A fluorescent sensor based on RAFT polymerization for the early diagnosis of non-small cell lung cancer

Wenwen Liu,* Ligang Ma,** Zhuangzhuang Guo,** Tao Liu,** Yanju Liu,**† Dazhong Wang,*† Jinming Kong***†

*Outpatient Integrated Systems Division, People's Hospital of Zhengzhou, 33 Yellow River Road, Zhengzhou 450053, P. R. China

**Department of Mathematical and Chemical, Pharmacy College, Henan University of Chinese Medicine, Zhengzhou 450046, P. R. China

***School of Environmental and Biological Engineering, Nanjing University of Science and Technology, Nanjing 210094, P. R. China

†To whom correspondence should be addressed.
E-mail: Liuyanju886@hactcm.edu.cn (Yj. L.); wdzlion_fox@163.com (Dz. W.); j.kong@njust.edu.cn (Jm. K.)
Abstract

We proposed a novel, ultrasensitive and low-cost sensor using reversible addition-fragmentation chain transfer (RAFT) polymerization as a signal amplification strategy for the detection of CYFRA 21-1 DNA fragment, a tumor marker of non-small cell lung carcinoma. The peptide nucleic acid (PNA) probes were firstly immobilized on magnetic beads (MBs) to capture the CYFRA 21-1 DNA specifically. After hybridization, CPAD was tethered to the hetero duplexes through the carboxylate-Zr⁴⁺-phosphate chemistry. Subsequently, a number of fluorescent tags were introduced to the heteroduplexes through RAFT polymerization, leading to an amplification of the fluorescence signal. The sensor demonstrates a low limit of detection (LOD) of 0.02 fM. It has great selectivity with respect to base mismatch DNA, and high anti-interference ability in the normal human serum. Overall findings of the study suggest that proposed sensor holds enormous potential to be used as a tool for the early-stage diagnosis of the lung cancers.

Key words: CYFRA 21-1 DNA, RAFT, fluorescence, non-small cell lung carcinomas
Introduction

Lung cancer, including non-small cell lung carcinomas (NSCLC) and small cell lung cancer (SCLC), has been reported to have highest incidence in the world. NSCLC contributes approximately 80% of total lung cancer patients along and has a five-year disease-free survival rate below 50-70%.\textsuperscript{1,2} Early screening or diagnosis has been shown to have a positive influence at the survival rates and the treatment outcomes.\textsuperscript{3} Unfortunately, existing diagnostic techniques such as chest x-ray, magnetic resonance imaging, and flow cytometry suffer from the well-known drawbacks in terms of being invasive, time-consuming and dependent upon expert operators.\textsuperscript{4,5} Fast, ultrasensitive detection of novel tumor markers such as circulating DNAs and proteins can provide a novel route for the early diagnosis.\textsuperscript{6} These biomarkers have been shown to be suitable for molecular recognition sensors (eg., nucleic acid hybridizations, and antigen-antibody interactions).\textsuperscript{7} CYFRA 21-1 DNA segment has been projected as an exclusive biomarker of NSCLC.\textsuperscript{8,9} Hence, having a sensitive, convenient, and selective sensor for the detection of CYFRA 21-1 DNA segment could contribute immensely to the early screening/diagnosis of NSCLC.

With attributes such as high sensitivity, stability, simple operating principles, and lower cost, the sensor technology has contributed immensely to the fields of clinical diagnosis, food safety, environmental monitoring and many other areas.\textsuperscript{10-13} The remarkable flexibility in sensor design is reflected by its interdisciplinary nature combining with electrochemistry, quartz crystal microbalance (QCM), electroluminescence, fluorescence, surface plasmon resonance (SPR) and other technologies.\textsuperscript{14-17} Especially, the fluorescent sensors are gaining great attention by virtue of their high sensitivity, non-destructive nature, rapid, and the ability
for real-time monitoring.

The pursuit of higher detection sensitivity has allowed integration of signal amplification strategies in different sensing platforms. Diverse signal amplification strategies such as controlled radical polymerization (CRP), hybridization chain reaction, rolling circle amplification, ligase chain reaction, as well as nanomaterials-based signal amplification strategy, had been applied in sensing for the detection of sequence-specific DNA or protein.\textsuperscript{18-23} Sensors based upon controlled radical polymerization as signal amplification strategies not only have the advantages of broad reactive monomers and controllable reactions,\textsuperscript{24} but they are also more convenient and inexpensive with respect to a sensor utilizing nanomaterials and enzymes as components for signal amplification.\textsuperscript{25} Classical CRPs such as atom transfer radical polymerization (ATRP) and reversible addition-fragmentation chain transfer (RAFT) polymerization, have been used widely for the detection of sequence-specific DNA in recent time.\textsuperscript{26,27} Kong and co-workers have proposed various kinds of electrochemical sensor based on RAFT polymerization or ATRP polymerization for detection of sequence-specific DNAs and suggested that RAFT has better bio-compatibility as transition-metal, Cu\textsuperscript{2+} of ATRP can cause toxicity.\textsuperscript{28,29} Because RAFT polymerization is harmless to biomolecules\textsuperscript{30}, it has great application prospects as a simple and effective signal amplification technique for biosensing for biomolecule detection. However, fluorescence detection DNA based on RAFT polymerization signal amplification has not been reported yet.

In this article, we demonstrate the fabrication of an ultrasensitive and inexpensive fluorescent sensor using RAFT as a signal amplification strategy for the CYFRA 21-1 DNA detection. The fabrication procedures of the proposed sensor are illustrated in Scheme.1.
Thiolated peptide nucleic acids (PNA) are connected to the surface of aminated magnetic beads (MBs) through SSMCC for capturing CYFRA 21-1 segment. After PNA and tDNA hybridization, the dithiobenzoates, 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPAD), which is the chain-transfer agent (CTAs), are connected to PNA/DNA heteroduplexes through the bridge of phosphate-Zr\(^{4+}\)-carboxylate chemistry. Subsequently, in the presence of FA as the monomer of RAFT and VA-044 (the azo free-radical initiator of RAFT), abundant fluorescence tags are labeled on the PNA/DNA heteroduplexes, which extremely amplified detection signal, leading to distinct improvement of sensor sensitivity. In addition, the sensor shows great selectivity for even single base mismatched ssDNA, and outstanding anti-interference ability in the 5% normal human serum samples, revealing tremendous application prospect in the early diagnosis of lung cancer.

Scheme. 1

**Experimental**

**Reagents and chemicals**

Aminated magnetic beads (MBs, 10 mg mL\(^{-1}\)) solutions were purchased from PuriMag Biotech Co. Ltd. (Xiamen, China). 4-(N-Maleimidomethyl)cyclohexane-1-carboxylic acid, 3-sulfo-N-hydroxysuccinimide ester sodium salt (sulfo-SMCC or SSMCC), zirconium dichloride oxide octahydrate (ZrOCl\(_2\)) and N, N-Dimethylformamide (DMF) were ordered from J&K Scientific Ltd. (Beijing, China). 4-cyano-4-(phenylcarbonothioylthio)-pentanoic acid (CPAD) was collected from Aladdin Co., Ltd. (Shanghai, China). Fluorescein-o-acrylate
(FA) was bought from Sigma-Aldrich (St. Louis, USA). 2,2′-azobis[2-(2-imidazoline-2-yl)propane] dihydrochloride (VA-044) was brought from TCI Development Co., Ltd. (Shanghai, China). Other chemicals and reagents were of analytical grade or higher and ultrapure water was used in all experiments. PBST buffer (0.1 M phosphate buffered saline, 0.05% Tween-20, pH 7.4) was prepared and used as washing buffer in the assays. Normal human serum (NHS) and thiolated peptide nucleic acid (PNA) were obtained from Shanghai YiJi Industrial Company, Ltd. (Shanghai, China) and Panagene Inc. (South Korea), respectively. All DNA fragments were bought from Sangon Biotechnology Co., Ltd. (Shanghai, China). Analyzed DNA sequences are shown in Table S1.

Apparatus

Fluorescence experiments were performed on an FLS-1000 fluorescence spectrophotometer (Edinburgh, UK). All modification processes of magnetic beads were conducted on a constant temperature shaker (Sigma, USA). Energy dispersive X-ray spectroscopy (EDS) was performed on a Quanta 250 field-emission scanning electron microscope (FEI, Hillsboro, OR). The modified MBs were observed by a FV1200 confocal microscope (Olympus, Japan).

Experimental Methods

20 μL MBs were washed thrice before resuspending them in PBST buffer (180 μL). Sulfo-SMCC (20 μL, 0.5 mM) was added later and incubated for 2 hours at 37 ℃ with gentle shaking. To remove the remaining reaction solution, MBs-SSMCC complex was magnetically separated and washed with PBST buffer and, then distributed in 190 μL PBST. Subsequently,
PNA (10 μL, 0.5 μM) was added and the reaction was performed at 37°C overnight for obtaining the PNA coated MBs (MBs/SSMCC/PNA).31,32

The prepared MBs/SSMCC/PNA was incubated with the tDNA (20 μL, 0.1 nM) in PBST buffer (180 μL, pH 7.4) for 1.5 hours at 37 °C. After washing with PBST buffer and magnetically separated, the MBs/SSMCC/PNA/tDNA was formed. Following the ZrOCl₂ solution (20 μL, 5 mM, prepared with 15% EtOH) was added and the reaction was further conducted for 30 minutes at 37 °C. Further, after washing, the MBs suspended in 180 μL PBST interacted with CPAD solution (20 μL, 0.5 mM) under similar conditions.

MBs/SSMCC/PNA/tDNA/Zr⁴⁺/CPAD generated from the above reaction were washed with PBST buffer (0.1 M, pH 7.4) and magnetically separated. The FA solution (10 μL, 10 mM), VA-044 (20 μL, 40 μM), and 15% DMF (170 μL) were added respectively and the reaction was performed using a constant temperature shaker at 47 °C for 2 hours.

The above-mentioned solution was magnetically separated, washed adequately, and finally resuspended in 3000 μL PBST buffer (0.1 M, pH 7.4) as a sample. The amplified fluorescent signal was acquired using Fluoro-spectrophotometer at an excitation wavelength of 489 nm, emission at 512 nm and a slit-width of 2 nm.

**Results and discussion**

**Feasibility study**

The modified MBs were observed by confocal microscope. As shown in Fig. 1, No obvious fluorescence was observed on the PNA/Zr⁴⁺/CPAD/FA modified MBs and obvious
green fluorescence could be observed on the MBs/PNA/DNA/Zr\textsuperscript{4+}/CPAD/FA, due to RAFT polymerization triggered on the surface of MBs. Next, we used energy dispersive X-ray spectroscopy (EDS) to track the elemental mappings of the modified MBs. It is necessary to understand the element composition of each part of the sensor before discussing the EDS results. The SSMCC, PNA, and CPAD mainly consisted of C, N, O, H and S, tDNA is made up of C, H, O, N and P, FA has C, H and O, and Zr\textsuperscript{4+} is composed of Zr. EDS images shown in Fig 1C and Fig 1D further corroborate this observation as P and Zr, the two unique elements in DNA and Zr\textsuperscript{4+} can be clearly seen. Overall these preliminary investigations revealed a successful sensor’s fabrication process, that is, the RAFT process was carried out successfully.

Fig. 1

Further to verify the feasibility of employing RAFT polymerization as a signal amplification strategy for the fluorescence detection of CYFRA21-1, the emission spectra of MBs at different modification steps were acquired in Fig. 2. Lowest emission intensity was observed in MBs added only with PBS buffer (curve h) and without added FA (curve g). Also, in the absence of PNA (curve e), tDNA (curve f), Zr\textsuperscript{4+} (curve d), CPAD (curve b) and VA-044 (curve c) weak fluorescence signals were observed. A strong fluorescence emission peak was observed in SSMCC/PNA/tDNA/Zr\textsuperscript{4+}/CPAD/VA-044/P(FA)-modified MBs (curve a) even after being washed multiple times. This can be primarily attributed to the accumulation of FAs onto MBs due to RAFT polymerization. High signal-to-noise (S/N) ratio also suggests high sensitivity for CYFRA21-1. These results strongly support that the strategy
based on RAFT amplified signal can be successfully applied to the detection of CYFRA21-1.

**Fig. 2**

*Principle of signal amplification strategy using RAFT strategy*

In this work, we have used RAFT-Polymerization as an amplification strategy to improve the sensitivity of the proposed sensor. The detailed principle of RAFT strategy is shown in Fig 3. It can be described in the following steps: (1) At the 43 °C temperature two C-N bonds of VA-044 are heated decomposition to produce alkyl radicals (I·) and N₂. (2) The fluorescence monomers (i.e., FA) react with alkyl radicals to form oligomeric radicals (Pn·). (3) Owing to the high activity of C=S bond of CPAD (a), oligomeric radicals react with them to generate the radical intermediates (b), these not only can split back to the original CPAD and the Pn· radicals, but can also split to the surface-tethered reinitiating radicals (c) and the dormant chains (d). (4) Polymer chain radicals (Pm·) continually grow by the surface-tethered reinitiating radicals (c) by repeatedly reacting with FA monomers. (5) Pm· during grow process react with the dormant chains (d) to produce the radical intermediates (e). Similarly, these can split back to Pm· radicals and the dormant chains or split to thiocarbonylthio-group-capped polymer chain and Pn· radicals. As the reaction in step (4) and step (5) occurs repeatedly, a great deal of FA monomers can be introduced into CPAD sites in the PNA/DNA heteroduplexes resulting in an enhancement of the fluorescence signal.33,34

**Fig. 3**

*Effect of the reaction time of RAFT and concentration of VA-044 of RAFT*
The results of condition optimization are detailed in supporting information.

**Fluorescence detection of CYFRA21-1 DNA**

To prove that the sensor can be used for quantification of tDNA, the fluorescence response of CYFRA21-1 DNA at various concentrations were detected under optimized conditions. Gradual enhancement in the fluorescence signal gradually increased with the increasing of the concentration of tDNA can be clearly seen from Fig 4A. A linear relationship between fluorescence signal change and tDNA concentration in the range of 1 fM to 1 nM was observed. This relationship can be described by the following equation

\[ F=2334 \log(C_{tDNA}) +16407 \quad (R^2=0.9985) \]

and the limit of detection is 0.02 fM (Fig 4B). Compared to existing DNA detection methods (table S2), the proposed sensor has a broad linear range and a low limit of detection, along with a simple fabrication procedure.

![Fig. 4](image)

**Selectivity, Reproducibility and Anti-interference Capability**

The selectivity of the sensor was evaluated by computing the fluorescent intensity of the tDNA along with three different mismatched ssDNA. This included single base mismatched ssDNA (SBM), three bases mismatched ssDNA (TBM), and control mismatched DNA (CON). As shown in Fig. S3, the mismatched DNA has lower fluorescence signal intensity as compared to tDNA. These values were quantified and found to be 28.6% (SBM), 21.3% (TBM) and 15.8% (CON) of the tDNA intensity. These findings suggest an excellent selectivity of the proposed sensor, majorly due to the high specificity and affinity of the PNA.
probe to the tDNA.

We have further studied the reproducibility of sensors. Under 1 nM tDNA conditions, the fluorescence intensity coefficient of variation obtained from five parallel experiments was 7.1%. It shows that this sensor has good reproducibility.

The desired sensor needs to perform well in a complicated and uncertain media such as real body fluid samples. Therefore, we have conducted an assay to evaluate the anti-interference efficacy of the sensor in diluted human serum. The serum samples were composed of 5% NHS and a certain concentration of tDNA along with PBS (0.1 M, PH 7.4). The control experiments were performed in PBS. As shown in Fig. S4, as compared to PBS, the fluorescence signal intensity of serum samples was relatively lower. The fluorescence intensity of 5% NHS samples with respect to PBS at 1 nM, 10 pM, and 1 fM tDNA concentrations were 89.59%, 90.46% and 98.09%, respectively. The decrease in fluorescence intensity may be due to the complex serum environment affecting the hybridization efficiency of DNA. It can be concluded that the proposed fluorescence DNA sensor could work efficiently even in a complicated biological matrix. This further supports its potential utility in clinical bioanalysis.

**Conclusion**

In conclusion, we have demonstrated a novel, ultrasensitive and cost-effective fluorescent sensor based on RAFT as a signal amplification strategy for the detection of CYFRA 21-1 DNA segment. To the best of our knowledge, this is the first time that RAFT as a signal amplification strategy has been applied to a fluorescence sensor. On one hand, a great
deal of PNA probes can be fixed to the surface of MBs owing to the large specific surface area of nanomaterials. On the other hand, single CYFRA 21-1 DNA was labeled by numerous fluorescence monomer via RAFT. Thus, the sensor displays excellent in a broad liner range from 1 fM to 1 nM along with a low limit of detection of 0.02 fM. Moreover, RAFT is not only an extremely effective and simple signal amplification strategy, but it also reduces the fabricating cost of the sensor as it requires only inexpensive reaction regents. What's more, we also demonstrated high specificity of sensor and a favorable accuracy in the 5% NHS samples, suggesting a valuable application in the early diagnosis of the lung cancers.

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Supporting Information

The Supporting Information provides condition optimization, selectivity, anti-interference and other data (PDF). This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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**Figure Captions**

Scheme. 1 Schematic illustration of the fabrication procedures of the developed sensor.

Fig. 1 Confocal fluorescence figures of PNA/DNA/Zr⁴⁺/CPAD/FA modified MBs (A) and PNA/Zr⁴⁺/CPAD/FA modified MBs (B), EDS figures of PNA/DNA/Zr⁴⁺/CPAD/FA modified MBs (C and D).

Fig. 2 Fluorescence spectra of SSMCC/PNA/tDNA/Zr⁴⁺/CPAD/VA-044/P(FA) modified MBs (curve a) and in the absence of CPAD (curve b), VA-044 (curve c), Zr⁴⁺ (curve d), PNA (curve e), tDNA (curve f), FA (curve g), MBs in PBS (curve h). (the concentration of tDNA is 1 nM).

Fig. 3 The principle of signal amplification strategy through RAFT.

Fig. 4 (A) Effect of different tDNA concentrations on the fluorescence signal. The concentration is 1 fM, 10 fM, 100 fM, 1 pM, 10 pM, 100 pM and 1 nM, respectively. (B) A linear relationship between the fluorescence signal change and the logarithm of the concentration of tDNA. (error bars show the standard deviations, n = 3).
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Fig. 3 The principle of signal amplification strategy through RAFT

Fig. 4 (A) Effect of different tDNA concentrations on the fluorescence signal. The concentration is 1 fM, 10 fM, 100 fM, 1 pM, 10 pM, 100 pM and 1 nM, respectively. (B) A linear relationship between the fluorescence signal change and the logarithm of the concentration.
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