CHAPTER III

LITERATURE REVIEWS

In this chapter, the literature reviews are summarized into four parts as follows:

1. Characterization and processing of silk fibroin.
2. *In vitro* and *in vivo* studies of silk fibroin.
3. Preparation and characterization of silk fibroin blends.
4. Preparation and characterization of hydroxyapatite-deposited biomaterials.

3.1 Characterization and processing of silk fibroin

Tsukada, M. *et.al.* [40]

In 1994, Masuhiro Tsukada *et.al.* studied the structural changes of two different kinds of silk fibroin membranes obtained by casting from native and regenerated silk fibroin solutions. The structural changes of these membranes were discussed as a function of immersion time in methanol and methanol concentration. X-ray diffractometry and infrared spectroscopy results showed that transition from random coil to β-sheet structure, which became water insoluble, is independent to immersion time. Only native silk membrane treated with pure methanol for 2 min, maintained its original amorphous structure, as demonstrated by differential scanning calorimetric (DSC) curves. SDS-PAGE pattern showed that the molecular weight of native silk fibroin was 350 kDa, while the regenerated sample was formed by a large number of polypeptides in the range of 50-200 kDa.

Chen, X. *et.al.* [41]

In 2001, Xin Chen *et.al.* studied differences in the rheological behavior of regenerated silk fibroin and secondary structure of membranes in four solvent systems: $CaCl_2$-EtOH-H_2O; $Ca(NO_3)_2$-MeOH-H_2O; LiBr-EtOH (with and without water); and LiBr-H_2O. The results demonstrated that $Ca(NO_3)_2$-MeOH-H_2O and LiBr-EtOH-H_2O had the strongest salvation on silk fibroin chains, as shown in an almost constant viscosity (Newtonian behavior) over most of the shear rate range (0.1 to $500s^{-1}$). In contrast, 9.5M aqueous LiBr appeared to have the weakest salvation with similar effects on the silk molecules to pure water. FTIR results also showed that the silk fibroin membranes prepared using all four solvent systems showed mainly random coil conformation with a small proportion of β-sheet.

Li, M. *et.al.* [42]

In 2001, Ming Zhong Li *et.al.* studied the relationship between freeze-drying conditions, structural characteristics and physical properties of silk fibroin aqueous solution. Porous silk fibroin materials, with average pore size of 10 to 300μm, pore density at 1 to $2000mm^{-2}$, and porosity at 35 to 70%, were prepared by freeze-drying aqueous solution obtained by dissolving silk fibroin in ternary solvent $CaCl_2$-CH_3CH_2OH-H_2O. Pore size distribution of such materials mostly accorded with logarithmic normal distribution. Freezing temperature and concentration of silk fibroin solution were adjusted to control structural parameter (pore size, pore density, and porosity) and the physical properties of moisture permeability, compressibility, strength, elongation, etc. Above glass transition zone (-34 to -20°C) of silk fibroin, the freezing temperature has more significant effect on the structure and properties of porous silk fibroin materials.

Yamada, H. *et.al.* [43]

In 2001, Hiromi Yamada *et.al.* examined the factors affecting the molecular mass of fibroin in the dissolved process, and describes a procedure for obtaining

fibroin solutions from cocoons without incurring molecular degradation. The molecular mass of fibroin solutions prepared from silk was analyzed by SDS-PAGE. It was found that fibroin molecule was degraded during reeling, degumming, and dissolution of silk fiber. A method for the preparation of fibroin solution conserving its native molecular size was offered: (1) use only fresh cocoons, after the completion of spinning without reeling as a starting material, (2) degum without breaking the fibroin molecule, and (3) dissolve by saturated aqueous lithium thiocyanate at room temperature.

Wang, H. *et.al.* [44]

In 2005, Hong Wang *et.al.* investigated the influences of temperature and silk fibroin concentration on the flow stability of the solution to obtain optimal storing conditions for silk fibroin aqueous solution. It was found that the flow stability decreased quickly with the increase of solution concentration and temperature. X-ray diffraction, Fourier transform infrared (FTIR) and Raman spectroscopy analysis showed that silk fibroin in aqueous solution was mainly in random coil conformation. After gelation process, it turned into α-helix and β-sheet conformation. The investigation implied that the original dilute regenerated silk fibroin aqueous solution should be stored under low temperature and concentration.

Kim, U.J. *et.al.* [1]

In 2005, Ung Jin Kim *et.al.* developed a new strategy for silk fibroin processing that avoided the use of organic solvents or harsh chemicals. A new mechanism was employed to promote water solubility and then stability of the assembled silk fibroin without the use of hexafluoroisopropanol (HFIP) or methanol. The result of this process showed that adjusting the concentration of silk fibroin in water, and the particle size of granular NaCl used in the process, leads to the control of morphological and functional properties of the scaffolds. The aqueous-derived scaffolds had highly homogeneous and interconnected pores with pore sizes ranging from 470 to 940μm, depending on the particle size of granular NaCl. At 10% silk

fibroin aqueous solutions, the porosity of scaffolds obtained was more than 90% and compressive strength and modulus up to 320±10 and 3330±500 kPa, respectively. The aqueous-derived scaffolds fully degraded upon exposure to protease during 21 days, while HFIP-derived scaffolds showed slow degradation. This new process offered an entirely new window of material properties when compared with traditional silk fibroin-based materials.

Horan, R.L. *et.al.* [45]

In 2005, Rebecca L. Horan *et.al.* demonstrated *in vitro* proteolytic degradation of silk fibroin and its predictable mechanical behavior including tensile and fatigue properties. *Bombyx mori* silk fibroin yarns were incubated in 1 mg/ml Protease XIV at 37°C to create an *in vitro* model system of proteolytic degradation. Control samples were incubated in phosphate-buffered saline. Scanning electron microscopy (SEM) indicated increasing fragmentation of individual fibroin filaments from protease-digested samples with time of exposure to the enzyme. Particulate debris was presented within 7 days of incubation. Gel electrophoresis indicated a decreasing amount of silk 25 kDa light chain and the molecular weight of the heavy chain shifted to a lower molecular weight range with increasing incubation time in protease.

3.2 *In vitro* and *in vivo* studies of silk fibroin

Minoura, N. *et.al.* [46]

In 1995, Norihiko Minoura *et.al.* investigated the cell attachment and growth on silk fibroin from *Bombyx mori* domestic silkworm (DSF) and *Antheraea pernyi* wild silkworm (WSF) and compared the performance to that on collagen. Two kinds of silkworm had a difference in their amino acid sequences. The sequence of WSF contains not only a large number of basic amino acids same as DSF but also the tripeptide sequence arginine-glycine-aspartic acid (RGD). The results on the culture of L929 cells showed that cell attachment and growth on DSF was as good as on collagen. The cells attached to DSF were extensively spread out and their filopodia

were observed in SEM micrographs. On the other hand, WSF displayed much better cell attachment and growth than DSF. The cells attached on WSF became virtually flat and their filopodia could be seen, indicating that they were strongly held on the surface.

Sofia, S. *et.al.* [47]

In 2001, Susan Sofia *et.al.* studied the attachment, growth, differentiation, and bone-forming capacities of human osteoblast cells (Saos-2 line) on *Bombyx mori* silk films. Silk films were prepared to be free of sericin and decorated by covalently coupled peptides included GRGDS containing the adhesion ligand RGD. Respondence of saos-2 to the decorated silk films indicated that the proteins served as suitable bone-inducing matrices. Osteoblast-like cell adhesion was significantly increased on RGD-modified silk film and parathyroid hormone (PTH) compared to plastic, a modified PTH (mPTH), and the control peptide RAD. At 2 weeks of culture, alkaline phosphatase levels were similar on all substrates but were greatest on RGD-modified silk film by 4 weeks. At 2 weeks of culture, α1(I) procollagen mRNA was elevated on silk film, RGD-modified, RAD-modified, and PTH-modified, and hardly detectable on mPTH and plastic. However, by 4 weeks RGD-modified silk film demonstrated the highest level of α1(I) procollagen mRNA compared to the other substrates. Osteocalcin levels detected by RT-PCR were greatest on RGD-modified at both 2 and 4 weeks of cultures. Calcification was also significantly elevated on RGD-modified compared to the other substrates with an increase in number and size of the mineralized nodules in culture. Therefore, RGD covalently decorated silk appears to stimulate osteoblast-based mineralization *in vitro*.

Panilaitis, B. *et.al.* [48]

In 2003, Bruce Panilaitis *et.al.* examined the direct activation of the innate immune response by silk fibers in order to understand the relationship between fibers and biological responses. The results indicated that silk fibers are largely immunologically inert in short- and long-term culture with murine macrophage cell

line while fibroin particles induced significant tumor necrosis factor release. Sericin proteins extracted from native silk fibers did not induce significant macrophage activation. While sericin did not activate macrophages by itself, it demonstrated a synergistic effect with bacterial lipopolysaccharide. The low level of inflammatory potential of silk fibers makes them promising candidates in future biomedical applications.

Kim, U.J. *et.al.* [49]

In 2004, Ung Jin Kim *et.al.* studied the environmental factors that influence silk fibroin sol-gel transitions. Using osmotic stress to generate highly concentrated fibroin aqueous solutions provided the opportunity to explore sol-gel transitions. It was found that gelation of silk fibroin aqueous solutions was affected by temperature, Ca^{2+}, pH, and polyethylene oxide (PEO). Gelation time decreased with an increase in protein concentration, decrease in pH, increase in temperature, addition of Ca^{2+}, and addition of PEO. No change of gelation time was observed with the addition of K^{+}. Upon gelation, a random coil structure of the silk fibroin was transformed into a β-sheet structure. Hydrogels with fibroin concentrations >4wt% exhibited network and spongelike structures. Pore sizes of the freeze-dried hydrogels were smaller as the silk fibroin concentration or gelation temperature was increased. Freeze-dried hydrogels formed in the presence of Ca^{2+} exhibited larger pores as the concentration of the ion was increased. Mechanical compressive strength and modulus of the hydrogels increased with an increase in protein concentration and gelation temperature. The results provided an insight into the silk fibroin sol-gel transitions which is the important insight in the *in vitro* processing of these proteins into useful new materials.

Meinel, L. *et.al.* [36]

In 2004, Lorenz Meinel *et.al.* examined porous silk scaffolds for tissue engineered human bone using human mesenchymal stem cells (hMSCs). The differentiation of hMSCs along osteogenic lineage, and the formation of bonelike tissue *in vitro* on porous scaffolds made of silk (slow degrading), silk-RGD (slow

degrading, enhanced cell attachment), and collagen (fast degrading) in control and osteogenic medium was studied. Histological analysis and microcomputer tomography showed the development of up to 1.2mm long interconnected and organized bonelike trabeculae with cuboid cells on the silk-RGD scaffolds. X-ray diffraction pattern of the deposited bone corresponded to hydroxyapatite presented in native bone. Biochemical analysis showed increased mineralization on silk-RGD scaffolds compared with either silk or collagen scaffolds after 4 weeks. Expression of bone sialoprotein, osteopontin, and bone morphogenetic protein 2 was significantly higher for hMSCs cultured in osteogenic than control medium both after 2 and 4 weeks of culture. The results illustrated that RGD-silk scaffolds are particularly suitable for autologous bone tissue engineering because of their stable macroporous structure, mechanical properties matching those of native bone, and slow degradation.

Meinel, L. *et.al.* [50]

In 2005, Lorenz Meinel *et.al.* studied the inflammatory reactions by hMSCs seeded on silk films and silk films covalently decorated with cell attachment sequences (RGD) *in vitro*. *In vitro* responses of hMSCs on silk were compared with the responses on tissue culture plastic (TCP; negative control), TCP with lipopolysaccharide (LPS) in the cell culture medium (positive control), and collagen films. After 9 h, it was found that the rate of cell proliferation was higher on silk films than either on collagen or TCP. *In vivo*, films made of silk, collagen or PLA were seeded with rat MSCs, implanted intramuscularly in rats and harvested after 6 weeks. Histological and immunohistochemical evaluation of silk explants revealed the presence of circumferentially oriented fibroblasts, few blood vessels, macrophages at the implant-host interface, and the absence of giant cells. Inflammatory tissue reaction was more conspicuous around collagen films and even more around PLA films when compared to silk. These data illustrated that purified degradable silk is biocompatible and the *in vitro* cell culture model (hMSC seeded and cultured on biomaterial films) gave inflammatory responses that were comparable to those observed *in vivo*.

Kim, H.J. *et.al.* [37]

In 2005, Hyeon Joo Kim *et.al.* compared two types of silk fibroin scaffolds prepared from hexafluoro-2-propanal (HFIP) and water, on human bone marrow stem cells (hMSCs) responses toward osteogenic outcomes. hMSCs were seeded on the scaffolds and cultured up to 28 days. It was found that hMSCs seeded onto the water-based silk scaffolds showed a significant increase in cell numbers compared to HFIP-based silk scaffolds. The results demonstrated that three-dimensional aqueous-derived silk fibroin scaffolds provided improved bone-related outcomes in comparison to the HFIP-derived systems. These data illustrated the importance of materials processing on biological outcomes of the same silk fibroin.

Tamada, Y. *et.al.* [51]

In 2005, Yasushi Tamada *et.al.* presented the new processes to form the fibroin sponge. The process involves freezing and thawing fibroin aqueous solution in the presence of a small amount of an organic solvent. The new process did not require freeze-drying, chemical cross-linking, or other polymeric materials. Solvent concentration, fibroin concentration, freezing temperature, and freezing duration affected sponge formation, porous structure, and mechanical properties. XRD and FTIR results indicated that silk I and silk II crystalline structures exist in the fibroin sponge and that the secondary structure of fibroin is transformed from a random coil to a β-sheet during this process. The tensile strength of fibroin sponge decreased slightly because of autoclave treatment. Prior to *in vitro* culture, the fibroin sponge was sterilized using an autoclave. At 3 weeks of culture, MC3T3 cells could proliferate in the sterilized fibroin sponge. Thus, the fibroin sponge formed by this new process is applicable as a tissue-engineering scaffold because it is formed from biocompatible pure silk fibroin and offers both porous structure and mechanical properties that are suitable for cell growth and handling.

Wang, Y. *et.al.* [52]

In 2006, Yongzhong Wang *et.al.* studied the attachment, proliferation and differentiation of adult human chondrocytes (hCHs) in three-dimensional aqueous silk fibroin scaffolds. The results were compared with those using human bone marrow stem cells (hMSCs). Cell adhesion, proliferation and redifferentiation on the scaffolds based on cell morphology, levels of cartilage-related gene transcripts, and the presence of a cartilage-specific extra cellular matrix (ECM). The hCH-based constructs were significantly different than those formed from MSC-based constructs with respect to cell morphology, structure and initial seeding density needed to successfully generate engineered cartilage-like tissue. These results illustrated fundamental differences between stem cell-based (MSC) and primary cell-based (hCH) tissue engineering, as well as the importance of suitable scaffold features, in the optimization of cartilage-related outcomes *in vitro*.

Meechaisue, C. *et.al.* [53]

In 2007, Chidchanok Meechaisue *et.al.* fabricated the ultra-fine fibers of silk fibroin (SF) from cocoons of Thai silkworm (Nang-Lai) and Chinese/Japanese hybrid silkworms (DOAE-7). The results showed that the average diameter of electrospun (e-spun) SF fibers increased as increasing the solution concentration and the electrostatic field strength (EPS) value. The average diameter of the e-spun Thai SF fibers was between 217 and 610nm while that of the DOAE-7 fibers was between 183 and 810nm. *In vitro* cell culture was tested using mouse osteoblast-like cells (MC3T3-E1). MTT assay showed a monotonic increase in the absorbance values with the increase in the time in culture implying that the number of cells on the surface of the Nang-Lai SF fibrous scaffolds increased with the increase in the cell culture time. After 5 days of culture, cells appeared to fully cover the surface of the scaffold. This result showed that cells could adhere and proliferate on the surface of Nang-Lai SF fiber mats. The e-spun Nang-Lai SF fiber mats could be used as scaffolding materials for bone cell culture.

3.3 Preparation and characterization of silk fibroin blends

Kweon, H.Y. *et.al.* [54]

In 2001, Hae Yong Kweon *et.al.* reported the structural characteristics and thermal properties of *Antheraea pernyi* silk fibroin (SF)/chitosan blend films prepared by solution casting. The results demonstrated that the conformation of blend films was revealed to be a β-sheet structure, due to the effect of acetic acid used as a mixing solvent. According to the Fourier Transform Infrared (FTIR) spectra, NH groups of SF and C=O and NH_2 groups of chitosan have participated in a specific intermolecular interaction among themselves. The exotherm of SF was not exhibited in blend films due to the precrystallization of SF induced by acetic acid. The blend films showed decomposition temperatures at around 294°C (chitosan component) and 369°C (*A.pernyi* component) which could be indirect evidences of phase separation. This was comfirmed by scanning electron microscopy (SEM) results. Blending with *A.pernyi* SF could enhance the thermal decomposition stability of chitosan.

Lv, Q. *et.al.* [2]

In 2005, Qiang Lv *et.al.* developed a new preparation for silk fibroin scaffolds with uniform pore distribution, controllable pore size and functional features by freeze–drying method. Collagen was added to fibroin solution to restrain unwanted fibroin aggregation in preparation processes. The results demonstrated that when collagen was added to fibroin solution, the viscosity of blend solution increased, and then it restrained unwanted fibroin leaf formation in freezing process. With methanol treatment, fibroin/collagen scaffolds became water-stable, following the transition from random and α-helix to β-sheet conformation. Aqueous-fibroin porous scaffolds had highly homogeneous and interconnected pores with pore sizes ranging from 127 to 833μm, depending on the fibroin concentration. The porosity of scaffolds was more than 90%, and the yield strength and modulus were up to 354±25 kPa and 30±0.1 MPa, respectively. Scanning electron microscopy (SEM) and MTT analyses

demonstrated that the adding of collagen evidently facilitated Human hepatocellular liver carcinoma (HepG2) attachment and proliferation *in vitro*.

Gil, E.S. *et.al.* [55]

In 2006, Eun S. Gil *et.al.* studied the effect of SF crystallization on properties of silk fibroin (SF)/type A gelatin (GA) blended membranes. When co-casting of solution, amorphous blends of these polymers appeared homogeneous, as discerned from visual observation, microscopy, and FTIR spectroscopy. After immersing in aqueous MeOH, the conformation of SF transformed from random coil to β-sheet. According to X-ray diffractometry and thermal calorimetry, this transformation occurred in pure SF as well as in each of the GA/SF blends. Thermal gravimetric analysis revealed that the presence of β-sheets in SF and GA/SF blends improves thermal stability. Extensional rheometry confirmed that SF crystallization enhanced the tensile properties of the blends. The formation of crystalline SF networks in GA/SF blends could be used to stabilize GA-based hydrogels for biomaterial and pharmaceutical purposes.

3.4 Preparation and characterization of hydroxyapatite-deposited biomaterials

Taguchi, T. *et.al.* [56]

In 1998, Tetsushi Taguchi *et.al.* introduced a method of hydroxyapatite (HAp) formation on/in a three-dimensional PVA hydrogel matrix. From the past study, the biomimetic process was used for HAp formation on/in hydrogel. The biomimetic method is basically divided into two stages: (1) nucleation, in which the substrate is immersed in a synthetic solution of simulated body fluid (SBF) and bioactive CaO–SiO_2 based glass particulates as a nucleating agent, and (2) precipitation and growth of the apatite layer. It can effectively prepare HAp composites on the surface of various kinds of material but it takes a long period of time (4 days for the first step

and another 4 days for the second step) to form a large amount of HAp on/in hydrogels. As a result, a novel HAp formation process was developed. This process was based on the widely-known wet synthesis of HAp. It employed alternate soaking process in 200mM $CaCl_2$/Tris-HCl (pH 7.4) at 37°C for 2 h and 120mM Na_2HPO_4 aqueous solutions at the same condition. The results illustrated that the HAp crystals could be formed on/in a PVA gel for a short amount of time (5 cycles correspond to 20 h).

Furuzono, T. *et.al.* [57]

In 2000, Tsutomu Furuzono *et.al.* applied alternate soaking process to silk fabric to prepare a composite of silk fabric and apatite and studied apatite deposited on the silk fabric. It was found that apatite weight increased with alternated soaking repetitions in calcium solution [200mM aqueous calcium chloride solution buffered with tris-(hydroxymethyl) aminomethane and HCl (pH 7.4)] and phosphate solution (120mM aqueous disodium hydrogenphosphate). Fresh solutions were used for each soaking. SEM showed that apatite deposited after 21 or more repeated soakings was over 20μm thick. XRD showed that the apatite crystals deposited on silk fabric elongated along the c axis. FTIR and XPS indicated the existence of carbonate, HPO_4^{2-}, and Na^+ ions in addition to constituent ions of hydroxyapatite. HPO_4^{2-} ions converted into PO_4^{3-} ions to form more stable hydroxyapatite crystals, which were associated with increasing apatite crystallinity. Apatite deposited on silk by the alternate soaking process was a deficient apatite containing carbonate, HPO_4^{2-}, and Na^+ ions as in a natural bone tissue. Thus, this apatite–silk composite material might be potentially bioactive.

Bigi, A. *et.al.* [10]

In 2002, Bigi *et.al.* reported that the presence of hydroxyapatite (HAp) inside the gelatin sponges could promote the deposition of apatite crystals from simulated body fluid (SBF). *In vitro* bioactivity of gelatin sponges and HAp/gelatin sponges was tested through evaluation of the variations in their composition and morphology after

soaking in SBF for periods up to 21 days at 37°C. It was found that HAp promotes the deposition of bonelike apatite crystals on gelatin sponges. The deposits were laid down as spherical aggregates with mean diameters increasing from about 1–2μm after 4 days of soaking in SBF solution and up to about 3.5μm in the samples soaked for 21 days. In addition, an amount of inorganic phase increased about 56wt% leading to a composite material with a composition quite close to that of bone tissue. The inorganic phase was a poor crystalline carbonated apatite similar to trabecular bone apatite.

Wang, L. *et.al.* [9]

In 2004, Li Wang *et.al.* prepared hydroxyapatite (HAp)–silk fibroin (SF) composites with various amounts of SF by a co-precipitation method in order to obtain well-dispersed HAp nanoparticles in a SF matrix. The effects of SF content on the microstructure and hardness of the composites were studied. A co-precipitation method, Phosphoric (H_3PO_4) solution was added into calcium hydroxide ($Ca(OH)_2$) suspension containing various amounts of SF powder. The mixture was stirred vigorously at room temperature for 3 h, followed by centrifugation and water-washing alternately for three cycles. The precipitates were dried in vacuum at 50°C for 24 h and subsequently ground into fine powders using an agate mortar. Meanwhile, pure HAp without SF was prepared as a control sample by the same process. When SF content increased up to 40wt%, the particle size distribution became narrower and the Vickers microhardness of the composites increased. The composites exhibited the porous microstructure with open porosity around 62–74%. About 70% of interconnective pores were between 40 and 115μm in diameter. The composite containing 30wt% of SF showed homogeneous form and a well dispersed state of HAp crystallites together with a highly developed three-dimensional network.

Taguchi, T. *et.al.* [11]

In 2004, Tetsushi Taguchi *et.al.* developed a novel bifunctional scaffold with calcium phosphate using alternate soaking process to form calcium phosphate in a

cartilage-like matrix of type II collagen gels. Characterization of calcium phosphate formed in type II collagen matrices was analyzed using X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR), thermogravimetric and differential thermal analysis (TG-DTA), and scanning electron microscopy (SEM). The results from XRD and FT-IR analysis indicated that calcium phosphate formed in the matrix was hydroxyapatite (HAp), whose phosphate ions were partially replaced by carbonate ions. TG-DTA analysis showed that HAp content increased with increasing immersion cycle in calcium and phosphate solutions. SEM image showed that a calcium phosphate layer was deposited on one side of type II collagen gels. The composite with gradient calcium phosphate crystals should be useful in regenerating bone-cartilage interface.

Ijima, H. *et.al.* [12]

In 2004, Hiroyuki Ijima *et.al.* studied the characteristics of hydroxyapatite (HAp) formed on glass plates covered with polyvinyl alcohol (PVA) gel by the alternate soaking process and optimized the conditions of the alternate soaking process for animal cell culture with regard to cell attachment and proliferation. The results illustrated that HAp formation ratio depended on the reaction cycle number but was independent of the alternate soaking period per cycle. The Ca/P molar ratio of HAp formed at 10 reaction cycles was very close to the theoretical value of HAp, 1.67. From *in vitro* study using Chinese hamster ovary cell line (CHO-K1), the cell number was counted using the nucleus counting. Cell adhesion, proliferation and maximum cell density on HAp plates formed at two or five reaction cycles using 200mM $CaCl_2$ and 120mM Na_2HPO_4 solutions were better than on plates formed under other conditions. Furthermore, the adhesion ratio of CHO-K1 cells on HAp plates formed at 10 reaction cycles was about 60% of those at two or five reaction cycles. HAp provided an adequate substratum for CHO-K1 cell adhesion and proliferation.

Wang, L. *et.al.* [58]

In 2005, Li Wang *et.al.* prepared hydroxyapatite (HAp)/intact silk fibroin (SF) and HAp/alkali pretreated SF nanocomposite sol via a wet-mechanochemical route. The influence of the alkali pretreatment of SF on chemical states, microstructure, Vickers microhardness of the composite and gelation behavior of the composite sol was studied. A wet-mechanochemical route, Phosphoric (H_3PO_4) solution was added into calcium hydroxide ($Ca(OH)_2$) suspension containing various amounts of SF powder. The mixture was stirred vigorously at room temperature for 1h, and then milled by a multi-ring mill at 1250 rpm for 3 h. After that, the milled sol was centrifuged and water-washed alternately for three cycles. The precipitates were dried in vacuum at 50°C for 24 h and subsequently ground into fine powders using an agate mortar. It was found that when the alkali pretreated SF involved, Vickers microhardness of the composite increased 57% and enhanced three-dimensional porous network with a homogenous particle form and a uniform pore size distribution. Not only, the alkali pretreatment of SF increased the viscosity and the rigidity of the composite sol but also promoted its gelation process, which was favorable for healing bone defects by an injection technique.