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Paper-Based Colorimetric Sensor Utilizing Peroxidase-Mimicking Magnetic Nanoparticles Conjugated with Aptamers

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***Abstract*—**We developed a paper-based colorimetric sensor utilizing magnetic nanoparticles conjugated with aptamers (MNP-Apts) against *E. coli* O157:H7. The MNP-Apts were applied to a test sample solution containing the target cells, and the solution was simply dropped onto PVDF (polyvinylidene difluoride) membrane. The membrane moves the sample radially to form the sample spots of different compounds as concentric rings, thus the MNP-Apts on the membrane enabled specific recognition of the target cells through a color ring generation by MNP-promoted colorimetric reaction of TMB (3,3',5,5'-tetramethylbenzidine) and H_2O_2. This method could be applied to rapidly and visually detect various bacterial pathogens in less than 1 h without cell culturing.

***Keywords*—**Aptamer, colorimetric sensor, *E. coli* O157:H7, magnetic nanoparticle, polyvinylidene difluoride.

I. Introduction

MAGNETIC nanoparticles (MNPs) possess intrinsic enzyme mimetic activities similar to those of natural peroxidases so that recently received significant attention owing to their effective applications in colorimetric sensors for various biomolecules [1]-[4]. MNPs could be suitable to be utilized in point-of-care testing (POCT) based on their superior characteristics that include controlled low cost large scale synthesis, catalytic stability over a wide range of temperatures and pHs, and convenient separation by application of a magnetic field.

Radial chromatography (RC) is one of the paper-based chromatography procedures which run on a piece of specialized filter paper. RC system acts as a stationary phase on which the separation of compounds occurs. The principle of RC system can be adsorption chromatography between solid and liquid phases, wherein the stationary phase is the solid surface of paper and the liquid phase is of mobile phase under the capillary action of pores in the paper. Therefore, RC is one of the most efficient tools for the fast analytical and preparative separation of complex samples, especially advantageous in the preparation of biological samples and initial separation of complex samples [5]-[7].

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Bacterial pathogens seriously threaten public health worldwide and they are responsible for the majority of illnesses and deaths. The detection of bacterial pathogens is vital for food safety, potable water, clinical diagnosis, and strategies for combating bioterrorism agents [8]. For early prevention of bacterial pathogen's infections, new alternative approaches are demanded to develop simple and time-saving methods. With the aim of developing new method, we have devised a colorimetric sensing system that rapidly detects *E. coli* O157:H7 by using colorimetric signals generated from peroxidase-mimicking MNPs on PVDF membrane. The MNPs, conjugated with aptamers against *E. coli* O157:H7, were found to exhibit excellent selectivity for the target cells. Moreover, by employing the new method that utilizes RC, the presence of the target cells in a test sample can be visually determined by the blue color ring generation arising from the MNPs-promoted color production on the membrane.

II. Experimental Section

A. Synthesis of Fe_3O_4 MNPs

MNPs were synthesized using the previously reported co-precipitation method, a simple and convenient way to synthesize iron oxides (Fe_3O_4) [9], [10]. Briefly, 1 M sodium hydroxide solution was rapidly added to a mixture of 0.25 M $FeCl_2$ and $FeCl_3$ ($Fe^{3+}/Fe^{2+} = 2$) in water with stirring at 80 °C for 40 min (pH 10). After cooling to room temperature, the precipitate was collected, washed several times with water and then with 70% ethanol, and dried at 70 °C under vacuum. Iron (II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), Iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

B. Surface Modification and Functionalization of MNPs

Amine-modified MNPs were synthesized following the procedure described by [11]. 5 g MNPs were suspended in 10 mL of methanol and a toluene solution (volume ratio 1:1) containing 10 µL of 3 mM 3-(aminopropyl)triethoxysilane (APTES) (Sigma-Aldrich, St. Louis, MO, USA) was added followed by vigorous stirring at 80 °C for 20 h under a N_2 atmosphere. The precipitate was collected and washed several times with methanol by using magnetic separation and dried at 50 °C under vacuum. To introduce glutaraldehyde moieties into the amine-modified MNPs, the standard protocol given by Bangslabs (Bangs Laboratories, Inc., Fishers, IN, USA) was followed. First, 10 mg of amine-modified MNPs was washed several times with 5 mL of PBS solution (Sigma-Aldrich, St.

Louis, MO, USA). The particle pellet was then re-suspended in 5 mL of 10% glutaraldehyde solution (Sigma-Aldrich, St. Louis, MO, USA) (final concentration of 2 mg/mL) and incubated for 1 h at room temperature with shaking. Glutaraldehyde-functionalized particles were collected, thoroughly washed with PBS solution to completely remove unreacted glutaraldehyde,

C. Immobilization of Streptavidins

The glutaraldehyde-functionalized MNPs were incubated with 10 mg of streptavidin in PBS solution at 4 °C overnight. The streptavidin-immobilized MNPs were then collected and washed several times with PBS solution containing 1% BSA (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 1 h to block nonspecific binding sites. The size and morphology of the synthesized MNPs were determined by using high-resolution transmission electron microscopy (JEOL, Tokyo, Japan) operated at an accelerator voltage of 200 keV.

D.Preparation of Aptamer-Conjugated MNPs

Biotin-conjugated aptamers (5′-biotin-CCG GAC GCT TAT GCC TTG CCA TCT ACA GAG CAG GTG TGA CGG-3′) were purchased from Bioneer (Deajeon, Korea). In order to conjugate the aptamers to the streptavidin-immobilized MNPs, 100 μL of 100 μM biotin-conjugated aptamers were incubated with 900 μL of streptavidin-conjugated MNPs (10 mg/mL) for 30 min at room temperature. The aptamer-conjugated MNPs (MNP-Apts) were then collected and washed several times with PBS solution by using magnetic separation. Particle size distribution analysis of the MNP-Apts was performed by measuring dynamic light scattering (Zetasizer Nano ZS, Malvern, UK). One milliliter of particle solution (1 mg/mL) was placed in a polystyrene cuvette, and the sample was scanned for 9 min (three runs) to obtain one set of raw data. The average values of the particle diameters were finally determined with at least three repeated measurements per sample. Bare MNPs and MNP-Apts were stained with DNA fluorometric dye, SYBR Green II (1X) (Molecular Probes, MA, USA), to confirm the conjugation of the aptamers to MNPs. The fluorescence intensities of 500 μg/mL of bare MNPs and MNP-Apts were measured using a Tecan Infinite M200 pro-microplate reader (Mnnedorf, Switzerland) and transparent 96-well plate (Corning, NY, USA).

E. Peroxidase Activity and Selectivity Tests

The peroxidase activities of both bare MNPs and MNP-Apts were examined by measuring absorbance after adding a solution containing 100 μL of 100 mM TMB (3,3',5,5'-tetramethylbenzidine) (Sigma-Aldrich, St. Louis, MO, USA) and 50 μL of 3 M H_2O_2 (Sigma-Aldrich, St. Louis, MO, USA) to the corresponding wells containing each bare MNPs and MNP-Apts (0, 40, 80, 160, and 320 μg/mL), followed by incubation for 20 min at room temperature. 10^3-10^8 cells of *E. coli* O157:H7 were mixed with 500 μg/mL of MNP-Apts in 1.5 mL tube, followed by incubation for 30 min at room temperature. The cells bound with the MNP-Apts were then collected by application of an external magnetic field, and they were used for PCR using primer sets (5'-CTT CTG CTG GCG CGA AGT TAA-3' and 5'-GAA GCT GAA TCA GCA GCC TGC-3'). 1 mL solution containing 10^4 cells incubated with 500 μg/mL of MNP-Apts passed through nitrocellulose membrane filter which has 13 mm of diameter and 0.45 μm of pore size (Millipore, MA, USA). After washing the filter with 1 ml of 1X PBS solution, the filter was absolutely dried for 48 hours and analyzed by field emission scanning electron microscopy using PHILIPS-XL30SFEG microscope (Philips, Amsterdam, Netherlands), operated at an accelerating voltage of 10 keV.

F. Paper-Based Colorimetric Detection of E. coli O157:H7

100 μL of a test sample solution containing 2 x 10^8 cells and 100 μL of 100 μg/mL MNP-Apts were mixed in 1.5 mL tube and incubated for 30 min at room temperature. 10 μL of the mixture solution was then dropped onto the center of a PVDF membrane which has 13 mm of diameter and 0.45 μm of pore size (Millipore, MA, USA). 10 μL of a solution containing TMB and H_2O_2 was then dropped onto the center of the previously dropped area. After incubation of the membrane for 20 min at room temperature, the colorimetric change was observed by the naked eyes. The cells on the membrane were stained with propidium iodide (LIVE/DEAD BacLight Bacterial Viability Kit, Molecular Probes, MA, USA) which is red-fluorescent nucleic acid stain and penetrates only bacteria.

III. Results and Discussion

A. Overall Procedures of the New Colorimetric Sensing System

To develop the new colorimetric sensing system that simply and rapidly detects *E. coli* O157:H7, MNPs as peroxidase mimics were conjugated with aptamers against *E. coli* O157:H7. The aptamer-conjugated MNPs (MNP-Apts) were introduced to a test sample containing the target cells and incubated for 30 min at room temperature. A mixture solution of the cells and MNP-Apts was then dropped onto the center of a circular PVDF membrane and absorbed toward outside of the membrane under the capillary action. After few minutes for drying the membrane, a mixture solution of TMB and H_2O_2 was dropped onto the same site of the previously dropped area. By the MNP-promoted blue color producing reaction, the presence of the target cells was visually recognized by the blue ring generation along with a mobile phase of the cells on the membrane (Fig. 1).

B. Characterization of the MNP-Apts

MNPs used in the sensing system were synthesized by using a co-precipitation method and modified with APTES to introduce reactive amine groups on the surface. The resulting MNPs were then treated with glutaraldehyde, a cross-linker that reacts with the amine groups on the activated MNPs and then enables immobilization of streptavidins on the surface of the MNPs through formation of stable amide linkages. The streptavidin-immobilized MNPs were then conjugated with biotin-conjugated aptamers as receptors for *E. coli* O157:H7. The morphology of streptavidin-immobilized MNPs were observed by TEM imaging (Fig. 2 (a)), and the particle size

distributions of MNP-Apts were analyzed by measuring dynamic light scattering (Fig. 2 (b)). As a result, MNP-Apts showed narrow particle size distributions around 100 nm in water, indicating that MNPs are well dispersed in aqueous solution without any significant aggregation. In order to investigate the conjugation of aptamers to the streptavidin-immobilized MNPs, bare MNPs and MNP-Apts were stained using a DNA fluorometric dye (SYBR Green II) and fluorescence spectroscopy was employed. The dye has been known to emit fluorescence signal at λmax=520 nm when forming dye-DNA complex, and was thus used for single-stranded DNA tracing. Fig. 1 (c) shows significantly high fluorescent intensity of MNP-Apts depending on the conjugation of aptamers.

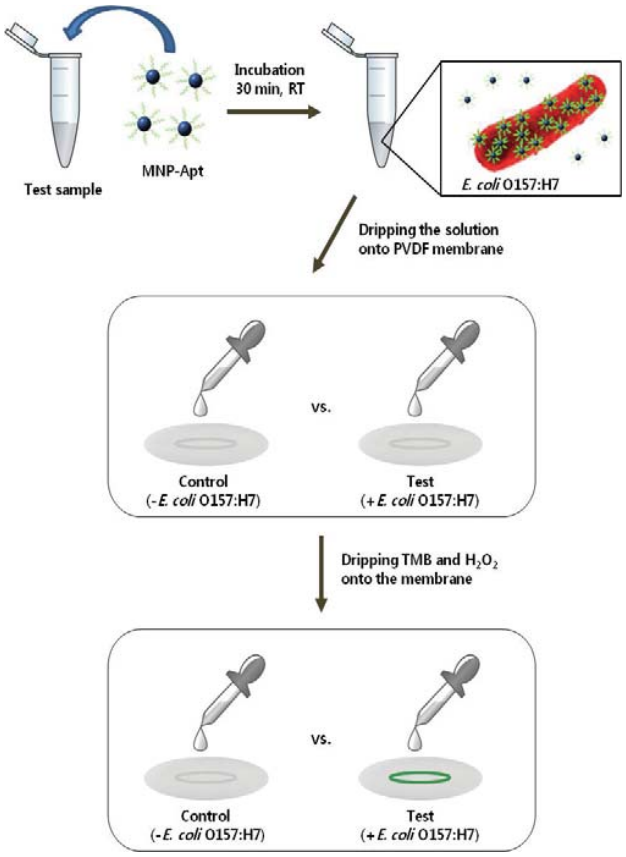

Fig. 1 Overall scheme of paper-based colorimetric detection of *E. coli* O157:H7

C. Evaluations of Peroxidase Activity and Selectivity of the MNp-Apts

We confirmed that blue color signals were generated when TMB substrate and H_2O_2 were introduced into the wells containing various concentrations (0, 40, 80, 160, and 320 ug/ml) of both bare MNPs and MNP-Apts (Fig. 3). As a result, signal intensities of the MNP-Apts were nearly equal to the intensities of the bare MNPs. The results demonstrate that the conjugation of aptamers on the surface of MNPs would not resist MNPs-induced colorimetric reaction of TMB and H_2O_2. SEM and energy dispersive X-ray (EDS) analyses revealed the selectivity of MNP-Apts for *E. coli* O157:H7 (Fig. 4). SEM image shows the aggregation of MNP-Apts on the surface of *E. coli* O157:H7 (Fig. 4 (a)). The composition of which was further probed using EDS analysis and EDS of two selected areas (cell and membrane surface) of the SEM image contained a peak associated with Fe along with other bands that can be assigned to the cells, membrane, and reagents (Fig. 4 (b)). The presence of peaks for Fe on the surface of the cell indicates that the MNP-Apts were selectively attached to the cell surface.

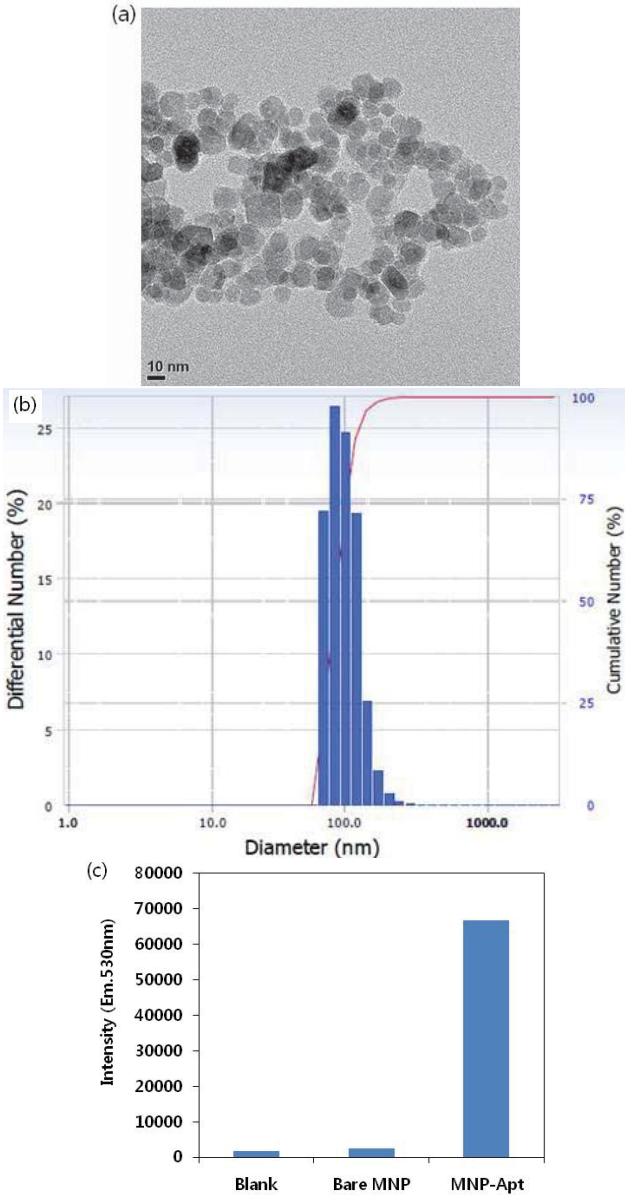

Fig. 2 (a) TEM image of streptavidin-immobilized MNPs (scale bar = 10 nm), (b) Particle size distribution analysis by measuring dynamic light scattering of aptamer-conjugated MNPs (MNP-Apts), (c) Fluorescence intensity results of blank, bare MNPs, and MNP-Apts at 520 nm

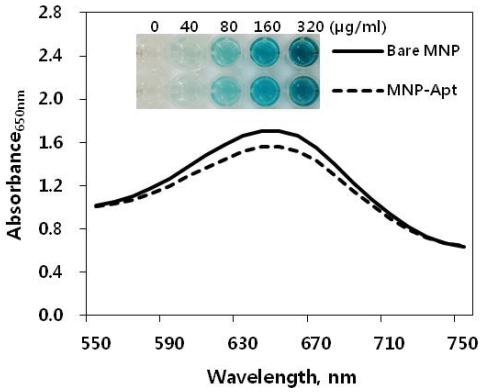

Fig. 3 Absorption spectra of 320 ug/mL of bare MNPs and MNP-Apts. The well plate image (inset) for the colorimetric reaction of various concentrations (0, 40, 80, 160, and 320 ug/ml) of bare MNPs and MNP-Apts

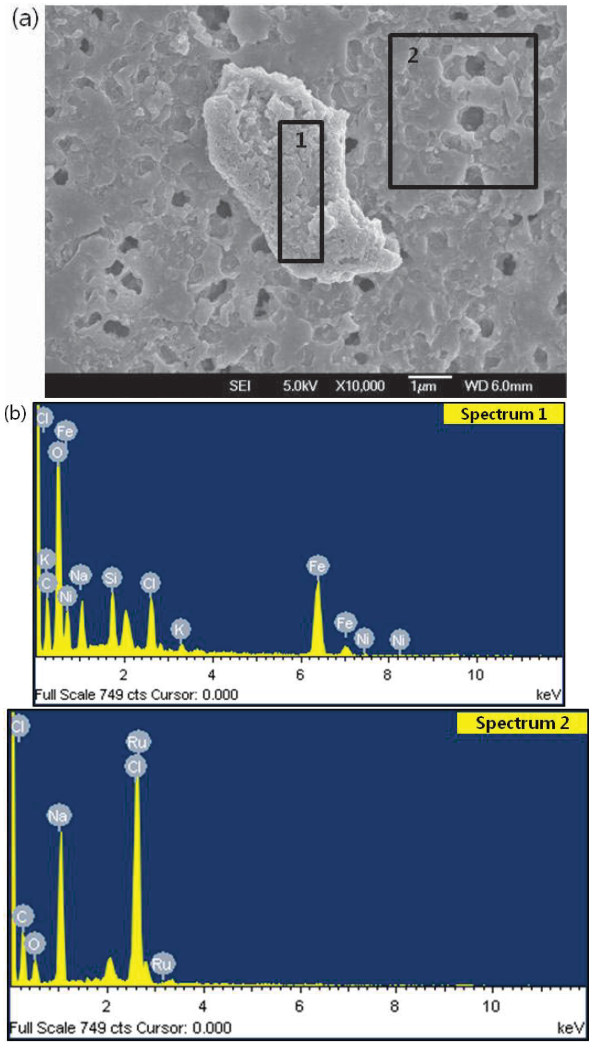

Fig. 4 (a) SEM image of *E. coli* O157:H7 cell bound with MNP-Apts, (b) EDS pattern of the cell surface with MNP-Apts (spectrum 1) and the membrane surface (spectrum 2)

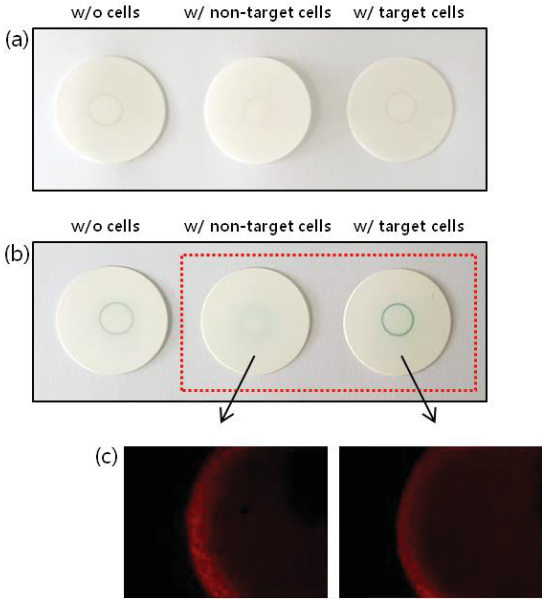

Fig. 5 (a) The circular PVDF membrane image after dripping MNP-Apts without cells, MNP-Apts with non-target cells, and MNP-Apts with target cells onto the membranes, (b) The circular PVDF membrane image after dripping a mixture solution of TMB and H_2O_2 onto the membranes of Fig. 5 (a), followed by colorimetric reaction for 20 min at room temperature, (c) Fluorescent image analysis after cell staining of the non-target cells and target cells on the corresponding membranes of Fig. 5 (b)

D.Paper-Based Colorimetric Detection of E. coli O157:H7

To demonstrate ability of the sensor system detecting *E. coli* O157:H7 on PVDF membrane by utilizing RC, three different samples, blank, non-target cells, and target cells, were incubated with MNP-Apts for 30 min at room temperature, and each sample was then dropped onto the corresponding membranes (Fig. 5 (a)). After further dropping of s mixture solution of TMB and H_2O_2, followed by incubation for 20 min at room temperature, blue color ring was generated on the membrane containing MNP-Apts with the target cells (Fig. 5 (b)). Interestingly, by using cell staining and fluorescent image analysis, the dense cells forming ring shape were observed on both membranes containing non-target cells and target cells (Fig. 5 (c)). However, blue color ring generation by MNP-promoted colorimetric reaction did not occur on the membrane containing non-target cells as shown in red box of Fig. 5 (b). These results demonstrate that MNP-Apts applied to the non-target cells randomly spread all over the paper with liquid phase of the dropped solution without specific binding with the cells. On the other hand, pale blue ring was generated on the membrane containing MNP-Apts only, since the particles could uniformly spread with forming the ring shape without any hindrance by large mass such as cells.

IV. Conclusions

The studies described above have led to the development of a new paper-based colorimetric sensor that is composed of aptamer-conjugated MNPs and PVDF membrane. We

demonstrated that by using this methodology *E. coli* O157:H7, serving as a model target, may be selectively identified through a color-generating reaction of TMB with H_2O_2 promoted by the peroxidase mimicking MNPs. This paper-based sensor system displayed highly simple and rapid detection of the target cells, along with the production of a color signals at the paper that can be recognized by the naked eyes. The advantageous features of the new system suggest that it will have potential applications as a POCT sensor for the verification of various bacterial pathogens.

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References

[1] K. S. Park, M. I. Kim, D. Y. Cho, and H. G. Park, "Label-free colorimetric detection of nucleic acids based on target-induced shielding against the peroxidase-mimicking activity of magnetic nanoparticles," *Small*, vol. 7, pp. 1521-1525, Apr. 2011.

[2] M. I. Kim, Y. Ye, B. Y. Won, S. Shin, J. Lee, and H. G. Park, "A highly efficient electrochemical biosensing platform by employing conductive nanocomposite entrapping magnetic nanoparticles and oxidase in mesoporous carbon foam," *Adv. Funct. Mater.*, vol. 21, pp. 2868-2875, May 2011.

[3] M. I. Kim, J. Shim, T. Li, M. A. Woo, D. Cho, J. Lee, and H. G. Park, "Colorimetric quantification of galactose using a nanostructured multi-catalyst system entrapping galactose oxidase and magnetic nanoparticles as peroxidase mimetics," *Analyst*, vol. 137, pp. 1137-1143, Mar 2012.

[4] M. A. Woo, M. I. Kim, J. H. Jung, K. S. Park, T. S. Seo, and H. G. Park, "A novel colorimetric immunoassay utilizing the peroxidase mimicking activity of magnetic nanoparticles," *Int. J. Mol. Sci.*, vol. *14*, pp. 9999-10014, May 2013.

[5] A. I. Liapis, "Theoretical aspects of affinity chromatography," *J. Biotechnology*, vol. 11, pp. 143-160, Aug. 1989.

[6] T. Sun, G. Chen, and Y. Liu, "Purification of human prothrombin from Nitschmann fraction III: using DEAE membrane radial flow chromatography," *J. Chromatogr. B*, vol. 742, pp. 109-114., May 2000.

[7] C. Cabanne, M. Raedts, E. Zavadzky, and X. Santarelli, "Evaluation of radial chromatography versus axial chromatography, practical approach," *J Chromatogr B.*, vol. 845, pp. 191-199, Jan. 2007.

[8] R. R. Hu, Z. Z. Yin, Y. B. Zeng, J. Zhang, H. Q. Liu, Y. Shao, S. B. Ren, and L. Li, "A novel biosensor for *Escherichia coli* O157:H7 based on fluorescein-releasable biolabels," *Biosensors and Bioelectronics*, vol. 78, pp. 31-36, Apr. 2016.

[9] R. V. Mehta, R. V. Upadhyay, S. W. Charles, and C. N. Ramchand, "Direct binding of protein to magnetic particles," *Biotechnol. Tech.*, vol. 11, pp. 493-496, July 1997.

[10] A. H. Lu, E. L. Salabas, and F. Schüth, "Magnetic nanoparticles: Synthesis, protection, functionalization, and application," *Angew. Chem. Int. Ed. Engl.*, vol. 46, pp. 1222-1244, Feb. 2007.

[11] F. Gao, B. F. Pan, W. M. Zheng, L. M. Ao, and H. C. Gu, "Study of streptavidin coated onto PAMAM dendrimer modified magnetite nanoparticles," *J. Magn. Magn. Mater.,* vol. 293, pp. 48-54, Mar. 2005.