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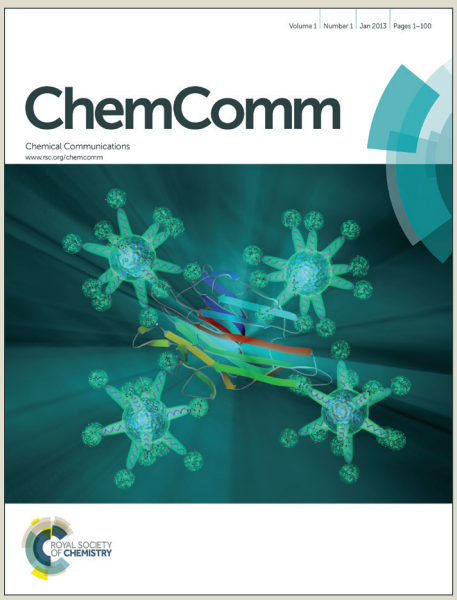

ROYAL SOCIETY OF CHEMISTRY

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Cite this: DOI: 10.1039/c0xx00000x

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ARTICLE TYPE

Detection of Telomerase on Upconversion Nanoparticle Modified Cellulose Paper

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

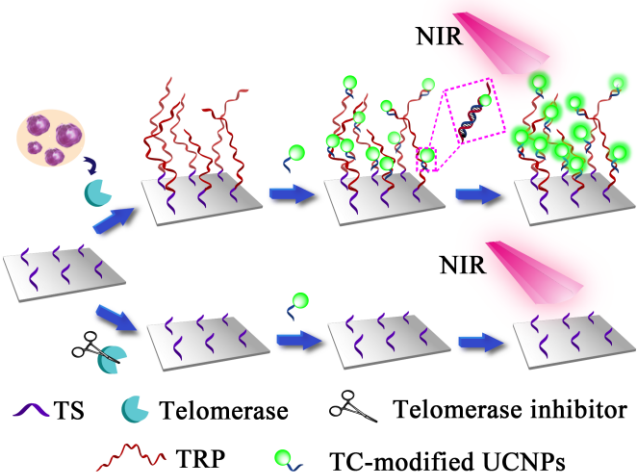
DOI: 10.1039/b000000x

Herein we report a convenient and sensitive method for detection of telomerase activity based on upconversion nanoparticles (UCNPs) modified cellulose paper. Compared with many solution-phase systems, this paper chip is more stable and easy to store the test results. What's more, the low background fluorescence of UCNPs increases the sensitivity of this method, and the low telomerase levels in different cell lines are clearly discriminated by naked eyes.

Telomeres are unique nucleic acids occurring at the ends of eukaryotic chromosomes, which are composed of constant repeat sequences $(TTAGGG)_n$. During cell division process telomeres protect chromosomes from degradation, recombination, and fusion by sacrificing themselves (about 50-200 nt per cycle).[1] This progressive shortening of telomeres leads to cellular senescence and apoptosis. Human telomerase, a ribonucleoprotein reverse transcriptase, catalyzes the addition of the telomeric repeats onto 3′-end of chromosomes.[2, 3] Overexpression of telomerase was found in a large majority of the known human tumors (85-90%), but not neighboring normal cells, which can prevent telomere from shortening during cell division and may be account for cancer cells' infinite life. The strong association between telomerase and cancer renders the enzyme a valuable target for early cancer diagnosis.[4]

Up to now, researchers have established various methods to analyze the activity of telomerase. The most common one is telomeric repeat amplification protocol (TRAP) based on polymerase chain reaction (PCR). Although quite sensitive, TRAP suffers the similar shortcomings of all PCR-related assays, for example, the limits of polymerase, laborious post-PCR processing and time consuming.[5] To overcome above disadvantages, some alternative PCR-free methods have been developed for the detection of telomerase activity, such as electrochemical strategy,[6-8] colorimetric method,[9-11] magnetic and fluorescence technique.[12-14] These efforts, however, mostly focused on solution-phase reactions, or needed expensive fluorescent labels and complex instruments. These solution-phase reactions are unstable and not practical for handling outside of the lab. The complex instruments are expensive and not suitable for point-of-care (POC) diagnostics, which is essential to on-site medical care for the prevention and control of serious diseases.[15]

In this context, we have developed a convenient and sensitive visual method for detection of telomerase activity based on cellulose paper and functionalized upconversion nanoparticles (UCNPs). Cellulose paper is an ideal material for bioassays. It can act as high-capillary-action matrix to keep small-volume substances and carry out biochemical reactions.[16, 17] So far, several paper-based biosensors have been developed in the sensing of glucose, nuclear acid and so on.[18, 19] There are some great advantages of paper bioassay systems, such as intrinsic filtering, biocompatibility, and ease of surface treatment. Compared with solution-phase based method, paper based sensors are simple, portable, disposable, and inexpensive, allowing us to run multiple bioassays simultaneously. Bioactive paper is easy-to-use product modified with biologically active chemicals. Therefore, the introduction of paper platform for detection of telomerase activity may be avoid of using the complex instruments and solve the stability problems.



Scheme 1 The illustration of our paper-based visual assay of telomerase activity coupled with TC-modified UCNPs.

However, previously reported paper-based detection methods utilizing quantum dots (QDs) and some fluorescent labels are limited due to the background fluorescence and light scatter from a sample matrix.[20-22] As the low concentration of telomerase in cells, choosing a probe with high signal-to-noise ratio for telomerase detection is necessary. Recently, UCNPs as an appealing new material has gained wide attention.[23-25] UCNPs are excited by NIR, avoiding generating any interfering bio-background luminescence. They do not suffer from size-dependent color and are resistant to photo-bleaching or biochemical degradation.[26, 27] UCNPs can be an ideal probe for

our paper-based telomerase detection system to suppress the background fluorescence and increase the sensitivity. This work utilizes UCNPs in telomerase detection for the first time.

Our assay was illustrated in Scheme 1. In the strategy, the telomerase substrate (TS) primer was modified on the surface of the sterile cellulose paper, which could be extended by telomerase (extracted from HEK-293T cells), and the products were denoted as telomerase reaction products (TRP). The telomere complementary oligonucleotides (TC) were modified on the surface of UCNPs (TC-UCNPs). The TC-oligonucleotides could hybridize with the TRP according the principle of complementary base pairing, so that the TC-UCNPs could be solidified on the paper. The signal intensity of the UCNPs on paper was related with the amounts of TRP, which reflected the activity of the telomerase. Besides, the results could be observed by naked eyes or recorded by a cell phone camera easily with the help of a portable NIR laser. In our design, this paper-based assay was portable, handy, low-cost and visual with the help of NIR laser. More importantly, this solid-phase system was more stable than most solution-phase methods and more practical for storing the test results. The application of UCNPs in our system successfully decrease interference and increase sensitivity owing to its innate advantages including no auto-fluorescence from samples and no photo-bleaching. We believe this platform was suitable for POC diagnostics in the clinic, private health in future.

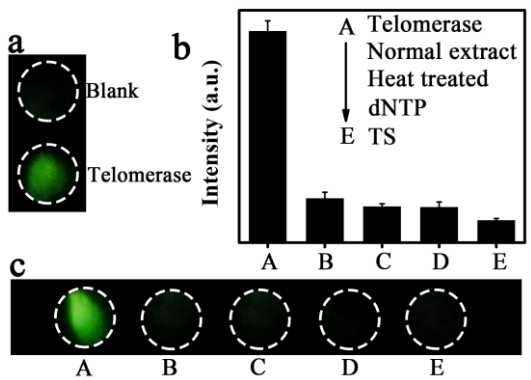


Fig. 1 a) The upconversion luminescence of the paper substrates: the enhanced fluorescence signal was observed by eyes on the substrate incubated with test solution (down) compared with the blank solution (up); b) The fluorescence intensity of substrate that incubated with the telomerase, normal cell extract, heat-treated telomerase, dNTPs, and TS. c) The photos of A to E in sequence.

In our experiments, Yb, Er doped upconversion nanophosphor ($NaYF_4$:Yb, Er) was prepared via the hydrothermal method according to the literature. In order to act with DNA, the UCNPs were modified with poly acrylic acid (PAA), denoted as PAA-UCNPs. (Details in Supporting information).[28, 29] The Fourier-transform infrared spectra (Figure S1) indicated that the PAA has been successfully introduced on the surface of UCNPs. The further characterization of the PAA-UCNPs was shown in Figure S2. After that, the amine-modified TC was conjugated to PAA-UCNPs via EDC-NHS mediated amide bond formation. The DNA-modified UCNPs probe was fabricated for telomerase activity detection, denoted as TC-UCNPs. To examine whether the DNA have been successfully attached on the surface of UCNPs, the UV-vis spectra of PAA-UCNPs and TC-UCNPs were measured (Figure S3a). Compared with the PAA-UCNPs, the TC-UCNPs exhibited a new absorption band centered at 260 nm, which was the absorption band of DNA. Besides, the Zeta Potentials of PAA-UCNPs and TC-UCNPs were studied (Figure S3b). After modification with DNA, the Zeta Potential of UCNPs decreased from -7.47 mV to -24.4 mV due to the negative charge of DNA.

Next, we carried out the construction of paper substrate. As illustrated in Scheme S2, the paper was dealt with periodate to oxidize the hydroxyl group of cellulose to aldehyde group for next reaction.[25] Afterwards, the chemically treated paper was sterilized and the amine-functionalized TS-oligonucleotides were immobilized on the paper through the formation of secondary amine. Then, the 5 μL 1 × TRAP buffer and 1 mM dNTP mixtures were dropped on the paper and lyophilized. When the telomerase samples were added to the paper, the TS would be elongated by telomerase.

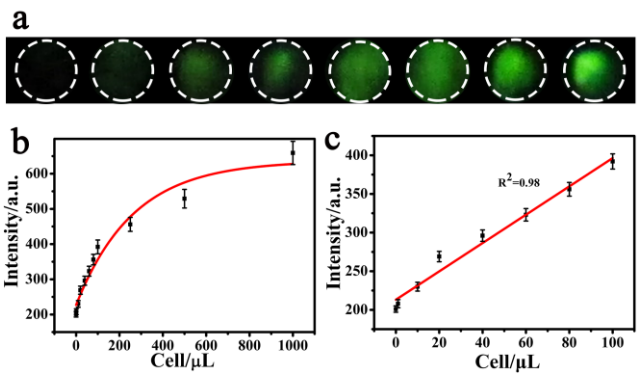


Fig. 2 a) Visual photo images of the paper substrate incubated with telomerase extract from different concentrations of HEK-293T cells: 0, 1, 10, 50, 100, 250, 500, 1000 cells/μL successively. b) The fluorescence intensity of the UCL spectra at 544 nm of the substrate incubated with telomerase extract from 0 to 1000 cell/μL. c) The linear relationship between fluorescence intensity of the UCL spectra at 544 nm and the concentration of telomerase.

To demonstrate the feasibility of our method, we designed one preliminary experiment. We added the test solution (extracted from 250 HEK-293T cells) and blank solution (H_2O) onto the constructed paper substrates. After incubated the substrates at 37 °C, the TC-UCNPs were introduced onto the paper through sequence-specific hybridization with TRP. Then, after a thoroughly washing with buffer, the upconversion luminescence (UCL) of the whole substrates were acquired with a 980 nm portable laser, reflecting the average level of each spot. Compare with blank solution, the substrate incubated with test solution emitted an obvious fluorescence signal (Figure 1a). The SEM images of the two paper substrates were shown in Figure S4. This result directly proved that many UCNPs were attached to the paper treated by telomerase. To further clarify the mechanism of fluorescence signal increment, a series of control experiments were designed. To this end, the heat-treated telomerase, dNTPs, and TS were separately incubated with the substrate. As shown in Figure 1b, the fluorescence intensity of these controls was at least 5 times low than that of the telomerase-treated ones. These results indicated that the heat-treated telomerase, dNTPs and TS had no influence on the signal of the paper substrate and the increased fluorescence signal was essentially related to the telomerase extension. In addition, the substrate was also incubated with the

sample extracted from normal cells, which also exhibited little fluorescence signal. The results agreed with previous study that telomerase was overexpressed in a large majority of cancer cells, but not normal cells. Taking the hybridization dynamics into consideration， previous studies have demonstrated that solid surface hybridization rates are usually about 20-fold slower than solution-phase rates for identical sequences and conditions. In general, surface suppression is likely caused by steric and electrostatic hindrance present in the DNA probe film as well as conformational restriction of the surface-tethered strands. Nevertheless, the hybridization in our system could complete owing to that our probe DNA was excessive and the 15 time was enough to reach the equilibrium. So the hybridization dynamics did not affect our results.[30]

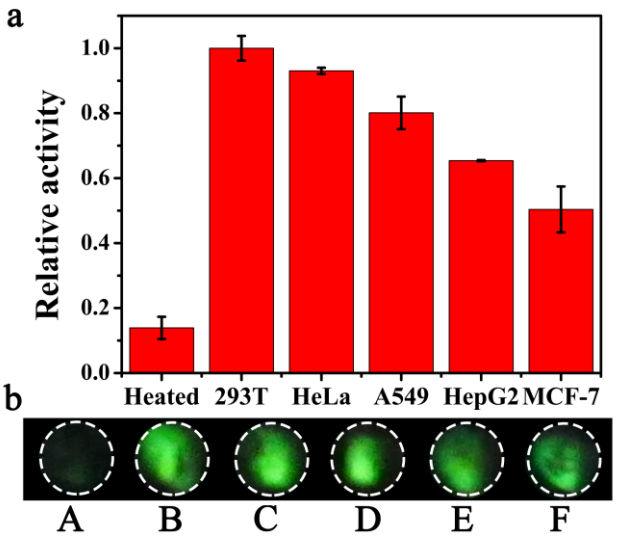


Fig. 3 The analysis of telomerase activities in different cell lines. a) The relative telomerase activity of each cell line and negative control. b) Visual photo images of the paper substrate incubated with telomerase extract from different cell lines and the negative control. A–F represent the heated-treated negative control, HEK-293T, HeLa, A549, HepG2, MCF-7, respectively.

We next tested telomerase activity in various concentrations using this method. In our design, TS was elongated by the different concentrations of telomerase, producing different amount of TRP. The fluorescent intensity of substrate was proportional to the amount of UCNPs that reacted with the TRP. In order to prove this, the telomerase extracted from different numbers of HEK-293T cells were incubated with the paper substrate. As shown in Figure 2a, with the 0 cell extract, negligible fluorescence was observed from the substrate. As the concentration of telomerase increased, the fluorescence of the substrates gradually enhanced. This phenomenon could be distinguished with naked eyes. Because of the low background signal by NIR excitation, this method had a low detection limit of 10 cells/μL by the naked eyes, which was enough for POC diagnostics. Furthermore, the fluorescence changes of the paper substrates were also monitored by the upconversion fluorescence spectrometer. As shown in Figure 2b, the emission intensity increased with the concentration of telomerase. The fluorescence at 544 nm exhibited a nearly linear dependence with the telomerase concentration in the range from 10 to 100 cells/μL (Figure 2c). With the aid of the fluorescence spectra, the limit of detection (LOD) could down to 1 cell/μL (Figure S5). The sensitivity is better than that of most previous PCR-free methods. Besides, the UCNPs based platform was extremely stable compared with many solution-phase systems. Such as, Au-based colorimetric system was easy to become invalid and restricted in the strict detection time. It was not convenient for long-term use and storage of test results.[10] For our system, even after one month, the fluorescence intensity was almost not changed and still could be discriminated by naked eyes (Figure S6).

When it came to evaluate our method in practical detection, we investigated the telomerase activities in five different kinds of cell lines and negetive control. The telomerase activity of each cell line was normalized according to HEK-293T cell. This result suggested that the telomerase activity in 293T and HeLa cells was higher than that in HepG2 and MCF-7 cells (Figure 3). This was in accordance with previous reports, which showed this method could discriminate the different telomerase levels in different cell lines.[6]

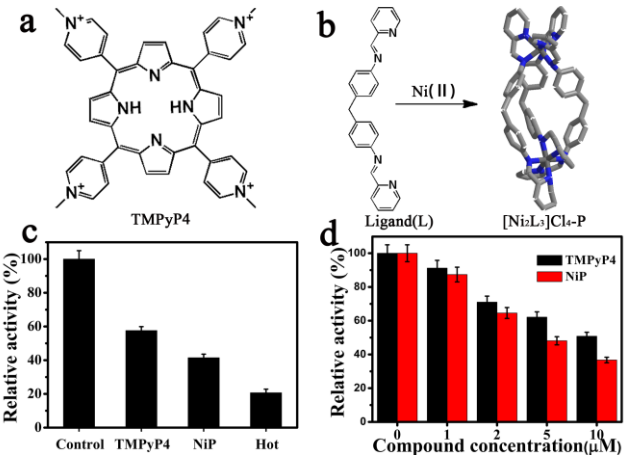


Fig. 4 a, b) The chemical structures of TMPyP4 and $[Ni_2L_3]Cl_4$-P. c) Assess the inhibition of telomerase activity by G-quadruplex ligand TMPyP4, $[Ni_2L_3]Cl_4$-P and heat with our assay. d) The inhibition effect of TMPyP4 and $[Ni_2L_3]Cl_4$-P at different concentrations.

To further verify the reliability of this method, we assessed telomerase inhibition experiments with this designed method. As mentioned above, telomerase played an important role on the infinite life of cancer cells. The agents which could suppress telomerase activity hold promise as potential anticancer agents. What's more, a complete endogenous RNA template and an unfolded single-stranded telomeric overhang are necessary for optimal telomerase activity.[31] The endogenous RNA template might be destroyed under certain external stimulations and the telomeric DNA could form G-quadruplex (an ineffective substrate for telomerase) in the special cationic atmosphere. Therefore, some compounds that can selectively bind to and stabilize telomeric G-quadruplex structures have strong inhibition effect of telomerase activity. Here, two compounds proved to inhibit telomerase activity were estimated by the method, $[Ni_2L_3]Cl_4$-P (L = $C_{25}H_{20}N_4$) and tetra-(N-methyl-4-pyridyl) porphyrin (TMPyP4). The strucutres of the two G-quadruplex compounds were shown in Figure 4a, b. The obvious decrease in the fluorescence intensity of substrate suggested that the telomerase activity was suppressed by TMPyP4, $[Ni_2L_3]Cl_4$-P and

heating (Figure 4c, d). From the results we could deduce that $[Ni_2L_3]Cl_4$-P (NiP) had stronger inhibitory activity of telomerase than TMPyP4. The IC_{50} values (half-maximal inhibitory concentration) for the two compounds were reported in Table S1. Considering that the TRAP assay overestimated the inhibiton effect of many quadruplex binding ligands,[32] our system was PCR-free and more reliable in initial screening of telomerase inhibitors.

In summary, we have constructed a paper-based platform with UCNPs as probe for detection of telomerase activity. The UCNPs can conjugate with the telomerase reaction products (TRP), and emit strong fluorescence when excited by NIR light. In our system, cellulose paper served as a high-capillary-action matrix readily stored and distributed these embedded materials. Compared with many solution-phase system, the solid-phase platform is extremely stable at room temperature and easy to store the test results. Further more, the less bio-background interferences and the higher photostability of UCNPs guarantee the sensitivity and reliability of our method. The telomerase levels in different cell lines could be be directly discriminated by naked eyes with this assay via NIR. Additionally, with the aviliable excitation source and avoiding using complex instruments, this method is easy to hand outside of the lab. Thus, we believe this ready-to-use paper-based telomerase detection assay will show great potential for clinical and POC testing in the future.

Acknowledgements

This work was supported by 973 Project (2011CB936004, 2012CB720602), and NSFC (21210002, 21431007, 91413111).

Notes and references

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† Electronic Supplementary Information (ESI) available: Experomental details, FT-IR spectra, TEM image, SEM image, UV-vis spectra, X-ray diffraction, upconversion luminescence spectra, visual photos of the substrate. See DOI: 10.1039/b000000x/

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