CHAPTER II
LITERATURE REVIEW

This thesis attempts to develop a novel drug delivery scaffold used for regenerating dental root sockets or other bone lesions. Many fields of knowledge have been contemplated in the study such as; tissue engineering, controlled drug release, polymeric biomaterials, and molecular biology of which fundamental information has been extensively reviewed in many articles elsewhere. In order not to overwhelm the document, literature review concentrates mainly the information relevant to bone regeneration particularly what have been progressed in research, except the part of biomaterial in which basic knowledge is more concerned. Therefore, literature review comprises the following aspects.

2.1 Protein delivery in tissue engineering
2.2 Use of growth factor in bone regeneration
2.3 Crude bone protein extracts
2.4 Scaffold in bone regeneration
2.5 Drug delivery scaffolds
2.6 Biomaterials of choice

2.1 Protein Delivery in Tissue Engineering

Growth factors are extracellular polypeptide signaling molecules that promote the growth, proliferation, differentiation, or survival of cells in animal tissue, usually act as local mediators at very low concentration (about 10^{-9}-10^{-11} M) (Alberts *et al.*, 2002). Growth factors are physiologically bound in extracellular matrix and released by enzymatic digestion of their anchors. The ideal delivery system in tissue engineering approach should imitate this scheme which is barely attainable by the traditional drug delivery approaches. Direct application of growth factors to the site of defects, specifically by injection, may not perfectly induce tissue to regenerate because the total potential of growth factors could not be accomplished due to rapid dissolution (Baldwin and Saltzma, 1998; Schliephake, 2002). Furthermore, since growth factors mostly act upon multiple tissue types, systemic approach of growth

factors may cause the unpredicted side effects. The localized delivery system, therefore, has been in a high preference to systemic application. Such delivery system must also be best suitable for each application according to the number of clinically relevant protein and diversity of the target tissues (Tessmar and Gopferich, 2007).

Protein delivery systems have been designed in several profiles and configurations. However, most systems base on the encapsulation or entrapment of proteins in biocompatible polymeric devices (Baldwin and Saltzma, 1998), which can be categorized regarding the diversity of their designs as (Baldwin and Saltzma, 1998; Tessmar and Gopferich, 2007).

2.1.1 Protein Reservoir

In this case, protein is stored in a reservoir created by a polymer coating i.e., poly(ethylene-co-vinyl acetate) that regulates the rate of release. The difficulties of this system are that large molecule like protein and growth factors diffuse very slowly through polymer films so the "dose-dumping" could occur and the massive release of the entrapped material is also possible due to membrane failure.

2.1.2 The Release from Polymer Matrices

The matrix devices contain dispersing proteins within a solid continuous matrix of polymer. Protein may be directly loaded into the matrix during fabrication or later coated onto the prefabricated matrix. This device gives the unpredictable release profile due to the detriment of protein integrity by organic solvent or high temperature during the process of matrix fabrication and the only small amount of protein adsorbing onto the scaffold in an uncontrolled fashion.

2.1.3 Microscopic Particles

The microscopic particles can be produced in the range of micro or even nanometer (Vadnelli *et al.*, 2001; Vandervoort and Ludwig, 2004), The protein-loaded particles behave as the separated release system when they are incorporated within the polymer matrix. The protein could be protected thereby from most of the chemical and physical extremes of the matrix-fabricating process where the controlled release of protein can be assured subsequently.

2.1.4 Hydrogel Carrier

Protein may release by different mechanisms correspondingly to types of hydrogel which are the physically entangled, the chemical crosslinked and the swelling hydrogel. For the physically entangled hydrogel, protein release simultaneously with polymer dissolution. The chemical crosslinked hydrogel degrade due to hydrolysis or enzymatic digestion so that the release of protein depends on polymer degradation. The hydrogel that swell in water allows macromolecule to diffuse throughout the entire matrix and release through a porous structure which expands during swelling. Therefore, the release of protein is determines by the pores size of the hydrogel network.

Among numerous applicable drug delivery devices, Gelatin microspheres have been frequently studied with several therapeutic agents such as antihypertensive drug (clonidine hydrochloride) in the study of Vandelli in 2001 (Vadnelli *et al.*, 2001). The gelatin microspheres crosslinked with at least 0.5% (w/v) glutaraldehyde produced a more gradual and sustained systolic blood pressure reduction in rats when injected subcutaneously and the antihypertensive effects was maintained until 52-72 h. In 2004, Vandervoot and Ludwig succeeded in encapsulating a hydrophilic (pilocarpine HCL) and hydrophobic (hydrocortisone) drug in gelatin nanoparticles by desolvation method. A sustained release for both drugs was observed comparing to the aqueous drug solution. The study also found that preparation parameters i.e. type of gelatin, pH and type of drug do not affect to the release characteristic (Vandervoort and Ludwig, 2004).

Apart form medication, the signaling proteins such as albumin (Lee *et al.*, 2007), bFGF (Kimura *et al.*, 2003), IGF and TGF-β (Holland *et al.*, 2005) or even the plasmid DNA (Kasper *et al.*, 2005) can also be encapsulated in gelatin microspheres in which the release profiles was under control. These studies demonstrated satisfactory results of release and tissue regeneration in animal test (Brown *et al.*, 1998). Gelatin microsphere apparently is a utility drug delivery device. In this thesis, the controlled release of crude bone protein in gelatin microspheres encapsulation is thus studied and expected, both in the condition of simply microspheres and combination with a porous scaffold

2.2 Use of Growth Factor in Bone Regeneration

The process of bone formation is complicated in which numerous growth factors are necessitated to generate a cascade of bone producing signals inside the cells (Lee and Shin, 2007). A number of growth factors have been studied in vitro and known for their functions in bone tissue growth (table 1) (Tessmar and Gopferich, 2007).

Table 2.1 Growth factor commonly used in bone regeneration

Growth factor		Known activities
Transforming growth factor-β	(TGF-β)	Recruitment, proliferation and differentiation of bone
Bone morphogenetic protein	(BMP)	Differentiation of bone forming cells
Insulin-like growth factor	(IGF-1)	Stimulates proliferation and migration of osteoblasts and the synthesis of bone matrix
Fibroblast growth factor-2	(FGF-2)	Proliferation of osteoblasts
Platelet-derived growth factor	(PDGF)	Proliferation of undifferentiated cells
Vascular endothelial growth factor	(VEGF)	Migration and differentiation of osteoblasts

However, clinical application of the growth factors in maxillofacial reconstruction has not been yet presenting an accurate potential in enhancing bone regeneration (Schliephake, 2002). For instance, PDGF itself did not significantly promote bone regeneration in skeletal reconstructive surgery but combination of PDGF with IGF, TGF-β, or BMP showed the positive results on the periodontal and peri-implant bone regeneration. Local application of the only IGFs did not potentially regenerate bone in craniofacial skeletal defects either. The use of unmixed TGF-β in skeletal reconstructions also showed uncertain results, whereas the conjunction with BMPs may achieve enhancement of bone formation. Although BMP-2, BMP-4, and BMP-7 appeared to be the most effective growth factors in term

of osteogenesis and osseous defect repair, the studies of these growth factors have illustrated indistinct results in reconstruction of the facial skeleton.

Evidently, clinical use of the growth factors in maxillofacial reconstruction requires the synergy of multiple growth factors' potentials for an auspicious result. Growth factors are typically stored in bone extracellular matrix. The proteins extracted form demineralized bone, so called the crude bone proteins, is therefore a considerable mixture of numerous bone growth factors (Urist, 1965; Somerman *et al.*, 1983; Hauschka *et al.*, 1986; Hou *et al.*, 2000) which is valuable to be studied for its potential in bone regeneration.

2.3 Crude Bone Protein Extracts

Bone is physically a hard tissue abundant with mineralized extracellular matrix which makes up about 90% of the total weight of compact bone. Bone extracellular matrix comprises 60% of the microcrystalline calcium phosphate resembling hydroxyapatite, 27% of fibrillar type I collagen and 3% of the minor collagen types and other bone proteins including osteocalcin, osteonectin, phosphoproteins, sialoproteins and glycoproteins, as well as proteoglycans, glycoaminoglycans, and lipids (Hauschka *et al.*, 1986).

Bone extracellular matrix apparently is a depository of bioactive proteins and peptides. In healthy tissue, bone extracellular matrix controls the presentation and distribution of growth factors accurately for any specific condition (Hauschka *et al.*, 1986). Bioactivities of the existent growth factors in bone extracellular matrix retain even in the demineralized condition. In the mid-60s, Urist (Urist, 1965) demonstrated the induction of new ectopic bone formation from a decalcified bone matrix and proposed the existence of osteoinductive molecules in the matrix that direct differentiation of precursor cells into bone forming osteoblasts. The study of Somerman et al. in 1983 (Somerman *et al.*, 1983) confirmed the proposition. They reported that demineratized bone matrix extracted with 0.05 M Tris HCl, pH 7.2 containing 4 M guanidine HCL contained all the information necessary for bone formation. The extracts illustrated chemotactic activity to osteoblast-like cells and itself was heat-labile and sensitive to trypsin, which therefore is a protein-like

material. The demineаralized bone powders induced new bone formation when implanted subcutaneously.

Bone extracts form the demineralized bovine bone powder was investigated the growth factors in a quantitative fashion by Hauschka et al. in 1986 (Hauschka *et al.*, 1986). The result illustrated abundant growth factor activity as high as 570 GFU/g of dry bone (200 pg protein/GFU) in the extracts obtained by 0.5 M EDTA, 0.05 M Tris-HCl, pH 7.5 extractions. The isolated crude bone extracts and growth factors significantly stimulated mitogenic activity on osteoblast of the newborn rat calvaria while depressing alkaline phosphatase specific activity by 2-3 folds.

Extracts from bone contain a lot of factors for chemotaxis, attachment, proliferation and differentiation of bone cells. In 2000, Hou LT et al (Hou *et al.*, 2000) studied the effects of bone extracts on behaviors of human periodontal fibroblasts in vitro and revealed that the periodontal fibroblasts cultured with bone extracts possess osteoblastic characteristics by presenting genetic expression of alkaline phosphatase, osteocalcin, osteonectin, sialoproteins and bone morphogenetic protein-7 and were also capable of forming mineralized foci in vitro. The results corresponded to those of the previous studies which also demonstrated that extracts of bone matrix regulate osteoblast function (Syftestad and Caplan, 1984; Cho *et al.*, 1992).

Crude bone extracts, therefore, evidently encompasses enormous active proteins and growth factors which facilitates new bone formation. Extraction of the crude bone protein with the intricate procedures seems not to obliterate bioactivities of those factors. These presumptions initiate the plan to exploit crude proteins extracted from demineralized bone in regenerating bone tissue in this thesis.

2.4 Scaffolds in Bone Regeneration

Bone is hard and ductile, functions typically in the load-bearing conditions (Currey, 2004). Most bone lesions heal well under the conventional treatment due to the high regenerating potential of bones (Wiesmann *et al.*, 2004). However, a bone graft is often required in maxillofacial surgery to enhance healing of large traumatic or post-surgical defects. Scaffold in bone regeneration needs a high elastic modulus

in order to provide adequate space to the tissue for cell growth and to be retained in the space they were designated for, or even to support mechanical load without failure.

The internal architecture of bone scaffold constructs should be composed of a network of interconnected pores (Mikos *et al.*, 2004). The porous structure facilitates transportation of nutrients, gas and bye-products into and out of the center of a scaffold and influences on vascularization. Macropores which are about 200-400 (Boyan *et al.*, 1996) or 300-500 μm (Hutmacher, 2000; Wiesmann *et al.*, 2004; Karageorgiou and Kaplan, 2005) enhance such transportation rate and are favorable for the formation of blood circulation (angiogenesis) and thus new bone (osteogenesis). On the contrary, micropores (< 200 μm) restrict diffusion rate and vascular ingrowth, thereby generate low oxygen condition in which the formation of cartilage (chondrongenesis) is preferred (Boyan *et al.*, 1996; Karageorgiou and Kaplan, 2005). However, from mechanical standpoint, large pores diminish mechanical properties of the scaffold so the upper functional limit of pore size must be concerned.

2.5 Drug Delivery Scaffolds

With an aim to enhance tissue engineering, scaffolds have been recently designed and fabricated as drug delivery scaffolds. Models of incorporating a drug delivery device in a scaffold have been broadly studied for their efficacy in controlled release and tissue engineering enhancement. In 2003, Kimura et al achieved in engineering adipose (fat) tissue in mice with the collagen sponge incorporating with the bFGF contained gelatin microsphere. The scaffold significantly induced angiogenesis and adipose tissue formation. Control release of the bFGF from gelatin microspheres was a key for success (Kimura *et al.*, 2003).

The sustained release of therapeutic agents can be encouraged by embedding a delivery device in a scaffold. For instance, Kasper et al, in 2005 illustrated that the composites of oligo(poly(ethylene glycol)fumurated) (OPF) and cationized gelatin microspheres were able to prolong the availability of plasmid DNA in mice relative to cationized gelatin microshperes alone (Kasper *et al.*, 2005).

The study of Lee et al. in 2007 also demonstrated a sustained release bovine serum albumin (BSA) and Histone for over 15 days by encapsulating proteins into poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres and further embedding on a porous polycarpolactone (PCL) scaffold with collagen coating, in which the porous structure was not obstructed (Lee *et al.*, 2007). The releases profiles of IGF-1 and TGF-β were also successfully manipulated in the study of Holland et al. in 2005 which showed the dramatic reduction of the burst release from 48% to be 14% by encapsulating microparticles in a network of OPF. Dual release of those both two growth factors was also achieved by the system (Holland *et al.*, 2005).

The ongoing mechanism of the controlled release in drug delivery scaffold may be elucidated with the study of Ungaro et al. in 2006 on the release of albumin from PLGA microspheres embedded in collagen-based scaffolds. It was considered that, in the case, protein release occurred by a diffusion-erosion process contributed by activity of water in the environment, which affected water uptake into the microspheres and the ability of protein transportation in the gel. Microspheres formulation mainly controls the induction time necessary to achieve protein release while polymer scaffold composition controls the release rate. Therefore, a temporal and spatial control of signaling molecules may be obtained by the combination of the appropriated microspheres and scaffold formulations (Ungaro *et al.*, 2006).

In accordance with the concept of cellular guidance and multi-functional scaffold, controlled release of protein at bone regeneration site may be achieved by the integration of protein encapsulated drug delivery devices into the designated scaffold.

2.6 Biomaterials of Choice

2.6.1 Gelatin (Tabata and Ikada, 1998; Young *et al.*, 2005; Chaplin, 2007)

Gelatin is a heterogeneous mixture of single or multiple stranded polypeptides (and their oligomers) each of which contains about 300-4000 amino acids. Gelatin is typically derived from the triple helix type I collagen from skin and bones. Gelatin can be produced by two different processes (Figure 2.1) depending on the method in which collagen are pretreated prior to be extracted. The alkaline

process (or liming) targets the amide groups of asparagines and glutamine residues, hydrolyses them into carboxyl groups, and eventually converts them to be aspartate and glutamate. The alkaline treated gelatin possesses more of carboxyl groups (so call acidic gelatin or type B), thus charges negatively and presents lower isoelectric point (*pI*) at about 3-5. On the contrary, the acidic process hardly reacts to the amide groups of collagen. As a result, acid treated gelatin (so call basic gelatin or type A) posses *pI* similar to that of collagen at about 7-9.

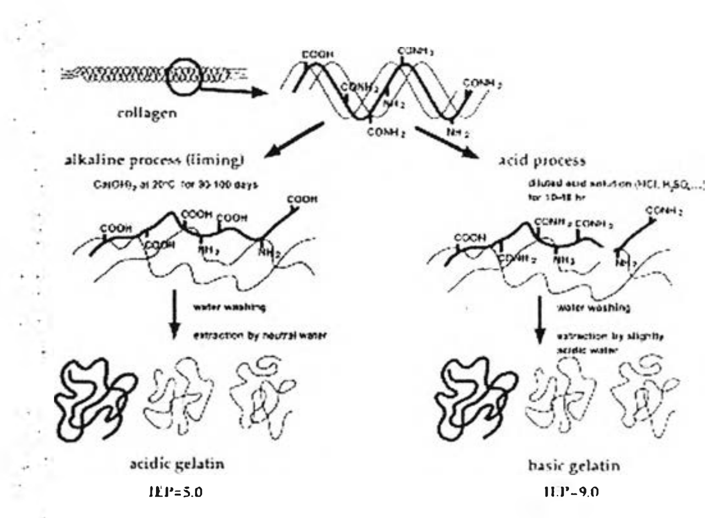

Figure 2.1 Process of gelatin production (Tabata and Ikada, 1998).

Gelatin structurally contains many glycine, proline and 4-hydroxyproline residues. A typical structure is -Ala-Gly-Pro-Arg-Gly-Glu-4Hyp-Gly-Pro- in which glycine is almost 1 in 3 residues, and arranged at every third residue (Figure 2.2).

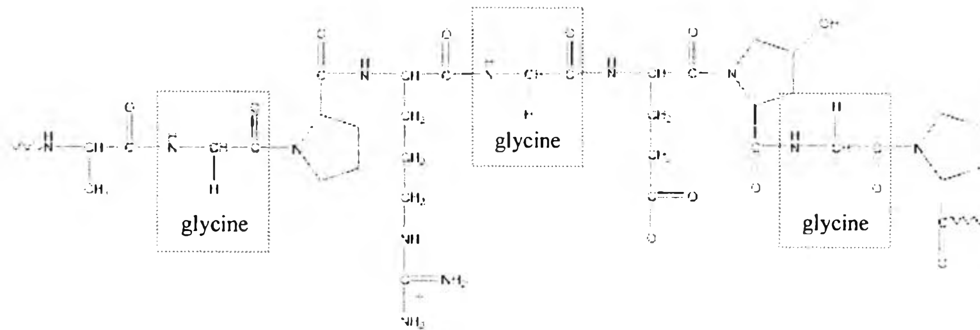

Figure 2.2 Chemical structure of gelatin (Chaplin, 2007).

Gelatin, a natural polymer, is commonly used in pharmaceutical and medical application due to the biocompatibility and biodegradability. The diversity of gelatin's PI can be selectively used to from complex with the oppositely charged molecule like proteins, to be the polyion complexation which is quite stable and can function as a matrix for sustained release of therapeutic proteins.

2.6.2 Hyaruluronan (HA)

HA is a member of a group of similar polysaccharides of the extracellular matrix that have been termed "glycosaminoglycan", "mucopolysaccharides" or "connective tissue polysaccharides". HA structurally is a straight chain, high molecular weight polymer composed of repeating units of the disaccharide [-D-glucuronic acid-β1,3-*N*-acetyl-D-glucosamine-β1,4-]$_n$ (Figure 2.3).

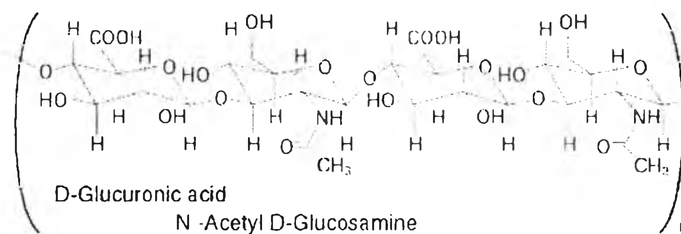

Figure 2.3 A tetrasaccharide fragment of hyaluronan showing the disaccharide repeat units (Prestwich, 2001).

HA possesses various biological properties favorable to tissue regeneration. Functions of HA are vast in range depending on its size. Apart form promoting cell quiescence and supporting tissue integrity, the high molecular size of HA play an essential role at the initial stage of tissue injury by taking part in blood clot formation, anti-inflammation, immune-suppression and anti-inflammation which facilitate responses and functions of inflammatory cells. Then, the fragments of HA induce migration and differentiation of endothelium for the formation of blood circulation, proliferation of fibroblast, collagen synthesis and immune-stimulation. Fragments of HA also have extraordinarily wide-ranging biological functions at the cellular level which have been reported in the literatures (Stern *et al.*, 2006).

HA is a polyelectrolyte polymer. It was reported that the shape of HA chains is affected from ionic strength and environmental pH. The chains contracted with the increasing ionic strength and decreasing pH (Cleland, 1968). Nevertheless, the conformation and interactions of HA in the dissolved state are still controversial. The polyelectrolytic property of HA may assist in an interaction between HA and the other charged molecules such as peptide. Therefore, HA may be fabricated as a drug carrier for the sustained release of the charged therapeutic proteins (Lubomir Lapčk *et al.*, 1998).

2.6.3 Chitin Whisker

Chitin, poly (b-(1-4)-N-acetyl-D-glucosamine), is a natural polysaccharide synthesized by an tremendous number of living organisms. Chitin occurs in nature as ordered crystalline microfibrils forming structural components in the exoskeleton of arthropods or in the cell walls of fungi and yeast. Chitin serves in many functions where reinforcement and strength are required. Chemical structure of chitin and its correspondent deacetylated chitosan are shown in Figure 2.4.

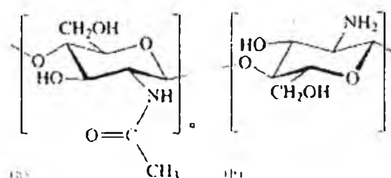

Figure 2.4 Chemical structure (a) of chitin poly(N-acetyl-b-Dglucosamine) and (b) of chitosan (poly(D-glucosamine) repeat units (Rinaudo, 2006).

Chitin whisker is the fiber of high-purity single crystals prepared by disrupting and eliminating amorphous regions of chitin under acid hydrolysis (Rinaudo, 2006; Samir *et al.*, 2008). Many studies have demonstrated that chitin whisker is effective strengthening filler for polymers. The over 10% by weight of chitin whisker increased significantly the shear modulus of poly(styrene-co-butyl acrylate) (Paillet and Dufresne, 2001). Morin and Dufresne (2002) found that above 5 wt% of chitin whisker stabilized mechanical properties of poly(caprolactone) for the temperature higher than poly(carpolaction)'s melting temperature (Morin and Dufresne, 2002). The study of Lu et al. (2004) demonstrated that 20 wt% of chitin whiskers increased tensile strength and Young's modulus of Soy protein isolate/chitin whisker nanocomposites from 3.3 to be 8.4 MPa and from 26.4 to be 158 respectively. In addition, water resistance of the nanocomposites was also improved (Lu *et al.*, 2004). Mechanism of such reinforcement may ascribed to the formation of rigid networks of the whiskers which are highly ordered structure, as well as their high strength crystallinity, and the intermolecular hydrogen bonding between whiskers (Paillet and Dufresne, 2001; Morin and Dufresne, 2002; Samir *et al.*, 2008).

2.6.4 Poly(1,4-Butylene Succinate) Extended with 1,6-Diisocyanatohexane

Poly(1,4-Butylene Succinate) or PBSu is a biodegradable polymer. It is a high molecular weight aliphatic thermoplastic polyester, synthesized through a condensation polymerization of succinic acid and 1,4-butanediol (Han *et al.*, 2002). PBSu is commercially used for the production of film, foamed sheet, blown bottles, or highly expanded foam.

PBSu was proven to be biocompatible with osteoblasts. The study of Li *et al.* in 2005 presented that PBSu film could support both the proliferation and differentiation of the osteoblast cell in vitro (Li *et al.*, 2005). However, the high melting temperature (T_m) of PBSu which is in the range of 90-120 °C can decelerate its biodegradability. PBSu with a low T_m is anticipated to posses an enhanced biodegradability. This could be achieved by introduction of a certain type of non-crystallizable units into the polymer chains, which causes both the T_m and the crystallinity to decrease, when compared with those of the pure polymer (Nikolic *et al.*, 2003). The 1,6-diisocyanatohexane-extended PBSu (or PBSu-DCH) is one such

modification that was developed by coupling PBSu with hexamethylene diisocyanate as a chain extender (Nikolic and Djonlagic, 2001). Its chemical structure is presented in Figure 2.5.

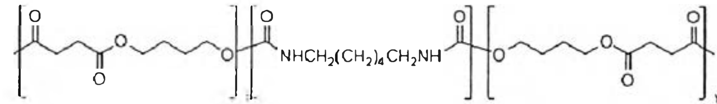

Figure 2.5 Chemical structure of Poly(1,4-Butylene Succinate) extended with 1,6-Diisocyanatohexane (Sigma-Aldrich, 2009).

PBSu-DCH, the same as PBSu, is applied for producing bags, rope, fibers, bottles, foamed cups and cushions (Sigma-Aldrich, 2009). Unfortunately, literature regarding PBSu-DCH is unfound even of the industrial relevance. Nevertheless, the properties of PBSu-DCH are expected to be enhanced over those of the pure PBSu in the tissue engineering point of view. And it is of the great interest of this thesis to study the functionality of PBSu-DCH as a drug delivery scaffold for the regeneration of bone in dental socket or in the other bone lesions.