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Development of superparamagnetic functional carriers and application for affinity separation of subtilisin Carlsberg

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Abstract

Superparamagnetic functional carriers to obtain high selectivity were reported in this study. Magnetic carriers with epoxy groups were synthesized by spraying suspension polymerization (SSP). The measurement of scanning electron microscopy (SEM) and vibrating sample magnetometer (VSM) showed that the magnetic carriers have a narrow size distribution and displayed superparamagnetic characteristics. The magnetic carriers with epoxy groups were modified by various affinity ligands to magnetic functional carriers such as copper-IDA carriers, benzamidine carriers and phenylboronic acid carriers. The effects of medium pH and subtilisin Carlsberg concentration on adsorption capacity were investigated. It was found that phenylboronic acid and benzamidine immobilized magnetic carriers were effective magnetic carriers for affinity adsorption of subtilisin Carlsberg, and the maximum adsorption capacity (about 65 mg/g) was obtained at pH 9.5. The adsorbed subtilisin Carlsberg was also desorbed successfully by using dissociation agents, and the recovery of the enzyme activity was still around 85%.

Keywords: Magnetic functional carriers; Subtilisin Carlsberg; Affinity ligand

1. Introduction

With the rapid development of modern biomedicine and biotechnology, magnetic bioseparation technique plays an important role in those areas in recent years, especially for controlled drug delivery, cell labeling and separation, probing the local viscoelasticity of living cell, or protein and enzyme purification [1–4]. There have been different approaches to the preparation of magnetic microspheres with pro and cons to each approach, such as emulsion polymerization [5–8], seeded emulsion polymerization [9], microemulsion polymerization [10], in situ polymerization [11], dispersion polymerization [12,13], suspension polymerization [14,15], and two-step swelling method [16]. However, the magnetic microspheres with both a narrow size distribution and high magnetite content are very necessary in bio-applications, especially for the drug delivery system, because the accumulated locations of the particles containing drugs also depend on the size of the particles and magnetite content. Furthermore, in the applications for the magnetic microspheres of proteins and enzymes, DNA and RNA, and cells, magnetic microspheres with a narrow size distribution and high magnetite content are also required. If the size distribution of particles is narrow and their magnetite content is high, the particles can guarantee uniform behavior in a solution and under a magnetic field, and can be separated from a solution very quickly. Therefore, developing a method, which can provide the magnetic polymer microspheres with a narrow size distribution and higher magnetite content is a new challenge currently.

Spraying suspension polymerization (SSP) technique is a promising technique which was first proposed by our group [17,18] to prepare magnetic polymer microspheres with a narrow size distribution and higher magnetite content. The SSP technique involves the spray of dispersed phase containing magnetite nanoparticles through a nozzle into continuous phase. The small droplets in continuous phase are directly formed from the nozzle by applying appropriate pressure. Compared with the conventional mechanical stirring method, the SSP technique can provide small droplets with a narrow size distribution. In addition, the size of droplets can be controlled easily by using nozzle with different pore sizes.

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Because of the narrow size distribution of the droplets, break-up and coalescence between droplets rarely occur during polymerization, and therefore, much higher magnetite content can be expected.

Subtilisin consists of a single polypeptide chain of about 275 amino acid residues (27.3 kDa) and has no disulfide bonds, and it is of considerable interest not only scientifically but also industrially, for they are used in such diverse applications as meat tenderizers, laundry detergents, and proteolytic medicines [19,20]. Furthermore, their catalytic efficiency and specificity in organic media would enhance practical uses related to synthetic applications [21]. There have been many researches about their stability [22,23] as stabilization normally improves their properties for applications, but there was no research on the affinity separation of subtilisin.

In this study, subtilisin Carlsberg was used as a model enzyme, and the affinity separation of subtilisin Carlsberg by magnetic functional carriers was investigated. The magnetic carriers with epoxy groups were synthesized by spraying suspension polymerization (SSP). Then, the magnetic carriers were modified by various affinity ligands to magnetic functional carriers such as copper carriers, benzamidine carriers and phenylbenze acid carriers. Furthermore, the affinity adsorption and desorption of subtilisin Carlsberg on the magnetic functional carriers were investigated.

2. Experimental

2.1. Materials

Styrene (St), glycidyl methacrylate (GMA) and divinyl benzene (DVB) were commercial grade, distilled under reduced pressure to remove inhibitors. All these treated monomers were stored in a refrigerator prior to use. Polyvinyl alcohol (PVA-217, degree of polymerization 1700, degree of hydrolysis 88%) was used as a stabilizer. Benzoyl peroxide (BPO), reagent grade, was used as an initiator in polymerization. Subtilisin Carlsberg, iminodiacetic acid, ethylenediamine, *m*-aminophenylboronic acid and *p*-aminobenzamidine were purchased from Sigma Chemical Company. Water was purified by distillation followed by deionization using ion exchange resins. Other chemicals were reagent grade and used as received.

2.2. Synthesis of magnetic carriers

A spraying suspension polymerization (SSP) apparatus for synthesis of magnetic carriers was already presented in our previous work [17,18]. Magnetic Fe_3O_4 nanoparticles coated with oleic acid were prepared by co-precipitation method [24]. Thirty grams of polyvinyl alcohol (PVA) dissolving 2000 mL deionized water was used as an aqueous phase. The oil phase, a mixture of 50 mL St, 35 mL GMA, 5 mL DVB and 5 g BPO dissolving 15 g magnetic Fe_3O_4 nanoparticles, was stored in the dispersion phase storage tank. By applying nitrogen pressure of 0.2 MPa, the oil phase was sprayed through the spraying nozzle into the aqueous phase to form uniform droplets, which were polymerized for 1.5 h with gentle stirring under a nitrogen atmosphere. The resulting magnetic carriers with epoxy groups were separated by permanent magnet and washed with deionized water and ethanol for several times.

2.3. Immobilization of affinity ligands

2.3.1. Iminodiacetic acid (IDA) immobilized magnetic carriers

Ten grams magnetic carriers were incubated in 18 mL of 0.1 M sodium hydroxide and 2.0 M iminodiacetic acid pH 9 at 80 °C for about 24 h under very gently stirring, the IDA magnetic carriers were washed with an excess of distilled water and stored at 4 °C.

2.3.2. Copper-IDA immobilized magnetic carriers

Ten grams IDA immobilized magnetic carriers was incubated in 60 mL of distilled water containing 2 g of $CuSO_4$ under very gently stirring. After 2 h, the magnetic carriers were washed with an excess of distilled water. This treatment should modify 100% of the IDA groups in the magnetic carriers. After the Cu^{2+} was released from the magnetic carriers by treatment with EDTA, the quantification of the copper ions by UV spectroscopy was utilized to quantify the degree of modification of the epoxy groups with the IDA groups.

2.3.3. Phenylboronic acid immobilized magnetic carriers

Ten grams magnetic carriers were incubated in 30 mL of 5% w/v *m*-aminophenylboronic acid in 20% dioxane at pH 8 and 75 °C. After 24 h, the samples were washed with excess distilled water and stored at 4 °C.

2.3.4. Benzamidine immobilized magnetic carriers

Ten grams magnetic carriers were incubated in 33 mL of 5% w/v *p*-aminobenzamidine in 20 mL of 0.1 M sodium hydroxide at pH 11 and 80 °C. After 24 h, the samples were washed with excess distilled water and stored at 4 °C.

2.4. Affinity adsorption of subtilisin Carlsberg

The affinity adsorption of subtilisin Carlsberg was carried out batchwise in the media at different pH values. The effects of medium pH and subtilisin Carlsberg concentration on the adsorption capacity of different ligands immobilized magnetic carriers were investigated. The different ligands immobilized magnetic carriers were dispersed in Tris–HCl buffer with identical concentrations of 5 g/L. Different subtilisin Carlsberg concentrations were added into 10 mL solutions of magnetic carriers, respectively. The mixtures were incubated at 25 °C for 1 h. At the end of adsorption, the solid phase was magnetically separated and the supernatant was analyzed for residual subtilisin Carlsberg concentration. Adsorption capacity of subtilisin Carlsberg was calculated by mass balance.

2.5. Desorption of subtilisin Carlsberg

The desorption of subtilisin Carlsberg from the different ligands immobilized magnetic carriers was investigated. These conditions were as followed:

The copper-IDA immobilized magnetic carriers released the adsorbed subtilisin Carlsberg by incubation in 0.1 M imidazole.

The phenylboronic acid immobilized magnetic carriers released the adsorbed subtilisin Carlsberg by incubation in 0.1 M ammonium acetate pH 6 containing 0.05 M $MgCl_2$.

The benzamidine immobilized magnetic carriers released the adsorbed subtilisin Carlsberg by incubation in 0.1 M glycin–HCl buffer pH 6 containing 0.02 M $CaCl_2$.

2.6. Analytical methods

The diameter and surface features of magnetic carriers were investigated by scanning electron microscopy (SEM, JSM-6700F, JEOL. Japan). The magnetization curves of the samples were measured at room temperature with a vibrating sample magnetometer (VSM, model-155, Digital Measurement System, Inc.). The concentrations of subtilisin Carlsberg and tyrosine were determined from the absorbance at 280 and 275 nm, respectively, by using UV spectrophotometer (Beckman, Fullerton, CA, USA). The dried magnetic carriers were re-dispersed in distilled water and measured by laser diffraction using a Coulter LS 230 (Coulter Electronics, USA).

Fibrinolytic activity of subtilisin Carlsberg was measured by the hydrolysis of fibrin [25]. The incubation mixture contained 2.5 mL of 12 g/L fibrin solution (pH 7.8), 6.5 mL of 0.1 M Tris–HCl buffer (containing 10 mM $CaCl_2$, pH 7.8), and 1 mL enzyme solution with suitable dilution. The incubation was carried out at 37 °C for 15 min and was stopped by adding 5 mL of 0.11 M trichloroacetic acid containing 0.22 M sodium acetate and 0.33 M acetic acid. The absorbance at 275 nm of the supernatant obtained after centrifugation was determined. A fibrinolytic unit was defined as the amount of enzyme that gave an increase in absorbance at 275 nm equivalent to 1 μg of tyrosine per minute at 37 °C.

3. Results and discussion

3.1. Synthesis and characterization of magnetic functional carriers

Magnetic carriers with epoxy groups were synthesized by spraying suspension polymerization (SSP). The morphology and size distribution of the resulting magnetic carriers are shown in Figs. 1 and 2. It can be seen that the magnetic carriers prepared by this method have a narrow size distribution with the average size of about 9 μm. In suspension polymerization, the initiator is soluble in the monomer phase, which is dispersed by agitation into the dispersion phase to form droplets. The coalescence and break-up of monomer droplets will occur continuously during polymerization and the size distribution of droplets is usually very broad. Thus, the polymer particles made by the conventional suspension polymerization are most in the size range of several hundred micrometers with very broad size distribution [14,15]. In this work, the dispersion phase containing monomer St, functional monomer GMA, cross linker DVB, initiator BPO and

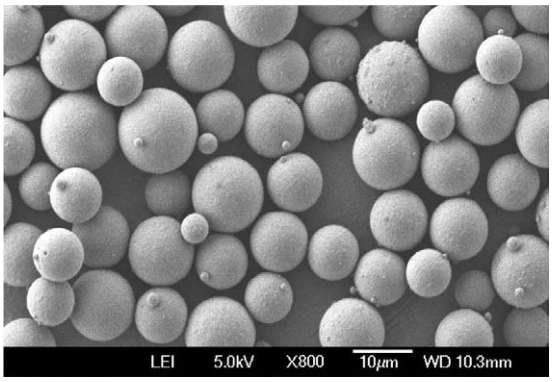

Fig. 1. SEM micrograph of overall appearance of magnetic carriers.

magnetite nanoparticles coated with oleic acid was sprayed by a particular pressure of nitrogen gas into the aqueous phase to form uniform droplets, which were stabilized by stabilizer PVA dissolved in the aqueous phase. After the temperature of the aqueous phase was controlled at polymerization temperature of the monomers, the droplets were polymerized to magnetic carriers. Compared with conventional suspension polymerization, the spraying suspension polymerization (SSP) can make the droplets disperse uniformly by spraying dispersion instead of the conventional mechanical stirring dispersion. Therefore, the magnetic carriers prepared by SSP are small with a narrow size distribution.

The magnetic carriers with epoxy groups are very reactive, which can be directly coupled with active compounds containing amino groups. Subtilisin is a serine protease, which consists of a single polypeptide chain of about 275 amino acid residues. Because *m*-aminophenylboronic acid and *p*-aminobenzamidine are the inhibitors of the serine protease, they can be considered as affinity ligands for separation of subtilisin. At the same time, the subtilisin consists of about 5 histidines, so copper-IDA can also be used as affinity ligand for separation of subtilisin. The immobilization of different ligands on the magnetic carriers was carried out by the ring-opening reaction of epoxy groups with amino groups in alkaline

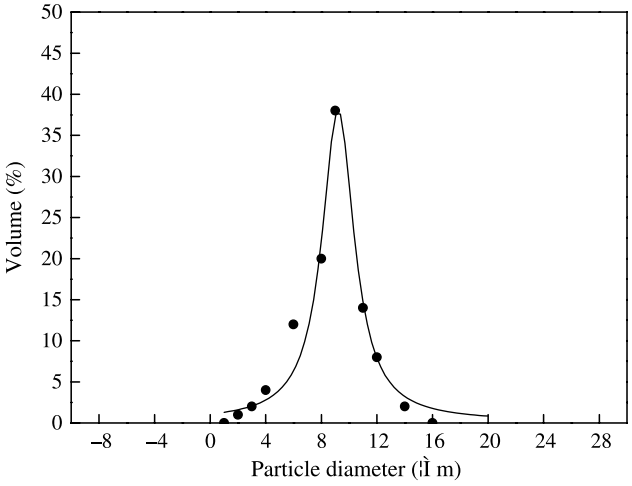

Fig. 2. Size distribution of magnetic carriers prepared by spraying suspension polymerization.

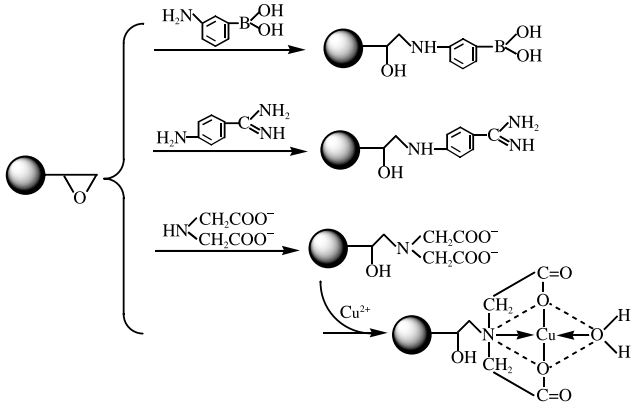

Fig. 3. Formation of magnetic functional carriers.

condition. The formation of phenylboronic acid, benzamidine and copper-IDA immobilized magnetic functional carriers are shown in Fig. 3. Compared with the other particles containing methoxy (–OCH_3) groups [26,27] and phenyl groups [28], the magnetic carriers with epoxy groups can be coupled with ligands directly without any further modification.

The magnetite content of the magnetic carriers could be measured by thermo-gravimetric analysis under a nitrogen atmosphere to minimize the mass increase due to iron oxidation. Herein, the polymer was completely decomposed at temperature above 600 °C and the magnetite content of the unmodified magnetic carriers was evaluated to be 19.4%, which is higher than those of particles prepared by the conventional suspension polymerization method [14,15]. It is well known that when magnetic carriers are employed for bioseparations, permanent magnetic carriers agglomeration should be avoided at all cost because it severely hinder magnetic carriers reusability and ease of product elution. Fig. 4 shows a hysteresis loop from vibrating sample magnetometer (VSM) measurements of the different ligands immobilized magnetic carriers and unmodified magnetic carriers. But no residual magnetism could be detected and hence, the loop is completed closed. These functional carriers are classical examples of superparamagnetic behavior [29]. The saturation magnetizations of benzamidine, phenylboronic acid and Copper-IDA immobilized magnetic functional carriers, which were found to be 11.8, 10.4 and 9.8 emu/g, respectively, are lower than that of unmodified carriers (12.3 emu/g). The results showed that the affinity ligand immobilization had a little impact on the magnetism of the carriers in the present condition, especially for the copper-IDA immobilized magnetic carriers. It can be explained that the affinity ligand immobilization was carried out at high temperature (> 70 °C), the magnetite (Fe_3O_4) nanoparticles in the carriers could be oxidated to ferric oxide (Fe_2O_3), which has lower magnetism than those of magnetite (Fe_3O_4). In addition, A large amount of affinity ligands was immobilized on the surface of the magnetic carriers, so the content of magnetite in the carriers decreased somewhat, resulting in a little lower saturation magnetization of affinity ligand immobilized magnetic carriers.

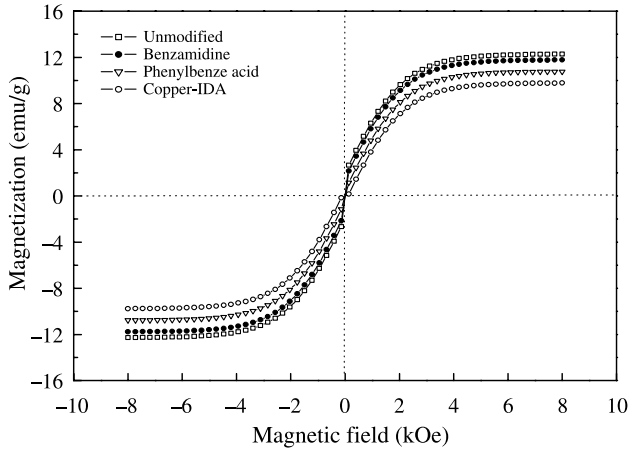

Fig. 4. Magnetization curves of magnetic functional carriers obtained by VSM.

3.2. Affinity adsorption and desorption of subtilisin Carlsberg

3.2.1. Effect of pH

The effect of the medium pH on the adsorption capacity of subtilisin Carlsberg onto the phenylboronic acid, benzamidine and copper-IDA immobilized magnetic carriers were investigated. Fig. 5 shows the change of subtilisin Carlsberg affinity adsorption against the medium pH in the initial subtilisin Carlsberg concentration of 2.0 mg/mL. The pH value was varied from 7 to 12. The maximum adsorption of subtilisin Carlsberg for phenylboronic acid, benzamidine and Copper-IDA immobilized magnetic carriers were all observed at pH 9.5, which is the isoelectric point of subtilisin Carlsberg [30].

With the increase of pH above or below the isoelectric point, the subtilisin Carlsberg adsorption capacity decreased. The decrease in the subtilisin Carlsberg adsorption capacity can be attributed to electrostatic repulsion effects between the identically charged groups. At pH different from the isoelectric point, the subtilisin Carlsberg molecules are charged and repel from each other. In addition, the neutral ligand immobilized magnetic carriers surface acquires a positive or negative charge, which prevents the further subtilisin Carlsberg adsorption.

3.2.2. Equilibrium adsorption of subtilisin Carlsberg

The results of the present study aimed at identifying conditions for preparation of benzamidine, phenylboronic

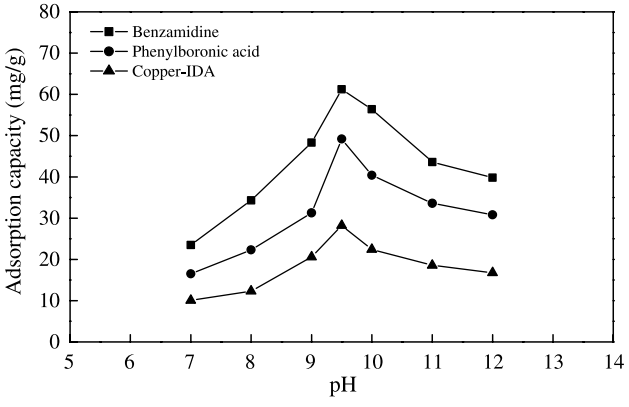

Fig. 5. Effect of medium pH on subtilisin Carlsberg adsorption capacity.

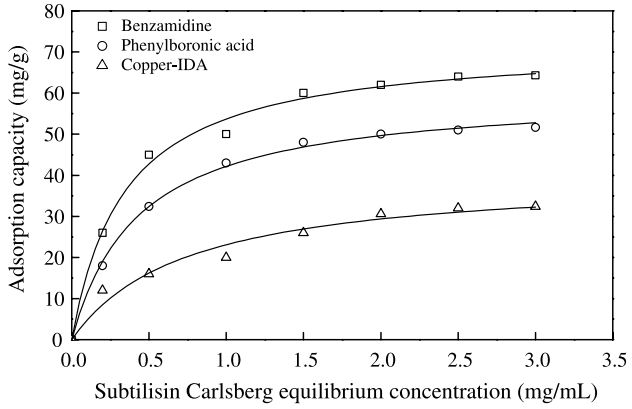

Fig. 6. Isotherms of subtilisin Carlsberg on magnetic functional carriers.

acid and copper-IDA immobilized magnetic carriers for affinity adsorption of subtilisin Carlsberg are presented in Fig. 6 and Table 1. In the first instance, three different magnetic affinity carriers were selected and the adsorption capacity of subtilisin Carlsberg were compared by an indirect method which involved analyzing supernatant for residual subtilisin Carlsberg concentration and its initial concentration. Various conditions for preparation of benzamidine, phenylboronic acid and copper-IDA immobilized magnetic carriers were screened by studying the adsorption behavior of various magnetic affinity carriers using subtilisin Carlsberg as a model enzyme.

The resulting data were fitted to the simple Langmuir model [Eq. (1)], in which Q and C represent the equilibrium concentrations of the adsorption and bulk phase target molecule, respectively, Q_{max} is the maximum capacity for the adsorbed target and K_d is the dissociation constant.

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

Although, the mechanistic assumptions upon which the model is based are not met by the present system, it has been widely used in characterizing the affinity adsorption of protein to ion exchanger [31] and affinity adsorbents [32]. Very good mathematical correlations between the experimentally obtained data point and Eq. (1) obtained, which make quantitative comparisons of the different magnetic affinity carriers possible.

Table 1
Effect of different ligands immobilized magnetic carriers on subtilisin Carlsberg adsorption

Langmuir parameters for affinity adsorption of subtilisin Carlsberg				Activity recovery (%)
Magnetic carriers	Q_{max} (mg/g)	K_d (mg/mL)	Initial slope (L/g)	
Benzamidine carrier	64.37±1.80	0.34±0.03	0.19	85.4
Phenylboronic acid carrier	52.34±0.98	0.43±0.03	0.12	82.5
Copper-IDA carrier	33.25±3.27	0.74±0.18	0.05	80.8

It is clear from Table 1 that the benzamidine and phenylboronic acid immobilized magnetic carriers exhibit much better adsorption capacity of subtilisin Carlsberg than that of copper-IDA immobilized carriers. As for benzamidine immobilized magnetic carriers, the initial slope of the isotherm, i.e. Q_{max}/K_d was about threefold higher than that of the Copper-IDA immobilized magnetic carriers. The very poor adsorption capacity for the copper-IDA immobilized magnetic carriers is most likely attributed to its less amount of histidine in the subtilisin Carlsberg. Benzamidine and phenylboronic acid are competitive inhibitors of serine protease and subtilisin, respectively, and has been used as ligands for affinity separation of serine protease such as urokinase, trypsin and chymotrypsin [33,34]. In this work, Benzamidine and phenylboronic acid immobilized magnetic carriers also exhibit better results for affinity adsorption of subtilisin Carlsberg. It can be concluded that benzamidine and phenylboronic acid are both suitable affinity ligands for the separation of subtilisin Carlsberg.

The desorption of subtilisin Carlsberg from magnetic functional carriers were accomplished using different dissociation agents in a matter of minutes. A number of different desorption conditions were investigated throughout the course of this work. It was found that the recovery of the enzyme activity was still around 85% as shown in Table 1.

4. Conclusion

Magnetic carriers with epoxy groups were modified by various affinity ligands to magnetic functional carriers such as Copper-IDA carriers, benzamidine carriers and phenylboronic acid carriers. The affinity adsorption and desorption of subtilisin Carlsberg showed that phenylboronic acid and benzamidine immobilized magnetic carriers were effective magnetic carriers for affinity adsorption of subtilisin Carlsberg, and the maximum adsorption capacity (about 65 mg/g) was obtained at pH 9.5. Magnetic carriers with epoxy groups can be considered as a promising way to be used in biological applications such as enzymes separation, cell isolation and purification of proteins.

Acknowledgements

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