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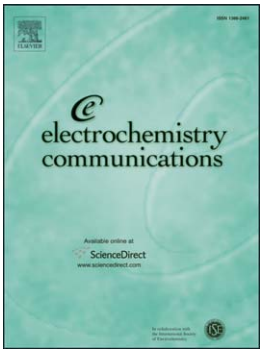

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PII: S1388-2481(16)30165-5
DOI: doi: 10.1016/j.elecom.2016.07.012
Reference: ELECOM 5742

To appear in: *Electrochemistry Communications*

Received date: 13 July 2016
Revised date: 25 July 2016
Accepted date: 26 July 2016

Please cite this article as: Gergely Lautner, Mónika Plesz, Gyula Jágerszki, Péter Fürjes, Róbert E. Gyurcsányi, Nanoparticle displacement assay with electrochemical nanopore-based sensors, *Electrochemistry Communications* (2016), doi: 10.1016/j.elecom.2016.07.012

Nanoparticle displacement assay with electrochemical nanopore-based sensors

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Abstract: The proof of concept of a nanoparticle displacement assay that enables the use of large diameter nanopores for the detection of targets of smaller molecular dimensions is presented. We hypothesized that an inherent signal amplification should arise from the selective displacement of nanoparticles preloaded in a nanopore by a much smaller molecular target. The method is demonstrated using peptide nucleic acid (PNA)-functionalized gold nanopore arrays in which short DNA-modified gold nanoparticles are anchored by weak interaction. Complementary microRNAs are detected via the resistance change caused by competitive displacement of nanoparticles from the PNA-functionalized nanopores.

Keywords: solid state nanopore, PNA, nanoparticle, displacement assay, microRNA, NASBA

Introduction

Electrochemical nanopore sensors are based on detecting changes caused by target species in the physical-chemical properties, most often electrical conductivity, of the minute space delimited by the nanopore interior [1-5]. This sensing concept with single species detection capability has been studied for the determination of a wide variety of targets ranging from small ions [6] up to nanoparticles [6-12]. While the size resolution of nanopore-based resistive pulse sensors can reach subnanometer levels [13], their analytical use, i.e. selective detection of target species in complex samples, still requires the use of selective receptors, either added to the sample solution [10, 14] or confined to the nanopore interior [15, 16]. In this latter approach either stochastic sensing [17, 18] based on reversible binding of the target [19, 20] or the more conventional approach in which the target is bound "irreversibly" into the nanopore sensing zone is used [21-25]. While there are exceptions [20], nanopore sensing requires nanopores with sizes that are comparable to, but larger than, that of the target. For solid state nanopores this requirement means that the smaller the target species the more demanding becomes the nanopore fabrication process in terms of reliability and cost. Therefore, we became interested in exploring sensing schemes that are able to address this problem, i.e., which have larger diameter pores adapted for the detection of much smaller macromolecules. Here we report the proof of concept of a novel nanoparticle-based displacement assay using solid-state nanopore arrays to amplify the signal of oligonucleotides, taking as a model the detection of a 22-mer microRNA, miR-208a [26], a potential biomarker of acute myocardial infarction. For this purpose we functionalized gold nanopore arrays made by focused ion beam (FIB) milling with a thiolated 18-mer peptide nucleic acid (PNA) probe that is uncharged and binds with high affinity to the complementary miR-208a [27]. Before analysis the PNA-functionalized nanopores were filled with 13 nm diameter gold nanoparticles (AuNPs) modified with 10-mer thiolated DNA (DNA-AuNP) - complementary to PNA - to block the ion current through the nanopores. The shorter DNA strands were designed to have a weak interaction with the PNA layer so that DNA-AuNPs can be released by the competitive action of the miR-208a strands. We anticipated that the release of the nanoparticles, which are much larger than the molecular dimensions of miRNA, would result in an inherent signal amplification.

Scheme 1.

Experimental

Thiolated PNA probe (HS-PNA: N'-Thiol-C6-OO-GCTTTTTGCTCGTCTTAT-C') was from Eurogentec. All natural nucleic acids were custom synthesized and HPLC purified (Sigma-Aldrich); targets: miR-208a microRNA (5'-AUAAGACGAGCAAAAAGCUUGU-3'), its DNA analogue (DNA-208a: 5'-ATAAGACGAGCAAAAAGCTTGT-3'), a 22-mer random RNA as negative control (NC RNA, 5'-AGUACUAAUUCGUCUCUGUUCU-3'). For the modification of the AuNPs a short complementary thiolated DNA (HS-DNA) with T_{10} spacer, 5'-Thiol-C6-T_{10}-ATAAGACGAG-3') and non-complementary (HS-NC DNA, 5'-Tiol-C6-TTGACCAACAAGCTTTTTTT-3') DNA strands were used. All solutions were made of highest grade chemicals for molecular biology and with DI water (18.2 MOhm·cm) or RNase-free water (Sigma-Aldrich).

Fabrication of solid-state gold nanopore arrays

Gold nanopore arrays consisting of 7 nanopores were fabricated by combined micromachining and FIB milling as described earlier [28, 29]. In essence, slightly conically-shaped nanopores were drilled by FIB in thin silicon nitride (200 nm, SiN_x) supported gold membranes (150 nm, Au). We used nanopores with 40, 60 or 80 nm smallest pore diameters as determined by a secondary electron yield map generated by Ga^+ ions passing through the pore and reaching a metal target behind the membrane (Zeiss Leo 1540 XB SEM, Canion FIB nanoprocessing system, Carl Zeiss).

Preparation of HS-DNA probe modified gold nanoparticles

HS-DNA probe modified AuNP solution was prepared as described earlier [30]. First, 13±1 nm diameter gold nanoparticles were prepared by reducing $HAuCl_4$ with a citrate salt and determining their concentration by measuring the absorbance at 524 nm ($\varepsilon = 2.7\times10^8$ L $mol^{-1}\cdot cm^{-1}$) [31]. The AuNP solution was concentrated tenfold to 120 nM by centrifugation at 16000 g for 10 min. Conjugation of AuNPs with HS-DNA probes was performed by adding 21 µL of 100 µM HS-DNA solution to 400 µL of AuNP solution. To increase the DNA probe density on the AuNPs, the ionic strength was increased in three consecutive steps: first 46.8 µL 100 mM NaCl solution was added and vortexed for 30 min, then 19.7 µL of 1 M NaCl and vortexed for another 30 min, and finally 27 µL of 1 M NaCl to reach a final NaCl concentration of 100 mM. DNA-AuNP solution was aged at 4°C overnight before adding 57 µL aqueous solution of (11-mercaptoundecyl)tetra(ethylene glycol) (HS-TEG) and vortexing for 30 min. The unreacted HS-TEG was removed by replacing the supernatant with 1 mM NaCl at least six times after centrifugation at 16000 g for 10 min.

Functionalization of the gold nanopore arrays

Prior to functionalization, the nanopore chips were cleaned with piranha solution for 30 min, thoroughly rinsed with DI water and dried. Gold nanopores were functionalized with HS-PNA probes in a pre-hybridized form with a complementary DNA to ensure self-regulated surface coverage of PNA on gold by concomitant minimization of the non-specific strand adsorptions [27]. The nanoporous membranes were modified by placing 5 µM miR-208a specific PNA prehybridized with DNA-208a (20 µL) in borate buffered saline onto their surface, covered to avoid evaporation and incubated at 4°C overnight. The membranes were then rinsed with DI water and blocked with 1 mM HS-TEG solution for one hour. Before use the immobilized PNA layer was activated by dehybridizing the complementary DNA strands using 50 mM NaOH (2×1 mL). The optimized procedure to load the DNA-AuNPs into the nanopores implied forcing 5 µM DNA-AuNP solution in 50 mM NaCl into the nanopore membrane by applying 16 kPa pressure difference for 60 min.

Electrochemical measurements

The resistance of the nanopore membrane was determined by impedance spectroscopy using a Reference 600-Potentiostat (Gamry Instruments) and a custom-made transport cell with the two compartments separated by the nanoporous membrane. Each compartment was filled with 1 mL of 100 mM NaCl solution and electrically connected by inserting an Ag/AgCl reference electrode (RE). Impedance spectra were recorded in the frequency range 100 kHz to 0.1 Hz using an excitation amplitude of 30 mV (vs. OCP). The experimental data were fitted with an equivalent circuit as shown in the inset of **Scheme 1**. The nanopore was considered by its resistance and a constant phase element that is connected in series with the rest of the circuit, comprising the capacitive and resistive contributions of the reference electrodes and the solution resistance. In some experiments the DNA-AuNP displacement during microRNA measurements was facilitated by applying a constant potential of 100 mV so that the RE in the compartment in contact with the larger base of the nanopores was positively polarized while the one in the compartment in which the microRNA samples were introduced was negatively polarized.

Results and discussion

For this study we used gold nanopore arrays consisting of seven nanopores giving a cumulative response because we expected single nanopore sensors to have a narrow dynamic range and sluggish responses at low target concentration [32]. The diameter of the pores (40, 60, 80 nm) was chosen:(i) to be larger than the largest pore sizes reported for oligonucleotide detection ca. 25 nm [22, 33], (ii) to be of a size range easily manageable by FIB milling and (iii) to be accessible for nanoparticle insertion. The resistance(R_p) and capacitance(C_p) of the different nanopores used in this study ranged between 5-25 MΩ and 0.9-1.9 nF, respectively.

First we investigated the direct binding of 100 nM miR-208a to PNA-functionalized nanopores with different pore diameters. Even at this very high concentration the resistance change (ΔR_p) of the nanopore arrays was less than ca. 10%, which is a small but significant effect compared with that of NC-RNA (**Figure 1**A). The pore diameter had little influence on the resistance change, with a slight trend towards decreased specific and increased nonspecific response as the pore size increased. The nanopores could be effectively regenerated by 50 mM NaOH (**Figure 1**B).

Figure 1.

While the limit of detection of the direct assay is obviously not sufficient we continued with the nanoparticle displacement assay as pictured in **Scheme 1**. First DNA-AuNPs were anchored in the PNA-functionalized nanopores (ø 60 nm) to achieve maximal load (maximal ΔR_p). Solely by incubation in DNA-AuNP the resistance increased gradually, reaching saturation after ca. 60 min at a maximum ΔR_p of 32(±0.6)%. We assume that the binding occurs dominantly at the entrance of the nanopore as the nanoparticles are highly charged (ζ=-10.60 mV) and the electrostatic repulsion would impede the accession of further DNA-AuNPs. To increase the driving force a negative pressure was applied, carefully optimized to find the maximum value that avoids membrane rupture, i.e. 16 kPa, which resulted in a more efficient obstruction of the nanopores ΔR_p=59(±1.6)%. Further experiments revealed that by increasing the ionic strength (50 mM NaCl; ζ(DNA-AuNP)=-3,59 mV) and the concentration of DNA-AuNPs to 5 μM the ΔR could be maximized to 85(±5)% (**Figure 2**A). By comparison, using NC DNA-AuNPs resulted in a ΔR of only up to 17%, suggesting that the AuNPs are selectively withheld by hybridization in the PNA-functionalized nanopores.

Figure 2.

We found that nanopore arrays with 60 nm pore diameter gave the largest displacement response (data not shown) and therefore we used these in our further experiments. During displacement assays 0.1 V was applied to facilitate the entrance of the target microRNA and release of DNA-AuNP. The hybridization of miR-208a targets in the nanopore array resulted in a concentration-dependent resistance change (Figure 2A). The small ΔR_p of 3.3(±0.7)% at 0 nM concentration is likely to be caused by the electrophoretic release of the DNA-AuNPs from the nanopores. The amplification effect is shown by the 10-fold lower microRNA concentration required to reach the ca. 10 % ΔR_p achieved in the direct assay. While this paper focuses on exploring the feasibility of nanoparticle displacement in nanopore arrays without an immediate analytical application, we applied an isothermal NASBA amplification [35] to concentrate the miR-208a in order to demonstrate the possibility of assessing extremely low microRNA levels in circulation [34]. NASBA does not require a thermal cycler, nor very precise temperature control, and thus can be potentially integrated in microfluidic addressed nanopore platforms [29]. As proof of concept, 100 fmol miR-208a target molecule was amplified by NASBA reaction in only 20 µL and the reaction mixture injected directly in the measurement cell. The miR-208a amplicon gave a -25.5(±1.0)% pore resistance change (Figure 3A), compared to the similarly amplified negative control which resulted in only -9.2(±2.9)%.

Figure 3.

While the use of PNA probes was motivated primarily by itheir high affinity and uncharged nature, important to overcome charge repulsion in the pore, they also have excellent thermal and (bio)chemical stability. Indeed, PNA-functionalized nanopore arrays and the displacement assay were found to give very consistent results, even over longer time periods. **Figure 3**B shows that PNA-functionalized nanopores with -31.1(±0.5)% resistance change in response to 100 nM miR-208a after regeneration and storage at 4°C for 30 days gave a response of -32.2(±0.7)% .

Overall, the results show the feasibility of performing a nanoparticle displacement assay with electrochemical nanopore sensors with remarkably good reproducibility, reversibility and stability. The concept may be extended to the detection of proteins by using aptamer-based receptors as well as for a wide range of nucleic acids. In this latter case nucleic acid targets of a well-defined length, such as microRNAs[36], are preferred for readily quantifiable resistance changes during nanoparticle displacement assays. While in this study we used impedance spectroscopy to detect changes in the pore resistance, it would probably be feasible to use simple DC measurements instead.

Acknowledgement

This work was supported by the Lendület program of the Hungarian Academy of Sciences (LP-2013/63) and ENIAC(CAJAL4EU) project. The authors gratefully acknowledge the technical assistance of Magda Erős and Levente Illés in nanopore fabrication.

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Figure captions

Scheme 1 (A) Schematics of the nanoparticle displacement assay with nanopore sensors for the detection of microRNA. (I) PNA-functionalized nanopore. (II) Nanopores after anchoring DNA-AuNPs by hybridization (corresponds to the ready-to-measure state). (III) The target microRNA by binding to the PNA displaces DNA-AuNPs decreasing the pore resistance. (B) Probe design for displacement assay (C) Secondary electron yield map of the nanopore arrays. (D) Equivalent circuit for fitting impedance spectra.

Figure 1 (A) Direct effect of 100 nM miR-208a and NC-RNA onto the resistance of PNA-functionalized nanopore arrays with different pore diameters. (B) Pore resistance during a measurement cycle: (a) PNA-functionalized nanopore (ø 40 nm), (b) 100 nM miR-208a, (c) after regeneration, (d) 100 nM NC RNA.

Figure 2 (A) Pore resistances during nanoparticle displacement assay: (a) PNA-functionalized nanopore array (ø 60 nm); (b) after immobilization of DNA-AuNP; (c) after incubation with 100 nM miR-208a; (d) after regeneration with 50 mM NaOH. (B) Concentration dependent resistance change.

Figure 3 (A) Representative Nyquist plots: (a) PNA-functionalized nanopore arrays; (b) after DNA-AuNP immobilization; (c) NASBA amplified miR-208a detection. (B) Reliability of the displacement assay.

Figure 1

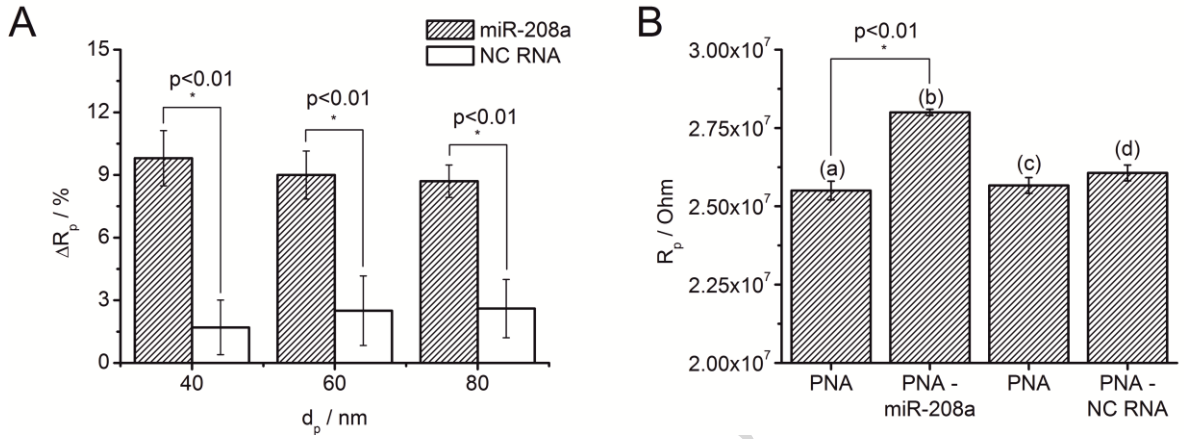


Figure 2

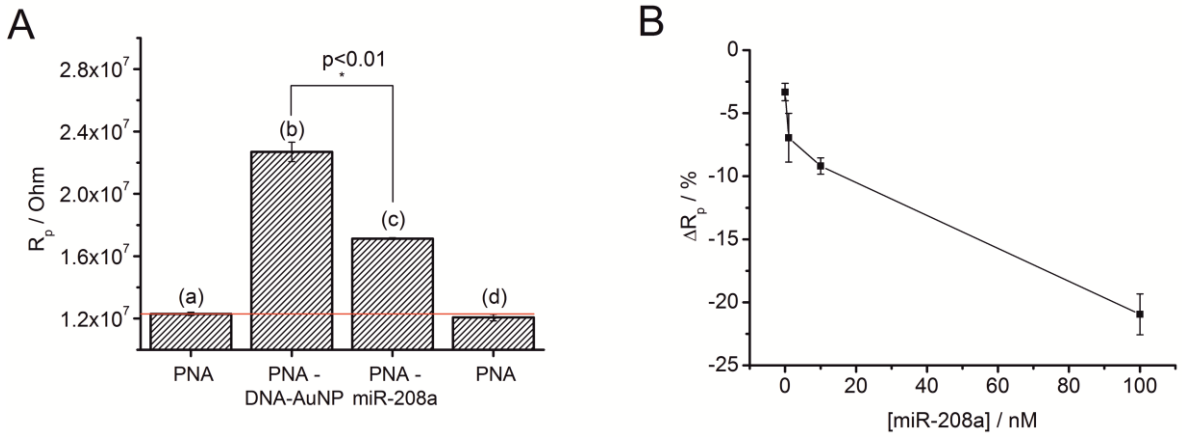
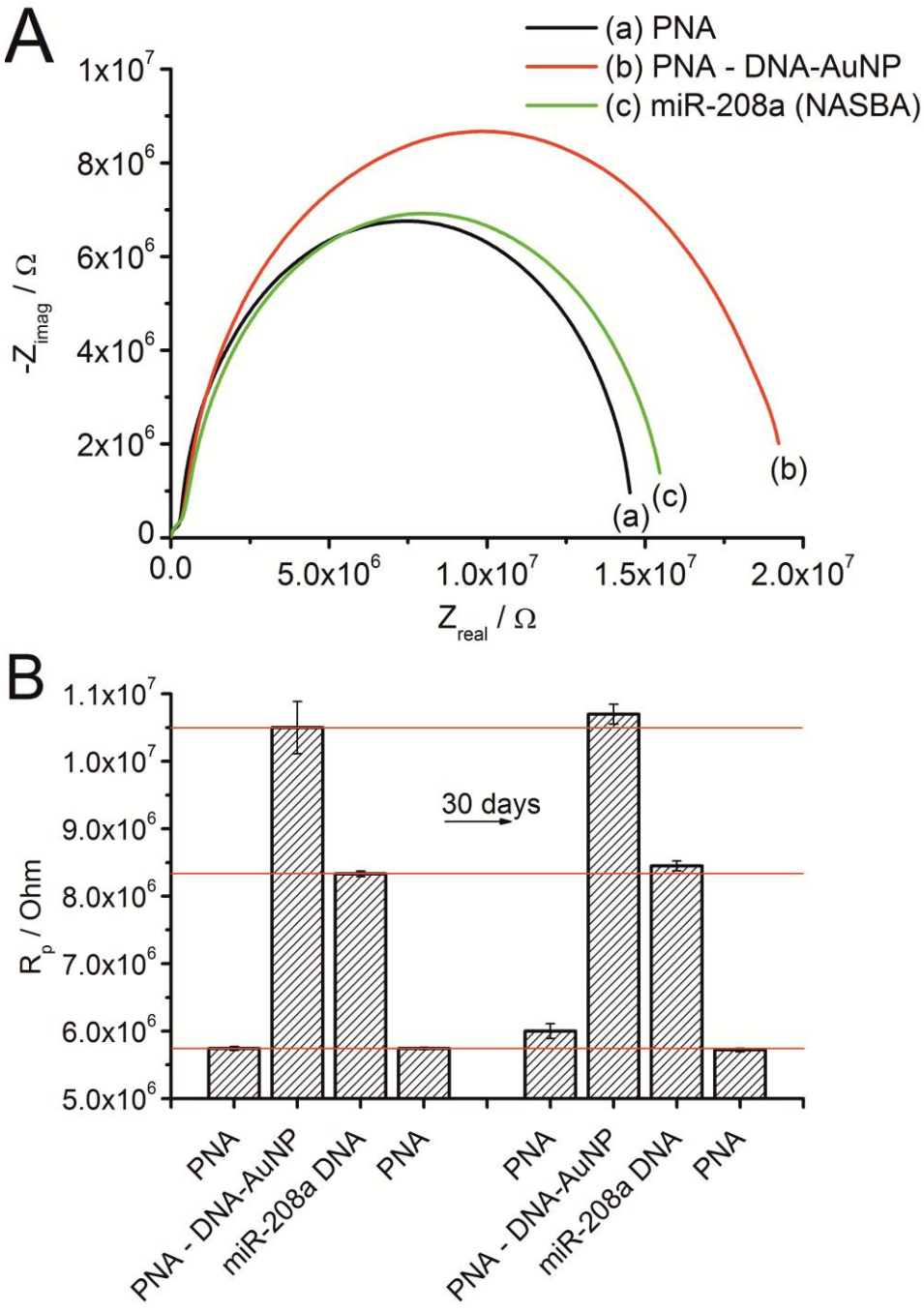
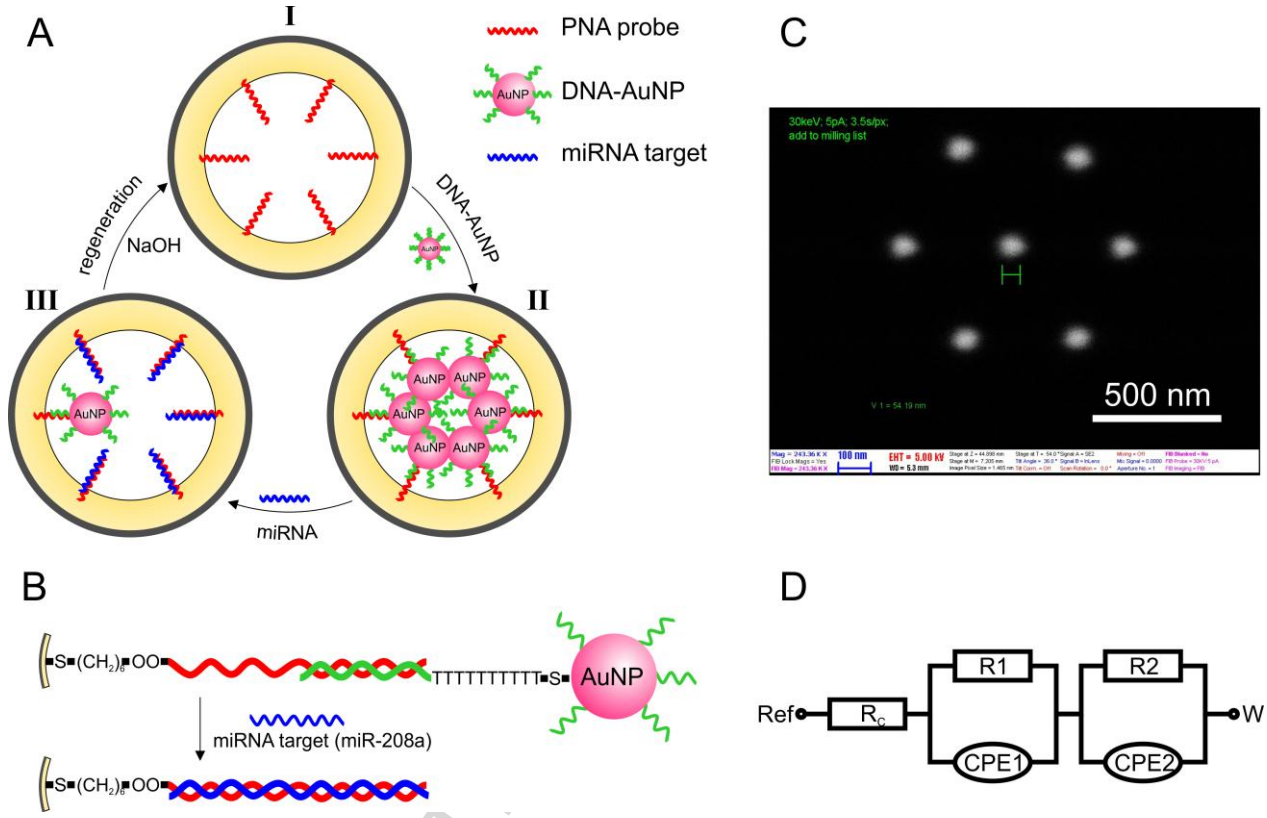


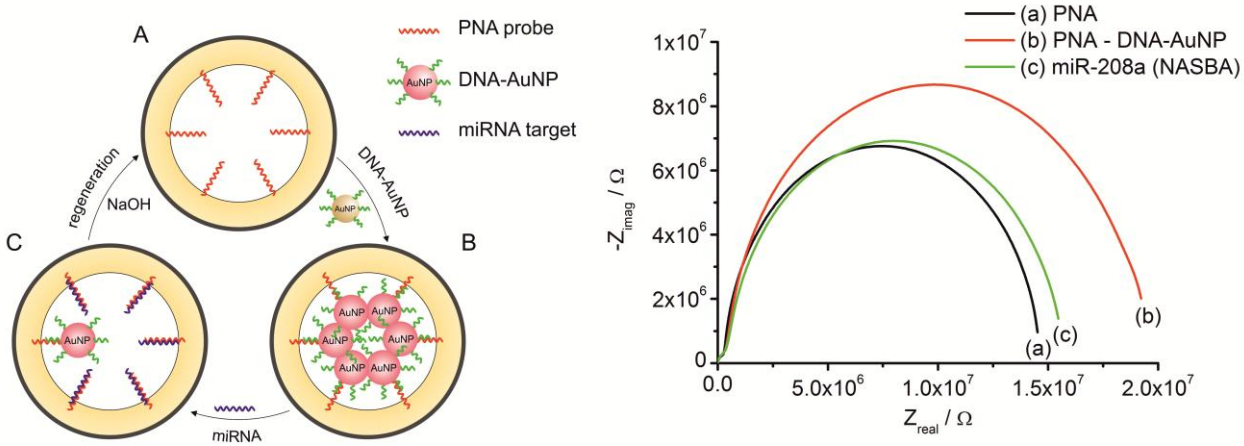
Figure 3



Scheme 1



Graphical Abstract



Highlights

- A novel nanopore-based sensing concept suitable for nucleic acid detection is introduced
- Nanoparticles are immobilized reversibly in nanopores
- A nanoparticle displacement assay with nanopores is demonstrated