENGTH (nm)	ABSORBANCE
245	0.003
250	0.272
255	0.7018
260	1.242
265	1.7531
270	2.0297
275	1.9657
280	1.7006
285	1.2061
290	0.598
295	0.4321
300	0.3221
305	0.1323
310	0.0071
315	0.0034
320	0.0001

Table 7: UV VISIBLE SPECTRA OF TYROSINE

WAVELENGTH (nm)	ABSORBANCE
240	1.1433
245	0.2969
250	0.2115
255	0.3140
260	0.5175
265	0.8214
270	1.2030
275	1.5189
280	1.5100
285	0.4107
290	0.0592
295	0.0129
300	0.0075
305	0.0061
310	0.0053
315	0.0048
320	0.0045

Table 8: UVVISIBLE SPECTRA OF HYDROLYSATE

WAVELENGTH (nm)	ABSORBANCE
240	1.3182
245	1.0859
250	1.0256
255	1.0259
260	1.0467
265	1.0431
270	1.0502
275	1.0351
280	1.0005
285	0.9283
290	0.7696
295	0.6605
300	0.5951
305	0.5418
310	0.4953
320	0.4259
325	0.3898
330	0.3668
340	0.3106
345	0.2839
350	0.2579

Table 9: FLUORESCENCE SPECTRA OF TRYPTOPHAN

WAVELENGTH (nm)	INTENSITY (a.u)
290	0.2635
300	0.1339
350	38.620
400	19.5929
450	3.2160
500	0.4866
550	0.0535
600	0.0974
650	3.322
700	4.9592
750	2.5477

Table 10: FLUORESCENCE SPECTROSCOPY OF TYROSINE

WAVELENGTH (nm)	**INTENSITY** (a.u)
220	0.9617
250	0.98814
300	82.3643
350	11.1336
400	3.7843
450	4.7424
500	12.2075
550	10.3199
600	25.4958
650	8.9197
700	0.0000
750	0.9940
800	0.0000

Table 11: FLUORESCENCE SPECTRA OF HYDROLYSATE

WAVELENGTH (nm)	**INTENSITY** (a.u)
290	139.888
300	301.7686
350	75.2850
400	134.2001
450	250.7043
500	260.7043
550	88.7992
600	136.1007
650	44.8516
700	10.4419
750	7.2444
800	4.4295

Table 12: FLUORESCENCE SPECTRA OF THE SOLID MATERIAL

WAVELENGTH (nm)	**INTENSITY (a.u)**
290	139.888
300	301.7686
350	75.285
400	134.2001
450	392.7556
500	260.7043
550	88.7992
600	136.1077
650	44.8516
700	10.4419
750	7.2444
800	4.4295

FIGURES:

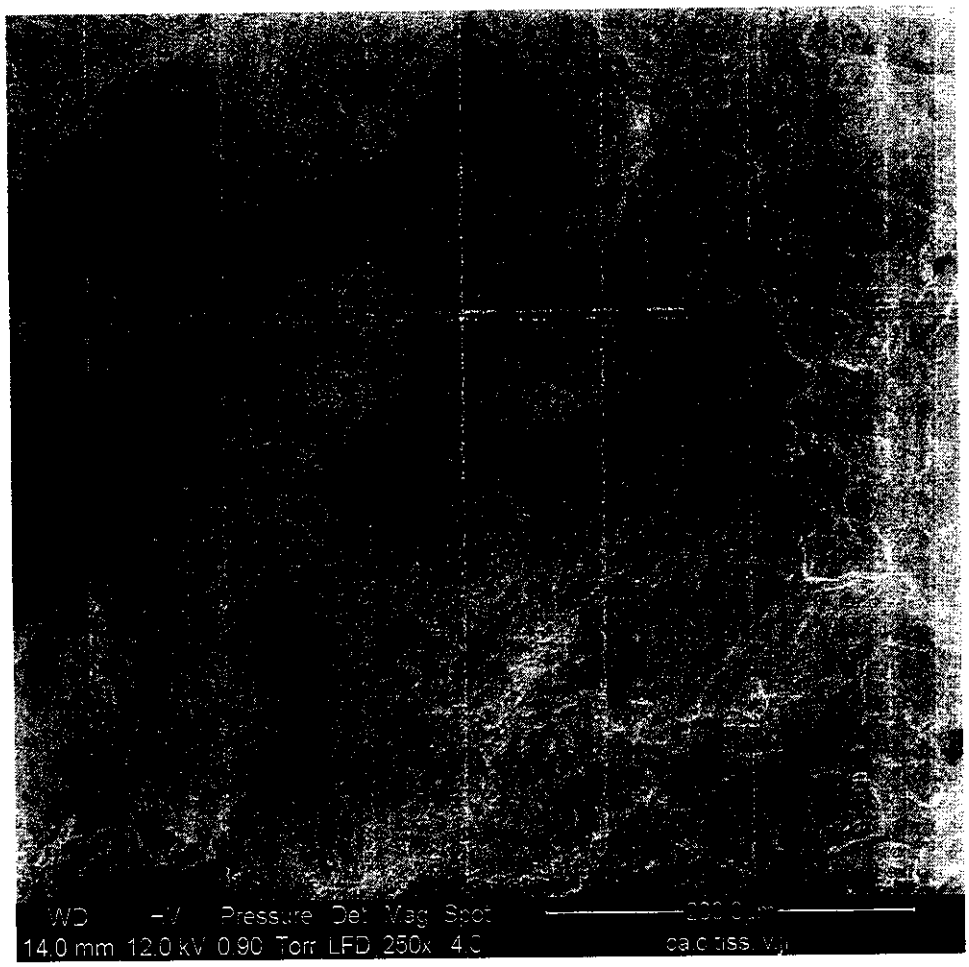


Figure1: POROUS STRUCTURE OF HYDROXYAPATITE MATRIX

Figure2: UNLOADED HYDROXYAPATITE MATRIX

Figure 3: PENICILLIN G LOADED HYDROXYAPATITE MATRIX

Figure 4: UV VISIBLE SPECTRA OF PENICILLIN-G IN WATER

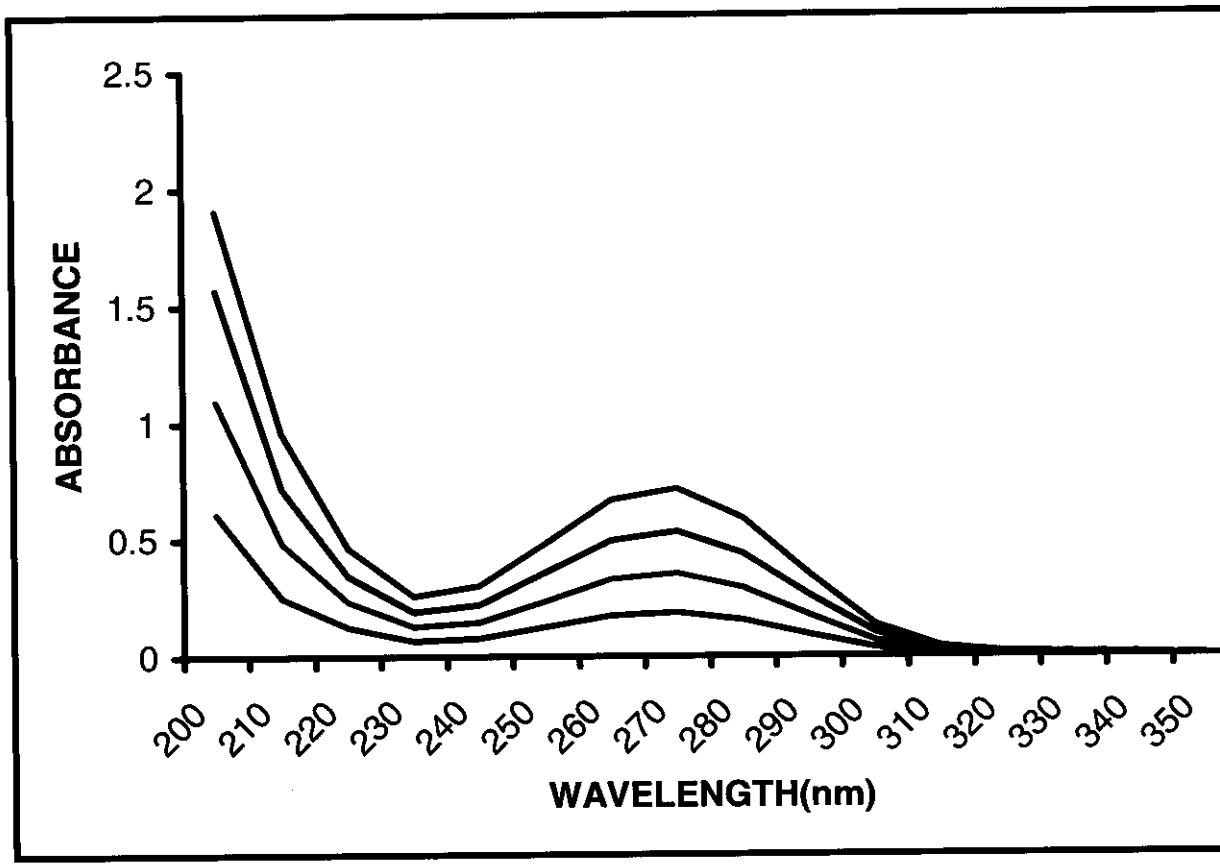


Figure 5: UV VISIBLE SPECTRA OF PENICILLIN G IN BUFFER

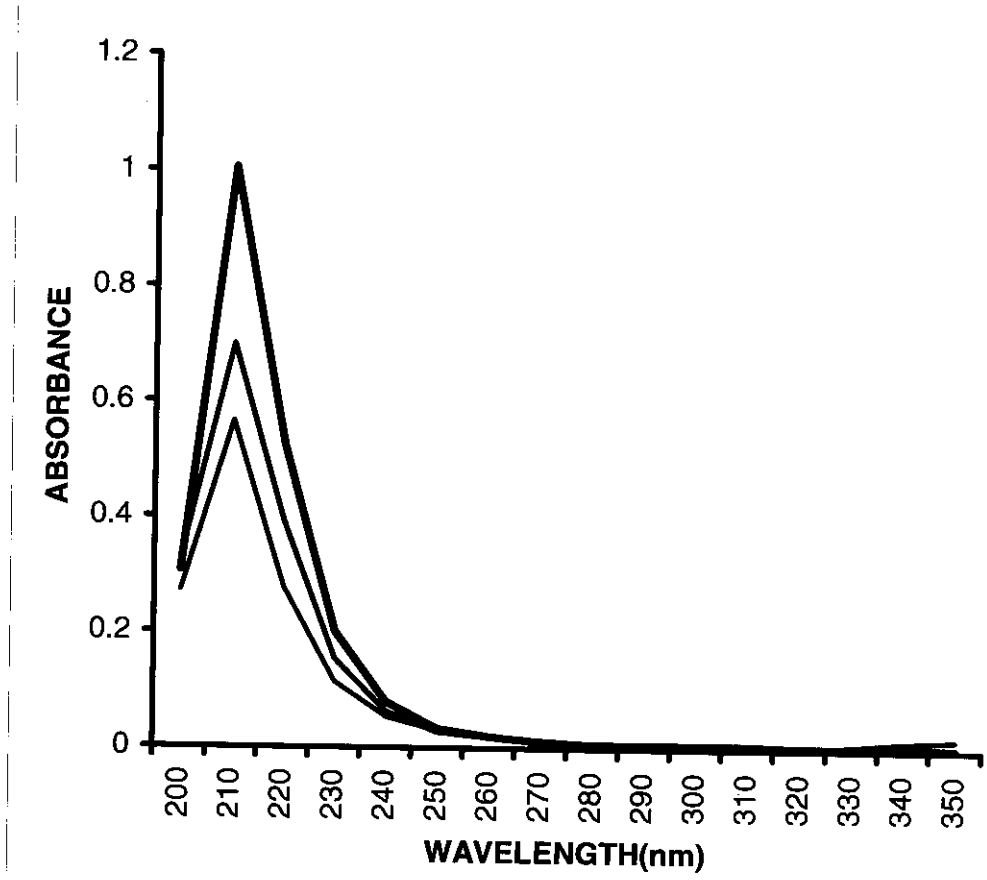


Figure 6: UV VISIBLE SPECTRA OF THE RELEASE PROFILE OF PENICILLIN-G

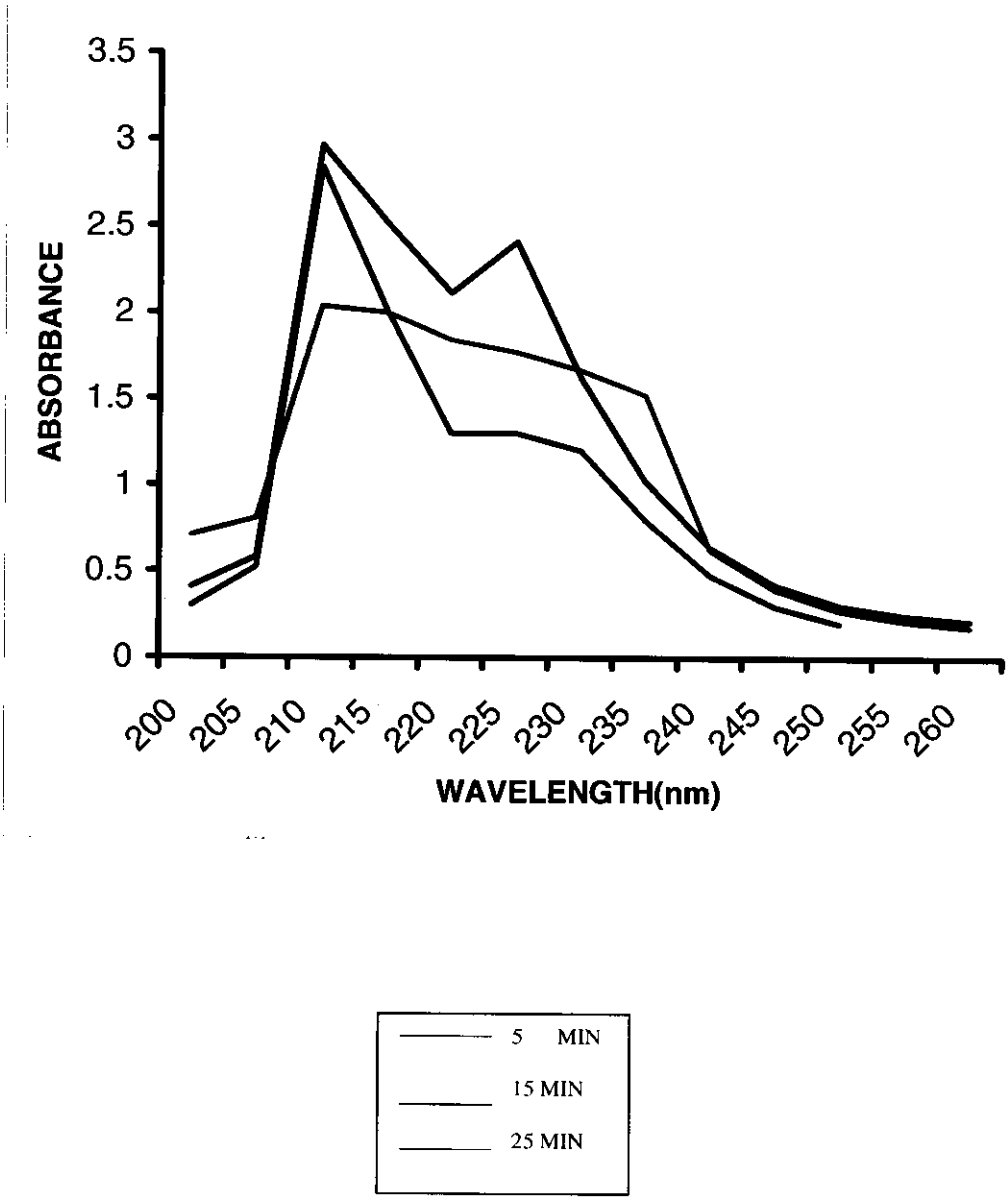


Figure7: RELEASE PROFILE OF PENICILLIN-G

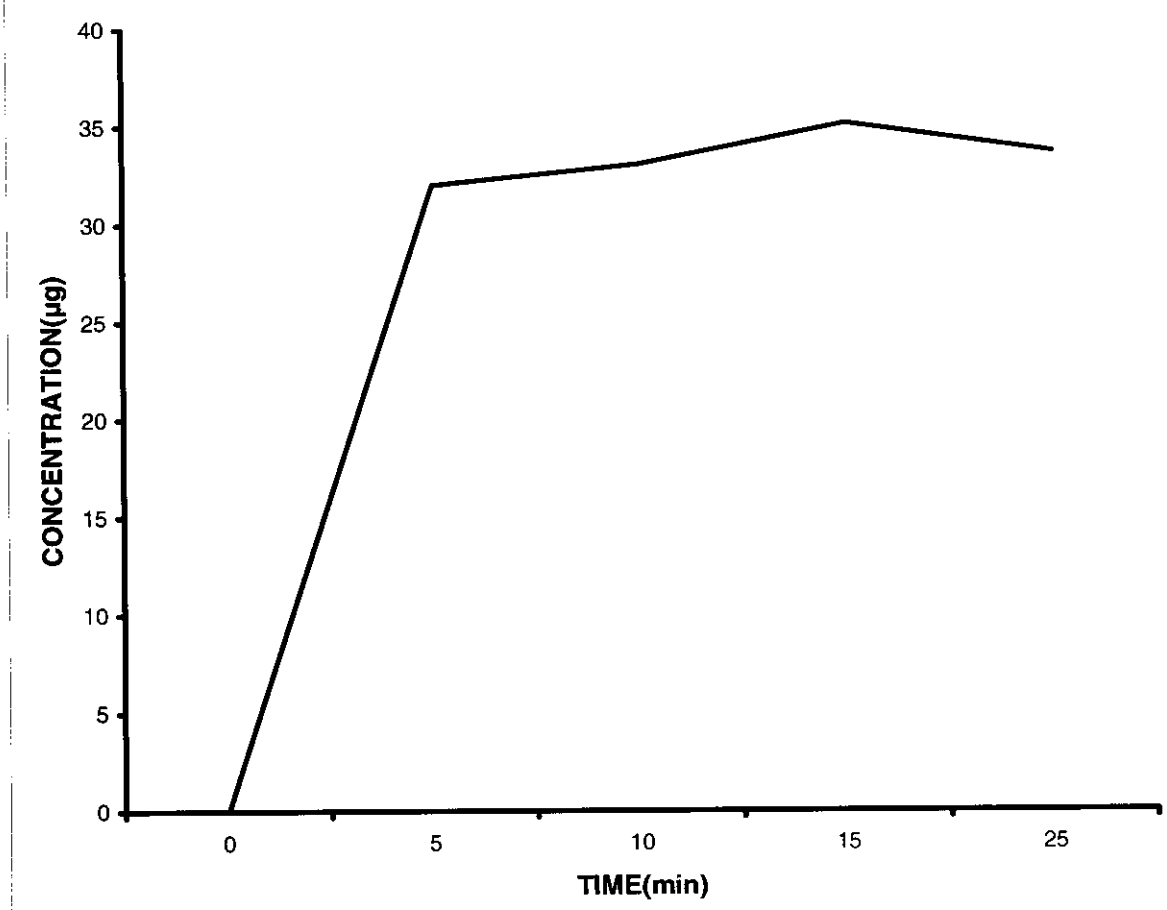


Figure 8 ESTIMATION OF HYDROXY PROLINE

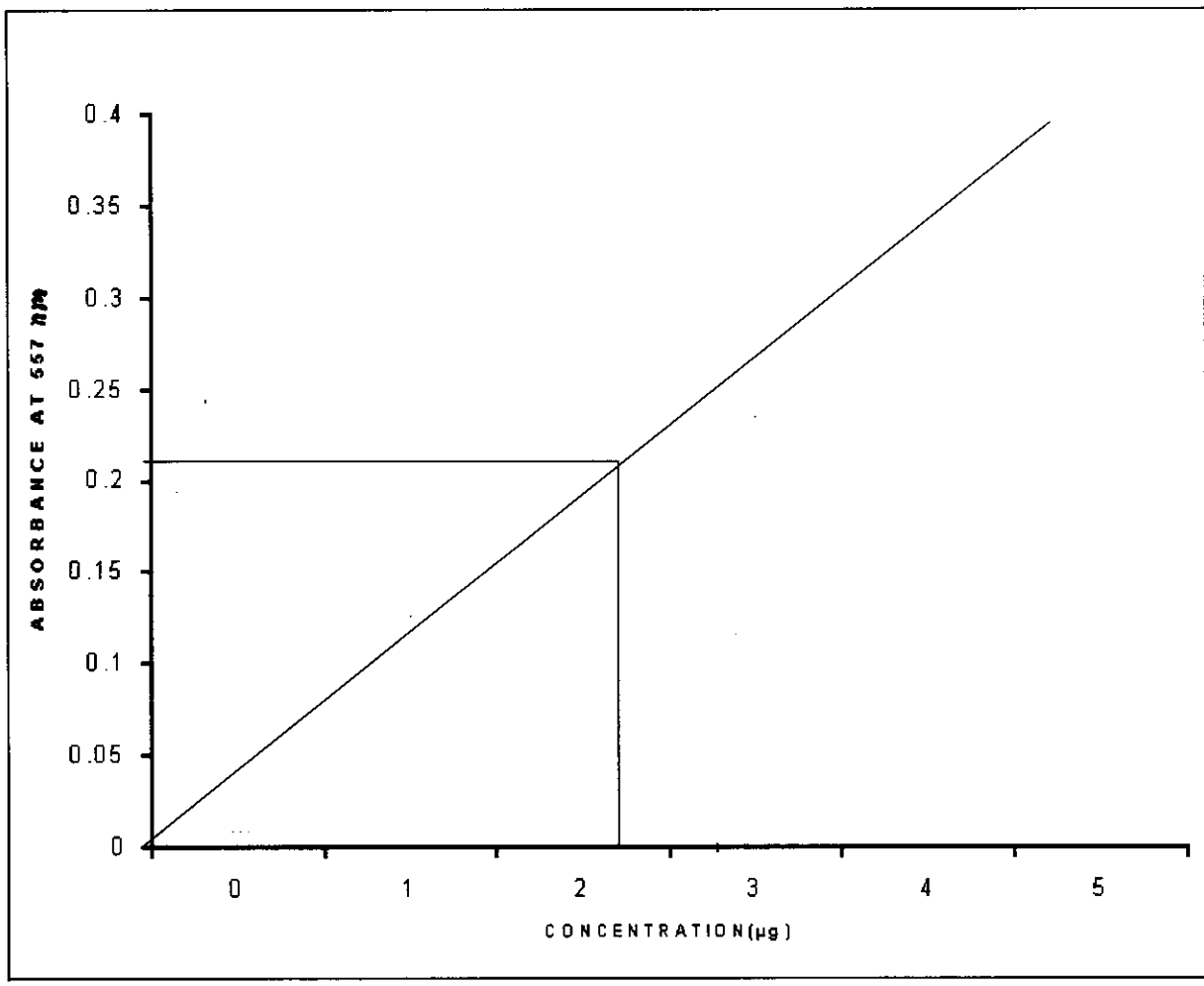


Figure 9 UV VISIBLE SPECTRA OF TRYPTOPHAN

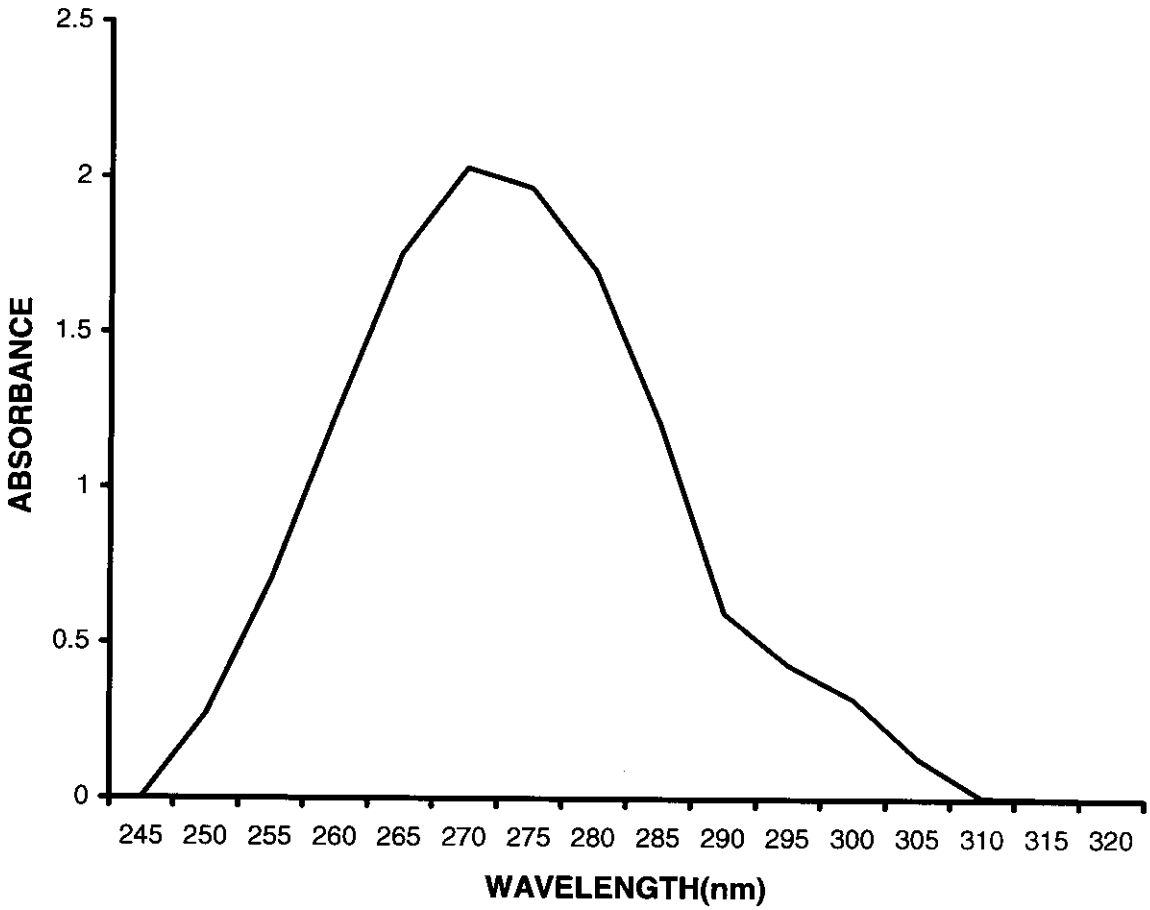


Figure 10: UV VISIBLE SPECTRA OF TYROSINE

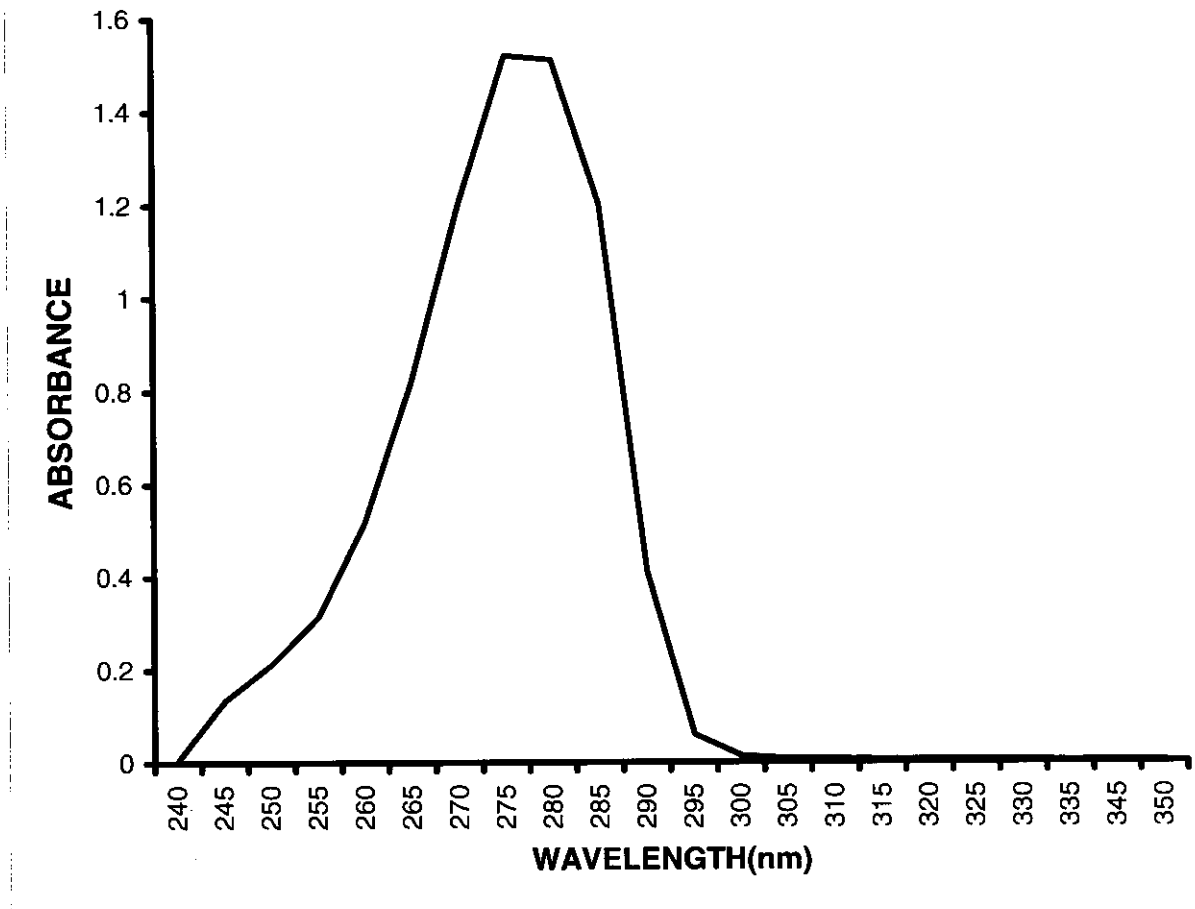


Figure 11: UV VISIBLE SPECTRA OF HYDROLYSATE

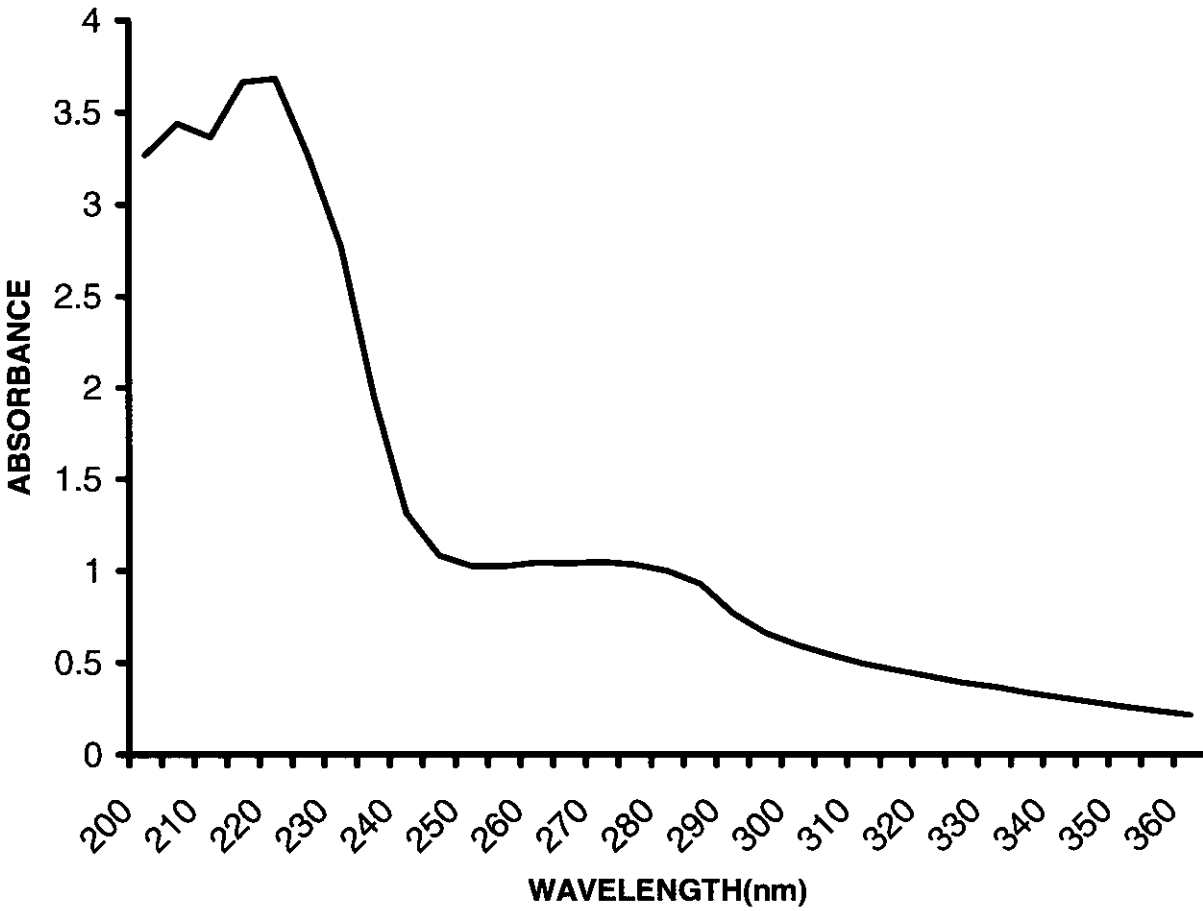
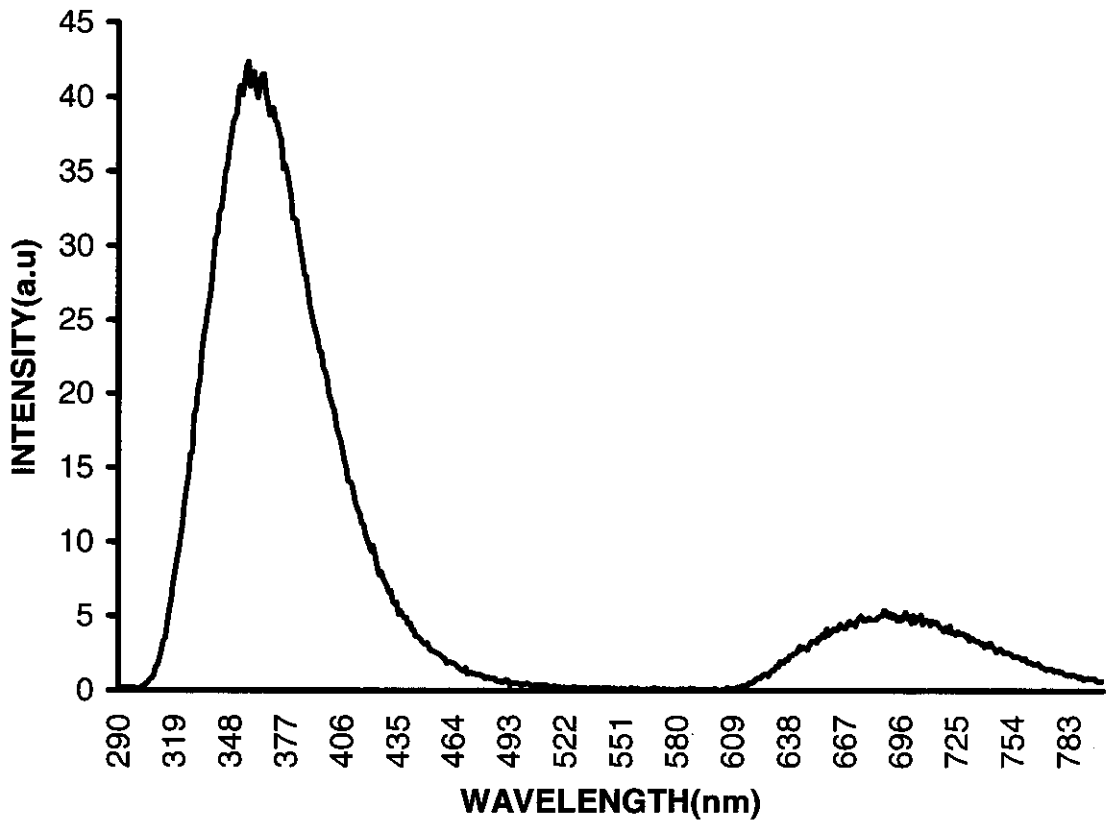


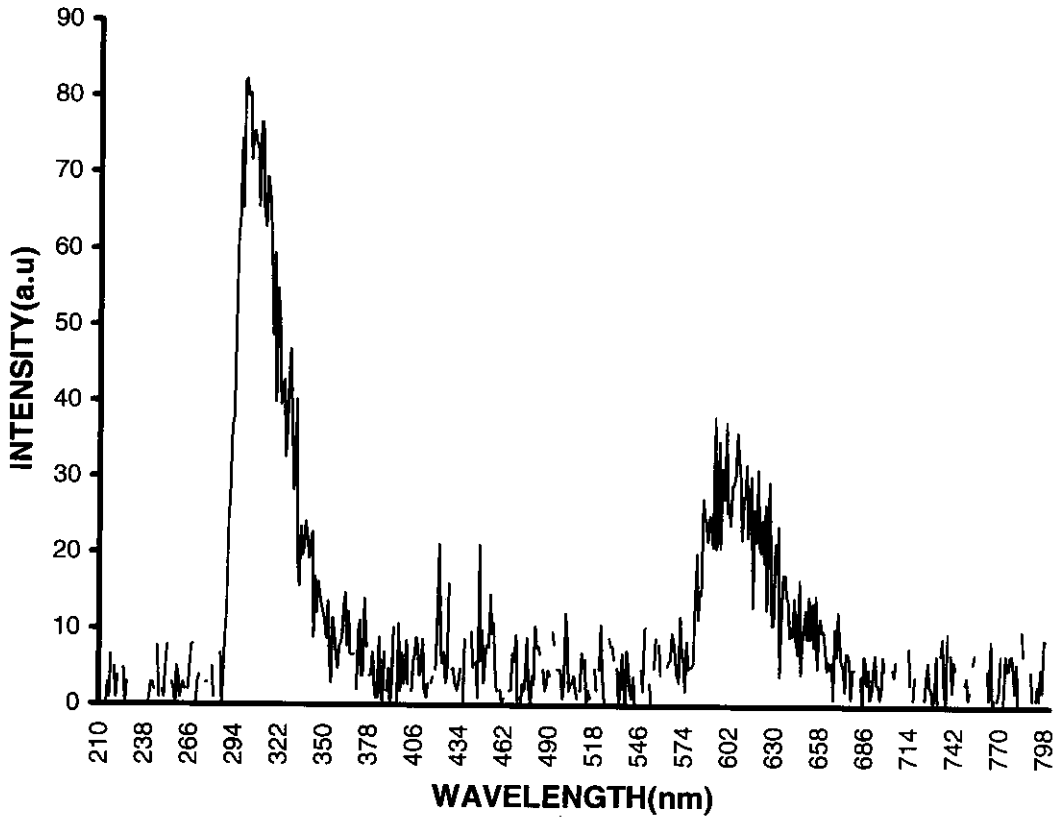
Figure 12: FLUORESCENCE SPECTRA OF TRYPTOPHAN



EXCITATION WAVELENGTH: 270nm

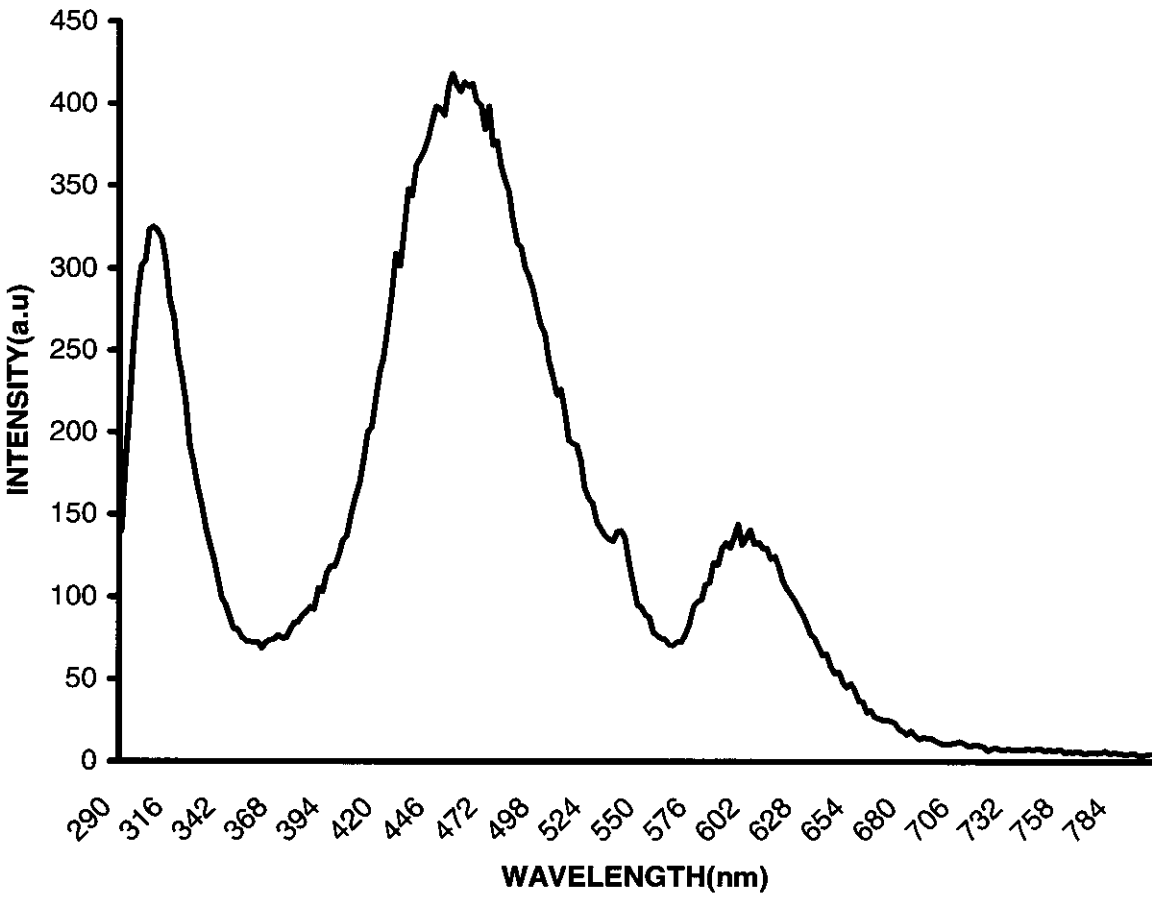
EMISSION WAVELENGTH: 370nm

Figure 13: FLUORESCENCE SPECTROSCOPY OF TYROSINE



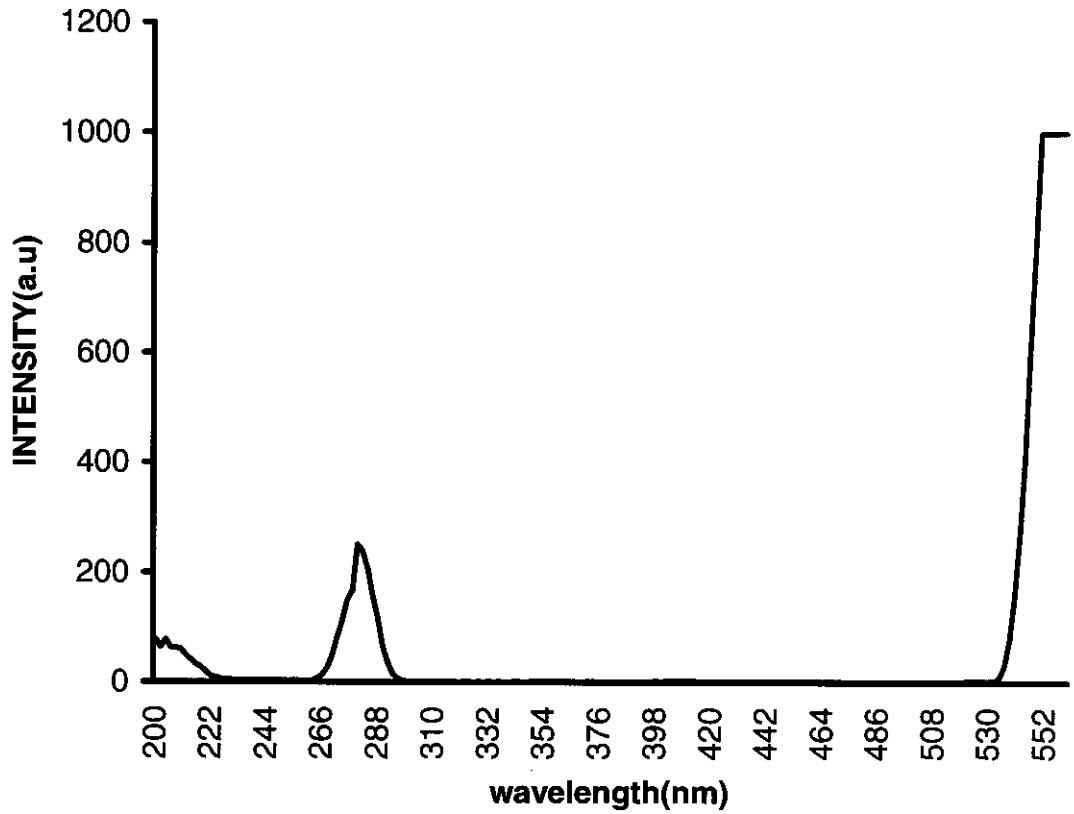
EXCITATION WAVELENGTH: 270nm
EMISSION WAVELENGTH: 310nm

Figure 14: FLUORESCENCE SPECTRA OF HYDROLYSATE

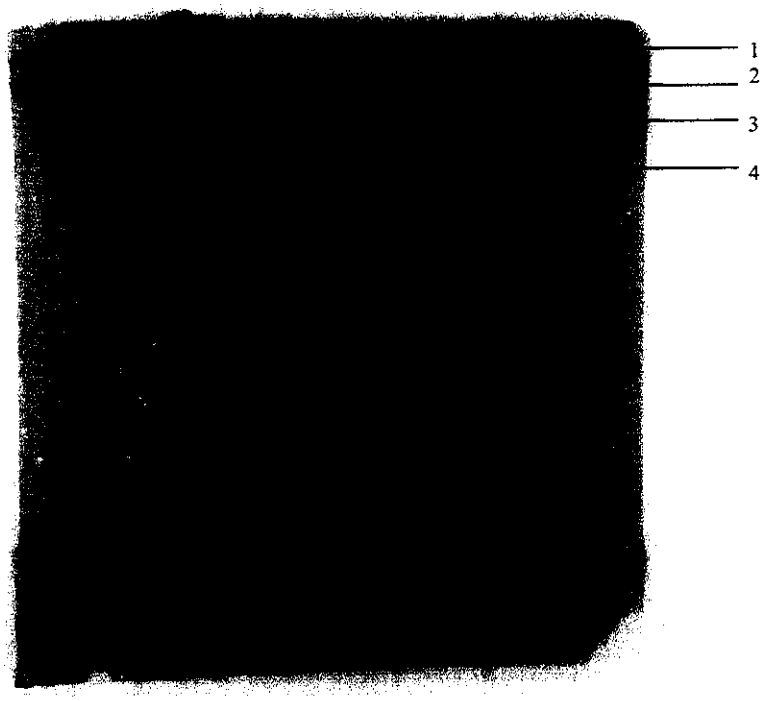


EXCITATION WAVELENGTH: 270nm
EMISSION WAVELENGTH: 313nm, 459nm, 602 nm

Figure 15: FLUORESCENCE SPECTRA OF THE SOLID MATERIAL



EXCITATION WAVELENGTH: 180nm
EMISSION WAVELENGTH: 278nm



1-β 1

2-β 2

3-α 1

4-α 2

Figure 16: SDS GEL ELECTROPHORESIS OF TYPE I COLLAGEN ISOLATED FROM RAT- TAIL TENDON

DISCUSSION:

MORPHOLOGY OF THE TURTLE SHELL:

The turtle shell after removal of the organic matrix by ashing was analyzed by SEM.The picture showed the presence of highly porous matrix. The porous matrix also seemed to possess a high degree of interconnectivity, indicating that this may serve as an ideal biomatrix for delivery of drugs. (Figure 1)

INCORATION OF PENICILLIN G INTO THE MATRIX:

Incorporation of Penicillin-G into the matrix was carried out by simple vacuum infiltration method, where the material was weighed before and after infiltration to find out the amount of antibiotic that had entered the material. Three pieces were infiltrated which contained respective amounts of 35 μg, 200 μg and 50 μg incorporated into it. The morphology of the infiltrated pieces was analyzed by SEM. Particulate materials of Penicillin G in the pores of the shell matrix was observed indicating efficient infiltration of the antibiotic. (Figures: 2,3)

RELEASE OF PENICILLIN G FROM THE SHELL MATRIX:

In order to study the release of Penicillin G from the matrix, a method of estimating the Penicillin G was required. For this, the UV- spectra of Penicillin-G recorded in the presence of water as well as PBS (pH 7.4). The UV-spectra in the presence of water indicated a peak at 270 nm,and this increased with increasing concentrations of Penicillin G.(Table 1 ;Figure 4).The UV- spectra of Penicillin G in the presence of PBS indicated the absorbance maxima at 210 nm.This may indicate the form of sodium salt of Penicillin G .The UV spectra for increasing concentrations of Penicillin G in the PBS also indicated increase in the value of the absorbance at 210 nm. . (Table 2; Figure 5)

The molar extinction coefficient value was found out for Penicillin G in PBS from the value of absorbance obtained at 210 nm.This was carried out in order to estimate the amount of Penicillin G that may be released into PBS.For finding out the release of Penicillin G from the matrix, the antibiotic infiltrated piece was put into PBS,and the spectra was recorded in every 10 minutes interval(Table 3,Figure 6). From the absorbance values obtained at 210 nm and extinction coefficient values calculated for Penicillin G in PBS, the amount of Penicillin G released from the matrix could be calculated. (Table 4, Calculation1, Figure 7)

From the results obtained, it is seen that the release of Penicillin-G increased with increasing time and after 15 minutes the slope of the graph became zero indicating no further release of Penicillin G.

CHARACTERIZATION OF THE ORGANIC MATRIX:

The organic matrix of the shell was investigated after decalcification of the turtle shell with 0.5M EDTA.The organic matrix was subjected to acid hydrolysis and the hydrolysate was made up to a known volume with water.Inorder to investigate if collagen is present,the hydroxyproline content of the hydrolysate was measured using known concentrations of hydroxyproline as standards.A standard graph was obtained and the amount of hydroxyproline in the hydrolysate was obtained.(Table 5,Calculation 2,Figure 8)

From the hydroxyproline content,it is possible to calculate the collagen content by multiplying the value by a factor 7.14,since collagen contains 14% hydroxyproline.It is found that the amount of collagen present in the organic matrix amounts to 54.52%

From the percentage of collagen obtained,it can be inferred that other non-collagenous components are also present in the organic matrix.

INVESTIGATION OF NON-COLLAGENOUS COMPONENTS:

The acid hydrolysate has also been investigated for the presence of other aminoacids such as Tyrosine and Tryptophan since the presence of non-collagenous proteins was indicated.

For this the UV-spectra of authentic samples of Tyrosine and Tryptophan was measured and compared with the UV- spectra of the acid hydrolysate(Table:6,7,8;Figures 9,10,11)

The amino acids Tyrosine and Trypophan also exhibit fluorescence and hence the fluorescence spectra of authentic samples of Tyrosine and Tryptophan were compared with that of the hydrolysate It can be seen that the acid hydrolysate has absorbance and fluorescence characteristics as that of Tyrosine. The fluorescence emission maximum for Tyrosine was found to be 310 nm when excited at 270 nm while Tryptophan has an emission maximum at 370 nm.The hydrolysate when excited at 270 nm,showed emission maxima at 313nm,459nm and 602 nm(Table: 9,10,11;Graph: 12,13,14).The emission maxima obtained at 459nm may possibly represent compounds which are cross links of collagen.

The solid material itself was polished and checked for fluorescence, which showed emission maxima at 278nm when excited at 180nm. This may be possibly due to the fluorescent components present in the material, which could be actually identified by further analysis through chromatographic methods. (Table 12,Figure 15)

ISOLATION OF TYPE I COLLAGEN FROM RAT-TAIL TENDON (RTT)

In addition,an attempt to purify Type I Collagen, from Rat –tail tendon was also carried out. The SDS PAGE- pattern of the purified RTT

Collagen indicates the presence of an α1 and α2 chain, in a ratio of 2:1.The molecular weight of the α chain is around 100,000.Also β components with a molecular weight of 200,000 could be seen in the picture. (Figure 16)

Further investigation need to be carried out to actually identify the collagenous and non-collagenous components and also to study the physio-chemical and biochemical properties of the material in detail. This would be followed by the formation of a composite bilayer matrix by means of cross-linking two biomaterials using a suitable agent, which could be further used as an ideal implant.

CHAPTER 6

CONCLUSION

CONCLUSION:

This study is carried out to characterize and assess the efficacy of a highly porous biomaterial from the carapace of turtle shell, in serving as drug delivery system.The drug component chosen here is an antibiotic, namely Penicillin G for its specific usage in treating bone infections which are predominant after an orthopedic surgery.Also,the collagen content and the non-collagenous proteins present in the material were investigated. The fluorescence emission of the material at its specific excitations was also studied. The results showed the percentage of collagen present,presence of Tyrosine in the non-collagenous portion of the material and the rate of release of Penicillin G from the biomaterial suitable for drug delivery.

Further studies need to be carried out to understand the physio-chemical and biochemical nature of the material in detail. After characterization of the material, the collagen has to be made to gel around physiological conditions and cross linked with appropriate chemicals to the material. An attachment to a layer of chitosan would also be effected at the same time resulting in a bilayer material. The composite matrix has to be processed further to be used as a suitable biomaterial.

CHAPTER 7

APPENDICES

Appendix1: SDS – PAGE ELETROPHORESIS

Electrophoresis is the migration of charged molecules in solution in response to an electric field. Their rate of migration depends on the strength of the field; on the net charge, size and shape of the molecules and also on the ionic strength, viscosity and temperature of the medium in which the molecules are moving. As an analytical tool, electrophoresis is simple, rapid and highly sensitive. It is used analytically to study the properties of a single charged species, and as a separation technique.

SDS-PAGE has a number of uses, which include:

- Establishing protein size
- Protein identification
- Determining sample purity
- Identifying disulfide bonds
- Quantifying proteins
- Blotting applications

Cross-linked polyacrylamide gels are formed from the polymerization of acrylamide monomer in the present of smaller amount of N, N'- methylene bis acrylamide. Bis acrylamide is essentially two acrylamide molecules linked by a methylene group and is used as a cross linking agent. Acrylamide monomer is polymerized in a head to tail fashion into long chains and occasionally a bis acrylamide molecule is built into the growing chain. The polymerization is initiated by the addition of APS and the base TEMED. TEMED catalyses the decomposition of the persulphate ion.

Acrylamide gels are defined in terms of the total percentage of acrylamide present in it. The pore size of the gel can be varied by changing the concentrations of both acrylamide and bisacrlyamide. Thus low percentage gels have large pore size and are used to separate high molecular weight proteins and high percentage gels having smaller pore size are used to separate low molecular weight proteins.

SEPARATION OF PROTEINS UNDER DENATURING CONDITIONS:

Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures proteins by "wrapping around" the polypeptide backbone - and SDS binds to proteins fairly specifically in a mass ratio of 1.4:1. In so doing, SDS confers a negative charge to the polypeptide in proportion to its length - i.e.: the denatured polypeptides become "rods" of negative charge cloud with equal charge or charge densities per unit length. It is usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size: this is done with 2-mercaptoethanol or dithiothreitol. In denaturing SDS-PAGE separations therefore, migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight.

DETERMINATION OF MOLECULAR WEIGHT

This is done by SDS-PAGE of proteins - or PAGE or agarose gel electrophoresis of nucleic acids - of known molecular weight along with the protein or nucleic acid to be characterized. A linear relationship exists between the logarithm of the molecular weight of an SDS-denatured

polypeptide, or native nucleic acid, and its *Rf*. The Rf is calculated as the ratio of the distance migrated by the molecule to that migrated by a marker dye-front. A simple way of determining relative molecular weight by electrophoresis *(Mr)* is to plot a standard curve of distance migrated vs. logMW for known samples, and read off the log*Mr* of the sample after measuring distance migrated on the same gel.

CONTINUOUS AND DISCONTINUOUS BUFFER SYSTEMS:

There are two types of buffer systems in electrophoresis, continuous and discontinuous. A continuous system has only a single separating gel and uses the same buffer in the tanks and the gel. In a discontinuous system, a non-restrictive large pore gel, called a stacking gel, is layered on top of a separating gel called a resolving gel. Each gel is made with a different buffer, and the tank buffers are different from the gel buffers. The resolution obtained in a discontinuous system is much greater than that obtained with a continuous system

Appendix 2: ESTIMATION OF HYDROXY PROLINE:

Hydroxyproline is unique to collagen and it offers a very useful marker for identifying collagen in the presence of non-collagenous proteins.

Soluble collagen (500µl) was hydrolysed in sealed hydrolysis tubes with 6N HCl for 16 hours.The hydrolysates were evaporated to dryness in a porcelain dish over a water bath to remove excess acid.The residue,free of acid,was made up to a known volume and hydroxyproline content was estimated.

PRINCIPLE:

This method involves oxidation of hydroxyproline to pyrrole-2-carboxylic acid which complexes with p-dimethylamino benzaldehyde exhibiting maximum absorption at 557 nm.

%Hydroxyproline = {conc. In/weight of sample} X dilution factor.

%Soluble collagen=% hydroxyproline X 7.4

REAGENTS:

Buffer (pH 6.0): 50g citric acid,12 ml of acetic acid,120 g of Sodium acetate ,34 g of NaOH was made up to 1 L and p H adjusted to 6.0.

Chloramine-T:

0.5M Chloramine –T was prepared by dissolving 1.41 g of the same, in 20 ml of water, 30 ml of methyl cellosolve and 50 ml of buffer (p H 6.0)

Perchloric acid:

10.8ml of 70% Perchloric acid made up to 40 ml.

p-dimethlaminobenzaldehyde(pDAB):

A 20 % solution of pDAB in methyl cellosolve was prepared freshly before use.

Hydroxy proline standard:

A stock solution was prepared by dissolving 10 mg of L-hydroxy proline in 100 ml of 0.001N hydrochloric acid.Standards were prepared by diluting the stock with water to obtain a concentration of 1 to 5 µg/2ml.

Sample preparation:

Known weight/volume of protein was hydrolyzed with 6N HCl,in sealed tubes at 110°C for 16 hrs.After hydrolysis ,the sample was evaporated to dryness the residue was dissolved in water and made up to a known volume(5ml).

PROCEDURE:

2ml portions of the samples were placed in test tubes.A series of standards were prepared and Chloramine T was added to it.It was allowed to stand for 20 min.Then 1 ml of Perchloric acid was added to destroy any excess of Chloramine-T followed by the addition of 1ml of Pdab.The solution was then heated at 70°C for 20 min,cooled well and absorbance read at 557nm.The hydroxy proline in the unknown sample was calculated using the calibration curve.

APPENDIX 3: SCANNING ELECTRON MICROSCOPY

In a typical SEM electrons are thermionically emitted from a tungsten or lanthanum hexaboride (LaB_6) cathode and are accelerated towards an anode; alternatively electrons can be emitted via field emission (FE). Tungsten is used because it has the highest melting point and lowest vapour pressure of all metals, thereby allowing it to be heated for electron emission. The electron beam, which typically has an energy ranging from a few hundred eV to 50 keV, is focused by one or two condenser lenses into a beam with a very fine focal spot sized 1 nm to 5 nm. The beam passes through pairs of scanning coils in the objective lens, which deflect the beam in a raster fashion over a rectangular area of the sample surface. As the primary electrons strike the surface they are inelastically scattered by atoms in the sample. Through these scattering events, the primary electron beam effectively spreads and fills a teardrop-shaped volume, known as the interaction volume, extending about less than 100 nm to 5 μm depths into the surface. Interactions in this region lead to the subsequent emission of electrons which are then detected to produce an image. X-rays, which are also produced by the interaction of electrons with the sample, may also be detected in an SEM equipped for energy dispersive X-ray spectroscopy or wavelength dispersive X-ray spectroscopy.

DETECTION OF SECONDARY ELECTRONS:

The most common imaging mode monitors low energy (<50 eV) secondary electrons. Due to their low energy, these electrons originate within a few nanometers from the surface. The electrons are detected by a scintillator-photomultiplier device and the resulting signal is rendered into a two-

dimensional intensity distribution that can be viewed and saved as a Digital image. This process relies on a raster-scanned primary beam. The brightness of the signal depends on the number of secondary electrons reaching the detector. If the beam enters the sample at a perpendicular the activated region is uniform about the axis of the beam and a certain number of electrons "escape" from within the sample. The higher the angle of incidence of the beam with the sample one side of the axis of the beam will have a much smaller "escape" distance and more secondary electrons will be emitted. Thus steep surfaces and edges tend to be brighter than flat surfaces resulting in images with good three-dimensional appearance. Using this technique, resolutions less than 1 nm are possible.

DETECTION OF BACKSCATTERED ELECTRONS:

In addition to the secondary electrons, backscattered electrons can also be detected. Backscattered electrons may be used to detect contrast between areas with different chemical compositions. These can be observed especially when the average atomic number of the various regions is different.

Backscattered electrons can also be used to form an electron backscatter diffraction (EBSD) image. This image can be used to determine the crystallographic structure of the specimen.

There are fewer backscattered electrons emitted from a sample than secondary electrons. The number of backscattered electrons leaving the sample surface upward might be significantly larger than those that follow trajectories toward the sides. Additionally, in contrast with the case with secondary electrons, the collection efficiency of backscattered electrons cannot be significantly improved by a positive bias common on Everhart-

Thornley detectors. This detector positioned on one side of the sample has low collection efficiency for backscattered electrons due to small acceptance angles. The use of a dedicated backscattered electron detector above the sample in a "doughnut" type arrangement, with the electron beam passing through the hole of the doughnut, greatly increases the solid angle of collection and allows for the detection of more backscattered electrons.

RESOLUTION OF THE SEM

The spatial resolution of the SEM depends on the size of the electron spot which in turn depends on the magnetic electron-optical system which produces the scanning beam. The resolution is also limited by the size of the interaction volume, or the extent of material which interacts with the electron beam. The spot size and the interaction volume are both very large compared to the distances between atoms, so the resolution of the SEM is not high enough to image down to the atomic scale, as is possible in the transmission electron microscope (TEM). The SEM has compensating advantages, though, including the ability to image a comparatively large area of the specimen; the ability to image bulk materials (not just thin films or foils); and the variety of analytical modes available for measuring the composition and nature of the specimen. Depending on the instrument, the resolution can fall somewhere between less than 1 nm and 20 nm. In general, SEM images are much easier to interpret than TEM images, and many SEM images, beyond their scientific value, are actually beautiful

CHAPTER 8

REFERENCES

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