# Slight Temperature Changes Affects Protein Affinity and Cellular Uptakes/Toxicities of Nanoparticles

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**Synthesis of $Fe_3O_4$ nanoparticles and characterization.** Analytical grade of iron salts (iron chloride) and sodium hydroxide (NaOH) were purchased from Merck (Darmstadt, Germany) and were employed without further purification. Dextran with average $M_W$ of 5000 Da, dimethylsulfoxide, sodiumperiodate, potassium cyanide, and ammonium persulfate were purchased from Sigma-Aldrich (MO, USA). Carboxylated-dextran and amino-dextran were prepared according to the procedures reported by Usher et al. [1, 2] and Saboktakin et al.[3], respectively.

In order to ascertain de-oxygenation of de-ionized (DI) water, the DI water underwent bubbling with neutral gas (argon) for a time period of 30 minutes. The iron salts ($FeCl_3$ ((0.086 M (3 mL))) and $FeCl_2$ (0.043 M (3 mL))) were dissolved in separate beakers containing the de-oxygenated DI water. Obtained iron salt solutions were blended together, with a molar fraction adjusted to 2 ($Fe^{3+}/Fe^{2+}$). Dextrans (carboxylated-dextran, plain dextran, and amino conjugated dextran) were dissolved in the 10 mL of de-oxygenated DI water. The dextran/iron mass ratio was fixed at 2.[4] The dextran solutions were mixed with iron salt solutions (50:50 V:V (total volume of 10 mL)), and introduced into a three-neck flask equipped with a homogenizer stirring at 10,000 rpm. This procedure was done separately for the 3 types of dextrane solutions in order to prepare $Fe_3O_4$ NPs with various surface coatings and thus zeta potentiasl (*i.e.* negative (carboxylated), neutral (plain), and positive (amine conjugated) NPs). Actual formation of NPs was triggered *via* drop wise addition of base medium (NaOH) to the prepared dextran-iron salt mixtures with vigorous stirring under the argon protection.

In order to achieve a good size distribution the NPs in the flask were transferred to an ultrasonic bath (100 Watt) which created a turbulent flow.[5] In this case, the mass transfer rate[5] which may allow NPs to combine and build larger polycrystalline NPs, was decreased. After one hour, the black NP suspension was magnetically washed using a strong magnetic field gradient produced by a permanent Nd-Fe-B magnet and the dextran coated $Fe_3O_4$ NPs were recovered. The supernatant was completely removed and the $Fe_3O_4$ NPs were re-dispersed in DI water for multiple times. As additional step for the removal of excess ammonia (there was no excess ammonia in case NaOH was used, but if ammonium hydroxide was used there was ammonia), unreacted iron cations, and free dextran macromolecules, the obtained ferrofluid was dialyzed using a membrane bag with a 50 kDa molecular weight cut-off (MWCO) for 24 h. The resulting purrified ferrofluid was kept at 4°C.

The sizes of the inorganic core and the core-shell materials were determined with transmission electron microscopy (TEM) and atomic force microscopy (AFM), respectively (*cf.* Figure S1). Dynamic light scattering (DLS) measurements were conducted utilizing a Malvern zetasizer. The number distributions of the hydrodynamic diameter $d_h$ are shown in Figure S2 and Table S1.

**Interaction of SPIONs, with various physiochemical properties, with FBS protein at various temperatures.** There are many methods that have been developed for investigation of protein corona on the surface of various NPs.[6] Among these, centrifugation route has been recognized to provide reliable information on the gradient of the absorbed proteins on the surface of NPs.[7] However, the centrifugation method is suffering from the detachment of loose-attached proteins.[8] Therefore, the distinguished feature of our setup together with the magnetic properties of SPIONs is that there is no removal of loose-attachment proteins and the precise gradient of protein coronas could be achieved. The interactions of negative-, plain-, and positive-SPIONs with fetal bovine serum (FBS) at various temperatures were probed. Typically 100 µl of particles (with concentration of 100µg/ml were mixed with 900 µl of FBS, followed by incubation at 5, 15, 25, 35, 37, 39, 41, 43, and 45 centigrade degree for 1 h. Since the previous reports confirmed that the protein corona is formed in a relatively stable manner over a period of one hour[9], we have selected one hour for evaluation of protein coronas in our samples. In order to obtain SPIONs-"hard corona" composition, we have used magnetic separation method, after incubation time at desired temperatures. The proteins-SPIONs solutions were run through a strong magnetic field using magnetic-activated cell sorting (MACS®) system (see Figure S3a for details). In this case, the SPIONs were fixed inside the magnetic column and the flow-through fraction was removed. The fixed NPs were washed by 500 µl of PBS buffers and the fraction removed; it is notable that the washing process was designed in order to remove the excess (unbound or loosely bound) proteins. The fixed NPs were again washed by 500 µl of PBS buffers and the fraction stored. Finally, the column was removed from the magnetic field and SPIONs-hard corona

were fully removed and stored. It is notable that all stages were done in a low protein attachment Eppendorf tube.

**Definition of protein's affinities in hard corona composition at various temperatures.** In order to define protein's affinities in hard corona composition at various temperatures, variety of washing solutions, with various capability of protein removal, were employed. More specifically, the proteins with SPIONs were run through MACS® system. In this case, the SPIONs were fixed inside the magnetic column and the flow-through fraction was removed. The fixed NPs were washed by 500 µl of varies washing solutions, including PBS, 0.01, 0.1, 0.5, and 1 molar of KCl, respectively and stored. Finally, the column was removed from the magnetic field and NPs were fully removed and stored. Figure S3b shows the schematic of the experimental setup and the washing steps.

**SDS-PAGE.** In order to define the protein profiles of formed hard coronas on the surface of various SPIONs, 1D SDS-PAGE was employed. In this case, the SPIONs-HCs were re-suspended 40 µl of fresh PBS followed by addition of 20 µl loading buffer, containing 10% DTT.

**Differential centrifugal sedimentation (DCS) method.** Differential centrifugal sedimentation (DCS) experiments were performed with a CPS Disc Centrifuge DC24000. The analyzer measures particle size distributions using centrifugal sedimentation within an optically clear spinning disc which is filled with sucrose fluid, which has gradient of 8-24% sucrose in PBS at 22 000 rpm, during the measurements. The iron concentration of 100µg/ml was selected as optimal amount of SPIONs for DCS.

In order to define the thickness of protein corona at the surface of nanoparticles (NPs) using DCS data, a simple core-shell method was used, with dextran-coated SPIONs as the core and protein biomolecules as the shell, to calculate 2 densities using the following equation [10]:

$$\frac{(\rho_c-\rho_s)}{(\rho_c-\rho_f)}\frac{{D_c}^3}{D_s}+\frac{(\rho_s-\rho_f)}{(\rho_c-\rho_f)}{D_s}^2={D}^2 \qquad (1)$$

where $\rho_c$ is the density of the inner core, $\rho_s$ is the density of the shell, $\rho_f$ is the density of the fluid, $D_c$ is the diameter of the core, $D_s(D_c+2\delta)$ is the total diameter of the core-shell particles, and D is a diameter measured by the DCS method.

**Liquid chromatography mass spectrometry (LC-MS/MS) technique.** Samples were loaded onto a nanoAcquity UPLC system (Waters) equipped with a nanoAcquity Symmetry $C_{18}$, 5 µm trap (180 µm x 20 mm Waters) and a nanoAcquity BEH130 1.7 µm $C_{18}$ capillary column (75 µm x 250 mm, Waters). The trap wash solvent was 0.1% (v/v) aqueous formic acid and the trapping flow rate was 10 µL/min. The trap was washed for 5 min before switching flow to the capillary column. The separation used a gradient elution of two solvents (solvent A: 0.1% (v/v) formic acid; solvent B: acetonitrile containing 0.1% (v/v) formic acid). The flow rate for the capillary column was 300 nL/min Column temperature was 60°C and the gradient profile was as follows: initial conditions 5% solvent B, followed by a linear gradient to 30% solvent B over 125 min, then a linear gradient to 50% solvent B over 5 min, followed by a wash with 95% solvent B for 10 min. The column was returned to initial conditions and re-equilibrated for 30 min before subsequent injections. The nanoLC system was interfaced with a maXis LC-MS/MS System (Bruker Daltonics) with a nano-electrospray source fitted with a steel emitter needle (180 µm O.D. x 30 µm I.D., Proxeon). Positive ESI- MS & MS/MS spectra were acquired using AutoMSMS mode. Instrument control, data acquisition and processing were performed using Compass 1.3 SR3 software (microTOF control, Hystar and DataAnalysis, Bruker Daltonics). Instrument settings were: ion spray voltage: 1,500 V, dry gas: 6 L/min, dry gas temperature 160 °C, ion acquisition range: *m/z* 50-2,200. AutoMSMS settings were: MS: 0.5 s

(acquisition of survey spectrum), MS/MS (CID with $N_2$ as collision gas): ion acquisition range: *m/z* 300-1,500, 0.1 s acquisition for precursor intensities above 100,000 counts, for signals of lower intensities down to 1,000 counts acquisition time increased linear to 1s, the collision energy and isolation width settings were automatically calculated using the AutoMSMS fragmentation table:, 5 precursor ions, absolute threshold 1,000 counts, preferred charge states: 2 – 4, singly charged ions excluded. 1 MS/MS spectrum was acquired for each precursor and former target ions were excluded for 30 s. Tandem mass spectral data were submitted to database searching using a locally-running copy of the Mascot program (Matrix Science Ltd., version 2.3), through the Bruker ProteinScape interface (version 2.1). All spectra were search against the Swiss-Prot database restricted to mammalia sequences only (63,676 sequences). Search parameters specified: Enzyme; Trypsin, Peptide Mass Tolerance; 10 ppm, Fragment Mass Tolerance; 0.1, Fixed Modifications; Methylthio (C), Variable Modification; Oxidation (M). All peptide identifications were filtered to only accept expect values of 0.05 or lower. Empirically derived estimates of peptide false discovery rate are calculated from searches against a Mascot generated decoy database.

In order to obtain the total number of the MS/MS spectra for all of the peptides that are attributed to a matched protein, a semi-quantitative assessment of the protein amounts was conducted through application of spectral counting method. The normalized SpC amounts of each protein, identified in the MS study of smooth and jagged surfaces, were calculated by applying the following equation[11]:

$$NpSpC_k = \left( \frac{\left( SpC / (M_w)_k \right)}{\sum_{i=1}^{n} \left( SpC / (M_w)_i \right)} \right) \times 100 \qquad (2)$$

where $NpSpC_k$ is the normalized percentage of spectral count for protein k, SpC is the spectral count identified, and Mw is the molecular weight (in kDa) of the protein k. Using equation 1, one can expect to obtain the protein size and to evaluate the real contribution of each protein to the hard corona composition.[9]

**Cell culture and incubation.** HeLa cells were obtained from American Type Culture Collection (Mannassas, USA) and cultivated with RPMI1640 in a $CO_2$-incubator (37°C, 5% $CO_2$, water-saturated atmosphere).

**Nanoparticle uptake.** For uptake studies 75,000 HeLa cells per well were seeded in 12-well plates on cover slips. 24 hours later cells were intensively washed with PBS and incubated with 5 µg/ml NPs and 500 µl/ml Lucifer Yellow (Sigma-Aldrich) for 24 hours at 37°C. The cover slips were removed and transferred to poly-Lysine-coated glass slides and finally sealed with ImmuMount (Thermo Scientific). Lucifer Yellow incorporation was analysed with a fluorescence microscope with an excitation wavelength of 430 nm. Emission was recorded at 520 nm.

**Cell viability.** The viability of HeLa cells in response to the incubation with NPs was determined with the CellTiter-Glo-Assay Luminescent Cell Viability Assay (Promega). 15,000 HeLa cells were seeded into 96-well plates and cultivated overnight. After intensive washing the cells were incubated with culture medium and the NPs (10 µg/ml) for 6 hours. 100 µl of CellTiter-Glo reagent per well was added and shacked (orbital) for 2 minutes. After a 10-minute incubation at room temperature luminescence was measured with a plate reader infinite M200 (Tecan).

**ROS production.** For the determination of intracellular ROS levels the fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate ($H_2$DCF-DA) (Invitrogen) was used.[12] In the presence of intracellular esterases this nonpolar component is converted into the nonfluorescent polar derivative $H_2$DCF. This intermediate is membrane impermeable and rapidly oxidized to fluorescent 2',7-dichlorofluorescein (DCF) by ROS. For the experiments cells were seeded in 96-well plates overnight. Cells were incubated with the NPs (10 µg/ml) for 6 hours, and 50 µM $H_2$DCF-DA was added. After 30 minutes the intracellular DCF fluorescence was measured with a plate reader infinite M200 (Tecan) with an excitation wavelength of 485 nm. Emission was recorded at 535 nm. All data were corrected for background fluorescence.

**Nanoparticles uptake/Lysosome labeling.** HeLa cells were labeled with Image-iT™ LIVE lysosomal and nuclear labeling kit (Molecular Probes, Invitrogen), which provides a red-fluorescent LysoTracker® Red DND-99 dye for lysosome staining, and a blue-fluorescent Hoechst 33342 dye for staining the nucleus. Cells were seeded on coverslips andexposed to the culture media containing SPIONs at a concentration of 55.845 µg/ml (1 mM of iron). Cells-NPs were incubated at different temperatures (35, 37, and 39°C) for 24 hours. Control cells were incubated at the absence of SPIONs. After rinsing the cells with Hanks Buffered Salt Solution (HBSS), they were labeled with Image-iT™ LIVE lysosomal and nuclear labeling kit according to the supplier's instructions. Briefly, the cells were incubated for 5 minutes with 2 µg/ml of Hoechst 33342 solution, followed by 1-minute incubation with 100 nM of LysoTracker Red DND-99®. Cells were rinsed with HBSS (2X) after each dye. The living cells in HBSS were finally mounted on microscope slides and examinedusing a DM2000 Leica microscope (Leica Microsystems). Images were acquired by a Leica DFC 290 camera.

**Bio-TEM.** To further investigate the effect of temperature changes on SPIONs uptake, HeLa cells which were interacted with positive-SPIONs at various temperatures were examined using TEM. As shown in Figures S7, SPIONs were internalized by the HeLa cells, accumulating in the lysosomes. According to the TEM results, we conclude that the shape of lysosoms and their correspondence amount of NPs are strongly related to the incubation temperature. Moreover, the incubation temperature could be also considered as crucial factor for prediction of the interacellular fate of NPs. For instance, at the incubation temperature of 39°C, majority of NPs were accumulated in mitochondria.

**TABLE S1.**Inorganic core diameters $d_c$ and hydrodynamic diameters $d_h$ (in deionized water and PBS) as determined by TEM and DLS, respectively, of negatively charged, neutral, and positively charged $Fe_3O_4$ NPs.

| NP charge | $d_c$ [nm] | DI Water | | PBS | |
|---|---|---|---|---|---|
| | | $d_h$ [nm] | zeta potential [mV] | $d_h$ [nm] | zeta potential [mV] |
| - | 15.1 ± 4.9 | 43.8 ± 10.5 | -20.8 ± 0.3 | 32.7 ± 7.9 | -13.9 ± 0.6 |
| 0 | 22.1 ± 7.2 | 28.2 ± 6.0 | -10.5 ± 0.6 | 32.7 ± 9.7 | -6.8 ± 0.5 |
| + | 16.9 ± 5.2 | 43.8.7 ± 6.2 | +18.9 ± 0.4 | 78.8 ± 7.2 | +10.9 ± 0.7 |

**TABLE S2.** Representative Detached Proteins from Hard Corona Proteins Associated with Positive-SPIONs Which Are Prepared at 39°C, After Washing with PBS Solution, As Identified by LC MS/MS[a]

| Protein Name | Mw | NSpC |
|---|---|---|
| Alpha-2-macroglobulin | 164325 | 0.119058 |
| Serotransferrin | 79450 | 5.171169 |
| Serum albumin | 70858 | 65.16081 |
| Alpha-fetoprotein | 70015 | 1.397148 |
| Fibrinogen beta chain | 60055 | 2.663173 |
| Vitamin D-binding protein | 54595 | 1.075058 |
| Fibrinogen gamma chain | 51276 | 0.356018 |
| Alpha-1-antiproteinase | 46351 | 5.909249 |
| Alpha-2-HS-glycoprotein | 39038 | 12.02782 |
| Apolipoprotein A-I | 30258 | 1.939744 |
| Alpha-1-acid glycoprotein | 23398 | 4.180754 |

[a] Normalized spectral count (NSpC) values were calculated for each protein hit according to equation 2.

**TABLE S3.** Representative Detached Proteins from Hard Corona Proteins Associated with Positive-SPIONs Which Are Prepared at 39°C, After Washing with 0.01KCL Solution, As Identified by LC MS/MS[a]

| Protein Name | Mw | NSpC |
|---|---|---|
| Serum albumin | 70858 | 4.850053 |
| Fibrinogen beta chain | 60055 | 36.38537 |
| Fibrinogen gamma chain | 51276 | 5.944322 |
| Alpha-2-HS-glycoprotein | 39038 | 52.82008 |

[a] Normalized spectral count (NSpC) values were calculated for each protein hit according to equation 2.

**TABLE S4.** Representative Detached Proteins from Hard Corona Proteins Associated with Positive-SPIONs Which Are Prepared at 39°C, After Washing with 0.1KCL Solution, As Identified by LC MS/MS[a]

| Protein Name | Mw | NSpC |
|---|---|---|
| Complement C3 | 188377 | 0.171867 |
| Pregnancy zone protein | 164924 | 0.021812 |
| Alpha-2-macroglobulin | 164325 | 0.021891 |
| Ubiquitin carboxyl-terminal hydrolase | 147986 | 0.024308 |
| Collagen alpha-1 | 139681 | 0.025754 |
| Inter-alpha-trypsin inhibitor heavy chain | 106738 | 0.30332 |
| Plasminogen | 93364 | 0.15412 |
| Complement factor B | 86462 | 0.083211 |
| Serotransferrin | 79450 | 7.697199 |
| Prothrombin | 71610 | 0.100469 |
| Serum albumin | 70858 | 43.15268 |
| Alpha-fetoprotein | 70015 | 1.74689 |
| Kininogen-1 | 69720 | 0.361176 |
| Fibrinogen alpha chain | 67385 | 0.106769 |
| Fibrinogen beta chain | 60055 | 0.376454 |
| Cytochrome P450 2C5 | 55791 | 0.064478 |
| Alpha-2-antiplasmin | 54906 | 0.327588 |
| Vitamin D-binding protein | 54595 | 2.174397 |
| Alpha-1B-glycoprotein | 53980 | 1.33283 |
| Antithrombin-III | 52728 | 0.068224 |
| Factor XIIa inhibitor | 51875 | 0.069346 |
| Angiotensinogen | 51410 | 0.069973 |
| Alpha-1-antiproteinase | 46351 | 5.665542 |
| Endopin-1 | 46300 | 0.310783 |
| Pigment epithelium-derived factor | 46292 | 0.233127 |
| Fetuin-B | 43280 | 1.745459 |
| AMBP protein | 39945 | 0.630396 |
| Beta-2-glycoprotein 1 | 39284 | 0.366287 |
| Alpha-2-HS-glycoprotein | 39038 | 18.70623 |
| Lumican | 39008 | 0.09222 |
| Ecto-ADP-ribosyltransferase 4 | 36130 | 0.099566 |
| Apolipoprotein A-I | 30258 | 3.566636 |
| Alpha-1-acid glycoprotein | 23398 | 6.303515 |
| Tetranectin | 22498 | 0.159895 |
| Hemoglobin fetal subunit beta | 15941 | 2.482303 |
| Transthyretin | 15809 | 0.227548 |
| Hemoglobin subunit alpha | 15175 | 0.94822 |

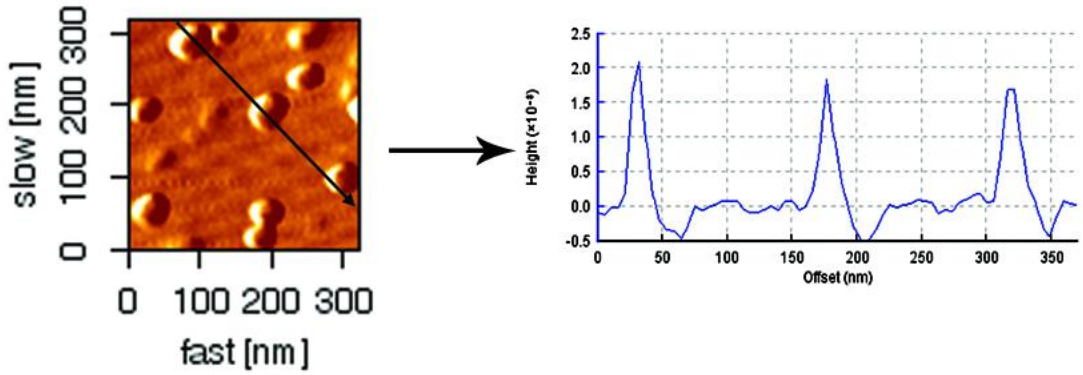
[a] Normalized spectral count (NSpC) values were calculated for each protein hit according to equation 2.

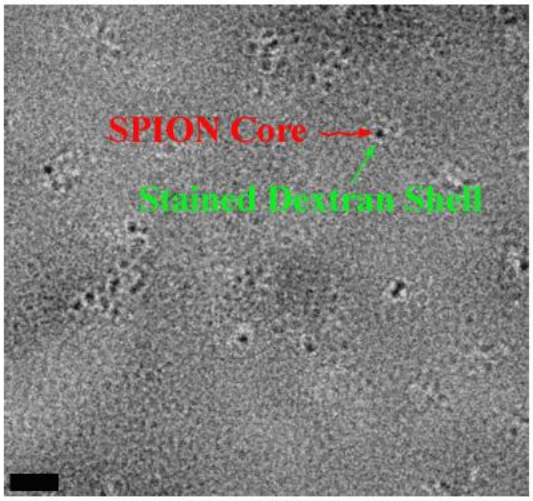
**TABLE S5.** Representative Detached Proteins from Hard Corona Proteins Associated with Positive-SPIONs Which Are Prepared at 39°C, After Washing with 1KCL Solution, As Identified by LC MS/MS[a]

| Protein Name | Mw | NSpC |
|---|---|---|
| Serotransferrin | 79450 | 2.249487 |
| Serum albumin | 70858 | 25.22252 |
| Alpha-1-antiproteinase | 46351 | 3.855834 |
| Alpha-2-HS-glycoprotein | 39038 | 68.67222 |

[a] Normalized spectral count (NSpC) values were calculated for each protein hit according to equation 2.

(a)

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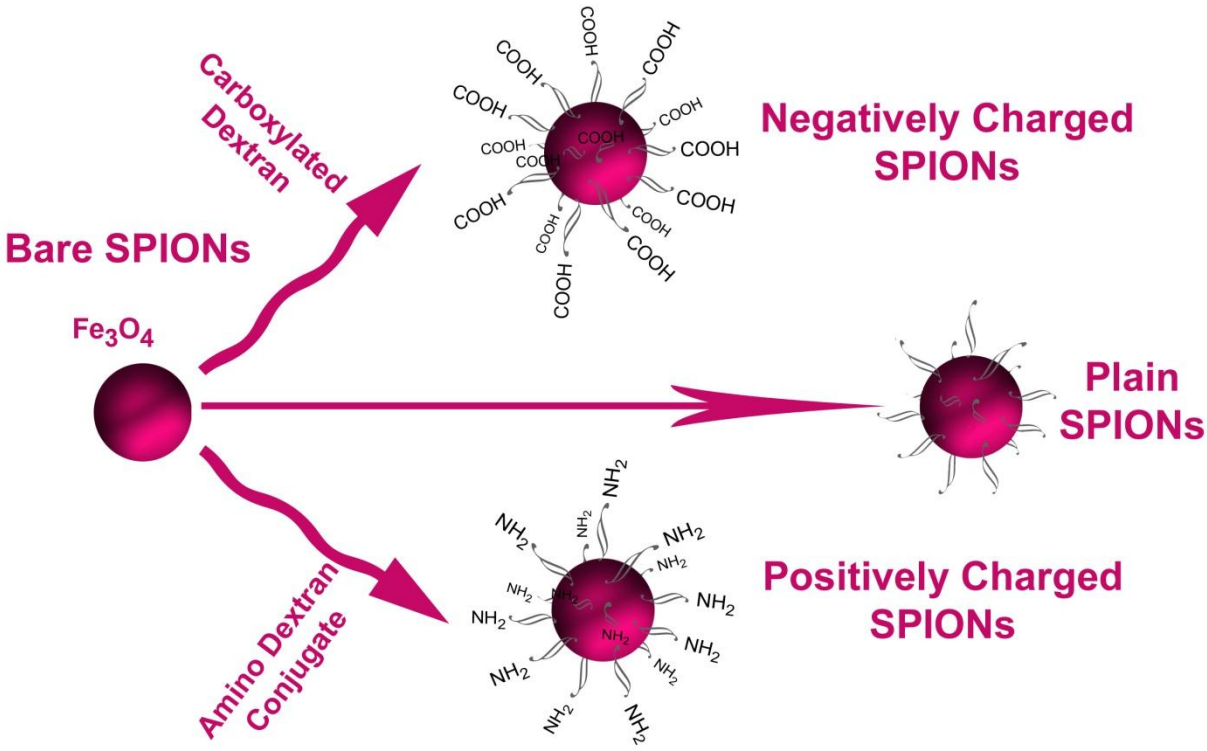



**(c)**

**Figure S1:** (a) AFM and its correspondence image profile of single coated SPIONs showing the formation of monodispersed dextran coated SPIONs; (b) TEM image of single-coated SPIONs (the scale bar is 20 nm). (c) Scheme showing the various prepared SPIONs. In-solution tryptic digestion was performed after reduction with Tris(2-carboxyethyl)phosphine hydrochloride and alkylation with S-methyl thiomethanesulfonate. Protein mixtures were digested with 0.5 μg of modified porcine trypsin (Promega), and incubation overnight at 37°C.

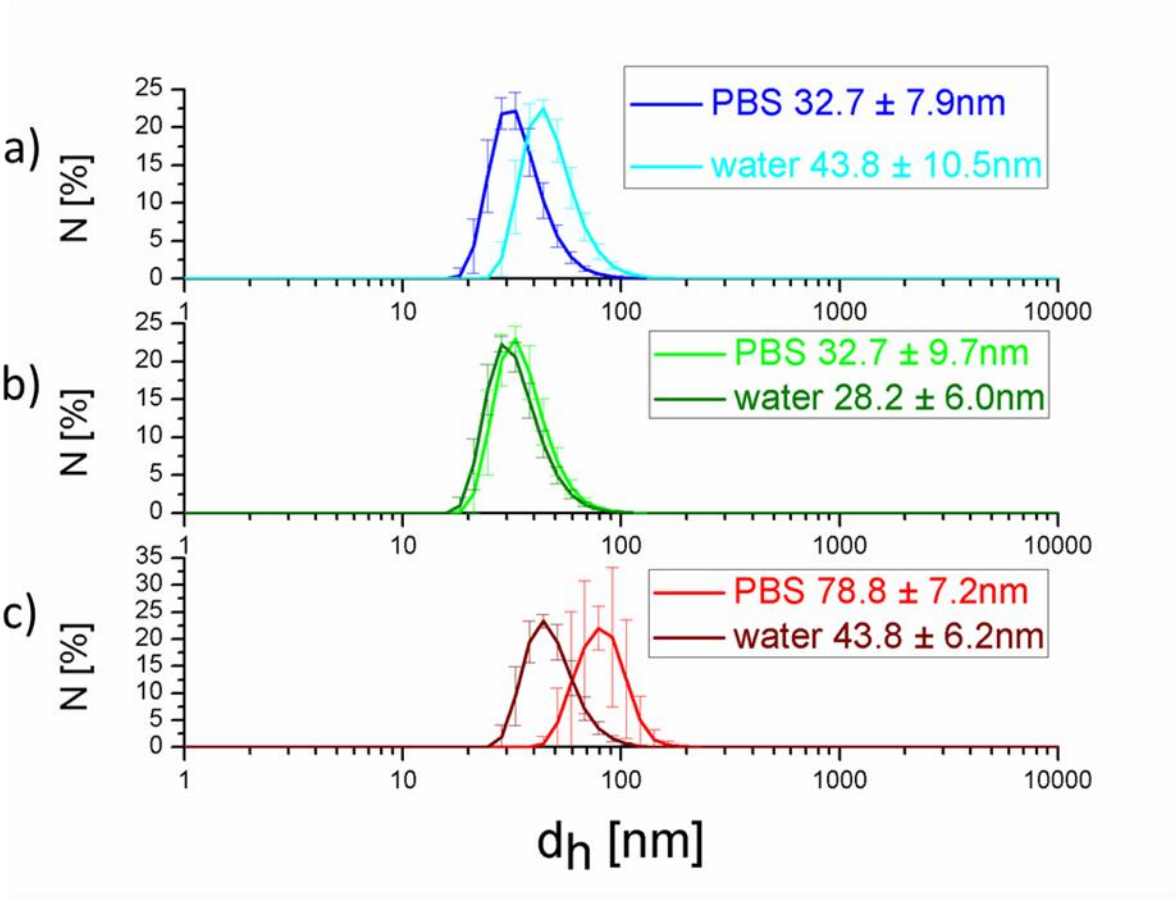
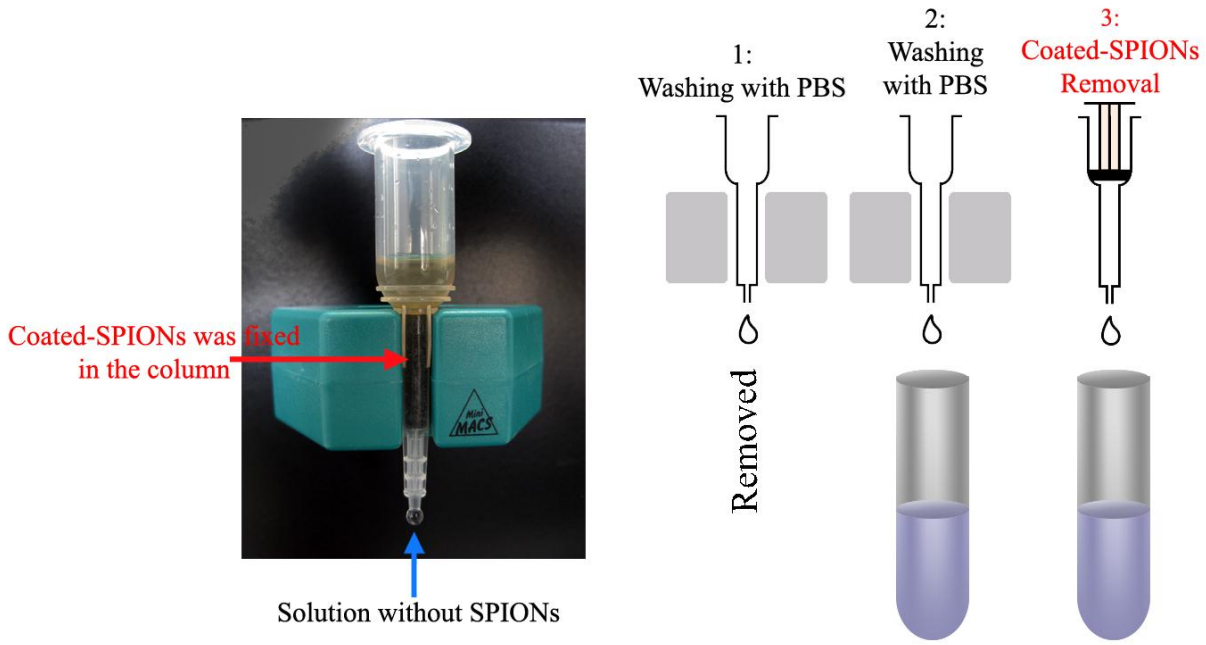
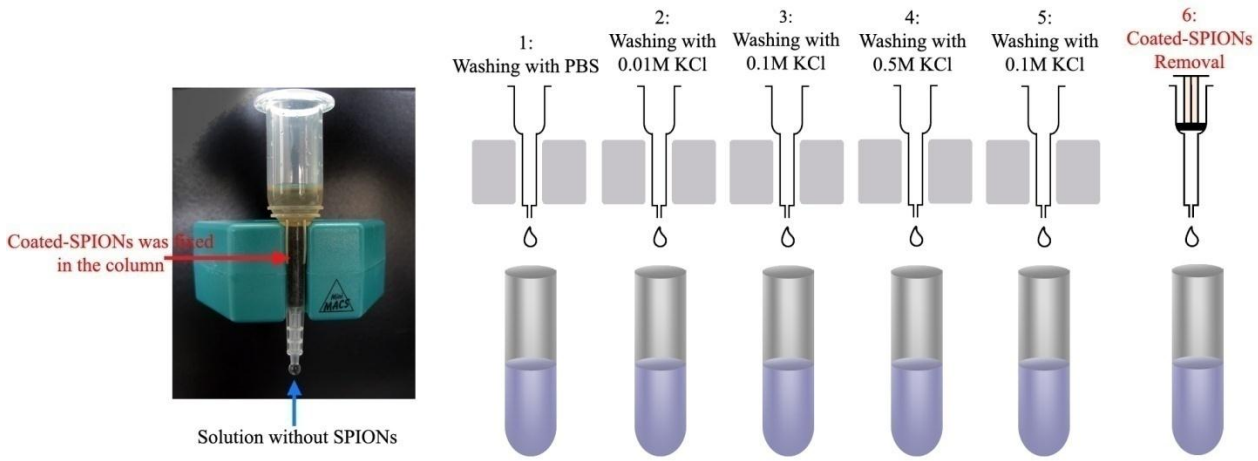



**Figure S2:** DLS size distributions of the hydrodynamic diameters $d_h$ (intensity distribution) of negative (drawn in blue), neutral (drawn in green), and positive (drawn in red) $Fe_3O_4$ NPs as dispersed in PBS.

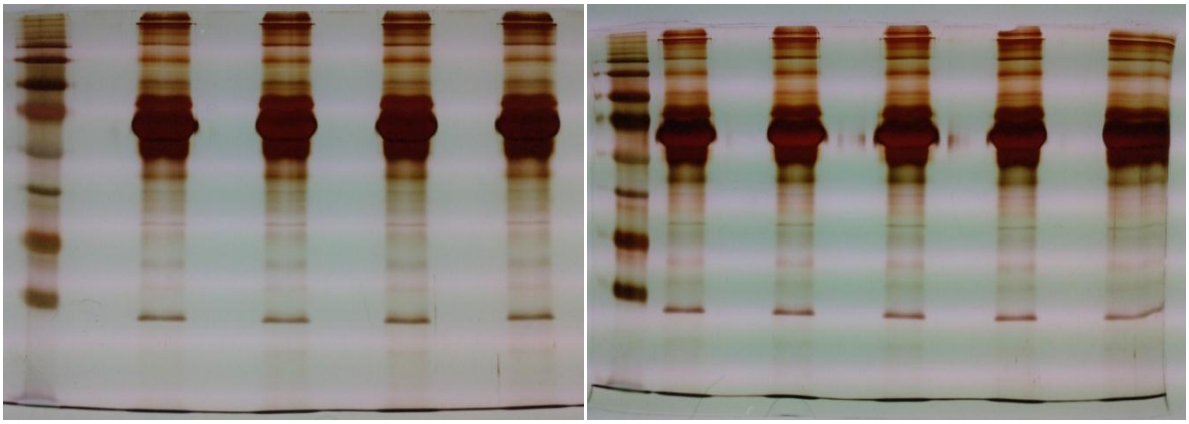



(a)



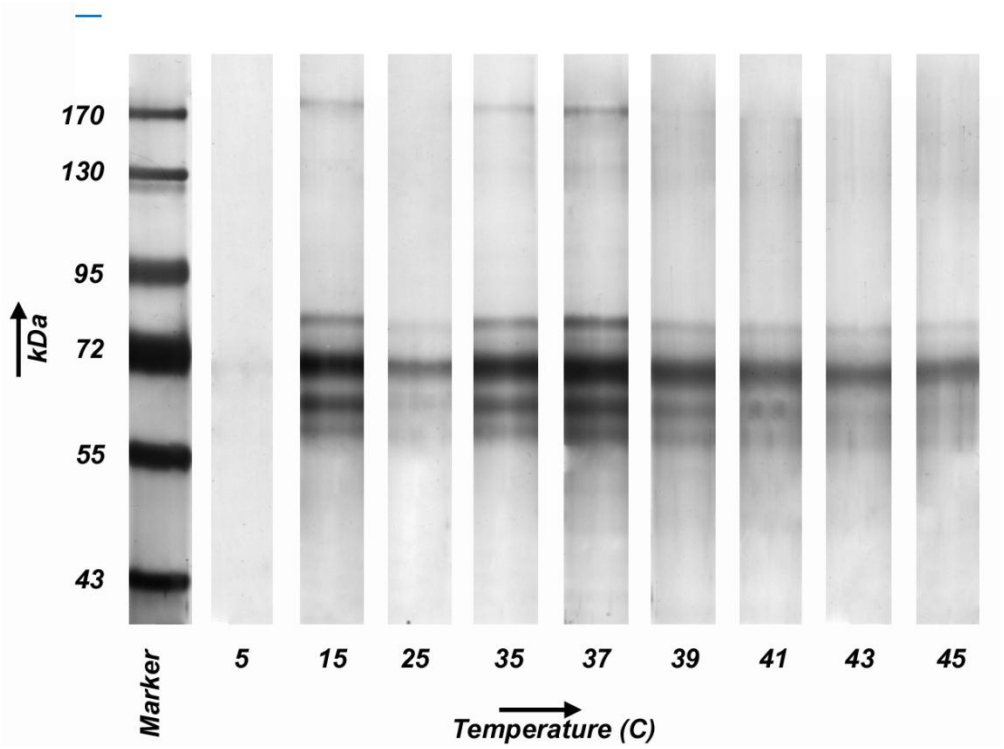

(b)

**Figure S3:** (a) Schematic representations of the magnetic separation method and employed washing steps for obtaining hard corona coated NPs. (b) Schematic representations of the magnetic separation method and employed various washing steps for definition of protein affinities to the surface of NPs.



(a) (b)

**Figure S4:** Effect of temperature on pure FBS: From left to right: (a) Marker, FBS (1%), 5, 15, 25, and 35°C; (b) Marker, FBS (1%), 37, 39, 41, 43, and 45 °C

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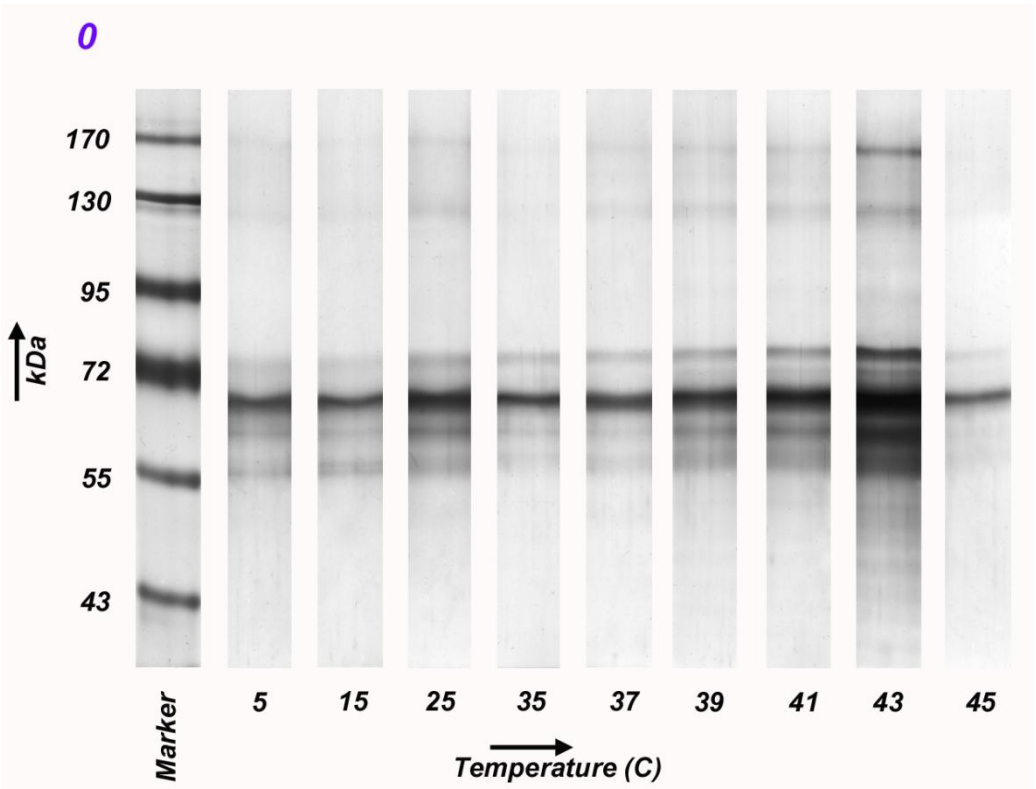


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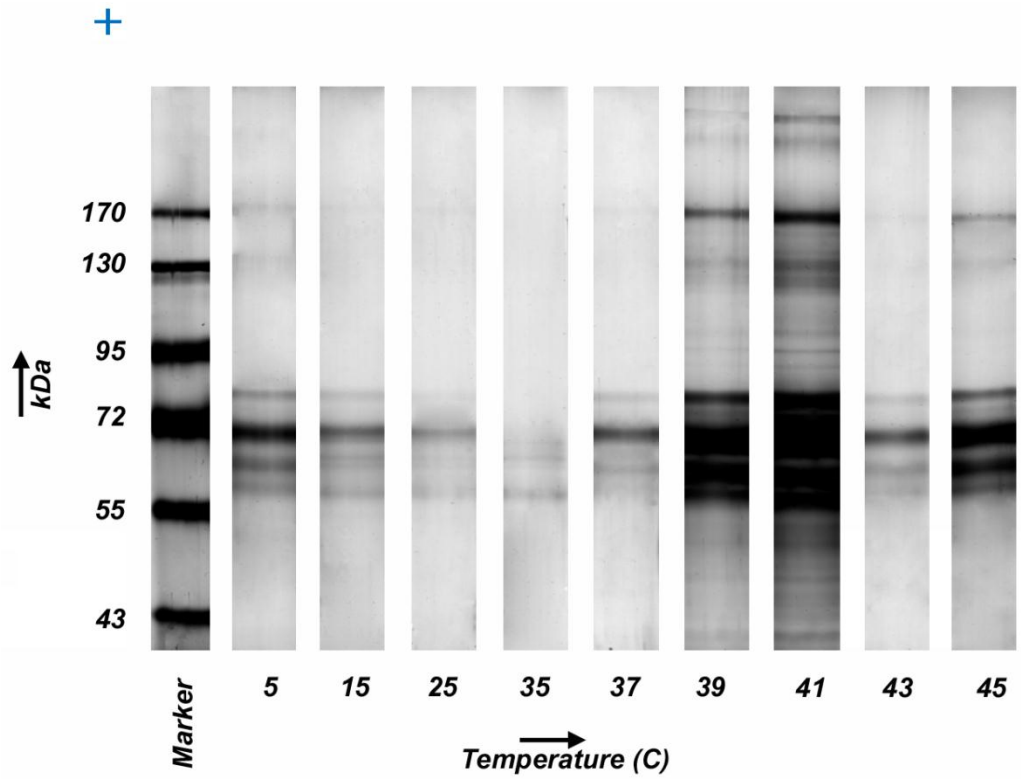


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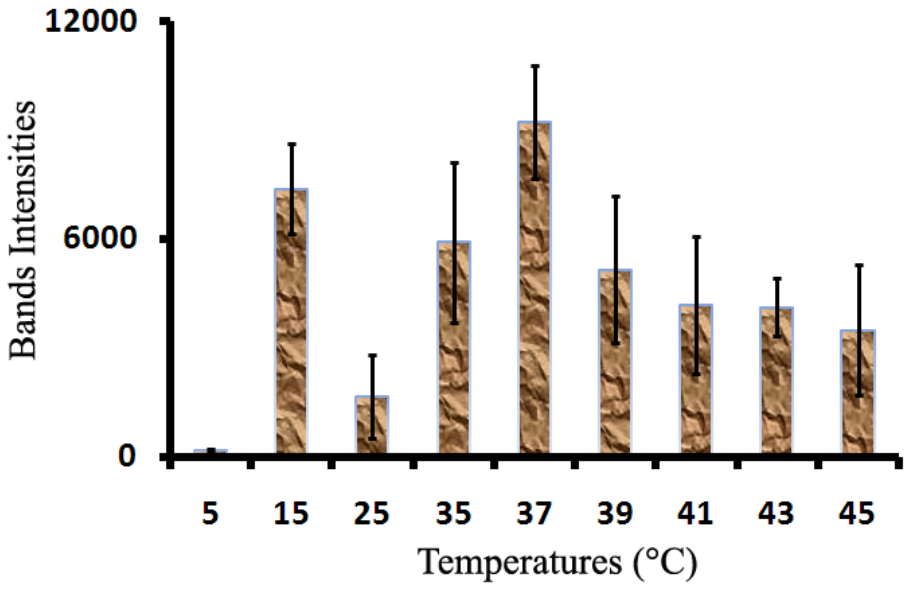


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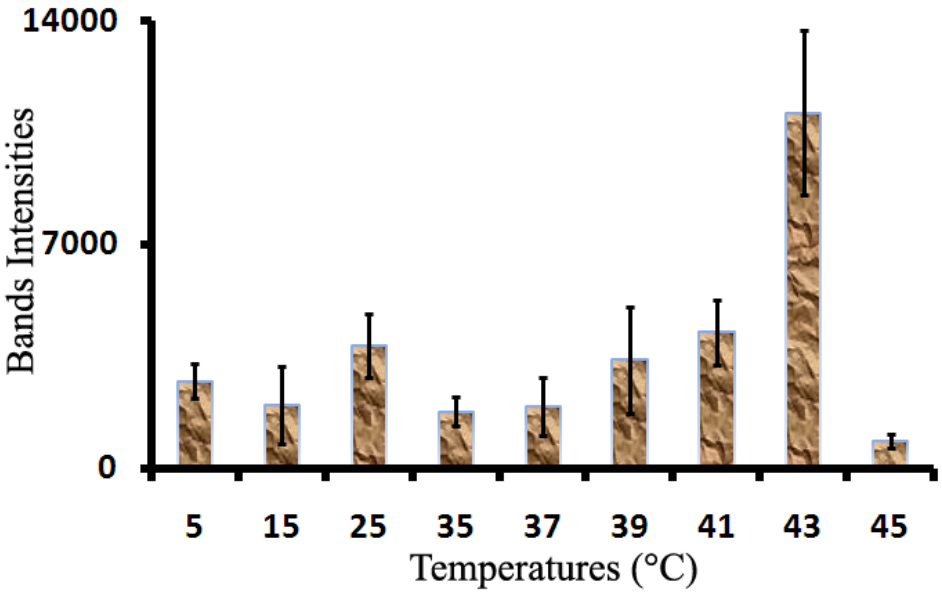


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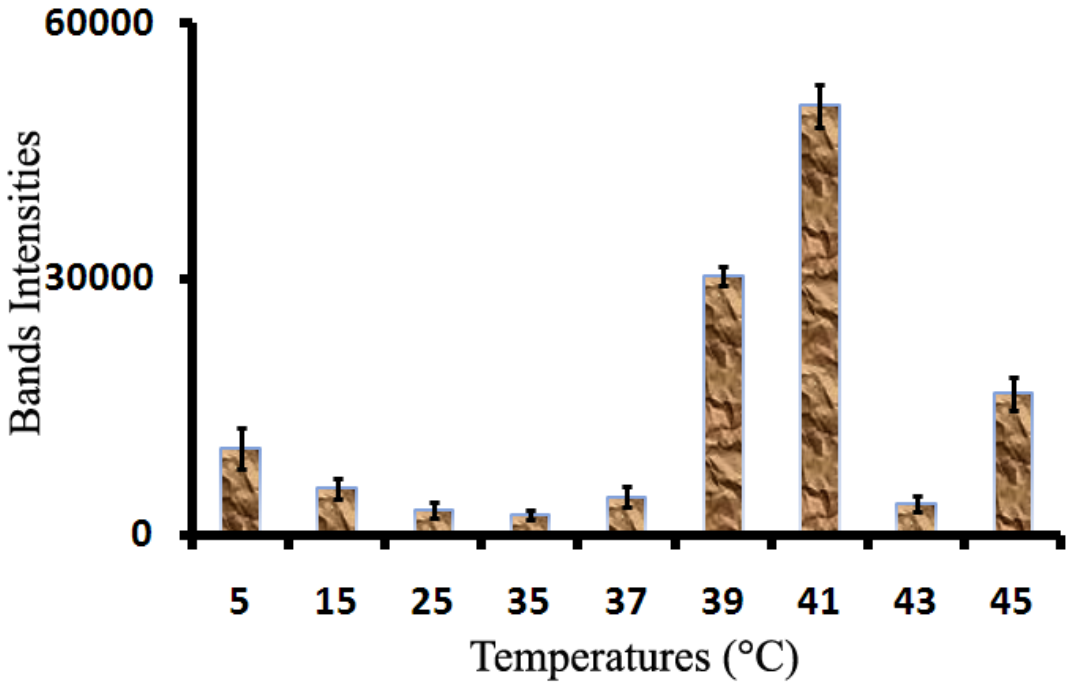


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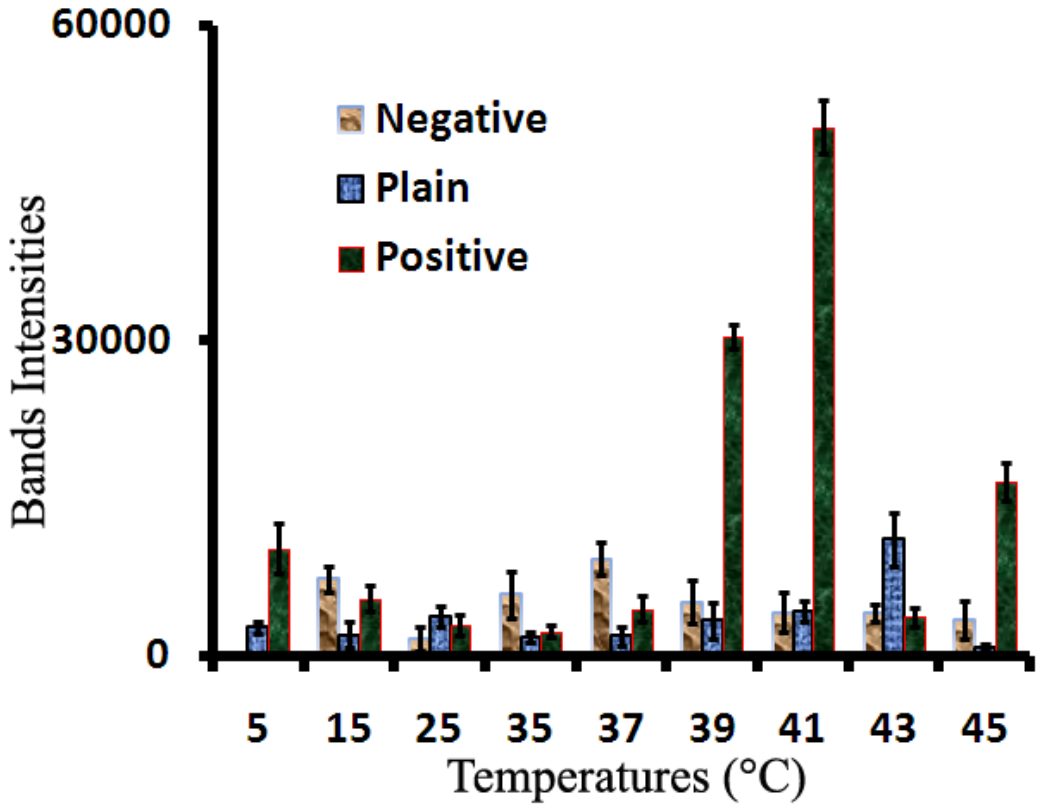


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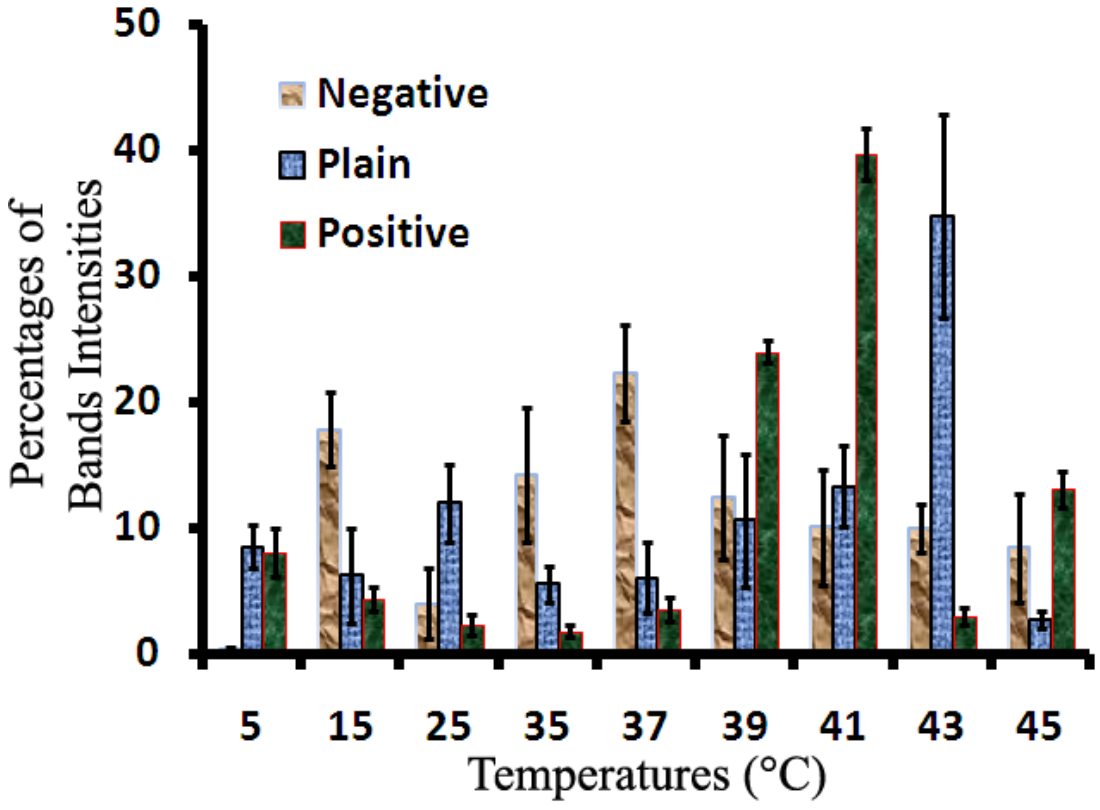
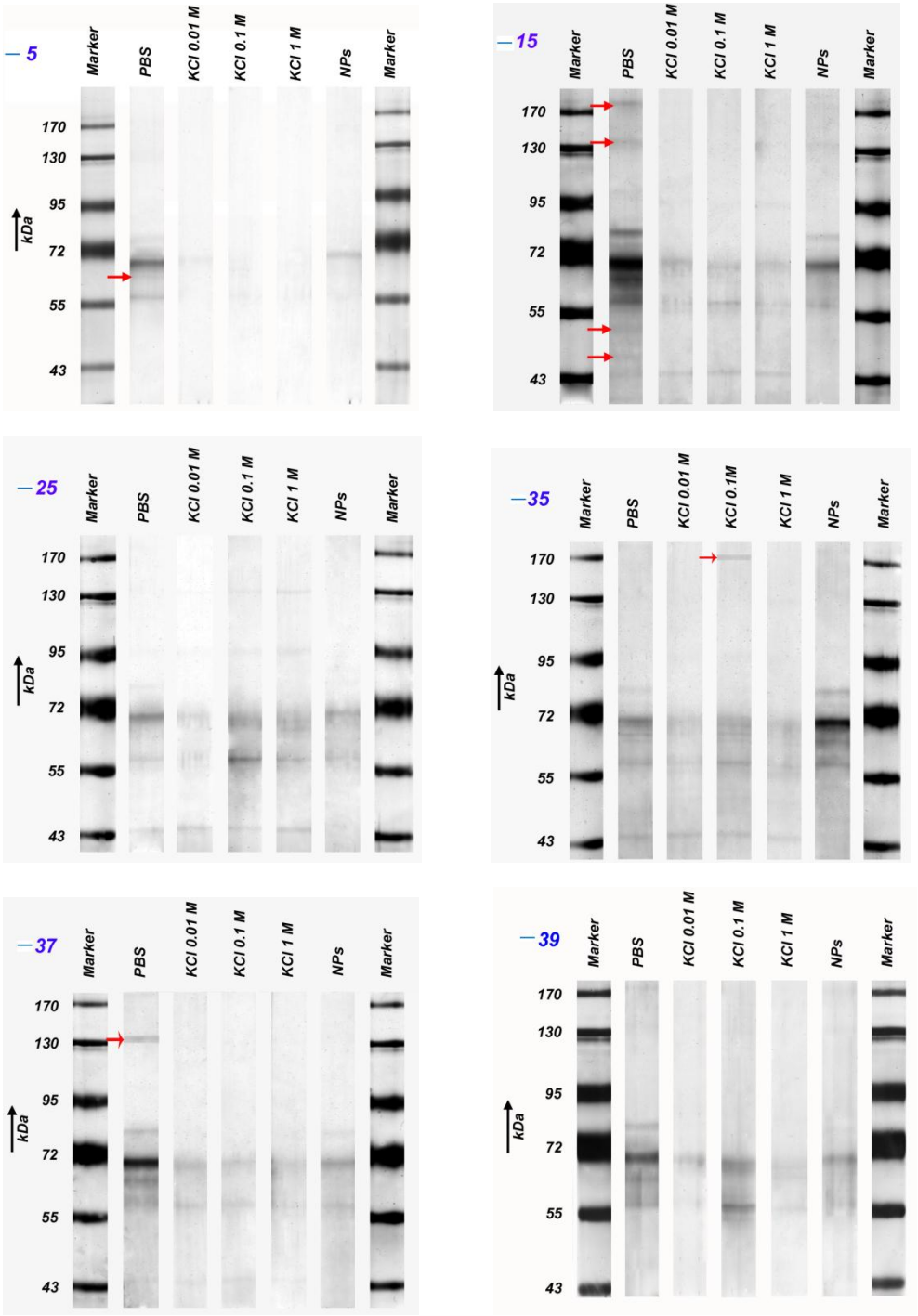
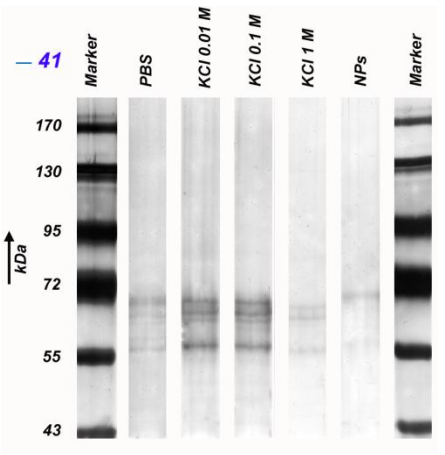


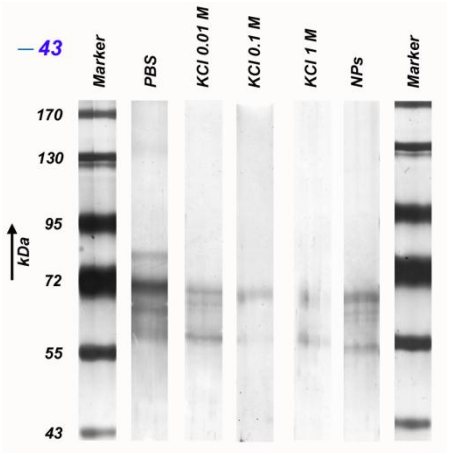

**(h)**

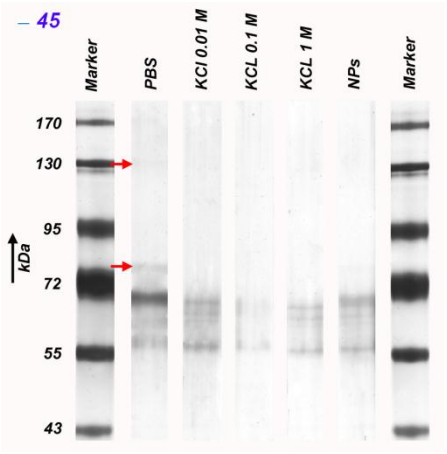
**Figure S5.** SDS-PAGE gel of FBS proteins obtained from (a) negative-, (b) plain-, and (c) positive-SPIONs-protein complexes free from excess plasma following incubation at different temperatures. The molecular weights of the proteins in the standard ladder are reported on the left for reference. Histograms representing the total band intensity of proteins recovered from (d) negative-, (e) plain-, and (f) positive-SPIONs incubated at various temperatures; the error bars are expressed as the SD of the values obtained from four different gels (independent measurements); (g) comparison between band intensities of negative-, plain- and positive-SPIONs from FBS solutions at different temperature after 1 h of incubation in FBS; (h) comparison between percentages of band intensities of negative-, plain- and positive-SPIONs from FBS solutions at different temperature after 1 h of incubation in FBS.

**(a)**



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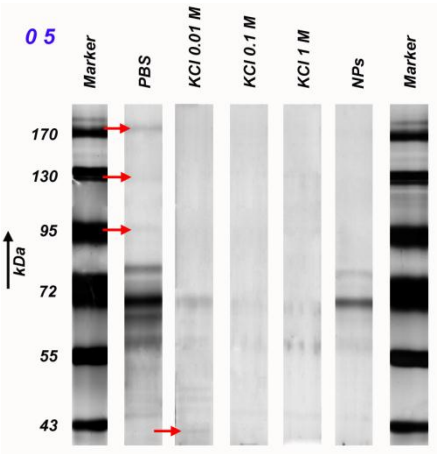


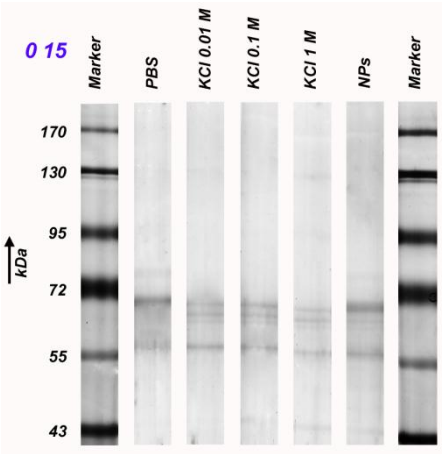

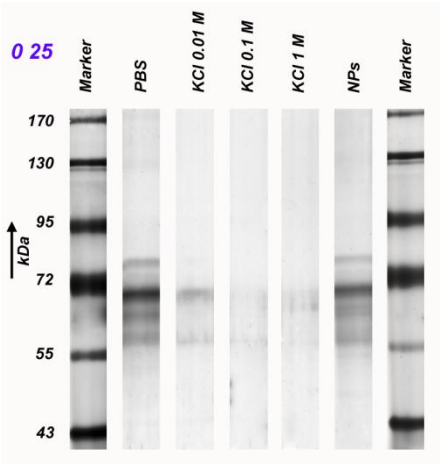


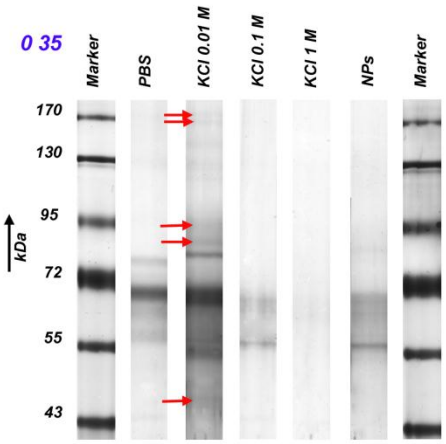


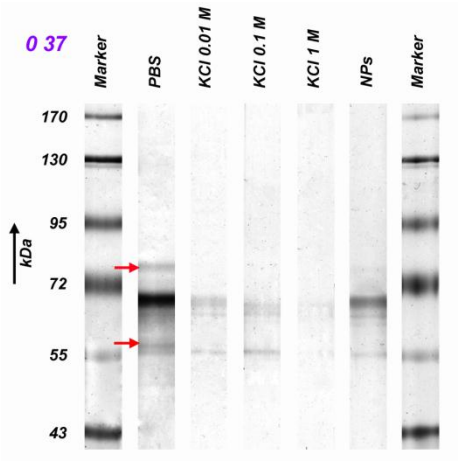


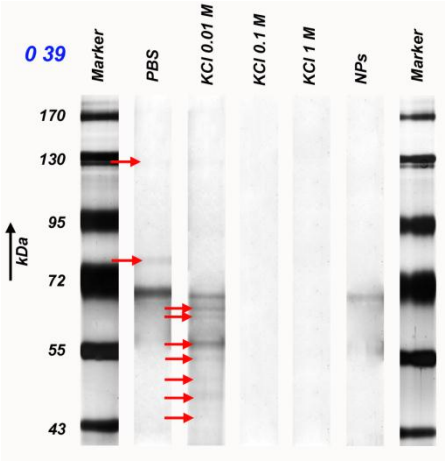


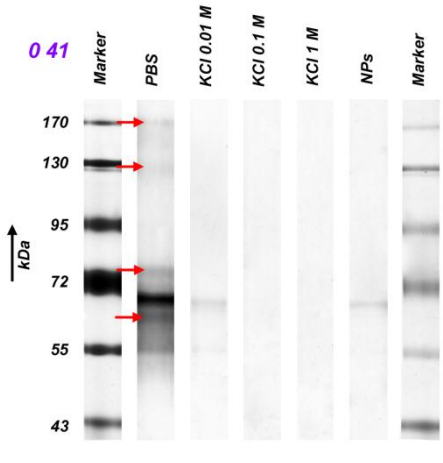


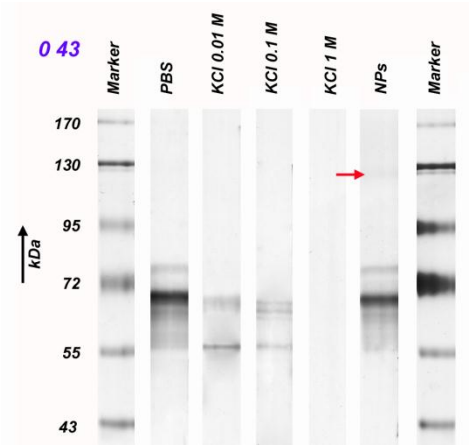

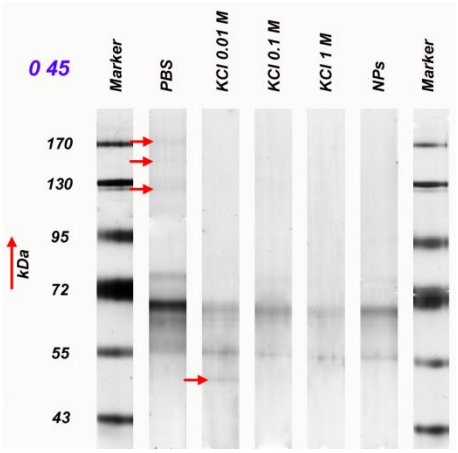
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Marker
PBS
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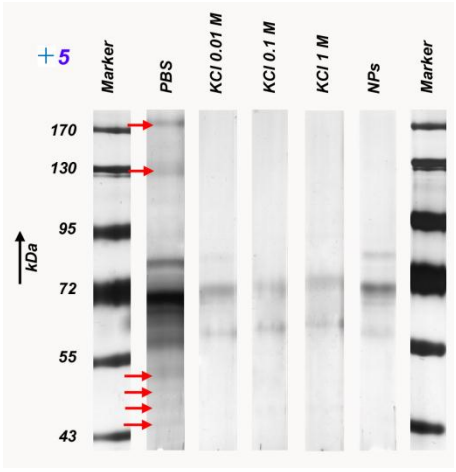
KCl 0.1 M
KCl 1 M
NPs
Marker
170
130
95
72
55
43
kDa

**(c)**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NPs
Marker
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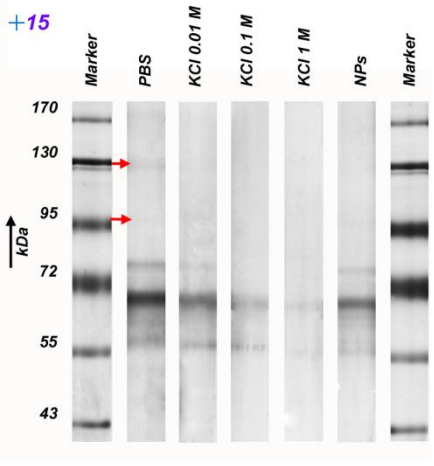
130
95
72
55
43
kDa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NPs
Marker
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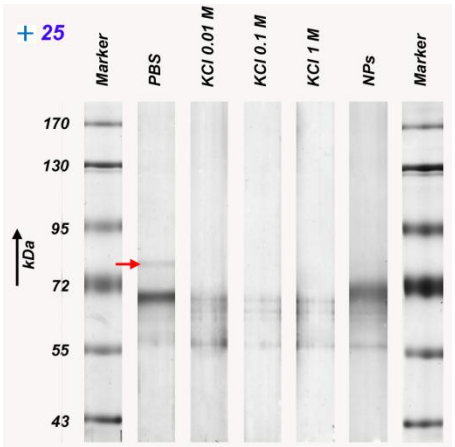
130
95
72
55
43
kDa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130
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72
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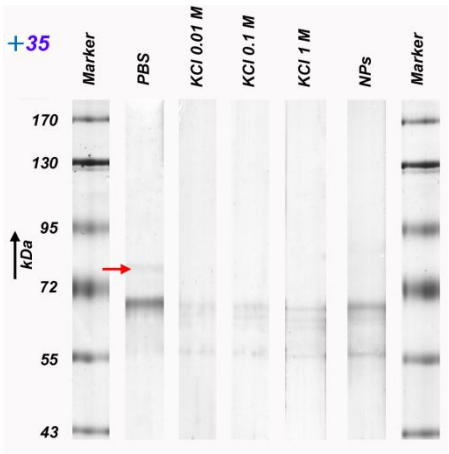
43
kDa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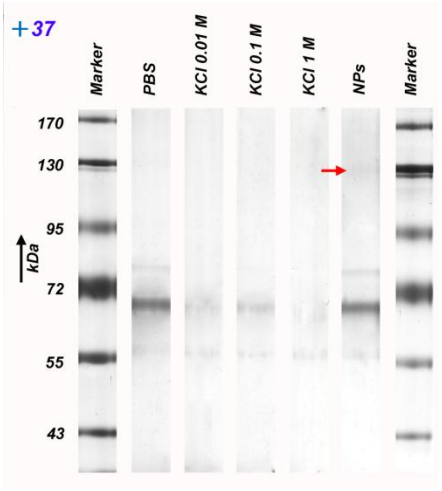
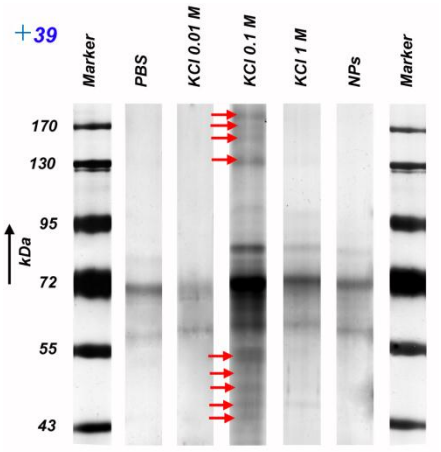
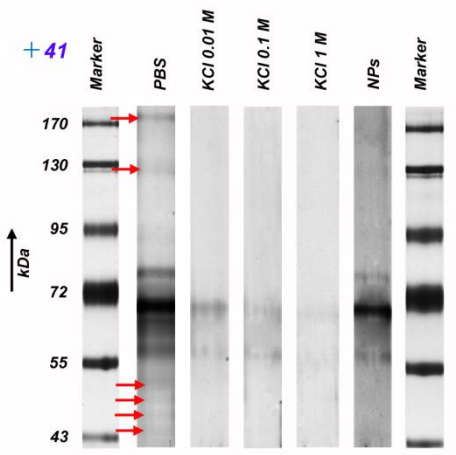
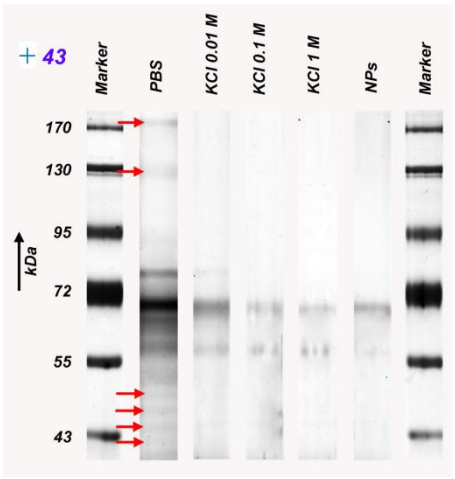
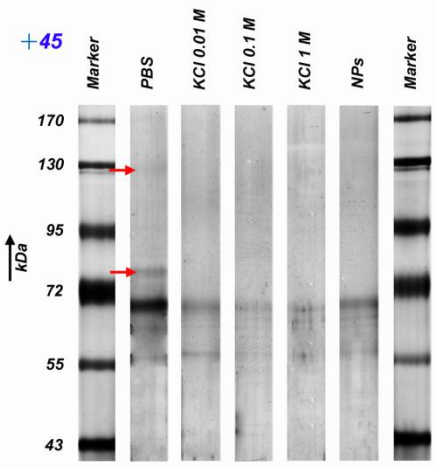
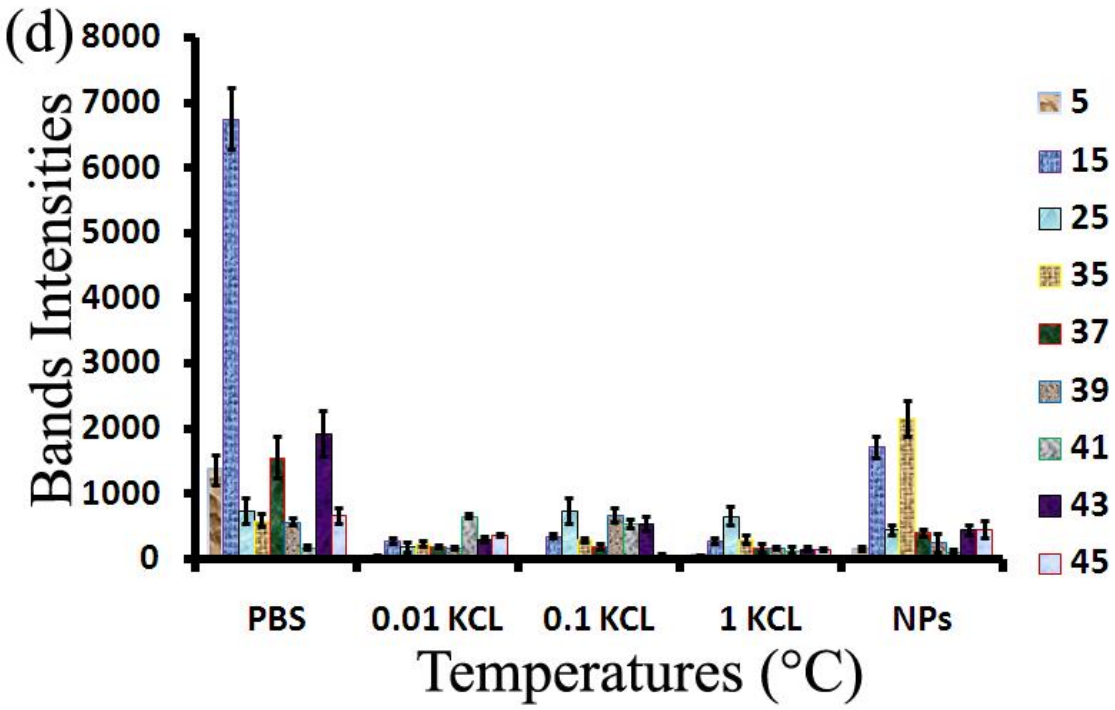


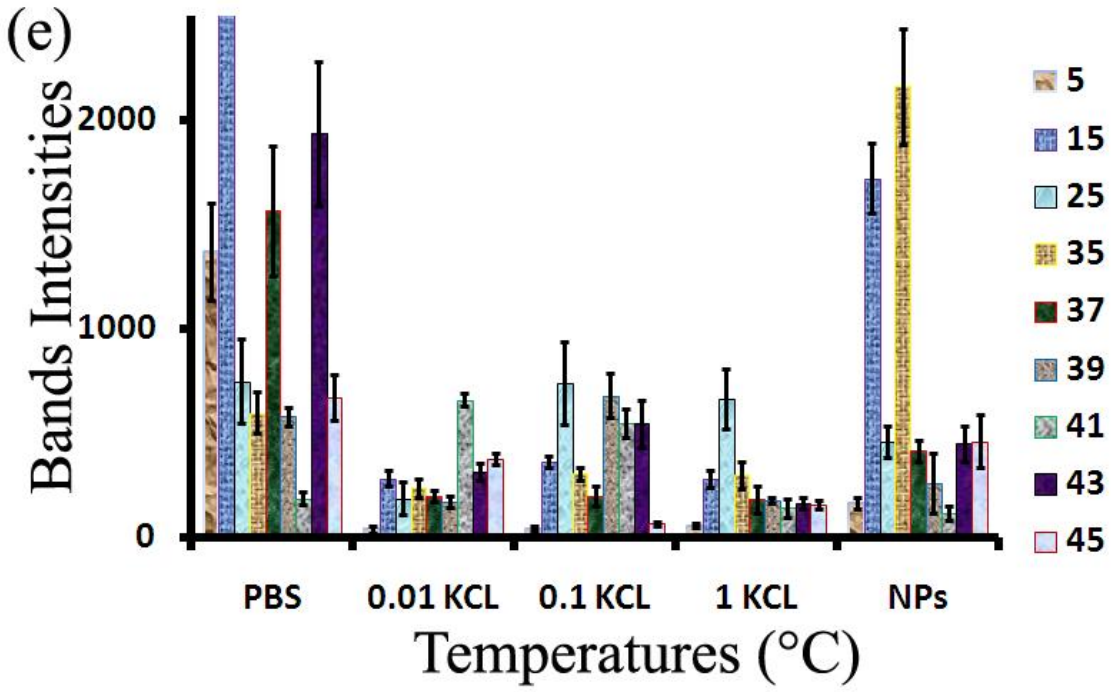



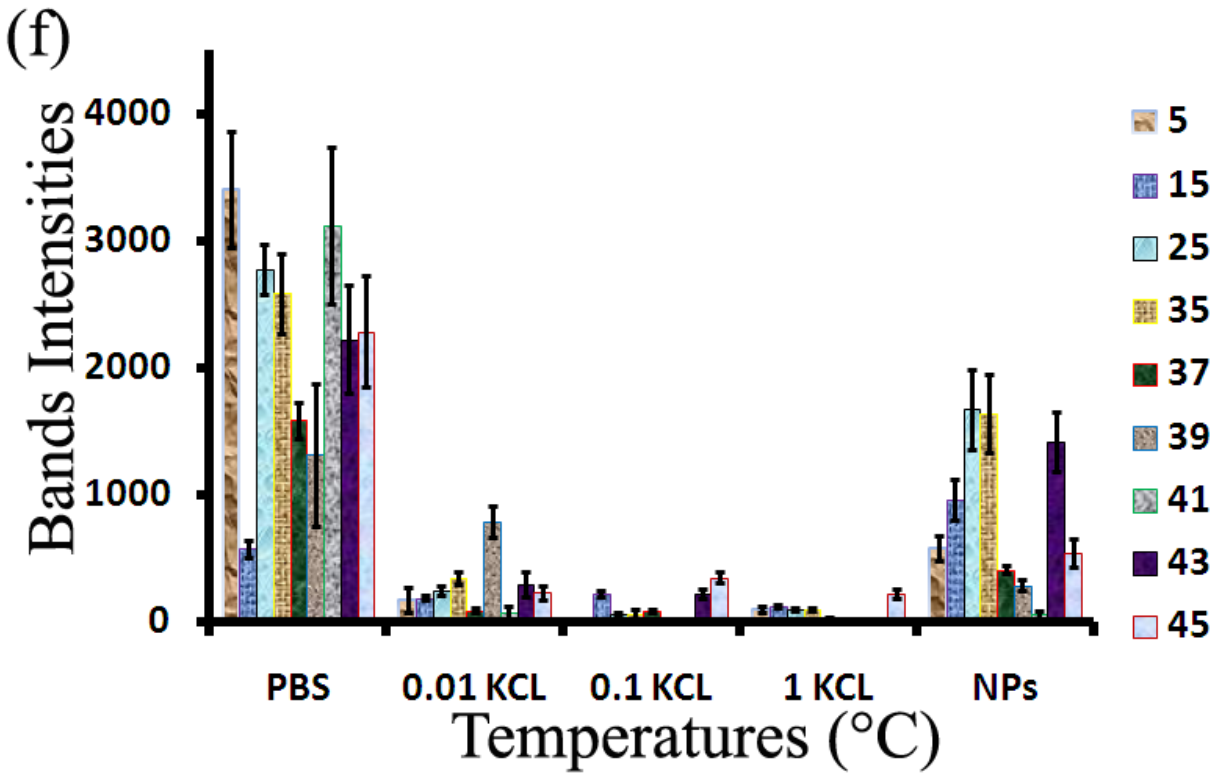



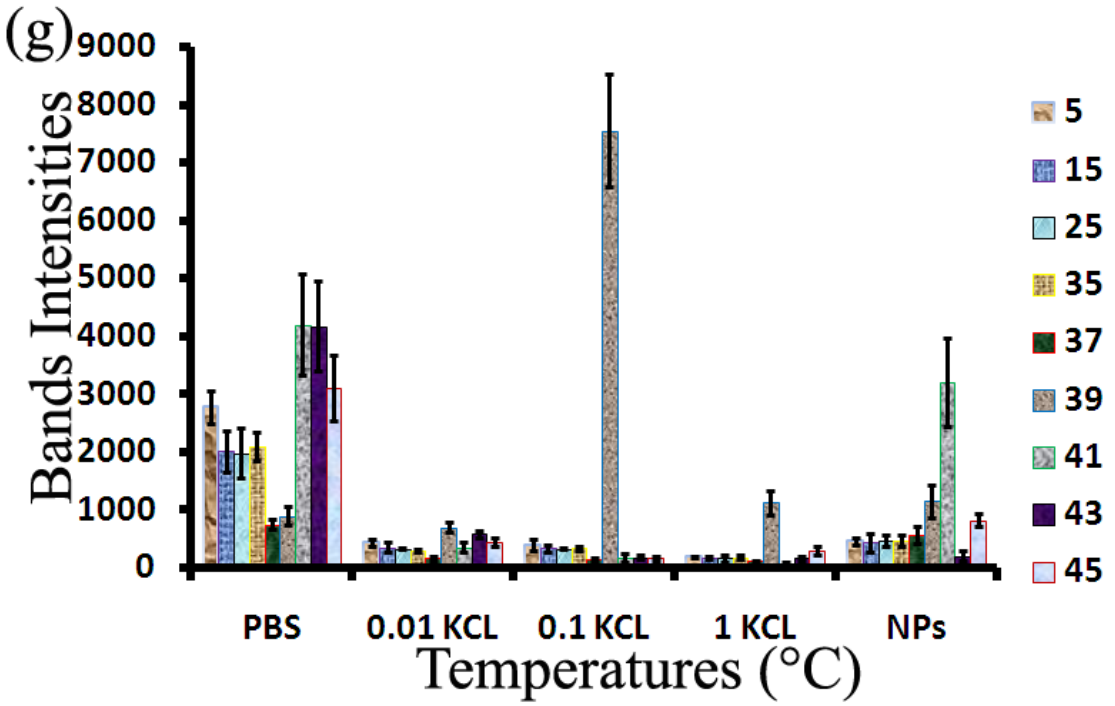



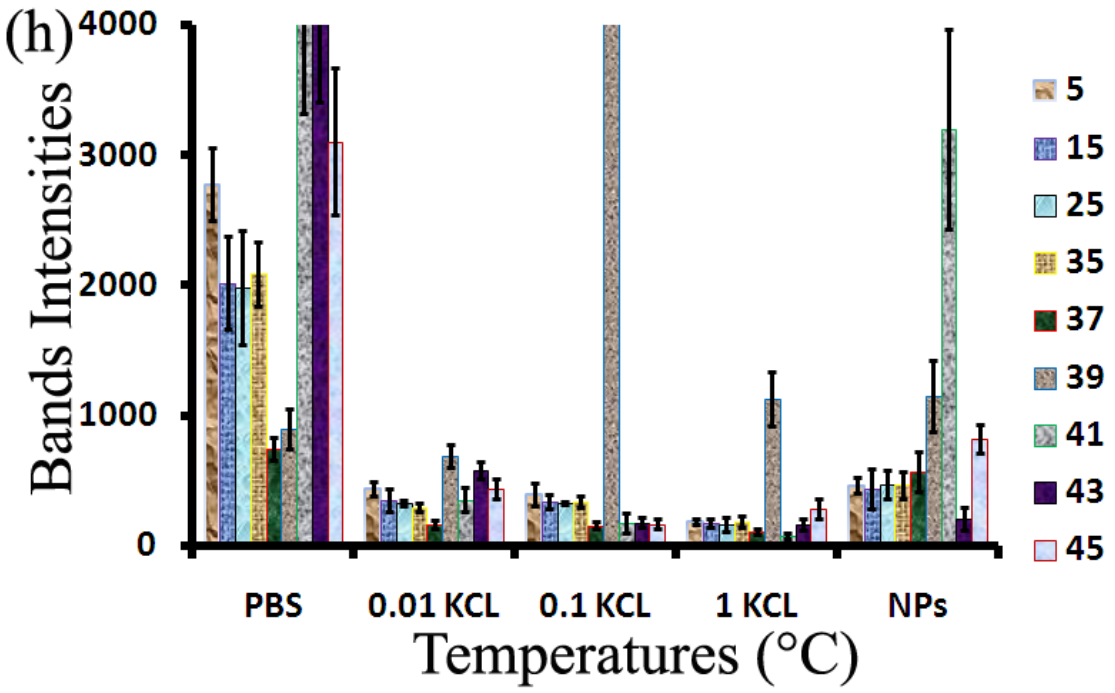





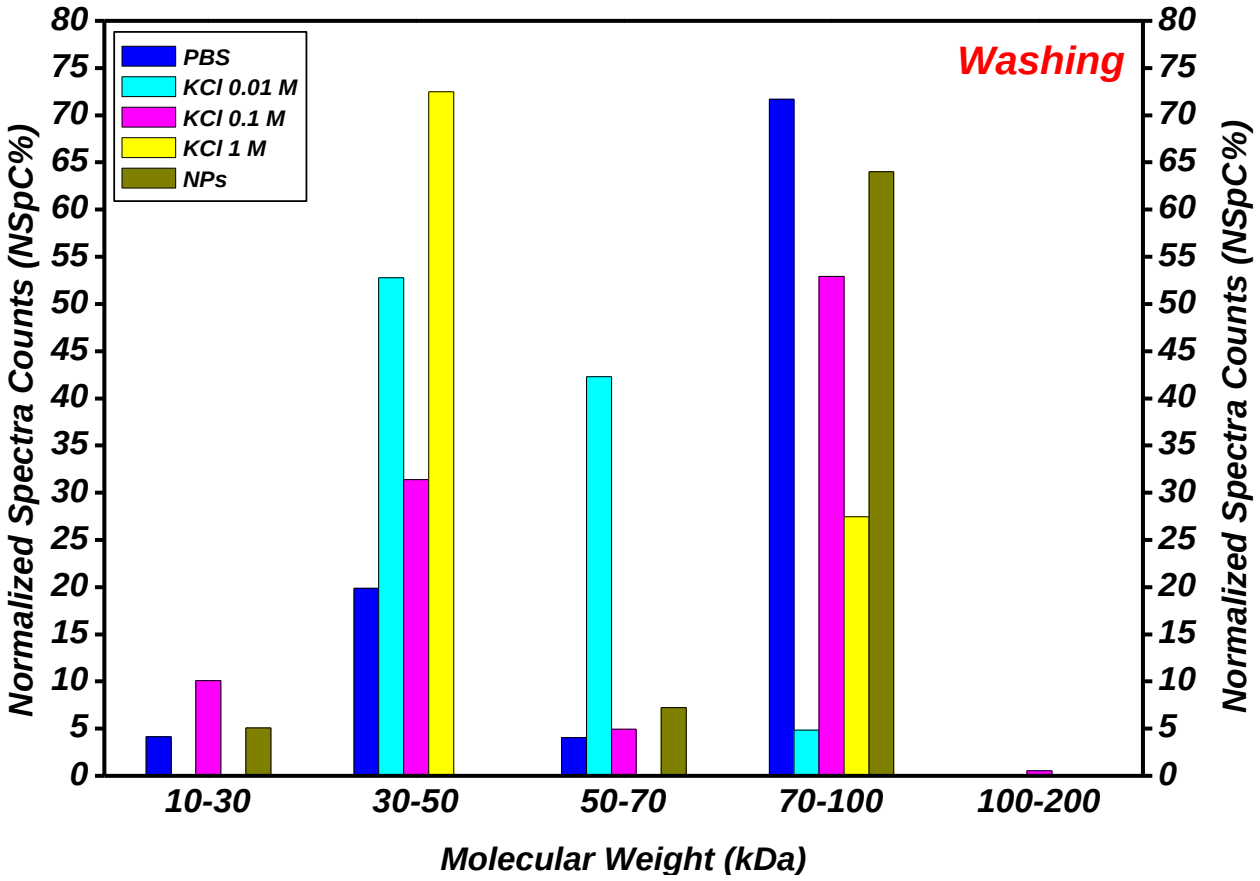







**(i)**




**(j)**

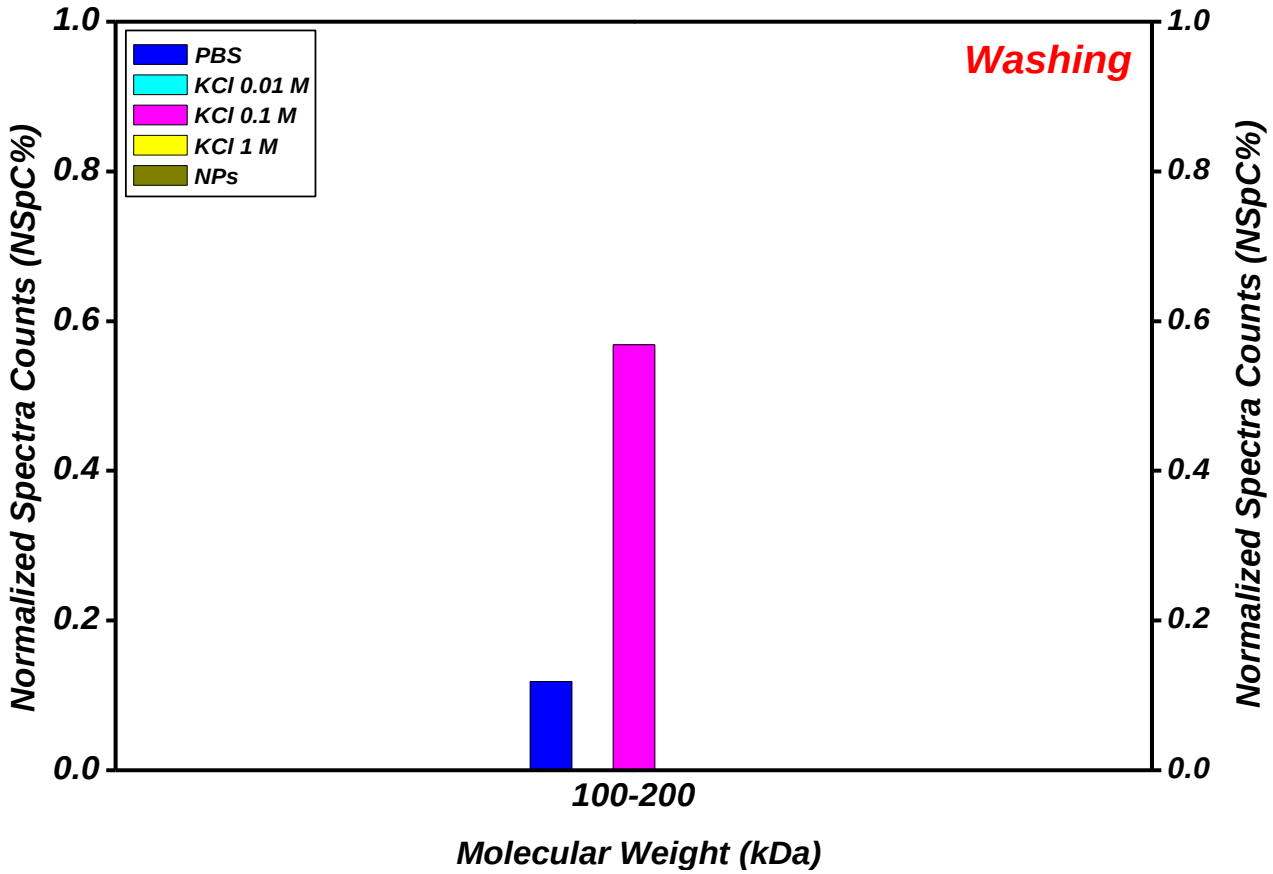



**Figure S6:** SDS-PAGE gel of various washing steps and final washed SPIONs-protein complexes at different temperatures for (a) negative-, (b) plain-, and (c) positive-SPIONs, respectively. The molecular weights of the proteins in the standard ladder are reported on the both left and right for reference. (d-h) Histograms representing the total band intensity of proteins recovered from (d-e) negative-, (f) plain-, and (g-h) positive-SPIONs incubated at various temperatures; the error bars are expressed as the SD of the values obtained from four different gels (independent measurements). (i) Normalized spectral counts (NSpC) of proteins of different Mw ranges contained in the hard corona from various washing steps of positive-SPIONs, which incubated at 39°C with FBS for 1 hour; (j) Enlargement of the proteins with Mw range of 100-200 kDa from (i).

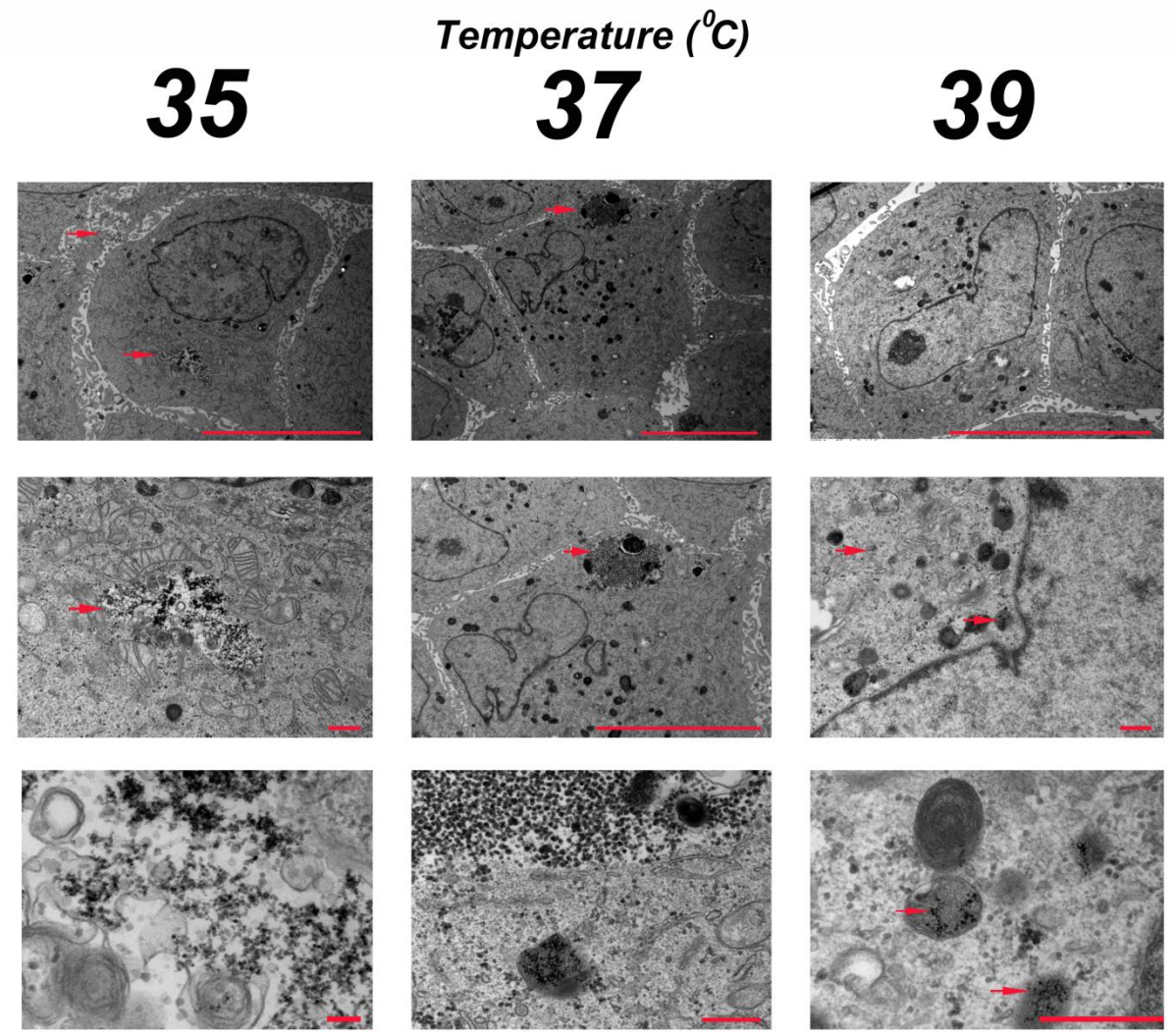



**Figure S7:** TEM images of HeLa cells after incubation with SPIONs-protein complexes at the same incubation temperatures as indicated showing the formation of lysozoms filled by SPIONs inside the cells.

# Reference


1 Usher, T. C. & Wallis, S. H. Process of Making Carboxilated Dextran. *US Patent* **Patent Number US6 703 499** (2004).

2 Martwiset, S., Koh, A. E. & Chen, W. Nonfouling Characteristics of Dextran-Containing Surfaces. *Langmuir* **22**, 8192–8196 (2006).

3 Saboktakin, M. R., Tabatabaie, R. M., Maharramov, A. & Ramazanov, M. A. A synthetic macromolecule as MRI detectable drug carriers:Aminodextran-coated iron oxide nanoparticles. *Carbohydrate Polymers* **80**, 695-698 (2010).

4 Mahmoudi, M., Simchi, A., Milani, A. S. & Stroeve, P. Cell toxicity of superparamagnetic iron oxide nanoparticles. *Journal of Colloid and Interface Science* **336**, 510-518 (2009).

5 Mahmoudi, M., Simchi, A., Imani, M., Milani, A. S. & Stroeve, P. Optimal design and characterization of superparamagnetic iron oxide nanoparticles coated with polyvinyl alcohol for targeted delivery and imaging. *Journal of Physical Chemistry B* **112**, 14470-14481 (2008).

6 Maiorano, G. *et al.* Effects of cell culture media on the dynamic formation of protein-nanoparticle complexes and influence on the cellular response. *ACS Nano* **ASAP Article; doi: 10.1021/nn101557e** (2010).

7 Lundqvist, M. *et al.* Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 14265-14270 (2008).

8 Mahmoudi, M., Sardari, S., Shokrgozar, M. A., Laurent, S. & Stroeve, P. Interaction of superparamagnetic iron oxide nanoparticles with human transferrin: Irreversible changes in human transferrin conformation. *Nanoscale* **inpress** (2010).

9 Walczyk, D., Bombelli, F. B., Monopoli, M. P., Lynch, I. & Dawson, K. A. What the cell "sees" in bionanoscience. *Journal of the American Chemical Society* **132**, 5761-5768 (2010).

10 Mahmoudi, M.; Lynch, I.; Ejtehadi, M. R.; Monopoli, M. P.; Bombelli, F. B.; Laurent, S., Protein−Nanoparticle Interactions: Opportunities and Challenges. *Chemical Reviews* **2011,** *111* (9), 5610-5637.

11 Monopoli, M. P. *et al.* Physical-Chemical Aspects of Protein Corona: Relevance to in Vitro and in Vivo Biological Impacts of Nanoparticles. *Journal of the American Chemical Society* **133**, 2525-2534, doi:10.1021/ja107583h (2011).

12 Wang, H. & Joseph, J. A. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radic. Biol. Med.* **27**, 612-616 (1999).