Effect of Particle Size of Various Inorganic Milled Particles on Protein Adsorption Behavior

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Abstract: The size of inorganic particles used as adsorbents and drug carriers affects protein adsorption behavior. In this study, the protein adsorption behaviors of various inorganic milled particles, including activated bamboo charcoal (ABC), silica (SiO_2), and hydroxyapatite (HAp), were investigated. Various inorganic particles were milled for different periods to reduce the particle size and enhance protein adsorption. A bicinchoninic acid test (BCA) kit was employed to assess the adsorption and desorption behaviors of bovine serum albumin (BSA) on inorganic particles. The increased amount of BSA adsorbed on the inorganic particles was correlated with the smaller sizes of the inorganic particles. Specifically, the maximum BSA adsorption capacity reached 10.18 mg/g for HAp, 4.47 mg/g for ABC, and 1.92 mg/g for SiO_2. Owing to the electrostatic interaction between the negatively charged carboxylate (COO^-) groups of BSA and Ca^{2+} ions on the surface of HAp, which is essential for the adsorption of acidic BSA molecules, HAp showed a higher ability to bind BSA. Desorption studies in phosphate buffer solution (PBS) showed a gradual release of protein from all inorganic-protein complexes; however, the cumulative release from HAp–BSA was markedly lower (13.14 ± 0.36 %) than that from ABC–BSA (22.69 ± 3.53 %%) or SiO_2–BSA (50.80 ± 4.70 %), indicating stronger immobilization on HAp. These findings demonstrate that both particle size and physicochemical surface properties significantly influence the adsorption and release behavior of acidic proteins.

Keywords: Adsorbent; Bovine serum albumin; Electrostatic interaction; Inorganic particles; Particle size

1. Introduction

Recent progress in the application of inorganic particles in various fields has attracted considerable scientific interest, particularly in biomedical applications. Owing to their unique physicochemical properties and biocompatibility, inorganic particles are frequently employed as diagnostic agents and for drug delivery[1-5]. When employed *in vivo*, inorganic particles are exposed to various biomolecules, such as proteins, lipids, and nucleic acids in the physiological environment[6-9]. Therefore, it is necessary to quantitatively estimate the interaction between biomolecules (such as proteins) and inorganic particles to select and evaluate materials for drug administration or cell interaction assessment. Improving the physicochemical properties of inorganic particles allows them to exert control over their interactions with proteins, making them suitable for biomedical applications[10-13].

Protein adsorption on inorganic particle surfaces is primarily driven by van der Waals, electrostatic, hydrogen-bonding, and hydrophobic interactions[14-17]. For instance, carbon materials have extensive biomedical applications owing to the surface properties of carbon compounds, and their ability to disperse individually in biological fluids is crucial for biological applications[18-22]. The diameter size of carbon materials' capability to infiltrate cells and tissues shows great potential for drug delivery and other biomedical applications[22]. Globular proteins, such as bovine serum albumin (BSA), have a larger molecular size (~66 kDa) and multiple binding sites, making them particularly suitable for protein adsorption studies by using various inorganic particles with different surface charges. Despite BSA's structural advantages, BSA has been used in drug delivery and enzyme formulations due to its high stability, biocompatibility, and well-characterized binding behavior[23-24]. Furthermore, BSA demonstrate a significant

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affinity for both hydrophobic and hydrophilic interfaces with inorganic particles, such as silica, attributable to the localized hydrophobic and hydrophilic characteristics of their surfaces[25-26]. The dominance of ionic or hydrophilic/hydrophobic interactions depends on molecular properties[26]. At physiological pH, silica surfaces have a net negative charge. Electrostatic attraction significantly influences the adsorption of positively charged protein molecules on a solid surface[27].

In addition, immobilizing bisphosphonates[28] and pyrophosphate ions[29] onto the surface of hydroxyapatite (HAp) particle's significantly increases protein absorption. Previous studies have reported the use of various shapes of HAp[30] and amino acid-functionalized HAp[31] to enhance protein adsorption and have shown a lower kinetic release of proteins. According to their findings, the shapes and polarity of the HAp particles influenced the attachment of proteins to the surfaces of different shapes of HAp and amino-acid-functionalized HAp. Therefore, a better understanding of the interactions among inorganic particles with varying physicochemical properties and interactions with proteins is necessary to enhance drug carrier design and enable precise targeting of specific protein sites in a natural environment. The particle sizes of the adsorbents and drug carriers affect the protein adsorption behavior. Rouhi et al. reported that HAp nanopowder with a particle size of 100 nm significantly adsorbs more proteins than 1 μm because smaller nanoparticles have higher specific surface areas than micro-scale HAp particles[32]. In this study, we used dry-milling treatment to reduce the particle size and enhance the protein adsorption properties of inorganic particles. Dry-milling is a simple and environmentally benign technology for reducing bulk materials to micro- or nanoscales.

Hence, the size and surface characteristics of inorganic particles can be deliberately manipulated to influence protein adsorption and release under physiological conditions. Previous studies have primarily examined protein adsorption on single inorganic particles or the effects of HAp particle size. However, there is a limitation to comparing protein adsorption behavior among inorganic particles with markedly different surface charges, specifically HAp, activated bamboo charcoal (ABC), and silica, regardless of whether their particle size was reduced through dry milling. This study addresses this gap by using particle size reduction as a controlled variable to evaluate its effect on protein adsorption, with BSA as a model globular protein. Our study reveals changes in the microstructure of a material in response to alterations in its surface chemistry. This provides a better understanding of how materials and proteins interact, which is crucial for biomedical applications.

2. Materials and Methods

2.1. Materials

Unless otherwise stated, all chemicals were of reagent grade and obtained from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan). Bamboo charcoal (BC) powder was obtained from Taketora, Japan, and a bicinchoninic acid assay (BCA) kit was provided by Takara, Japan.

2.2. Preparation of inorganic particles

The silica particles were used without chemical treatment. The preparation of ABC was followed by the method of Banat et al.[33], in which a brief amount of BC with an impregnation weight ratio of BC to 30 wt% KOH solutions 1:3 at 130 ºC for 15 h and the residue was washed with hot Milli-Q water several times to adjust the pH to neutral and dried the sample at 120 ºC overnight. The powder was activated at 800 ºC for 1 h to obtain a 26.366±4.439% (N=15) of the ABC powder.

HAp synthesis was performed using the wet precipitation method, as previously described[34-35]. An aqueous solution of $Ca(NO_3)_2{\cdot}4H_2O$ (0.42 M, 800 mL) at pH 10 using a 28% NH_3 solution in a 1 L Erlenmeyer flask with a magnetic stirrer at room temperature (RT). Subsequently to the reaction mixture, a 1 M aqueous solution of $(NH_4)_2HPO_4$ (200 mL) was incrementally introduced into the flask and stirred for 24 h at RT. HAp dispersions were subjected to centrifugal washes several times until neutral pH. Next, the sample was dried at 135 ºC for overnight. Subsequently, the sample was pulverized to obtain a fine powder. The prepared powders were calcined at 800 ºC for 1 h at a heating rate of 10 ºC/min. This process produced HAp powder with a yield of approximately 77±1% (n=14).

2.3. Dry-ball-milling of inorganic particles

The inorganic particles were milled at RT in an 80-ml pot of zirconia pot with 5 mm zirconia balls in a planetary laboratory mill (FRITSCH-Pulverisette 6, Germany) at 300 rpm for 1, 15, 30, and 45 min. Seven grams of inorganic particles were employed for all the experiments, with a balls/inorganic particle weight ratio of 5:1. The milled powder was collected for analysis of physicochemical characteristics and protein adsorption behavior.

2.4. Physico-chemical properties of inorganic milled particles

The microstructure of the particles was observed using scanning electron microscopy (SEM, S4800, Hitachi High-Tech Corp., Tokyo, Japan), and the average particle size was determined from the SEM images using ImageJ software. X-ray diffraction (XRD, Miniflex 600, Rigaku Corp., Japan) with Cu-K radiation (λ= 1.54060 Å) at a scan rate of 10°/min and 2θ range 2-70° at RT was used to analyze the inorganic crystal structure. The particles'

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specific surface area (SSA) was determined by the single-point Brunauer-Emmett-Teller (BET, Macsorb HM model-1208, Mountech Co., Ltd., Tokyo, Japan) method using N_2 adsorption. Fourier-transform infrared (FT-IR) spectra were acquired using an IRAffinity-1S system (Shimadzu Corp., Kyoto, Japan) employing the KBr pellet method (4 cm^{-1}, 32 scans at RT).

2.5. Protein adsorption on inorganic milled particles

The adsorption behavior of BSA on each particle was evaluated at RT by measuring the solute concentration in the powder/solute dispersion supernatant. Briefly, 200 mg of inorganic milled particles were immersed in 2 mL of BSA solution for different periods. The amount of BSA adsorbed by the supernatant was measured for isotherm and kinetic adsorptions using the BCA protein assay kit (λ= 562 nm). The precipitate in the centrifuged solution was filtered, washed extensively with distilled water, and dried for several days at RT. Subsequently, FT-IR analysis was conducted to analyze protein adsorption on the inorganic particles.

2.6. Release kinetics of BSA from inorganic milled particles adsorbed BSA

The kinetic release of BSA adsorbed on inorganic particles (200 mg) was assessed by dispersing the particles in 2 mL phosphate buffer solution (PBS) at pH 7 for 24 h. BSA release was quantified using a BCA assay.

2.7. Statistical analysis

GraphPad Prism (version 10.0; GraphPad Software, CA, USA) was used for statistical analyses, including one way ANOVA and Tukey-Kramer tests. The alpha level was set to 0.05.

3. Results and Discussion

Figure 1 shows SEM and histogram images of the inorganic particles after ball milling for different periods. The milling process for each particle decreased the particle size and showed a microstructure in each particle's irregular shape after milling for different periods. Ball milling can effectively reduce the particle size of inorganic particles due to the reduced surface morphology of the

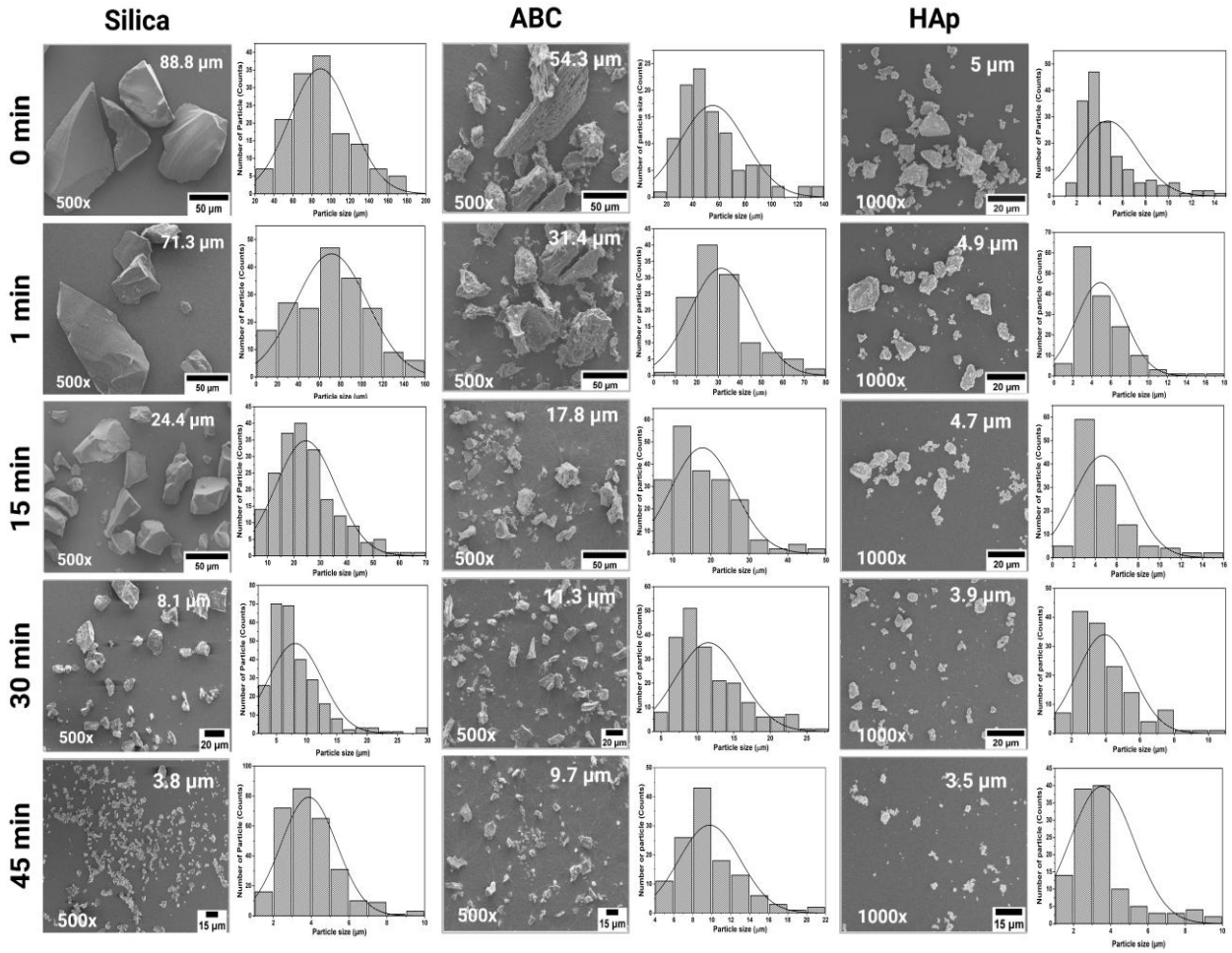

Fig. 1: SEM and histogram images of inorganic particles after ball milling for different periods

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inorganic particles during the milling process.

The decrease in the size of the inorganic particles after milling for different periods showed no significant difference in the crystal structure of the inorganic particles based on the X-ray diffraction peaks (Figure 2a-c). The smaller sized inorganic particles adsorbed more BSA (Figure 3a). The BSA adsorption on silica milled only increased after 45 min of milling. In contrast, protein adsorption for ABC increased in each milling treatment period. Moreover, BSA adsorption mostly increased on HAp milled for 30 and 45 min. Smaller inorganic particle sizes have a higher saturation surface concentration and a larger number of active site densities, and, consequently, more adsorption sites to adsorb more BSA[36].

The Langmuir and Freundlich models (Figure 3b) explain the adsorption equilibrium behavior of BSA in aqueous solutions onto inorganic particles. We employed pseudo-first- and second-order kinetic models (Figure 3c) to assess the adsorption dynamics, and they demonstrated strong consistency with the adsorption process of BSA in aqueous solution onto inorganic particles. The SSA of ABC (488.5 m2/g) was higher than those of HAp (15.7 m2/g) and silica (1.8 m2/g) after ball-milling for 45 min. However, the amounts of BSA adsorbed onto HAp at 45 min were larger than those adsorbed onto ABC and silica (Figure 3b-c). Specifically, the maximum BSA adsorption capacity reached 10.18 mg g^{-1} for HAp, 4.47 mg g^{-1} for ABC, and 1.92 mg g^{-1} for SiO_2.

The adsorption of BSA on the inorganic particles was confirmed by FT-IR spectroscopy. The characteristic of BSA structure, specifically amide I (1650 cm-1) and amide II (1540 cm-1) bands in Figure 4, were identified, along with changes in surface functional group vibrations after adsorption. Proteins are adsorbed onto inorganic particles via electrostatic interactions between the amino groups of the protein and negatively charged sites on the inorganic crystals.

The electrostatic interaction of the inorganic particle surfaces with BSA played an essential role in this study. HAp exhibited greater BSA adsorption than silica and ABC. This is because HAp particles have two different charge planes on their surfaces: C-sites (Ca2+, positively charged) and P-sites (PO43-, negatively charged). (Figure 4c & 5c). These results indicate that the electrostatic interaction of -COO- groups with Ca2+ ions on HAp surfaces is dominant for the adsorption of acidic BSA molecules.

While FTIR analysis in this study provides conclusive evidence of BSA adsorption on the inorganic particles, future studies incorporating zeta potential measurements will be essential to assess surface charge modifications upon protein binding quantitatively and to distinguish electrostatic contributions from other interaction forces more definitively.

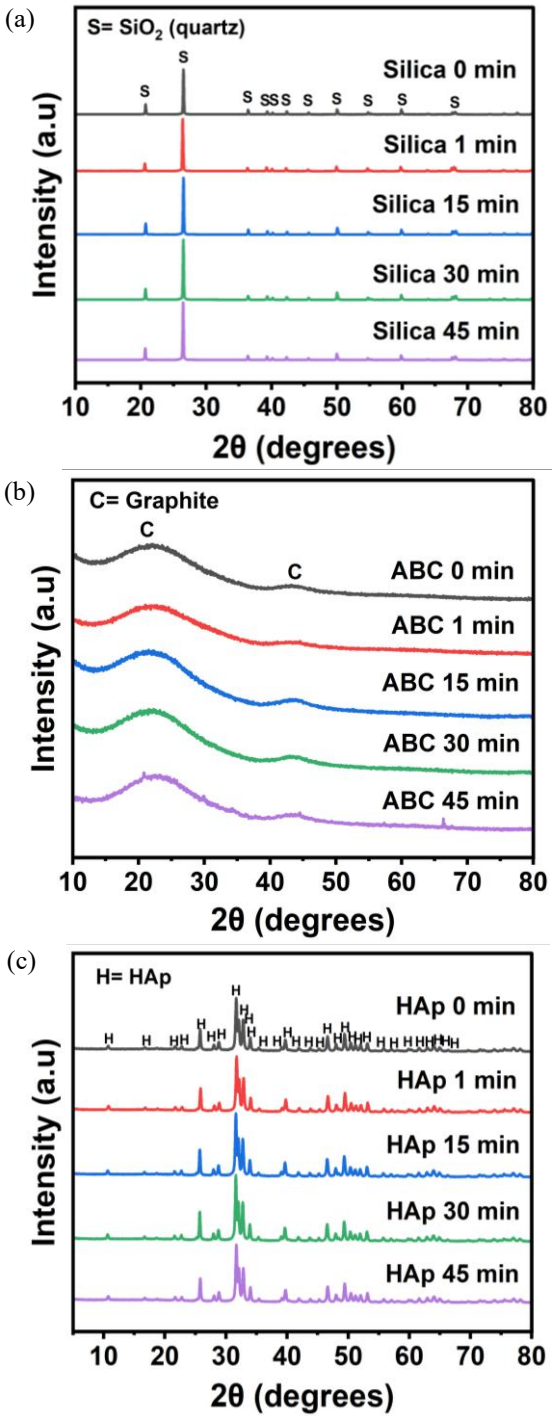

Fig. 2: XRD patterns of inorganic particles after ball-milling for different periods: (a) silica, (b) ABC, and (c) Hap

The adsorption capacity of BSA on ABC, as illustrated in Figures 4b and 5b, is determined by the internal pore structure, presence of functional groups on the pore surfaces, and electrical charge of the materials, which may lead to either attraction or repulsion[37]. In addition, the mechanism of protein adsorption onto a silica surface (Figure 4a & 5a) is a frequently observed phenomenon that is primarily controlled by the electrostatic attraction

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between the negatively charged silica surface and the positively charged biomolecule[38-40]. The above results indicate that the particle size and physicochemical characteristics of the adsorbents greatly affected the adsorption of acidic protein adsorbates.

Figure 6 illustrates the BSA release profiles plotted as a function of time for 24 h. HAp-BSA had the lowest cumulative percentage of BSA release (13.148%) compared to Silica-BSA (50.80%) and ABC-BSA (22.69%). The immobilization of HAp-BSA demonstrated a more significant interaction between the C-site of HAp and acidic BSA molecules compared to Silica-BSA or ABC-BSA. The positive charges of the biomolecules interact with the negatively charged silica surface, whereas systems with BSA in ABC-BSA interact without covalent bonds.

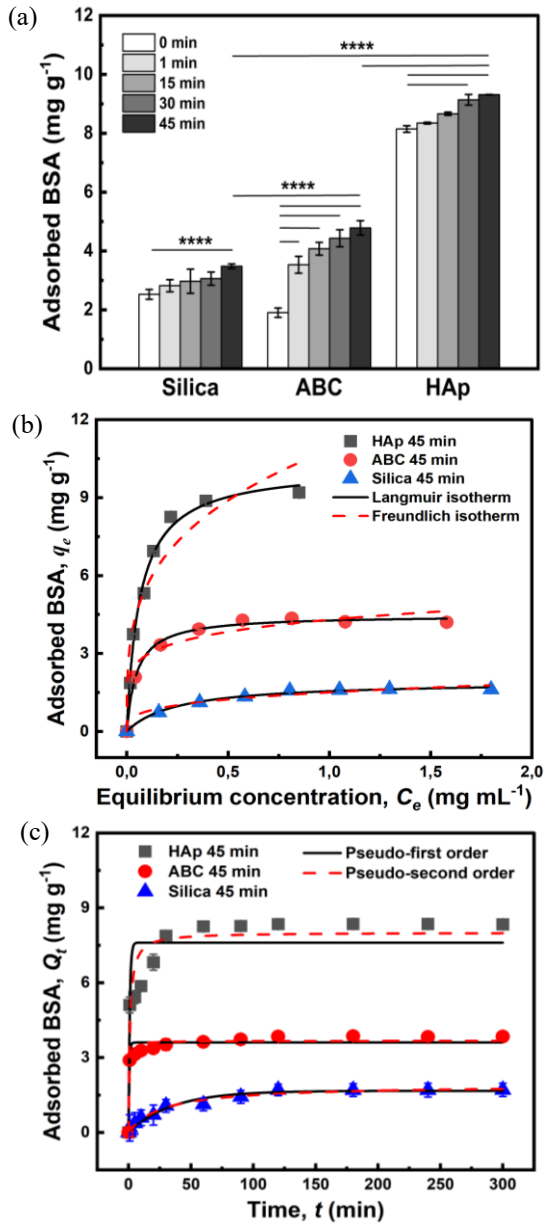

Fig. 3: (a) The adsorbed amounts of BSA onto inorganic particles after ball-milling for different periods (0-45 min) (b) Adsorption isotherms and (c) adsorption kinetics of BSA onto inorganic particles after ball milling for 45 min. Statistical analyses were performed with one-way ANOVA and Tukey-Kramer tests between inorganic particle before milling treatment and after milling treatment at different periods.; n= 4; ****p<0.0001.

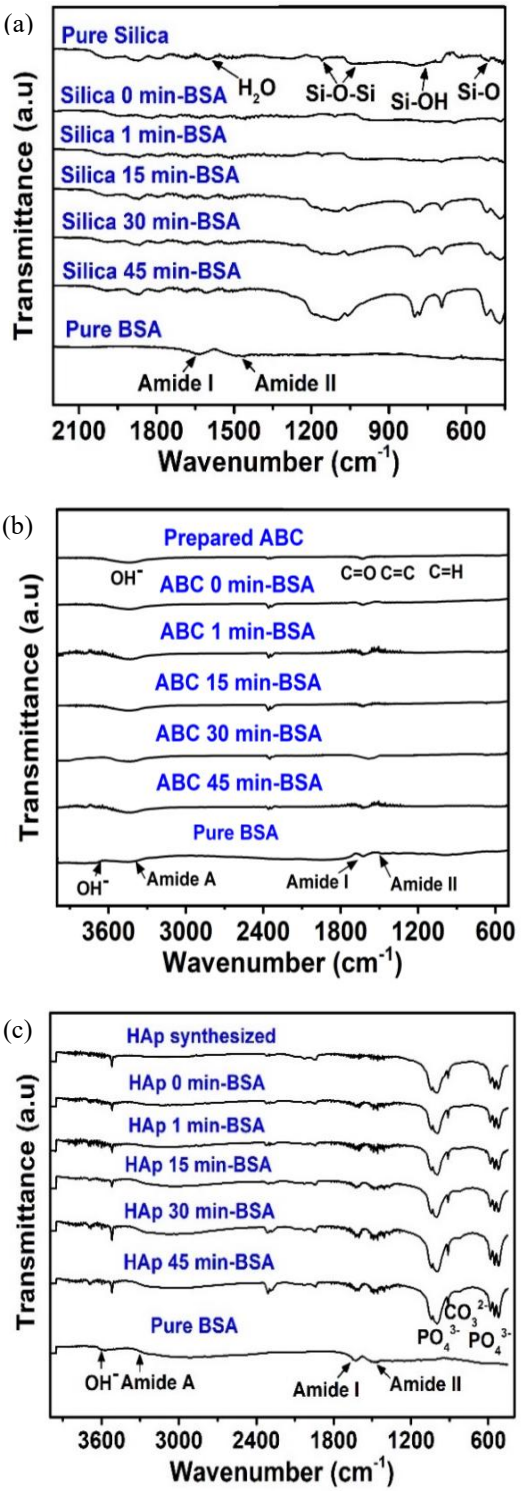

Fig. 4: FT-IR spectra of inorganic particles adsorbed BSA; a) Silica-BSA, b) ABC-BSA, and c) HAp-BSA.

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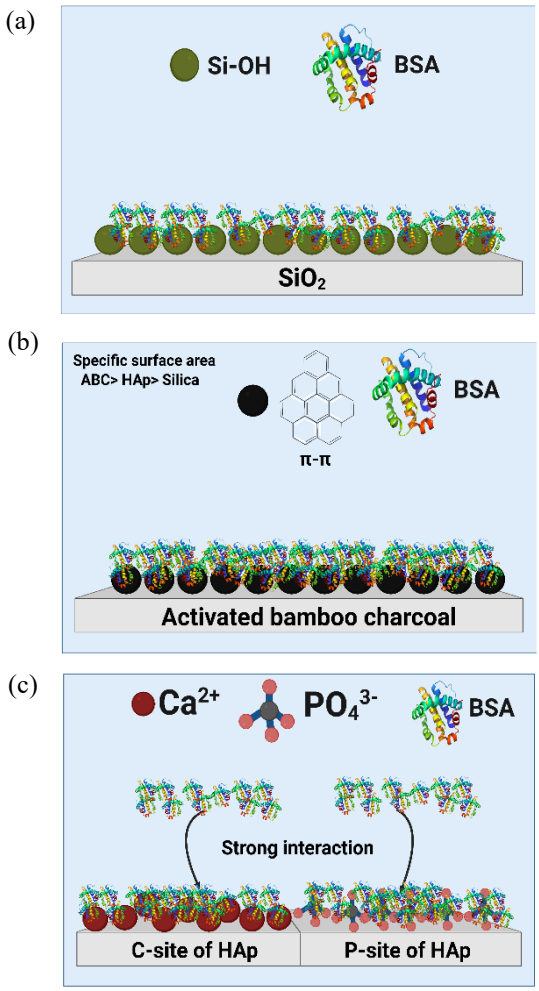

Fig. 5: BSA adsorption mechanism on (a) silica-BSA, (b) ABC-BSA, and (c) HAp-BSA.

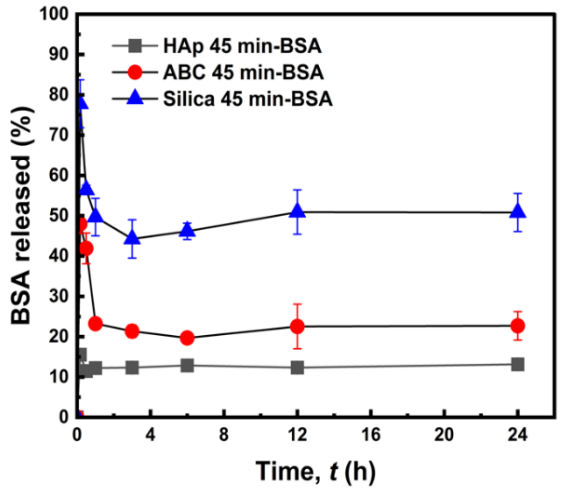

Fig. 6: Release kinetics of protein from inorganic-milled-45 min adsorbed protein (n= 3) for 24 h in PBS (pH 7.0).

4. Conclusions

The ball-milling treatment significantly decreased the particle size and modified the microstructure of the inorganic particles, resulting in enhanced adsorption of BSA. The Langmuir and Freundlich models effectively described the adsorption equilibrium behavior, whereas the pseudo-first-order and pseudo-second-order kinetic models aligned with the adsorption dynamics. The physicochemical characteristics of the inorganic particles significantly affect the adsorption of BSA. HAp exhibits strong electrostatic interactions with BSA molecules because HAp particles possess two different charge planes, C-sites (Ca^{2+}, positively charged) and P-sites (PO_4^{3-}, negatively charged), on their surface. These results highlight the significance of particle size and surface characteristics in influencing the adsorption capacity of inorganic particles for acidic protein adsorbates.

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