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Kinetic Characterization of Tetracycline Binding and Release by Demineralized Bone - DFDBA

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KINETIC CHARACTERIZATION OF TETRACYCLINE BINDING AND RELEASE BY DEMINERALIZED BONE - DFDBA

by

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ABSTRACT

KINETIC CHARACTERISTICS OF TETRACYCLINE BINDING AND RELEASE BY DEMINERALIZED BONE

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The microbial etiology of periodontal diseases has led to widespread research in the development of methods and local delivery systems to increase the efficacy of antibiotic therapy. Several drug delivery systems employing biodegradable and nonbiodegradable carriers have been shown to release antibiotics directly into periodontal pockets. The purpose of this study was to determine the binding and release kinetics of tetracycline by demineralized bone. Further aspects of the study include *in vitro* evaluation of DFDBA (demineralized freeze-dried bone allografts) as a tetracycline carrier system for periodontal therapy. Experiments were performed which employed different tetracycline concentrations with constant amounts of bone and constant tetracycline concentrations with different amounts of bone to determine the kinetics of tetracycline binding. Time course studies of tetracycline binding and release by bone were performed to assess binding capacity and release as a function of time. DFDBA was demonstrated to maximally bind 0.014 mg tetracycline per mg dry weight of DFDBA. Thirty percent of the tetracycline bound to DFDBA was released in a time dependent manner over a four hour incubation period. The rate of tetracycline release was curvilinear over the

incubation period studied. The rate of release exhibited a rapid linear decrease for the first 40 minutes and followed a very slow and nonlinear decrease over the remaining 200 minutes suggesting DFDBA may act as a slow release device for the tetracycline.

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INTRODUCTION

The basic rationale for the concept of controlled drug delivery is to promote the therapeutic efficacy of a drug by augmenting delivery of a drug to the target site while eliminating or reducing drug exposure of healthy tissues. Controlled drug delivery is especially important in antibiotic therapy where the therapeutic level and duration of the agent in the diseased area become essential parts of an effective therapy. In the treatment of periodontal diseases, including regenerative therapy, elimination of pathogenic microbial flora has to be achieved. Antibiotics such as tetracycline, metronidazole and chlorhexidine have been widely used for this purpose in periodontal treatment. Tetracyclines have been the antibiotics of choice since the demonstration that in addition to their antibacterial effects they also inhibit destructive enzymes such as collagenase hence reducing bone destruction. In regenerative therapy, they have acid conditioning-like effects on the bone structure (1, 2).

Systemic administration of tetracycline has been avoided due to the gastric side effects and the risk of producing resistant bacterial flora and the risk of elimination of normal flora. A second difficulty associated with systemic tetracycline use is the establishment of a therapeutically effective concentration levels at the diseased area without producing toxic levels systemically.

Various local controlled delivery systems have been introduced and clinically evaluated as a means of prolonged release of the agent at a therapeutically effective levels.

The journal model used for this thesis is the journal *Biochemistry.*

These systems have been applied specifically for antibiotic delivery in periodontal and orthopedic therapy. Local drug carrier devices are primarily based on polymer technology. Drug is dispersed within a solid polymer matrix in the form of strips or fibers and implanted or placed into the periodontal pocket in the case of periodontal therapy. Nonbiodegradable polymers such as polymethylmethacrylate (PMMA) as matrices or beads and biodegradable polymeric carriers such as polyanhydrides have been introduced to deliver antibiotics such as gentamycin, fucidin and clindamycin in the treatment of wound infection (3).

Application of a local drug delivery system in periodontal therapy was first reported by Goodson et al. (4). In their study, permeable hollow fibers of cellulose acetate filled with tetracycline solution were placed in periodontal pockets. Tetracycline-containing nonbiodegradable ethylene vinyl acetate (EVA) monolithic fibers (5) have also been reported as a useful slow release device. Recently tetracycline immobilized collagen matrix has been introduced for clinical use in periodontal therapy (6).

Tetracycline interaction with bone matrix has been known since 1957 when tetracycline deposition in the bone structure was observed during clinical trials. After long term tetracycline therapy, discoloration of teeth and retardation of skeletal development were noticed as primary side effects of tetracycline use (7). Since then tetracyclines have been used as a useful marker to assess the rate of bone formation and destruction in histological studies.

In this study, a fluorometric tetracycline assay was used for the quantitative determination of tetracycline in solution and an experimental procedure was designed to identify the kinetics of tetracycline binding and release by demineralized bone (DFDBA-

demineralized freeze-dried bone allograft). DFDBA was evaluated *in vitro* as tetracycline carrier system for periodontal therapy.

LITERATURE REVIEW

A compound is considered as a therapeutic agent and used if its toxic effect is minimal compared to its beneficial effect. Absorption and body distribution of a therapeutic agent is in general dependent on the physicochemical properties of the agent and the existence of certain barriers to the agent. Solubility reactivity and stability of a given agent determine the distribution pattern. Indeed for the most of the therapeutic agents, administration for a particular local condition results in drug exposure of nontarget tissues and a large amount of administered agent may not be able to reach the diseased area failing to meet the minimal required agent concentration at the deseased area. This being the reason generally high doses have to be administered to achieve the effective concentration. Another undesired event associated with conventional drug administration is that, especially with the drugs having a short half-life, frequent administration is required and this results in maximum highs and lows of drug concentration.

To overcome these problems, development of methods and techniques by which drugs could be concentrated at the diseased areas or the function of the agent could be restricted to the target have to be developed. Such a system would minimize the side effects by eleminating the exposure of nontarget sites to the agent. The concept of drug targeting was stated by Paul Ehrlich (8) almost a century ago. He used the term "magic bullet", a technology whereby drugs were imported with the ability to localize at the diseased site in the body.

Methods and techniques for controlled drug delivery have been the subject of intense research since the 1950s. Delivery systems with unique success for the elimination

of certain problems in drug administration have been developed and have employed drug carriers, macromolecular conjugates, and prodrugs. However particular systems to meet all properties of intended " Controlled Drug Delivery" are not yet available.

2. 1 CONTROLLED DRUG RELEASE FROM POLYMERIC SYSTEMS

In 1964 Folkman et al., while studying heart valves, noticed that silicon elastomer used to fabricate the valves absorbed and released dyes during the fabrication process. They later showed that lipid soluble dyes easily diffused from the silicon membrane (9). These studies represented pioneer developments for the use of polymeric drug delivery systems. By confining deposition of a therapeutic agent within a polymeric capsule, Long et al. (10) was able to demonstrate that only small amounts of agent diffused out through the capsule wall with time providing a controlled release rate. The ability of capsules to slowly release steroid hormones was shown by Dzuk et al. (11). Using silicon capsules Dzuk et al. achieved the slow release of testosterone and a consequent long term control of fertility (11). Subcutaneous implantation of silicon capsules containing fertility inhibitor norprogesteron provided 100% inhibition of fertility for a long period of time in female mice although only 10% of the agent had been released from the implant (12). Chang et al. (13) reported that the efficacy of the agent increased when implanted in silicon capsules, and that dose requirements were lower as compared to oral administration and/or subcutaneous injection. Silicon elastomer was later used as delivery vehicle for slow release of many therapeutic agents such as medroxyprogesterone (14), norgestrel, and norethindrone (15). Besides polymeric capsules, matrix type polymeric devices were

developed by Duncan et al. (16) and extended to subcutaneous implantation by Chien et al. (17).

Hydrophilic copolymer of poly(hydroxy ethyl methacrylate) with ethylene glycol dimethacrylate as a matrix type drug delivery system for estrus synchronization was developed in 1968s. The therapeutic agent, either encapsulated in a polymeric membrane in capsule type delivery or homogeneously dispersed in a polymer in a matrix type system, diffuses through the polymer phase to the surrounding fluid. The release is almost constant from the capsule type system but is dependent on drug content in the matrix type system (18). The use of synthetic nonbiodegradable polymers, such as silicon and polyethylene, as drug delivery vehicles requires a second surgery to remove the implant.

The first use of a biodegradable synthetic polymer as a matrix type delivery system was by Yolles et al. (19) who described the release of cyclozocine from a poly(lactic acid) (PLA) implant. It was found that about two months after implantation the polymer matrix had disappeared (19, 20). The degradation product of PLA is lactic acid, which represents a naturally occuring compound in the body.

Polymeric matrix type drug delivery systems involve incorporation of a drug into the polymer matrix and implantation of the device. Via some modifications in the preparation process of agent-polymer matrix, composites shaped as films or pellets can be achieved. For the preparation of composite polymer materials, drugs are mixed with the polymer and the mixture is combined with tributyl citrate as plasticizer and dissolved in dichloromethane. After removal of the solvent the remaining material can be processed to obtain different kinds of composites (21). After implantation the drug diffuses from the interior of the polymeric composite to the outer surface where it is taken away by body

fluids. Experiments by Yolles et al. (22) demonstrated that implanted cyclazocine-PLA (molecular weight of 45,000-70,000) excreted 70-80 % of the administered dose in 49 days. After approximately five days, during which the release rate was high, the drug was released at an almost constant rate of ~300 μg/day. The use of poly(lactic acid) (PLA) with anticancer agents, cyclophosphamide (2-[bis(2-chlorethyl)mino]-tetrahydro-2H-1, 3, 2-oxaz aphosphorine 2-oxide), doxorubicin and *cis*-pta2Cl2 were tested by Yolles et al, (23). They implanted the agent loaded PLA films in rats and demonstrated that 67% of the administered dose was released over a 34-day period. They reported that the blood level of released agent was active against the tumors tested.

In 1978, a new polymeric system in which drug is covalently attached to the polymeric chains was developed (23). Release of the drug in this system occurs by hydrolysis of the linkage. They bound anticancer agent methotrexate on hydroxypropylcellulose via amide bonds and observed that hydrolysis of the amide bond liberated the agent.

Incorporation of antibiotics in acrylic cement was described where the antibiotic gentamycin was incorporated in a polymethyl-methacrylate copolymer cement (24). Antibiotic loaded polymeric cements have been used in the treatment of joint arthroplasty since 1972 (25). Release of the antibiotics clindamycin, erythromycin, and tetracycline from acrylic cements were also tested (26). Using antibiotic (gentamycin) loaded polymer bead implants in the medullary canal in the treatment of posttraumatic osseous and tissue infections, Vecsai et al. (27) observed effective local concentrations and minimal systemic concentrations of gentamycin. Application of antibiotic incorporated polymers has also been applied in the treatment of chronic osteomyelitis (28). One of the problems in the

treatment of osteomyelitis is the difficulty in obtaining bactericidal levels of antibiotic in infected bone without achieving toxic effects at the systemic level. Use of delivery systems employing polymeric matrices released antibiotic in effective levels while resulting in undetectable systemic levels have been described (25).

2. 2 MICROPARTICULATE DRUG DELIVERY SYSTEMS

Microparticulate drug delivery systems include microspheres (29), polymeric beads (30), macromolecular complexes (31), nanocapsules (32) and liposomes (33). These carriers are small colloidal particles and they are typically administered locally. Macromolecules such as proteins, cellulose and polymeric macromolecules such as polylactic acid are suitable substances for the construction of these particles. These particles are available for intravenous injection if the macromolecule is biodegradable. The use of nonbiodegradable particles is limited to implantation devices. Microcapsulation of therapeutic agents, polymer coating of a solid particle or a liquid droplet, via various materials such as albumin, ethylcellulose and poly(LD-lactic acid) have been introduced. Microencapsulation of cytotoxic agents in albumin was first suggested by Kramer et al., (34). Polymers with hydrophobic and hydrophilic moieties form micelles in aqueous media resulting in nanoparticles. Preparation of microspheres and nanoparticles differ in the agent addition step. In microcapsulation the agent is added dropwise to the media containing the polymer solution. For the preparation of nanocapsules (35) drug precipitation occurs in early stages of the process and addition of the agent in late stages results in microcapsulation. After the process is completed

particles with the desired size can be chosen (36). As an example, the construction of gelatin nanoparticles is as follows: The solution of gelatin and active ingredient is added to a solution of sodium sulfate until the system gains a faint permanent turbidity. An alcohol, such as isopropanol, is then added until the turbidity disappears and then gluteraldehyde is added as a stabilizing agent. With the addition of sodium metabisulfide solution the process is terminated and the system is freeze dried. By controlling the desolvation process colloidal particles with desired size could be obtained (37).

Microparticulate drug carriers are potential drug carrier systems in cancer chemotherapy. The primary advantage in the use of these small colloidal embolic particles is that they show very little diffusion when injected intravenously. The particles are trapped by arterioles at the vicinity of the tumor. When trapped they reduce the blood flow and enhance the drug penetration into the tumor interstitium. This system also takes advantage of the characteristics of tumor blood vessels which tend to lack the smooth muscle layer and have a rather fenestrated endothelial cell lining to the arteriol.

Antitumor/antibiotic mitomycin-C administration by albumin microspheres infusion into the hepatic artery of rats with hepatic tumors revealed high local antibiotic concentration and a cytotoxic response (36). Microspheres of mitomycin-C and doxorubicin (38) microcapsulated with albumin were studied in rats and rabbits, respectively. Mitomycin-C was also microcapsulated in ethylcellulose by Keto et al. (39). By infusing these microsphere delivery systems in the renal artery of dogs, Keto et al. (39) demonstrated that microcapsules are trapped in small arteries and agent was released into the surrounding tissue. Arterial injection supplied a high level of local agent concentration while systemic levels were undetectable. Microspheres of poly-DL-lactate-co-glycolide-

ampicilin administration via intramedullary injection in rabbits with osteomyelitis resulted in a 30.7% reduction of the disease (40) as compared to control treatments.

Encapsulation of polar and nonpolar therapeutic agents in liposomes was also described. It was observed that polar drugs, such as methatroxate were entrapped within the internal compartment of liposomes while nonpolar drugs such as actinomycin-D were bound to the lipid membrane. The blood stability of liposomes loaded with anticancer drugs such as methotrexate, actinomycin-D, cytosine arabinoside, and duanomycin in rats were studied and it was reported that liposome encapsulated drugs were available in the blood stream much longer than when free drugs were used (21). Since liposomes are able to fuse with cells or are taken up by endocytosis, they are potential drug delivery systems to target cells. Systemic delivery of antiparasitic agents (41), chelating agents to treat metal poisoning, enzymes to treat lysosomal storage disease (42), and radiopharmaceutical agents in nuclear medicine have been studied. Richardson et al. (43) noticed that liposomes were primarily found in the lymph nodes when injected into the footpads of rats. This study suggested the use of liposomes as carriers for imaging agents. Targeting liposomes to a special cell type or location requires liposome specific cell or tissue interaction. Attachments of antibody and carbohydrate determinants to liposomal membrane were studied for targeting the liposome to particular cells as a function of specific recognition of the receptors on the target cells. The use of antibodies as guiding agent was first reported by Gregoriadis et al. (44) who conjugated the IgG fractions to liposomes and achieved an increased cytotoxic response.

2. 3 CONTROLLED DRUG DELIVERY BY PRODRUGS AND MACROMOLECULE-DRUG CONJUGATES

The concept of prodrug as a drug delivery vehicle was first suggested by Albert et al. (45) and Harper et al. (46). The terms "prodrug" and " drug latentation" were used to describe the concept that a drug is modified giving a new compound after administration with in vivo liberation of parent drug. Once produced, the prodrug is a new compound with novel physicochemical properties in the sense of absorption, solubility, stability, and bioavailability. Classic examples of prodrug are methenamine and aspirin. Methenamine is a prodrug of formaldehyde and aspirin is a prodrug of salicylic acid. Salicylic acid is toxic whereas aspirin is minimally toxic. Formaldehyde can not be used as a urinary-tract antiseptic but is generated from methenamine during excretion into acidified urine. Prodrug can be a chemically modified therapeutic agent or a compound resulting from attachment to a carrier molecule. There is some confusion in the term prodrug. Those systems where drug remains associated with the carrier molecule and is still able to function therapeutically are considered as drug carrier system rather than prodrug. In the prodrug concept, the agent is liberated (21).

With an appropriate prodrug design, dissociation or activation of parent drug can be restricted to specific sites. Dissociation/activation can be via enzymatic cleavage by an enzyme found only in the target site. Dopamine has been used in treatment of Parkinson's disease, however due to its poor stability in the gastrointestinal tract only a small amount of administered drug is able to reach circulation. The blood brain barrier is also impermeable to this agent. A prodrug of dopamine, L-Dopa, is actively absorbed in the

gastrointestinal tract and can actively pass the blood brain barrier by a L-amino acid active transport system. Once in the brain L-Dopa is converted to dopamine by dopa decarboxylase. However, the method is not able to supply site specificity since some of the administered L-dopa is converted to parent drug by peripheral decarboxylase. For this reason in L-Dopa therapy, inhibitors to bloc the peripheral dopa decarboxylase have been used to achieve side specific drug action (21).

Macromolecules including synthetic polymeric units such as ethylene glycol, polylysine, glutamic acid and peptide polymers of DL-alanine and L-tyrosine have been studied. Carriers with prescribed specificity such as antibodies, lectins and hormones have been studied for drug targeting to tumor cells. In later years anticancer agent methotrexate was directed to L1210 leukemia cells using a tumor specific antibody (21). They bound the drug to the antibody via a diazo-linkage. Increased efficacy of the agent via this method was observed in their study. Development of hybridoma technology and availability of monoclonal antibodies resulted in extensive studies describing the production of antibody/drug conjugates. Some of the drug carrier systems employing macromolecules as carriers are shown in Table 1.

Drug is attached to a carrier molecule which protects the drug from degredation and from reacting with other molecules in the body fluid. Carrier molecule directs the drug to the target recognized by the carrier. The interaction of carrier and the agent can be covalent or noncovalent. Ghose et al.(47) succeeded in achieving noncovalent binding of antitumor agent chlorambucil to a tumor specific antibody. Dissociation of the agent at the vicinity of the tumor cells and resulting site specific cytotoxic function was reported by the same group.

Table 1: Carrier Conjugate Systems (21).

Therapeutic agent	Carrier molecule	Conjugation
Methotrexate	Antibody	Mixed anhydride
	Albumin	Carbodiimide
	Fibrinogen	Diazotization
	Polylisine	Carbodiimide
Chlorambucil	Antibodies	Carbodiimide, Adsorption
	IgG	Noncovalent binding
	Serum proteins	Noncovalent binding
Adriamycin	Antibody	Periodate oxidation
	Fab′ dimers	Carbodiimide
Duanomycin	Antibody	Carbodiimide
	Fab′ dimers	Carbodiimide
	Albumin	Mixed anhydride
	Dextran	Periodate oxidation
	Polyglutamate	Carbodiimide (trapped in liposomes)
Actinomycin D	Albumin	Binding
	DNA	Noncovalent binding
Phospholipase C	Antibody	Gluteraldehyde
Diphteria toxin	Antibody	Gluteraldehyde
Superoxide dismutaze	Dextran	Cyanogen bromide

Following binding of anticancer agents, 5-fluorouracil, chlorambucil and the antibiotic tetracycline to antibodies, cellular toxication toward cancer cells were studied. It was reported that antibody coupled with drug still retained its ability to bind to antigen. Antibody conjugates of the cytotoxic antibiotics duanomycin (48) and adriamycin (49), and alkylating agents for cancer chemotherapy such as trenimon (50) have been described.

Concanavalin-A, a lectin, was used as a macromolecular carrier for L-asparaginase by Shier et al. (51). They reported that 80% of the activity of the enzyme was retained in the gluteraldehyde cross-linked conjugate. Dextran was first used by Bernstein et al. (52). They conjugated duanomycin with dextran and coupled this conjugate to antibody. The resulting prodrug was highly cytotoxic against YAC cells (52). Dextran/duanomycin conjugates were also studied by Levi-Schaffer et al. (53) who reported that toxic effects to heart tissue and reduction of bone marrow cell counts, which are the side effects of this agent when used alone, were not observed.

The antibiotic mitomycin-C-dextran conjugates were made by Kojima et al. (54). Duanomycin- poly-L-lysine conjugate was reported by Arnord et al. (55).
Binding of therapeutic agents to albumin and other plasma proteins such as fibrinogen and globulin have been studied as drugs active against tumor cells. Conjugates of methotrexate-albumin against L1210 leukemia cells (56), and L-asparaginase-albumin against 6C3HED lymphosarcoma in mice and human pancreatic tumor cells have been studied. It was reported that the L-asparaginase-albumin conjugates prolonged survival in mice and inhibited the tumor growth in humans (57). All albumin conjugates, in which the agent is liberated by enzymatic cleavage, were reported to be effective in the treatment of cancer (58). Binding of anticancer agents to fibronectin and globulin were reported by Szekerke et al. (59). In their study conjugation of compounds, actinomycin-D, cytosine arabinoside, 5-azacytine, and dichloromethotrexate with plasma proteins was achieved and therapeutic effectiveness was tested.

2. 4 CURRENT METHODS AND DELIVERY SYSTEMS IN PERIODONTAL THERAPY

The application of local antibiotic therapy using several methods has been investigated. Tetracycline solution treatment to dehydrated lyophilized bone prior to implantation (60), the use of hollow fiber devices (4), and a combination of tetracycline and demineralized freeze dried bone allografts were some early methods studied for local administration to periodontal lesions. Hollow fibers of cellulose acetate filled with tetracycline solution were shown to release about 90% of the fixed tetracycline within two hours (4). In a clinical study the effectiveness of hollow fibers containing tetracycline was shown to be similar to that of scaling and root planing (61).

Polymeric devices employing monolithic fibers of ethylene vinyl acetate (62, 63), ethylcellulose-polyethylene glycol and, ethyl cellulose (64) and polymethyl-methacrylate strips (65) have been used to achieve slow release of the antibiotics in periodontal pockets. Monolithic fibers, especially ethylene vinyl acetate, have been reported as a useful system for prolonged tetracycline release. These fibers retained tetracycline at a high concentration in the periodontal pocket for ten days and clinical studies revealed that there were improvements in the parameters associated with periodontal health (63).

Tetracycline immobilized acetocollagen films have been tested as a local drug delivery system by Minabe et al. (6). They used gluteraldehyde to cross-link the agent to collagen and stabilize the device. The resulting film (1 g tetracycline powder / 1g collagen) was placed into periodontal pockets and studied for efficacy. Cross-linked tetracycline dissolved in the periodontal pockets. In the tenth day the tetracycline

concentration measured in the periodontal pockets was reported to be 17μg/mL. This was higher than the minimally required therapeutic level which was reported as 8 μg/mL (6).

Recently tetracycline hydrochloride containing fibers have been produced and marketed under different brand names for use in combination with scaling and root planing as a treatment. Periodontal fibers of ethylene/vinyl acetate copolymer, with evenly dispersed tetracycline, have been manufactured by ALZA laboratories (ALZA Corporation, Palo Alto, CA 94304) under the brand name "ACTISIDE". The device releases tetracycline and results in periodontal pocket concentrations of 1590 μg/mL through 10 days. The device must be removed after ten days, as recommended by manufacturer. Use of ethylene vinyl acetate fibers combined with scaling provided a better treatment than scaling alone (65).

A new method, ATRIGEL Drug Delivery System, a polymeric (poly (DL-lactide)) drug carrier matrix has been developed by Atrix Laboratories Inc. They used solid polymer and mixed it with a liquid carrier. The mixture gives a viscous fluid which has the ability to slowly release the drug that was added. Various drugs have been added in this formulation. Dropwise addition of this viscous fluid into an aqueous solution releases the carrier while the drug coprecipitates with the polymer. The polymer used is biodegradable and releases the drug as it bioabsorbs (Atrix Laboratories, Inc. Colorado). This delivery systems are reportedly able to release therapeutically effective tetracycline concentrations throughout 10 days of implantation (66).

2. 5 TETRACYCLINES AND THEIR CHEMISTRY

Tetracyclines are a group of broad-spectrum antibiotics. The basic structure is composed of four six-membered rings (Figure 1). The structure possesses many hydroxyl and oxygen groups important for activity. Different tetracycline homologues and their derivatives have different groups on the basic structure and they are slightly different in their chemical behaviors. In the 1940s, the first tetracycline isolated from *Streptomyces aureofaciens* was chlorotetracycline. Isolation of oxytetracycline, tetracycline, dimethychlorotetracycline, metacycline, doxycydine and minocycline were reported in the late 1940s. Tetracyclines have been used against a broad spectrum of gram positive and gram negative bacteria. The bacteriostatic effect of tetracyclines is specifically due to the interference with bacterial protein synthesis and phosphorylation. Tetracycline binds to the 30S ribosomal particle and binding results in inhibition of translocation of the amino acid-transfer RNA from "donor site" to the "acceptor site". Binding of tetracycline to protein structures was suggested to occur through L-alpha-amino acids, especially glutamate, since the surface area on a tetracycline molecule exhibits a perfect match for such an interaction (67). The chelating properties of tetracycline reside in the ring BCD, beta-dicarbonyl function, the acid character of C3-OH group with a pK value of approximately 3 and the basic character of the dimethyl-amino function at C4 forms the contact points.

Tetracyclines also form insoluble complexes with metal ions, such as calcium, magnesium, copper and cobalt. Multiple metal ion binding sites on the structure allows tetracyclines to bind more than one metal ion at a time. Metal ions neutralize the charge

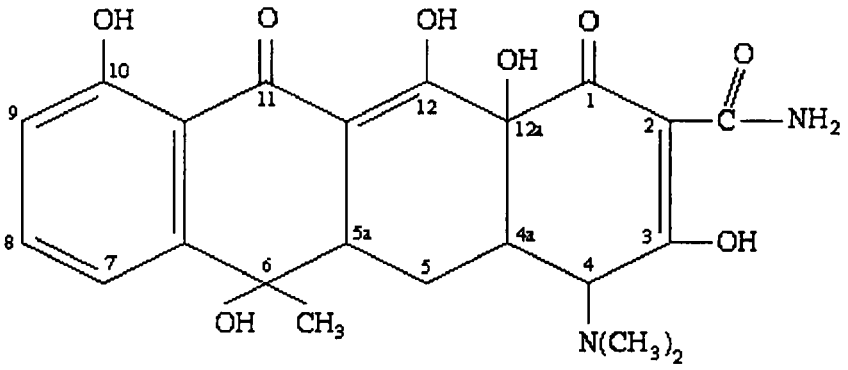

Figure 1: Molecular structure of tetracycline.

of the tetracycline molecule which effects solubility (68). Nuclear magnetic resonance studies by Williamson, et al. (69) identified specific sites on tetracycline and determined their affinity constants for metal ions. For example, for a one to one tetracycline/calcium complex, ring-A oxygen is the most favored site for calcium to bind. However the ratio of tetracycline calcium complex is not restricted to a one to one ratio, and when calcium concentrations are high further reactions take place and the ratio of calcium to tetracycline can be 1:2, 1:3 or higher (70).

2. 6 DISTRIBUTION, STORAGE AND EXCRETION OF TETRACYCLINES

Studies designed to examine the distribution, storage and excretion of tetracyclines. Bottiger et al. (71) showed that right after administration in clinical applications, tetracyclines can be found in various soft tissues. The reticuloendothelial

system, liver and kidney, seemed to have the highest concentrations of tetracycline, however this tissue distribution typically disappeared within 72 hours. Moreover tetracycline homologues seemed to have slightly different distribution patterns (72) and the authors suggested that tetracycline type should be chosen depending on the diseased organ. Tetracyclines are bound reversibly to serum proteins and the extent of binding differs among tetracycline members (Table 2). Binding of tetracyclines to serum proteins has been described as a "reservior" of the drug since the binding is reversible and the antibiotic is still therapeutically active after dissociation from the serum proteins (67).

Studies using radioactive tetracycline in model animals, showed that about 90% of the administered tetracycline were excreted by urinary and fecal routes (73). The rest of the tetracycline was deposited in the bone structure. Tetracycline incorporated in the bone matrix remains there for months and is removed during bone remodeling (7).

Table 2: Serum Binding and Serum Half Life of Tetracyclines (67).

Tetracycline	%serum protein binding	Serum half life (hours)
Tetracycline (TC)	55-56	8.5
Chlortetracycline (CTC)	55	5.5
Oxytetracycline (OTC)	27.35	9.5
De-methylchlortetracycline (DMCTC)	78	17

2. 7 TETRACYCLINE AND BONE INTERACTION

Tetracycline has been used in medicine since the early 1940s against bacterial infections. Based on observations during clinical use, it was noticed and then reported that long term and high dose tetracycline use resulted in discoloration of the teeth. Discoloration was more intense when the antibiotic was used during bone development, during pregnancy, infancy and childhood. It was reported that tetracycline deposits in bone structure in the sites that are currently undergoing mineralization (7). Later, in 1960, similar results were reported and discoloration of teeth by tetracycline antibiotics was confirmed. It was concluded that tetracyclines were deposited in actively mineralizing new bone, hyaline cartilage and teeth (74). According to the experiments by Lambrou et al. (75) already mineralized bone sections did not bind tetracycline. These findings led researchers to identify the interaction of tetracycline with bone material and eventually opened a new area for tetracycline use in periodontal and orthopedic therapy. Although the actual chemistry of the interaction was not clear, except for the affinity of tetracyclines for metal ions, by taking the advantage of observed tissue distribution patterns the researchers began to use tetracycline as a marker of bone formation. For both normal bone physiology and in transplantations the rate of new bone formation can be detected by measurement of localized tetracycline deposition since newly forming bone will bind tetracycline. Bone biopsy and the use of fluorescence microscopy shows the newly formed bone as yellow bands stained with tetracycline.

Studies were done to identify the actual interaction of tetracycline with bone matrix. Its deposition in bone matrix have not yet been clearly attributed either to

tetracycline-calcium complexes or tetracycline-organic matrix interactions. Since tetracycline deposition and mineralization patterns overlap the belief is that the tetracycline-calcium complex is responsible for its localization in newly formed bone. Experiments on new-born dogs were in agreement with previous studies. New born dogs were given tetracycline on a daily basis. From the teeth of these dogs, dentin and enamel were separated. From these experiments it was shown that tetracycline deposition in the enamel was much more than that deposited in dentine(76). These findings were in agreement with those reported by Bale et al. (77). Bale et al. using roentgen-ray diffraction, compared the structures of dentine and enamel and found enamel to be richer than dentine in terms of surface area of hydroxyapatite. Studies by Epker (78) also support the suggestion that tetracycline calcium complexes are the primary basis for tetracycline localization in bone matrix. He did not see fluorescence in undemineralized predentine suggesting that tetracycline does not bind to protein matrix.

Early suggestions that tetracycline-organic matrix interaction were possible were made by Milch et al.(7). They believed that tetracycline deposition involves interaction with both calcium and collagen proteins. They further stated that interaction of tetracycline with collagen is via the oxygen atom on the D ring of the tetracycline. Further studies also supported this mechanism that where ever tetracycline binds in the bone matrix, the interaction involves metallic cations.

It was also suggested that polysaccharides are involved in tetracycline binding to bone and that binding occurs prior to hydroxyapatite formation (79). Studies involving *in vitro* bone staining with tetracycline revealed that it was possible to sain and destain the bone surface with tetracyclines. This suggested that further mineralization cements

tetracycline in the bone matrix (74). There also was some results which were contrary to previous studies. There was no fluorescence in the completely mineralized enamel sections from patients treated with tetracycline suggesting that it is the organic matrix which binds up the tetracycline and the tetracycline-calcium complex is not a sufficient for tetracycline deposition in bone structure (80).

From the overall review of the literature on tetracycline orientation in bone structure, three possible models for tetracycline deposition are: tetracycline-apatite crystals-polysaccharides-collagen, apatite-polysaccharides-tetracycline-collagen, and apatite-tetracycline-polysaccharides-collagen (81).

2. 8 BONE MATRIX CHEMISTRY AND MINERALIZATION

Bone matrix is composed of inorganic and organic components. Inorganic ingredients are mainly calcium, phosphate, and carbonate. Calcium and phosphate are in the form of hydroxyapatite crystals. Other minor inorganic elements are magnesium, fluoride, potassium, and sodium. About 85% of the mineral content is hydroxyapatite crystals. Surface ions of the hydroxyapatite crystals are hydrated and the resulting water shell enhances ion exchange of calcium and phosphate between bone matrix and body fluids.

The organic matrix is composed of collagen, mainly type-I, glycoproteins, proteoglycans (negatively charged glycosaminoglycan chains that are covalently attached to core protein) and noncollagenous proteins such as regulatory bone growth factors. Organic matrix is synthesized and secreted by osteoblast cells as membrane bound vesicles

containing procollagen and ground substance. Vesicles also contain phospholipids, phosphate, alkaline phosphatase, magnesium, and ATP. Procollagen is cleaved and converted into collagen by a procollagen aminoprotease and a carboxyprotease. Collagen is stored in the matrix as tropocollagen macromolecules. Within the collagen fibers tropocollagen macromolecules associate with each other longitudinally. The space between the end of one macromolecule and the beginning of the next serves as nucleation sites for mineralization. A tropocollagen macromolecule is a righthanded triple helical polypeptide containing three left handed helices.

The presence of proline and hydroxyproline enhance the stability of polypeptides by limiting the rotation of the polypeptide backbone. Collagen fibers are responsible for the orderly organization of bone matrix.

The hardness of the bone is gained by mineralization. Initiation and deposition of inorganic salts on/between collagen fibers and formation of hydroxyapatite crystals is not clearly understood. At the beginning of the mineralization process calcium binds to matrix first (82) and then further calcium and phosphate deposition occurs. Calcium is presumed to be released by osteoblast cells. A transporting enzyme Ca-ATPase is required for calcium transportation into the matrix (83). Alkaline phosphatase originating from osteoblast cells hydrolyses organic esters releasing inorganic phosphate. Calcium lies in the mineralizing matrix before the formation of hydroxyapatite crystals. Calcium can bind tightly to proteins. It can bind to negatively charged and uncharged oxygen atoms, and its ability to bind up to eight oxygen atoms makes it an excellent cross-linking agent for proteins. However calcium deposition does not occur throughout the body, but only in bone matrix. This is due to the negatively charged proteoglycans common in the bone

matrix. Sialoprotein and osteocalcin, containing sialic acid and gamma-carboxyglutamic acid respectively, are strong calcium chelators. Hyaluronic acid and chondroitin sulphate are also involved in calcium deposition (82).

2. 9 EFFECT OF TETRACYCLINE USE IN BONE GRAFTING

It is known that tetracyclines normally interfere with and partially inhibit bone matrix mineralization (84), which is one of the side effects of these antibiotics and is frequently seen in children after long term or high dose use. Tetracyclines were first used for their antimicrobial effect during periodontal therapy. Soon it was observed and reported that independent of their antimicrobial effects, the use of tetracyclines in bone grafting provided a better healing response compare to the cases where other antibiotics were used. Thus the effects of tetracyclines independent of their antimicrobial activity were discovered. Following studies pointed several mechanism by which tetracyclines played the role to partilly block tissue degeneration. These included their inhibitory effects against matrix metaloproteases such as collagenase (1), gelatinase and stromelysin (2) and inhibition of pathogenic tissue destruction (85). Today, chemically modified non-antimicrobial tetracyclineas are being used in experiments designed to assess the basis of these effects.

In all cases related to bone defects, restoration of the lost tissue is the main aim in therapy. The therapeutic precedure includes the induction of osteogenesis and initiation of new bone formation. After the implantation of a graft material, infiltration of the graft by recipient mesenchymal tissue via chemotaxis, proliferation and differentiation of

mesenchymal cells into progenitor cells are the key events to be accomplished for a successive new bone formation. In periodontal therapy another important step is new connective tissue attachment to previously diseased root surface. There are two main factors which interfere with the attachment resulting in incomplete new cement formation and incomplete repair of osseous defect. First, mesenchymal cell adhesion to diseased tooth root surface is unlikely. Second, migration of apical epithelial cells through the space between root surface and connective tissue surrounding the root blocs new connective tissue attachment (86, 87).

Tetracyclines enhance new bone formation and resolve the attachment problems in some degree by their non-antibacterial activities. Tetracycline treatment results in partial demineralization and exposure of matrix proteins providing a matrix structure resembling embryonic matrix induced bone formation. Exposure of collagen and other osteogenic proteins in the collagenous matrix provides a natural receptive substrate and enhances the chemotaxic events and attachment of fibroblasts and attachment proteins such as fibronectin, which result in better healing of soft tissue-tooth surface (86). Fibroblasts have affinity for fibronectin and attachment of fibronectin to these cells promotes their proliferation (88). Tetracycline treatment leaves a smear free surface that enhances the new connective tissue attachment and blocks epithelial cell migration. Firstly, tetracycline conditioned root surface enhances the formation of fibrin-collagen junctions which blocs the epithelial downgrowth between fibrin and the root. Secondly, epithelial cells lose their ability to attach to tetracycline-HCl treated dentine surfaces (89). Laminin, another attachment protein which is used by epithelial cells, adheres more readily to nondemineralized surfaces (90). Root surface conditioning and demineralization by means

of tetracycline provides continuing effects since release of tetracycline is slow. A study by Wikesjo et al. (91) showed that tetracycline removes the amorphous surface layer and exposes dentine with open tubules.

Tetracyclines have been suggested to prevent bone resorption (92) by inhibiting destructive enzymes such as collagenase (1) and by reducing the acid phosphatase activity (93). Tetracyclines also have influence on osteoblast and osteoclast activity. They activate osteoblast cells and enhance the synthesis of collagen and proteoglycans. Tetracycline also stimulates alkaline phosphatase and calcium transporting-ATPase (Ca-ATPase) synthesis pathways (94), which all are essential for matrix synthesis and mineralization. A number of studies have been reported that tetracyclines modify the activities of both osteoblasts and osteoclasts,. Rats with induced osteopenia were treated with tetracycline to observe the changes in the activity of osteoclasts and osteoblasts (93, 94). Osteopenia is a condition of osteoid deficiency and is a complication of diabetes (95). It is characterized as abnormal osteoblast and osteoclast structure and function. These studies showed that tetracycline treatment restored the reduced osteoblast activity and normalized the acid phosphatase activity of these cells.

A correlation between tetracycline concentration and alveolar bone loss induced by infecting rats with *Porphyromonas gingivalis* (*Bacteroides gingivalis*) was also reported where bone loss was reduced by increasing concentration of tetracycline. The disease, alveolar bone loss, is the result of high collagenase activity. The rationalization for these results was that tetracycline reduced alveolar bone loss by inhibiting collagenase (96).

Anti-inflammatory properties of tetracycline have also been reported. Tetracycline has been shown to be protective against tissue injury in osteoarthritis (OA) by down

regulating the synthesis and inhibiting the release of nitric oxide (NO) from diseased cartilage (2). Nitric oxide is a mediator of inflammation and enhances the degradation processes by activating proteases including collagenase. The down-regulation of nitric oxide by tetracycline probably reduces the effect of inflammatory phase, a transient inflammatory phase experienced shortly after implantation of the graft material (97).

MATERIALS AND METHODS

3. 1 DEMINERALIZED BONE-DFDBA

DFDBA (Demineralized Freeze-Dried Bone Allografts)used in this study was supplied by LifeNet transplant services (LN, 5809 Ward Court, Virginia Beach, VA 23455) and was equivalent to clinically used demineralized bone. Specific details of production of demineralized ground bone are considered to be propriety to LifeNet and are not available for disclosure in this document. In general the procedure involves the following steps. Bone procured from donors within 24 hours after death was stored at -84 °C. For processing the frozen bone was thawed at room temperature, debrided of soft tissues and the shafts (primarily contained cortical bone) were cut into small fragments and ground. The ground bone was freeze dried and sized. Size distribution of DFDBA used in this experiment is shown in Figure 2. Sizing was studied by using six different sieve size. For demineralization, the frozen ground bone was thawed and washed in 100% ethanol for about 12 hours prior to demineralization. Dilute hydrochloric acid (0.5 N) solution was applied to the ground bone to effect demineralization. After demineralization bone materials was washed in distilled/deionized water for 30 minutes. The demineralized/washed bone was then washed in potassium phosphate buffer solution to achieve a slightly acid to neutral pH. The resulting bone materials were then freeze-dried under vacuum. Percent calcium of DFDBA (reported by LifeNet and redetermined in our laboratory) used in this study as "demineralized bone" was 0.058.

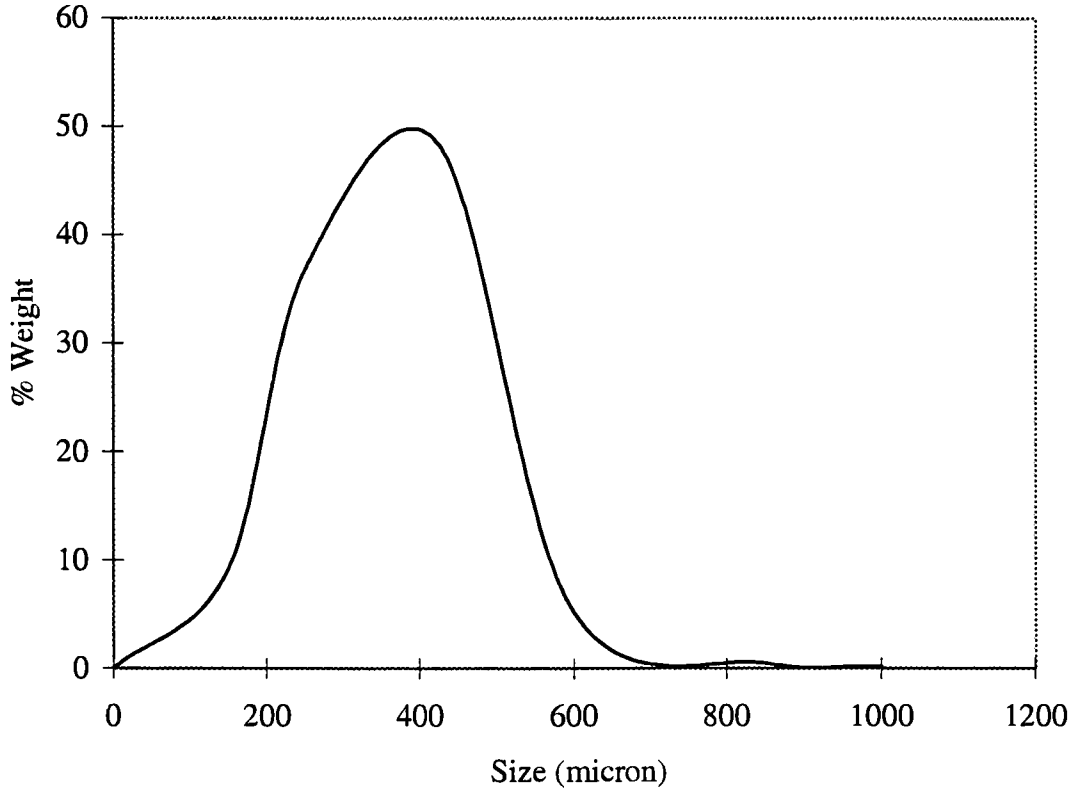

Figure 2: Particle size distribution of ground DFDBA.

3. 2 REAGENTS AND EQUIPMENT

Sodium barbital (Fisher Scientific Co.) was used in the procedures without further purification. A 0.9 M aqueous solution of sodium barbital is stable for 2-3 weeks at room temperature. Trichloroacetic acid and ethyl acetate (Sigma) were used as they were received. Powdered form of tetracycline in 250 mg commercially produced capsuls (LifeNet) were used in the experiments.

Fluorescence intensities was measured by means of a Turner model 111 fluorometer equipped with a general mercury vapor lamp and a RCA 1P21 multiplier phototube. Two 405-mμ interference filters were used in the incident light path and a 530-mμ interference filter was used in the emitted light path.

3. 3 TETRACYCLINE ASSAY

The tetracycline assay was developed based on the fluorescence of tetracycline-calcium-barbital complexes in ethyl acetate (98). The structure of the tetracycline-calcium-barbital complex is shown in Figure 3. The procedure for the assay is as follows: 100 μl sample including tetracycline was added to 2.5 ml of water and vigorously agitated to achieve a homogenous distribution of tetracycline molecules. To the resulting solution was then added 2 mls 0.44 N trichloroacetic acid, 2 mls 0.04 M $CaCl_2$, 3 mls ethyl acetate and 3 mls 0.9 M sodium barbital, in sequence. The samples were then shaken vigorously for two minutes and allowed to stand for one minute or until the two phases separated. Ethyl acetate which contains the fluorescence complexes, forms a top organic layer over the

aqueous solution. The top layer was carefully removed and placed in a small glass tube for determination of fluorescence.

For the generation of standard curve, tetracycline-HCl solutions with concentrations of 0.0156 mg/mL, 0.0185 mg/mL, 0.03125 mg/mL, 0.04167 mg/mL, 0.05 mg/mL, 0.0555 mg/mL, 0.0625 mg/mL, 0.0883 mg/mL, 0.1 mg/mL, 0.125 mg/mL, and

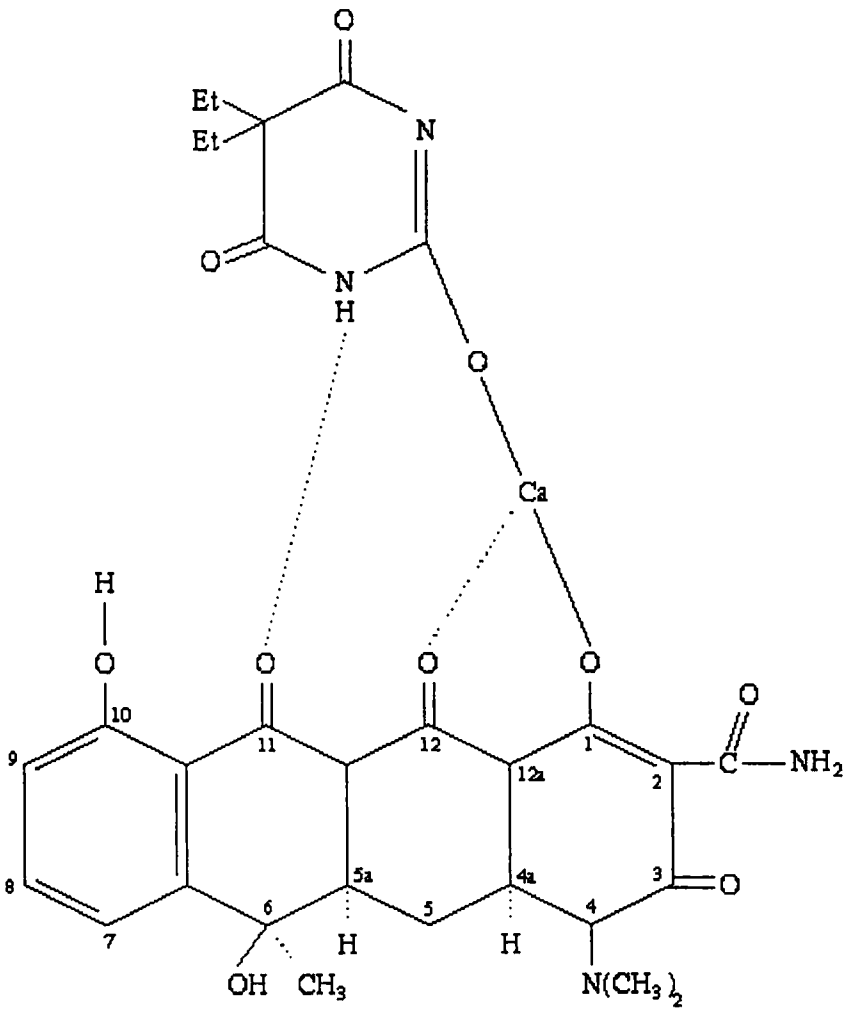

Figure 3: Structure of fluorescent complex of tetracycline-Ca-barbital.

0.166 mg/mL were prepared in water and the tetracycline assay was applied to measure the corresponding fluorescence for each concentration. As illustrated in Figure 4, standard curve is applicable to relative fluorescence readings between 10 and 90 which correspond to tetracycline concentrations of 0.0156-0.166 mg/mL. Concentrations of tetracycline higher than 0.166 mg/mL results in breakdown of the linear relationship between tetracycline concentration and fluorescence (a limitation of Lambert Beer's law).

3. 4 DETERMINATION OF BOUND TETRACYCLINE

Demineralized bone material was incubated in tetracycline solution to allow the binding of tetracycline to bone. The mixture then was centrifuged at 5000 rpm for five minutes to separate the bone material from supernatant. Aliquots of the supernatant were quantitatively assayed for the determination of free tetracycline. The difference between tetracycline concentrations of the initial tetracycline solution (prepared concentration) and that of the supernatant of bone-tetracycline mixture (assayed concentration) was assumed to represent bound tetracycline. As controls, prepared tetracycline solutions and bone water mixtures (not containing tetracycline) were processed and assayed along with the samples containing bone and tetracycline to preclude possible factors that might complicate quantitation of tetracycline concentrations during experimental procedures.

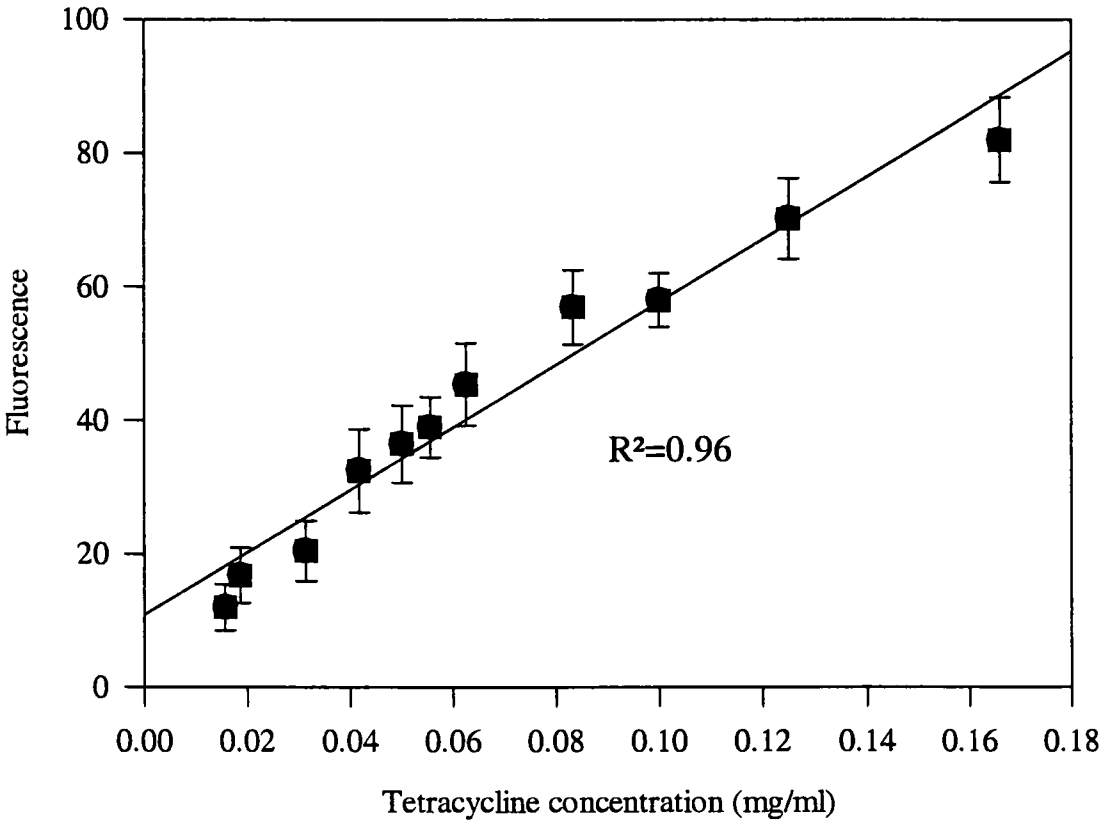

Figure 4: Tetracycline standard curve. Tetracycline concentrations ranging from 0.0156 mg/mL to 0.166 mg/mL were prepared in water and tetracycline assay was applied to measure the corresponding fluorescence. Each data point presented consists of at least five replicate assay.

3. 5 DETERMINATION OF RELEASED TETRACYCLINE

Tetracycline solution and bone were mixed together and binding of tetracycline to bone was allowed to occur. The supernatant including unbound free tetracycline molecules was removed and assayed for tetracycline content. By this method the amount of tetracycline bound per unit weight of ground bone was determined. A second incubation of these tetracycline bound bone materials with water at 37 °C resulted in release of bound tetracycline into the supernatant. Sampling and assaying the supernatant for tetracycline concentration allowed calculation of tetracycline released versus time.

3. 6 STATISTICAL ANALYSIS

Each data point in the figures provided consists of at least three replicate assays. The data were analyzed using student T-test and Tukey test. Replicants in each data point presented in grafts are normally distributed. Differences among groups were tested and results are presented in figures. Linear regression analyses were used to derive slopes and intercepts for standard curve. B_{max}, K_{aff}, $R_{max,}$ and K_{diss} values were obtained using Sigma Plot- Scientific Graphing Program. Significance level in this study was assigned at 0.05.

RESULTS

4. 1 TETRACYCLINE BINDING TO DFDBA

The amount of tetracycline-hydrochloride that could bind to DFDBA and the time required to achieve an equilibrium in tetracycline binding was determined by quantitative determination of tetracycline concentrations in solution. Demineralized bone was incubated in tetracycline hydrochloride solution for 30 minutes at room temperature. Demineralized bone was separated from the solution by centrifugation. Quantitative determination of tetracycline in the supernatant was sampled and assayed for tetracycline concentration. The relative fluorescence readings were converted to quantity of tetracycline by using the slope equation generated from the standard curve. The difference between the original (starting tetracycline concentration) and final tetracycline concentrations (tetracycline concentration in the supernatant after incubation with DFDBA) was assumed to represent that tetracycline bound to the DFDBA. In addition, tetracycline binding to DFDBA under different tetracycline concentrations versus constant amounts of DFDBA and constant tetracycline concentrations versus different amounts of DFDBA were evaluated for their effects on tetracycline binding.

Time Dependence of Tetracycline Binding:

To determine the required incubation time to achieve an equilibrium for tetracycline binding to DFDBA, experiments were performed by varying the incubation time while the amounts of DFDBA (10 mg dry weight of DFDBA) and the tetracycline

concentration (0.5 mg/mL) were constant. Ten mg DFDBA was incubated in 1 ml of tetracycline solution (0.5 mg/mL) at room temperature and allowed to stay for predetermined time intervals. Aliquots of supernatant were taken after 10, 20, 30, 40, 80, 120, 160 and 240 minutes. DFDBA was separated from the solution using five-minute-centrifugation at 5000 rpm. Aliquots of supernatants were assayed for quantitative determination of free (unbound) tetracycline. The amount of tetracycline bound to the DFDBA was calculated as the difference in the tetracycline concentration between starting concentration and the assayed concentration. It took about 40-80 minutes for the maximum amount of tetracycline to be bound by 10 mg DFDBA from a 0.5 mg/mL tetracycline solution (Figure 5). As illustrated in Figure 5 a rapid increase in the amount of tetracycline bound by DFDBA was observed over the first 30-40 minutes followed by a very slow increase in binding over the following 30 minutes. The maximum tetracycline bound to 10 mg dry DFDBA was found to be ~ 0.232 mg and 84.7% of this amount was bound during first 30-40 minutes. Binding of the remaining 15.3% of the tetracycline to DFDBA occurred over the next 30 minutes of the incubation time. After 80 minutes of incubation period, there was no additional binding of tetracycline to DFDBA. Percent tetracycline recovery in the control groups is shown in Table 3.

Dependence of Tetracycline Binding on Tetracycline Concentration:

By varying tetracycline concentration (0.125 mg/mL, 0.166 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 0.6 mg/mL, 0.75 mg/mL and 1 mg/mL) against constant amounts of DFDBA (10 mg), the saturation conditions for the two reactants were determined. DFDBA and tetracycline solutions were incubated for 30 minutes at room temperature. The bound

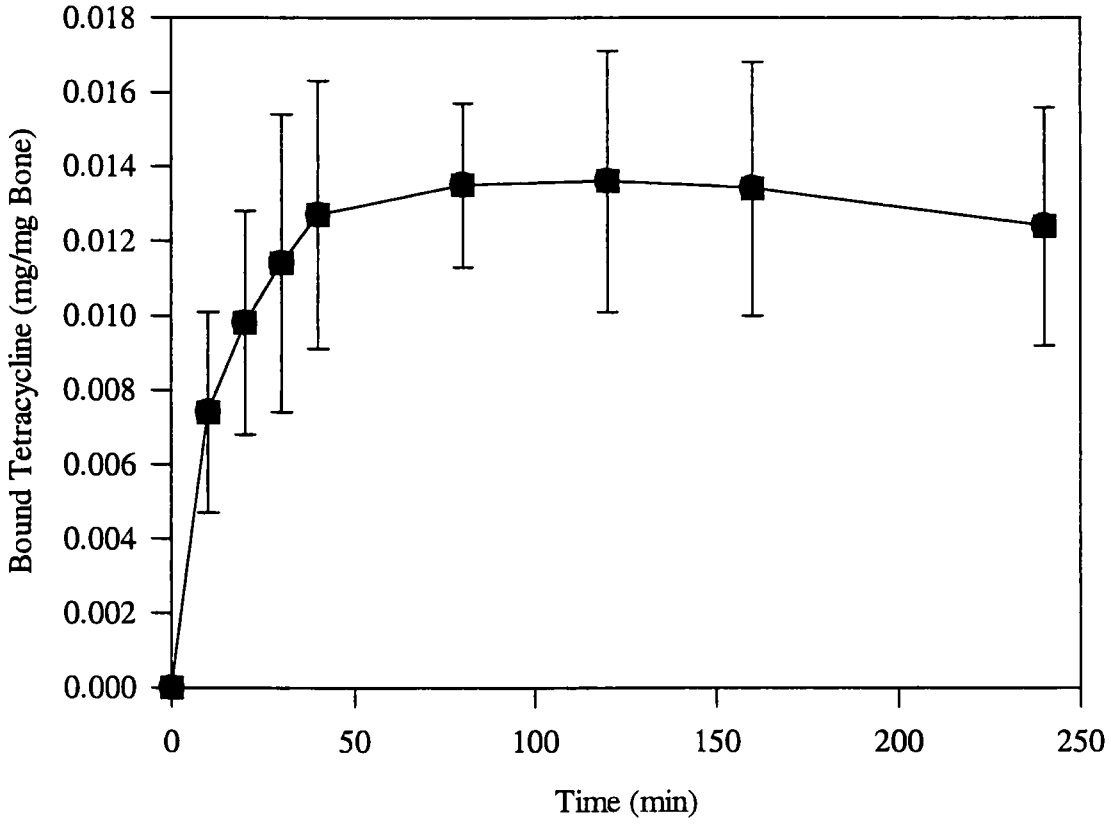

Figure 5: Time dependence of tetracycline binding to DFDBA. One mL of 0.5 mg/mL tetracycline solution was mixed with 10 mg of DFDBA at room temperature and the amount of bound tetracycline was determined after different incubation times.

Table 3: Tetracycline recovery in control groups		
Time (minute)	% Recovery[i]	Recovery % Range[ii]
10	99.4	4
20	101	9
30	100	9
40	101	15
80	102	19
120	100	18
160	97	15
240	98	10

*i.*Percent recovery: Percent of added tetracycline (0.5 mg/mL) that determined by analysis. *ii.*Recovery % Range: Difference in percent recovery among replicate assays.

tetracycline was determined as described under materials and methods. The amounts of bound tetracycline (bound tetracycline/mg bone) were plotted against their respective tetracycline concentrations and the resulting curve showed a pattern, similar to plots obtained for enzymatic reactions (Michaelis-Menten) with a linear increasing phase, an intermediate phase and a saturation phase (Figure 6). From this study it was determined that it requires a tetracycline concentration of ~0.8 mg/mL to saturate 10 mg DFDBA. B_{max} and K_{aff} values were determined by fixing these data into Michaelis-Menten equation using SigmaPlot-Scientific Graphing Program. B_{max} and K_{aff} values were 0.0232 mg tetracycline/mg DFDBA and 0.57 mg tetracycline/mL respectively. Percent tetracycline recovery in the control groups for the data in figure 6 is shown in Table 4.

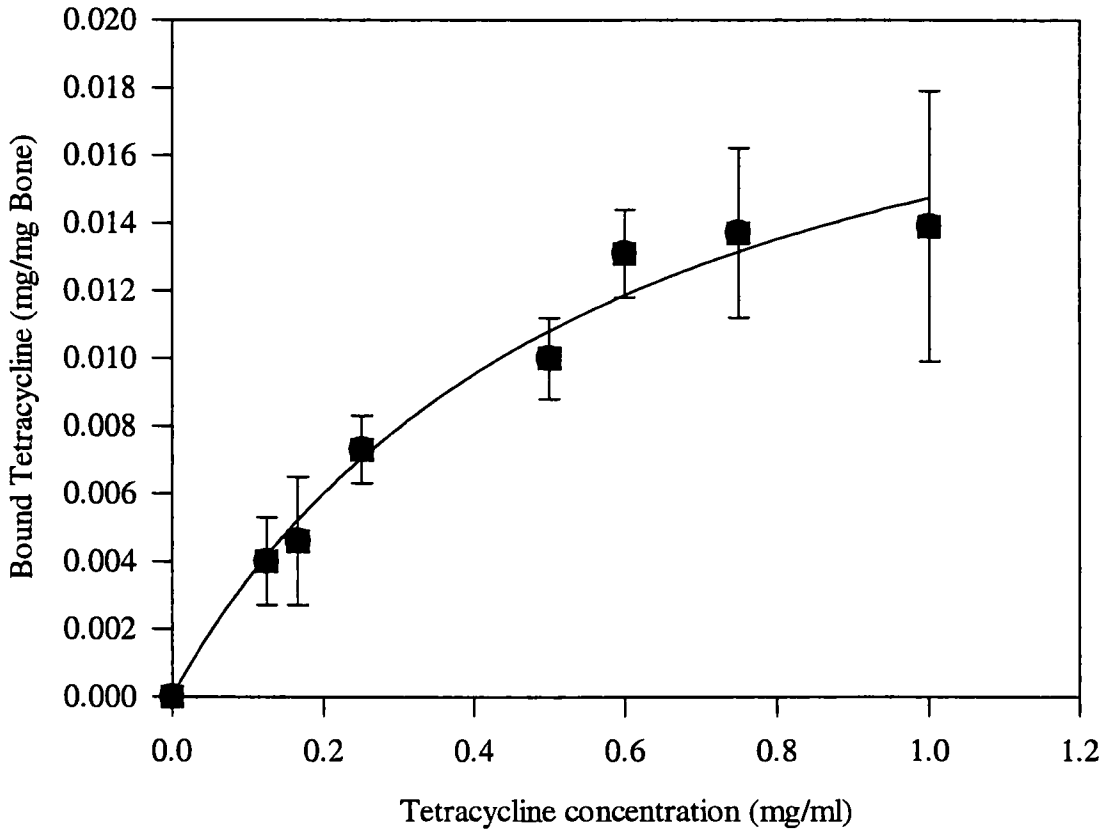

Figure 6: Kinetics of tetracycline binding to DFDBA. Tetracycline binding to a fixed quantity of DFDBA with increasing concentrations of tetracycline.

Table 4: Concentration dependence of tetracycline recovery

Tetracycline Concentration (mg/mL)	% Recovery at 30 minutes[i]	Recovery % Range[ii]
0.125	96	11
0.166	97	29
0.25	98	22
0.5	97	15
0.6	98	26
0.75	98	26
1	103	28

*i.*percent recovery: Percent of tetracycline added determined by analysis.
ii. Recovery percent range: Difference in percent recovery among replicates.

Dependence of Tetracycline Binding on DFDBA:

To determine the effects the amount of DFDBA on tetracycline binding, different amounts of bone (5 mg, 10 mg, 15 mg and 20 mg) were mixed with 1mL of 0.5 mg/mL tetracycline solutions and incubated for 30 minutes at room temperature. The samples then were centrifuged at 5000 rpm for five minutes to separate DFDBA from the supernatant. Quantitative determination of tetracycline in the supernatant revealed that there was an increase in the amount of bound tetracycline with increasing amounts of DFDBA (Figure 7). It was expected that the variable quantities of DFDBA would alter the equilibrium kinetics of tetracycline binding by reducing the effective tetracycline concentration in solution. However as illustrated in Figure 8, the amounts of tetracycline bound per mg DFDBA remained constant when 10, 15, and 20 mgs of DFDBA were used. Significantly more tetracycline was bound to per mg of DFDBA when 5 mg of DGDBA was used in the assay compared to 10, 15, and 20 mgs of DFDBA samples.

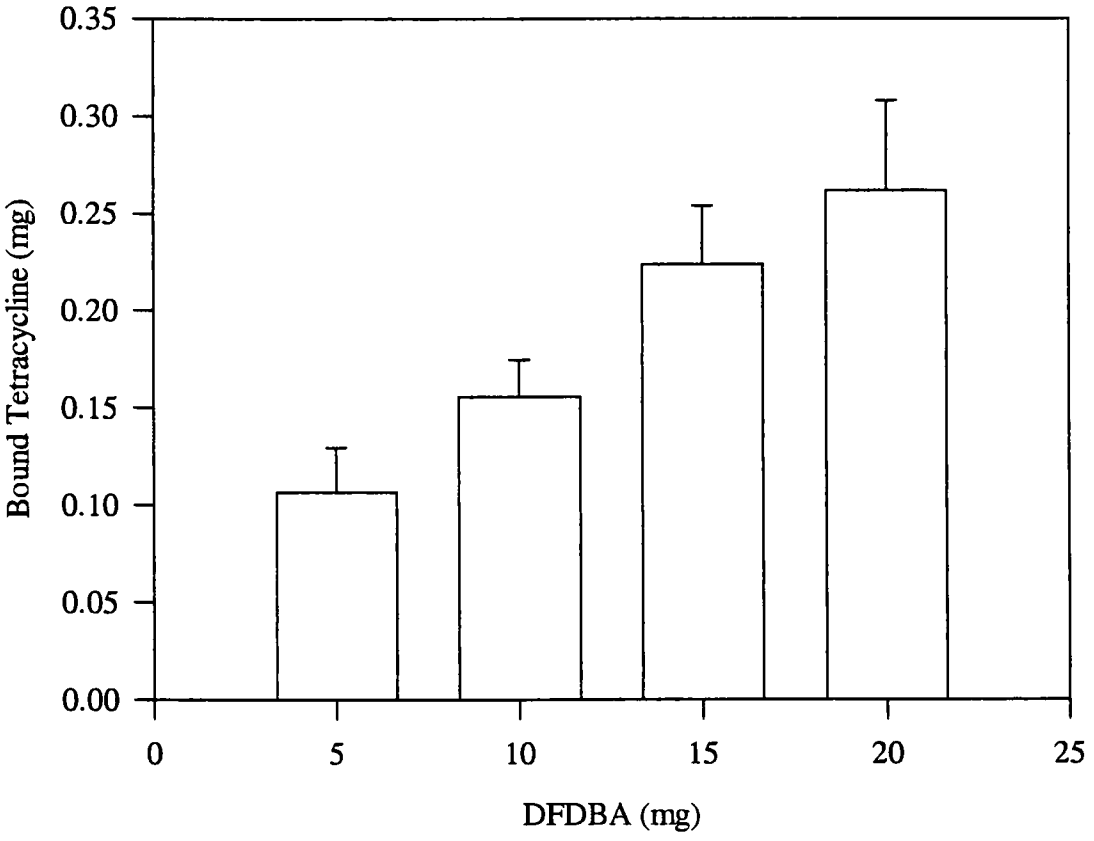

Figure 7: Dependence of tetracycline binding on DFDBA. Bound tetracycline was determined when 1 mL of 0.5 mg/mL tetracycline solution was mixed with different amounts of DFDBA for 30 minutes at room temperature.

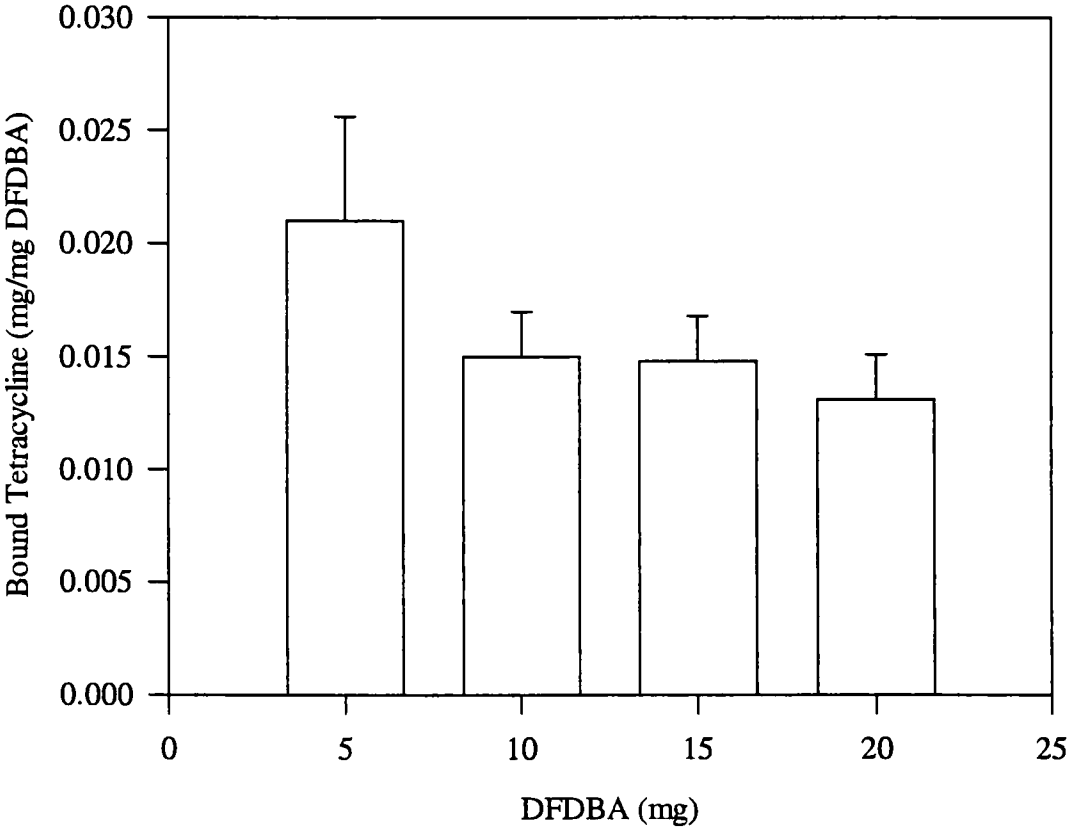

Figure 8: Tetracycline binding to per unit of DFDBA. Bound tetracycline to per mg DFDBA at a constant 0.5 mg/ mL tetracycline solution versus increasing amounts of DFDBA. Each data points consists of at least three replicate assay and difference between these means are not statistically significant.

4. 2 TETRACYCLINE RELEASE FROM DFDBA

Release experiments were performed using demineralized bone with bound tetracycline. DFDBA was incubated in tetracycline hydrochloride solution of determined concentration for 30 minutes at room temperature. The mixture was allowed to equilibrate with respect to tetracycline binding. The mixture was then centrifuged at 5000 rpm for 5 minutes to separate the DFDBA and supernatant was removed. Tetracycline concentration of supernatant (unbound tetracycline) was determined and used to calculate the bound tetracycline which is expressed as mg tetracycline bound per mg dry weight of DFDBA. DFDBA was left to dry. Tetracycline bound DFDBA materials were then added to 1 ml of water and allowed to incubate for the release of tetracycline to occur. The release of bound tetracycline from the bone was determined by assaying for tetracycline concentrations in supernatant.

Time Dependence of Tetracycline Release:

For the determination of time dependence of tetracycline release, tetracycline bound DFDBA materials were incubated with water for different periods of time and the release was determined by assaying supernatant for tetracycline concentration. Ten mg dry weight of bone was mixed with 1 ml of 0.5 mg/ mL of tetracycline solution and allowed to incubate for 30 minutes at room temperature. The supernatants were removed and assayed to determine the amount of unbound tetracycline and the amount of tetracycline bound to 10 mg bone was calculated as 0.1 mg/10 mg DFDBA. Tetracycline bound DFDBA materials (10 mg) were added to 1 ml of water and allowed a second

incubation for 10, 20, 30, 40, 80, 120, 160, and 240 minutes. At these time intervals, aliquots of supernatants were assayed for the concentration of tetracycline. During the four-hour incubation period the maximum amount of tetracycline released from 10 mg of DFDBA was 0.031379 mg/mL which is equivalent to a release of 31% of the bound tetracycline (Figure 9 and Figure 10). Release of bound tetracycline was essentially complete by 50 minutes with an initial release rate of 0.8×10^{-3} mg tetracycline/min/10 mg DFDBA. The rate of release varied over the four-hour-incubation period as illustrated in Figure 11. The rate of tetracycline release becomes linear with time on a semi-log plot (Figure 12).

Dependence of Tetracycline Release on Tetracycline Concentration:

In this study 10 mgs of DFDBA were mixed with 1 mL of tetracycline solution at 0.125 mg/mL, 0.166 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 0.6 mg/mL, 0.75 mg/mL and 1 mg/mL tetracycline for 30 minutes at room temperature. DFDBA was removed and the supernatants were assayed for the calculation of the amount of tetracycline bound per unit weight of DFDBA. The tetracycline bound DFDBA were then added 1 mL of water and incubated for 30 minutes at 37°C. Aliquots of the supernatants were assayed to determine the quantity of tetracycline released. As illustrated in Figure 13, the amount of tetracycline released increased as the amount of tetracycline bound to DFDBA increased. The data illustrated in Figure 13 suggest that at some higher concentrations of bound tetracycline, the rate of release does not increase, and becomes independent of concentration. This observed independence of concentration is analogous to velocity versus substrate concentration data plots for enzymatic reactions.

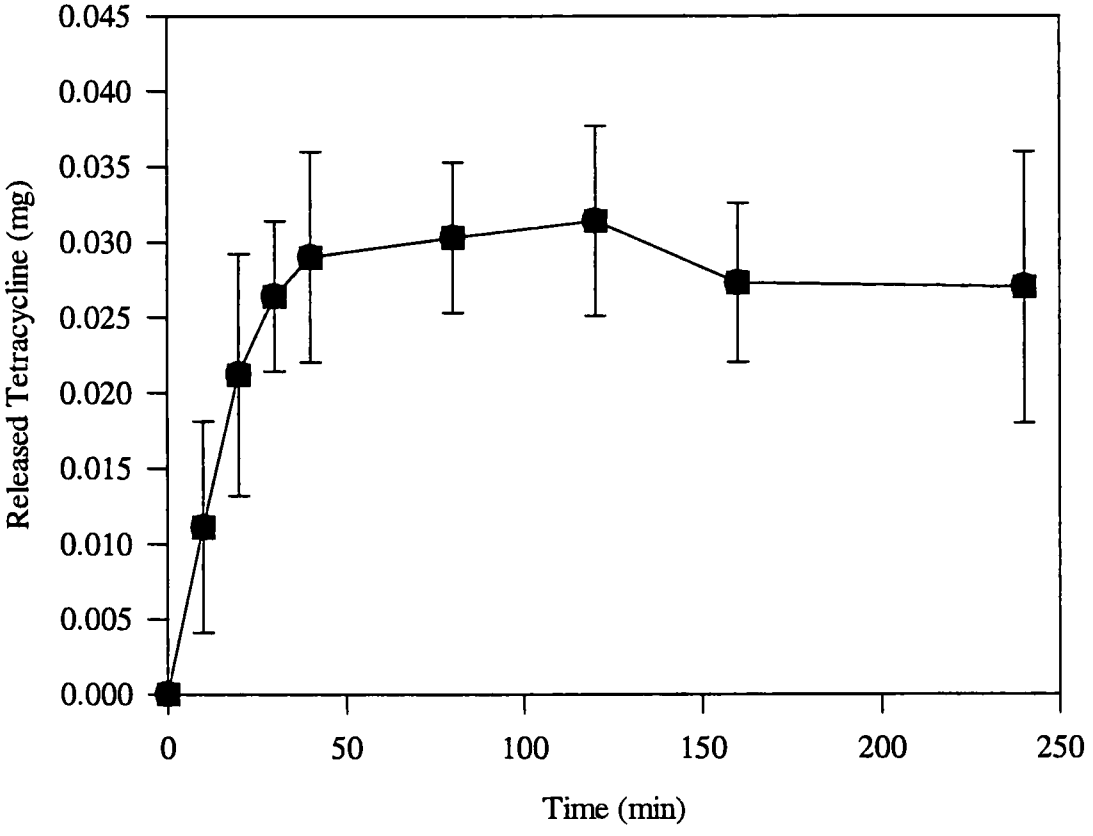

Figure 9: Time dependence of tetracycline release from DFDBA. Time course of tetracycline release was determined over a 4 hour incubation period. Ten mg DFDBA with 0.1 mg bound tetracycline was incubated with one mL of water at 37 celcius degree and the released tetracycline was determined at different incubation times.

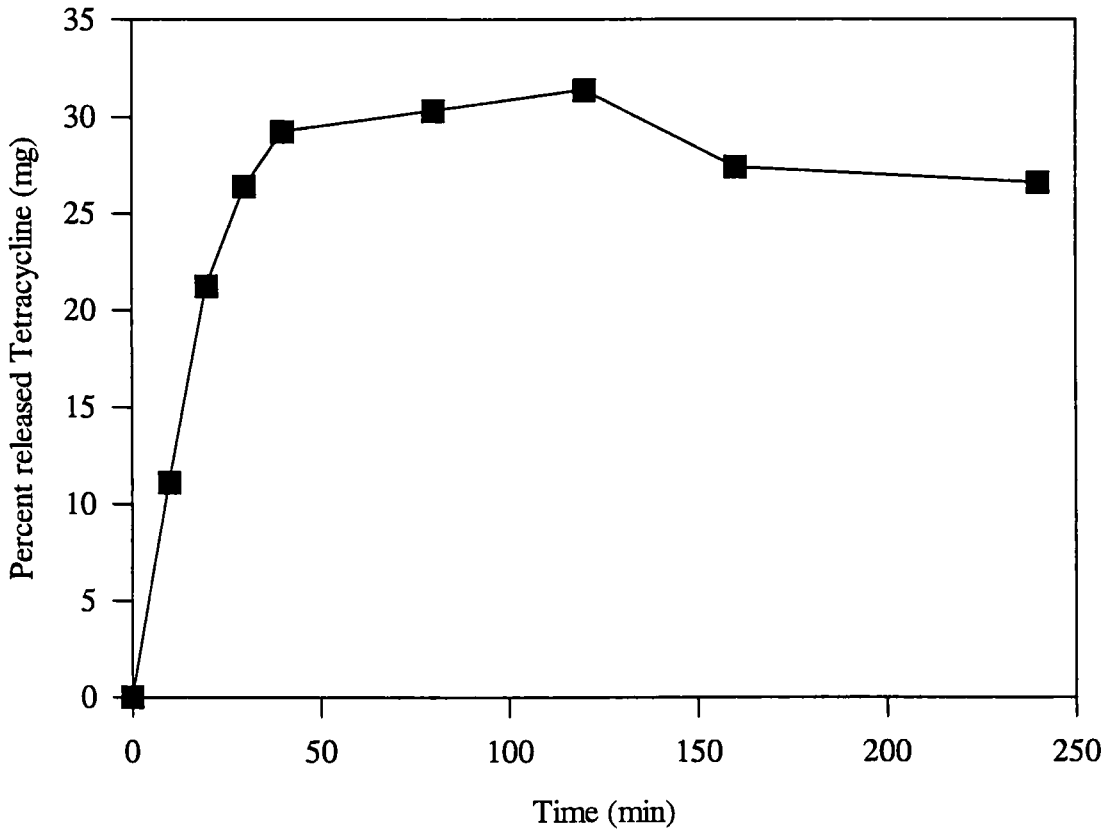

Figure 10: Percent tetracycline release from DFDBA. 10 mg DFDBA with 0.1 mg bound tetracycline was used. Tetracycline bound DFDBA was added 1 mL of water and released tetracycline was determined as percent of bound tetracycline.

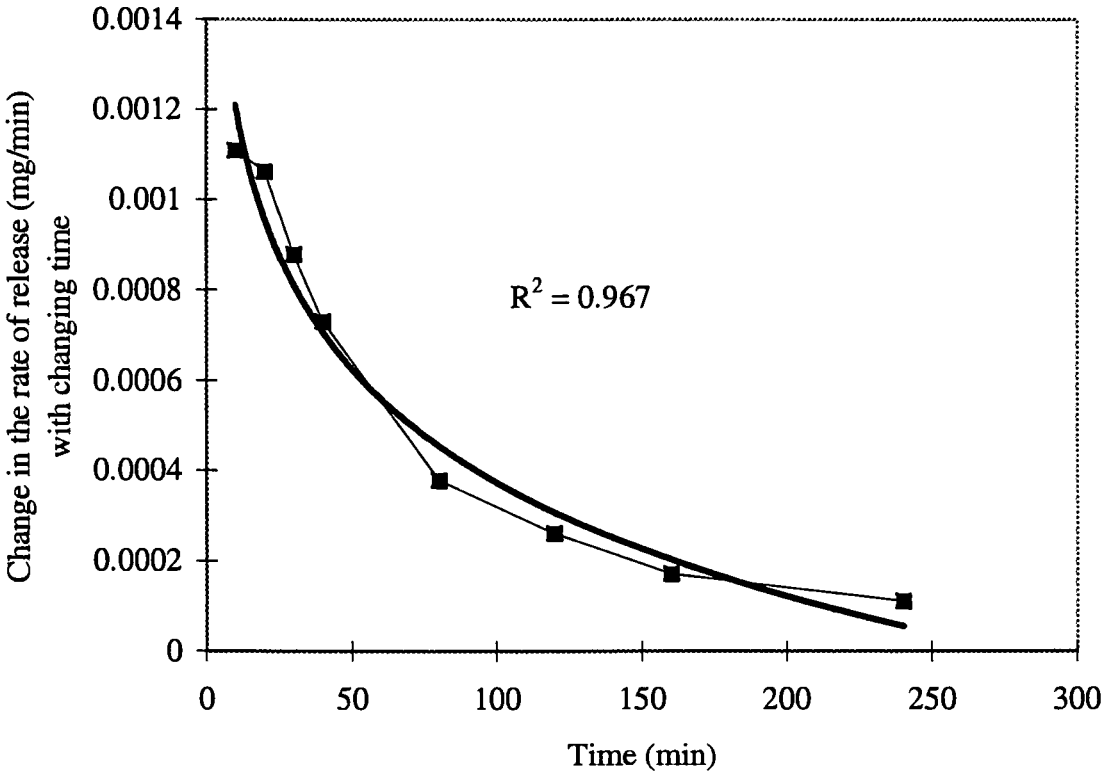

Figure 11: Time dependence of the rate of tetracycline release. Ten mgs of DFDBA containing 0.1 mg bound tetracycline was used to determine the rate of the release as a function of time.

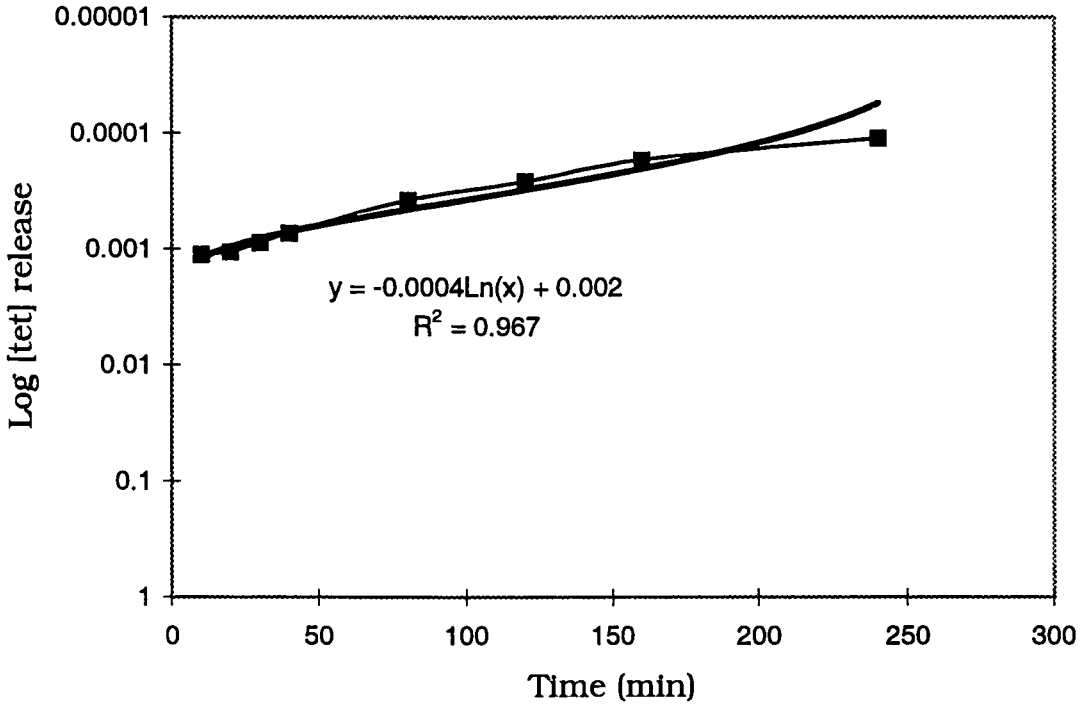

Figure 12: Rate of tetracycline release. Logarithmic scale regression analysis was used to show the correlation between time and the decrease in the rate of tetracycline release from DFDBA (Note: The values on the y axes are in reverse orientation).

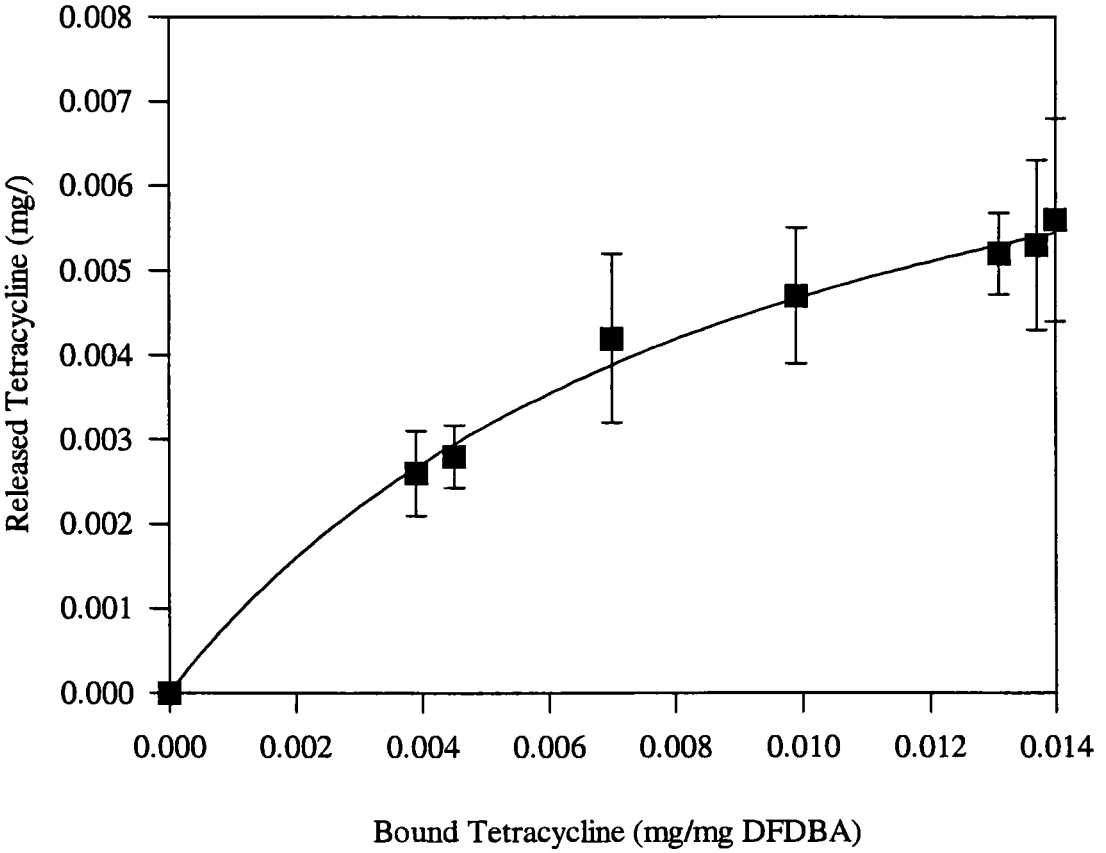

Figure 13: Kinetics of tetracycline release from DFDBA. For the kinetics of tetracycline release, DFDBA with different amounts of bound tetracycline was used. Tetracycline bound DFDBA was incubated with 1 mL of water for 30 minutes at 37 celcius degree. Released amounts of tetracycline were determined and expressed as mg released tetracycline/mg DFDBA.

To determine the maximal rate of release at high concentrations of bound tetracycline and a value similar to a Michaelis constant (K_m). Thus the data were fitted into the Michaelis-Menten equation to determine maximum release (R_{max}) and dissociation constant(K_{diss}). R_{max} and K_{diss} were $9.13x10^{-3}$ and $9.4x10^{-3}$ mg tetracycline/mL, respectively.

Dependence Tetracycline Release on DFDBA:

In order to determine the effects of the amounts of DFDBA on tetracycline release, 1 ml of 0.5 mg/mL tetracycline solution was mixed with different amounts of bone (5 mg, 10 mg, 15 mg and 20 mg). After 30 minutes incubation time (for binding of tetracycline to DFDBA to occur) the supernatants were assayed for free tetracycline and bound tetracycline was calculated. The DFDBA was removed from their respective tetracycline solutions, added to 1 mL of water, and incubated for 30 minutes at 37°C. The amount of tetracycline released increased linearly (R^2=0.9917) as the quantity of DFDBA (and hence bound tetracycline) increased (Figure 14). However the quantity of tetracycline released per mg DFDBA decreased as the amount of DFDBA increased (Figure 15). In that rebinding of released tetracycline might increase with increasing amounts of DFDBA, it might be suggested that release rates *in vivo* might be reduced in clinical applications by using greater quantities of DFDBA as a carrier.

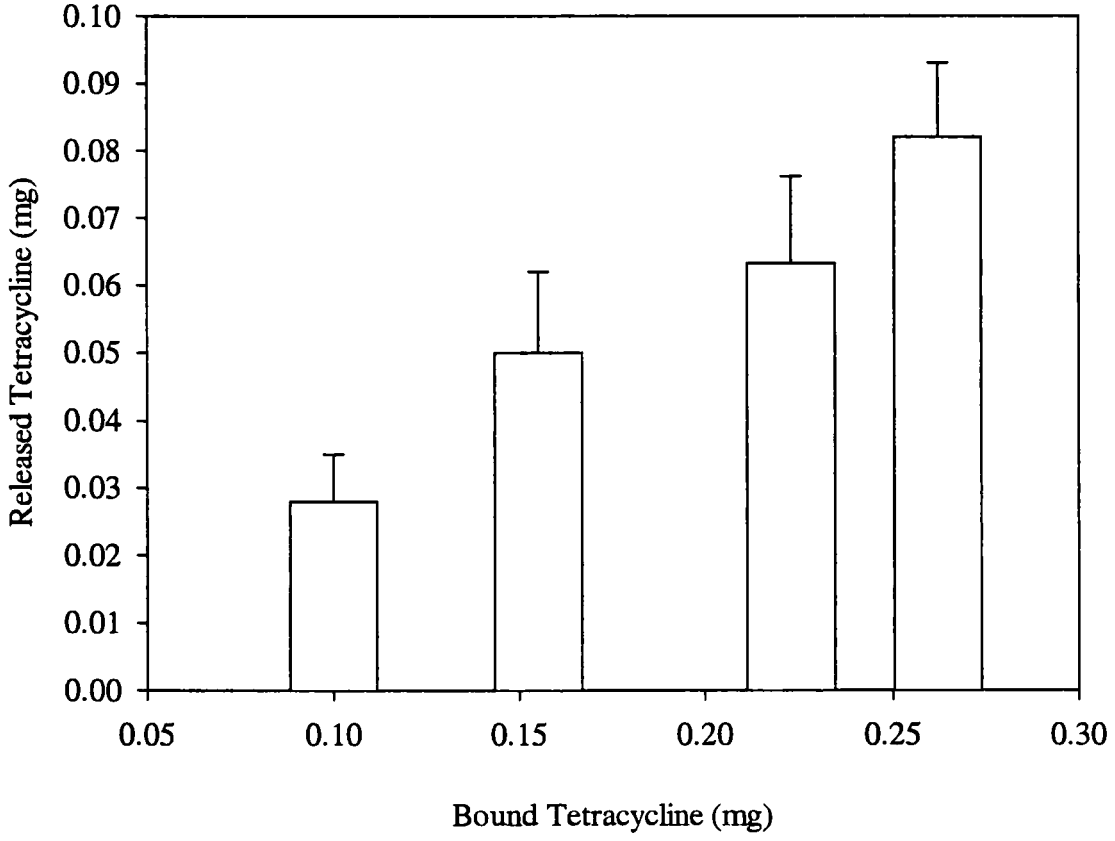

Figure 14: Dependence of tetracyline release on DFDBA. Tetracycline release from different amounts of DFDBA was determined. 5 mg, 10 mg, 15 mg, and 20 mg have bound tetracycline of 0.1 mg, 0.155 mg, 0.223 mg, and 0.262 mg respectively.

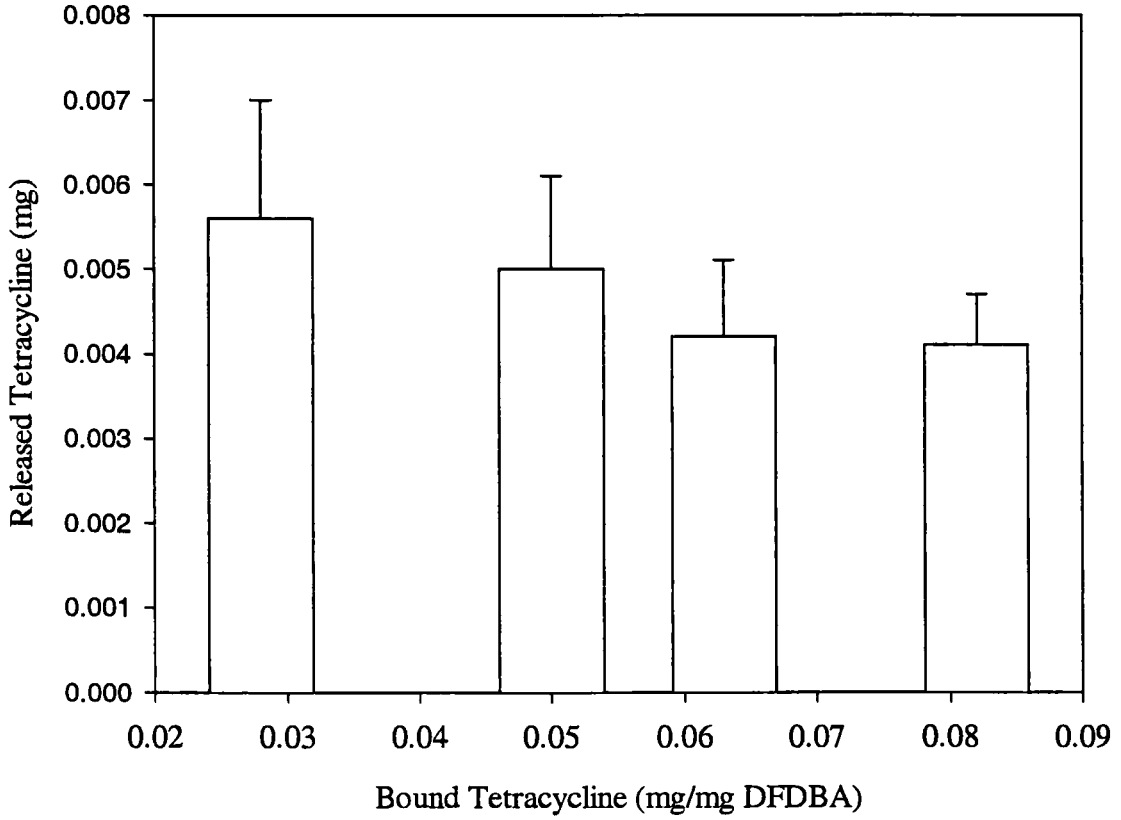

Figure 15: Tetracycline release from per unit of DFDBA. Released tetracycline (mg tetracycline/mg DFDBA) from different amounts of DFDBA, 5 mg, 10 mg, 15 mg, and 20 mg with bound tetracycline of 0.022 mg, 0.015 mg, 0.014 mg, and 0.0135 mg tetracycline/ mg DFDBA respectively.

4. 3 TETRACYCLINE BINDING TO BONE WITH DIFFERENT PERCENT RESIDUAL CALCIUM

For the determination of target sites for tetracycline binding (i.e. the organic phase or the mineral phase of bone matrix), amounts of bone (10 mg dry weight) with percent calcium contents of 8.85, 6.87, 4.74, 2.49, 1.27, 0.559, 0.241 and 0.058 were mixed with 1 mg/mL of tetracycline solution for 30 minutes. The amount of tetracycline bound by 10 mg bone decreased as the residual calcium increased suggesting that tetracycline binds to the organic phase of bone matrix. Although overall decrease in the amount of bound tetracycline was not linear, the bound amount of tetracycline linearly decreased (R^2= 0.99) where the calcium content was higher than 5% (Figure 16). When the calcium content is higher than 5% the formation of calcium phosphate restricts tetracycline binding as a linear function of weight % calcium phosphate and the greater the demineralization the greater is tetracycline binding.

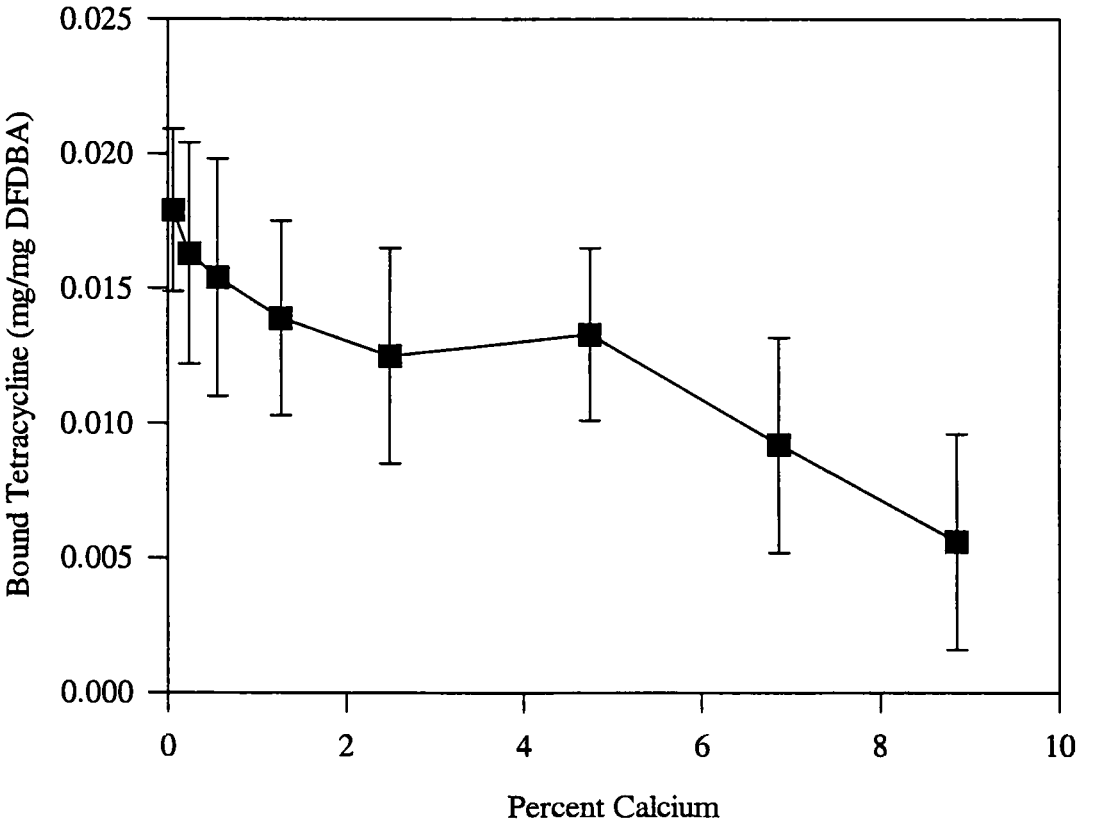

Figure 16: Tetracycline binding to DFDBA with different calcium content. 10 mg dry weight of DFDBA with different percent calcium ranging from 8.85 to 0.058 were mixed with 1 mL of 1 mg/mL tetracycline solution at room temperature for 30 minutes.

DISCUSSION

For a controlled drug delivery system to become an effective means of periodontal treatment some essential requirements should be met: 1) Therapeutically effective concentrations of the agent should be released for a prolonged time period, and 2) preparation of the device should be easy enough to handle for clinical use. Various controlled drug delivery devices have been introduced and clinically evaluated according to the degree of sustained drug release, effectiveness in the reduction of pathogenic microflora in the periodontal pocket, control over plaque formation and in the case of regenerative periodontal therapy these also include the degree of repair of osseous defects.

Most of the controlled drug delivery systems applied in periodontal therapy employee polymeric materials as drug carriers. Nonbiodegradable and biodegradable polymeric materials have been introduced and tested to some degree of success. The disadvantage of using nonbiodegradable biomaterials as drug carriers is that the carrier has to be removed after the fixed therapeutic agent is released. This requires a second operation resulting in disturbance of the tissue under healing process, and a second patient visit.

Hollow fibers of cellulose acetate filled with tetracycline supplied local delivery of antibiotics with minimal control over the rate of release. When such a device was placed into periodontal pocket, 95 percent of the fixed tetracycline was released within two hours (4). Delivery systems in which the therapeutic agent is dispersed in a polymer matrix in the form of strips or fibers have been reported to be effective for the sustained release. Polyethylmethacrylate strips (acrylic strips) coated with tetracycline-HCl was

introduced and reported that the released amount of tetracycline decreased and after two days the concentration of the antibiotic in the periodontal pocket was found to be lower than the therapeutically effective level (65).

Monolithic fibers of several polymeric substances such as ethylene vinyl acetate (EVA) and polycaprolactone were developed and used as slow delivery device. Tetracycline coated monolithic fibers of EVA have been reported to be a useful device for sustained release of tetracycline. Following the placement of the device into the periodontal pocket, EVA fibers is able to release the therapeutically effective levels of the antibiotic for ten days and the tetracycline concentration throughout ten days was reported to be 600 μg/mL. (5). All these delivery systems require the removal of the device from the periodontal pocket after use.

Biodegradable hydroxypropyl cellulose strips (99) have poor control over the rate of release in vitro but in vivo studies showed that the release of the tetracycline from the device continued for 24 hours liberating the total amount of the antibiotic (100). Collagen matrix with immobilized tetracycline is able to release tetracycline for ten days, and the rate of the release can be controlled by the degree of cross-linking. Clinical studies showed that on the tenth day the concentration of tetracycline in the periodontal pocket was 17 μg/mL (101). Slow release of tetracycline from a poly(lactide/glycolide) film strips was also used in clinic therapy (66).

This study showed that tetracycline binds to the protein phase of bone matrix. The bound amount of tetracycline decreases with the increase in the percent residual calcium in the matrix. In this study, binding and release kinetics of tetracycline-HCl by DFDBA has been determined. Different tetracycline concentrations were employed and the maximum

amount of the antibiotic bound per unit mg bone was found to be 0.0232 mg (23 μg) from 1 ml of a 0.5 mg/mL tetracycline solution. Complete saturation required 80 minutes. However a rapid increase in the amount of tetracycline bound by DFDBA was observed over the first 30-40 minutes and this is followed by a very slow increase in binding over the next 30 minutes. Tetracycline binding to bone was found to be dependent on both the amount of bone and the concentration of tetracycline-HCl. 31% of the bound tetracycline released during a four-hour-incubation period. The maximum released amount of tetracycline (0.0092 mg/mg bone/hr) was achieved by incubating the tetracycline bound bone with dH_2O. The time course of tetracycline release showed a sustained release pattern.

Evaluation of the data obtained from this study of tetracycline release from DFDBA during four-hour period showed that the release of bound tetracycline is slow and the release of all bound tetracycline is going to take a long time since the rate of release linearly decreased (R^2= 0.97) during four hour incubation period. This study evaluates DFDBA as a potential carrier system for tetracycline slow release in periodontal therapy. In early studies, tetracycline-bone interaction has been reported as a parameter contributing to the slow release of tetracycline-HCl from a white petrolatum device in the periodontal pocket (102). The author reported that tetracycline extruded from the carrier device into periodontal pocket attached to the teeth and contributed the resulting sustained release and retardation of the tetracycline into periodontal pocket.

Tetracycline bound DFDBA may overcome some difficulties associated with other tetracycline delivery systems. DFDBA has been already in use as a grafting material in periodontal diseases and in orthopedics. Tetracycline bound DFDBA will eliminate the

difficulties associated with nonbiodegradable tetracycline carrier devices which in most cases require a second operation to remove the device. Production of tetracycline bound DFDBA does not require a complicated process and is easy to handle.

In this study we report that DFDBA is able to bind and release therapeutically effective concentrations of tetracycline *in vitro,* and the release of the antibiotic shows a slow pattern. Further studies should be done for the *in vivo* evaluation of DFDBA as tetracycline carrier for periodontal treatment. Placement of tetracycline bound DFDBA into a periodontal pocket and determination of the concentration of released tetracycline for different times would be a valuable study to test the *in vivo* capacity of DFDBA for tetracycline delivery system. Studies should also include the determination of the changes in the bacterial microflora in the periodontal pocket during tetracycline local delivery via a DFDBA carrier. A decrease in the bacterial microflora would also indicate the presence and effectiveness of tetracycline released from DFDBA.

SUMMARY

Demineralized bone is a major product being used in periodontal therapy. Tetracycline delivery systems using different types of beads have been reported. Each of these systems such as polymer devices and collagen matrices has some disadvantages such as the capacity of the device to bind enough tetracycline or controlled delivery of the bound substance. Removal of a nonbiodegradable device brings another difficulty which interferes with the healing.

The biodegradable and biologically active-osteoinductive material, ground demineralized bone (DFDBA) was used as a carrier system for delivery of the antibiotic tetracycline. DFDBA was evaluated as an easy to handle device and a potential delivery device for tetracycline slow release. Ten mgs of bone material is capable of binding and delivering 31 micrograms/ml of tetracycline under the condition used in this study. Complete saturation of ground demineralized bone with tetracycline takes 80 minutes from a 0.5 mg/ml tetracycline solution. Tetracycline binding to demineralized bone is dependent on both tetracycline concentration and on the amount of bone used. Tetracycline release studies demonstrated that 31% of the bound tetracycline is released during the four-hour incubation period. Tetracycline release begins almost immediately and the release is essentially complete within the first hour. The quantity of tetracycline bound to the delivery vehicle can be controlled by controlling the extent of demineralization of the ground bone.

Different from other delivery systems is the osteoinductive properties of the demineralized bone material. Osteoinductive affects of DFDBA has been demonstrated by laboratory bioassays.

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