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Preparation and Characterization of Molecularly Imprinted Polymeric Nanoparticles for Atrial Natriuretic Peptide (ANP)

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Abstract

Natriuretic peptide receptor A (NPRA), the receptor for the cardiac hormone atrial natriuretic peptide (ANP), is expressed abundantly on cancer cells and disruption of ANP-NPRA signaling inhibits tumor burden and metastasis. Since antagonists of NPRA signaling have not provided reproducible results, we reasoned that a synthetic neutralizing antibody to ANP, which has high selectivity and affinity for ANP, could be used to regulate ANP levels and attenuate NPRA signaling. In this study, we prepared molecularly imprinted polymer nanoparticles (MIPNPs) for ANP using a short peptide of ANP as the template and determined their binding affinity and selectivity. The MIPNPs were prepared by precipitation polymerization using NH₂-SLRRSS-CONH₂, which is a short peptide from ANP as template, methacrylic acid (MAA) and N-isopropylacrylamide (NIPAm) as functional monomers, bis-acrylamide (BIS) as crosslinker. The average diameter of MIPNPs and non-imprinted nanoparticles (NIPNPs) in water is 215.8 ± 4.6 nm and 197.7 ± 3.1 nm respectively. The binding isotherm analysis showed that MIPNPs have a much higher binding affinity for template peptide and ANP than NIPNPs. Scatchard analysis gave an equilibrium dissociation constant, K_d of 7.3 μM with a binding capacity 106.7 μmol/g for template peptide and K_d of 7.9 μM with a binding capacity of 36.0 μmol/g for ANP. Measurements of binding kinetics revealed that MIPNPs reach protein adsorption equilibrium in 30 min. MIPNPs found to have high specificity for ANP with little affinity for BSA or scrambled ANP peptide. MIPNPs also recognized and adsorbed ANP in cell culture media spiked with ANP and human plasma. Taken together, these results indicate that MIPNPs have high affinity and selectivity for ANP and can be used as a synthetic antibody for modulating ANP-NPRA signaling in cancers.

Keywords

Atrial natriuretic peptide (ANP); natriuretic peptide receptor A (NPRA); molecularly imprinted polymer; cancer; ANP-NPRA signaling

1. Introduction

Human atrial natriuretic peptide (ANP) is a 28-amino acid polypeptide hormone released from the cardiac atria in response to atrial stretch that regulate sodium levels and blood volume homeostasis [1]. ANP's receptor, natriuretic peptide receptor A (NPRA), is expressed on cells in many different tissues of various organ systems such as vasculature,

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kidneys, adrenal glands, terminal ileum, adipose, aorta, and lung [2–6]. ANP-NPRA signaling has been implicated in many inflammatory conditions including allergic disease and cancer [7]. For example, both ANP and NPRA are abundantly expressed by lung cancer cells, and over-secretion of ANP has been linked with hyponatremia [8–10]. Recently, our lab has reported that the expression and signaling of NPRA is important for tumor growth and that the NPRA signaling axis could be effective target for cancer therapy [11, 12]. NPRA consists of an extracellular binding domain for ANP and brain natriuretic peptide (BNP), a single transmembrane-spanning helical domain, an intracellular protein kinase-like homology domain, a dimerization domain, and guanylyl cyclase [13]. ANP or BNP binding to the NPRA extracellular domain promotes activation of second messenger system, converts GTP to cyclic guanosine monophosphate, which in turn activates protein kinase G that regulates phosphorylation of proteins, ion channels and transcription of genes, and together regulates cellular growth, apoptosis, cell cycle and inflammation [14, 15]. A number of approaches have been used to block NPRA signaling including small interfering RNA to decrease NPRA synthesis and by decreasing binding of ANP to NPRA using either peptide antagonists or small molecular inhibitors [7, 16]. We reasoned that a synthetic neutralizing antibody to ANP which has high selectivity and affinity for ANP can be used to sequester ANP and attenuate ANP-NPRA signaling in cancer cells.

Molecularly imprinted polymers (MIPs) are synthetic materials produced by cross-linking the functional monomers in the presence of the template molecules. Following removal of the template, the cavities generated possess the size, shape, and functional group orientation complementary to the target molecules [17, 18]. MIPs have considerable promise for applications in clinical analysis, medical diagnostics, environmental monitoring and drug delivery [19, 20]. The majority of MIPs characterized so far have been against low molecular weight templates in organic solvents. The aqueous molecular imprinting of biomolecules such as peptides and proteins has been largely unsuccessful [21, 22]. The main difficulties in imprinting proteins are their large molecular dimensions, the flexibility of the chain and the low mass-transfer kinetics, which limit the polymer's molecular recognition capacity and selectivity [23–24].

A number of strategies for creating MIP nanoparticles targeting proteins and peptides have been developed, such as utilizing functional groups to form strong electrostatic and metal-chelating interactions [25, 26]. For example, MIP nanoparticle synthesis with a functional monomer optimization strategy using hydrophobic and electrostatic interactions could generate MIPs with binding affinity and selectivity for the biological target comparable to those of natural antibodies [27]. Such particles have recently been studied as functional materials for antibodies to capture and neutralize peptide toxins [27–29], and for selective protein separation and discrimination [30]. The recognition of antigen by antibody depends on the specificity between the antibody and an antigenic site on the protein, the epitope. The epitope is a short amino-acid sequence that is complementary to the binding site of the antibody. Using a small sequence of amino acids from the large protein eliminates the problem of size and steric hindrance in the target and has been used successfully to create artificial antibodies. For example, the epitope approach can be used to produce MIP that can successfully bind oxytocin by imprinting Tyr-Pro-Leu-Gly amino acid sequence [31, 32].

Herein, we demonstrate the creation of MIPNPs that specifically bind ANP using a small epitope sequence of amino acids from ANP. The hydrodynamic size of the MIPNPs, binding isotherm and adsorption kinetics of MIPNPs for template and ANP has been measured. The selectivity of the MIPNPs and the recognition of ANP in the cell culture medium with spiked ANP and human plasma are also studied.

2. Results and Discussion

2.1 Preparation and characterization of MIPNPs

Aqueous molecular imprinting of macromolecules such as peptides and proteins is known to be hindered by steric and thermodynamic effects. It is very difficult for proteins or other bulky macromolecular templates to move freely in and out of a polymer matrix [33]. The thermodynamic considerations indicated that the flexible templates yield less well-defined recognition sites in the MIP matrix [35]. Recently, an epitope approach has been developed for imprinting with proteins and peptides [31, 32, 35]. In this technique, a short peptide from the larger target (as an epitope represents an antigen) is selected for use as template to create the imprint, which in turn can efficiently recognize both the template and larger target [32]. ANP is a 28-amino acid peptide with a 17-amino acid ring formed by a disulfide bond between two cysteine residues at positions 7 and 23 in the middle of the molecule (Fig. 1a). To explore the epitope approach for imprinting ANP, an N-terminal sequence of the hormone ($\text{NH}_2\text{-SLRRSS-CONH}_2$, Fig. 1a) was chosen as template, since it is outside of the ring and has six residues of which three are positively charged, three are uncharged but polar (-OH), and one has a hydrophobic side chain. The monomers used for the MIP synthesis included Methacrylic acid (MAA) and ethyl alcohol and N-isopropylacrylamide (NIPAm) as hydrogen bonding, negative charged, and hydrophobic functional monomers, and bis-acrylamide (BIS) as a cross-linker. The strong electrostatic interactions between the functional monomers and the template molecules should create molecularly imprinted receptors. Fig. (1a) and Fig.(1b) illustrate the epitope approach for imprinting ANP and the chemical functions of the imprinting, respectively.

The precipitate polymerization method was employed to prepare MIPNPs at room temperature using water as solvent. BIS was dissolved in water with the assistance of a little of surfactant sodium dodecyl sulfate (SDS). The polymerization was performed with a small amount of template (1 μmol) which possesses 3 μmol potential positive charged sites and 1 μmol of the potential hydrophobic site. We employed the functional monomers MAA at 3 mM to give 3000 μmol positive charged sites; NIPAm at 1 mM facilitated 1000 μmol hydrophobic sites. After studying different ratios of the functional monomers to cross-linking agent (data not shown), the functional monomers MAA and NIPAm to cross-linking agent (BIS) ratio was optimized to 1:3:10. The free radical polymerization was initialized by ammonium persulfate (APS) and N,N,N',N'-tetramethylethylenediamine (TMED) at room temperature. As a weak polycarboxylic acid with $\text{pK}_a=6 \sim 7$, polymethacrylic acid carried different negative charges at different pH due to different degree of ionization of its carboxylic acid groups [36]. When the pH is higher than the pK_a , the amount of negative charge is greater than that of positive charge. Therefore, a greater electrostatic interaction of functional groups on MIPNPs with the template will be expected when the pH is > 7 . In contrast, when pH is lower than 6, the electrostatic interaction between the MIPNPs and template will be weakened, which is helpful to remove the template from the imprinted cavities of the MIPNPs. This pH sensitive adsorption and desorption characteristic is useful for the separation of ANP from the other peptides. The hydrodynamic size of the MIPNPs and NIPNPs was characterized by a DynaPro DLS plate reader. Fig. 2 shows the particle size distribution of MIPNPs and NIPNPs in water. The average diameter of MIPNPs ($215.8 \pm 4.6 \text{ nm}$) is a little bit larger than NIPNPs ($197.7 \pm 3.1 \text{ nm}$).

2.2 Binding isotherm for template and ANP

To investigate the affinity of MIPNPs for template peptide and ANP targeting peptide, adsorption isotherm measurements and subsequent Scatchard analysis were carried. The adsorption isotherm of MIPNPs for template peptide was determined in the concentration range of template from 30 to 340 $\mu\text{g/ml}$. As shown in Fig. 3a, the amount of template

peptide bound to the MIPNPs at equilibrium Q_e increased with increasing initial concentration of template. The binding capacity of MIPNPs is much higher than NIPNPs. The saturation binding data were plotted according to the Scatchard equation to estimate the binding properties of MIPNPs. The Scatchard relationship can be obtained using the following equation.

$$Q_e/C_s = (Q_{\max} - Q_e)/K_d \quad (1)$$

where $Q_{\max}$ is the saturated adsorption capacity, Q_e is the amount of protein bound to MIPNPs at equilibrium, C_s is the free concentration after adsorption equilibrium, K_d is the dissociation constant.

By plotting Q_e/C_s versus Q_e (Fig. 3b), the dissociation constant K_d and the apparent maximum binding capacity can be calculated from the slope and intercept. The obtained Scatchard plot of MIPNPs for template consisted of one straight line, indicating there is one kind of binding site formed in the MIPNPs. K_d and $Q_{\max}$ is 7.3 μM and 106.7 $\mu\text{M/g}$, respectively.

The adsorption isotherm of MIPNPs for targeting peptide ANP and the following Scatchard analysis were also carried out in the concentration range of ANP from 20 to 286 $\mu\text{g/ml}$. As illustrated in Fig. 4a, the adsorption capacity of MIPNPs for ANP also increased with increasing initial concentration of ANP. Compared to the adsorption capacity of NIPNPs for ANP, the adsorption capacity of MIPNPs is much higher. The Scatchard analysis (Fig. 4b) also gave a single straight line suggesting that only one kind of binding site present in the MIPNPs. K_d and $Q_{\max}$ is 7.9 μM and 36.0 $\mu\text{mol/g}$, respectively. The dissociation constant of MIPNPs for target ANP peptide is slightly higher than that of template peptide.

2.3 Adsorption kinetics

The kinetics of the template peptide and targeting peptide binding to the MIPNPs were explored. It took about 30 min to reach the adsorption equilibrium of template peptide or ANP peptide on MIPNPs (Figs. 5a and 6a). In addition to providing information about how long it will take to reach the binding equilibrium, these experiments provide another way to determine the value of association constants by experimentally measuring the association rate. The first-order rate equation of Lagergren is used for the analysis of the adsorption kinetics of template peptide and targeting peptide on MIPNPs.

$$\ln(Q_e - Q_t)/Q_e = -kt \quad (2)$$

[37]

The plots of $\ln(Q_e - Q_t)/Q_e$ versus t for template peptide and target peptide gave the linear regressions as shown in Fig. 5b and 6b, respectively. The apparent rate constants characterizing the speed of peptide adsorption were 0.15 for template peptide or 0.08 for ANP peptide, respectively. The adsorption rate of ANP onto MIPNPs is slower than that of template peptide. This is reasonable since the size of ANP is larger than that of the template peptide, which results in slower moving and orientation rates.

2.4 Selectivity of MIPNPs

The selectivity of MIPNPs compared to the NIPNPs was carried out with the same concentration of ANP, scrambled ANP and bovine serum albumin (BSA) solutions. Fig. 7 demonstrates that MIPNPs have higher binding affinity for ANP, but lack significant affinity

for scrambled ANP and BSA. Compared to control polymer NIPNPs, MIPNPs show excellent affinity for ANP, but not for BSA or scrambled ANP.

2.5 Recognition of MIPNPs in the spiked cell culture medium and human plasma

The purpose of generating MIPNPs capable of neutralizing ANP is to attenuate the ANP-NPRA signaling in cancer cells in vitro and in vivo. Therefore, the recognition properties of MIPNPs for ANP peptide in cell culture media spiked with ANP and human serum were studied here. Fig. 8 shows the adsorption capacity of MIPNPs and NIPNPs in the spiked cell culture media containing ANP and 0.5% FBS. It can be seen that MIPNPs bind more ANP than NIPNPs in cell culture media containing FBS. We also examined MIPNPs binding to ANP in human plasma. MIPNPs absorbed significantly more ANP in human plasma than NIPNPs, as shown by a loss of ANP peak following absorption by HPLC chromatograms (Fig. 9b). The Q_e of MIPNPs for ANP in two different purified plasma samples (with ANP concentration: 6.5 and 8.8 $\mu\text{g/ml}$) is shown in Fig. 10. MIPNPs demonstrated higher adsorption of ANP than NIPNPs (Fig. 10). These results suggest the capacity of MIPNPs to specifically recognize ANP peptide in the human plasma which comprise many different proteins and peptides.

3. Conclusion

The plastic antibody ANP (MIPNP) was prepared by precipitation polymerization using the epitope approach. The average diameter of prepared MIPNPs in water was 215.8 ± 4.6 nm which is a little bit higher than NIPNPs 197.7 ± 3.1 nm. According to the binding isotherm results, the MIPNPs exhibited a much higher binding affinity for template molecule and ANP than NIPNPs. Scatchard analysis implied that there was a single binding site in MIPNPs with an equilibrium dissociation constant, K_d of 7.3 μM , and a maximum binding capacity 106.7 $\mu\text{mol/g}$ polymer for template peptide, and a dissociation constant of 7.9 μM and a maximum binding capacity 36.0 $\mu\text{mol/g}$ for target ANP. The binding kinetics studies showed that MIPNPs quickly (~ 30 min) reach the protein adsorption equilibrium. The selectivity results demonstrated that MIPNPs had high affinity for ANP, but low affinity for BSA or scrambled ANP. Our synthetic ANP antibody, MIPNP also showed higher affinity and selectivity for ANP in the spiked cell culture medium and human plasma. Collectively, our results demonstrate the potential of MIPNPs to recognize specifically ANP peptide in the cell culture supernatants and in human plasma. The functional effects of using the MIPNPs to reduce ANP levels and thereby disrupt ANP-NPRA signaling will be the subject of future studies.

4. Experimental

Materials

MAA, ethyl alcohol and NIPAm were obtained from Acros Organics. Ammonium persulfate (APS), sodium bicarbonate and BIS were purchased from Fisher Scientific. SDS, 2-mercaptoethanol and albumin from bovine serum were purchased from Sigma Aldrich. TMED was obtained from Fisher Bioreagent. Human or porcine atrial natriuretic peptide (ANP) was obtained from Ana Spec Inc. Scrambled ANP peptide (NH₂-SLRRSSCFGGRMDRIGAQSGLCNSFRY-OH) and template peptide were purchased from Biosynthesis. Standard Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum were obtained from Invitrogen.

Measurement of template peptide, ANP, BSA and scrambled ANP

Determination of single peptide using fluoroldehyde (OPA) reagent—The measurement of template peptide, ANP, BSA and scrambled ANP was performed using

OPA reagent solution. OPA reagent was prepared by dissolving 5 mg of OPA, 100 μ l of absolute ethanol, and 5 μ l of β -2-mercatoethanol in 10 ml of 50 mM carbonate buffer pH10.5. The OPA reagent was protected from direct light, and used within 2 hrs. Protein/peptide standards of known concentration were prepared by dissolving the standard protein/peptide under the same conditions as the unknown sample. First, 10 μ l of each sample was added to an opaque 96- well plate, then 100 μ l of OPA reagent was added and mixed well with the samples. PBS and supernatant of MIPNPs or NIPNPs suspension after filtered with 0.45 μ m PVDF syringe filter (Whatman, Germany) were used as blanks respectively of standard, MIPNPs and NIPNPs samples. After 5 min, the fluorescence was measured at excitation of 330–390 nm and emission of 436–475 nm using a Synergy H4 plate Reader (Biotek Instruments Inc. USA). The blank's fluorescence was subtracted from the fluorescence emission values of the sample and standards to determine net fluorescence. The net relative fluorescence of the standards versus concentration was plotted. The concentration of the unknown was calculated using the standard curve.

HPLC determination of ANP in cell culture medium spiked with ANP and in human plasma—ANP in cell culture medium was determined by HPLC with a Shimadzu system controller, UV-vis detector, and Kinetex C18, 100 $\times$ 4.60 mm column (Phenomenex, USA). The mobile phase was run at 0.5 ml/min in gradient mode with mobile phase A: 98% water and 2% acetonitrile with 0.1% TFA and mobile phase B: 98% acetonitrile and 2% water with 0.1% TFA. The calibration curve was obtained by plotting the integration area of the peak at different concentrations of ANP standard solutions (100, 50, 25, 10, 1 μ g/ml) versus the concentration. The concentrations of the unknown samples were calculated by calibration curve.

Synthesis of MIPNPs

To prepare MIPNPs, first 10 mmol (0.1582 g) BIS was dissolved in 50 mL SDS (10 mg) aqueous solution in a 250 mL two-neck round bottom flask. After 1 mmol (0.1100 g) NIPAm, 3 mmol (30 μ l) MAA and 1.5072 g APS were dissolved in the BIS solution, the template peptide (0.7 mg, 1 μ mol) was added to the solution. After purging the solution with argon gas for 5 min, TEMD (40 μ l) was added. The solution was protected from oxygen with argon gas at room temperature under stirring at 1000 rpm. After 15 min, white precipitate was seen in the flask. After 24 hrs reaction, the suspension was centrifuged at 3000 rpm for 15 min. After removal of the supernatant, the white product was washed with 10 wt% acetic acid until no peptide can be determined by OPA assay. The prepared MIPNPs were freeze-dried for further use. The NIPNPs were prepared under identical conditions as MIPNPs in the absence of template.

Size Measurement

The hydrodynamic diameter of the purified MIPNPs and NIPNPs was determined using a DynaPro DLS Plate reader (Wyatt Technology) and a minimum of thirty measurements were taken for each sample. The MIPNPs and NIPNPs were dispersed in de-ionized water by sonicating for one minute using Sonic Dismembrator (Fisher Scientific) at 5 watts.

Binding experiments

Binding isotherm experiments of MIPNPs and NIPNPs were carried out using a batch-wise adsorption method. The bulk template peptide or ANP solutions (1 mg/ml) were prepared by dissolving the peptide in autoclaved de-ionized water. MIPNPs or NIPNPs were suspended in autoclaved PBS solution and ultra-sonicated for 1 min with 5 watts to obtain a 0.05 mg/ml suspension. Different amounts of peptide solution were mixed to MIPNP or NIPNPs suspension, and the suspensions were oscillated in a shaking incubator at 4 $^{\circ}$ C for 30 min to

reach adsorption equilibrium. After adsorption, the peptide concentration in the supernatant was determined after filtration with a 0.45 μm syringe filter. The equilibrium adsorption capacity Q_e ($\mu\text{g}/\text{mg}$) was calculated by mass balance according to the following equation:

$$Q_e = (C_0 - C_s)V/m, \quad (3)$$

where m is the mass (mg) of nanoparticles, V (ml) is the volume of protein or peptide solution, and C_0 ($\mu\text{g}/\text{ml}$) and C_e ($\mu\text{g}/\text{ml}$) are the protein or peptide concentrations of the initial solution and the supernatant at equilibrium, respectively. In dynamic adsorption experiments, the MIPNP and template or ANP peptide suspensions in the 1.5 ml centrifuge tubes were shaken at 4 $^{\circ}\text{C}$. The tubes were taken out at specific intervals and the template concentration in the supernatant was measured. The absorbance Q_t ($\mu\text{g}/\text{mg}$), i.e. dynamic adsorption capacity at time t , was calculated according to the following equation:

$$Q_t = (C_0 - C_t)V/m, \quad (4)$$

Where C_t ($\mu\text{g}/\text{ml}$) is the template concentration in the supernatant at time t .

Selectivity tests of MIPNPs

Selectivity tests were carried out by adding 32 μl of 1 mg/ml BSA, scrambled ANP or native ANP to 100 μl of 0.05 mg/ml MIPNP suspension in PBS in a 2 ml centrifuge tubes. The centrifuge tubes were agitated in a shaking incubator at 4 $^{\circ}\text{C}$ for 30 min. After adsorption, the peptide or protein concentration in the supernatant was measured.

Recognition tests of MIPNPs in cell culture media and in human plasma

The recognition tests of MIPNPs were studied in both cell culture media spiked with ANP. For the recognition experiments of MIPNPs in cell culture media (DMEM with 0.5% of fetal bovine serum (FBS)), three different amounts of 1 mg/ml of ANP solution (1 μl , 5 μl and 10 μl) were added separately to 100 μl DMEM with 0.05 mg/ml MIPNP or NIPNP in centrifuge tube. For the control experiment, ANP solution was added to DMEM without nanoparticles. After adsorption, the peptide concentration in the supernatant was measured by HPLC. The experiment was performed in triplicate. For the recognition experiment of MIPNPs in the human plasma, the plasma samples collected from asthmatic patients (kindly provided by Dr. Shyam Mohapatra) were purified using a microconcentrator, 10 kDa mwco (Amicon corporation, MA) by centrifuging at $4000 \times g$ for 2 hrs at 4 $^{\circ}\text{C}$ (Sorvall Legend XTR centrifuge, Thermo Scientific). After purification, 50 μl of 0.15 mg/ml MIPNPs or NIPNPs PBS suspension was added to 100 μl of plasma. The samples were run in duplicate.

Statistical analysis

Each experiment was repeated three times. Data were analyzed using IBM SPSS statistical 19 software and values shown are the mean $\pm$ standard deviation (SD).

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Figure 1. Preparation of polymer nanoparticles. (a) Scheme of the synthesis of MIPNPs; (b) Chemical interactions in preparation of MIPNPs.

Figure 2.
The size distribution of MIPNPs (a) and NIPNPs (b) in water.

Figure 3.

(a) Adsorption isotherm curves (Q_e (μg/mg) vs. C_0) of MIPNPs (◆) and NIPNPs (×) for template peptide. (b) Scatchard plot of MIPNP for template peptide, Q_e/C_s vs. Q_e .

Figure 4.

(a) Adsorption isotherm curves (Q_e ($\mu\text{g}/\text{mg}$) vs C_0) of MIPNPs (◆) and NIPNPs (×) for ANP. (b) Scatchard plot of MIPNP for ANP (Q_e/C_s vs. Q_e).

Figure 5.

(a) Dynamic adsorption profile of template peptide on MIPNPs, Q_t ($\mu\text{g}/\text{mg}$) vs time. (b) Plot of $\ln (Q_e - Q)/Q_e$ vs time for template peptide.

Figure 6.

(a) Dynamic adsorption profile of ANP on MIPNPs, Q_t ($\mu\text{g}/\text{mg}$) vs time. (b) Plot of $\ln (Q_e - Q_t)/Q_e$ vs time for ANP peptide.

Figure 7.
ANP binding to MIPNPs in solution.

Figure 8. ANP binding to MIPNPs or NIPNPs in a cell culture medium supplemented with 0.5% FBS and ANP.

Figure 9. HPLC chromatograms obtained after the absorption of a 6.5 $\mu\text{g/ml}$ of ANP in human serum without nanoparticles (a), with 0.05 mg/ml MIPNPs (b) or NIPNPs (c) at 214 nm.

Figure 10.
MIPNPs or NIPNPs recognition of ANP in human plasma samples.