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Pei-Fen Yang[a]; Meng-Csu Chin[a]; Cheng-Kang Lee[a]
[a] Department of Chemical Engineering, National Taiwan University of Science and Technology, Taipei, Taiwan

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Interaction of Hyaluronic Acid (HA) with Organosilicon (Si-QAC) Modified Magnetite for HA Recovery

Pei-Fen Yang, Meng-Csu Chin, and Cheng-Kang Lee
Department of Chemical Engineering, National Taiwan University of
Science and Technology, Taipei, Taiwan

Abstract: Cetylpyridinium chloride (CPC) is commonly used to precipitate hyaluronic acid (HA) from crude extract in a HA purification process. 3-(Trimethoxysilyl)-propyldimethyl octadecyl ammonium chloride (Si-QAC) has a structure very similar to CPC when it is bonded to surfaces through its silane base. By taking advantage of its easy surface bonding property, Si-QAC was bonded to the silica-coated magnetite to facilitate HA recovery. The effects of pH, salt concentration, and the temperature on HA adsorption to Si-QAC-magnetite were studied. Not only the positively charged nitrogen but also hydrophobic long tail of Si-QAC contributed to the strong interaction between HA and Si-QAC-magnetite. The maximum HA adsorption capacity was 38 mg/g-dry magnetite and the equilibrium dissociation constant was 0.016 g/l at pH 4 under 37°C. The adsorbed HA could be effectively desorbed by 1 N NaCl supplemented with 0.1% Triton X100.

Keywords: Hyaluronic acid, hydrophobic interaction, quaternary ammonium chloride, Si-QAC, magnetite

INTRODUCTION

Hyaluronan (HA) is a naturally occurring high molecular weight, nonsulfated glycosaminoglycan that has important structural and biological roles in animal tissue. It is composed of 2,000–25,000 repeating disaccharide subunits of

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Address correspondence to Cheng-Kang Lee, Department of Chemical Engineering, National Taiwan University of Science and Technology, 43 Keelung Rd. Sec. 4, Taipei, Taiwan 106. Fax: 886-2-27376644; E-mail: cklee@ch.ntust.edu.tw

β-(1–4)-D-glucuronic acid and β-(1–3)-N-acetyl-D-glucosamine. It has high capacities for lubrication, water sorption and water retention, which influences several cellular functions, such as migration, adhesion and proliferation (1, 2). Recent biomedical applications of HA have included ophthalmic surgery, arthritis treatment, scaffolds for wound healing, tissue engineering, and the use as a component in implant materials (3–5). It is also used in eye drops, cosmetics, and food products (6). Commercially, HA is produced through extraction from rooster combs or via microbial fermentation. In a typical HA purification process, precipitation of HA with quaternary ammonium chlorides (QAC) is a most commonly employed primary recovery method (7–9). QAC are cationic surface-active agents with antimicrobial activity (10, 11). Cetylpyridinium chloride (CPC), an antiseptic agent used alone or in combination with other drugs for oral and throat care, is one of the most often used QAC for precipitating HA. To isolate the HA-CPC precipitate and remove the CPC from the HA required several separation steps. If the CPC could be covalently coupled to a solid support, the HA purification through forming complex with immobilized the CPC would be much easier than with free CPC. However, CPC structure as shown in Fig. 1, has no functional group available for easy activation and coupling to a solid surface.

The quaternary amine-containing organosilicon salt, 3-(trimethoxysilyl)-propyldimethyloctadecyl ammounium chloride (Si-QAC), as shown in Fig. 1, can be easily bonded to surfaces through its silane base. It exhibits its antimicrobial activity against a broad range of microorganisms (12) and antiviral activity (13) while chemically bonded to a variety of surfaces. Si-QAC has been used as a bactericidal agent in many consumer products (14). The major mode of action of surface-bonded organosilicon QAC was identified as the positively charged nitrogen that attracts the negatively charged microorganisms which comes to contact with the long molecular chain which pierces the cell membrane and leads to cytolytic damage. In addition to the

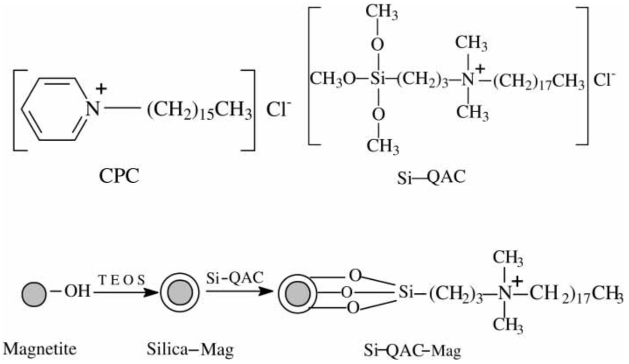

Figure 1. Chemical structure of CPC and Si-QAC; and the scheme of Si-QAC-magnetite preparation.

positively charged nitrogen, Si-QAC has 18 carbons long tail very similar to the 16 carbons long molecular chain possessed by CPC. Therefore, the aim of this work is to explore the feasibility of using the surface bonded Si-QAC for HA isolation. To recover the QAC precipitated HA, the operation of filtration or centrifugation is required. Compared with filtration or centrifugation, magnetic separation is particularly attractive because lower energy is required and a strong magnetic force can be easily generated. By taking advantage of its easily retrievable property, in this work, magnetite prepared by alkaline precipitation and subsequently surface modified with Si-QAC was employed for studying its interactions with HA. The acidified or denatured bovine serum albumin (BSA) has been employed to form a precipitate with the undigested HA in the hyaluronidase activity assay (15). The complex formation between BSA and HA has also been characterized (16). Therefore, in this work, BSA was also immobilized onto the magnetite surface to study its interactions with HA in comparison with that of Si-QAC. The factors such as temperature, pH, and salt concentrations affecting the adsorption and desorption of HA were investigated.

MATERIAL AND METHODS

Materials

3-(Trimethoxysilyl)-propyldimethyloctadecyl ammounium chloride (Si-QAC) also known as AEGIS Antimicrobial Shield was obtained from Widtex Co (local distributor of AEGIS Environments). Sodium hyaluronate was obtained from the Fine Chemical Division of Q. P. Co. (Tokyo, Japan). All other chemicals were purchased from either Acros or Sigma Chemical.

Preparation of Si-QAC Modified Magnetite

$FeCl_2$ tetrahydrate of 3.79 g and $FeCl_3$ hexahydrate of 10.81 g were dissolved in 360 ml deionized water under nitrogen gas with vigorous stirring at 80°C. After adding 45 ml NH_4OH of 25% (w/v) into the solution, the color of the solution turned from orange to black immediately and that indicated the formation of magnetite. The magnetite was recovered by a powerful neodymium-iron-boron magnet block (Magtech Co., Taipei, Taiwan). The magnetite precipitate was washed thoroughly with deionized water and then stored in ethanol. Before it was modified with Si-QAC, the magnetite was coated with silica by hydrolyzing tetraethylorthosilicate (TEOS) in an ethanol medium in the presence of ammonium hydroxide. Magnetite of 10 g (wet weight) was dispersed in an ethanol solution made up by 80 ml 95% ethanol and 24 ml deionized water. After homogenization in a bath type ultrasonicator (NEY Ultrasonik 104H, Yucaipa, CA) for 10 min, 28 ml NH_4OH of

25% (w/v) and 30 ml TEOS were added and mechanical stirring was applied for 12 h. After thorough washing with deionized water, the silica-coated magnetite of 15 g (wet weight) was dispersed in a 300 ml 50% (v/v) glycerol ethanol solution in a bath type ultrasonicator for 30 min. Si-QAC of 10 ml was added into the homogenized mixture to modify the silica-coated magnetite at 80°C for 3 h. The resultant Si-QAC-magnetite was thoroughly washed deionized water and ethanol, then stored in ethanol. The schematic diagram of preparing Si-QAC-magnetite is shown in Fig. 1. The size and zeta potential of the prepared magnetite were determined by a dynamic laser light scattering apparatus (Otsuka, Photal LPA-3000/3100) and a zeta potential meter (Malvern, Zetamaster), respectively.

Preparation of Bovine Serum Albumin Immobilized Magnetite

Instead of using Si-QAC, 3-aminopropyltrimethoxysilane (APTMS) was employed to modify the silica-coated magnetite following the same procedure as Si-QAC. The APTMS modified magnetite of 1 g (wet weight) was dispersed in 10 ml 2% glutaraldehyde solution prepared in pH 4, 0.1 M acetate buffer at 37°C for 1 h. Denatured BSA of 2 mg/ml prepared in acetate buffer of 1ml was mixed with 0.1 g glutaraldehyde treated magnetite for 30 min at room temperature. After washing twice with 2 ml acetate buffer, the magnetite was mixed with 1 ml 3 M enthaolamine to block the reactive aldehyde groups exposed on surface of the magnetite. The immobilized BSA magnetite was stored in pH 4, 0.1 M acetate buffer at 4°C for HA adsorption. The denatured or acidified BSA was prepared by dissolving BSA in pH 2 HCl solution and heated to 90°C for 30 min.

HA Concentration Determination

The carbazole method (17) was employed for HA determination. Briefly, a serial dilution of HA standard or sample of 150 μL was added into 900 μL of a solution of 25 mM sodium tetraborate in sulfuric acid. The mixture in the test tube was heated for 10 min at 100°C in an aluminum heating block. After cooling in an ice bath for 15 min, 30 μL of 0.125% carbazole in absolute ethanol were carefully added. After heating at 100°C for 10 min and cooling in an ice bath for 15 min, the absorbance of the reaction solution was measured at a wavelength of 525 nm. The HA concentration was determined form the established calibration curve.

HA Adsorption to Si-QAC-Magnetite

For the study of the HA adsorption time course, sample tubes each containing 1 ml of 0.5 mg/ml HA solution prepared in pH 4, 0.1 M acetate buffer were mixed with magnetite of different modifications at 37°C by shaking at 170 rpm on an orbital shaker. At different times, sample tubes were taken out to separate the magnetite in a side-pull permanent magnet racks (Chemagen Biopolymer-Technologie AG, Baesweiler, Germany). The supernatant was analyzed for the remaining HA concentration. To study the pH effect on HA adsorption, 100 mM buffers of pH 4∼7 (acetate buffer for pH 4 and 5; phosphate buffer for pH 6 and 7) were employed to prepare 0.5 mg/ml HA solutions. The HA solutions were mixed with magnetite at 37°C for 30 minutes with constant shaking before analyzing HA concentration in the supernatant. The equilibrium adsorption isotherm was obtained by measuring the remaining HA concentration in the supernatant after mixing 1 ml HA solution of concentration 0.1∼1.0 mg/ml prepared in pH 4, 0.1 M acetate buffer with 0.1 g of magnetite for 30 min. The amount of HA adsorbed by the magnetite was calculated based on the balance of initial and equilibrium HA concentration. The adsorption efficiency was defined as the ratio of the amount adsorbed HA to that of initially loaded.

HA Desorption from Si-QAC-Magnetite and Repeat Use

After the adsorption was carried out in 1 ml of 0.5 mg HA/ml pH 4, 0.1 M acetate buffer at 37°C for 30 min, the collected Si-QAC magnetite of 0.1 g was washed twice with 2 ml acetate buffer. HA was desorbed from the washed magnetite by mixing with 1 ml desorption solution under ultrasonication for 30 min. The desorption solution consisted of 1% CPC and 1 N NaCl in pH 7 0.1 M phosphate buffer. After washing twice with 2 ml pH 7 phosphate buffer, the magnetite recovered by magnetic force was used for the next cycle of HA adsorption. The desorption efficiency was defined as the ratio of the amount of HA desorbed to that adsorbed on magnetite during adsorption. For the electrophoretic desorption HA from magnetite, ElectroPrep System (CZ-02892, Cole-Palmer, USA) with two linker chamber was employed. A filter paper and a dialysis membrane were used to separate the magnetite sample chamber and the HA collecting chamber, respectively. The electrophoretic desorption buffer of pH 8.5 was consisted of 50 mM Tris-base and 50 mM acetate. Voltage of 120 V was applied across the chambers for 2 h. The HA concentration in the HA collecting chamber was used to calculate the electrophoretic desorption efficiency.

RESULTS AND DISCUSSION

HA Adsorption

Before the HA adsorption was carried out, the prepared magnetite was characterized. The average size of the magnetite was about 1 μm and modification with TEOS to prepare silica-coated magnetite slightly increased its size. However, as shown in Fig. 2 the size of the particle tripled when the silica-coated magnetite was further modified with Si-QAC. The zeta potentials of the magnetite with different modifications measured in deionized water varied significantly. The average zeta potential for magnetite, silica-coated magnetite, and Si-QAC-magnetite were −2.8 mV, −46.6 mV, and 35.4 mV, respectively. As expected, the acidic silanol groups of silica contributed to the significant negative charges carried by the silica-coated magnetite. Once modified with the positively charged Si-QAC, the silica-coated magnetite turned into positively charged. When it was measured in 0.1 M buffers (acetate for pH 4.5; phosphate for pH 6, 7, and 8), the zeta potentials of the silica-coated magnetite still maintained its negative charge but became less negatively charged as compared with that measured in dionized water. On the other hand, the zeta potential of the Si-QAC-magnetite could maintain its positive value (40 mV) at pH 4.5 but decreased to about −5 mV as pH increased from 6 to 8. The significant decrease of the zeta potential probably resulted from the phosphate buffer employed in which the negatively charged phosphate ions might bind with the positively charged Si-QAC that neutralized the charge of the Si-QAC-magnetite. Evidently, the observed increase of particle size and zeta potential indicates that the silica-coated magnetite was successfully modified with Si-QAC.

The ability of undigested HA to form complex with acidified BSA has been used to measure the hyaluronidase activity of HA hydrolysis (15). Based on its complex forming ability with HA, the acidified BSA was also employed to immobilize on the surface of the magnetite for the HA adsorption

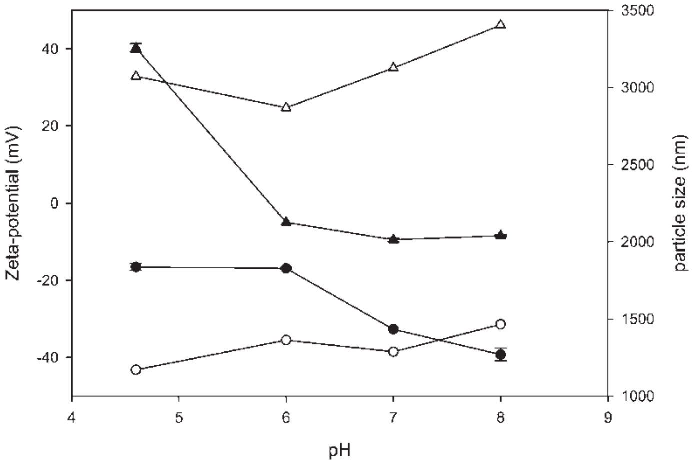

Figure 2. Particle size (open symbol) and zeta potential (closed symbol) of silica-coated magnetite and Si-QAC-magnetite at 0.1 M buffers of different pH. (●;○) silica-coated magnetite; (▲;△) Si-QAC-magnetite.

study. The time courses of HA adsorption to acidified BSA, Si-QAC, and ethanolamine-modified magnetite as well as the silica coated-magnetite were studied at pH 4 in 0.1 M acetate buffer. A significant HA concentration decrease was observed (Fig. 3) for Si-QAC, BSA, and ethanolamine modified magnetite at first 10 min. The concentration started to level off after 30 min and that implies that the equilibrium adsorption could be reached around 30 min. The adsorption capacity of these magnetites for HA follows the order of Si-QAC > BSA> ethanolamine. The control, silica coated-magnetite could also adsorb the HA but to a much less extent. The HA molecule possesses its anionic character at pH 4 because of its much lower isoelectric point (pI = 2.5) (18), whereas the silica-coated magnetite carries negative charges which originate from the relative acidic silanol groups. With the same electrostatic charge, the observed HA adsorption to the silica-magnetite may result from the hydrogen bonding between the hydrophilic moieties in HA such as carboxylic acid or hydroxyl groups with silanol or hydroxyl groups on the silica surface. A similar observation has also been reported by Suh et al. (19) in the study of the HA film directly immobilized to silicon and other dioxide solid surface. At pH 4, all the compounds (Si-QAC, BSA, and enthanolamine) used to modify the silica-coated magnetite are positively charged. Therefore, the significant HA adsorption onto these magnetites observed in Fig. 3 can be attributed to the electrostatic interaction. The pH effect on the HA adsorption was studied for the silica-coated magnetite with modification of Si-QAC and BSA. As shown in Fig. 4, the HA adsorption efficiency of the BSA-magnetite decreased with pH. The adsorption efficiency, about 70%, was attained at pH 4 and 20% was reached as the pH was increased to 7. The lower efficiency at a higher pH was mainly because

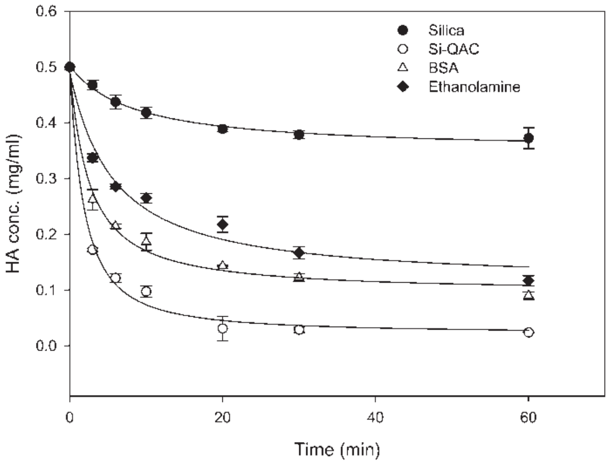

Figure 3. Time courses of HA concentration during adsorption to various silica-coated magnetite at pH 4 and 37°C.

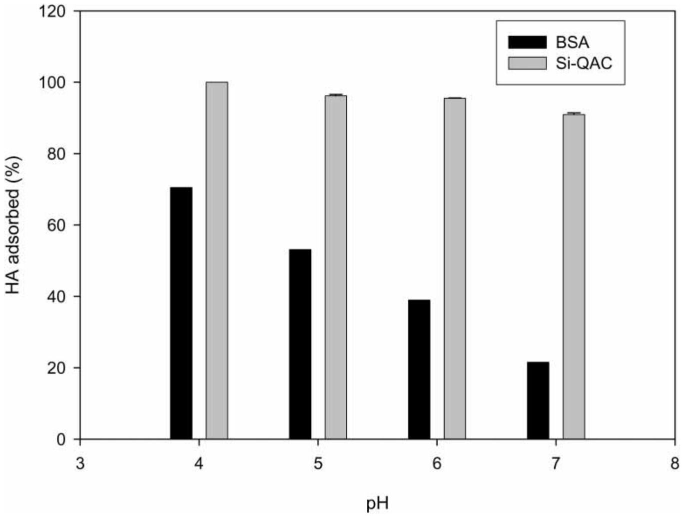

Figure 4. pH effect on HA adsorption to BSA and Si-QAC modified magnetite.

BSA became more negatively charged as the pH increased above its pI (ca 4.7). The repulsive electrostatic interaction reduced the amount of HA adsorbed by the BSA-magnetite. Other interactions such as hydrophobic or hydrogen bonding may contribute to the remaining HA adsorption efficiency attained at a pH higher than BSA's pI. In contrast, the Si-QAC-magnetite demonstrated its high HA adsorption efficiency (ca 90%) independent of the pH from 4 to 7. Electrostatic interaction between Si-QAC-magnetite and HA contributed a part of this high adsorption efficiency because the quaternary amine group of Si-QAC has a strong cationic property, and it is supposed to remain positively charged over a wide pH range (pH 3∼11). However, when modified on the silica-coated magnetite, the strong cationic properties of the Si-QAC was not observed. As shown in Fig. 2, the highly positively charged zeta potential of Si-QAC-magnetite at pH 4.5 decreased to a slightly negative charge in 0.1 M phosphate buffer of pH 6, 7, and 8. Interactions other than electrostatic should contribute to the observed high adsorption efficiency at pH 6 and 7. HA is generally known as an anionic hydrophilic biopolymer but it also has extensive hydrophobic patches, about 8 CH units in its secondary structure (20). Si-QAC, in addition to its positively charged nitrogen group, also carries a hydrophobic 18 CH_2 unit. Therefore, the hydrophobic interaction between HA and Si-QAC-magnetite was explored in the presence of hydrophobic interaction enhancing salt, $(NH_4)_2SO_4$. As shown in Fig. 5, the HA adsorption efficiency of the Si-QAC-magnetite decreased from about 90% to 70% as $(NH_4)_2SO_4$concentration increased to 1.0 M. In contrast, the HA adsorption efficiency of BSA-magnetite decreased significantly from 65% to 10%. The decrease of the HA adsorption efficiency is

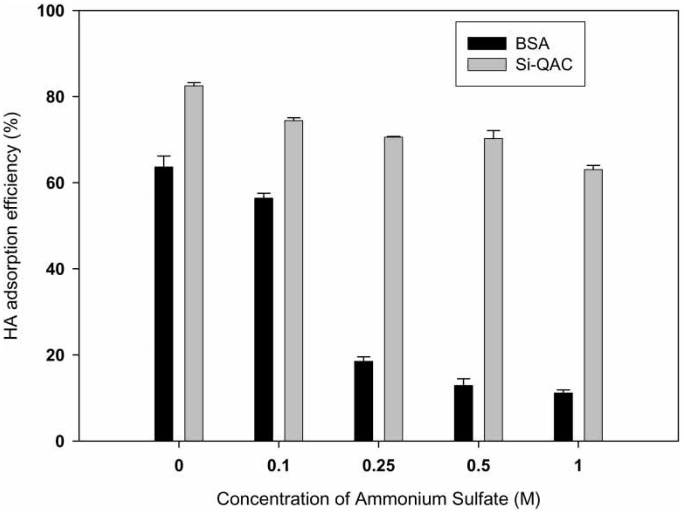

Figure 5. Effect of ammonium sulfate concentration on HA adsorption to BSA and Si-QAC modified magnetite.

expected because the electrostatic attractive interaction was reduced by the screening effect, an effect which arises from the high salt concentration employed. On the other hand, the hydrophobic interaction between the hydrophobic patches of the HA and the long CH_2 chain of Si-QAC promoted by $(NH_4)_2SO_4$ enhanced the HA adsorption. The balance between the reduced electrostatic interaction and enhanced hydrophobic interaction contributes to the less extent of the adsorption efficiency decrease of Si-QAC-magnetite in the presence of 1 M $(NH_4)_2SO_4$. Hydrophobic patches also exists on the BSA surface which should be increased after BSA is denatured. Compared with that of Si-QAC, the hydrophobicity of the denatured BSA may be lower. Therefore, the HA adsorption enhanced by hydrophobic interaction could not compensate that reduced by the weakened electrostatic attraction so that a significant HA adsorption decrease was attained for the BSA-magnetite in the presence of high concentration of $(NH_4)_2SO_4$.

HA Adsorption Isotherm

Figure 6a shows the effect of temperature on HA adsorption to Si-QAC-magnetite at pH 4. It can be seen that the initial slope of the adsorption isotherm, which reflects tightness of adsorption increased with temperature. This indicates that the increase of temperature benefits the HA adsorption. The adsorption isotherms were fitted to the Langmuir model [Eq. (1)]:

$$Q^* = \frac{Q_{max} C^*}{C^* + K_d} \tag{1}$$

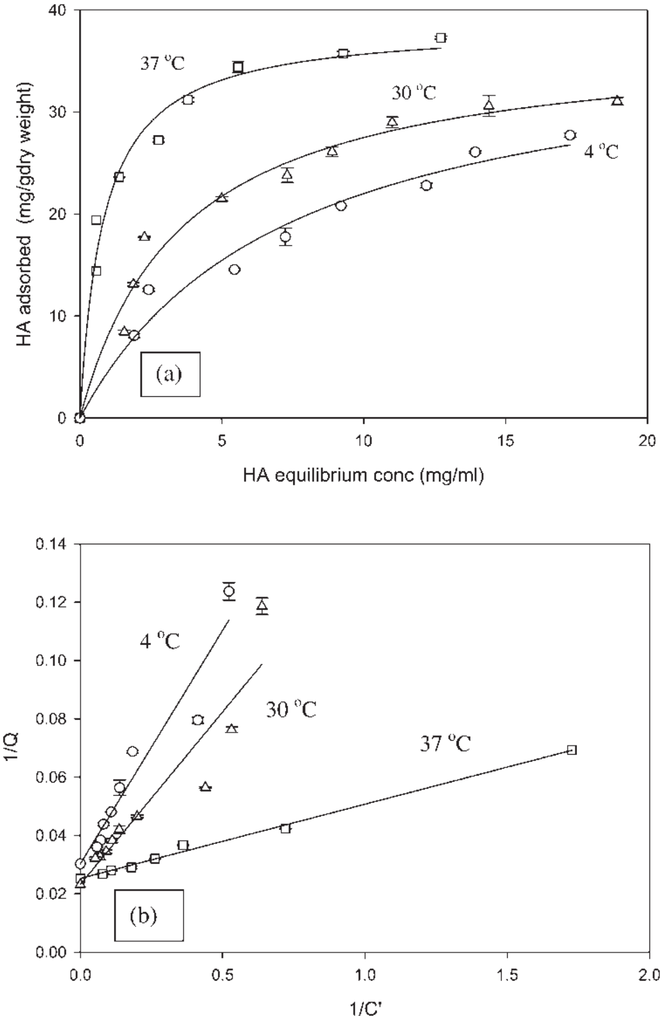

Figure 6. (a) Adsorption isotherms of HA to Si-QAC-magnetite at pH 4; (b) reciprocal plot of adsorbed HA versus equilibrium concentration.

where Q^* is adsorbed HA at equilibrium, Q_{max} maximum adsorption capacity, C^* the equilibrium HA in solution, and K_d the dissociation constant. The reciprocal plots of $Q^*$and C^* are shown in Fig. 6b. The straight lines indicate that the adsorption isotherm can be well described by the Langmuir model. The values of the equilibrium adsorption parameters, Q_{max} and K_d, were estimated by using the non-linear regression of SigmaPlot 2000 (SPSS Inc., USA) as shown in Table 1. The temperature does not seem to affect Q_{max} much. It was about 38 mg HA/g dry magnetite for a temperature from 4°C

Table 1. Temperature effect on Langmuir adsorption parameters of HA to Si-QAC-magnetite at pH 4

	37°C	30°C	4°C
Qmax (mg/g)	38.6	37.5	38.5
Kd (g/l)	0.016	0.076	0.150
Qmax/Kd (l/g)	2.412	0.493	0.256

to 37°C. In contrast, K_d was strongly affected by the temperature. It was 0.016 g/l at 37°C and increased about 10 fold to 0.15 g/l at 4°C. Hence, K_d is a direct measure of the strength of the interaction between the adsorbate and the adsorbent; the smaller the value of K_d indicates the stronger interaction between HA and Si-QAC-magnetite which mainly resulted from electrostatic and hydrophobic interactions. However, with respect to the applicability in HA isolation, both Q_{max} and K_d are important, and therefore, the initial slope of the binding isotherm (Q_{max}/K_d), which reflects the tightness of the binding are also listed in Table 1. The tightness of the binding increased about 10-fold as the temperature increased from 4°C to 37°C. Since the hydrophobic interaction is known to be enhanced by temperature, the significant increase of binding tightness demonstrates again that the hydrophobic interaction plays an important role in HA adsorption to Si-QAC-magnetite.

Desorption of HA and Repeated use of Si-QAC-Magnetite

To dissociate the electrostatic and hydrophobic interactions between HA and Si-QAC, various methods have been carried out in pH 7, 0.1 M phosphate buffer. As shown in Table 2, surfactant such as Triton or CPC alone could not desorbe HA effectively. The desorption efficiency could only reach 25% and 10% for using 0.1% Triton and 1% CPC as desorption solutions, respectively. However, the desorption efficiency increased significantly to about 70%

Table 2. HA desorption efficiency from Si-QAC-magnetite by using desorption solutions and electrophoretic method

Methods	Desorption efficiency(%)
0.1% Triton X100	25
0.1% Triton X100 + 1 N NaCl	65
1% CPC	10
1% CPC + 1 N NaCl	70
Electrophoretic desorption for 2 hr	62

when 1 N NaCl was employed. This indicates that NaCl at concentration of 1 N is very effective to suppress the electrostatic interaction between the anionic HA and cationic Si-QAC. It seems that the hydrophobic interaction, which usually can be suppressed by employing the surfactant, is not as important as the electrostatic interaction in the mechanism of HA adsorption to Si-QAC-magnetite. In order to avoid using chemicals in the HA isolation process, electrophoretic desorption was employed to recover the HA. As shown in Table 2, about 62% of the adsorbed HA can be recovered in a not optimized electrophoretic desorption condition. Compared with the desorption efficiency of using the surfactant-containing desorption solution, the electrophoretic method for HA recovery seems feasible and can provide a surfactant-free HA isolate.

Repeated adsorption/desorption operations were performed to examine the reusability and HA recovery yield of the Si-QAC-magnetite. As shown in Fig. 7, the Si-QAC-magnetite had a HA adsorption efficiency about 85% and 60% of the initially loaded HA was recovered by a desorption operation at the first cycle. As was expected, the HA adsorption efficiency and recovery yield increased with the operation cycle. The adsorption efficiency about 95% and the recovery yield about 85% were reached at the 4th cycle. The increased HA recovery yield evidently resulted from the increased adsorption efficiency because the recovery yield is based on the fixed amount of the HA loaded into each adsorption cycle. It is not clear about the exact mechanism involved in the increased adsorption efficiency yet. But, an interesting flocculation phenomenon observed for the first and the following adsorption is worth being noted. During the first cycle of adsorption, a nearly homogeneous particles solution was observed and the magnetic field

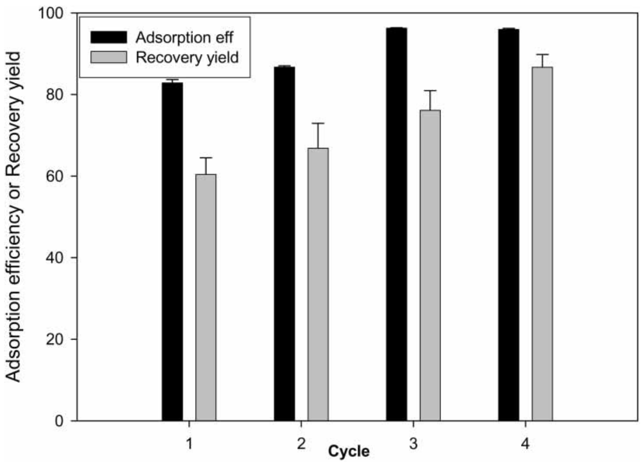

Figure 7. Effect of repeated use of Si-QAC-magnetite on HA adsorption yield and recovery yield.

had to be applied in order to collect the precipitate. After washing and the HA desorption, the Si-QAC-magnetite returned to its very dispersive form. However, during the second and the following cycle of the HA adsorption, the formation of larger flocks of the HA-magnetite complex was noted and the flocks could precipitate in the absence of the magnetic field. Probably, after first use the morphology of the Si-QAC-magnetite was changed due to the chemisorbed HA on its surface which could not be desorbed. As a consequence, in the second and the following cycle of the HA adsorption, not only the covalently bonded Si-QAC but also the chemisorbed HA could contribute to the HA adsorption through electrostatic, hydrophobic, and possibly through the hydrogen bonding interactions. The synergic effect of those interactions probably made the HA adsorption efficiency increase with the adsorption cycle.

CONCLUSION

Silica-coated magnetite after surface modification with denatured BSA and Si-QAC were very effective for the HA adsorption at pH 4. However, the HA adsorption efficiency of the BSA-magnetite decreased significantly while the Si-QAC-magnetite remained at the same level as the pH increased from 4 to 7. Ammonium sulfate concentration also decreased the HA adsorption efficiency of the Si-QAC-magnetite but with a lesser extent as compared with BSA-magnetite. The temperature that enhanced the HA adsorption efficiency confirmed that, other than electrostatic interaction, the hydrophobic interaction played a very important role in the HA adsorption to Si-QAC-magnetite. The adsorbed HA could be effectively desorbed from the Si-QAC-magnetite by employing surfactant-supplemented 1N NaCl solution as desorption solution. The Si-QAC-magnetite could be repeatedly used for HA recovery for its high HA adsorption efficiency and easy retrieval from the solution.

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