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The immobilization of Chinese herbal medicine onto the surface-modified calcium hydrogenphosphate

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Abstract

To accelerate the healing of bone defects or to enable to heal at all, it is often necessary to fill them with suitable substance. Various artificial materials defects have been developed. Among these, calcium phosphates and bioactive glass have been proven to be biocompatibile and bioactive materials that can chemically bond with bone, and have been successfully used clinically for repair of bone defects and augmentation of osseous tissue. However, those bioceramics have only the property of osteoconduction without any osteoinduction.

Many ligands have been physicochemically absorbed onto substrates to enhance cell-substrate interactions. Although it has been widely developed, it is still limited to use in long-term implantation because of short half-life period. Thus, some interfacial modification will be required for enhancing the efficacy of delivery system. These models involve the immobilization of biologically active ligands of natural and synthetic origin onto various substrates to produce an interface with stronger chemical bond between ligand and substrate. The advantage of covalently immobilizing a ligand is that a chemical bond is present to prevent ligand or medicine from desorption.

In our study, two-step of chemical immobilization was performed to surface-modified calcium hydrogenphosphate powders. The first was to modify the surface of calcium hydrogen-phosphate (CHP) with coupling agent of hexanmethylene diisocyanate (HMDI). The linkage between CHP and HMDI will be characterized by FTIR. The second step was to immobilize chemically Gusuibu onto MCHP. Moreover, the sorption and desorption of Gusuibu was evaluated and quantitative analyzed by spectrophotometer and HPLC.

From the results, bioceramic CHP was surface-modified by two-step of chemical immobilization. Firstly, successfully modified the surface of calcium hydrogen-phosphate (CHP) with coupling agent of hexanmethylene diisocyanate (HMDI). The first step was also activated the surface of CHP to induce primary amine terminator. The reaction of this functional group with Gusuibu was the second step. We confirmed simultaneously that Gusuibu could be immobilized chemically onto the surface of MCHP. Although some immobilized Gusuibu also released rapidly at the first 12 hours, the degree of released Gusuibu was lower than both by Gusuibu-adsorbing MCHP and Gusuibu-adsorbing CHP.

1. Introduction

Bone grafts are required in about 15% of all reconstructive surgical operations on the locomotor system. Autografts are preferably used because of their superior efficacy and for avoiding transmission of infection by the grafting. However, the amount of autograft available in a patient is limited, and they need a secondary operation with the possibility of complications at the removal site [1-2]. Allografts and xenografts are used as alternatives. However, grafting of the bone grafts involves numerous problems; in particular, the possible transmission of disease due to bacterial or viral contamination and infection is a major factor.

Early studies in the use of bioactive ceramics as hard tissue implants revolved around the hope that local release of calcium ions would stimulate osteogenesis. Bioactive ceramics such as hydroxyapatite, tricalcium phosphate, and bioglass have been proven to be biocompatibile that can chemically bond with bone, and have been successfully used clinically for repair of bone defects and augmentation of osseous tissue [3-4]. However, there is no conclusive evidence that any of bioactive ceramics are osteoinductive. No bone ingrowth will occur if the implant is inserted into muscle or subcutaneous tissue. There also may be a limit to the rate and depth of bone ingrowth when they are inserted into osseous defects. If these bioactive ceramic materials are to serve as more than just osteoinductive scaffolds for bone ingrowth, adjunctive osteoinductive factors must be added [5].

More recently, research in different scientific disciplines is coming together with the expectation of being able to provide osteogenic substitute for bone grafts within the foreseeable future [6]. Therefore, much effort has been directed to the development of osteoconductive and osteoinductive materials by the addition of polypeptides or protein factors to carrier. Several bone growth factors, like bone morphogenetic protein, insulin-like growth factor-I [7], fibroblast growth factor [8-9], platelet-derived growth factor [10], or transforming growth factor [10-11] have been shown to be potent stimulators of bone healing and bone formation. It is expected to promote bone tissue to growth and differentiation through effective biodegradation delivery system.

Calcium phosphate ceramics have been used as drug carriers and delivery systems for numerous drugs: bisphosphonates [12], chlorhexidine [13], antibiotics [14-17], insulin [18], aspirin [19], hormones [20], coumadin [21], anticancer drugs [22-23], indomethacin [24], epinephrine [25], and azidothymidine [26]. Calcium phosphate ceramics loaded with bone growth factors are of interest for developing biomaterials. However, it is very difficult to reach a goal of long-term delivery of osteoinductive agents in the living body just by blending osteogenic factors and calcium phosphates together. We hope immobilize osteogenic factors on the surface of osteoconductive materials, where osteoprogentitor and osteoblast will be attracted to the defect sites during the healing period. Our group has developed a multi-phase system bone implant that consists of a biodegradable ceramic with surface modified by organic molecules, which will play a role as a connector between osteogenic factors and biodegradable ceramics. The organic molecules will provide an effective means to manipulate the surface properties of the biodegradable ceramic. From our previous study, the surface of calcium hydrogenphosphate ($CaHPO_4$, CHP) has been successfully modified by Hexamethylene diisocynate (HMDI). CHP has been proven

to bridge with HMDI molecule through a urethane linkage and would leave an amine group in the tail after modified. The HMDI-surface-modified CHP is as so-called MCHP. In this study, the amine tail will be designed to graft with a potent Chinese herbal medicines to promote osteogenesis.

There are many kinds of Chinese herbal medicines proven to be effective for bone regeneration. Among these, Guosuibu has been proven a very effective herb to stimulate proliferation of human fibroblasts, fetal rat periosteal osteoprogenitor cells, fetal rat calvarias osteoblasts, and human osteoblast [28-29]. The effect of Gusuibu (Drynaria fortunei) on calcium deposition in animal experiments has reported by Ma KC[30]. It was found that Gusuibu significantly accelerated the synthesis of proteoglycan. This may be one of the factors promoting calcium and phosphate deposition and accelerate tissue calcification.

In the study, the Gusuibu will be grafted onto the surface of MCHP as an osteoinductive bone substitute. The adsorption and desorption of Gusuibu from Gusuibu-immobilized MCHP (GMCHP) will be analyzed by HPLC and UV-VIS spectrophotometer.

2. Materials and Methods

Hexamethylene diisocyanate (HMDI) was purchased from Aldrich and used in the experiments without further purification. Calcium hydrogen-phosphate ($CaHPO_4$, CHP) powder was prepared by heating calcium hydrogen-phosphate dibasic ($CaHPO_4.2H_2O$) at 200°C for about 8 hours, which has been proven as pure CHP both by Fourier transformation infrared (FTIR) and X-ray diffraction (XRD) spectroscopy. Dimethyl formamide (Aldrich, DMF) was purified with distillation and stored over molecular sieves of 4Å. Both hydroquinone and dibutyltin dilaurate were purchased from Acros without further purification.

Gusuibu, Rhizoma Drynariae, was purchased from a local Chinese medicine store that would be used as raw materials to prepare Gusuibu solution. Naringin and 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide (EDAC) were acquired from Sigma without further purification.

2-1 Prepared and charactered surface-modified calcium hydrogenphosphate

A typical procedure for CHP surface modification was briefly described as follows. 12.0 g of dried $CaHPO_4$ powder with an average grain size of about 0.1 μm, 150 ml of DMF, dibutyltin dilaurate (0.12 ml) and hydroquinone (200 mg) were put into a 250ml flask. In the system, dibutyltin dilaurate and hydroquinone were used as catalyst and inhibitor, respectively. The flask was then stirred for 1 hour in N_2 atmosphere with a flow rate of 100 ml/min to make sure the reaction in water free condition. 6 ml of HMDI was added to the flask subsequently. The reaction was kept at 50°C under N_2 protection for 4 hours to graft HMDI onto the surface of CHP, which was as so-called surface-modified CHP (MCHP). The MCHP powder was filtered and washed with DMF for three times to remove excess HMDI and HMDI-oligomer. MCHP was then further washed with acetone for three times to

remove the residual DMF.

2-2 Preparation of Gusuibu solutions

500 g of local Gusuibu was extracted with 10 liter of 70% acetone for three times. The extracted solution was collected and then filtered out solid fragment by using Whatman No 1 filter paper. After condensing with reduced pressure at 40°C and frozen drying solvent was removed. The condensate was stored in freeze before use. The stock extract was diluted by water to an appropriate concentration of Gusuibu solution for later experiment.

2-3 Gusuibu onto MCHP

The following two methods will be used to graft Gusuibu onto CHP and MCHP. One was to soak 10 g of CHP or MCHP powder in 100 ml Gusuibu solution with a concentration of 1366 ppm for 4 hours at 25^{o}C with a slow rate of magnetic stir. The filtered solution is collected for further analysis. By this way, we could obtain **Gusuibu-adsorbed CHP powder** and **Gusuibu-adsorbed MCHP powder**, respectively. The other way is to immobilize Gusuibu onto the MCHP. 10 g of MCHP powder is soaked in 100 ml of 1366 ppm Gusuibu solution at 25^{o}C. 0.5 g of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, EDAC, is then added to the solution to initiate the reaction of Gusuibu with the primary amine that existed on the surface of MCHP powder. After 4 hours, the reaction is terminated by addition of 0.5 M NaOH solution. **Gusuibu-immobilized MCHP powder** will be acquired by filtration of the mixture. The solution will collect for further analysis. Niringin is the major compound in the Gusuibu. In order to examine the amount of Gusuibu onto the CHP or MCHP, narigin will be used as the marker compound for UV and HPLC analysis.

2-4 Qualitative analysis of Gusuibu onto MCHP

All the filtered solution is analyzed by UV-VIS spectrophotometer and high performance liquid chromatography (HPLC) to determine how much Gusuibu on the MCHP. A double beam, self-recording (PERKIN ELMER, Norwalk, CT06859) will be used to obtain spectrums of Gusuibu and naringin aqueous solution. The spectrophotometer was with 1 cm matched quartz cell. The spectral bandwidth was 2 nm and the wavelength scanning speed was 200 nm min^{-1}; $\Delta\lambda$=4 nm and scaling factor was 6. The response time was 0.02 s in the spectrum mode.

HPLC apparatus used in this study to separated effectively naringin from filtered solutions. It was consisted of a Waters 2487 HPLC with a 515 HPLC pump, an injector, a variable wavelength detector (Dual λ absorbance detetor) with optical unit upgrade and software (PC 800 Integrator version 2.0). The separation was carried out with a μBondapak^{TM} C18 column (3.9×300 mm) from Waters. Sample and standards were injected using a Rheodyne 10-μl loop. Data acquisition and treatment were accomplished by using a Minichrom data acquisition system. The eluent consisted of methanol-acetic acid-water 35/4/65 (v/v/v) filtered through a 0.45μm nylon filter. The

flow rate was 1 ml/min. The absorption of the eluate was measured at 279 nm (direct UV detection). All experiments were performed at room temperature (25°C).

2-5 Quantitative analysis of Gusuibu sorbed into MCHP

In the spectrum of Gusuibu and naringin aqueous solution it is found that the wavelength of maximum UV absorbance at 279 nm. The stock Gusuibu aqueous solution was diluted to appropriate concentration ranges, 1-257 ppm for lower concentration and 124-4133 ppm for higher concentration, for the construction of calibration curves. A correlation between the observed UV-absorption (y) and the concentration of raw Gusuibu (x) was analyzed by the method of least-squares regression. Then, all of Gusuibu aqueous solution and filtered solution were quantitative analyzed in according to the correlation equation.

2-6 Desorption of Gusuibu from MCHP

For desorption experiments, 1.0 g of the CHP or MCHP powders of each series were immersed in 10 ml of water alone and incubated at 37^{o}C in the water bath. Because the water was periodically exchanged by the fresh solution at the same volume, the released Gusuibu was collected independently for different periods of time during two months. At different time intervals, the concentration of desorbed Gusuibu was determined on UV-VIS spectrophotometer to evaluate the time profile of gusuibu desorption and the percent desorbed was expressed as the ratio of the weight of desorbed Gusuibu to the total weight of sorbed Gusuibu.

3. Results

3-1 Characterization of surface modified calcium hydrogenphosphate

Fig.1 (a) is the FTIR spectrum of CHP, which shows a typical CHP spectrum. Fig.1 (b) is the spectrum of MCHP without 10% nitric acid treatment. There are several extra peaks appeared on the MCHP pattern but the intensity of the extra peaks are very weak. In this study, ceramic part of MCHP will be removed with 10% nitric acid treatment and then analyzed by FTIR. We cannot observe any absorption band of isocyanate group (O=C=N-R) on the spectrum of MCHP, as arrowhead on Fig.1 (b). It can be realized that all the isocyanate groups are involved in grafting reaction.

Fig.1 (c) is the FTIR pattern of MCHP with 10% nitric acid treatment. The bands corresponding to urethane can be traced at 3138, 1716, 1580, 1479, 1255, and 1077 cm^{-1}. The peak at 3138 cm^{-1} is assigned to N-H stretching vibration. 1580 cm^{-1} is the combination of N-H deformation and C-N stretching vibration. The absorption band at 1255 cm^{-1} is the combination of C-N and C-O stretching vibration. A weak peak at 1716 cm^{-1} was the urethane carbonyl group. It is worthy note that a very clear peak can be observed at the position of 1077 cm^{-1}, which is resulted from asymmetric stretching vibration of P-O-C group.

Some peaks at 3327, 1615 and 773 cm^{-1} are assigned to deformation and stretching vibration of NH_2. The absorption bands at 2930, 2857, 1461 and 773 are asymmetrical vibration, symmetrical vibration, skeleton vibration, and deformation vibration of $-CH_2$, respectively. The other one distinct absorption band at 1402 cm^{-1} is responsible for $-CH_2$ deformation band with an adjacent phosphorous group.

3-2 Calibration curves of Gusuibu aqueous solution

Although the specific efficacy of Gusuibu on boned regeneration was well known, little information about the constituents and healing component of Gusuibu was reported [31-33]. Among these, it was reported that less than 4.78 % naringin was found containing in Gusuibu and the naringin is usually presented as the marker compound for Gusuibu [34-35]. Therefore, the UV absorption by photometer was considered to be suitable to describe herbal medicine entirely. Firstly, appropriate concentration Gusuibu aqueous solution was characterized by UV-VIS spectrophotometer from 200 to 600 nm as shown in Figure 2 (a). It was found that the wavelength of maximum UV absorbance at 279 nm. The pure naringin aqueous solution was also assayed by a photometer. Figure 2 (b) showed their UV spectra and illustrated the wavelength of maximum UV absorbance was the same as Gusuibu. Therefore it was suitable to quantity the content of Gusuibu containing in solution by determining the UV absorbance at 279 nm.

Both calibration curves of Guisuibu aqueous solution in the range 1-257 and 124-4133 ppm follow Beer's law. A good correlation was found between the observed UV-absorption (y) and the concentration of raw Gusuibu (x). A least-squares regression analysis gave the regression line $y=0.0012x+0.0005$ ($r^2=0.9998$) and $y=0.0009x+0.0228$ ($r^2=0.9995$), which was used respectively as low and high concentration of Gusuibu solution.

3-3 Quantitative analyzed the immobilized Gusuibu by photometer

Whether by physical or chemical immobilization, reaction would introduce to change the concentration of Gusuibu in filtered solution. Therefore, it was reasonable to quantity the degree of sorbed Gusuibu by measuring the concentration of Gusuibu containing in the filtered solution. Using UV spectrophotometer the values of the adsorbed-Gusuibu and immobilized-Gusuibu was determined indirectly. Table 1 showed concentrations of Gusuibu containing in the filtered solution and amounts of adsorbed-Gusuibu onto CHP or MCHP powders. From the result, there were 12.4 and 79.8 % of Gusuibu adsorbed onto the CHP and MCHP, respectively, through physical treatment. It was clear to promote the adsorption of Gusuibu by mixing Gusuibu with MCHP. By chemical treatment the amount of immobilized-Gusuibu was also be determined. Although the amount of immobilized-Gusuibu (71.3 %) by chemical method was a little less than Gusuibu-adsorbing MCHP, it was higher than Gusuibu-adsorbing CHP. It means that the chemical method was efficient to bond chemically Gusuibu on the surface of MCHP.

3-4 Qualitative analyzed the sorbed Gusuibu by HPLC

Reversed-phase HPLC has been widely utilized for the determination and separation of naturally occurring flavonoids in crude plant materials and food products. For evaluated the effect of MCHP on sorption of Gusuibu, reversed-phase HPLC with UV detector was also used for determination of naringin. Figure 3 (a) showed a typical HPLC profile of HPLC chromatogram of standard naringin solution. The retention time was at 10.1 min. Under the same chromatographic conditions, the Gusuibu aqueous solution was also analyzed by HPLC. Figure 3 (b) showed their typical HPLC profile. It was indicated that a small peak due to naringin was observed at the retention time of 10.5 min and the analysis could be completed within 15 min. The peak was well resolved with a baseline separation from all analogues. The amount of naringin in Gusuibu solution was calculated with the peak area. There was 0.976 wt % of naringin in the Gusuibu aqueous solution. The result was agreement to some researches. It was deserved to mention that several minor peaks was also observed near the peak of naringin.

After mixed CHP or MCHP with Gusuibu for four hours physically, the filtered solution was qualitative analyzed by the same chromatographic conditions in order to understand the effect of naringin. Figure 3 (c) and Figure 3 (d) showed the profile of filtered solution for Gusuibu-adsorbing CHP and Gusuibi-adsorbing MCHP, respectively. In both of profile (c) and (d) their peak area due to naringin were almost the same as original Gusuibu solution. However, the peak due to mirror compound decreased especially in Gusuibi-adsorbing MCHP. Furthermore, in the Gusuibu-immobilizing MCHP, the HPLC profile of the filtered solution was shown as Figure 3 (e). The composition of filtered solution for Gusuibu-immobilizing MCHP differed to Gusuibi-adsorbing CHP and Gusuibi-adsorbing MCHP. The peak heigh not only due to naringin was decreasing but also mirror compound.

Therefore, we focus our interest on the marker compound, naringin, whether MCHP own the higher chemical reactivity to immobilize naringin than the other mirror compound. The chromatogram of standard naringin aqueous solution and filtered solution of naringin-adsorbing CHP, naringin-adsorbing MCHP, naringin-immobilizing MCHP and naringin-immobilizing CHP were illustrated as shown in Figure 4 (a), (b), (c), (d) and (e), respectively. The naringin peak caused by preparation of naringin-adsorbing CHP, naringin-adsorbing MCHP and naringin-immobilizing CHP were not different to original naringin solution. It means that the degree of adsorbed naringin was lower by physical treatment. However, a lower naringin peak was found in the HPLC profile of filtered solution for naringin-immobilizing MCHP shown as Figure 4 (d).

3-5 Assessment of Gusuibu desorbed from Gusuibu-immobilizing MCHP

In order to elucidate the effect of surface-treatment on desorption, we assessed the release of the adsorbed Gusuibu for Gusuibu-adsorbing CHP, Gusuibi-adsorbing MCHP and Gusuibu-immobilizing MCHP at 37.5^{o}C in water during two months. Figure 5 showed the time-release curves of Gusuibu-adsorbing CHP, Gusuibi-adsorbing MCHP. The amount of Gusuibu released from the surface of CHP or MCHP powder is expressed as a percentage of the quantity of adsorbed Gusuibu. From the result, both of release was significant during the first 12 hours. Then it

showed down until the 14th day.

For Gusuibu-adsorbing CHP, the adsorbed Gusuibu was almost released completely at the end of the experiment. Most of adsorbed Gusuibu, 90%, was release during the first 12 hours, as show as Figure 5 (a). But, for Gusuibi-adsorbing MCHP the release tendency was very differed to Gusuibu-adsorbing CHP. Only 65% of the adsorbed Gusuibu was released during two months. Among these, 60% was released rapidly during the first 12 hours as shown as Figure 5 (b). This means that much of Gusuibu still adsorbed onto the MCHP after the two months release in water. In spite of the amount of released Gusuibu decrease with soaking time increase after first 12 hours, it still release in water albeit very slowly.

After immobilized Gusuibu onto MCHP for 4 hours isothermally, some of Gusuibu was bonded chemically onto the surface of MCHP powder. Gusuibu release was also investigated and compared with Gusuibu-adsorbing CHP and Gusuibu-adsorbing MCHP. Figure 5 (c) showed the time-release curves for Gusuibu-immobilizing MCHP. The release tendency of Gusuibu- immobilizing MCHP was also very differ to Gusuibu-adsorbing CHP and Gusuibu-adsorbing MCHP. Gusuibu- immobilizing MCHP released about 20% within 12 hours, but thereafter no substantial release was observed until the two months. After soaking for two months, the amount of residual Gusuibu on Gusuibu- immobilizing MCHP was much more than Gusuibu-adsorbing CHP and Gusuibu-adsorbing MCHP.

4. Discussion

Many ligands have been physicochemically absorbed onto substrates to enhance cell-substrate interactions, such as cell-membrane receptor fragments, antibodies, adhesion peptides, enzymes, adhesive carbohydrates, lectins, membrane lipids, and growth factors. Although it has been widely developed, it is still limited to use in long-term implantation because of short half-life period. Thus, some interfacial modification will be required for enhancing the efficacy of delivery system. These models involve the immobilization of biologically active ligands of natural and synthetic origin onto various substrates to produce an interface with stronger chemical bond between ligand and substrate. The advantage of covalently immobilizing a ligand is that a chemical bond is present to prevent ligand or medicine from desorption.

The isocyanate group employed surface modification has high reactivity. Generally speaking, the reaction of isocyanate group with hydroxyl groups can form a urethane linkage, which is characterized by a secondary amide absorption bands in FTIR (Fig.1 (c)). The presence of the functional group provides the evidence of P-O-CO-NH bond formed between HMDI and CHP. The other one absorption band appeared at 1080 cm^{-1} in Fig.1 (c) is one of evidence of P-O-C formation.

After CHP reacted with HMDI, the product will be put into de-ionized water to convert the terminal group of MCHP (as underlined of CHP-O-CO-NH-$(CH_2)_6$-N=C=O) into primary amine group (-NH_2). The three-amine absorption bands were clearly identified at 3327, 1615 and 773 cm^{-1}. Fig.7 shows no isocyanate absorption band at 2200 cm^{-1}. We can tell that all the isocyanate groups do

not exist in the MCHP any more. It indicates that the final product of CHP reacted with HMDI should be CHP-O-CO-NH-$(CH_2)_6$-NH_2, which is the formation of phosphate urethane and has a terminal amine group.

In the study, we successfully modified the surface of calcium hydrogen-phosphate (CHP) with coupling agent of hexanmethylene diisocyanate (HMDI). The linkage between HMDI and the surface of CHP is a urethane linkage as CHP-O-CO-NH-$(CH_2)_6$-N=C=O. After further treatment, the terminal group of MCHP will be converted into a primary amine group as the formula of CHP-O-CO-NH-$(CH_2)_6$- NH_2. Some detail information about the thermal property and spectrum of ^{31}P and ^{13}C-NMR was presented as our preliminary research.

In adsorption and desorption of Gusuibu onto Gusuibu-adsorbing CHP, fewer adsorbed Gusuibu and rapid release revealed that the interaction between Gusuibu and CHP was too weak to avoid Gusuibu hydrolyzing or degrading. The release is just only due to the simple diffusion of Gusuibu. On the other hand, Figure 4 showed that the marker component naringin also didn't adsorb onto CHP because the affinity of naringin to CHP was poor. Therefore, a little of naringin should be observed to adsorb onto the CHP. The result is agreed with Figure 3 why the naringin peak still appeared in the HPLC profile of the filtered solution. These findings indicate either release is due to the simple diffusion of Gusuibu or selectivity was poor for adsorption of naringin.

In an attempt to improve the interface between Gusuibu and ceramic we developed a surface-modified CHP. Some active amine group was confirmed to exist on the surface of MCHP. The amine groups play an important role for adsorbed Gusuibu. In the study, the functional group acts not only as a acceptor to adsorb medicine but also could be a controller to adjust delivery by changing of pH condition. Some researchers have presented that primary amines are good nucleophiles when unprotonated (pKa~9); moderately basic pH (8-10) ensures their reactivity. Therefore, it is expect to prepare a carrier to adsorb more Gusuibu by blended Gusuibu with MCHP powder.

Table 1 shows there are 79.8% Gusuibu to adsorb onto the MCHP. It is much more than onto the CHP. We extrapolated that stronger interaction strength formed due to the presence of nucleophiles. Figure 5 shows their release was slow down clearly and about 35% of adsorbed Gusuibu still exist onto the Gusuibu-adsorbing MCHP in soaking for two months. Furthermore, the HPLC profile of filtered solution indicated that the adsorption of naringin was very low either in pure aqueous naringin and Gusuibu solution as shown in Figure 3 and Figure 4. It seems reasonable to suppose that no stronger interaction formed due to poor of nuclear donor, naringin. Although the presence of amine group on the surface of MCHP could induce most of Gusuibu components to adsorb onto the MCHP, the method could not promote more naringin to do.

Table 1 shows relative more Gusuibu was immobilized onto MCHP. It was probably caused by two ways to absorb Gusuibu. One was adsorption of Gusuibu and another was a chemical action on interface due to acylation. Although some immobilized Gusuibu also released rapidly at the first 12 hours, the degree of released Gusuibu was lower than both by Gusuibu-adsorbing MCHP and Gusuibu-adsorbing

CHP. Figure 5 shows there are only 20% of Gusuibu released at the first step and it was caused just by diffusion of adsorbed-Gusuibu. Until two months soaking, about 75% of immobilized Gusuibu existed onto the surface of Gusuibu-immobilizing MCHP. Therefore, we could affirm that the chemical treatment by addition of EDC could introduce Gusuibu to immobilize covalently on the surface of MCHP. Accidentally, naringin was observed with nice affinity to MCHP in the chemical immobilization. Figure 3 shows after chemical treatment naringin decreased contained in filtered solution. In the HPLC profile a relative small peak due to naringin was obtained. Additively, pure naringin was used to react chemically with MCHP. Pure naringin appear higher reactivity toward to amine group than by physical method. Although hydrolysis of naringin into naringenin was able to induce naringin decreasing, the doubt was eliminated by experiment the reactivity of pure narigin to CHP in the same condition for chemical treatment. It was excited to observe lower reactivity of naringin toward to CHP. It means that naringin could be reacted with amine group containing on the surface of MCHP but couldn't with CHP. Therefore, we considered in chemical treatment not only Gusuibu but also naringin could covalently bond to MCHP.

5. Conclusion

In our study, bioceramic CHP was surface-modified by two-step of chemical immobilization. Firstly, successfully modified the surface of calcium hydrogen-phosphate (CHP) with coupling agent of hexanmethylene diisocyanate (HMDI). The first step was also activated the surface of CHP to induce primary amine terminator. The reaction of this functional group with Gusuibu was the second step. We confirmed simultaneously that Gusuibu could be immobilized chemically onto the surface of MCHP.

Moreover, the properties about sorption and desorption of Gusuibu offered us many information to develop a multifuntion carrier. The initial release of adsorbed Gusuibu would be able to induce osteoblast to adhesion and immobilized Gusuibu was a cell membrane receptor. There was enough half-life more thane two months to regenerate boned tissue.

6. Acknowledgement

The author would be grateful to National Science Council of ROC for their kindly financial support on the research.

7. Reference

1. E. Gendler. Perforated demineralized bone matrix: A new form of osteoinductive biomaterial. J. Biomed. Mater. Res. 1986;20:687-697.
2. J. E. Lemons. Bioceramics: Is there a difference? Clinical Orthopaedics and Related Research 1990;261:153-8.
3. T. Kitsugi, T. Yamamura, T. Kokubo. Bone bonding behavior of MgO-CaO-SiO_2-P_2O_5 glass. J. Biomed. Mater. Res. 1989;23:631-5.

4. M. Ogino, L. L. Hench. Formation of calcium phosphate films on silicate glasses. J. Non-Cryt. Solids 1980;38:673-7.
5. F. H. Lin, C. J. Liao, H. C. Liu, K. S. Chen, J. S. Sun. Behavior of fetal rat osteoblasts cultured in vitro on the DP-bioactive glass substratum. Mater. Chem. and Phys. 1997;49:270-6.
6. T. M. Chen, C. H. Yao, H. J. Wang, G. H. Chou, T. W. Lee, F. H. Lin. Evaluation of a novel malleable, biodegradable osteoconductive composite in a rabbit cranial defect model. Mater. Chem. and Phys. 1998;55:44-50.
7. Philippe Laffargue, Patrice Fialdes, Patrick Frayssinet, Mohamed Rtaimate, Hartmut F. Hildebrand, Xavier Marchandies. Adsorption and release of insulin-like growth factor-I on porous tricalcium phosphate implant. Journal of biomedical material research 2000;49:415-21.
8. Yasuhiko Tabata, Atsuhiro Nagano, Md. Muniruzzaman, Yoshito Ikada. In vitro sorption and desorption of basic fibroblast growth factor from biodegradable hydrogels. Biomaterials 1998;19:1781-9.
9. Yasuhiko Tabata, Yoshito Ikada. Vascularization effect of basic fibroblast growth factor released from gelatin hydrogels with different biodegradabilities. Biomaterials 1999;20:2169-75.
10. H.D. Kim, R.F. Valentini. Human osteoblast response in vitro to platelet-derived growth factor and transforming growth factor-β delivered from controlled-release polymer rods. Biomaterials 1997;18:1175-84.
11. Boonsri Ongpipattanakul, Tue Nguyen, Thomas F. Zioncheck, Rita Wong, Gary Osaka, Leo DeGuzman, Wyne P. Lee, L. Steven Beck. Development of tricalcium phosphate/amylopectin paste combined with recombinant human transforming growth factor beta 1 as a bone defect filler. Journal of biomedical material research 1997;36:295-305.
12. Denissen H, van Beek E, Lo¨ wik C, Papapoulos S, van den Hooff A. Ceramic hydroxyapatite implants for the release of bisphosphonate. Bone Miner 1994;25:123–134.
13. Sodhi RNS, Grad HA, Smith DC. Examination by X-ray pho-toelectron spectroscopy of the adsorption of chlorhexidine on hydroxyapatite. J Dent Res 1992;71:1493–1497.
14. Tung IC. *In vitro* drug release of antibiotic-loaded porous hy-droxyapatite cement. Artif Cells Blood Substit Immobil Bio-technol 1995;23:81–81.
15. Kano S, Yamazaki A, Otsuka R, Ohgaki M, Akao M, Aoki H. Application of hydroxyapatite-sol as drug carrier. Biomed Mater Eng 1994;4:283–290.
16. Yu D, Wong J, Matsuda Y, Fox JL, Higuchi WI, Otsuka M. Self-setting hydroxyapatite cement: a novel skeletal drug de-livery system for antibiotics. J Pharm Sci 1992;81:529–531.
17. Shinto Y, Uchida A, Korkusuz F, Araki N, Ono K. Calcium hydroxyapatite ceramic used as a delivery system for antibi-otics. J Bone Joint Surg 1992;74B:600–604.
18. Otsuka M, Matsuda Y, Suwa Y, Fox JL, Higuchi WI. A novel skeletal drug-delivery system using self-setting calcium phos-phate cement. 3. Physicochemical properties and drug release rate of bovine insulin and bovine albumin. J Pharm Sci 1994;83:255–8.
19. Otsuka M, Matsuda Y, Suwa Y, Fox JL, Higuchi WI. A novel skeletal drug delivery system using self-setting calcium phos-phate cement. 4. Effects of the mixing solution volume on the drug-release rate of heterogeneous aspirin-loaded cement. J Pharm Sci 1994;83:259–63.

20. Bajpai PK, Benghuzzi HA. Ceramic systems for long-term de-livery of chemicals and biologicals. J Biomed Mater Res 1988;22:1245–66.
21. Mileti IF, Bajpai PK. Development of a hydroxyapatite ceramic matrix for the continuous delivery of coumadin. Biomed Sci Instrum 1995;31:177–182.
22. Uchida A, Shinto Y, Araki N, Ono K. Slow release of anticancer drugs from porous calcium hydroxyapatite ceramic. J Orthop Res 1992;10:440–445.
23. Otsuka M, Matsuda Y, Suwa Y, Fox JL, Higuchi WI. A novel skeletal drug delivery system using self-setting calcium phos-phate cement. 5. Drug release behaviour from a heterogenous drug-loaded cement containing an anticancer drug. J Pharm Sci 1994;83:1565–1568.
24. Otsuka M, Nakahigashi Y, Matsuda Y, Fox JL, Higuchi WI. A novel skeletal drug delivery system using self-setting calcium phosphate cement. 7. Effect of biological factors on indometh-acin release from the cement loaded on bovine bone. J Pharm Sci 1994;83:1569–1573.
25. Benghuzzi HA, England BG, Bajpai PK. Controlled release of hydrophilic compounds by resorbable and biodegradable ce-ramic drug delivery devices. Biomed Sci Instrum 1992;28:179– 182.
26. Cannon MR, Bajpai PK. Continuous delivery of azidothymi-dine by hydroxyapatite or tricalcium phosphate ceramics. Biomed Sci Instrum 1995;31:159–164.
27. Guo-Chung Dong, Feng-Huei Lin, Chun-Hsu Yao, George J Jiang, Chin-Wang Huang. Preparation and Characterization of Surface-Modified Calcium Hydrogenphosphate by Hexamethylene Diisocyanates. Biomaterials 2000;submitted.
28. Ma KC. Stimulative effects of gusuibu (Drynaria baronii) injection on chick embryo bone primordium calcification in vitro. Chung-Kuo Chung Yao Tsa Chih-China Journal of Chinese Materia Medica 1995;20:178-80.
29. Zhou XH. Therapeutic effect of Drynaria baronii Diels on experimental osteoarthritis. Chung Yao Tung Pao Bulletin of Chinese Materia Medica 1987;12:41-4.
30. Ma KC, Zhu TY, Wang FX. Stimulative effects of gusuibu (Drynaria baronii) injection on chick embryo bone primordium calcification in vitro. American Journal of Chinese Medicine 1996;24:77-82.
31. Liu S, Xiao Z, Feng R. A flavanol glycoside from Drynaria propinqua. Phytochemistry 1994;35:1595-96.
32. Liu S, Xian Z. Studies on the chemical constituents of Drynaria propinqua (wall) J. SM. Hua Si Yao Shue Tsa Chih 1991;6:125-8.
33. Zhou TS, Zhou RH. Original plants and their taxonomic studies of crude drug Gusuibu. Journal of China Pharmaceutical University Bulletin 1993;24:70-2.
34. Zhou TS, Lin DW, Li RZ, Zhou RH. Identification and determination of Flavonoids in Twelve types of Gusuibu (Rhizoma Drynariae). Journal of China Pharmaceutical University Bulletin 1996;27:540-3.
35. Zhou FP, Zhang ZQ. Quality evaluation of 3 kinds of rhizoma Drynariae. Chung-Kuo Chung Yao Tsa Chih-China Journal of Chinese Materia Medica 1994;19:261-3.

Table 1 The change of concentration of Gusuibu in filtered solution and the degree of immobilization

Sample	Concentration of Gusuibu before reaction (mg/L)	Concentration of Gusuibu after reaction (mg/L)	The amount of immobilized Gusuibu (mg/g CHP)	The degree of immobilization (%)
Gusuibu-adsorbing	1366	1197, 1250, 1129	1.69, 1.16, 2.37	12.408 ± 3.200
DCP		1211, 1165, 1227	1.55, 2.01, 1.39	
Gusuibu-adsorbing	1366	276, 298, 311	10.90, 10.68, 10.55	79.795 ± 3.250
MDCP		283, 189, 299	10.83, 11.77, 10.67	
Gusuibu-immobilizing	1366	390, 400, 387	9.76, 9.66, 9.79	71.266 ± 0.575
MCHP		392, 383, 403	9.74, 9.83, 9.63	

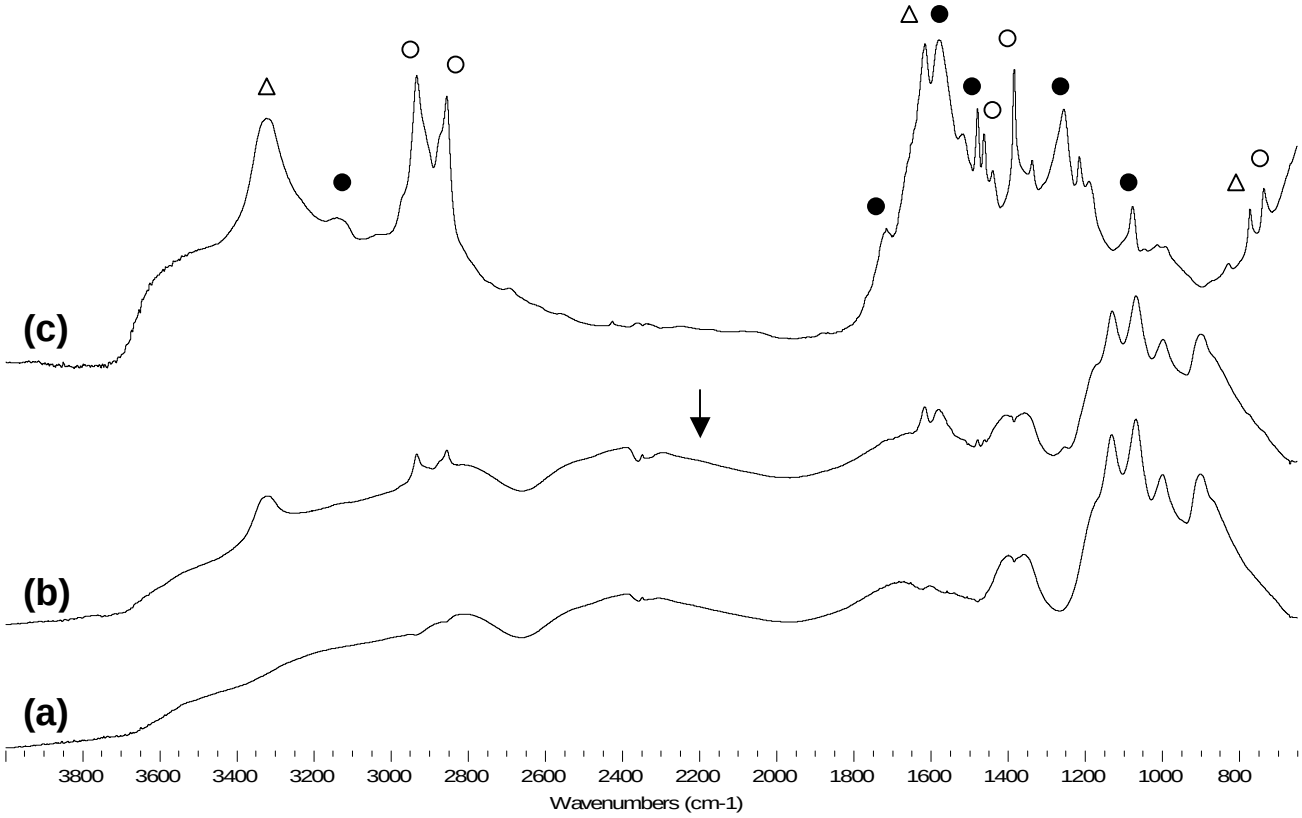

Figure1 FTIR spectra of (a) CHP powder, (b) MCHP without nitric acid treatment, and (c) MCHP with nitric acid treatment. Urethane (λ); Amine (Δ); methyl (O); isocyanate (↓).

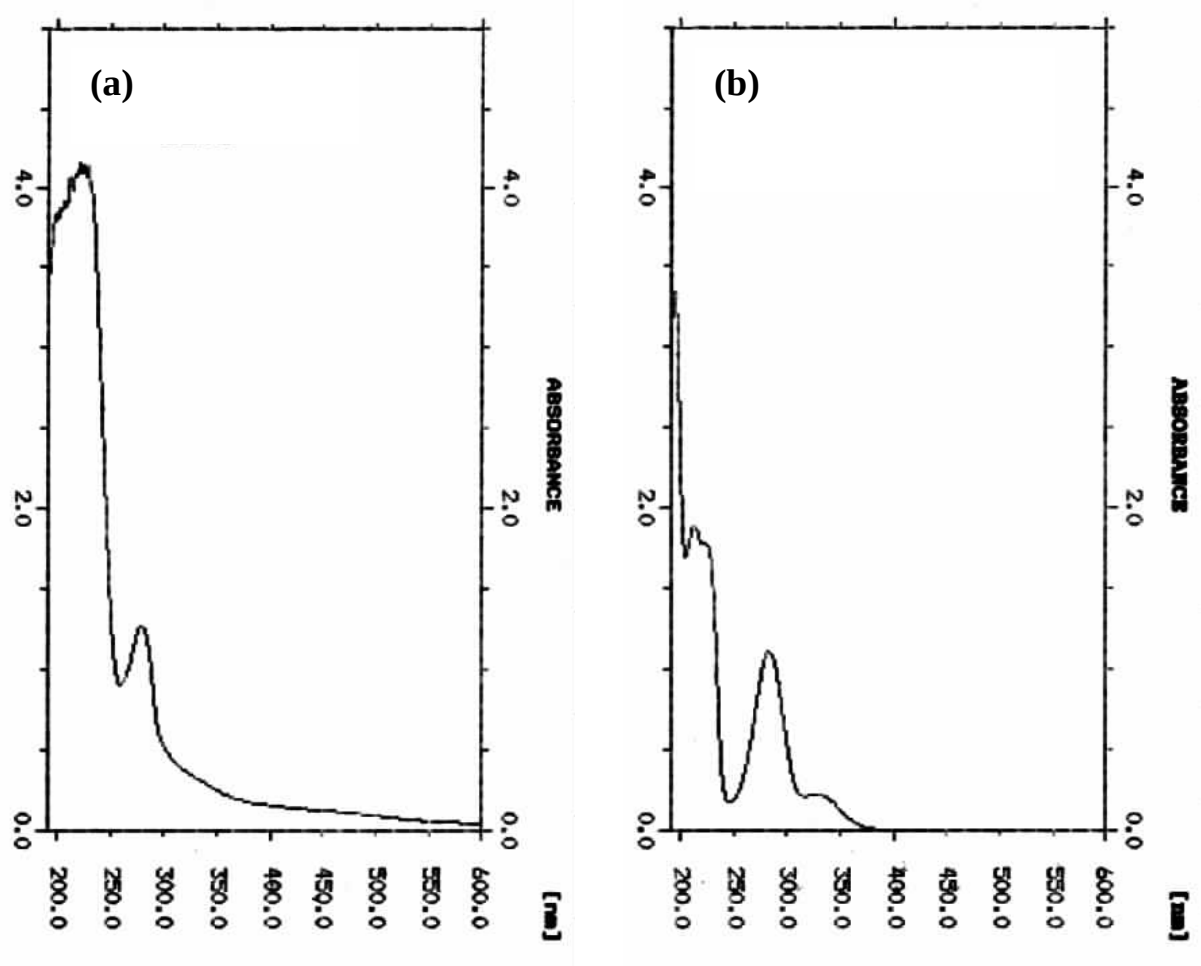

Figure 2 The UV-VIS spectrum of (a) Gusuibu and (b) naringin aqueous solutuin

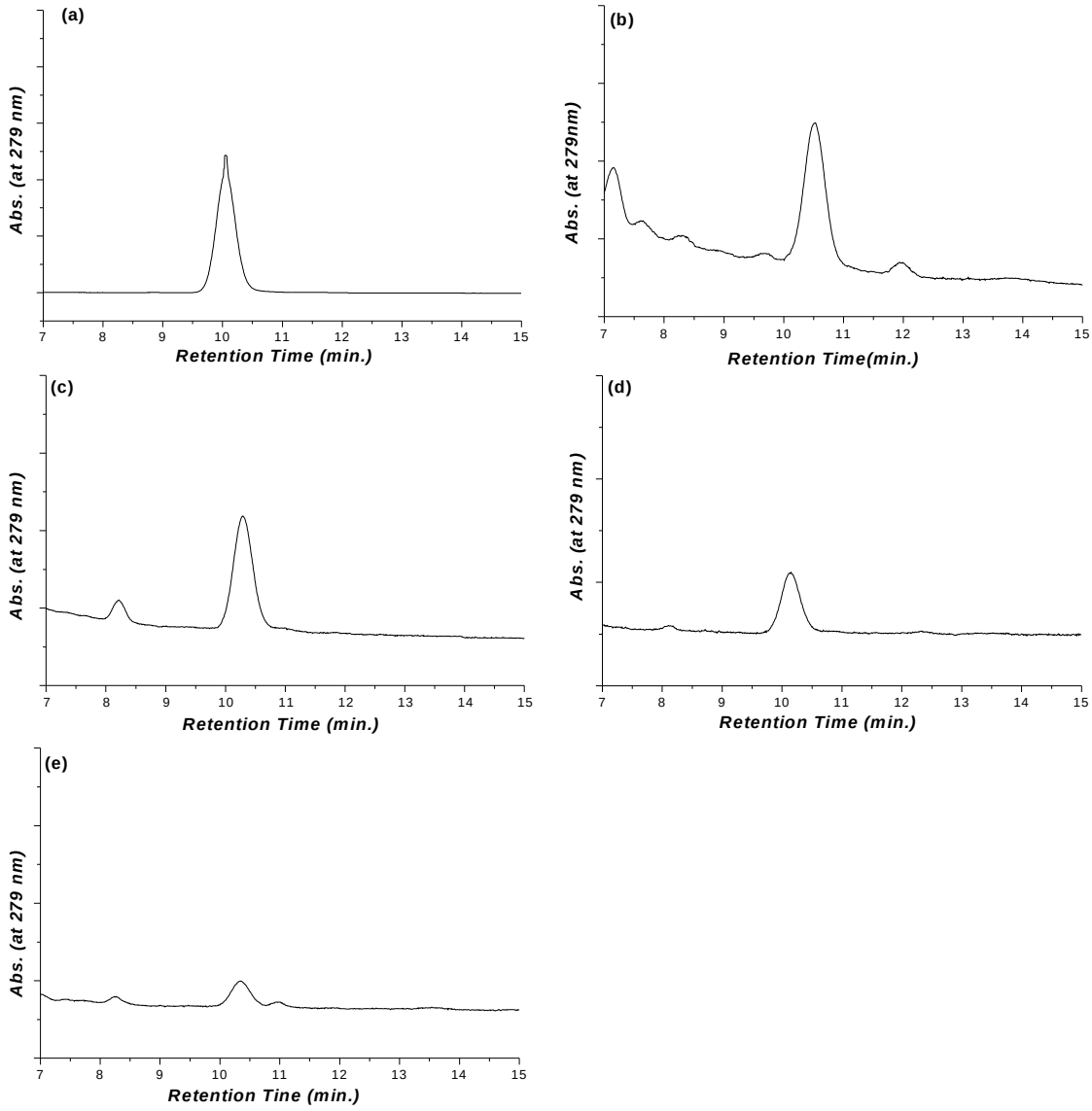

Figure 3 The HPLC profile of (a) pure naringin, (b) Gusuibu aqueous solution and the filtered solution of (c) Gusuibu-adsorbing CHP, (d) Gusuibu-adsorbing MCHP and (c) Gusuibu-immobilizing MCHP

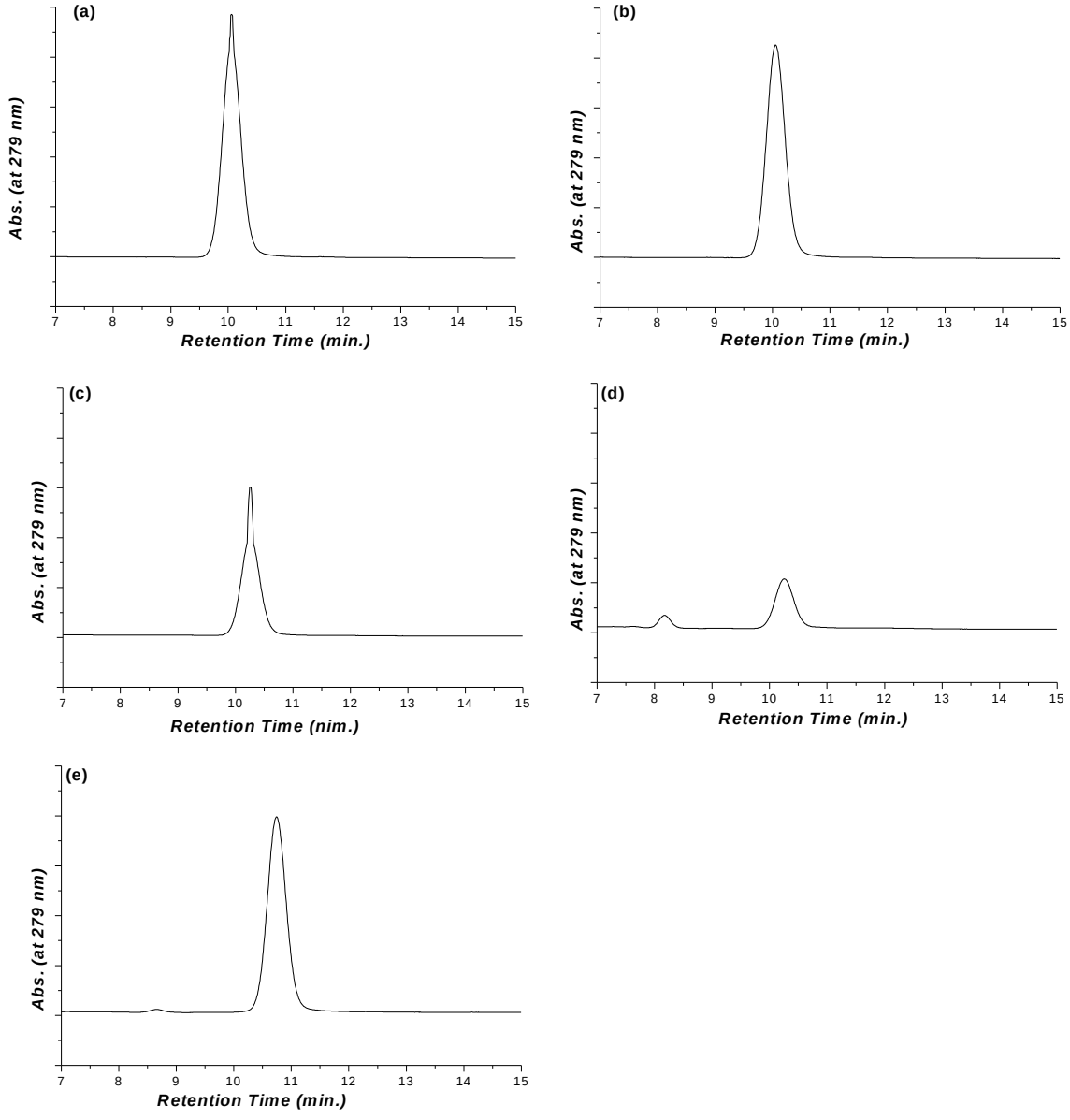

Figure 4 The HPLC profile of (a) pure naringin aqueous solution and the filtered solution of (b) naringin-adsorbing CHP, (c) naringin-adsorbing MCHP, (d) naringin-immobilizing MCHP and (e) naringin-immobilizing CHP

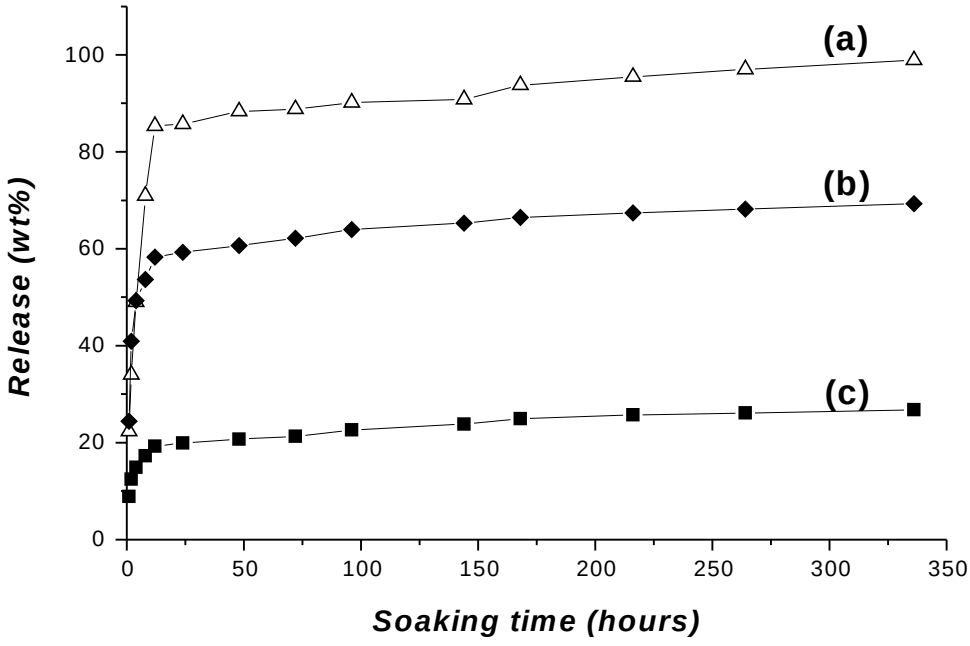

Figure 5 Time-release curves for (a) Gusuibu-adsorbing CHP, (b) Gusuibi-adsorbing MCHP and (c) Gusuibu-immobilizing MCHP at 37.5^{o}C in water during two months.