Chapter 8

Hybridization Chain Reaction Design and Biosensor Implementation

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Abstract

DNA biosensors could overcome some of the common drawbacks of lab-based techniques for nucleic acids detection for diagnostics purposes. One of the main impediments for such applications of DNA biosensors is their lack of sensitivity: this can prevent their full exploitation in the diagnostic analytical field. DNA nanotechnology could enhance DNA biosensors and let them perform at the required high sensitivity. Well-designed, programmable self-assembly reactions can be triggered by a specific nucleic acid target. The Hybridization Chain Reaction (HCR) is a self-assembly strategy in which the target nucleic acid sequence triggers the formation of long nicked double-stranded DNA nanostructures. This can be performed in solution or on a surface, and the process can be coupled to different signal transduction schemes. We here describe the methods to design and test HCR reactions for the detection of different nucleic acid targets in solution and the procedures to exploit this strategy on surfaces with an electrochemical biosensing platform.

Key words Hybridization chain reaction, Self-assembly, Biosensors

1 Introduction

Until the 80s, DNA has been always considered mainly as the molecule for data storage in lifeforms, with a poor repertoire of structures or applications in different contexts. In the past decades, starting with the pioneering work of Nadrian Seeman [1, 2], this polymer has been involved in quite different kinds of studies and consequent applications, suggesting a new way to think about DNA. DNA nanotechnology exploits the powerful features of nucleic acids, such as programmable base-pairing properties, a well-known structure and the great stability and flexibility towards radically different goals compared to their role in organisms. DNA may be nowadays considered a self-assembling building block for objects in the nanoscale, or more interestingly, it can be considered a versatile object, able to generate dynamic and automatic switchable devices thanks to metastable conditions in a dynamic

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disequilibrium [3]. An example of this kind of dynamic process is the toehold-mediated strand displacement (TMSD), in which alternative metastable secondary structures of nucleic acid species have an important role. The TMSD is an isothermal reaction involving nucleic acids in which one strand displaces a second strand in the interaction with a third one that is in some grade complementary to both. The thermodynamic stability determines the favorite interaction. In this way, DNA nanostructures can be also kinetically controlled by properly defining the involved sequences [4]. The Hybridization Chain Reaction (HCR), invented by Dirks and Pierce in 2004 [5], is a simple yet powerful implementation of this kind of tunable process. This reaction consists of the isothermal assembly of stable DNA monomers in bigger structures at the addition of a specific initiator. The monomers in the original reaction design have hairpin-like secondary structures, composed of a long stem (18 nt), a short loop and a short corresponding overhanging toehold (both 6 nt). The easiest way to design this kind of reaction involves just two species of hairpins in equal molar concentrations. The two species stably coexist in solution, thanks to their metastability, without any interaction in the experiment timescale. The equilibrium of the system is altered by the initiator with a proper sequence: this triggers a cascade reaction of subsequent hybridizations, or more precisely, a cascade of toehold-mediated strand displacements, leading to the formation of a long nicked double-stranded DNA. In absence of the initiator, the folded secondary structure of the monomers prevents the triggering of the reaction, since the toehold is closed within. The initiator activates the first hairpin, leading to the exposure of the short loop sequence, suitable for the binding to and opening of a second hairpin. The second hairpin, once incorporated, restarts the cycle with another copy of the first hairpin, and so on (*see* Fig. 1).

DNA biosensors could play an important role in the detection and quantification of nucleic acids targets and to this purpose, they can potentially replace some lab-based techniques such as qPCR and Next Generation Sequencing in applications where cost, easiness to use, size of the device and portability could become an issue. One of the main drawbacks of DNA biosensors is their lack of sensitivity in the detection of targets. An effective strategy to overcome this issue may be offered by DNA nanotechnology through the implementation of self-assembly reactions triggered by the recognition of the target, hence the interest to use HCR as detection method for the biosensing of nucleic acids targets (DNA or RNA). HCR is based on an enzyme-free isothermal process. It is very simple in principle and it could be very sensitive and specific. The product is quantitatively and qualitatively related to the amount of the specific target and, finally, HCR can be combined with various strategies of signal transduction. Therefore, the design and characterization of a highly specific and effective hybridization

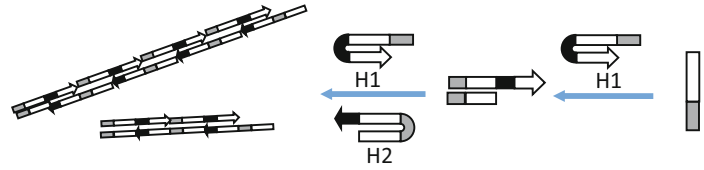

Fig. 1 Scheme of HCR in solution for the detection of microRNA

chain reaction would facilitate the application of biosensors. Recently, our group described the adaptation of HCR on surface for the plasmonic and electrochemical detection of pathogen DNA in samples [6]. Many publications show different strategies using HCR for diagnostic purposes thanks to its simplicity and suitability [7–10].

A general setup of a HCR-based detection method involves:

1. Election of the initiator, its sequence, depending on the goal and on the adopted strategy.
2. Selection of the convenient thermodynamic and structural features of the monomers to ensure the proper thermodynamic stability and kinetic properties of the system in the desired conditions. Ang and Yung proposed possible guidelines to design stable HCR monomers in order to get a highly effective HCR in solution [11]. The use of dedicated computer tools employing physical thermodynamic models is very useful.
3. Experimental analysis of the DNA oligonucleotides in the reaction conditions is needed. Electrophoresis gel analysis helps to investigate the efficiency of the formation of the intermediate complex and the final nanostructures. AFM could also be useful to study the general morphology of the products.
4. Once the reaction is well characterized and its efficiency is proved, the implementation on a biosensing platform is the next step, involving the choice of a strategy and methods for signal transduction coupled to the hybridization chain reaction amplification.

Here we describe methods for each step of the above-proposed pipeline. We have been able to design hybridization chain reactions specific for different targets such as short sequences, such as microRNA sequences, and sequences of pathogen DNA.

2 Materials

All solutions should be prepared in ultrapure water (resistivity >18 MΩcm at 25 °C, such as from a MilliQ apparatus from Millipore).

2.1 HCR in Solution and Reaction Mixtures

1. Single-stranded sequences of oligonucleotides, hairpins, and target DNA at 100 μM concentration in water. These are commonly stored at −20 °C. Aliquots are allowed to fully thaw before use and stored as working aliquots at 4 °C.
2. HCR buffer: 0.5 M NaCl, 50 mM Na_2HPO_4, pH 6.8.
3. A PCR thermal cycler (such as the Thermo Scientific Sprint PCR) to perform thermal treatment of the samples.

2.2 Nondenaturing PAGE for HCR Products Characterization

1. 40% Acrylamide solution (Acrylamide/bis-acrylamide 37.5:1), of a purity suitable for electrophoresis.
2. 10% Ammonium persulfate (APS) in water: prepare fresh before use.
3. N,N,N,N′-tetramethyl-ethylenediamine (TEMED).
4. 5× TBE buffer: 445 mM Tris–boric acid, pH 8.0, 10 mM ethylenediaminetetraacetic acid tetrasodium salt (EDTA-Na_4).
5. Loading Buffer: prepare 0.2% w/v solution of bromophenol blue (BB) and xylene cyanol FF (XC) in 50% v/v glycerol solution in 1× TBE.
6. SYBR Gold nucleic acid gel stain solution, 10,000× stock solution in DMSO (Invitrogen). Prepare 1× solution in 1× TBE buffer before use.
7. DNA ladder: pUC19 DNA/MspI (Hpa II) (store at 4 °C).
8. An apparatus for vertical PAGE electrophoresis (we use the PROTEAN II xi cell large format electrophoresis chamber and the Mini PROTEAN II, depending on the desired size of the gel, from Bio-Rad).
9. Electrophoresis power supply (such as the Powerpac 300, from Bio-Rad).
10. A gel documentation system (such as the Gel Doc 1000, from Bio-Rad).
11. Gel loading pipette tips appropriate for the thickness and length of the well in the PAGE gel.

2.3 AFM Characterization of HCR Products

1. AFM deposition buffer: 10 mM HEPES, 10 mM NaCl, 5 mM MgCl, pH 7.5.
2. 1× TE: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.
3. Centrifugal filter units, MWCO 100 kDa (like Amicon Ultra-0.5 mL centrifugal filters).
4. Freshly cleaved muscovite mica (EMS).
5. Atomic Force Microscope (such as a Multimode system, from Bruker) with proper cantilevers with probes to image in air sample, preferably in an intermittent contact mode (the technique name can vary for the AFM model in use).

2.4 Biosensor Surface Preparation and Characterization

1. TCEP solution: 300 μM Tris(2-carboxyethyl) phosphinehydrochloride (TCEP).
2. 1 mM solution of 6-mercapto-1-hexanol (MCH) in ultrapure water, freshly prepared.
3. 100 μM thiolated oligonucleotide probes (Eurofins MWG or Sigma-Genosys).
4. 99.99% pure gold wire (Electron Microscopy Sciences).
5. Muscovite mica discs or squares (to be cleaved just before use).
6. High-vacuum evaporator with a resistively heated specimen stage (from Edwards or other).
7. Round glass cover slides (12 mm in diameter).
8. EpoTek 377 (Epoxy Technology Inc., Billerica, MA, U.S.A.).
9. PBS buffer: 10 mM NaCl, 197 μM KCl, 291 μM Na_2HPO_4, 131 μM KH_2PO_4.
10. A plasma Cleaner, (Femto, Diener Plasma surface technology).

2.5 HCR Detection on Surface and Electrochemical Characterization

1. Oligonucleotide sequences were purchased from Eurofins MWG and Sigma-Genosys.
2. Costumer made 3-electrodes electrochemical cell, consisting of a single chamber that covers the gold electrodes.
3. KCl 0.1 M solution in ultra-pure water.
4. PBS buffer, 10 mM NaCl, 197 μM KCl, 291 μM Na_2HPO_4, 131 μM KH_2PO_4.
5. Hairpin oligonucleotides buffer 0.5 M NaCl, Na_2HPO_4 50 mM, pH 6.8.
6. Electrochemical workstation (μAutolab, Metrohm Autolab B.V., Utrecht, The Netherlands).

3 Methods

3.1 Design of DNA Sequences

We describe the general procedures adopted to design couples of hairpins specific for a selected target sequence of interest in diagnostics, such as microRNA and sequences for the detection of pathogens. First, special attention is required to elect the target sequence, taking into account the length and base composition of the candidate sequences. Bibliographic research, bioinformatics analysis or database mining are useful to this purpose (*see* **Note 1**). A proper set of oligonucleotide probes and hairpins can be consequently designed to detect the target sequence and consequently to perform HCR amplification. NUPACK represents a powerful instrument for the design of self-assembly reactions such as HCR. It is a software focused on the design and analysis of nucleic acids secondary structure in systems involving different

interacting strands [12] (http://www.nupack.org—as visited in July 2017). NUPACK includes two main tools: (1) the Design tool allows to design sequences for more strands at the same time, intended to adopt a specific secondary structure and to interact to form specific complexes [13–16]. (2) The Analysis tool can be used to perform the thermodynamic analysis of a theoretical dilute solution of interacting nucleic acid strands and gather information about the system in the elected conditions. It calculates the partition function and the minimum free energy secondary structure for possible complexes of an arbitrary number of interacting nucleic acids strands. Moreover, it shows the equilibrium concentration of the species in a diluted solution [17–19]. Other strategies may be adopted using different tools (*see* **Note 2**).

3.1.1 Generation of Candidate Sequences

The following procedures allow designing probes and hairpins using NUPACK. The software needs all the following information to generate candidate sequences. Parameters not mentioned here are used at their default settings. Below, the scripts used in NUPACK web application to generate candidate sequences for a specific target are presented and each step listed.

1. Specify the theoretical experimental conditions as follow:

   temperature = 25.0
   material = dna
   sodium = 0.5
   trials = 3 (number of designs).

2. Describe the target structures of hairpin-like monomers in DU + notation (*see* **Note 3**). Keep the toehold and the loop at 6 nt length. Input the elected length of the stem, as a function of the target sequences, in this case 16–17 nt stem. Specify the structure of the expected complexes in the same way.

   #
   # target structures
   #
   structure hp1 = U6D17(U6)
   structure hp2 = D17U6U6
   structure miR-17 = U23
   structure miR-17-hp1 = D23(U23+)
   structure miR-17-hp1-hp2 = D23(D23(+U23)+)

3. Input the sequence domains using IUPAC nucleic acid notation. The target sequence is sectioned into two domains, one corresponding to the 3′ portion complementary to the first hairpin 5′ toehold (domain b), and the other complementary to the adjacent stem portion of the hairpin (domain a). Leave the loop region (domain c) as a random sequence for NUPACK to generate (*see* **Note 4**). The sequences of the

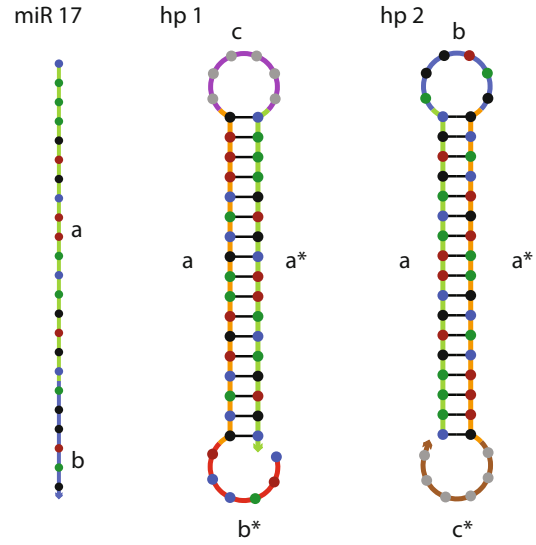

Fig. 2 Images from NUPACK design tool (www.nupack.org). Structures and domains depicted for monomers specific for miR17 detection

hairpins can be divided in four domains, where two of them are complementary, to form the stem.

```
#
# sequence domains
#
domain a = CAAAGTGCTTACAGTGC
domain b = AGGTAG
domain c = NNNNNN
```

4. Describe the sequences of the species defined in the structure section as a function of the previously defined domains. In Fig. 2, the target structures and corresponding domains are depicted. In this notation, "a*" is the sequence complementary to "a."

```
#
# strands (optional, used for threading sequence information
# and for displaying results)
#
hp1.seq = b* a* c a
hp2.seq = a b a* c*
miR-17.seq = a b
miR-17-hp1.seq = b* a* c a a b
miR-17-hp1-hp2.seq = b* a* c a a b a* c* a b
```

5. Insert the pattern of bases to avoid as shown (use IUPAC nucleic acid notation).

```
#
# prevent sequence patterns
```

Fig. 3 NUPACK results with candidate sequences (www.nupack.org) for HCR specific for miR17. The "Normalized Ensemble Defect" quantifies how much the generated sequence missed the inserted instructions: it is the average percent of nucleotides incorrectly paired at the equilibrium compared to the specified constrains and structures. Stop conditions for this parameter can be set in the design instructions

#
prevent = AAAA, CCCC, GGGG, UUUU, KKKKKK, MMMMMM, RRRRRR, SSSSSS, WWWWWW, YYYYYY

6. Check the design preview.
7. Run the Design and check the results (*see* Fig. 3).

3.1.2 Check of the Candidate Sequences

Probe, target and hairpins candidates should be then checked using the NUPACK analysis tool, testing for probe-target and hairpin-target binding in the theoretical reaction conditions and checking the predicted concentration of each species and complex at the equilibrium, as is shown in Fig. 4 (*see* **Note 5**).

1. Select DNA as starting material.
2. Select 25 °C as reaction condition temperature.
3. Set 3 as number of interacting strand to test the theoretical behavior of the two hairpins in solution in presence of the target or select 2 in absence of the target. The number of complex size can be changed as preferred to investigate complexes involving a variable number of species. Set 5, to investigate also complexes involving more monomers.
4. In advanced options, choose NaCl concentration 0.5 M and 0.0 M for Mg.
5. Input the sequences in the corresponding field. Input the target sequence and the sequences of hairpin-like monomers as shown in individual fields, specifying their unique names.
6. Set the concentrations of all species according to the elected reaction conditions. We set to 0.5 μM or below.
7. Keep all the other settings as default and run the analysis to obtain the results in the specified conditions.

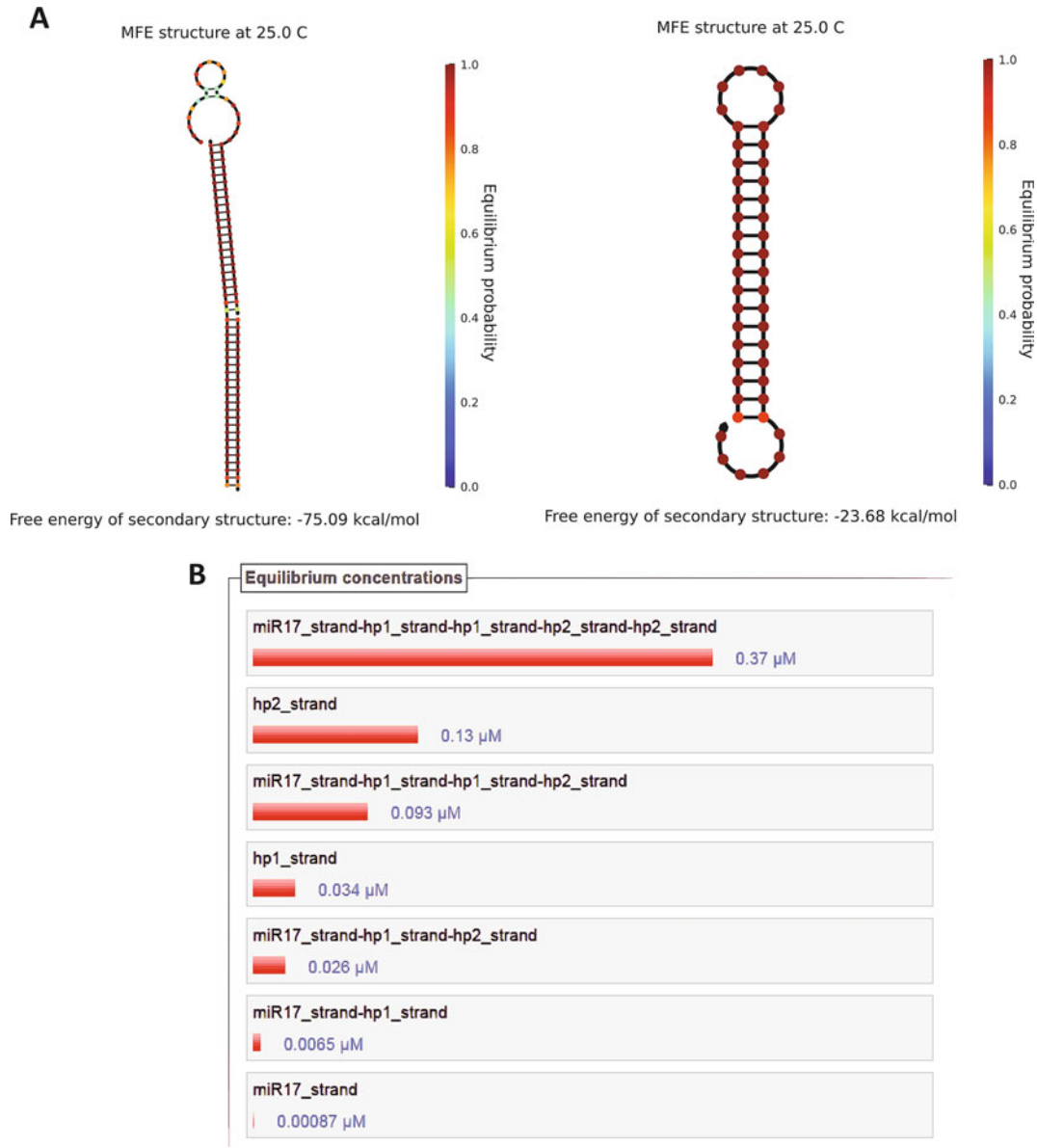

Fig. 4 NUPACK analysis: (**a**) Minimum free energy structures calculated by the analysis tool for hp1-hp2-miR17 complex (left) and hp1 (right), specific for miR17. (**b**) Concentration of the complex and different species at the equilibrium in the specified conditions

3.2 HCR Reaction in Solution

The samples are usually diluted in the HCR buffer. It is important to disrupt any possible alternative secondary structure in the nucleic acid species through a thermal treatment of the samples before setting up the reaction mixtures. During this step, each species should find the most energetically favored secondary structure. All species (hairpins and target) can be mixed in stoichiometric amounts by adding a volume of target solution to the hairpins

mixture and left to react spontaneously at room temperature. Different ratios of target to hairpins can be used in modeling the detection of lower concentrations of target.

1. Dilute the stock solution of oligonucleotides (target and hairpins) to 3 μM inHCR buffer in PCR tubes.
2. Do a thermal treatment with the PCR thermal cycler: 95 °C for 5 min, then cool down to 20 °C in 1 h (0.02 °C/s) (*see* **Note 6**).
3. Prepare the hairpin mixture by mixing 10 μL of each hairpin species in a 0.5 mL Eppendorf tube.
4. Prepare several dilutions of the target oligonucleotide making serial dilutions: for example from 3 μM to 0.003 μM.
5. Add 10 μL of target solution to the hairpin mixture (for a total reaching volume of 30 μL).
6. Keep the reaction at room temperature for at least for 1 h (*see* **Note 7**) and immediately characterize the reaction products (*see* **Note 8**).

3.3 Characterization of the HCR Product

3.3.1 Nondenaturing PAGE for Characterization of HCR Products in Solution

In this context, gel electrophoresis is the most convenient analysis method for the characterization of DNA nanostructures in solution. We use nondenaturing PAGE for the characterization of HCR products. The triggering of the reaction should lead to an amount of products easily distinguishable with gel electrophoresis, as they will form higher molecular weight bands (*see* **Note 8**). PAGE allows to check the stoichiometry and the success of the self-assembly reaction in the elected conditions. WARNING: acrylamide is neurotoxic. Avoid using acrylamide powder for preparing solutions. Use all precautions, gloves, goggles and protective garment to prevent skin contact with the acrylamide solution during the procedures. Use precast gels if available.

1. Set up the gel as specified by the PAGE apparatus manufacturer.
2. Under the chemical hood, prepare the needed amount of 10% acrylamide in a flask of volume equal to at least three times the required solution. For 50 mL: add 10 mL of 5× TBE solution to 20 mL of ultrapure water, then add 12.5 mL of 40% acrylamide solution and bring to 50 mL with ultrapure water (*see* **Note 10**). Stir the solution until homogeneous.
3. Add 250 μL of 10% APS and 25 μL of TEMED to the acrylamide solution, mix well by swirling the flask, then cast the gel according to the manufacturer's instructions or the preferred procedure (*see* **Note 11**).
4. Use a plastic Pasteur pipette to quickly but gently pour the acrylamide solution between the two glass plates and insert the comb. Avoid trapping air bubbles in the wells and in the body of the gel during this step. Make sure that no leakages occur

before gel polymerization. Leave at room temperature for at least 1–2 h for completing the polymerization and for best reproducibility (*see* **Note 12**).

5. When the gel is completely polymerized, use it immediately or store it at 4 °C in a sealed package (such a Ziploc bag or analogous) avoiding drying (do not keep for longer than 2–3 days).
6. Set up the polymerized gel in the electrophoresis cell. Add 1× TBE buffer (running buffer) to fill the chambers of the electrophoretic device, making sure that there are no leakages.
7. Prepare the samples for PAGE analysis: add 3 μL of nondenaturing loading buffer to 3 μL of HCR reaction mixture (*see* **Note 13**) and 7 μL of ultrapure water. Centrifuge the samples briefly before loading (*see* **Note 14**).
8. Remove the comb. Carefully rinse the wells using a syringe with a fine needle to squirt running buffer inside each of the wells to remove nonpolymerized acrylamide efficiently (*see* **Note 15**).
9. When the wells are clean, carefully load the samples using a pipette and gel loading pipette tips appropriate for the wells thickness and length. Load the reference DNA ladder as the last sample or separated from the samples by an empty lane to avoid marker contaminations (in case, load the empty lanes with 1× loading buffer).
10. Turn on the thermostat and circulating water, setting the temperature to 25 °C. Secure the lid of the gel box and connect the electrodes to a DC power supply (Powerpac 300 or similar) making sure the polarity is correct. Run the gel at 5 V/cm for 30 min or till the samples enter into the gel, than at constant voltage 10 V/cm for 3–4 h depending on the size of the investigated DNA complexes, temperature, and gel density (*see* **Note 16**).
11. Disassemble the electrophoresis box and stain the gel in 1× SYBR Gold solution in 1× TBE for 5 min (*see* **Note 17**). Keep it in the dark during the staining.
12. Acquire the gel image in fluorescence using an electrophoresis documentation system (like the Gel doc, Biorad). *See* Fig. 5 for example.

3.3.2 AFM Characterization of HCR Products

Characterization through AFM can provide information about the size and the shape of the HCR products, in order to get more information about the growth of the nanostructures, their morphology and length. The samples need to be diluted and cleaned-up before AFM imaging. Starting from the experimental conditions previously described, samples are diluted 1:100. After a wash step through centrifugal ultrafiltration, the HCR product is collected in the buffer for the deposition on mica for AFM imaging.

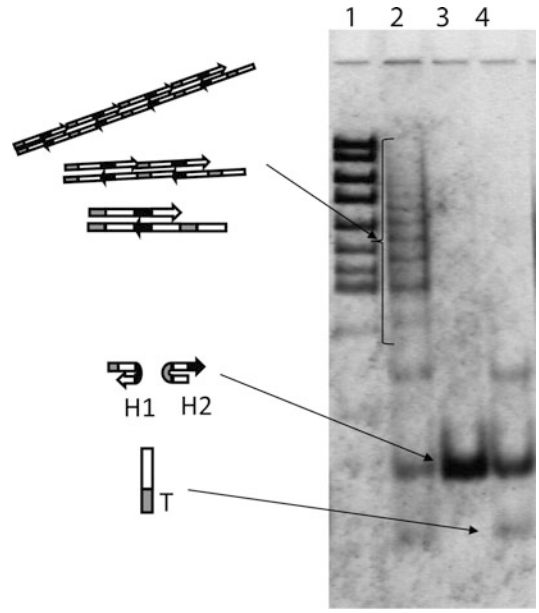

Fig. 5 Characterization of HCR specific for miR21 sequence. Molecular weight marker in Lane1. As also shown by the schemes on the side, the reaction of specific target with hairpins is in lane 2, the HCR monomers in absence of the target is in lane 3, while lane 4 shows the mix of the first hairpin with the miRNA sequence

1. Collect 5 μL of HCR reaction mixture and dilute it by adding 495 μL of 1× TE buffer.
2. Load the solution in the centrifugal filter device (100 kDa MWCO).
3. Centrifuge for 8 min at 11,200 × *g*.
4. Discard the filtrate and add 200 μL of AFM imaging buffer to the same filter unit and centrifuge for 5 min at 11,200 × *g*.
5. Collect the samples by inverting the filter unit and placing it in over a new tube. Centrifuge for 3 min at 100 × *g* to transfer the HCR product to the new tube.
6. Layer 10 μL of the filtered HCR product onto a freshly cleaved mica disc glued on a steel disk. Leave to adsorb for 5 min (*see* **Note 18**).
7. Wash with about 1 mL of ultrapure water added dropwise and dry with a gentle stream of nitrogen.
8. Load the disk on the AFM and scan the adsorbed sample according to the method in use (we currently prefer ScanAsyst mode in air for routine checking, available on Bruker AFM systems). *See* Fig. 6 for example AFM images of the HCR product.

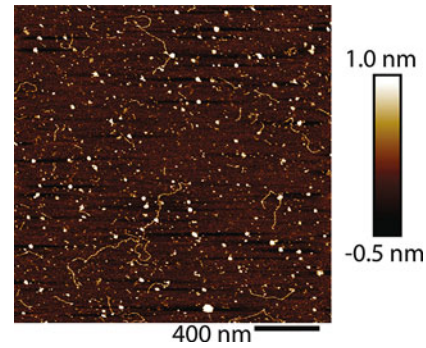

Fig. 6 Example AFM image of the HCR products. The average length of the products here has been measured as 200 nm, while structures up to 1 μm long are visible

3.4 Biosensor Surface Preparation and Derivatization for Electrochemical Measurements

Template-stripped gold disks are used to build the sensors. They are prepared according to the procedure outlined below (analogous to that described in Chapter 10 of this book), adapted from [20].

1. In a high-vacuum evaporator, deposit a 200 nm layer of ultra-pure gold (Electron Microscopy Sciences, 99.99% purity) onto muscovite mica which had been previously conditioned at about 300 °C overnight in the vacuum (10^{-6} Torr or less). We use resistive heating of a gold wire in an Edwards high-vacuum evaporator with a resistively heated temperature-controlled specimen stage.
2. After cooling, glue round glass cover slides (12 mm in diameter) on the evaporated gold with EpoTek 377 epoxy resin. Use about 10 μL each disc taking care of not trapping air bubbles. Cure the glue at 150 °C for 2 h.
3. Just before use, separate the glass cover slide with the ultra-flat gold layer from the mica by gently prying them apart by hand or with the help of a pair of tweezers. Make sure no mica flakes are left on gold (testing conductivity with a multi-meter could prove helpful).
4. Deprotect the thiolated oligos by adding concentrated oligonucleotide stock solution to achieve a 10 μM oligo solution in an about 300 μM solution of TCEP. Incubate for 1 h at 37 °C before use (oligo solution with TCEP can be stored at 4 °C for further use).
5. Dilute the 10 μM deprotected thiolated oligo stock to 2 μM in PBS buffer just before use.
6. Layer 10 μL of 2 μM deprotected thiolated oligonucleotide solution in PBS buffer on the surface of a freshly exposed template-stripped gold electrode disk and leave overnight at

room temperature (taking precautions for preventing the evaporation of the solution, such as incubating in a sealed humid chamber).

7. Wash the gold disc thoroughly but quickly with ultrapure water.
8. Passivate the gold surface by layering 20 μL of 1 mM 6-mercapto-1-hexanol (MCH) solution. Leave for 30 min at room temperature taking precautions for preventing the evaporation of the solution.
9. Wash quickly with ultrapure water. Preferably, install in the biosensor while still wet and use, or, alternatively, store at 4 °C in a small amount of PBS buffer or in a dry state (the reproducibility of results might depend on the storage conditions).

3.5 Electrochemical Capacitive Detection of HCR on Surface

HCR can be used in a simple electrochemical label-free detection system. We employed an HCR reaction on the surface for making a capacitive biosensor employing a "sandwich" format: the target DNA is specifically recognized by a probe immobilized on the gold surface. Later, the hairpins mixture is added to allow the amplification through HCR (*see* Fig. 7) anchored to the bound target DNA. The HCR product formed on surface leads to a change in capacitance on the interface. Measurements can be carried out using a custom-built three-electrode electrochemical cell. This can be simply designed to host an integrated reference electrode and a platinum wire auxiliary electrode in proximity of the working electrode. A cast polydimethylsiloxane (PDMS) cell is a good choice and it can achieve an efficient seal for fluidic control and minimize the volumes of the needed solutions. The gold surface functionalized with the thiolated probe represents the

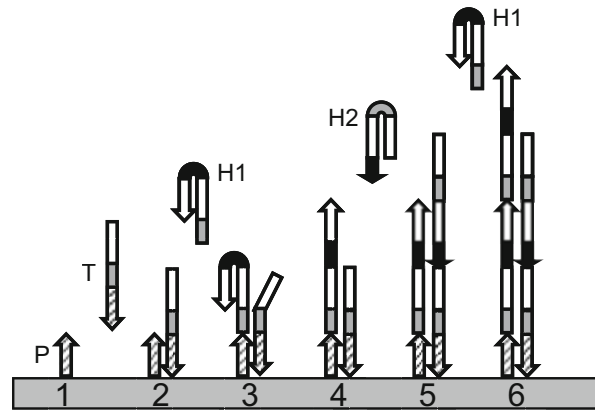

Fig. 7 Scheme of HCR on surface for electrochemical biosensor detection. Figure republished from ref. [6] with permission from Elsevier

working electrode. The cell is connected to an electrochemical workstation (in our case a μAutolab III, Metrohm Autolab B.V., The Netherlands).

3.5.1 Electrochemical Cell Assembly and Capacitive Measurements

The operations are merely sketched here, as they will depend on the electrochemical cell in use.

Capacitive measures can be performed by applying the method described by Berggren and coworkers [21, 22]. A gold surface with a SAM of alkanethiols is close to the ideal condition of an electrode without charge transfer across the electrode-solution interface when a potential is applied. This is an Ideal Polarizable Electrode (IPE) and it could be described as an equivalent RC circuit, involving a capacitance and a resistance in series (*see* Fig. 8).

The applied potential leads to a non-Faradaic current at the interface between surface and solution. This current decrease over time follows a known relation that can be used to calculate the values of resistance and capacitance of the interface. To calculate the capacitance, data are fitted with the RC model. The method needs a fast potentiostat and a relatively low concentration of electrolyte,

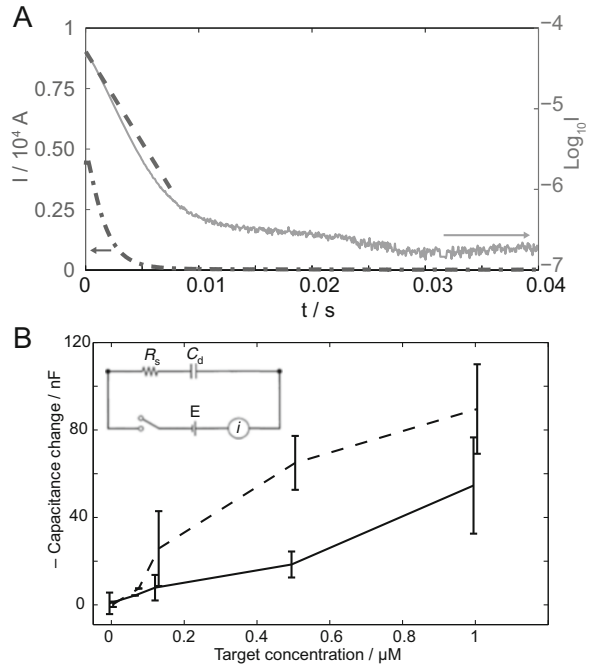

Fig. 8 (**a**) Current decay over time after the application of a 50 mV potential (solid line on the Log scale, dash-dot line on the linear scale). Fitting to RC model of the first points of the current decay Log10(I): the regression line is showed (dashed line). (**b**) Calibration curves of a capacitive DNA sensor before HCR amplification (points connected by the solid line) and after HCR amplification (points connected with dashed line); in the inset, the RC circuit used for the analysis of potentiostatic step experiments. Panel B modified from ref. [6] with permission from Elsevier

and requires the detection of the initial part of the current decay curve. Change in capacitance occurs after target hybridization and hybridization chain reaction and it is possible to evaluate these changes over time and target concentration. The measurements are performed acquiring the current decay in absence of the target, until a stable capacitance is reached. After the treatment with target and hairpins, the measurement is repeated and the capacitance variation upon the hybridization event can be calculated.

1. Open the cell and load the functionalized gold disc.
2. Close the cell making sure all electrodes are in place and connected to their respective wires for the electrochemical workstation. Avoid touching the surface of the gold disc.
3. Inject buffer in the cell to check for any leakage. Reassemble the cell if necessary. Avoid trapping air bubbles in the cell and make sure all electrodes are immersed in the measurement solution.
4. Connect the system to the potentiostat.
5. The system is now ready to measure.
6. Equilibrate the gold surface in the electrochemical cell in the PBS buffer.
7. Substitute the buffer solution with a new volume of the same PBS buffer, to perform the measurements.
8. Apply series of potential steps of +50 mV and −50 mV to the working electrode with respect to the open circuit potential. Measure the resulting current decay in the transient after the step. Use a sampling rate of at least 20 kHz until stable capacity is reached.
9. Inject 100 μL of target solution at the test concentration (and hybridization buffer). Wait for 30 min (or for the time defined in the desired protocol variation).
10. Substitute the cell (hybridization) buffer with PBS buffer.
11. Measure the current decay over time while applying potential steps, as described above, until stable capacitance is reached.
12. Wash with PBS buffer.
13. Add 100 μL of mixture of hairpins at 2 μM each in HCR buffer. Wait 30 min or the reaction time of choice.
14. Substitute the HCR buffer with PBS buffer.
15. Measure the current decay over time while applying potential steps, as described above, until stable capacitance is reached.

3.5.2 Data Analysis

The system can be described as an equivalent RC circuit involving a capacitance and a resistance placed in series (*see* Fig. 8). Current decreases over the time (t) through a relation described by the following equation:

$$i = \frac{E}{\mathrm{Rs}} \times \exp\left(-\frac{t}{\mathrm{RsCd}}\right) \quad (1)$$

where Rs is the resistance, Cd the system capacitance, E the applied potential step, i(t) the current as a function of time t after the step. Equation 1 can be used to calculate the values of resistance and capacitance of the interface. Equation 1 can be linearized as:

$$\log(i) = \log\left(\frac{E}{\mathrm{Rs}}\right) - \frac{t}{2.303\mathrm{RsCd}} \quad (2)$$

From the value of intercept, it is possible to determine Rs, while the capacitance (Cd) value can be obtained from the slope of the linear regression. This linear relation is valid only for the first points of the sampled current decay over time. To calculate the capacitance, perform the analysis on the first points of the current signal, and then fit with the RC model.

1. Collect the current decay data over time (*see* Fig. 8).
2. Calculate the Log_{10} of the current values.
3. Discard the first few points and select the following 10–15 points to calculate the linear regression to obtain the best trend-line fitting the data (R^2 should be >0.99). Calculate Rs value from the intercept and Cd from the slope of Eq. 2.
4. Plot the capacitance after addition of target DNA and HCR (*see* Fig. 8).

4 Notes

1. The DNA sequences of probes and hairpins were designed directly based on the selected targets, such as genes from pathogenic microorganisms (e.g. *Cryptosporidium parvum*) viruses and microRNA. Promising targets could be found through bibliographic searches and database mining. For example, specific circulating microRNAs of diagnostic interest have been found in various database, such as miRBase (www.mirbase.org—visited in July 2017) and miRandola (www.mirandola.iit.cnr.it—visited in July 2017). NCBI's BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi—visited in July 2017) was used to look for similarities between different microRNAs in order to identify competitors in the hybridization chain reaction. For pathogenous DNA detection, some regions of selected genes known to be specific for a strain of interest were chosen. We considered advantageous, especially for long sequences, to screen the entire sequence of an elected gene for candidate regions with good potential binding and low interfering secondary structure and dimerization with a custom-

made script (written in MATLAB) using RNAcofold ver.1.80 [23, 24]. Moreover, the NUPACK analysis tool and the RNAfold webserver (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi—visited in July 2017) were used to individually check the candidate sequences for self-complementarities and predicted secondary structures interfering with the target recognition. The candidate sequences with the lowest interfering secondary structure and dimerization were finally elected.

2. Other software packages are available for sequence design. We have previously also used NANEV to generate self-assembling sequences. NANEV is a software utility for the generation of random sequences starting from information about the complementarity and the composition of each strand [25]. It employs evolutionary methods and combines different design paradigms: (a) A negative design paradigm that is the sequence symmetry minimization (SSM) to eliminate as much as possible undesired interaction between the specified strands. (b) A positive design paradigm, such as the energy minimization aiming to keep at the maximum the specific desired interactions. The software permits to define subsequences involved in the assembly and combine them to generate the final strands. The entire set of interacting DNA strands is described as formed by complementary subsequences. Although NUPACK includes the same models, NANEV can be simpler to use.
3. The specified target structures must not be connected. In DU+ notation, Dx represents a duplex of length x, while Ux defines an unpaired region of length x. Each structure is described in 5′–3′ direction and each duplex is followed immediately by the substructure delimited by the duplex, enclosed in brackets. More parentheses are used if more elements are included in the substructure. The character "+" is used to break strands. (www.nupack.org, Zadeh, Ph.D. thesis, California Institute of Technology, 2011).
4. The easiest way to design couples of hairpins is to insert domains of the target sequence in the sequence of the hairpin-like monomers. This would be the main constrain of the system, since an entire portion of the hairpin has to hybridize with the target to trigger the reaction. This fact would affect the thermodynamics and the kinetics of the system, from target recognition to the self-assembly of the hairpins. If HCR reaction is designed to be triggered by a randomly generated sequence, such constrictions are avoided. Setting no specific domains in the NUPACK design scripts, thus describing strands just as composed of random bases (N), NUPACK randomly generates all the species involved, initiator included and better self-assembling sequences are obtained. In this ideal

(and idealized) case, this approach would help preventing self-complementarities and undesired secondary structures and a better hybridization chain reaction can be reached.

5. The results of the NUPACK thermodynamic analysis are very useful to prevent important undesired secondary structures and interactions between strands during the design, but the real behavior of the strands in the experimental observations may be different. Check the sequences as well as you can in these steps to avoid undesired interactions. Moreover, alternative uncommon interactions and structures are often not taken into account by NUPACK models. Therefore it is better to check the strands to look for sequence features that could lead to behaviors not predicted by the models, for example, peculiar bases patterns such as C or G repeats could lead to alternative structures of nucleic acids.
6. The sample can be left cooling for 1 h at room temperature after 5 min at 95 °C, according to the original HCR protocol [5].
7. Different reaction times have been tested and they might depend on the sequences. In most cases, in our hands, an incubation time of 1 h is enough to enable the characterization of the nanostructures.
8. After the fast formation of the products, the reaction keeps going slowly thanks to hairpins remaining in solution. This aspect should be considered when a later characterization is required. When later characterization is required, the HCR products can be stored at 4 °C. AFM images obtained from the HCR products after 3 days of storage at 4 °C showed assembled nanostructures completely analogous to the freshly prepared ones.
9. HCR products may also run as a smear in gel electrophoresis, probably due to background reaction noise that could be avoided, for example, by reducing the hairpin concentrations. The size of the HCR product depends on the stoichiometry of the reaction between hairpins and target. If the initiator is very diluted, the HCR product can reach a higher molecular weight, so that the molecular weight difference should be clearly visible in the electrophoretic gel.
10. For small format gel (ten wells) using miniProtean electrophoresis cell (Biorad), add 2.5 mL Acrylamide stock solution to 2 mL TBE 5× solution, then make 10 mL with ultrapure water.
11. For miniProtean electrophoresis cell, add 62.5 μL of APS 1% and 6.25 μL of TEMED to the entire 10 mL prepared solution (*see* **Note 9**), mix vigorously and cast the gel.

12. The time for polymerization could change with the room temperature. Sometimes less than 1 h is enough, but it could take longer. Be careful that all the gel is polymerized before proceeding with the electrophoresis.
13. It is convenient not to overload the wells. If comparison between lanes is needed, it is always better to load same amounts of DNA species in each wells, to be able to know the reagents consumption after the reaction.
14. For miniProten gel, a smaller volume is required: add 1 μL of loading buffer to 3 μL of HCR sample and 1 μL of ultrapure water.
15. Samples are prepared for loading when the gel is well polymerized, cast and set up in the buffer tank. When it is sure that the buffer chambers do not leak and the samples are ready, remove the comb, clean the wells, and load the samples immediately.
16. Usually, we run 15-wells large format gel at 25 °C, to maintain the room temperature condition during the analysis and to avoid overheating during the run. If room temperature is higher in the lab, it is convenient to run the gel at lower temperature to preserve the resolution and gel integrity. The running time depends on the running temperature, acrylamide percentage and buffer composition. Changing running buffer would need a revision of these conditions and of the running time. Small format gel (ten wells) in miniProtean II cell were usually run setting the voltage at up to 80 V.
17. Time of staining in SYBR Gold could vary. Each aliquot of SYBR Gold solution may be used a few times by adjusting the staining time and storing the solution at 4 °C between uses. For a fresh solution of SYBR Gold in 1× TBE, 5 min of staining is usually enough.
18. Since mica is negatively charged, multivalent cations would help shielding the negative charges of both DNA and surface, improving the adsorption. A magnesium or nickel solution can be used to treat mica for a few minutes before the DNA adsorption or, alternatively, DNA can be diluted in a buffer containing magnesium.

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