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1 Self-assembling Protein Platform for Direct
2 Quantification of Circulating MicroRNAs at
3 Femtomolar level in Serum with Total Internal
4 Reflection Fluorescence Microscopy

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10 **ABSTRACT**

11 MicroRNA (miRNA) has recently emerged as a new and important class of cellular
12 regulators. Strong evidences showed that aberrant expression of miRNA is associated
13 with a broad spectrum of human diseases, such as cancer, diabetes, cardiovascular and
14 psychological disorders. However, the short length and low abundance of miRNA
15 place great challenges for conventional techniques in the miRNA quantification and
16 expression profiling. Here, we report a direct, specific and highly sensitive yet simple
17 detection assay for miRNA without sample amplification. A self-assembled protein
18 nanofibril acted as an online pre-concentrating sensor to detect the target miRNA.
19 Locked nucleic acid (LNA) of complimentary sequence was served as the probe to
20 capture the target miRNA analyte. The quantification was achieved by the fluorescence
21 intensity measured with total internal reflection fluorescence microscopy. A detection
22 limit of 500 fM was achieved with trace amount of sample consumption. This assay
23 showed efficient single-base mismatch discrimination. The applicability of quantifying
24 circulating mir-196a in both normal and cancer patient's serums was also
25 demonstrated.

26 **1. Introduction**

27 MicroRNAs (miRNAs) are non-coding RNAs of ~ 18-25 nucleotides. They initially
28 exist in the cell nucleus as a long primary transcript, primary miRNAs (pri-miRNAs). By
29 enzymatic cleavages of pri-miRNA and precursor miRNAs, mature miRNAs are
30 produced. Mature miRNAs are found to be an important gene expression regulator, which
31 are involved in early tumorigenesis and tumor progression¹⁻³. Abnormal and distinctive
32 expression profile of miRNAs is found in different types of tumor tissues and cancers.
33 miRNAs expression profiling is therefore regarded as an important biomarker for cancer
34 diagnosis⁴⁻⁵.

35 Meanwhile, circulating miRNAs in body fluids, such as serum, blood plasma and urine,
36 are found to be well-preserved and highly correlated to pathological changes. These
37 endogenous miRNAs are highly stable in a range of temperature and pH and they are
38 robust against RNase digestion⁶⁻⁷. Although the mechanism behind the high resistance of
39 circulating miRNA against degradation is still not well-understood, researchers proposed
40 that the circulating miRNAs are protected by the RNA-induced silencing complex (RISC),
41 which forms an extra protection towards the fragile miRNAs⁸. With the potential that
42 circulating miRNAs can be collected non-invasively in serum, plasma, and urine,
43 circulating miRNAs are considered as a potential biomarker for clinical cancer diagnosis.

44 Since the level of circulating miRNAs is much lower (~ 20 pM)⁹ compared to those
45 in tissue and cells, a method of high sensitivity, specificity and high quantitative accuracy
46 is very critical for a reliable miRNAs expression profiling. However, it is always
47 challenging to determine directly and accurately the amount of miRNAs for their short
48 length and low abundance. The short sequence limited the design of complementary

49 probe used to capture the target miRNAs and confined the mismatch discrimination
50 efficiency. Quantitative reverse transcript PCR (qRT-PCR)¹⁰, northern blotting¹¹, and
51 microarray¹² are currently the golden methods to perform miRNAs expression profiling,
52 although these methods encounter complications like sample loss during the
53 amplification steps, large sample consumption, use of radioactive reagents, intensive
54 labor and long assay time. As a result, other sample pretreatment strategies aiming at
55 higher detection sensitivity, specificity, and minute sample consumption have been
56 developed recently, for example, electrochemical¹³⁻¹⁴, isothermal strand-displacement¹⁵,
57 and enzymatic amplification¹⁶ detection assays. Taking advantage of the signal
58 amplification technique, some of these detection assays are capable of quantifying
59 miRNAs at attomolar level. However, the reliability and reproducibility of the assay are
60 scarified by multiple manipulation and transversal steps. Our group previously reported a
61 direct miRNA detection method by single-molecule counting with total internal reflection
62 fluorescence microscopy (TIRFM)¹⁷. The miRNA detection assay is free of sample pre-
63 treatment and amplification. The detection was achieved by counting the fluorescent
64 analytes within the evanescent field layer in single-molecule level.

65 Over the recent decade, bio-sensing platform constructed from different novel
66 nanomaterials has been widely applied for detection of biomolecules like DNAs,
67 miRNAs proteins etc. Thaxton *et.al* developed a sensitive scanometric miRNA detection
68 assay using gold nanoparticles¹⁸. Other nanomaterials such as silver nanowires¹⁹, silicon
69 nanowires²⁰, and tin dioxide²¹, have also been incorporated into the detection assays. The
70 resulted signal enhancement dramatically improves the sensitivity of the detection assay,
71 shortens the assay time, and reduces the sample consumption by pre-concentrating the

72 analytes on the surface of the nanocomposites. However, most of the nanoparticles are
73 limited to the working conditions such as solution pH and ionic strength, possibly
74 resulting in self-aggregation and activity loss. Unlike the fabrication of nanoparticles and
75 nanowires which requires sequential chemical synthetic techniques, self-assembled
76 bionanocomposite with peptides simultaneously forms a well-structured, well-defined
77 and stable macromolecular structure²². Having peptides as the building blocks, the
78 protein scaffold is inherently stable and of high activity under physiological conditions.
79 Most importantly, the specificity and functionality of the protein scaffold can be
80 manipulated by simply modifying the functional moiety of the protein motif. Amyloid
81 fibril is well-known to be rich in β -sheet structure, which is rigid and stiff compared to
82 other natural protein-based material like collagen and silk. Functionalized $A\beta_{1-40}$ fibril
83 can be straightly obtained by co-incubating functionalized $A\beta_{1-40}$ monomer with native
84 $A\beta_{1-40}$ monomer. It is demonstrated that the functionalized $A\beta_{1-40}$ fibril is of high
85 potential to serve as biocompatible and highly stable pre-concentrating scaffold for
86 biomarker detection.

87 Our group previously developed a functionalized self-assembling beta-amyloid (1- 40)
88 ($A\beta_{1-40}$) fibril as a platform for biomolecules detection²³. However, the hybridization
89 efficiency of the surface-based assay is highly limited by diffusion kinetic of the analyte
90 molecules. Here, we developed a solution-based direct miRNAs detection assay with
91 self-assembling $A\beta_{1-40}$ fibril with TIRFM. As a proof-of-concept, hsa-mir-196a (mir-
92 196a) was chosen to be the miRNA target for detection. Mir-196a was found to be one of
93 the significant miRNAs elevated in nasopharyngeal²⁴, gastrointestinal²⁵, and pancreas²⁶
94 cancers. In the assay, self-assembling $A\beta_{1-40}$ fibrils were labeled with a mixture of Alexa

95 Fluor-555 streptavidin (AF 555 stv) and bare streptavidin (stv), to locate the fibrils. The
96 target miRNAs were pre-hybridized with the complementary 5' biotinylated locked
97 nucleic acid (LNA) probe in solution to achieve high hybridization efficiency and then
98 labeled with fluorescent dye YOYO-1. The hybrid complexes were then added to the AF
99 555 stv-labeled A β ₁₋₄₀ fibril, and were captured onto the backbone of the fibrils through
100 the biotin and streptavidin interaction. The fluorescent images of fibril were collected by
101 TIRFM. The target miRNAs was quantified with the fluorescent intensity of individual
102 fibrils. This assay provided a limit of detection (LOD) at around 500 fM, as well as a
103 high specificity as it substantially discriminated the two miRNAs within the same family,
104 target mir-196a and mir-196b, which differ by a single nucleotide. The developed assay
105 was applicable for the direct determination of the mir-196a concentration in both normal
106 and cancer patient's serum samples without sample pre-treatment. This direct, sensitive
107 and specific detection assay for miRNAs is of high potential for clinical application for
108 early stage cancer diagnosis.

109 **2. Material and methods**

110 **2.1 Coverslides pretreatment.** All the coverslides were prewashed prior to use.
111 Generally, No.1 22 × 22 mm square glass (Menzel-Glaser, Germany) were successively
112 sonicated for 15 min in household detergent, 15 min in absolute ethanol, 40 min in 1 M
113 sodium hydroxide, 15 min in acetic acid, then 5 min in absolute ethanol thrice. The
114 coverslides were rinsed thoroughly with filtered distilled water between every solvent
115 replacement. The cleaned coverslides were dried in the oven at 120 °C for 3 hrs and
116 stored for further use.

117 **2.2 Preparation of A β ₁₋₄₀ Fibrils for seeding.** Monomeric A β ₁₋₄₀ was purchased from
118 Invitrogen and used without further purification. The stock A β ₁₋₄₀ monomer solution was
119 prepared by dissolving 0.1 mg A β ₁₋₄₀ monomer with 400 μ L 0.02 % ice-cold ammonia
120 solution and stored at – 20 °C until use. The seed was prepared as mentioned elsewhere²⁷⁻
121 ²⁹. Briefly, by diluting the stock A β ₁₋₄₀ monomer to 57.7 μ M with 1 \times phosphate buffer
122 (50 mM sodium phosphate, pH 7.4); the solution was incubated at 37 °C for 20 hrs under
123 gentle shaking. The second generation of A β ₁₋₄₀ seeding was prepared by adding 1 μ L of
124 0.87 μ g/mL A β ₁₋₄₀ seeding from the previous step to 2 μ L of A β ₁₋₄₀ monomer and diluted
125 to 20 μ L with 1 \times phosphate buffer. The solution mixture was incubated at 37 °C for 1 hr.
126 The resultant fibrils were used as the second generation of seeding for further fibrillation.

127 **2.3 Preparation of Biotinylated A β ₁₋₄₀ Fibrils.** The stock biotin functionalized A β ₁₋₄₀
128 monomer (Anaspec, USA) was diluted with 0.02 % ice-cold ammonia solution to 100
129 μ M. The biotinylated A β ₁₋₄₀ fibrils were prepared by incubating 20 % of biotinylated A β ₁₋
130 ₄₀ monomer with native A β ₁₋₄₀ monomer in 0.5 \times phosphate buffer. The total
131 concentration of the A β ₁₋₄₀ monomer is 50 μ M. In order to accelerate the fibrillation
132 process, 1 μ L of 0.87 μ g/ mL A β ₁₋₄₀ seeding was added to the solution. The fibrils were
133 diluted to 200-fold with phosphate buffer prior to experiments.

134 **2.4 Preparation of Hybridization Buffer.** The 1 \times Tris-EDTA buffer with 250 mM
135 NaCl (TNE 250) was prepared by mixing 20 mM pH 8.0 Tris-HCl (Invitrogen, Carlsbad,
136 CA) with 1 mM EDTA (Sigma-Aldrich) and 250 mM sodium chloride in distilled water.
137 The pH of the buffer was adjusted to pH 7.4 with 2 M HCl. The buffer was filtered
138 through a 0.2 μ m nylon membrane filter and autoclaved prior to use.

139 **2.5 Preparation of Probes and Target MicroRNA Oligonucleotides.** The commercial
140 available LNA-modified oligonucleotide probes (miRCURY LNA™ microRNA
141 Detection Probes) LNA196a (5'-CCCAACAACATGAAACTACCTA-3') specific to
142 corresponding target microRNAs was purchased from Exiqon (Denmark). The HPLC-
143 purified synthetic mature microRNA oligonucleotides *hsa-mir-149* (5'-
144 UCGGCUCCGUGUCUUCACUCCC-3'), *hsa-mir-196a* (5'-biotin-
145 UAGGUAGUUUCAUGUUGUUGGG-3') and *hsa-mir-196b* (5'-
146 UAGGUAGUUUCCUGUUGUUGGG-3') were custom synthesized and purchased from
147 Integrated DNA Technology (USA), acting as the target microRNA strands. All
148 oligonucleotides were suspended in 500 µL DEPC-treated water (Ambion, USA) and
149 diluted to appropriate concentration with 1× TNE 250 buffer.

150 **2.6 Study of the hybridization efficiency.** Sealed flow cell channel was prepared by
151 combining prewashed coverslides with double-sided adhesive tape. Each channel is
152 around 3 mm wide. For detection, 10 µL of the diluted Aβ₁₋₄₀ fibrils was first flowed into
153 the channel; followed by adding excess stv/ AF 555-Stv mixture (molar ratio of 2:1). The
154 channels were successively washed with phosphate buffer between each consecutive
155 sample addition. In the study of the hybridization efficiency, surface base hybridization
156 duplex was prepared as reported previously²³. Briefly, excess poly (T)₁₅ probe (5'-biotin-
157 TTT TTT TTT TTT TTT-3', Invitrogen, HPLC-purified) was added to the channel that
158 contained the stv/ AF 555-stv labeled fibril and incubated for 15 min. Then 50 pM poly
159 (A)₁₅ target (5'-AAA AAA AAA AAA AAA-3', Invitrogen, HPLC-purified) was flowed
160 into the channel and hybridized for 30 min. Finally, fluorescent dye YOYO-1 was added

161 to label the hybrids for further quantification by TIRFM. The ratio of the YOYO-1 and
162 the hybridized base pair was 1:3 (dye/ bp).

163 The solution-based hybridization duplex was prepared by hybridization 500 pM
164 poly (A)₁₅ with 500 pM biotinylated poly (T)₁₅ probe in solution at room temperature for
165 30 min and fluorescent dye YOYO-1 was added to label the hybrids. The ratio of the
166 YOYO-1 and the hybridized base pair was 1:3 (dye/ bp). The hybrids were then added
167 into the channel with the AF 555-stv labeled fibril as mentioned in the previous step and
168 incubated for 1 hr to saturate the streptavidin site and for further detection by TIRFM.
169 Three experimental trials were performed, while the mean of mean and standard error of
170 the mean from the triplicates were taken to the plot unless specified.

171 **2.7 Hybridization of the MicroRNAs.** All the LNA probes and miRNA strands were
172 diluted to 1 nM with 1× TNE 250 buffer (pH 7.4), respectively. The hybridization
173 cocktail contained target miRNA strand at different concentrations (0, 0.5, 1, 5, 10, 25,
174 50, 100 pM respectively), 100 pM corresponding LNA probe and hybridization buffer.
175 The solution mixture was incubated in heating block (Labnet, USA) for 1 hr. The
176 hybridization temperature was set to 20 °C below the melting temperature (T_m) of the
177 LNA probe, which is 58 °C. The T_m was predicted based on the thermodynamic nearest
178 neighbor method³⁰.

179 **2.8 Labeling of MicroRNAs.** After incubation, YOYO-1 was added to label the hybrids
180 (biotinylated-LNA-miRNA duplex). The ratio of the YOYO-1 and the hybridized base
181 pair was 1:3 (dye/ bp). The YOYO-1 labeled hybrids were incubated in room temperature
182 for 5 min for equilibrium.

183 **2.9 Quantification of mir-196a with A β ₁₋₄₀ fibrils on the surface of the flow cell.**

184 Different concentrations of target microRNAs hybrid (0, 0.5, 1, 5, 10, 25, 50, and 100 pM
185 respectively) were added into each of the channels that contained the stv/ AF 555-stv
186 labeled fibril and incubated for 30 min for further quantification.

187 For the mismatch study, 50 pM hsa-mir-196b and hsa-mir-149 hybrids were used to
188 show the specificity of the assay. The fluorescent images of the fibrils detection platform
189 were captured under a home-built total internal reflection fluorescence microscopy
190 system (TIRFM) with an excitation 488 nm cyan laser (50 mW, CMA1-01983, Newport,
191 USA).

192 **2.10 Quantification of mir-196a in serum samples by TIRFM.** Serum samples were

193 obtained from the Nasopharyngeal Carcinoma Area of Excellence Research Tissue Bank
194 under the Center for Nasopharyngeal Carcinoma Research, the serum samples were
195 stored at -80 °C prior to use without further modification. Synthetic mir-196a, with a
196 final concentration of 0, 1, 2, 5, 10, and 15 pM respectively, was spiked into the standard
197 addition cocktail which contained 3.5 μ L of the 5-fold diluted serum and LNA probe of a
198 final concentration of 100 pM, and finally diluted to 20 μ L with TNE buffer. The solution
199 was incubated at 58 °C for 1 hr. Finally, the quantification was constructed as mentioned
200 above.

201 **2.11 Fluorescence imaging and data analysis.** The prism type total internal reflection

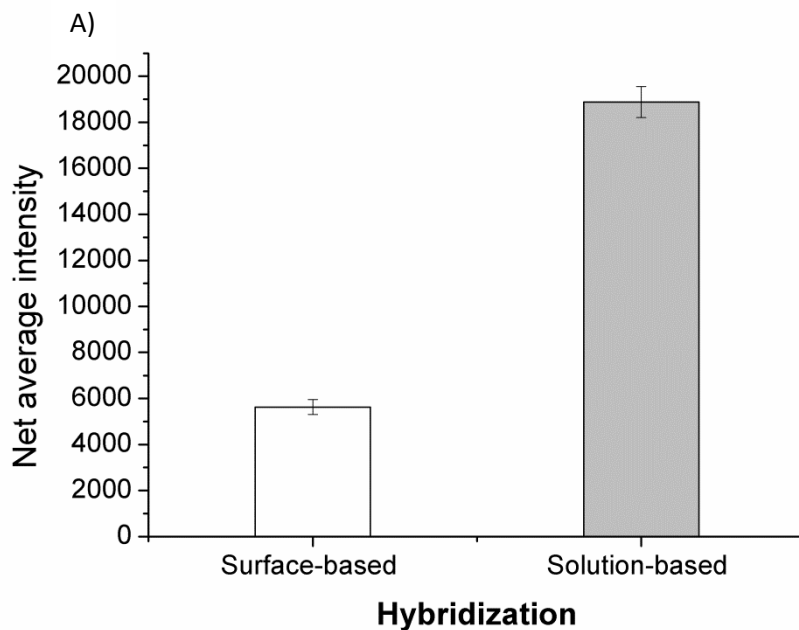
202 fluorescence microscopy was setup as mentioned before^{17, 23}. Briefly, the flow cell was
203 placed between a fused-silica isosceles prism (CVI, laser USA) and a 60 \times oil type
204 objective equipped with an Olympus IX71 inverted microscopy with a HQ 535/50
205 (Chroma) band-pass filter. A 488-nm diode laser (Newport, USA) was used as the

206 excitation source to excite the YOYO-1 dye. The fluorescence image of the $A\beta_{1-40}$ fibrils
207 was captured by an Electron Multiplying Charge Coupled Device (EMCCD) (PhotonMax
208 512, Princeton Instrument, USA) incorporated with a Uniphase mechanical shutter
209 (Model LS2Z2, Vincent Associates, USA) and a driver (Model VMM-T1, Vincent
210 Associates, USA) in external synchronization and frame-transfer mode. The exposure
211 time of both camera and shutter were set at 100 ms. The multiplication gain and the delay
212 time of the shutter drive were set at 4000 and 10 ms respectively. The fluorescence signal
213 of the fibrils was measured by the free-domain software *Image J*. The fluorescence
214 signals of the sensor was obtained by measuring net fluorescence intensity of 50
215 independent fibrils, which located randomly, in each condition with *Image J* (Region of
216 interest = 15×2 square pixels). Net intensity = $50 \times (15 \times 2$ square pixels of independent
217 fluorescent area on fibrils) – $50 \times (15 \times 2$ square pixels of independent background area
218 on image). Three experimental trials were performed, while the mean of mean and
219 standard error of the mean from the triplicates were used in the plot unless specified.

220 **3. Results and discussions**

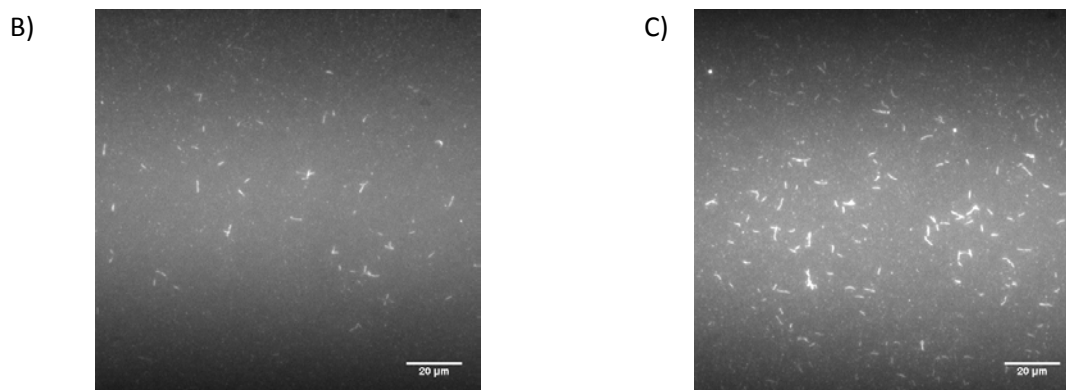
221 **3.1 Study of the hybridization efficiency.** The quantification of the target
222 oligonucleotide presented here is a hybridization-based assay. The hybridization
223 efficiency of the target and the complimentary sequence is very crucial for the overall
224 performance of the assay. We firstly compared the hybridization efficiency of the target
225 oligonucleotide and the complimentary probes where hybridization occurs in solution and
226 on surface. Figure 1 shows the net fluorescence intensity measured on the fibril loaded
227 with 500 pM solution-based and surface-based hybridized poly (A)₁₅ and poly (T)₁₅
228 complex. Under the same concentration of the target and the probe, higher hybridization

229 efficiency is indicated by a higher detected fluorescence signal. The average fluorescence
230 intensity of the fibrils loaded with solution-based hybridized complex was almost tripled
231 over the surface-based one. This result suggested that solution-based hybridization was
232 more efficient than surface-based. In surface-based hybridization, the detection probes
233 were first immobilized on the backbone of the fibrils. Then the target single-stranded
234 oligonucleotide reached the probes solely by diffusion. The overall hybridization rate is
235 limited by the diffusion kinetic of the target analyte, the electrostatic repulsion of both
236 negatively charged probes and targets, density of the surface-immobilized probe, probe
237 immobilization efficiency and the steric hindrance. On the other hand, in solution-based
238 hybridization, both the probe and the target are free in solution, the charge density is
239 diluted due to large diffusion volume and, therefore they are subject to higher chance to
240 interact and form hybrid. Comparing under the same hybridization time, solution-based
241 hybridization offers higher efficiency. Therefore, solution-based hybridization was
242 adopted for the miRNA detection assay in the following work.



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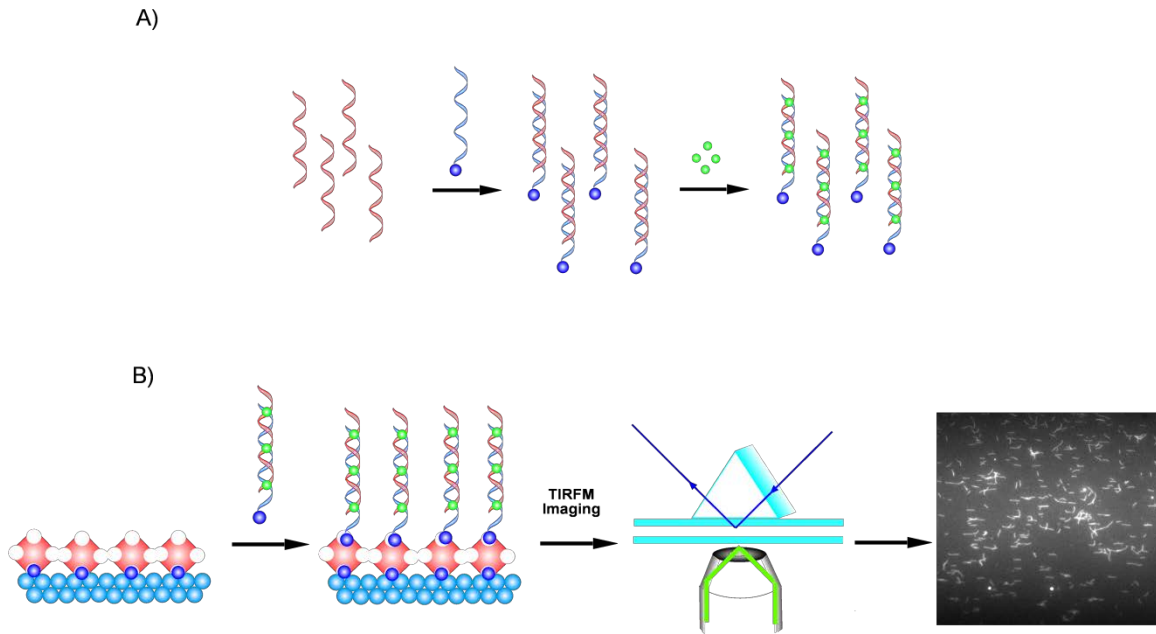
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250 **Figure 1.** A) The average of resulted fluorescence signal on individual biosensing fibril,
 251 as an indication of the hybridization efficiency, obtained by solution-based and the
 252 surface-based hybridization with 500 pM poly A and complementary poly T probe. Error
 253 bar, standard error of mean. $n = 3$. (Net intensity = $50 \times (15 \times 2$ square pixels of
 254 independent fluorescent area on fibrils) – $50 \times (15 \times 2$ square pixels of independent

255 background area on image).) The fluorescence images of the fibrillar sensors detecting
256 poly A/poly T duplex duplexes formed by (B) surface-based hybridization; and (C)
257 solution-based hybridization. The hybridization efficiency of solution-based is
258 significantly higher than that of surface-based.



259
260

261 **Figure 2.** Schematics of the direct quantification of miRNA with a self-assembling
 262 protein fibrillar platform. (A) A hybridization-based detection for mir-196a by using
 263 biotin functionalized LNA196a detection probe. The hybrids were labeled with
 264 fluorescent dye YOYO-1. (B) The hybrids were then conjugated onto the surface of the
 265 amyloid protein fibril by biotin/streptavidin interaction for fluorescence measurement and
 266 quantification.

267 **3.2 Detection assay.** As a proof-of-concept, mir-196a was chosen as the target miRNA in
268 this study. LNA modified nucleotide detection probe (LNA196a) with a biotin
269 functionalized 5' terminal was designed. The target and the LNA probes were mixed and
270 allowed for sufficient hybridization. The hybrids were then labeled with an intercalating
271 fluorescent dye YOYO-1. The binding affinity of the YOYO-1 dye towards the double-
272 stranded nucleic acid is around $6 \times 10^8 \text{ M}^{-1}$ with a fluorescence intensity enhancement of
273 ~ 400 -fold upon binding as compared to single-stranded.³¹ The resulted fluorescence
274 signal is employed for quantitative analysis.

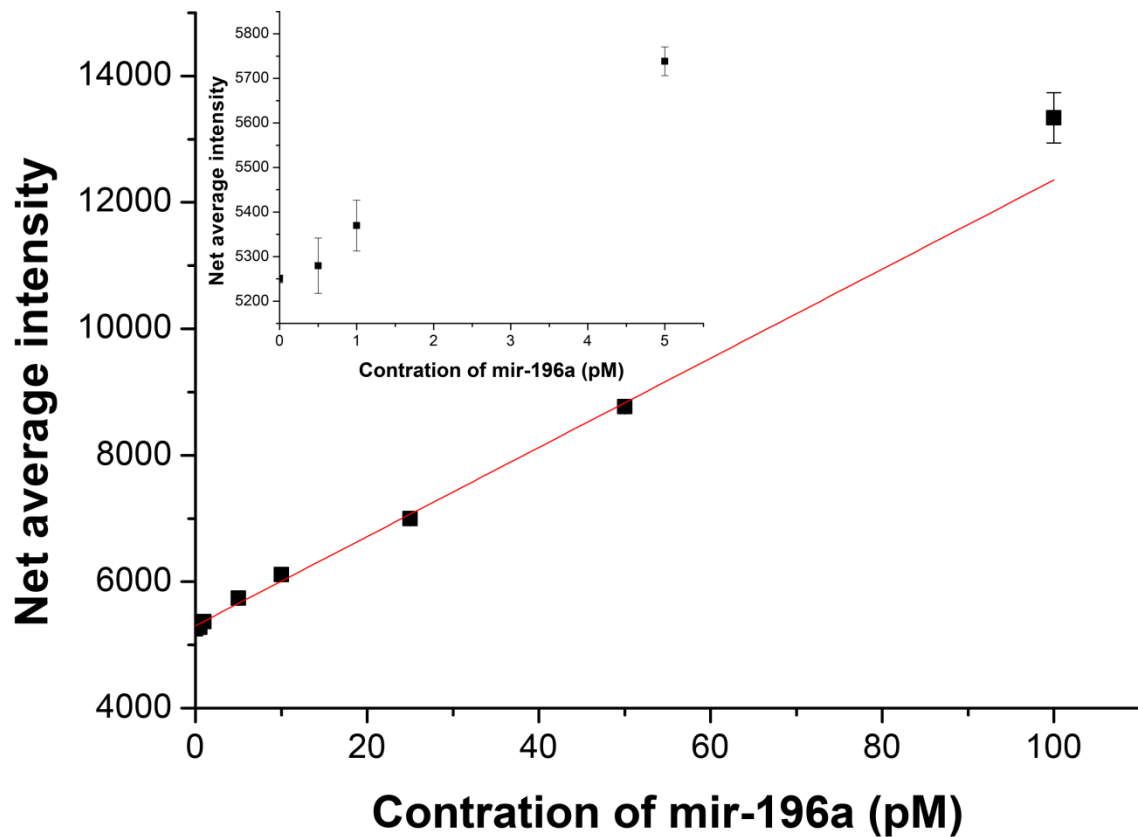
275 The principle of the detection assay is illustrated in figure 2. Biotinylated fibrils were
276 prepared by simply mixing and incubating the biotinylated amyloid monomer and native
277 amyloid monomer for an hour. Each of the as-formed protein fibril acted as an individual
278 platform for target analyte capturing. With the wide-view fluorescence imaging of large
279 number of fibrils simultaneously, a detection of high-throughput was accomplished. We
280 made use of the electrostatic and hydrophobic interaction between the protein fibrils and
281 the glass slide surface that the fibrils could be immobilized and stretched upon capillary
282 force once the fibrils were flowed into the channel which was comprised by two glass
283 coverslides and adhesive tape. Neither pretreatment of the glass surface nor chemical
284 modifications on the protein scaffold was required. As shown from the fluorescence
285 images, the fibrils were consistently well-stretched and physically adsorbed on glass
286 surface. After that, freely diffusing hybrids will then be captured onto the nanofibrils.
287 Fluorescence signal from the labeled hybrids was collected for analysis. According to our
288 previous study²³, the sensitivity of the detection assay was influenced by the density (or
289 concentration) of A β_{1-40} fibrils on the coverslide and the compositional percentage of the

290 biotin moiety on the fibrillar protein. As reported, the fibrils obtained by co-incubation of
291 native monomer with 20 % of biotinylated A β ₁₋₄₀ monomer gave optimal experimental
292 conditions for the strongest fluorescence signal and therefore it was adapted in this
293 current study. After the immobilization of the fibrils on the glass surface, the solution
294 mixture of excessive stv/AF 555-stv was injected into the home-made channel to capture
295 the biotinylated target hybrids as well as to label and locate the fibrils. It was found that
296 the highly negative double-stranded hybrid did not have substantial non-specific
297 adsorption onto the negative surface of the coverslips (at pH 7.4). Addition of the
298 blocking agent, for example, BSA, was therefore not necessary (See figure S1).

299 As one of the wide-view fluorescence imaging techniques, TIRFM is regarded as a
300 sensitive and powerful one to monitor chemical and biological systems at the glass/water
301 interface. The fact that only the fluorophores (the immobilized protein fibrils with
302 captured targets in this work) within the evanescent field layer was excited and hence
303 high signal-to-noise ratio was provided. The set-up of the TIRFM system is shown in
304 figure S2. A 488 nm laser was used to excite the YOYO-1 dye on the hybrids and the
305 AF555 stv on the fibril. The angle of incidence of the laser beam is $\sim 66^\circ$, the total
306 internal reflection occurred at the glass/solution interface and the penetration depth of
307 evanescent field layer (d) was calculated to be 190 nm by $(d = \lambda / 4\pi(n_1)^2 \sin^2 \theta - n_2^2)^{1/2}$
308 where λ is the wavelength of the excitation source, n_1 and n_2 are the refraction indices of
309 the solution and glass-surface, and θ is the angle of incidence. The fluorescence image of
310 the fibrils was captured by the EMCCD.

311 To illustrate the performance of the developed miRNA assay, the LNA196a-mir-196a
312 hybrids with different concentration of miRNA target (0 to 100 pM) were added to the

313 channel and captured on the surface of the fibrils through the strong biotin and
314 streptavidin interaction. Individual fibrils loaded with hybridization complex of 0 to 100
315 pM mir-196a with 100 pM LNA196a were visualized under the TIRFM imaging system
316 and the intensity was analyzed. A calibration curve of the fluorescence intensity collected
317 as a function of the target concentration was constructed as shown in figure 3. A good
318 linearity with a coefficient of determination of 0.994 was obtained. The limit of detection
319 (LOD) of 500 fM was achieved ($LOD = \text{mean of the blank} + 3 \times \text{standard error of mean}$
320 of blank) for miR196a. As literature reported, the level of miRNAs in the cancer patient's
321 serum sample is around 20 pM⁹. This as-developed assay is applicable to quantify the
322 level of miRNAs in serum sample.

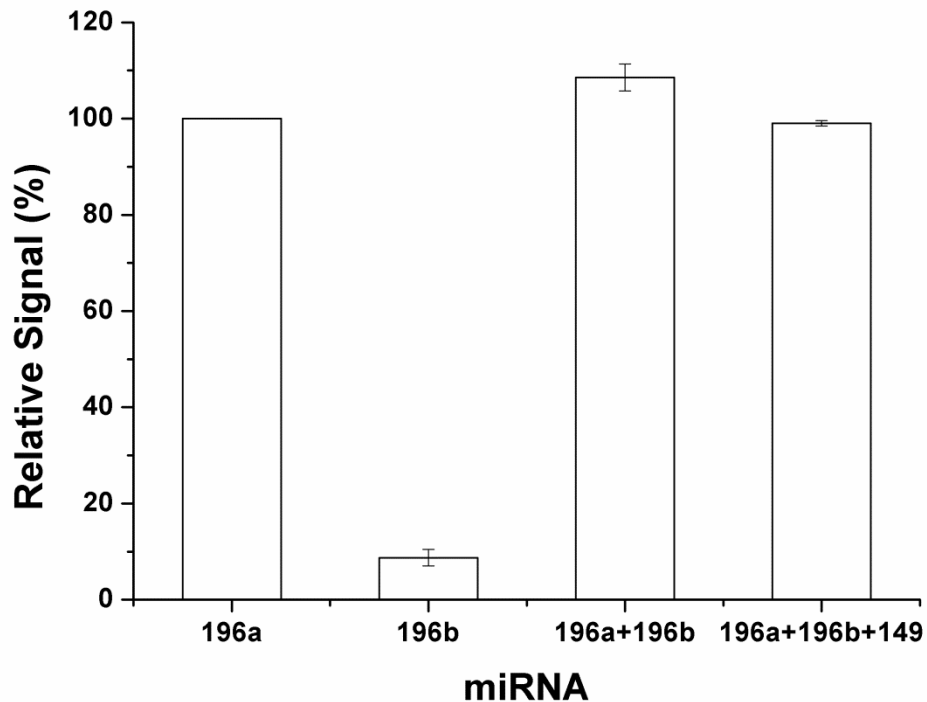


323

324 **Figure 3.** Calibration plot showing the linear relationship between net averaged
 325 fluorescence intensity generated from the YOYO-intercalated miRNA hybrids and the
 326 concentration of mir-196a, $R^2 = 0.994$. Detection limit of the detection assay is 500 fM.
 327 Error bars, standard error of mean, $n = 3$. (Net intensity = $50 \times (15 \times 2$ square pixels of
 328 independent fluorescent area on fibrils) – $50 \times (15 \times 2$ square pixels of independent
 329 background area on image).)

330 **3.3 Specificity study.** The sequences of the miRNAs within a family are very similar and
331 some of them differ only by a single nucleotide. This places another challenge to obtain
332 an accurate expression profiling of miRNAs from the same family. To demonstrate the
333 specificity of this detection assay, mir-196b and a non-complementary miRNA (mir-149)
334 were chosen. Literature reported that mir-149 also plays an important role in the
335 development of nasopharyngeal carcinoma (NPC)³². Mir-196b is a family member of the
336 mir-196 family, the optimal hybridization temperatures of the two miRNAs, mir-196a
337 and mir-196b, differ by 2 °C. In the recent decade, locked nucleic acid (LNA) has been
338 used as detection probe for a variety of miRNAs assay for significant increase in the
339 sensitivity and specificity of the detection probe compared to using RNA and DNA
340 probes. The methylene bridge connecting the 2'O and the 4'C of the ribose backbone
341 fixed the LNA modified nucleotide such that the nucleotides were locked in place after
342 hybridization. This provided greater stability as an increase in the melting temperature of
343 the hybridized complex³³.

344 As illustrated in figure 4, mir-196a can be clearly discriminated from mir-196b and mir-
345 149. Compared to mir-196a, mir-196b with a mismatch nucleotide showed detection
346 signal (8.7%). While the signal detected from sample matrices that contained 1) both mir-
347 196a and mir-196b, and 2) mir-196a, mir-196b, and mir-149 was 108 % and 99 %
348 respectively. It elucidated that LNA detection probe exhibits a high specificity to the
349 complementary target.



350

351 **Figure 4.** Study on the single-base mismatch discrimination efficiency (%) of the assay.

352 The relative signal produced by mir-196a is normalized to 100%, the relative detection of

353 the other miRNAs are calculated by the correlation equation in figure 3. The

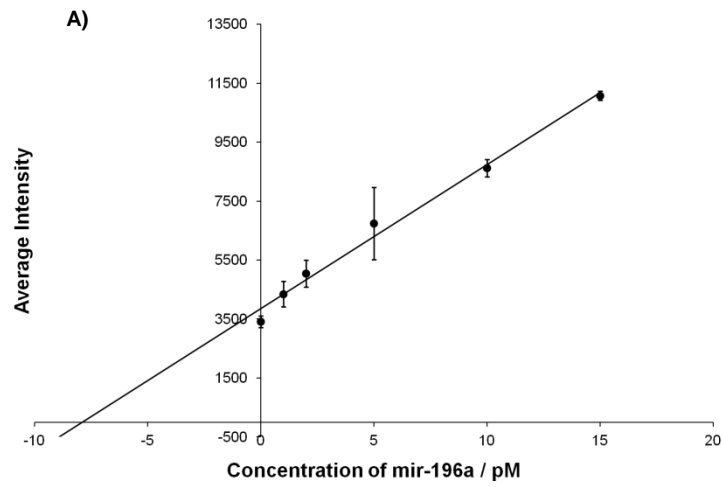
354 concentration of each miRNAs is 50 pM. The detection platform shows high specificity

355 towards the target analyte, and is able to discriminate sample which deviated by a single-

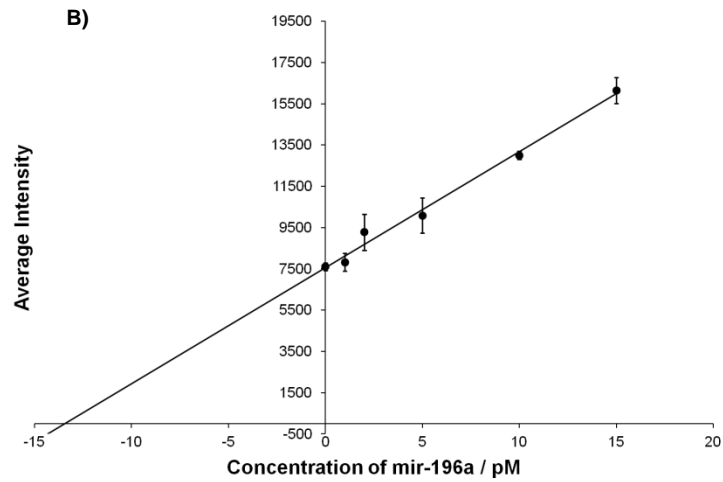
356 base mismatch. Error bars, standard error of mean, n = 3.

357 **3.4 Quantification of mir-196a in normal and cancer patient's serum samples.** We
358 then applied the developed assay to quantify the expression of mir-196a in both normal
359 and NPC cancerous serum samples. Since the fluorescent dye YOYO-1 has no selectivity
360 towards oligonucleotides like mRNA, tRNA and other kind of small RNAs in such a
361 complex sample matrix, in order to eliminate the unwanted signal from the background
362 matrixes, standard addition method was applied in a manner that total synthetic target
363 miRNA (mir-196a) was spiked into mixture of probes and the serum samples without any
364 sample pre-treatment to the serum. The concentration of the mir-196a in the serum
365 samples was obtained by extrapolation of the calibration curve. Two independent
366 calibration curves were prepared for the two serum samples and the concentration mir-
367 196a in each serum sample was determined. Both calibration curves showed good
368 correlation between the concentration of the mir-196a and the intensity of the fibril with
369 coefficient of determination above 0.989 (Figure 5). The content of mir-196a in normal
370 and cancerous serum samples were estimated to be 7.9 ± 0.2 pM and 13.5 ± 0.3 pM
371 respectively. The original concentration of mir-196a was then corrected by 5-fold dilution
372 factor to be 40 and 68 pM. The result agreed with literature report that mir-196a is the up-
373 regulated in nasopharyngeal carcinoma³⁴. While the entire assay could be completed
374 within 2 hrs, which is fast compared to traditional qRT-PCR, a direct quantification with
375 minute sample consumption (~ 5 μ L) was achieved. It is promising for constructing
376 miRNA expression profile in real sample.

377



378



379

380 **Figure 5.** Quantification of mir-196a in (A) normal serum and (B) cancer patient's serum
381 samples by standard addition method. Synthetic mir-196a was spiked into the serum
382 samples. Error bars, standard error of mean, n = 3.

383 **4. Conclusion**

384 In this work, we developed a sensitive and specific platform for quantification of
385 miRNAs. As a proof-of-concept, the concentration of mir-196a was quantified in both
386 normal and cancerous serum samples. Profiling of these small regulatory miRNAs
387 provides valuable information to diagnostics test developers as well as pharmaceutical
388 companies. The protein-based detection assay can be readily modified and applied in
389 immunoassays, in which the probe sequence on the fibrillar sensor can be converted from
390 oligonucleotide probes into antibodies for capturing disease-related antigens or other
391 acceptor molecules. Similarly, the LNA probes are also readily replaced by ATP aptamer,
392 cardiac disease markers, thrombin aptamer, tumor angiogenesis markers, etc., for
393 academic, biomedical researches and many other clinical applications.

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- 400 1. de Planell-Saguer, M.; Rodicio, M. C. Analytical aspects of microRNA in diagnostics: A
401 review. *Anal. Chim. Acta* **2011**, *699* (2), 134-152.
- 402 2. Kwak, P. B.; Iwasaki, S.; Tomari, Y. The microRNA pathway and cancer. *Cancer Sci.*
403 **2010**, *101* (11), 2309-2315.
- 404 3. Pritchard, C. C.; Cheng, H. H.; Tewari, M. MicroRNA profiling: approaches and
405 considerations. *Nat. Rev. Genet.* **2012**, *13* (5), 358-369.
- 406 4. Bartel, D. P. MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* **2004**,
407 *116* (2), 281-297.
- 408 5. Esquela-Kerscher, A.; Slack, F. J. Oncomirs - microRNAs with a role in cancer. *Nat. Rev.*
409 *Cancer* **2006**, *6* (4), 259-269.
- 410 6. Chen, X.; Ba, Y.; Ma, L. J.; Cai, X.; Yin, Y.; Wang, K. H.; Guo, J. G.; Zhang, Y. J.;
411 Chen, J. N.; Guo, X.; Li, Q. B.; Li, X. Y.; Wang, W. J.; Zhang, Y.; Wang, J.; Jiang, X. Y.; Xiang,
412 Y.; Xu, C.; Zheng, P. P.; Zhang, J. B.; Li, R. Q.; Zhang, H. J.; Shang, X. B.; Gong, T.; Ning, G.;
413 Wang, J.; Zen, K.; Zhang, J. F.; Zhang, C. Y. Characterization of microRNAs in serum: a novel
414 class of biomarkers for diagnosis of cancer and other diseases. *Cell Res.* **2008**, *18* (10), 997-1006.
- 415 7. Wark, A. W.; Lee, H. J.; Corn, R. M. Multiplexed detection methods for profiling
416 microRNA expression in biological samples. *Angew. Chem.-Int. Edit.* **2008**, *47* (4), 644-652.
- 417 8. Lin, S. L.; Chang, D.; Ying, S. Y. Asymmetry of intronic pre-miRNA structures in
418 functional RISC assembly. *Gene* **2005**, *356*, 32-38.
- 419 9. Tian, T.; Xiao, H.; Zhang, X. L.; Peng, S.; Zhang, X. E.; Guo, S.; Wang, S. R.; Liu, S.
420 M.; Zhou, X.; Meyers, C.; Zhou, X. Simultaneously sensitive detection of multiple miRNAs
421 based on a strand displacement amplification. *Chem. Commun.* **2012**, *49* (1), 75-77.
- 422 10. Bustin, S. A.; Mueller, R. Real-time reverse transcription PCR (qRT-PCR) and its
423 potential use in clinical diagnosis. *Clin. Sci.* **2005**, *109* (4), 365-379.
- 424 11. Valoczi, A.; Hornyik, C.; Varga, N.; Burgyan, J.; Kauppinen, S.; Havelda, Z. Sensitive
425 and specific detection of microRNAs by northern blot analysis using LNA-modified
426 oligonucleotide probes. *Nucleic Acids Res.* **2004**, *32* (22).
- 427 12. Lim, L. P.; Lau, N. C.; Garrett-Engele, P.; Grimson, A.; Schelter, J. M.; Castle, J.; Bartel,
428 D. P.; Linsley, P. S.; Johnson, J. M. Microarray analysis shows that some microRNAs
429 downregulate large numbers of target mRNAs. *Nature* **2005**, *433* (7027), 769-773.
- 430 13. Kilic, T.; Topkaya, S. N.; Ariksoysal, D. O.; Ozsoz, M.; Ballar, P.; Erac, Y.; Gozen, O.
431 Electrochemical based detection of microRNA, mir21 in breast cancer cells. *Biosens. Bioelectron.*
432 **2012**, *38* (1), 195-201.
- 433 14. Wu, X. Y.; Chai, Y. Q.; Yuan, R.; Su, H. L.; Han, J. A novel label-free electrochemical
434 microRNA biosensor using Pd nanoparticles as enhancer and linker. *Analyst* **2013**, *138* (4), 1060-
435 1066.
- 436 15. Li, C. P.; Li, Z. P.; Jia, H. X.; Yan, J. L. One-step ultrasensitive detection of microRNAs
437 with loop-mediated isothermal amplification (LAMP). *Chem. Commun.* **2011**, *47* (9), 2595-2597.
- 438 16. Yin, B. C.; Liu, Y. Q.; Ye, B. C. One-Step, Multiplexed Fluorescence Detection of
439 microRNAs Based on Duplex-Specific Nuclease Signal Amplification. *J. Am. Chem. Soc.* **2012**,
440 *134* (11), 5064-5067.
- 441 17. Chan, H. M.; Chan, L. S.; Wong, R. N. S.; Li, H. W. Direct Quantification of Single-
442 Molecules of MicroRNA by Total Internal Reflection Fluorescence Microscopy. *Anal. Chem.*
443 **2010**, *82* (16), 6911-6918.
- 444 18. Alhasan, A. H.; Kim, D. Y.; Daniel, W. L.; Watson, E.; Meeks, J. J.; Thaxton, C. S.;
445 Mirkin, C. A. Scanometric MicroRNA Array Profiling of Prostate Cancer Markers Using
446 Spherical Nucleic Acid-Gold Nanoparticle Conjugates. *Anal. Chem.* **2012**, *84* (9), 4153-4160.

447 19. Weizmann, Y.; Chenoweth, D. M.; Swager, T. M. DNA-CNT Nanowire Networks for
448 DNA Detection. *J. Am. Chem. Soc.* **2011**
449 *133* (10), 3238-3241.
450 20. Zhang, G. J.; Chua, J. H.; Chee, R. E.; Agarwal, A.; Wong, S. M. Label-free direct
451 detection of MiRNAs with silicon nanowire biosensors. *Biosens. Bioelectron.* **2009**, *24* (8), 2504-
452 2508.
453 21. Dong, H. F.; Lei, J. P.; Ju, H. X.; Zhi, F.; Wang, H.; Guo, W. J.; Zhu, Z.; Yan, F. Target-
454 Cell-Specific Delivery, Imaging, and Detection of Intracellular MicroRNA with a Multifunctional
455 SnO₂ Nanoprobe. *Angew. Chem.-Int. Edit.* **2012**, *51* (19), 4607-4612.
456 22. Gao, X. Y.; Matsui, H. Peptide-based nanotubes and their applications in
457 bionanotechnology. *Adv. Mater.* **2005**, *17* (17), 2037-2050.
458 23. Chan, H. M.; Li, H. W. Multifunctional Encoded Self-Assembling Protein Nanofibrils as
459 Platform for High-Throughput and Multiplexed Detection of Biomolecules. *Anal. Chem.* **2011**, *83*
460 (24), 9370-9377.
461 24. Pereira, P. M.; Marques, J. P.; Soares, A. R.; Carreto, L.; Santos, M. A. S. MicroRNA
462 Expression Variability in Human Cervical Tissues. *PLoS One* **2010**, *5* (7).
463 25. Niinuma, T.; Suzuki, H.; Nojima, M.; Noshio, K.; Yamamoto, H.; Takamaru, H.;
464 Yamamoto, E.; Maruyama, R.; Nobuoka, T.; Miyazaki, Y.; Nishida, T.; Bamba, T.; Kanda, T.;
465 Ajioka, Y.; Taguchi, T.; Okahara, S.; Takahashi, H.; Nishida, Y.; Hosokawa, M.; Hasegawa, T.;
466 Tokino, T.; Hirata, K.; Imai, K.; Toyota, M.; Shinomura, Y. Upregulation of miR-196a and
467 HOTAIR Drive Malignant Character in Gastrointestinal Stromal Tumors. *Cancer Res.* **2012**, *72*
468 (5), 1126-1136.
469 26. Szafranska, A. E.; Davison, T. S.; John, J.; Cannon, T.; Sipos, B.; Maghnouj, A.;
470 Labourier, E.; Hahn, S. A. MicroRNA expression alterations are linked to tumorigenesis and non-
471 neoplastic processes in pancreatic ductal adenocarcinoma. *Oncogene* **2007**, *26* (30), 4442-4452.
472 27. Chan, H. M.; Xiao, L. H.; Yeung, K. M.; Ho, S. L.; Zhao, D.; Chan, W. H.; Li, H. W.
473 Effect of surface-functionalized nanoparticles on the elongation phase of beta-amyloid (1-40)
474 fibrillogenesis. *Biomaterials* **2012**, *33* (18), 4443-4450.
475 28. Man, B. Y. W.; Chan, H. M.; Leung, C. H.; Chan, D. S. H.; Bai, L. P.; Jiang, Z. H.; Li, H.
476 W.; Ma, D. L. Group 9 metal-based inhibitors of beta-amyloid (1-40) fibrillation as potential
477 therapeutic agents for Alzheimer's disease. *Chem. Sci.* **2011**, *2* (5), 917-921.
478 29. Yang, W. G.; Wong, Y.; Ng, O. T. W.; Bai, L. P.; Kwong, D. W. J.; Ke, Y.; Jiang, Z. H.;
479 Li, H. W.; Yung, K. K. L.; Wong, M. S. Inhibition of Beta-Amyloid Peptide Aggregation by
480 Multifunctional Carbazole-Based Fluorophores. *Angew. Chem.-Int. Edit.* **2012**, *51* (8), 1804-
481 1810.
482 30. Xia, T. B.; SantaLucia, J.; Burkard, M. E.; Kierzek, R.; Schroeder, S. J.; Jiao, X. Q.; Cox,
483 C.; Turner, D. H. Thermodynamic parameters for an expanded nearest-neighbor model for
484 formation of RNA duplexes with Watson-Crick base pairs. *Biochemistry* **1998**, *37* (42), 14719-
485 14735.
486 31. Cosa, G.; Focsaneanu, K. S.; McLean, J. R. N.; McNamee, J. P.; Scaiano, J. C.
487 Photophysical properties of fluorescent DNA-dyes bound to single- and double-stranded DNA in
488 aqueous buffered solution. *Photochem. Photobiol.* **2001**, *73* (6), 585-599.
489 32. Luo, Z. H.; Zhang, L. Y.; Li, Z.; Li, X. Y.; Li, G.; Yu, H. B.; Jiang, C.; Dai, Y. F.; Guo,
490 X. F.; Xiang, J. J.; Li, G. Y. An in silico analysis of dynamic changes in microRNA expression
491 profiles in stepwise development of nasopharyngeal carcinoma. *BMC Med. Genomics* **2012**, *5*.
492 33. Nuovo, G. J. In situ detection of microRNAs in paraffin embedded, formalin fixed tissues
493 and the co-localization of their putative targets. *Methods* **2010**, *52* (4), 307-315.
494 34. TANG, S.-y.; HUANG, G.-l.; LU, Y.; HE, Z.-w. Relationship between hsa-mir-196a-2
495 gene polymorphism and susceptibility and clinicopathologic factors of nasopharyngeal carcinoma
496 in Hunan Han people. *Journal of Guangdong Medical College* **2013** (1), 8-10,14.