Biosensors and Bioelectronics 26 (2011) 3309–3312

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Cocaine detection via rolling circle amplification of short DNA strand separated by magnetic beads

Cuiping Ma, Wenshuo Wang, Qing Yang, Chao Shi*, Lijie Cao

State Key Laboratory Base of Eco-chemical Engineering, College of Chemistry and Molecular Engineering, Qingdao University of Science and Technology, 53 ZhengZhou Road, Qingdao 266042, PR China

ARTICLE INFO

Article history:
Received 26 September 2010
Received in revised form 4 December 2010
Accepted 4 January 2011
Available online 6 January 2011

Keywords:
Cocaine
Aptamer
Rolling circle amplification
DNA biosensor

ABSTRACT

A novel and sensitive fluorescence biosensor based on aptamer and rolling circle amplification for the determination of cocaine was developed in the present work. Here cocaine aptamers immobilized onto Au nanoparticles modified magnetic beads hybridized with short DNA strand. In the presence of cocaine, the short DNA strand was displaced from aptamer owing to cocaine specially binding with aptamer. Next, the short DNA strand was separated by magnetic beads and used to originate rolling circle amplification as primer. The end products of rolling circle amplification were detected by fluorescence signal generation upon molecular beacons hybridizing with the end products of rolling circle amplification. With rolling circle amplification and the separation by magnetic beads reducing the background signal, the new strategy was suitable for the detection of as low as 0.48 nM cocaine. Compared with reported cocaine sensors, our method exhibited excellent sensitivity. Our new strategy may provide a platform for numerous proteins and low molecular weight analytes to highly sensitively detect by DNA amplification.

1. Introduction

Amplification of nucleic acids is recently proving to be valuable for the quantitative detection of nucleic acids and indirect detection of other targets. Among DNA amplification methods, rolling circle amplification (RCA) has gained great attention for ultrasensitive DNA analysis (Nilsson et al., 2006; Nilsson, 2006; Fischer et al., 2008; Schweitzer and King-smore, 2001). In a typical RCA reaction, a primer hybridizes with a single strand nucleic acid circle containing 13–240 nucleotides (deVroom et al., 1988; Frieden et al., 1999; Capobianco et al., 1990), which is extended around the circle with DNA polymerase and the end product is a very long single strand DNA (ssDNA) molecule complementary to the nucleic acid circle template. Hence, a single molecule can be amplified hundreds to thousands of times of template as a fundamental characteristic of RCA (Jarvius et al., 2006; Schweitzer et al., 2002; Nallur et al., 2001; Cho et al., 2005). The end products can be detected by molecular beacons, cleavable probes, or SYBR Green (Di Giusto et al., 2005; Nilsson et al., 2002; Smolina et al., 2004). Moreover, RCA is a convenient DNA amplification tool without the need for sophisticated instrumentation. Shortly after RCA was discovered, it has been widely applied as an important DNA amplification method in biodetection and nanotechnology with functional nucleic acids (Zhao et al., 2008).

Aptamer-based sensors have been developed with its specific recognition properties toward targets (Zhang et al., 2008; Li et al., 2010a,b; Ren et al., 2010; Ding et al., 2010). Aptamers found growing interests as recognition materials for the detection of cocaine. In 2001, Stojanovic et al. adapted double-end labeled aptamer to signal the recognition of cocaine. Baker et al. (2006) proposed the electronic aptamer-based cocaine biosensor. Liu and Lu (2006) and Stojanovic and Landry (2002) reported colorimtric sensors based on a cyanine dye–aptamer complex and gold nanoparticles linked by aptamer, respectively. DNA ultrasensitive analysis with aptamer has been widely applied by DNA polymerase. Shlyahovsky et al. (2007) presented the amplified analysis of cocaine by an autonomous aptamer-based machine, which included DNA polymerase, the nicking enzyme Nt.BbvCI and a molecular beacon. The detection limit for the 70 min of incubation results was 5 μM. Recently, Yu et al. (2010) built a fluorescence aptameric sensor for strand displacement amplification detection of cocaine with SYBR Green I intercalating into the new DNA double helix. The detection limit of the method for 2 h was 1 nM.

In the present study, a highly sensitive fluorescence sensor for quantification of cocaine was developed based on aptamer identification and the application of rolling circle amplification of short DNA strand separated by magnetic beads as primer. In this protocol, DNA hybridization was performed by mixing the cocaine aptamer captured by Au nanoparticles modified magnetic beads with short

* Corresponding author. Tel.: +86 532 84023652; fax: +86 532 84022750.
E-mail address: qax169@hotmail.com (C. Shi).

0956-5663/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2011.01.003

Table 1
Sequences of sensing probes and primers.

Probes	Sequence (5′–3′)
cDNA	5′-*GTC TCC CCC TCA* GA **TAG CTT ATC AG A CTG ATG TTG A CC**-3′
Aptamer	5′-**TGA GGG GGA GAC** AAG GAA AAT CCT TCA ATG AAG TGG GTC TCC CTT TTT TTT-SH-3′
Padlock probe	5′-P-CTG ATA AGC TA TCA CT ATG GTC GC GCT AGG TCT ATT TAG TGG AGC CCA A GTAC TCACT ATG GTC GC GCT AGG TCA ACA TCA GT-3′
Molecular beacons	5′-6-FAM-CGA ATT CTA TTT AGT GGA GCC CAA TTC G-DABCYL-3′

DNA strand (cDNA). When cocaine was in present, aptamer was specially bound with cocaine so that short DNA strand was replaced from aptamer. The short DNA strand separated by magnetic beads was directly used to cyclize so-called padlock probe, and originated rolling circle amplification as primer. The RCA products were partially complementary with molecular beacons and the molecular beacons were opened to generate fluorescence signal. Therefore, the quantitative detection of cocaine was realized through the RCA products detection. This method exhibited excellent sensitivity due to DNA amplification by RCA, reducing background signal by magnetic beads, and improving DNA immobilization by Au nanoparticles.

2. Experimental

2.1. Reagents and materials

Cocaine was purchased from Beijing Institute for Drug Control (Beijing, China). T4 DNA ligase, deoxynucleotide triphosphates (dNTPs), *Rsa I* restriction enzyme and phi29 DNA polymerase were purchased from MBI Fermentas Crop. All oligonucleotides were supplied and high-performance liquid chromatography (HPLC) purified by Takara Biotechnology Co. Ltd. (Dalian, China) as shown in Table 1. The cDNA strand in italic portion was complementary with the boldface in the aptamer sequence. The boldface of cDNA strand matched the underlined sequence of padlock probe as the primer sequence of RCA. The circularizing padlock probe had phosphates at its 5′-end for ligation. Molecular beacon as reporter was labeled at the ends of the stem with the dyes, (N-3-fluoranthyl) maleimide (FAM) and 4-(4′-dimethylaminophenylazo) benzoic acid (DABCYL). Thio-modified magnetic beads (3–4 μm, diameter) were obtained from BaseLine Chromtech Research Centre (Tianjin, China). All chemicals were analytical grade to directly be used. Deionized water was used to prepare all of solutions.

2.2. Apparatus

Fluorescence experiments were performed by a Hitachi F-4500 spectrophotometer (Tokyo, Japan) equipped with a xenon lamp. Oligonucleotide quantitation was performed using a Cary 50 UV–vis–NIR spectrophotometer (Varian).

2.3. Hybridization of cDNA and aptamer on Au nanoparticles modified magnetic beads

10 μL (10 mg/mL) thio-modified magnetic beads were washed twice with 500 μL 10 mM PBS buffer (0.1 M NaCl, pH7.4). 1 mL 20 nm Au nanoparticles (0.01%) was added to the washed magnetic beads and gently shaked for 12 h at 37 °C. Aptamer strands in PBS buffer were denatured at 90 °C for 5 min and added to Au nanoparticles modified magnetic beads at a final concentration of 0.1 μM for 12 h. The same final concentration of cDNA was incubated with aptamer immobilized onto magnetic beads for 2 h at 37 °C with gentle shaking. The immobilized DNA double helix of cDNA and aptamer onto Au nanoparticles was washed three times with 200 μL cocaine action buffer (10 mM PBS, 0.3 M NaCl, pH7.0) and stored at 4 °C to use.

2.4. RCA detection

Cocaine solutions (10 mM PBS, 0.3 M NaCl, pH7.0) of different concentrations were mixed with the above prepared magnetic beads for 20 min at room temperature. The displaced cDNA was separated by magnetic beads for cocaine specially binding with aptamer. After collection, 10 μL of the resulting solution and 2 μL of 0.1 μM padlock probe were heated at 90 °C for 5 min and incubated for 20 min at room temperature. Subsequently, 2.5 U T4 DNA ligase was mixed in a 8 μL solution consisting of 1× phi29 buffer (33 mM Tris–acetate (pH7.9), 10 mM Mg-acetate, 66 mM K-acetate, 0.1% (v/v) Tween 20, 1 mM dithiothreitol (DTT)) and 2 μL ATP, followed by incubation in 22 °C for 1 h. Remaining RCA components including 0.5 μL phi29 DNA polymerase (10 units/μL), 1 mM of each dNTP and 1× phi29 buffer were added to ligated solution at 37 °C for 1 h. The end products of RCA were heated at 90 °C for 5 min and hybridized with 0.1 μM molecular beacons at 37 °C for 25 min. Then, the solutions were measured at an excitation wavelength of 480 nm and emission wavelength at 520 nm. The slit widths were set at 10 nm.

2.5. Polyacrylamide gel electrophoresis

15% polyacrylamide gel electrophoresis was used to directly show RCA replication induced by cocaine binding with aptamers. Polyacrylamide gel electrophoresis was stained by ethidium bromide (EB) and run in 1× TAE buffer (40 mM Tris, 20 mM glacial acetic acid, 1 mM EDTA, pH 8.0) at room temperature. The RCA amplification was performed by adding 5 U phi29 DNA polymerase, 2.5 U T4 DNA ligase, and 1.2 mM dNTPs with 10 μM cocaine in a final volume of 25 μL, followed by incubation at 37 °C for 1 h. The resulting solution was denatured at 90 °C for 5 min and hybridized with 5′-CCCAA**GTAC**TCACT-3′ (the boldface is a restriction endonuclease site), followed by incubation with 0.8 μL *Rsa I* restriction enzyme at 37 °C for 4 h. The same experiment conditions were used without 10 μM cocaine. 8 μL of each sample was loaded into the lanes and performed at a constant potential of 90 V for 40 min.

3. Results and discussion

3.1. Design of the aptameric sensor

The principle of aptamer-based RCA for cocaine detection is depicted in Fig. 1. The aptamer of cocaine was immobilized onto Au nanoparticles modified magnetic beads, which could cause the surface areas increase of magnetic beads to improve DNA immobilization. A short DNA (cDNA) hybridized with the immobilized aptamer of cocaine to form DNA double helix on magnetic beads according to the protocols described above. In the presence of cocaine, cDNA was displaced from aptamers by targets and separated by magnetic beads to anneal to a padlock probe template. Next, the padlock probe was circularized by T4 DNA ligase and RCA was performed by cDNA as primer. In RCA, phi29 DNA polymerase replicated the circular template hundreds to thousands of times with dNTPs. The DNA extension process could efficiently be monitored by molecular beacons. Conversely, no RCA products hybridized with molecular beacons to give fluorescence signal in the absence of cocaine.

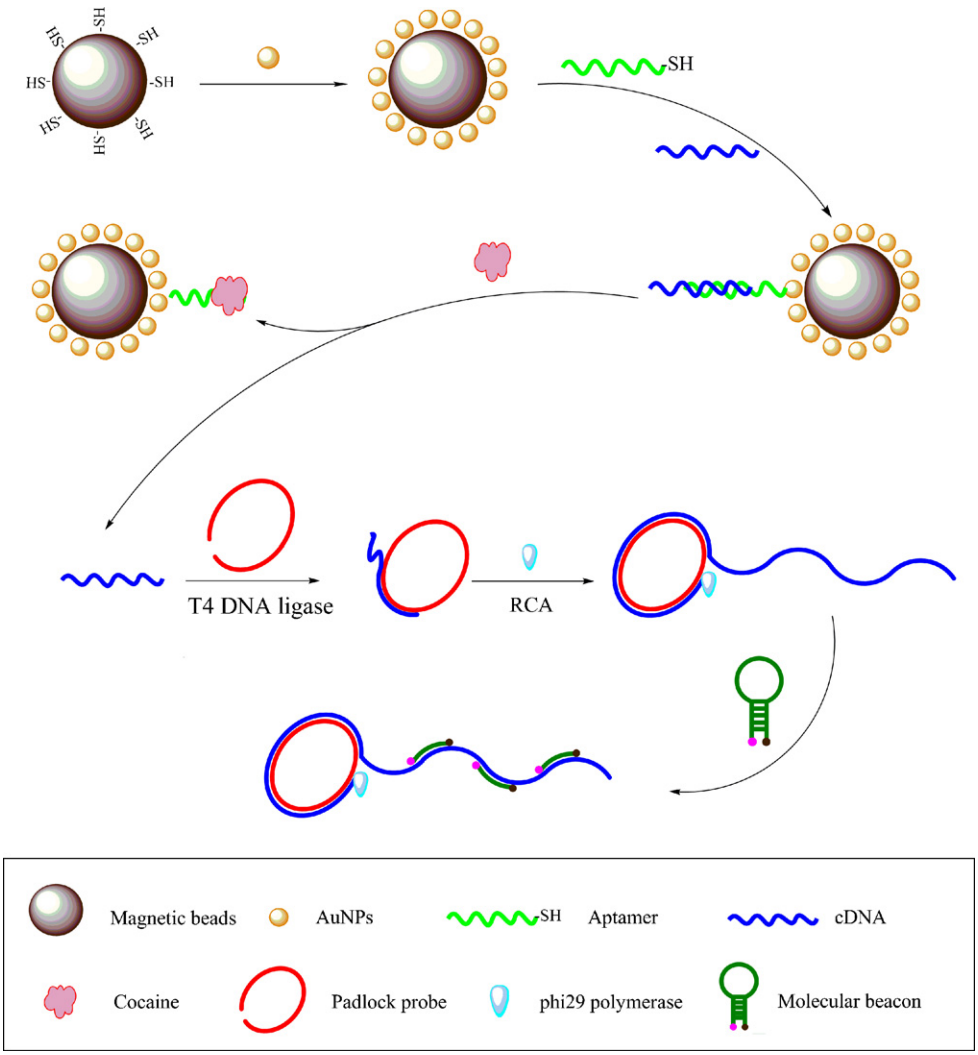

Fig. 1. Schematic outline of fluorescence biosensor to detect cocaine based on signal amplification under RCA and the separation by magnetic beads reducing the background signal.

3.2. *The verification of RCA reaction by polyacrylamide gel electrophoresis*

To directly show RCA replication induced by cocaine binding, electrophoresis analysis using a 15% polyacrylamide gel was performed (Fig. 2). The cDNA induced by cocaine binding hybridized with padlock probe, which was extended around the circularized padlock probe with phi29 DNA polymerase and the end product was a very long ssDNA molecule. As shown in Fig. 2, the anticipated high molecular weight of RCA product was confirmed in lane 2 in the presence of cocaine. Compared with lane 2, lane 1 displayed no bands in absence of cocaine due to no cDNA as primer of RCA. The concatenated RCA reaction product contained corresponding complementary regions with 5′-CCCAAGTACTCACT-3′ generating multiple equivalent duplex structures in the nascent product. As these duplex regions were designed to contain an *Rsa I* recognition site, the identity of the RCA product was confirmed by the presence of correctly sized fragments upon restriction digestion of assay products (Fig. 2, lane 3).

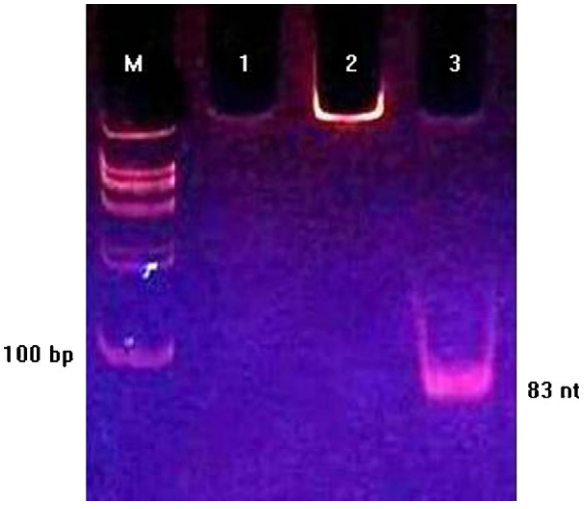

Fig. 2. Polyacrylamide gel (15%) with DL 2000 Marker (M) followed by cocaine binding with aptamers mediated RCA products generated in the presence of (1) 0 μM cocaine, (2) 10 μM cocaine and (3) 10 μM cocaine with subsequent *Rsa I* restriction enzyme digestion. No product was generated in the absence of cocaine, while high molecular weight products were observed when cocaine was present. These products could be digested using *Rsa I* restriction enzyme into fragments displaying the expected sizes.

3.3. *Characteristics of cocaine detection with RCA amplification of cDNA as primer*

Experiments were carried out by adding different amounts of cocaine to verify whether the fluorescence signal could be used for

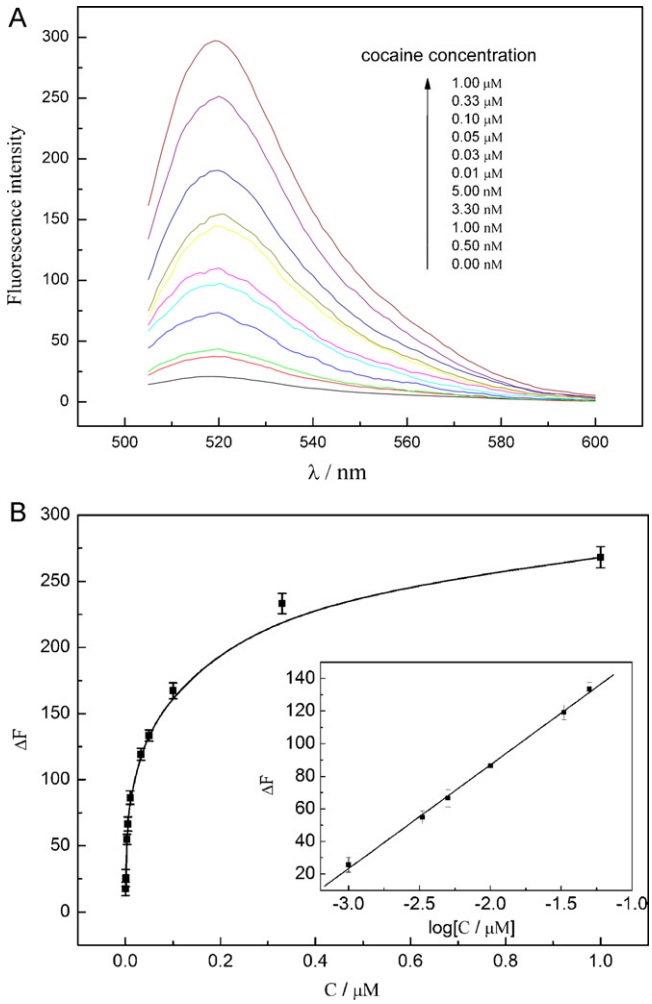

Fig. 3. Detection of different concentrations of cocaine based on aptamer and rolling circle amplification. (A) Fluorescence spectra recorded at different concentrations of cocaine. (B) The change fluorescence signal upon addition of cocaine by our strategy. The inset showed the linear relationship between the change fluorescence signal (ΔF was calculated by $F - F_0$, where F and F_0 are the fluorescence intensities observed in the presence and absence of cocaine, respectively) and the logarithm of cocaine concentration. Averages and the standard deviations were shown in figure.

cocaine quantitation according to the protocols described above. Fig. 3A shows the fluorescence intensities observed on the addition of different concentrations of cocaine. Fluorescence signal was detectable for cocaine in the range of 0.5 nM to 1.0 μM. As seen in Fig. 3A, the cDNA displaced from the cocaine–aptamer complex efficiently underwent RCA. Extension of the cDNA at increasing cocaine concentrations showed a corresponding increase in fluorescence signal. The background signal was induced by unquenched fluorescence signals and unwashed cDNA on magnetic beads that triggered DNA amplification as primer.

As shown in Fig. 3B, with the increase of cocaine concentration, the resulting fluorescence intensity increased owing to release of the cDNA to initiate RCA as primer until a plateau was reached. The change of fluorescence intensity (ΔF) revealed a good linear relationship with the logarithm of cocaine concentration ranging from 1.0×10^{-9} to 5.0×10^{-8} M. The regression equation could be expressed as $\Delta F = 63.585 \log C + 214.177$ (C was the concentration of cocaine, 10^{-6} M) with a correlation coefficient of 0.998 for the linear calibration curve shown in the inset. A detection limit of 4.8×10^{-10} M of cocaine could be further estimated using 3σ. This high sensitivity of cocaine detection contributed to the large RCA amplification initiated by displaced cDNA from cocaine–aptamer complex, the separation by magnetic beads reducing the background signal, and the improvement of DNA immobilization by Au nanoparticles.

4. Conclusions

In conclusion, we have developed a highly sensitive fluorescence biosensor for cocaine based on signal amplification under RCA and the separation by magnetic beads reducing the background signal. Moreover, magnetic beads were modified by Au nanoparticles to improve DNA immobilization. The detection limit of this strategy was 0.48 nM, which showed better sensitivity than the other reported fluorescent sensors of cocaine (Yu et al., 2010). Importantly, this strategy can be expected to provide a sensitive platform for amplified detection of numerous proteins and low molecular weight analytes by RCA because of the wide availability of aptamers. Additionally, according to our method, DNA strand could also be adapted to surfaces of the chips instead of Au nanoparticles modified magnetic beads for high throughput detection of analytes.

Acknowledgements

The work was supported by the National Basic Research Program of China (2010CB732404), and the Research Initial Funding Project for Doctors in Qingdao University of Science and Technology (No. 0022335).

References

Baker, B.R., Lai, R.Y., Wood, M.S., Doctor, E.H., Heeger, A.J., Plaxco, K.W., 2006. J. Am. Chem. Soc. 128, 3138–3139.
Capobianco, M.L., Carcuro, A., Tondelli, L., Garbesi, A., Bonora, G.M., 1990. Nucleic Acids Res. 18, 2661–2669.
Cho, E.J., Yang, L., Levy, M., Ellington, A.D., 2005. J. Am. Chem. Soc. 127, 2022–2023.
deVroom, E., Broxterman, H.J., Sliedregt, L.A., vanderMarel, G.A., vanBoom, J.H., 1988. Nucleic Acids Res. 16, 4607–4620.
Di Giusto, D.A., Wlassoff, W.A., Gooding, J.J., Messerle, B.A., King, G.C., 2005. Nucleic Acids Res. 33, e64.
Ding, C.F., Ge, Y., Zhang, S.S., 2010-5-2. Chem. Euro. J. (chem.201001173).
Fischer, N.O., Tarasow, T.M., Tok, J.B., 2008. Anal. Biochem. 373, 121–128.
Frieden, M., Pedroso, E., Kool, E.T., 1999. Angew. Chem. Int. Ed. 38, 3654–3657.
Jarvius, J., Melin, J., Goransson, J., Stenberg, J., Fredriksson, S., Gonzalez-Rey, C., Bertilsson, S., Nilsson, M., 2006. Nature 3, 725–727.
Li, X.M., Liu, J.m., Zhang, S.S., 2010a. Chem. Commun. 46, 595–597.
Li, X.M., Xia, J.P., Li, W., Zhang, S.S., 2010b. Chem. -Asian 5 (2), 294–300.
Liu, J., Lu, Y., 2006. Angew. Chem. 118, 96–100.
Nallur, G., Luo, C., Fang, L., Cooley, S., Dave, V., Lambert, J., Kukanskis, K., Kingsmore, S., Lasken, R., Schweitzer, B., 2001. Nucleic Acids Res. 29, e118.
Nilsson, M., Dahl, F., Larsson, C., Gullberg, M., Stenberg, J., 2006. Trends Biotechnol. 24, 83–88.
Nilsson, M., 2006. Histochem. Cell Biol. 126, 159–164.
Nilsson, M., Gullberg, M., Dahl, F., Szuhai, K., Raap, A.K., 2002. Nucleic Acids Res. 30, e66.
Ren, R., Leng, C.C., Zhang, S.S., 2010. Chem. Commun. 46, doi:10.1039/C002466J.
Schweitzer, B., King-smore, S., 2001. Curr. Opin. Biotechnol. 12, 21–27.
Schweitzer, B., Roberts, S., Grimwade, B., Shao, W., Wang, M., Fu, Q., Shu, Q., Laroche, I., Zhou, Z., Tchernev, V.T., Christiansen, J., Velleca, M., Kingsmore, S.F., 2002. Nat. Biotechnol. 20, 359–365.
Shlyahovsky, B., Li, D., Weizmann, Y., Nowarski, R., Kotler, M., Willner, I., 2007. J. Am. Chem. Soc. 129, 3814–3815.
Smolina, I.V., Demidov, V.V., Cantor, C.R., Broude, N.E., 2004. Anal. Biochem. 335 (2), 326–329.
Stojanovic, D.W., 2001. J. Am. Chem. Soc. 123, 4928–4931.
Stojanovic, M.N., Landry, D.W., 2002. J. Am. Chem. Soc. 124, 9678–9679.
Yu, R.Q., He, J.L., Wu, Z.S., Zhou, H., Wang, H.Q., Jiang, J.H., Shen, G.L., 2010. Anal. Chem. 82, 1358–1364.
Zhao, W., Ali, M.M., Michael, A., Brook, Li, Y.F., 2008. Angew. Chem. Int. Ed. 47, 6330–6337.
Zhang, S.S., Xia, J.P., Li, X.M., 2008. Anal. Chem. 80 (22), 8382–8388.