Bioactive Materials 6 (2021) 208–218

Contents lists available at ScienceDirect

Bioactive Materials

journal homepage: http://www.keaipublishing.com/biomat

Advanced protein adsorption properties of a novel silicate-based bioceramic: A proteomic analysis

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ARTICLE INFO

Keywords:
Silicocarnotite
Bioceramic
Protein adsorption
Proteomics
Pathway

ABSTRACT

Silicate bioceramics have been shown to possess excellent cytocompatibility and osteogenic activity, but the exact mechanism is still unclear. Protein adsorption is the first event taking place at the biomaterial-tissue interface, which is vital to the subsequent cellular behavior and further influence the biomaterial-tissue interaction. In this work, the protein adsorption behavior of a novel CPS bioceramic was evaluated using the proteomics technology. The results showed that CPS adsorbed more amount and types of serum proteins than HA. FN1 and IGF1 proteins selected from proteomics results were validated by Western-blot experiment. Pathway analysis also revealed mechanistic insights how these absorbed proteins by CPS help mediate cell adhesion and promotes osteogenic activity. Firstly, the dramatically enhanced adsorption of FN1 could greatly promote cell adhesion and growth. Secondly, IGF1 was uniquely adsorbed on CPS bioceramic and IGF1 could activate Rap1 signaling pathway to promote cell adhesion. Thirdly, the increased adsorption of FN1, IGF1 and COL1A2 proteins on CPS explains its better ability on bone regeneration than HA. Fourthly, the increased adsorption of IGF1, CHAD, COL2A1 and THBS4 proteins on CPS explains its ability on cartilage formation. Lastly, the increased adsorption of immunological related proteins on CPS may also play a positive role in bone regeneration. In addition, CPS had a much better cell adhesion ability than HA, proving that more adsorbed proteins really had a positive effect on cell behavior. The more adsorbed proteins on CPS than HA might indicated a better bone regeneration rate at early stage of implantation.

1. Introduction

It has been well documented that protein adsorption is the first step when biomaterials come into contact with biological environment *in vitro* and *in vivo* [1]. The category and quantity of adherent proteins play an important role in subsequent process like blood coagulation, inflammation, and cell behavior [2,3]. That is to say, adsorption proteins can mediate the tissue-biomaterial interaction and reflect the biocompatibility of biomaterials [4]. Moreover, the exploration of adsorbed protein on biomaterials is beneficial to understanding the molecular mechanism of their bioactivity.

With the development of technology, methods for characterization of adherent protein on biomaterials surface have been developed from measuring a particular or a few proteins to analyzing all proteins. Many technologies including Fourier transform infrared spectroscopy (FTIR) [5], enzyme-linked immunosorbent assay (ELISA) [6], polyacrylamide gel electrophoresis (PAGE) [7], mass spectra (MS) [8], X-ray photoelectron spectroscopy (XPS) [9] and atomic force microscopy (AFM) [10] have been used to investigate the information of adherent proteins. In 1994, Wilkins and Willian proposed the conception of proteome and then proteomics technology developed quickly [11]. Instead of studying one or more proteins, proteomics focuses on the study of all proteins and their interactions. In combination with protein identification techniques, proteomics can give the information of all proteins [12]. Moreover, proteomics technology can be used to compare the protein adsorption behavior on different materials, so as to predict and explain the subsequent biological effects [13,14], which has been attracted more attention in the field of biomaterials recently. Abdallah et al. revealed the information of extracellular matrix proteins adsorbed on different biomaterials including titanium (Ti), polycaprolactone

Peer review under responsibility of KeAi Communications Co., Ltd.
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https://doi.org/10.1016/j.bioactmat.2020.08.011
Received 30 May 2020; Received in revised form 8 August 2020; Accepted 12 August 2020

(PCL) and poly(methyl methacrylate) (PMMA) through proteomics technology [15]. Oughlis et al. compared the type and quantity of plasma proteins on surfaces of pure Ti and Ti grafted with poly (sodium styrene sulfonate) (PSS) using LC-MS/MS, and predicted the better protein adsorption and cell adhesion capacity of Ti grafted with PSS using proteomics technology [16]. Yang et al. investigated the serum protein adsorption on different TiN surfaces by proteomics technology and revealed the mediation mechanism of adsorbed protein on cell behavior [17].

In the field of orthopeadics, HA, as the chief inorganic composition of human bone tissue, has already been proposed for bone healing since 1920 [18], due to its good biocompatibility and osteoconductivity [19,20]. Nevertheless, the ability for HA bioceramic to stimulate the formation of new bone is limited, and the degradation rate of HA bioceramic is slow whether *in vitro* or *in vivo* [21]. In recent decades, silicate ceramics have been proved to possess good osteogenic activity, which can promote bone regeneration and vascularization [22–24]. Many works have been carried out to explore the mechanism of the bioactivity of silicate ceramics [25–27]. However, they are mainly focused on the expression of some specific osteogenic related genes and the exact mechanism are still unclear.

silicocarnotite (calcium phosphate silicate, CPS) ceramic, a novel silicon-containing calcium phosphate bioceramic with carnotite structure, has been attracted much attention in recent years [28]. It has been shown that CPS not only possesses superior apatite formation ability and osteogenic activity to HA [29], but also can induce the formation of new bone *in vivo* [30,31]. The crystal structure of CPS is similar to that of HA, in which SiO_4^{4-} substitutes one-third of PO_4^{3-} and OH^- disappears due to charge balance [32]. To date, sol-gel method [29,32], solid state reaction [33,34] and aqueous precipitation [35] have been used to obtain pure CPS powders.

To date, no studies have been reported on the protein absorption profiles of CPS or even Si containing calcium phosphate bioceramics using the proteomics technology. In this study, we used LC-MS/MS to determine proteins absorbed by CPS bioceramic. Since the similarity in chemical composition, hydroxyapatite (HA) was evaluated as a comparation to explore the bioactivity of silicon component. The results were further verified by Western-blot and cell adhesion experiments. More importantly, these proteins provide critical mechanistic insights how CPS bioceramic possess excellent cytocompatibility and osteogenic activity.

2. Materials and methods

2.1. Materials preparation

The CPS powder was prepared by a sol-gel method according to the literature [29] using calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$), triethylphosphate (TEP) and Tetraethoxysilane (TEOS) as starting materials, which was calcined at 1350 °C for 6h. The HA powder was obtained by a chemical precipitation method using calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$) and diammonium phosphate ($(NH_4)_2HPO_4$) as initial materials under a pH value of 10.8 followed by a calcination at 900 °C for 2h [29].

Porous CPS and HA bioceramics with same porosity (about 65%) were used in the present work to minimize the interference of porosity on protein adsorption. The obtained CPS and HA powders were ground and sieved through a 200-mesh and polyethylene glycol (PEG) particles between 40–100 mesh was used as the pore former.

After mixing with proper amount of pore former, CPS and HA powders were uniaxially pressed into disks with a diameter of 10 mm and a thickness of 1 mm in a stainless steel die. The pressed CPS and HA specimens were sintered at 1300 °C and 1200 °C respectively for 2 h in a muffle furnace with a heating rate of 2 °C/min, and then naturally cooled in the furnace.

2.2. Phase characterization

Phase composition of the starting CPS and HA powders were determined by X-ray diffraction (XRD, D/MAX-RBX, Rigaku, Japan) with Cu Kα X-ray (λ = 0.15418 nm) at a voltage of 30 kV and a current of 30 mA. XRD patterns were recorded in the 2θ range of 10°–80° using a size step of 5°/min. Morphologies of the ceramics were investigated by a tungsten filament scanning electron microscope (SEM, S–3400 N TypeI, HITACHI, Japan). Open porosity of the ceramics was measured using Archimedes method.

2.3. Protein adsorption kinetics determination

The protein adsorption assay was conducted using 10% Fetal Bovine Serum (FBS, Thermofisher). FBS solutions were diluted by phosphate-buffered saline (PBS) with a pH value of 7.4.

Protein adsorption experiments were carried out in 5 ml centrifuge tubes at a constant temperature oscillation incubator. CPS and HA disks were equilibrated with 1 ml of PBS for 24 h at 37 °C, then replaced with 1 ml 10% FBS solution at 37 °C for 10min, 2h, 6h and 24h separately with continuous shaking. After immersion, the disks were removed and washed 3 times for 20 min each in PBS to eliminate free or loosely bound proteins. Finally, the washed disks were eluted with 200 μL 2% (w/v) SDS solution for 2h at 37 °C to collect the adsorbed proteins. The elution protein concentrations were measured with a BCA™ Protein Assay Kit (Pierce, USA). Each time point was performed with three replicates to obtain a statistical result. Considering the CPS and HA bioceramics were porous, the amount of serum protein (mg) adsorbed on disc volume was calculated according to the equation:

$$Qe = Ct/V$$

Where Ct is the concentration of a certain time t, V is the volume of ceramic.

2.4. Measurement of pH value

The pH value of 10% FBS solution after incubating with bioceramics for different times was measured using a Mettler Delta 320 pH electrode.

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western-blot experiment

After adsorption, protein samples were resuspended under shaking in an SDS-PAGE loading buffer. The samples were centrifuged, transferred into a new vessel and heated at 100 °C for 5 min to denature the proteins. Subsequently, a 12% SDS polyacrylamide gel and the samples as well as serum positive controls were applied on the gel. The SDS-PAGE was performed using a constant voltage of 200V for 1h. The gel was then stained with Coomassie Brilliant Blue R-250 and de-stained in an acetic acid/methanol solution.

For Western-blot experiment, protein samples were separated on 12% SDS-PAGE at 200V for 1h. Then the separated proteins were transferred to 0.2 μm Immuno-Blot PVDF membrane for 1h at 300 mA. The membranes were treated with 5% BSA at room temperature for 1h to block nonspecific binding. The membranes were then incubated with primary antibodies at room temperature for 2h and washed with TBST. Secondary antibodies were added and the membranes were incubated for 2h at room temperature. Protein signals were revealed using ECL, and the intensities of scanned bands were performed with the ImageJ software.

2.6. Proteomics analysis

According to the process represented in the serum proteins

adsorption assay, the disk samples were soaked with 10% FBS for 24h, and then adsorbed proteins were collected by elution. For each kind of ceramic, adsorbed proteins collected from three independent samples were pooled to address biological variation. The eluted protein was lysed using a Filter Aided Sample Preparation (FASP) method for proteomics analysis. The prepared samples were analyzed using an Easy-nLC 1200 - Q Exactive Orbitrap mass spectrometer (LC-MS/MS) system. The LC-MS/MS data were searched with PEAKS Studio 8.5 for protein identification and quantification.

2.7. 7 Bioinformatics analysis

Functional category and KEGG pathway analyses of identified proteins were performed by DAVID software (http://david.abcc.ncifcrf.gov/).

2.8. 8 cell attachment and adhesion

MC3T3-E1 cells were cultured on CPS and HA disks for 2, 6 and 24h, respectively. At the end of each cultured period, cells on bioceramics were immobilized with 4% paraformaldehyde, penetrated by 0.1% TritonX-100 and blocked with 1 wt% BSA. Afterwards, DAPI and Rhodamine-phalloidin were used to stain nucleus and cytoskeleton, respectively. Cell morphology was observed using confocal laser scanning microscope (CLSM, TCS SP8, Leica, Germany). The samples used for SEM observation were fixed with 2.5% glutaraldehyde followed by a dehydration in series of ethanol solutions (30, 50, 75, 90, 95 and 100 v/v%).

3. Results

3.1. Characterization of the bioceramics

XRD patterns of as-prepared CPS and HA powders were shown in Fig. 1a, b. XRD patterns of CPS and HA powders were coincided well with the diffraction data of PDF #40–0393 and PDF #09–0432, respectively, indicating that single phase $Ca_5(PO_4)_2SiO_4$ and $Ca_{10}(PO_4)_6(OH)_2$ powders were obtained. Fig. 1c and d presented the fracture SEM morphology of porous CPS and HA bioceramics. It could be seen that both of them exhibited high-porosity structure with interconnected pores, and Archimedes method confirmed that the porosity for CPS and HA was 66.1% and 65.4%, respectively.

3.2. Protein adsorption kinetics

The protein adsorption kinetics of serum proteins on CPS and HA bioceramics were shown in Fig. 2a. The protein adsorption curves of CPS and HA bioceramics exhibited a similar trend, the amount of protein adsorption both gradually increased with the increase of incubation time. However, at each time point, the amount of serum proteins adsorption on CPS disks was much higher than that on HA ones. Fig. 2b showed the pH value of 10% FBS solution after immersion with ceramic disks for various times. The solution of HA bioceramic remained slightly alkaline and its pH value changed mildly during the whole immersion period. On the contrary, the pH value of 10% FBS solution immersed with CPS bioceramic increased with the increase of immersion time, and its value reached up to 8.46 especially at the time point of 24 h. The obvious change of pH value indicated a quite lively ion exchange occurred between CPS bioceramic and FBS solution during soaking.

3.3. Proteomics analysis of absorbed proteins

Results of proteomics analysis were shown in Fig. 3. We defined protein groups with $-10\lg P > 26$ as available results. A total of 368 and 298 kinds of serum proteins were detected from the CPS and HA samples, respectively, with 197 common ones. Moreover, 171 and 101 unique proteins were detected respectively in CPS and HA samples under the same condition. Among all proteins, 32 were found to have significant differences (significance > 20), which were shown in Supplementary Fig. S1. Among these 32 proteins, CPS showed 27 significantly higher ones, while HA possessed 5 ones. Detailed information and relative abundance value of the common and unique proteins were shown in Supplementary Tables S1–S3.

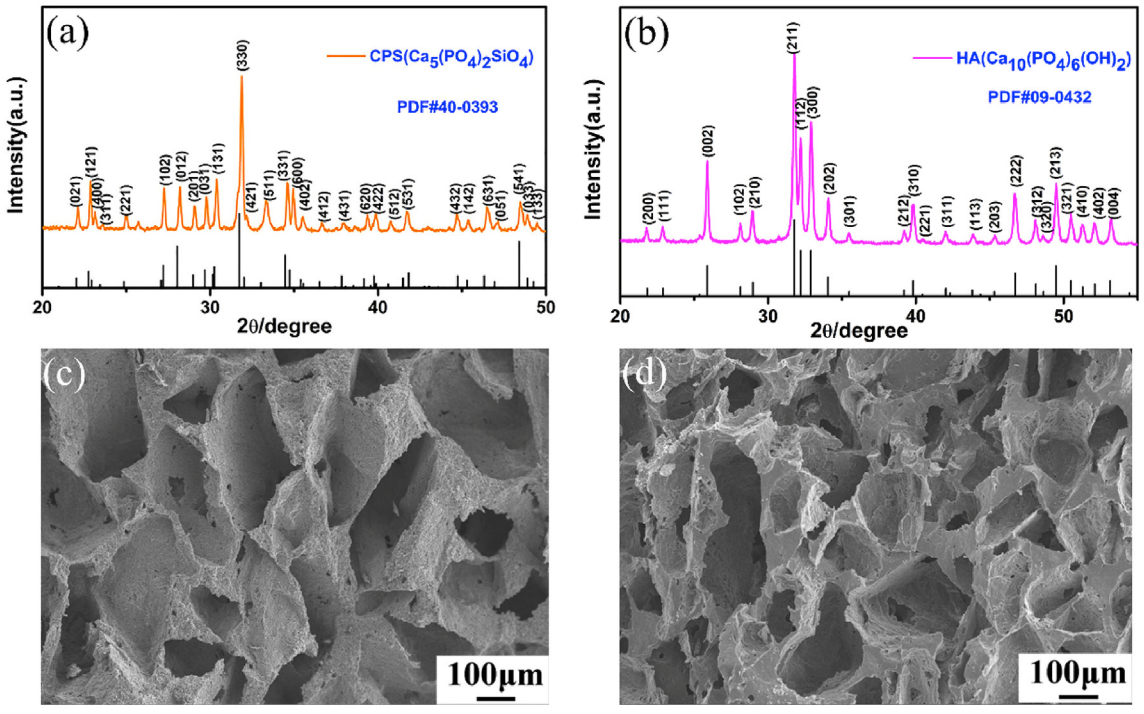

Fig. 1. (a, b) X-ray diffraction pattern of as-prepared (a) CPS and (b) HA powders, (c, d) SEM porous morphology of (c) CPS and (d) HA bioceramics.

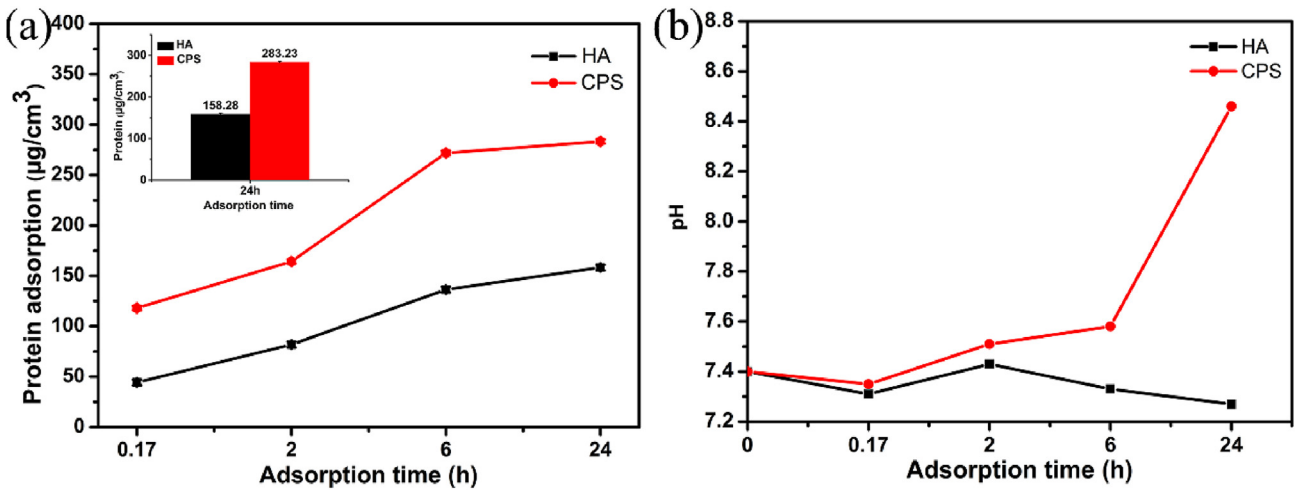

Fig. 2. (a) Protein adsorption onto CPS and HA bioceramics after incubated with PBS containing 10% fetal bovine serum for different time. Each value is the mean ± standard deviation of triplicate determinations, (b) the pH value of 10%FBS solution after different immersion time with CPS and HA bioceramics.

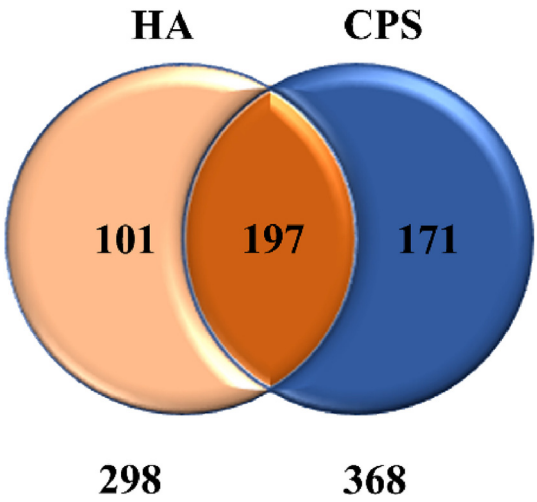

Fig. 3. Types of proteins adsorbed on CPS and HA bioceramics.

3.4. Bioinformatics analyses

3.4.1. Gene Ontology annotation and classification profiles

Gene Ontology (GO) includes biological process, cellular component and molecular function. To further classify adsorbed proteins, we performed GO analysis of unique and differentially adsorbed proteins on bioceramics, and we defined categories with fold enrichment > 2.0 and p-values < 0.01 as available results. Gene Ontology (GO) analyses showed in Supplementary Fig. S2, and the number on each sector represented the number of proteins involved. For biological process, most differential adherent proteins involved in oxidation-reduction process, proteolysis and cell adhesion process. 125, 81, 51 and 45 proteins belong to extracellular exosome, cytoplasm, extracellular space and cytosol in cellular component analysis. For molecular function, most differential adherent proteins related to poly (A) RNA binding, calcium ion binding, GTP binding and identical protein binding.

3.4.2. Functional category analyses

Supplementary Table S4 showed the functional category analysis of adherent proteins. Identified proteins from CPS and HA samples were involved in 76 and 80 enriched functional categories, respectively. Among them, 66 categories (such as secreted, signal, glycoprotein and so on) were common. For the same category, the number of adsorbed proteins on CPS was significantly higher than that on HA.

3.4.3. Biological pathway analyses

Identified proteins from CPS and HA samples were involved in a total of 41 KEGG biological pathways (20 common, 13 unique to HA and 8 unique to CPS). Result is summarized in Table S5. Among these, seven biological pathways are of particular interest, five cell adhesion related pathways and two immunological reaction related pathways could be activated. For cell adhesion relate pathways, they could be activated by the binding of the identified proteins as ligands to cell surface receptors (Fig. 4a). Interestingly, PI3K-Akt signaling pathway is correlated only with proteins adsorbed on HA bioceramic, while rap1 signaling pathway only contains proteins adsorbed on CPS sample. Other three pathways contain proteins from both samples, 11 from HA and 12 from CPS. Among these, 10 proteins are common. Unique proteins include secreted phosphoprotein 1 (SPP1) from HA sample; Thrombospondin-2 (THBS2) and IGF1 from CPS. In common proteins, Fibronectin 1 (FN1) participated in the activation of all 3 pathways: focal adhesion, ECM-receptor interaction and regulation of actin cytoskeleton. Other 8 common proteins, including Cartilage oligomeric matrix protein (COMP), chondroadherin (CHAD), Collagen alpha-1(I) chain (COL1A1), Collagen alpha-2(I) chain (COL1A2), Collagen alpha-1(II) chain (COL2A1), Thrombospondin-1 (THBS1), Thrombospondin-4 (THBS4) and von Willebrand Factor (VWF) activated 2 pathways: focal adhesion and ECM-receptor interaction. Prothrombin (F2) participated in the activation of regulation of actin cytoskeleton. Insulin-like growth factor I (IGF1), unique for CPS case, was involved in both focal adhesion pathway and rap1 signaling pathway.

Fig. 4b compared protein amount of the 10 common proteins on CPS and HA bioceramics related in activating the above three biological pathways. Seven of them, including FN1, CHAD, COL1A1, COL1A2, COL2A1, THBS4 and VWF, exhibited higher adsorption on CPS bioceramic than HA one. Specifically, the adsorption of these four proteins FN1, COL1A2, COL2A1, THBS4, were over 8-fold higher on CPS surface. The adsorption of COMP was comparable on both cases.

Furthermore, proteins adsorbed on HA and CPS bioceramics could activate immunological system pathways including complement and coagulation cascades pathway and platelet activation pathway (Fig. 5a). Fig. 5b compared protein amount of the 19 common proteins on CPS and HA bioceramics related in activating the above two biological pathways. Seventeen of them exhibited higher adsorption on CPS bioceramic than HA one. Specifically, the adsorption of these nine proteins coagulation factor XI(F11), coagulation factor XII(F12), complement factor D(CFD), complement factor H(CFH), serpin family A member 5(SERPINA5), kallikrein B1(KLKB1), carboxypeptidase B2(CPB2), fibrinogen gamma chain(FGG) and COL2A1, were over 10-

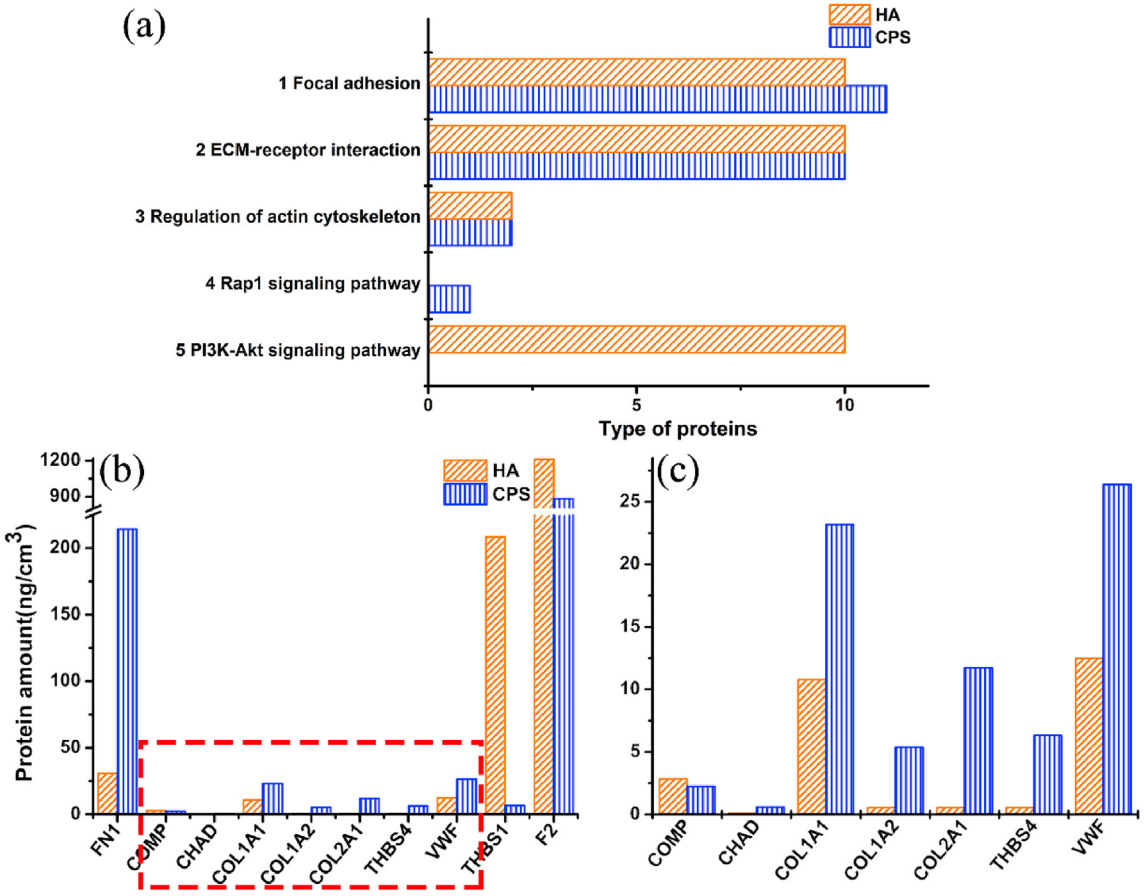

Fig. 4. (a) Five concerned KEGG pathways that could be activated by binding of the identified proteins as ligands to cell surface receptors, (b) protein amount for ten identified proteins, which participate in activating three biological pathways on CPS and HA bioceramics, and (c) is the magnification of the red region in (b).

fold higher on CPS surface.

3.5. SDS-PAGE and Western-blot verification

To verify the protein adsorption capacity on bioceramics, SDS-PAGE and Western-blot experiments of adsorbed proteins on CPS and HA bioceramics were performed and 10%FBS was used as control. The results were shown in Fig. 6, SDS-PAGE result revealed obviously different banding patterns between CPS and HA groups. With the increase of adsorption time, protein bands of CPS and HA turned darker and wider, indicating the augment of protein amount. The Western-blot result showed that the adsorption amount of FN1 and IGF1 on CPS bioceramic were significantly higher than that of HA group, which was coincided well with proteomics analyses.

3.6. Cell attachment and spreading verification

The adhesion of MC3T3-E1 on CPS and HA bioceramics was verified further. Fig. 7 showed the fluorescence and SEM morphologies of MC3T3-E1 cells on CPS and HA bioceramics. After 2h of culture, the morphology of MC3T3-E1 cells on CPS and HA bioceramics was similar, and most of them presented spherical. The Cell number on CPS and HA bioceramics both increased with the increase of adhesion time. When the adhesion time increased to 24h, the number of attached MC3T3-E1 cells on samples further increased, and cells all presented fibrous shapes. However, a distinctly different adhesion phenomenon was detected between CPS and HA bioceramics. The countable adhesion numbers and common spreading of MC3T3-E1 on HA bioceramic were observed while numerous actin cytoskeleton fibers and promoted cell adhesion and proliferation were detected on CPS bioceramic as shown in Fig. 8, indicating the better cell adhesion ability of CPS bioceramic.

4. Discussions

The interactions between proteins and biomaterials play an important role in subsequent cell-biomaterial behavior. The composition of biomaterials dominates the type and amount of adsorbed proteins, which will mediate the subsequent cell behavior on the biomaterials after implantation *in vivo*. Silicate bioceramics are considered as bioactive materials for bone regeneration. As a novel silicate bioceramic, CPS have been proved exhibiting superior cytocompatibility and osteogenic activity, which can enhance the expression of osteogenic markers, like ALP, Runx-2 and OPN. It is necessary to understand the underlying mechanism. In the current study, proteomic technology was used to explore the protein adsorption behavior on CPS compared with HA, which is expected to provide valuable information to understand its excellent bioactivity.

The pH value increased greatly especially after 6 h as shown in Fig. 2b, indicating a quite lively ion exchange occurred during soaking. Therefore, proteins adsorbed on bioceramic after 24 h incubation were selected for proteomic analysis.

Based on the LC-MS/MS analysis, the present study figured out a profile of differentially adsorbed proteins on CPS and HA bioceramics. A total of 272 uniquely adsorbed proteins were identified, 101 of them adsorbed on HA bioceramic while 171 on CPS one. Since bone regeneration is regulated by cell behavior and immunological system, in the present work, pathways and proteins related to cell adhesion and immune system were emphasized. The KEGG analysis revealed that 7 biological pathways on CPS and HA bioceramics could be activated, and 50 proteins were involved. Thirteen of these proteins, i.e. FN1,

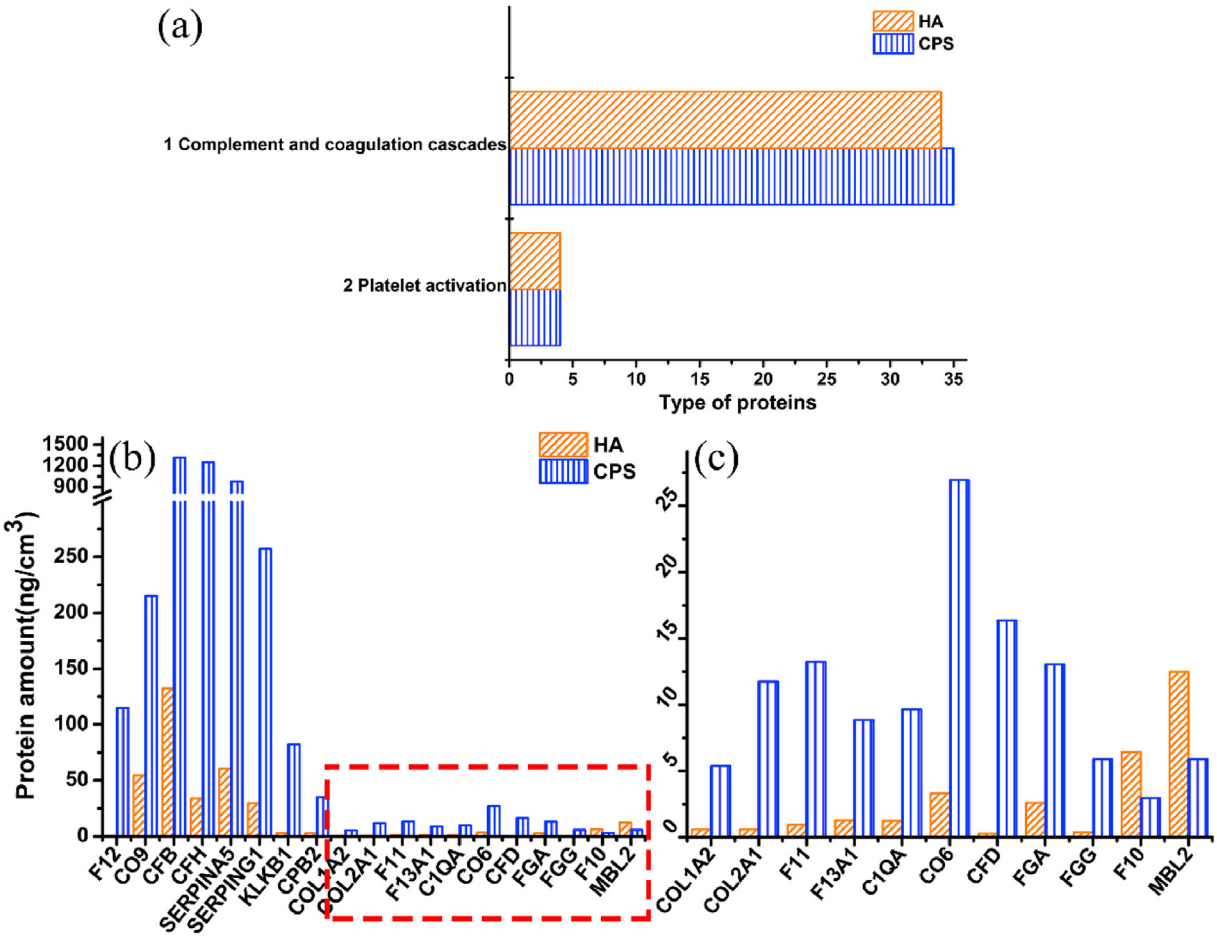

Fig. 5. (a) Two immunological system pathways, (b) amount for nighteen identified proteins adsorbed on CPS and HA bioceramics, which participates in activating two immunological system pathways, and (c) is the magnification of the red region in (b).

CHAD, COL1A2, COL2A1, THBS4, F11, F12, CFD, CFH, SERPINA5, KLKB1, CPB2 and FGG presented elevated adsorption on CPS than HA bioceramic. It implied that the enhanced osteogenic differentiation of rBMSC induced by CPS bioceramic may attributed to the increased adsorption of these proteins on it. To further confirm the proteomics analyses, FN1 and IGF1 proteins were chosen to perform Western-blot experiment, and the cell attachment results also indicated that a positive effect of adsorbed proteins on cell behavior.

Fibronectin (Fn) is a high molecular weight glycoprotein in extracellular matrix (ECM) and belongs to one of the major adhesion proteins [36]. Fn also plays an important role in other cellular activities including cell migration, proliferation and differentiation processes [37]. It has been demonstrated that the binding of Fn on nanoporous TiO_2 promote the adhesion and proliferation of human fetal osteoblast

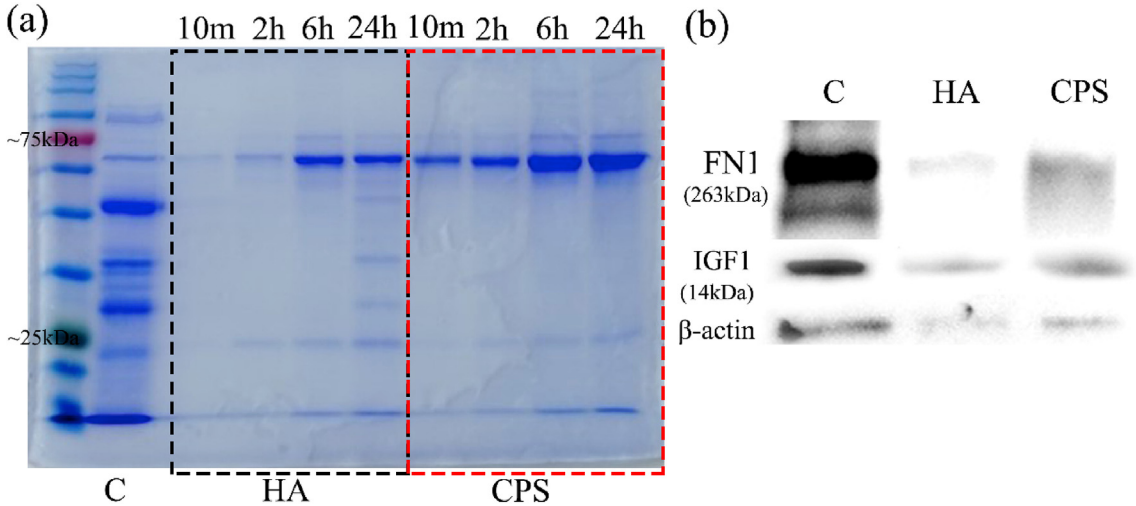

Fig. 6. (a) SDS-PAGE of 10%FBS proteins adsorbed on CPS and HA bioceramics with different time. (b) Western-blot analysis of FN1 and IGF1 adsorbed on CPS and HA bioceramics. 10%FBS solution was used as control.

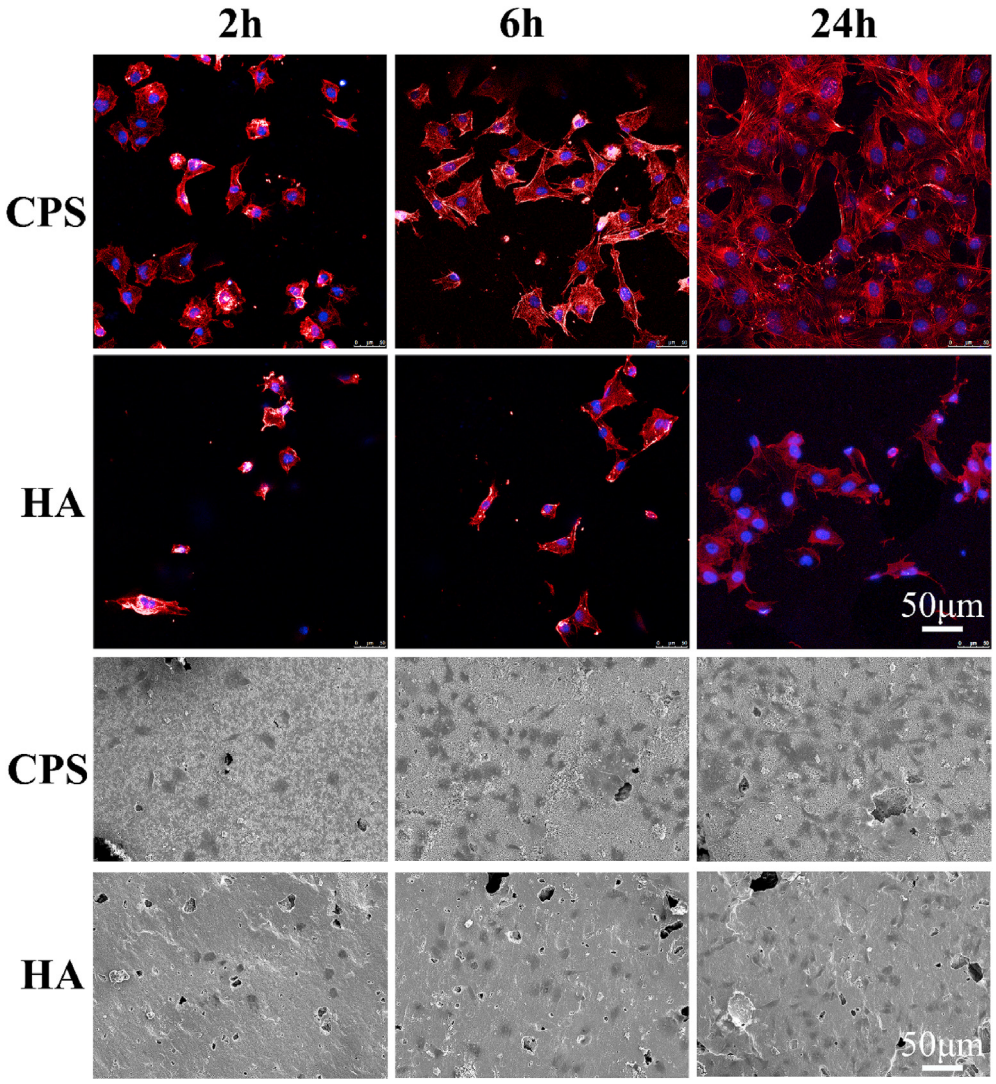

Fig. 7. Fluorescence microscope images and SEM images of MC3T3-E1 cultured on CPS and HA bioceramics for 2 h, 6 h and 24 h. Actin cytoskeleton and cell nuclei in MC3T3-E1 c were visualized by immunofluorescent staining of actin filaments (red) and nucleus (blue), respectively.

cells [38]. The enhancement adsorption of Fn on poly (NaSS) grafted Ti6Al4V also have stimulated the attachment of MC3T3-E1 osteoblastic cells [39]. Furthermore, the better adhesion and proliferation results of porous HAp treated with Fn and fetal calf serum than fetal calf serum alone have indicated the outstanding effect of Fn [40]. The *in vivo* results also have shown that the immobilization of Fn on HAp can promote the development of cell pseudopodia. In addition to promoting adhesion and proliferation of MC3T3-E1 cells on HAp, Kawashita et al. claimed that Fn adsorption might have an effect on inflammatory response to HAp [41]. In this work, both the proteomics analysis and Western-blot result proved that the CPS sample adsorbed significantly more FN1 than HA. The adsorbed FN1 may participate in activating focal adhesion pathway, ECM-receptor interaction pathway and regulation of actin cytoskeleton. The above three pathways are related to cell adhesion and motility, which is a strong evidence indicating the better cell adhesion property of CPS.

Multifunctional growth factor IGF1 is one of the most abundant growth factors in bone matrix. The absence of IGF1 of mice has shown a significant decrease of bone formation and mineralization [42]. The *in vivo* study has found that the total amount and local expression level of IGF1 in bone tissue obviously reduced with the increase of age [43,44]. In another work, the overexpression of IGF1 in aging bmMSCs has accelerated the appearance of cell clusters at the area of bone defect, and thus promoted the expression of osteogenic related genes and enhanced mineralization process [45]. Nandi et al. revealed that IGF1-loaded marine coral hydroxyapatite could stimulate early stage bone regeneration [46]. Besides, the effect of IGF1 on cartilage formation has been studied. The results have shown that IGF1 can monitor the phenotype and morphology of chondrocyte [47]. Longobardi et al. have found that IGF1 promote mesenchymal stem cell (MSC) cartilage formation through accelerating the expression of cartilage-related markers [48]. Scaffolds loading nanoparticles with IGF1 have presented favorable capacity to induce the regeneration of cartilage defect [49]. The investigation of *in vivo* implantation of IGF1-coupled collagen membrane onto full-thickness articular cartilage defects have proven that cartilage repair and bone repair can be achieved by different dose of IGF1 [50]. All results have implied that IGF1 plays an important role in bone and cartilage formation, and also is vital for differentiation and metabolism [51–53]. CPS bioceramic individually adsorbed IGF1 in this work, in addition to favorable effects on bone and cartilage formation, IGF1 could bind cell surface IGF1 receptor to activate focal adhesion pathway and rap1 signaling pathway and thus promote cell adhesion.

THBS4 is an extracellular Ca-dependent cell-adhesive glycoprotein that regulate cell-to-matrix interactions including cell migration and adhesion [45,54]. Both collagen type II (COL2A1) and chondroadherin (CHAD) are cartilage-related proteins. CHAD is a cartilage matrix

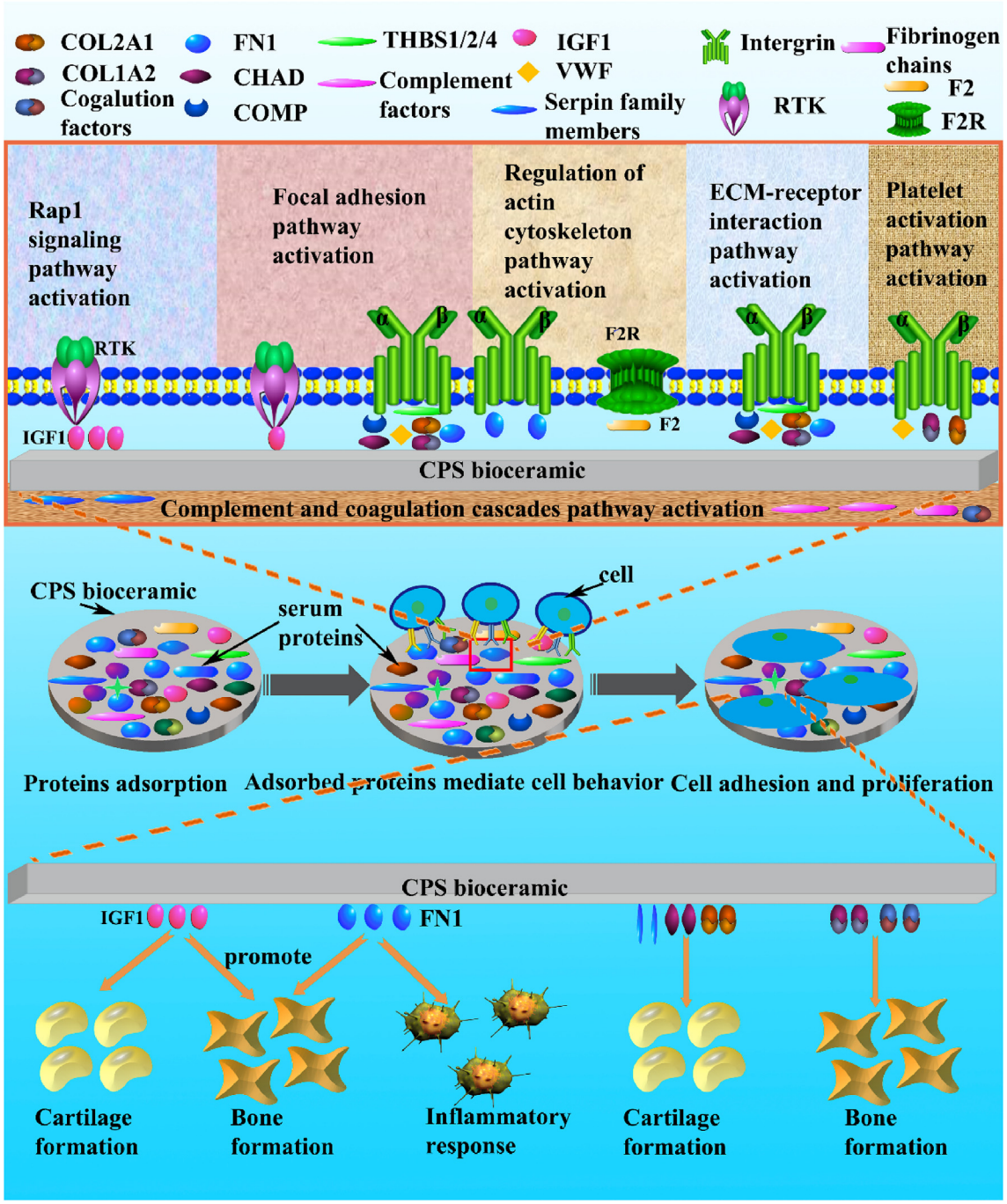

Fig. 8. Schematic diagram illustrating the mediation of adsorbed proteins on adhesion and immunological process (top), and subsequent cellular interaction and signaling (bottom) on the CPS bioceramic.

protein which has been shown to regulate attachment of isolated chondrocytes [55,56]. Meanwhile, COL2A1 is a vital cartilage-specific extracellular matrix protein which plays an important role in chondrogenesis [57]. Reticular fiber structure formed by collagen type II is essential to maintain the integrity of cartilage [58,59]. The results have found that collagen type II can stimulate the adhesion, growth and redifferentiation of chondrocytes [60–62]. Guo et al. have found that the addition of CPS into PLLA scaffolds could improve the construction of new cartilage and bone in tendon to bone integration [63]. The higher adsorption amount of IGF1, CHAD and COL2A1 proteins may explain the good ability of PLLA/CPS composites on new cartilage formation.

Type I collagen protein exists in most connective tissues, especially, it is the main component of extracellular matrix in bone tissue [64]. Several studies have shown that Col I can promote the proliferation of BMSCs, and also can induce its osteogenic differentiation. BMSCs cultured in type I collagen matrix have found high ALP activity (alkaline phosphatase) and collagen synthesis ability, osteoblast-specific protein including osteocalcin (OCN), osteosialin (BSP) and osteopontin (OPN) have also been detected [37,65–67].

Ten proteins (FN1, COMP, CHAD, COL1A1, COL1A2, COL2A1, THBS1, THBS4, SPP1 and VWF) on the HA bioceramic took part in activating focal adhesion pathway through ECM-receptor interaction, and also activating PI3K-Akt signaling pathway. while 11 proteins (FN1, COMP, CHAD, COL1A1, COL1A2, COL2A1, THBS1, THBS4, THBS2, VWF and IGF1) on the CPS bioceramic participated in

activating focal adhesion pathway via ECM-receptor interaction and Cytokine-cytokine receptor interaction. Compared with HA, the CPS bioceramic obviously increased the adsorption of FN1, CHAD, COL1A1, COL1A2, COL2A1, THBS4, VWF and IGF1, but decreased in THBS1 and SPP1 adsorption. It can be apparently inferred that the effect of 11 proteins of focal adhesion pathway would be significantly greater on CPS than HA bioceramic.

F2 and FN1 adsorbed on CPS and HA bioceramics could participated in activating regulation of actin cytoskeleton pathway by combining with F2 receptor and integrin, respectively [68]. The LC-MS/MS and Western-blot results verified that the content of FN1 adsorbed on CPS sample was much higher than that on HA. Thus, it can be deduced that the influence of 2 proteins on regulation of actin cytoskeleton pathway would be greater on CPS than HA bioceramic. Moreover, rap1 signaling pathway that can regulate cell adhesion and motility could be activated by the adsorbed IGF1 on the CPS bioceramic, while no adsorbed proteins on HA bioceramic participated in activating the pathway (Fig. 6a). Moreover, the content of IGF1 adsorbed on CPS was also much higher than that on HA bioceramic, indicating a better cell adhesion ability on CPS bioceramic.

Besides, platelet activation pathway could be activated through ECM-receptor interaction by COL1A1, COL1A2, COL2A1 and VWF proteins, and COL1A1, COL1A2, COL2A1 and VWF proteins were more adsorbed on CPS bioceramic than HA bioceramic. These proteins have a direct role in the activation of platelet activation pathway, and the activation of platelets would release growth factors to mediate bone formation [69]. The activation of platelet would also activate complement and coagulation cascade pathway. In parallel, complement cascade could be initiated by the classical and alternative pathways while coagulation cascade could be initiated by the intrinsic pathway [70]. The activation of complement cascade will result in the opsonization of pathogens, the recruitment of inflammatory and immunocompetent cells, and the direct killing of pathogens, and the activation of coagulation pathway will result in the formation of thrombin [71,72]. Within the 37 proteins detected of complement and coagulation cascades pathway, only the amount of alpha-2-macroglobulin (A2MG), coagulation factor IX(F9), coagulation factor X(F10), complement C3(CO3), complement component 4 binding protein alpha(C4BPA), fibrinogen beta chain(FGB) proteins adsorbed on CPS showed a little lower than those on HA, while the amount of other 31 proteins on CPS were much higher than those on HA, and the amount of F11, F12, CFD, CFH, SERPINA5, KLKB1, CPB2 and FGG were over 10-fold higher on CPS surface as shown in Fig. 5b. F11, F12, SERPINA5, KLKB1, CPB2 and FGG proteins play a role in the coagulation pathway. CFD and CFH, also known as complement factor, have an essential role in the regulation of complement activation [73]. It can be apparently inferred that the effect of 31 proteins of complement and coagulation cascades pathway would be significantly greater for CPS case.

More amount and types of proteins related to cell adhesion and immunological response adsorbed on CPS bioceramic, which indicated CPS bioceramic may possess better osteogenic and anti-inflammatory abilities. Cell adhesion experiments on HA and CPS bioceramics as shown in Fig. 8 verified that more cells achieved attachment on CPS bioceramic at the same culture time, proving that adsorbed proteins indeed have significantly positive effect on cell adhesion.

A schematic diagram is drawn to illustrate the mediation of adsorbed proteins on adhesion and subsequent cell behavior of cells over CPS and HA bioceramics, as shown in Fig. 8. The activated focal adhesion, ECM-receptor reaction, regulation of actin cytoskeleton and rap1 signaling pathways on CPS bioceramic will result in focal adhesion and regulate the actin cytoskeleton structure of subsequently attached cells, which is crucial for the 1st step during implant-protein-cell interactions. The activation of complement and coagulation cascades pathway and platelet activation pathway could promote blood coagulation and create a biocompatible microenvironment. In addition, the adsorbed proteins including FN1, IGF1, THBS4, COL1 and COL2 will have good effects on subsequent cell behavior and then promote bone or cartilage regeneration.

Many factors, such as chemical composition, surface properties and surrounding solvent system have impact on protein adsorption [74,75]. In this work, CPS and HA porous bioceramics with same porosity were used to minimize the effect of surface area. And the final pH value of FBS protein solution was changed due to ion exchanges between the material and surrounding solution. The pH difference between CPS and HA groups was caused by the deference in chemical composition, indicating the silicon component in CPS bioceramic should be responsible for its superior protein adsorption behavior and the subsequent cell behavior.

5. Conclusions

We characterized the protein adsorption profile of CPS bioceramic using the proteomics technique in this study. Our results revealed that CPS could adsorb more types and higher amount of proteins than HA. The numbers of protein types on CPS and HA bioceramics were 368 and 298, respectively.

The adsorbed proteins on CPS mediated osteoblast cell attachment mainly through focal adhesion pathway, ECM-receptor interaction pathway, regulation of actin cytoskeleton pathway and rap1 signaling pathway. Among these pathways, FN1, CHAD, COL1A1, COL1A2, COL2A1, THBS4, VWF and IGF1 presented elevated adsorption on CPS bioceramic than HA bioceramic. The elevated adsorption of immunological related proteins may also play an important role in the regulation of bone regeneration. The Western-blot results were consistent with proteomics analyses, the adsorption amount of FN1 and IGF1 were significantly higher than that of HA group. Osteoblast cells also exhibited much better spreading and growth on CPS bioceramic than on HA bioceramic. In addition to the effect on cell adhesion, the elevated adsorption of FN1, IGF1 and COL1A2 on CPS bioceramic than HA group may provide a reliable explanation of its outstanding ability on new bone formation, and the excellent adsorption capacity of IGF1, CHAD, COL2A1 and THBS4 on CPS bioceramic may provide a theoretical basis of its favorable ability on cartilage formation. The outstanding protein adsorption ability and cell adhesion capacity of CPS bioceramic indicated that CPS could induce more cells and then accelerate bone regeneration process after implantation.

CRediT authorship contribution statement

Fanyan Deng: Methodology, Investigation, Data curation, Formal analysis, Validation, Writing - original draft. **Wanyin Zhai:** Methodology, Investigation. **Yue Yin:** Formal analysis, Resources. **Chao Peng:** Formal analysis, Resources. **Congqin Ning:** Conceptualization, Resources, Funding acquisition, Supervision, Project administration, Writing - review & editing.

Declaration of competing interest

We declare that we do not have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by National Key Research and Development Program of China (Grant No. 2017YFC1103800), International Partnership Program of Chinese Academy of Sciences (Grant No. GJHZ1760) and National Natural Science Foundation of China (Grant No. 51672304). We thank Drs. Jing Chen and Haining Zhu at the University of Kentucky for productive discussion and technical suggestions. The SDS-PAGE and Western-blot experiments were

performed at Shanghai Daixuan Biotechnology Co., Ltd. The proteomics analysis was performed at National Center for Protein Sciences (Shanghai, China).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2020.08.011.

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