Anal. Chem. **2010,** *82,* 1186–1190

Letters to *Analytical Chemistry*

Thermally Addressed Immunosorbent Assay for Multiplexed Protein Detections Using Phase Change Nanoparticles

Liyuan Ma,[†] Chaoming Wang,[†,‡] Yan Hong,[†,‡] Minghui Zhang,[†,§] and Ming Su*[,†,‡]

NanoScience Technology Center and Department of Mechanical, Materials, and Aerospace Engineering, University of Central Florida, Orlando, Florida 32826, and College of Chemistry, Nankai University, Tianjin, 300071 China

Thermally addressed immunoassay is developed to detect multiple proteins using phase change nanoparticles as thermal barcodes. The solid to liquid phase changes of nanoparticles absorb heat energy and generate sharp melting peaks, which are used as thermal signatures to determine the existence and concentration of proteins. Multiple proteins can be detected by using different types of nanoparticles in order to create a one-to-one correspondence between one type of nanoparticle and one type of protein. The fusion enthalpy that is proportional to the amount of phase change materials has been used to derive the amount of protein. The melting temperatures of nanoparticles are designed to be higher than 100 °C to avoid interference from species contained in the fluid. Thus, the use of thermal nanoparticles allows the detection of multiple low concentration proteins in a complex fluid such as cell lysate regardless of the color, salt concentration, and conductivity of the sample.

Detecting protein biomarkers in complex fluids such as blood, urea, or cell lysate offers great benefits in sample preparation and target retention for early disease detection.[1−3] Enzyme-linked immunosorbent assay (ELISA) relies on antigen−antibody interaction to detect antibodies (i.e., proteins), and the binding events are readout through enzyme activities or the physical, chemical, or spectroscopic labels attached on the antibodies at different sites.[4−6] Because of the small sizes, nanoparticles have intimate contacts with target proteins in solutions and have been used to detect specific antigen−antibody interaction,[7−10] where the sensitivity is enhanced by amplifying the physical signatures of nanoparticles. Although widely used, ELISA has some limits: (1) it requires extensive efforts and expensive agents to produce testing samples that are free of colored species, suitable pH, and salt concentration; species that interfere with the activity of enzyme should be removed; (2) the multiplicity is often low, even if nanoparticles are used as probes, which makes it hard to detect multiple proteins from a small amount of sample. In this aspect, although optical absorbances of metallic and semiconductor nanoparticles can be controlled in the range of 400−900 nm by changing diameters,[11] the absorbance or emission peaks in this region are wide (∼150 nm), which limits the number of biomarkers that can be detected in one assay due to peak overlap.[12] Metal or magnetic nanoparticles cannot be used to detect multiple proteins because different nanoparticles are not distinguishable by their electric or magnetic properties.[13−16] The electrochemical method can detect a few types of nanoparticles but the peaks are wide and the voltage range is narrow (few voltages), which limits its detection multiplicity.[17]

We describe a novel technique that depends on the solid−liquid phase transitions of nanoparticles to detect multiple proteins. In this thermally addressed immunosorbent assay (TAISA), pure metal or alloy nanoparticles with a variety of compositions and melting temperatures are made by the colloidal method and modified by antibodies. A solid surface (i.e., aluminum) is also

* To whom correspondence should be addressed. E-mail: mingsu@mail.ucf.edu.

[†] NanoScience Technology Center, University of Central Florida.

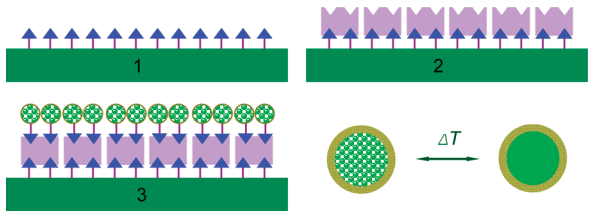
[‡] Department of Mechanical, Materials, and Aerospace Engineering, University of Central Florida.

[§] Nankai University.

(1) Jain, K. K. *Curr. Opin. Mol. Ther.* **2007**, *9*, 563.
(2) Diamandis, E. P. *J. Natl. Cancer Inst.* **2004**, *96*, 353.
(3) Sawyer, C. L. *Nature* **2008**, *452*, 548.
(4) Pita, M.; Cui, L.; Gaikwad, R. M.; Katz, E.; Sokolov, I. *Nanotechnology* **2008**, *19*, 375502.
(5) Nam, J. M.; Thaxton, C. S.; Mirkin, C. A. *Science* **2003**, *301*, 1884.
(6) Ferrari, M. *Nat. Rev. Cancer* **2005**, *5*, 161.
(7) Bao, Y. P.; Wei, T.-F.; Lefebvre, P. A.; An, H.; He, L.; Kunkel, G. T.; Muller, U. R. *Anal. Chem.* **2006**, *78*, 2055.
(8) Rosi, N. L.; Mirkin, C. A. *Chem. Rev.* **2005**, *105*, 1547.
(9) Alivisatos, P. *Nat. Biotechnol.* **2004**, *22*, 47.
(10) Han, M.; Gao, X.; Su, J.; Nie, S. *Nat. Biotechnol.* **2001**, *19*, 631.
(11) Zimmer, J. P.; Kim, S.-W.; Ohnishi, S.; Tanaka, E.; Frangioni, J. V.; Bawendi, M. G. *J. Am. Chem. Soc.* **2006**, *128*, 2526.
(12) Chan, W.; Maxwell, D.; Gao, X.; Bailey, R.; Han, M.; Nie, S. *Curr. Opin. Biotechnol.* **2002**, *13*, 40.
(13) Josephson, L.; Perez, J. M.; Weissleder, R. *Angew. Chem., Int. Ed.* **2001**, *40*, 3204.
(14) Park, S.-J.; Lazarides, A. A.; Mirkin, C. A.; Brazis, P. W.; Kannewurf, C. R.; Letsinger, R. L. *Angew. Chem., Int. Ed.* **2000**, *39*, 3845.
(15) Roberts, M. A.; Kelley, S. O. *J. Am. Chem. Soc.* **2007**, *129*, 11356.
(16) Zheng, G.; Patolsky, F.; Cui, Y.; Wang, W. U.; Lieber, C. M. *Nat. Biotechnol.* **2005**, *23*, 1294.
(17) Wang, J.; Xu, D.; Kawde, A.-N.; Polsky, R. *Anal. Chem.* **2001**, *73*, 5576.

10.1021/ac902724y
Published on Web 01/22/2010

Scheme 1. Thermally-Addressed Immunosorbent Assay



modified by antibodies;[18] the aluminum surface is immersed in a solution that contains the target antigen. After washing, the aluminum surface is immersed in a solution containing nanoparticle-labeled antibody (Scheme 1). In this sandwich detection configuration, the thermal property of the immobilized nanoparticles is directly measured rather than that of the antigen−antibody interaction. The signal transduction is based on a known but unexplored phenomenon: the temperature of a single component solid does not rise over its melting point until the entire solid is molten,[19] thus the melting peak of the solid is sharp after a linear temperature rise process. A one-to-one correspondence can be created between the melting point of one type of nanoparticles and one type of protein. The melting temperature and the heat flow are used to distinguish and quantify the nature and concentration of the protein. Because the melting temperature of the nanoparticles can be designed to be higher than those of the species contained in samples, the method is immune to colored or conductive species. In addition, the method can detect multiple proteins due to sharp melting peaks and multiple choices of phase change materials (i.e., metal, alloy, polymer, and salt). In a typical thermal scan from 20 to 700 °C, the number of different types of nanoparticles that can be detected in one run will reach 1000 if the peak width at half-maximum is 0.6 °C, meaning that the method has a high level of multiplicity.

EXPERIMENTAL SECTION

Synthesis of Phase Change Nanoparticles. All chemicals used in this experiment are obtained from Sigma-Aldrich. The phase change nanoparticles of pure metals and alloys such as indium, tin, and lead−tin alloy nanoparticles are prepared by thermal decompositions of organometallic precursors.[20−22] The precursors are dissolved, or two types of precursors are mixed at a stoichometric ratio and dissolved into ethylene glycol (EG) in the presence of polyvinyl-pyrrolidone (PVP). These precursors are decomposed at 200 °C under protection of nitrogen. After reacting for 30 min, the reactions are quenched by pouring the liquid into 200 mL of ethanol that is precooled at 0 °C. Then the nanoparticles are separated by using centrifugation, washed by ethanol three times, and dried with nitrogen flow for future use.

Nanoparticle Characterizations. A ZeissUltra 55 scanning electron microscope (SEM) working at an accelerating voltage of 10 kV is used to image nanoparticles that are dispersed from suspension onto a conductive silicon surface. The compositions of nanoparticles are confirmed by an energy dispersive X-ray (EDX) detector. A JEOL 1011 transmission electron microscope (TEM) that is operated at 100 kV is used to image the nanoparticles. The melting temperatures and the fusion enthalpies of nanoparticles are measured by using a differential scanning calorimeter (PerkinElmer DSC 7). A total of 5 mg of nanoparticles is sealed inside an aluminum pan and tested from 50 to 300 °C at a temperature ramp rate of 10 °C/min. An empty aluminum pan is used as reference to determine the difference in heat flow of the sample and the reference. The differential scanning calorimetry (DSC) experiments provide heat fluxes in both melting and crystallization processes, but only melting curves are used in this work. The enthalpies of fusion of the nanoparticles are derived from peak areas using the data analysis software of the DSC instrument. From the latent heats of fusion, the amount of phase change nanoparticles can be derived. The melting temperatures of indium, lead−tin, and tin nanoparticles are determined to be 156, 183, and 230 °C, respectively, which are the same as the bulk counterparts because of the large diameters.[23−25] Lead−tin alloy nanoparticles at the eutectic compositions have also been prepared by this method. Supporting Figure parts A−C in the Supporting Information show the TEM images of indium, lead−tin alloy, and tin nanoparticles. The crystallized structures of nanoparticles at room temperatures have been confirmed by selected area electron diffraction (SAED). Some of the characterization results such as SEM images, SAED patterns, and DSC curves can be found in our previous papers.[26]

Surface Modification of Nanoparticles. The surface modifications of nanoparticles are carried out as follows. The lead−tin nanoparticles are dried and heated to 100 °C in an oven in atmosphere, which produces thin layers of oxide around nanoparticles. The existence of a thin oxide layer has been verified by using energy dispersive X-ray analysis (EDX) (Supporting Figure part D in the Supporting Information). The oxide layer of the nanoparticle is amine-modified by incubating in 10% (aminopropyl)triethoxysilane (APTES) in toluene for 1 h at room temperature, which is followed by washing in toluene and in PBS solution. The surface modification of nanoparticles is confirmed by using fluorescent labeled bovine serum albumin proteins (BSA), which are covalently immobilized onto nanoparticles using a bifunctional cross-linker, disuccinimidyl suberate (DSS). Supporting Figure part E in the Supporting Information is a fluorescent image of BSA modified lead−tin nanoparticles. Furthermore, we have shown the surface oxidation of lead−tin thin films at the same heating conditions, where the thin film is patterned to indicate fluorescent contrast. Lead−tin thin films are deposited onto silicon surfaces using electron beam evaporation. Then thin layers of photoresist are spun onto the films and exposed with UV light through a photomask that have patterns. After development of exposed portions, the films are treated in APTES vapor at 100 °C, which is followed by removing unexposed photoresist. In the next,

(18) Sebra, R. P.; Masters, K. S.; Bowman, C. N.; Anseth, K. S. *Langmuir* **2005**, *21*, 10907.
(19) Zalba, B.; Marin, J. M.; Cabeza, L. F.; Mehling, H. *Appl. Thermal Eng.* **2003**, *23*, 251.
(20) Wang, F.; Yang, R.; Yu, H.; Gibbons, P. C.; Buhro, W. E. *Chem. Mater.* **2008**, *20*, 3656.
(21) Foos, E. E.; Stroud, R. M.; Berry, A. D.; Snow, A. W.; Armistead, J. P. *J. Am. Chem. Soc.* **2000**, *122*, 7114.
(22) Wang, Y. L.; Xia, Y. N. *Nano Lett.* **2004**, *4*, 2047.
(23) Olson, E. A.; Efremov, Y. M.; Zhang, M.; Zhang, Z.; Allen, L. H. *J. Appl. Phys.* **2005**, *97*, 034304.
(24) Jiang, H.; Moon, K. S.; Dong, H.; Hua, F.; Wong, C. P. *Chem. Phys. Lett.* **2006**, *429*, 492.
(25) Dippel, M.; Maier, A.; Gimple, V.; Wider, H.; Evenson, W. E.; Rasera, R. L.; Schatz, G. *Phys. Rev. Lett.* **2001**, *87*, 095505.
(26) Ma, L.; Hong, Y.; Ma, Z.; Kaittanis, C.; Perez, J. M.; Su, M. *Appl. Phys. Lett.* **2009**, *95*, 043701.

fluorescent BSA proteins are immobilized onto amine-modified surfaces by DSS. The micropattern on the lead−tin film can be seen under a fluorescent microscope, confirming the oxidization and modification of the lead−tin film (Supporting Figure part F in the Supporting Information). Similar modifications have been done on thin films of tin and indium.

Surface Modification of Aluminum Surfaces. In order to immobilize ligands on surface, the native oxide film on an aluminum surface is modified by putting the surface in a small vial that contains 0.1 mL of APTES. The vial is then heated in an oven at 100 °C for 1 h. The APTES vapor will condense and react with oxide surface to form an amine-terminated monolayer. The EDX spectrum confirms the oxidation and modification of aluminum (Supporting Figure part G in the Supporting Information). The surface modification has been further confirmed by immobilizing fluorescent BSA proteins on an aluminum surface with micropatterns. After the surface was washed with dimethyl sulfoxide (DMSO), the amine-terminated surface is immersed in a DMSO solution containing DSS for 1 h. The surface is washed three times using DMSO and phosphate buffered saline (PBS), respectively, and incubated with fluorescent labeled BSA protein in PBS for 2 h. The unreacted proteins are removed by washing with PBS. Supporting Figure part H in the Supporting Information shows the fluorescent micropatterns on the aluminum surface.

Preparation of Cell Lysate. The MDA-MB-231 human breast cancer cells are obtained from American Type Culture Collection (ATCC, Manassas, VA). These cells are grown to confluence in T-75 tissue culture flasks using a protocol suggested by ATCC. Then the culture medium is removed, and the cells are washed with PBS (pH 7.4). The cells are lysed in 3 mL/flask of lysis buffer for 15 min at 4 °C with gentle rocking, where the lysis buffer is composed of 10 mM Tris-HCl at pH 7.6, 100 mM NaCl, 1 mM EDTA, and 1% Triton X-100. The detergent (Triton) is able to break cell membranes. The cell lysate is collected and centrifuged at 12 000 rpm for 10 min at 4 °C to remove debris. The supernatant of cell lysate is collected and stored in small aliquots at −20 °C until use. The total protein concentration of as-made cell lysate is determined to be 1.1 mg/mL using the DC Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Thermal Detections of Avidin in Buffers. The thermally address method is used to detect avidin in buffer by using phase change nanoparticles of indium and lead−tin alloy. The nanoparticles are modified to have amine groups. The aluminum surface with native oxide is also modified to have amine groups. The amine-modified nanoparticles and the aluminum surfaces are conjugated with biotins by incubating with amine reactive biotinylation reagent (NHS-LC-biotins) in anhydrous DMSO for 1 h at room temperature, which is followed by washing in DMSO and in PBS. The avidin detection is carried out by incubating the biotin modified aluminum surface in solutions that contain avidin at certain concentrations for 1 h. The surface is then incubated in biotin modified nanoparticles for 1 h after rinsing. Unconjugated nanoparticles are rinsed away, and immobilized ones are readout using DSC. Figure 1A shows the DSC curves of indium nanoparticles immobilized on aluminum surfaces at different avidin concentrations, where the thermal scan is carried out from 50 to

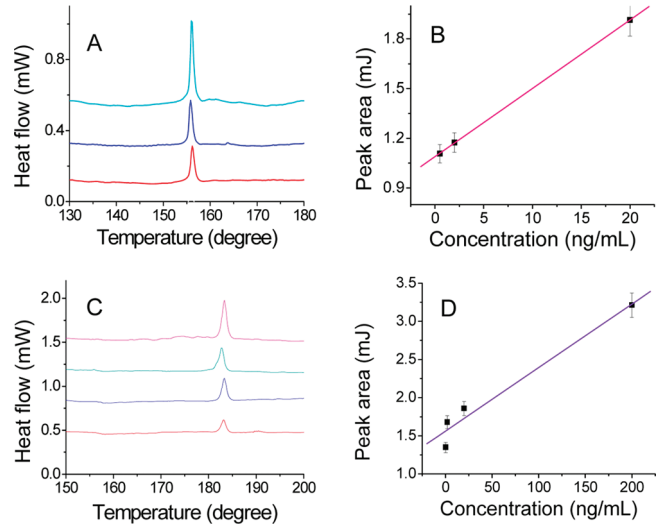


Figure 1. DSC curves (A) and concentration dependent heat flows (B) of indium nanoparticles that are immobilized on aluminum surfaces through biotin−avidin interaction, where the curves from up to down are at avidin concentrations of 20, 2, and 0.05 ng/mL, respectively; DSC curves (C) and concentration dependent heat flows (D) of lead−tin nanoparticles that are immobilized on aluminum surfaces through biotin−avidin interaction, where the curves from top to bottom are at avidin concentrations of 200, 20, 0.5, and 0.2 ng/mL, respectively.

300 °C at a ramp rate of 10 °C/min. The single melting peaks at 156 °C confirm that indium nanoparticles have been immobilized onto aluminum surfaces. The measured heat flows decrease as the concentrations of avidin decrease (Figure 1B). Similarly, we have also used lead−tin nanoparticles (atomic ratio of 63:37) to detect avidin in buffers. The avidin detection is carried out by incubating the biotin modified aluminum surface in solutions containing different concentrations of avidin for 1 h. The surface is incubated in biotin modified lead−tin nanoparticles for 1 h after rinsing. After the aluminum surface is rinsed, the thermal signatures of lead−tin alloy nanoparticles are read out using DSC at a ramp rate of 10 °C per min. Figure 1C shows the DSC curves of the lead−tin alloy nanoparticles immobilized on aluminum surfaces at different avidin concentrations, where nanoparticles melt at 183 °C, and the heat flows decrease linearly as avidin concentration reduces (Figure 1D).

Thermal Immunoassay in Buffers. The thermal immunoassay using phase change nanoparticles is carried out as follows. The amine-modified nanoparticles and the aluminum surface are conjugated to antirabbit IgG by incubating them with DSS in anhydrous DMSO in PBS (pH of 7.4) for 2 h. The nanoparticles and the aluminum surface are then washed by PBS to remove excess DSS. The antirabbit IgG modified aluminum surface is incubated in solutions containing rabbit IgG at a certain concentration for 1 h. The surface is incubated in antirabbit IgG modified lead−tin nanoparticles for 1 h after rinsing. After the aluminum surfaces are rinsed, the lead−tin nanoparticles are read out using DSC. Figure 2A shows the melting peaks of lead−tin nanoparticles immobilized at different concentrations of rabbit IgG. The cross-reactivity (selectivity) has been checked by human IgG (bottom curve), where 20 ng/mL human IgG does not lead to immobilization of nanoparticles. Figure 2B shows that the heat flow is proportional to the concentration of rabbit IgG.

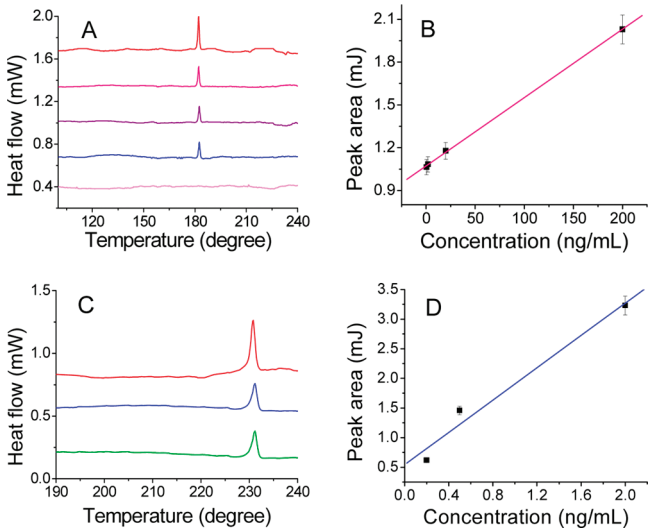


Figure 2. DSC curves (A) of lead−tin nanoparticles at 200, 20, 2, and 0.5 ng/mL (top to bottom) of rabbit IgG, 20 ng/mL of human IgG does not cause attachment of nanoparticles (flat line); the relation between the heat flow (peak area) and rabbit IgG concentration (B); DSC curves (C) of tin nanoparticles that are immobilized at 2, 0.5, and 0.2 ng/mL (top to bottom) of human IgG; the relation between the heat flow and human IgG concentration (D) after immobilization of tin nanoparticles.

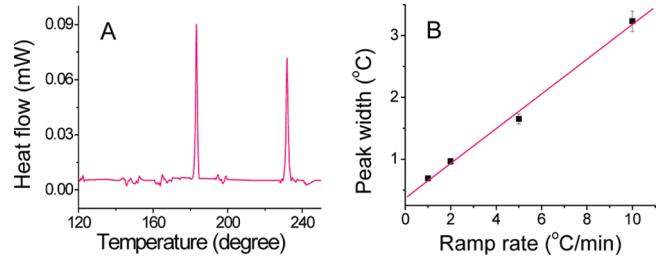


Figure 3. DSC curve of the multiplexed detection of 2 ng/mL of human IgG and 2 ng/mL of rabbit IgG using tin nanoparticles and lead−tin nanoparticles, respectively (A); ramp rate dependent peak width for indium nanoparticles (B).

In addition, human IgG in buffer is detected using tin nanoparticles in PBS (pH of 7.4). The surfaces of tin nanoparticles and aluminum surfaces are first modified with antihuman IgG. The antihuman IgG modified aluminum surface is incubated in solutions containing human IgG at a certain concentration for 1 h. The surface is incubated in antihuman IgG modified nanoparticles for 1 h after rinsing. Figure 2C shows the melting peaks of tin nanoparticles immobilized on aluminum surfaces at several human IgG concentrations, where the nanoparticles melt at 230 °C and the heat flows decrease as the concentrations of human IgG reduce (Figure 2D). Each DSC curve is flattened by using the commercial software of the DSC instrument to remove the slope, which is induced by a heat transfer difference between the sample cell and the reference cell.

Multiplexed Thermal Immunoassay in Buffers. The multiplicity of thermal detection is reflected in the simultaneous detection of rabbit IgG and human IgG, where lead−tin nanoparticles and tin nanoparticles are modified with anti-IgGs of the rabbit and human, respectively. In order to modify aluminum surfaces, both anti-IgGs of the rabbit and human are mixed at the same molar ratio and immobilized on amine-ended aluminum surfaces. The multiplexed detection is done by incubating the modified aluminum surface in a mixture that contains 2 ng/mL rabbit IgG and 2 ng/mL human IgG in PBS (pH 7.4) for 1 h. After the surface is rinsed, it is incubated in a mixture of two types of modified nanoparticles for 1 h. The aluminum surface is tested by DSC after rinsing for a second time. Figure 3A shows two melting peaks of tin and lead−tin nanoparticles at 183 and 230 °C, respectively. The difference in the heat flows of the two peaks may be induced by differences in latent heats of indium and lead−tin alloy with sizes of two types of nanoparticles or grafting densities of anti-IgGs on them. The one-to-one correspondence created between protein and melting temperature allows the detection of multiple proteins in one experiment.

The multiplicity of the thermal method is dependent on the sharpness of individual peaks due to the issue of peak overlap. Figure 3B shows the relation between the ramp rate and the peak width at half height of the indium nanoparticles, where the peak width is proportional to the ramp rate and the minimal peak width is 0.6 °C. Considering the composition-dependent melting temperatures of alloys, nanoparticles with different melting temperature can be designed to have a large number of melting peaks based on phase diagrams. The sharp melting peaks, combined with the large number of different melting peaks of nanoparticles, enhance the multiplicity of detection by operating DSC at a low ramp rate.

Thermal Immunoassay in Cell Lysate. The thermal immunoassay has been carried out in a complex cell lysate of human breast cancer cells. The cell lysate contains a certain amount of human IgGs released from cell membranes. At first, rabbit IgGs are added at a certain concentration in diluted cell lysates (total protein concentration is determined to be 1 μg/mL). The antirabbit IgG modified aluminum surfaces are incubated in solutions containing different concentration of rabbit IgG for 1 h. The surface is incubated with antirabbit modified nanoparticles for 1 h after rinsing. After the surface was rinsed again, the thermal signatures of the lead−tin nanoparticles are read out using DSC at a ramp rate of 10 °C/min. Figure 4A shows the DSC curves of lead−tin nanoparticles immobilized on aluminum surfaces at different rabbit-IgG concentrations, where the heat flows decrease as the concentration of rabbit IgG decreases (Figure 4B).

The concentration of human IgG in the diluted cell lysate (total protein concentration of 1 μg/mL) is determined by using antihuman IgG modified lead−tin alloy nanoparticles and aluminum surfaces. The surface modifications are carried out by using the same method as before. The antihuman IgG modified aluminum surface is incubated in the lysate for 1 h. The surface is incubated with antihuman IgG modified lead−tin nanoparticles for 1 h after rinsing. Then the aluminum surfaces are taken out, washed using PBS to remove excess nanoparticles, and analyzed using DSC at a ramp rate of 10 °C/min. Figure 4C shows the melting curve of lead−tin nanoparticles immobilized on the aluminum surface. From the peak area, the concentration of human IgG in the dilute lysate (1 μg/mL total protein) is determined to be 182 ng/mL. As a rough comparison, 20% of proteins contained in normal human serum are

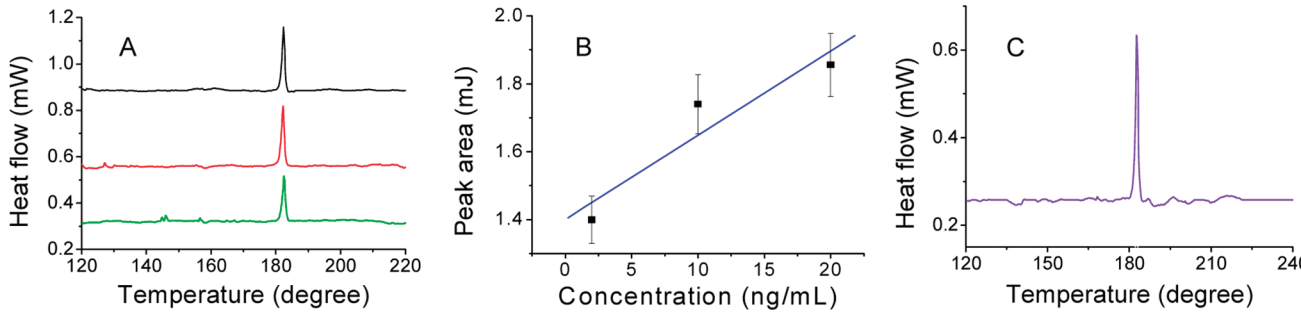


Figure 4. DSC curves (A) and concentration dependent heat flow (B) of lead−tin nanoparticles that are immobilized on aluminum surfaces by antirabbit-IgG and rabbit IgG interaction in diluted cell lysate, where rabbit IgG is intentionally added into the complex liquid at concentrations of 20, 10, and 2 ng/mL (top to bottom); DSC curve of lead−tin nanoparticles that are immobilized by human IgG contained in the diluted cell lysate (C).

IgG.[27] If this value is valid for the human cancer cells in this study, the estimated concentration of human IgG (with total protein concentration of 1 μg/mL) would be 200 ng/mL, which is at the same order as the value we have derived using lead−tin nanoparticles.

In conclusion, we have shown the multiplexed detection of proteins using solid−liquid phase change nanoparticles as thermally addressed barcodes. A one-to-one correspondence is established between the type of nanoparticles and the type of proteins, thus multiple proteins can be detected simultaneously by using a combination of nanoparticles. The sensitivity can be further enhanced using nanoparticles with a large latent heat of fusion and reducing the grafting density of the antibody on the nanoparticle. The melting peak and heat flow reflect the nature and concentration of the protein, respectively. The melting temperature of the nanoparticles can be designed to avoid interference from coexisting species in samples, thus bringing high sensitivity and multiplicity and sample preparation benefits to the early detection of proteins. At last, the method is generic and can be used for the detection of disease markers in various body fluids.

(27) Wang, L.; Mah, K. Z.; Ghosh, R. *Sep. Purif. Technol.* **2009**, *66*, 242.

ACKNOWLEDGMENT

This work is supported by National Science Foundation (Grant 0828466) and the King Biomedical Program of the Florida Department of Health. The characterizations have been done at the Materials Characterization Facilities (MCF) at the University of Central Florida (UCF).

SUPPORTING INFORMATION AVAILABLE

Characterization results of phase change nanoparticles. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review November 30, 2009. Accepted January 15, 2010.

AC902724Y