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RESEARCH ARTICLE

Paraoxonase 1-Bound Magnetic Nanoparticles: Preparation and Characterizations

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This is most probably the first time that covalently binding of Human serum paraoxonase 1 (PON1) to superparamagnetic magnetite nanoparticles via carbodiimide activation was investigated and presented in this study. PON1 was purified from human serum using ammonium sulfate precipitation and hydrophobic interaction chromatography (Sepharose 4B, L-tyrosine, 1-Napthylamine) and magnetic iron oxide nanoparticles were prepared by co-precipitation Fe^{+2} and Fe^{+3} ions in an ammonia solution at room temperature. X-ray diffraction (XRD) and the magnetic measurements showed that the nanoparticles are magnetite and superparamagnetic, respectively. Direct measurements by dynamic light scattering revealed that the hydrodynamic size was 16.76 nm with polydispersity index (PDI: 0.234). The analysis of Fourier transform infrared spectroscopy revealed that the PON1 was properly bound to magnetic nanoparticles replacing the characteristic band of $-NH_2$ at 1629 cm^{-1} with the protein characteristic band at 1744 cm^{-1} and 1712 cm^{-1}. Magnetic measurements determined that PON1-bound nanoparticles have also favorable superparamagnetic properties with zero coercivity and remanence though a slightly smaller saturation magnetization due to the decrease of magnetic moment in the volume friction. The kinetic measurements indicated the PON1-bound nanoparticles retained 70% of its original activity and exhibited an improved stability than did the free enzyme. The PON1 enzyme is seen to be quite convenient to bind superparamagnetic nanoparticles as support material.

Keywords: Human Serum Paraoxonase, Superparamagnetic Nanoparticles, Immobilization, Magnetite.

1. INTRODUCTION

Human serum paraoxonase 1 (PON1), primarily associated with HDL, is a member of a family of enzymes that has the ability to catalyze the hydrolysis of a broad range of carboxyl esters, carbonates, lactones, and toxic organophosphates.[1,2] In addition to its potential role in detoxification of toxic organophosphates, an emerging body of evidence has shown that PON1 possesses important anti-atherogenic actions.[3] The anti-atherogenic role of PON1 is further supported by the studies using PON1-knockout mice,[4] PON1 is suggested to exhibit its anti-atherogenic actions through several mechanisms including the attenuation of macrophage oxidative stress,[5] inhibition of oxidized LDL-induced MCP-1 production in endothelial cells,[6] and stimulation of HDL-mediated cholesterol efflux from macrophage.[7]

In the recent years, the nanosized magnetic particles receive increasing attention with the rapid development of nanostructured materials and nanotechnology in the fields of biotechnology and medicine.[8,9] Magnetite (Fe_3O_4) is one of the famous magnetic materials due to strong magnetic property and low toxicity. Many bioactive substances such as enzymes, proteins, antibodies, and anticancer agents have been bound to it.[9,10] Using magnetic nanoparticles as the support of immobilized enzymes has the advantages of the higher specific surface area was obtained for the binding of a larger amount of enzymes and the lower mass transfer resistance and less fouling, and followed by the immobilized enzymes can be selectively separated from a reaction mixture by the application of a magnetic field.[10] The binding is commonly accomplished through the surface coating with polymers, the use of coupling agents or crosslinking reagents, and encapsulation. Recently, a new method for the direct binding of proteins

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such as bovine serum albumin via carbodiimide activation was reported.[10]

Despite its traditional assignment as paraoxonase/arylesterase, evidences have been accumulated to indicate PON1 as lactonase. Previously, PON1 was shown to hydrolyze a variety of lactones.[11–13] Meanwhile, there is also a report that PON1 catalyzes the formation of many lactones[14] Structure-reactivity studies[16] and directed evolution experiments[15] further support the view that the native activity of PON1 may be lactonase activity. Although the physiological substrates of PONs are still unknown, they are likely to include lactones consumed as food ingredients, drug metabolites (statins, spironolactone, and glucocorticoid γ lactones), and derivatives of fatty acid oxidation processes, 13, 16 such as 5-HETEs lactone that resides in HDL.

The immobilisation of PON1 on silica gel support has been investigated using biosensor.[17] However, to our knowledge, its immobilization on magnetite nanoparticles has not been studied. The aim of this work is to immobilize the PON1 to superparamagnetic nanoparticles so that paraoxonase with variety different enzyme activities could maintain its activity in longer time periods in use of variety of applications such as chemical synthesis, detoxification of water reservoir. The properties, stability and activity of the covalently binding of PON1 to nanoparticles via carbodiimide activation were examined and also compared with the activity of free PON1 enzyme. The results showed that magnetic nanoparticles can be effectively used as a support for immobilization of PON1 enzyme.

2. EXPERIMENTAL DETAILS

2.1. Chemicals

The materials including sepharose 4B, L-tyrosine, 1napthylamine, paraoxon, protein assay reagents and chemicals for electrophoresis and carbodiimide were obtained from Sigma Chem. Co. Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$, >99%), aqueous ammonia (25% NH_3 in water, w/w), perchloric acid ($HClO_4$, %60) were obtained from Merck. Ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$, >99%) were purchased from Fluka. All other chemicals were the guaranteed or analytic grade reagents commercially available and used without further purification.

2.2. Preparation of Magnetic Nanoparticles

Magnetic nanoparticles were prepared by co-precipitating Fe^{+2} and Fe^{+3} ions by ammonia solution. 40 ml of a 1 M $FeCl_3 \cdot 6H_2O$ solution in water was combined with a 10 ml solution of 2 M $FeCl_2 \cdot 4H_2O$ in 2 M HCl. The chloride solutions were prepared quickly, then added to 500 ml of 0.7 M NH_4OH (purged initially with N_2 gas for 1 hour before adding salts) in an open vessel stirring at 1800 rpm for 30 minutes under a continuous flow of N_2. Magnetite precipitate formed in the reaction was deposited with a magnet placed under the vessel of the solution, and supernatant liquid was removed. The precipitate was washed several times with water finally dried in an oven at 75 °C.

2.3. Characterizations

The crystalline of magnetic particles was analyzed by using powder X-ray diffraction (XRD) of PANalytical's X'Pert PRO diffractometer using Cu-K_α radiation with a wavelength of 0.154 nm. Dynamic light scattering (DLS) was used in order to determine the mean particle diameter and size distribution (polydispersity index, PDI) of magnetite nanoparticles. To do that, magnetic sol was prepared using $HClO_4$ as depicted in the paper.[17] The measurement was performed by an ALV/CGS-3 Malvern, compact goniometer system. High resolution transmission electron microscope (HRTEM, FEI TECNAI G2 F30 model) with an accelerating voltage of 300 kV was used for micrograph. The magnetic properties of nanoparticles were studied by vibrating sample magnetometer (VSM-ADE EV9 Model). The direct binding of PON1 to the magnetic nanoparticles was checked using Perkin-Elmer FT-IR Spectrometer. All measurements were carried out at room temperature.

2.4. Purification of Paraoxonase from Human Serum by Hydrophobic Interaction Chromatography

Human serum was isolated from 35 ml fresh human blood and put into a dry tube. Serum paraoxonase was firstly isolated by ammonium sulfate precipitation (60–80%). The precipitate was collected by centrifugation at 15,000 rpm for 20 min, and redissolved in 100 mM Tris-HCl buffer (pH 8.0). Next, we synthesized the hydrophobic gel, including Sepharose 4B, L-tyrosine and 1-Napthylamine, for the purification of human serum paraoxonase.[16] The column was equilibrated with 0.1 M of a Na_2HPO_4 buffer (pH 8.00) including 1 M ammonium sulfate. The paraoxonase was eluted with an ammonium sulfate gradient using 0.1 M Na_2HPO_4 buffer with and without ammonium sulfate (pH 8.00).

2.5. SDS Polyacrylamide Gel Electrophoresis

Polacrylamide gel slab electrophoresis of purified enzyme was carried out according to the method of Laemmli.[18]

2.6. Binding of PON1 to Magnetic Nanoparticles

For the binding of PON1, 20–100 mg of magnetic nanoparticles were first added to 2 ml of buffer A (0.003 M phosphate, pH 6, 0.1 M NaCl). Then, the reaction mixture was sonicated for 10 min after adding 0.5 ml of carbodiimide solution (0.025 g/ml in buffer A). Finally, 2 ml of PON1 solution (0.4–13.6 mg/ml in buffer A) was added

and the reaction mixture was sonicated for 30 min. The supernatant was used for the protein analysis. The precipitates were washed with buffer A, then buffer B (0.1 M Tris, pH 8.0, 0.1 M NaCl), and then directly used for the measurements of activity and stability.

2.7. Paraoxonase Enzyme Assay

Paraoxonase enzyme activity towards paraoxon was quantified spectrophotometrically by the method described by Gan et al.[19] The reaction was followed for 2 min at 37 °C by monitoring the appearance of p-nitrophenol at 412 nm in Biotek automated recording spectrophotometer.

2.8. Stability Measurements

The storage stabilities of bound and free PON1 stored at 4 °C were examined by assaying their residual activities in buffer B at 37 °C. The stabilities of bound and free PON1 (4 °C) were investigated by measuring their residual activities up to 550 hours.

3. RESULTS AND DISCUSSION

3.1. Comparative Characterizations of the Magnetite and PON1-Bound Nanoparticles

Crystalline structure of XRD pattern of the magnetic nanoparticles is shown in Figure 1. Experimental d-spacings obtained from the peaks are very similar to the ASTM–XRD graphics of magnetite (JCPOS 19-0629), which corresponds to the same inverse spinal structure. Therefore, it can be said that the iron oxide particles is most probably composed of magnetite. With the XRD pattern, the average core size of the particles can be evaluated from Scherrer equation[20]

$$L = \frac{0.94\lambda}{\mathrm{B}(2\theta)\cos\theta} \quad (1)$$

where L is equivalent to the average core diameter of the particles, λ is the wavelength of the incident X-ray, $\mathrm{B}(2\theta)$ denotes the full width in radian subtended by the half maximum intensity width of the powder peak, for instance (311), and θ corresponds to the angle of the (311) peak. For the (311) peak in the XRD pattern shown in Figure 1, 2θ is observed as 35.7153, and $\mathrm{B}(2\theta)$ is 0.6788. With λ being 0.154 nm, L is obtained as 12.29 nm via Eq. (1). The size distribution of the resultant magnetic nanoparticles was measured. The hydrodynamic diameter of nanoparticles and PDI value was found to be 16.76 nm and 0.234, respectively. It is worth noting that the value for the particle diameter obtained from XRD pattern means the particle core size whereas the size detected using DLS system refers to a hydrodynamic diameter of particles. For TEM, the magnetite nanoparticles produced under the same experimental conditions were used and their micrograph was showed in Figure 2. The average particle diameter was found as 10.57 ± 2.03 nm which is consistent with the size values from obtained XRD and VSM (see below) as also reported many times.[21–23] PON1 was purified from human serum as indicated in material methods. The purity of enzyme was checked by SDS gel electrophoresis. Figure 3 indicates the pure PON1 that corresponds to 43 kDA. Figure 4 presented a comparison of FT-IR spectra's for the magnetite nanoparticles and PON1-bound nanoparticles. Main characteristic peak[24] of Fe–O is seen at 589 cm^{-1} in both spectra's. In the spectra of magnetite nanoparticles, a characteristic band of $-NH_2$ at 1629 cm^{-1} was observed due to the NH_3 used in co-precipitation but disappeared at PON1-bound particles because of the binding of PON1 to nanoparticles.[10] On the side, the characteristic bands[25] of protein at 1744 and 1712 cm^{-1} that appeared in the PON1-bound magnetite nanoparticles verified the binding of PON1 to magnetite nanoparticles. However, the weak characteristic bands of proteins for the PON1 bound magnetite may be owing to the low enzyme loading. To study the magnetic properties, magnetization curves of the magnetic nanoparticles and PON1-bound nanoparticles measured are illustrated in Figure 5. As seen in the inset of magnetite nanoparticles, the typical characteristics of superparamagnetic behavior are observed showing zero coercivity and remanence. The saturation magnetization, M_s that is 54.81 emu/g is significantly less than 92 emu/g of the bulk magnetization

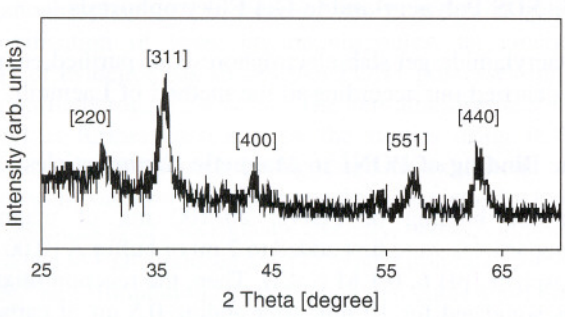

Fig. 1. X-ray powder diffraction pattern of the synthesized magnetic nanoparticles.

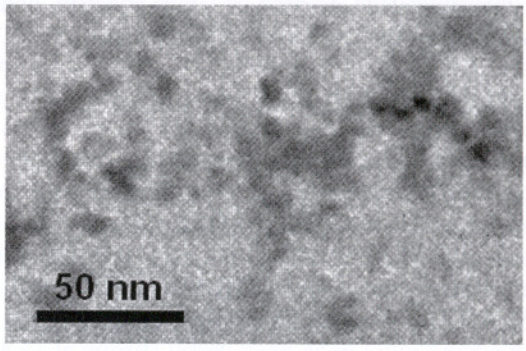

Fig. 2. TEM micrograph of magnetic nanoparticles.

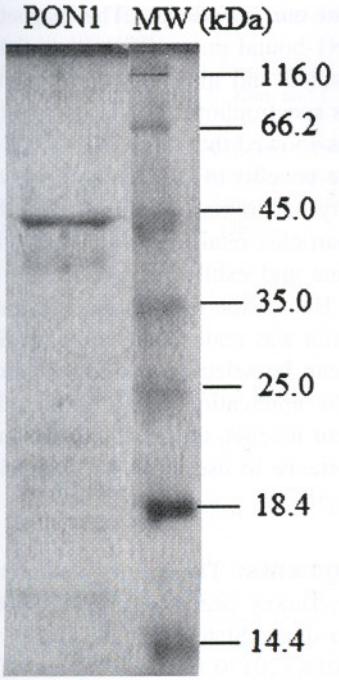

Fig. 3. SDS-PAGE of human serum PON1. The pooled fractions from ammonium sulfate precipitation and hydrophobic interaction chromatography (sepharose-4B, L-tyrosine, 1-napthylamine) were analyzed by SDS-PAGE (12% and 3%) and revealed by Coomassie Blue staining. Experimental conditions were as described in the method. Lane 1 hPON1; Lane 2 contained 3 μg of various molecularmass standards: β-galactosidase, (118.0), bovine serum albumin (79.0), ovalbumin (47.0), carbonic anhydrase, (33), β-lactoglobulin (25.0), lysozyme (19.5). Thirty microgram of purified bovine serum paraoxonase (lane 1) migrated with a mobility corresponding to an apparent Mw 43.0 kDa.

of magnetite.[20] The decrease in the saturation may be ascribed to the size effect as similar in the reports.[26, 27] The magnetic particle size and the standard deviation can also be obtained from the fitting of the hysteresis curve using the following formula.[28]

$$D_{\mathrm{Mag}} = \left[\frac{18\mathrm{kT}}{\pi m_s}\sqrt{\frac{\chi_i}{3M_sH_o}}\right]^{1/3}$$
$$\sigma = 1/3\left[\ln\left(\frac{3\chi_i}{M_s}/\frac{1}{H_o}\right)\right]^{1/2} \quad (2)$$

where M_s and m_s are the saturation magnetization of the nanoparticles and the bulk phase, respectively. χ_i is the initial susceptibility calculated at low fields, in the region where the variation of M against H is linear and $1/H_o$ is obtained by extrapolating M to 0 at high fields, in the region where the relationship between M and $1/H$ is a straight line. Hence, the mean magnetic particle size was calculated as 11.70 nm ($\sigma = \pm 0.42$), which is smaller than that observed from XRD measurement. Since a number of alternate mechanisms could result in the demagnetization of the particles, it was simplest to assume that the surface layer of magnetite atoms does not contribute to the magnetic properties of the particle. In magnetization

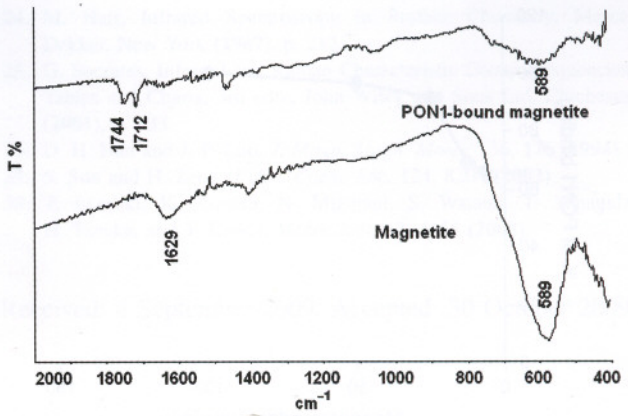

Fig. 4. FT-IR spectra of the magnetite and PON1-bound magnetite nanoparticles.

curve of PON1-bound nanoparticles, M_s are obtained to be 49.25 emu/g and they are also superparamagnetic with zero coercivity and remanence, which provided that the PON1 bound nanoparticles has the same magnetic advantages as free nanoparticles. It should also be noted that the PON1-bound nanoparticles is so small that they may also be considered to have a single magnetic domain for both samples. As accepted, M_s obtained from the curves decreased with the PON1 binding to the nanoparticles. This could be attributed to the binding of PON1 to the particle surface led to reduce the number of magnetic moment per weight in the volume friction measured by VSM and hence caused around 10% decrease in the M_s of free nanoparticles.

3.2. Binding Efficiency

By assaying the unbound protein in the supernatant after binding process, it was found that, with increasing the amount of Fe_3O_4 added at a constant PON1 amount of 3.4 mg/ml, the percentage of bound PON1 increased and then remained at approximately 100% when the amount of Fe_3O_4 added was above 25 mg/ml as indicated in Figure 6. Accordingly, the maximum weight ratio of bound PON1

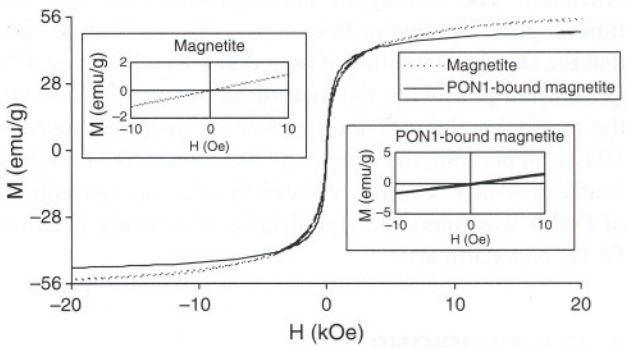

Fig. 5. The magnetization plots of the magnetite and the PON1-bound magnetite nanoparticles. Insets show the zero coercivity and remanence, which is the indication of superparamagnetism.

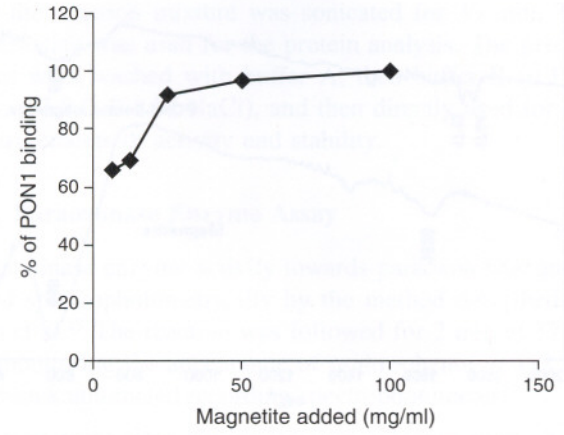

Fig. 6. Effect of the amount of nanoparticles added on the percentage of PON1 binding. [PON1] = 3.4 mg/ml.

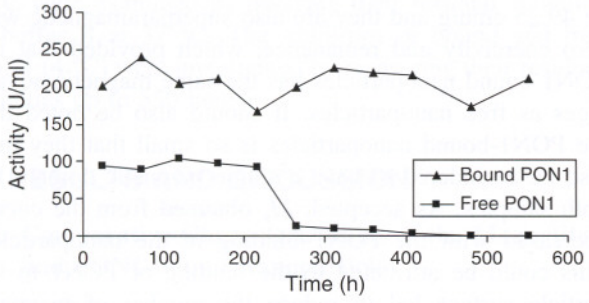

Fig. 7. Storage stabilities of the bound and the free PON1 at 4 °C.

to Fe_3O_4 nanoparticles could be determined to be 0.136. When the weight ratio of PON1 to Fe_3O_4 was below 0.136, PON1 could be completely bound to Fe_3O_4 nanoparticles. The binding of PON1 to Fe_3O_4 nanoparticles in this work has been achieved at a level of monomolecular binding.

3.3. Activity and Stability

Figure 7 indicates the activities of PON1 bound on 30 mg Fe_3O_4 nanoparticles. Under this condition, PON1 was completely bound according to investigation on binding efficiency. The activity of immobilized PON1 was two times higher than that of free enzyme. Figure 7 also shows that the storage stabilities of bound and free PON1 at 4 °C in semi-log plot. After the incubation time of 250 h, while the residual activity of the free enzyme was decreased to 10 EU/ml per min, the activity of the bound PON1 retained nearly 70% at 4 °C. This revealed that the storage stability of PON1 was improved significantly after being bound to Fe_3O_4 nanoparticles.

4. CONCLUSIONS

PON1 enzyme was directly bound to them via carbodiimide activation, which may be the first immobilization in literature to our knowledge. The magnetite nanoparticles and PON1-bound magnetite nanoparticles were comparatively studied and also the enzyme activity towards paraoxon was spectrophotometrically quantified. Magnetic measurements showed that the PON1-bound nanoparticles have the same benefits of superparamagnetic properties of zero coercivity and remanence. It is seen that the PON1 bound nanoparticles retained 70% of its original activity of free enzyme and exhibited significantly better storage and stability. Hence, the use of magnetic nanoparticles as support material was realized successfully. Since immobilized PON1 can be selectively separated from a reaction mixture by the application of a magnetic field produced by a permanent magnet, the results of the study has a significant importance to use in diverse potential application of PON1 enzyme.

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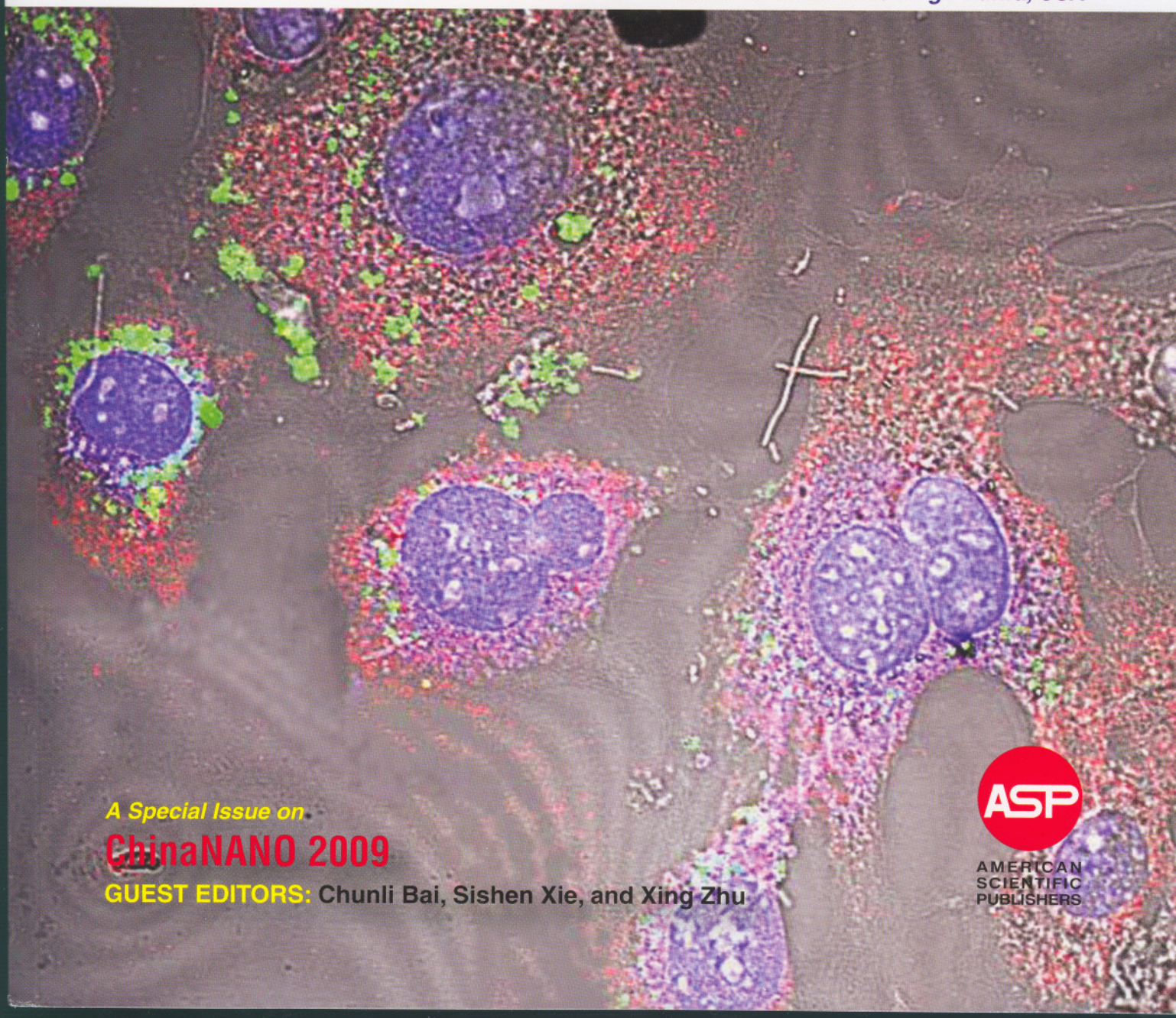

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