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TUTORIAL REVIEW

Assays for human telomerase activity: progress and prospects

Xiaoming Zhou and Da Xing*

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Human telomerase is a ribonucleoprotein complex that functions as a telomere terminal transferase by adding multiple TTAGGG hexamer repeats using its integral RNA as the template. There is a very strong association between telomerase activity and malignancy in nearly all types of cancer, suggesting that telomerase could be used not only as a diagnostic and prognostic marker but also as a therapeutic target for managing cancer. The significant progress in biomedical telomerase research has necessitated the development of new bioanalytical methods for the rapid, sensitive, and reliable detection of telomerase activity in a particular cell or clinical tissue and body fluids. In this review, we highlight some of the latest methods for identifying telomerase activity and inhibition and discuss some of the challenges for designing innovative telomerase assays. We also summarise the current technologies and speculate on future directions for telomerase testing.

1. Introduction

Quantitation-based biological science has undergone a revolution in the last two decades. Developments of new bioanalytical methods at the multidisciplinary interface have created tremendous opportunities for improving assay performance. Specifically, the study of telomerase, a continuously active research area, has stimulated enormous interest in the fields of biology, chemistry, medicine, materials science, and engineering, among others. Since the 1970s, unremitting efforts by the scientific community have led to great progress in understanding telomere biology. The demonstration of telomere later became important for the discovery of telomerase enzyme activity. Today, evidence from genetic association studies and functional analyses of telomerase have suggested an exciting potential link between telomerase activity and disease pathologies, such as cancer, age-related diseases, and premature ageing syndromes.[1,2] These fundamental findings have raised the possibility that telomerase could be used not only as a

MOE Key Laboratory of Laser Life Science & Institute of Laser Life Science, College of Biophotonics, South China Normal University, Guangzhou 510631, China. E-mail: xingda@scnu.edu.cn; Fax: +86 20 85216052; Tel: +86 20 85210089

Xiaoming Zhou

Xiaoming Zhou was born in Shaoyang, Hunan Province, China, in 1981. He graduated from Hunan University of Arts and Science, where he received his B.S. degree in life science in 2005. Then he started his PhD studies in the South China Normal University under the supervision of Prof. Da Xing, obtaining his PhD degree in 2010. He joined College of Biophotonics in SCNU in July 2010. His current research interests focus on the developments of new bioanalytical methods with an emphasis on gene probes and optical techniques for disease diagnosis, environmental monitoring, and food safety.

Da Xing

Da Xing received the Doctor degree in Engineering from Harbin Institute of Technology, China, and PhD in Physics from University of Electro-Communications, Japan, in 1989 and 1991. He joined the faculty of UEC from 1991 to 1995 with the Department of Electrical Engineering. He became the Director and professor of Institute of Laser Life Science, South China Normal University since 1996. He got Chinese Prime minister's Fund for Distinguished Young Scholars in 1997. He has published more than 300 peer-reviewed papers, and over 30 major invited talks of international conferences. His research activities include Biomolecular Spectroscopy, Noninvasive Functional Imaging and Biomolecular Sensing.

diagnostic and prognostic marker but also as a therapeutic target for managing cancer. However, it is still too early in the research process to know whether telomerase is the missing link between aging and cancer. The challenge is to learn how to exploit our increasing knowledge of telomere biology to diagnose and treat malignancies. Advances in assays for detecting telomerase will hopefully elucidate the relationship between telomerase and cancer.

The earliest developed telomerase activity measurements were performed in ciliated protozoans, as these model organisms express high levels of telomerase.[3] However, in humans, the abundance of telomerase is only approximately 100 molecules per cell, even in a telomerase-positive tumour cell.[4] Thus, early human telomerase assays required the use of large quantities of samples and radioactive material.[5] Amplification-based methods solved this problem but suffered from PCR-derived issues.[6] Because of continuing interest in telomerase in basic life science and medical science research, a more sensitive, simple, reliable method for detecting telomerase is a technological challenge that will also create new opportunities in cancer research. Currently, the goal of using telomerase as a common clinical cancer marker has not yet been achieved. Achieving this goal will encourage scientists to develop more robust assays for detecting telomerase to provide more reliable biomedical information. This objective has recently emerged as a focal point in the field. A Web of Science search revealed that approximately 500 papers related to telomerase activity are being published annually. Undoubtedly, the field of telomerase research would benefit from interdisciplinary and multidisciplinary collaboration. For example, physicists are developing novel optical tools for biosensors, engineers are crucial in the development of apparatuses such as point-of-care devices, materials scientists are creating a variety of nanoparticles that could streamline testing operations, and analytical chemists and medical doctors can contribute their wealth of experience to integrate the efforts of this diverse group of scientists.

Although there are no clinically-approved telomerase assays, several promising studies have been recently published. In this review, we will focus on the current state of telomerase assay development and will discuss on the questions that have arisen during this process to realise the potential of telomerase as a cancer biomarker. For the non-professional researchers who need to grasp the general structure and mechanisms of telomerase, we first briefly describe the biochemical properties of human telomerase. For discussion purposes, progress in telomerase assays is categorised into two sections: amplification-based assays and direct assays. Due to space constraints, we can only highlight the most important recent advances. Readers who desire further examples and references are directed to other published reviews.[7–9]

2. The basic biochemical properties of human telomerase

The 3′ end of linear duplex DNA cannot be fully copied by DNA polymerases, resulting in the shortening of the chromosomes with each cell doubling. This phenomenon has been called the ''end-replication problem''.[10–12] The mechanism behind a cell's ability to protect its chromosomal terminus from nucleolytic degradation and processing has been a long-standing puzzle for biologists. In the 1970s and 1980s, seminal observations were made by Elizabeth Blackburn, Carol Greider, and Jack Szostak, who won the Nobel Prize in Physiology or Medicine in 2009 for their fundamental discoveries in telomere and telomerase research.[13] These advances enabled scientists to understand that telomeres, which are located at the ends of eukaryotic chromosomes, are composed of repeated DNA sequences and protein assemblies and are essential for stabilising the ends of chromosomes. Telomerase, first identified in 1985 by Greider and Blackburn, is a ribonucleoprotein reverse transcriptase (RT).[3] Telomerase uses a template within its own RNA subunit to extend chromosome ends by synthesising single-stranded telomeric repeats, thus solving the ''end-replication problem''. Telomeres and telomerase were first identified in protozoan models, but were soon confirmed elsewhere, including human, yeast and plant cells.

Currently, the exact mechanism of telomerase recruitment is not fully known. The involvement of auxiliary proteins, such as dyskerin, in regulating the assembly, localisation and catalytic activity of telomerase is still controversial.[14] The core components of the human telomerase holoenzyme consist of a catalytic reverse transcriptase, hTERT (human telomerase reverse transcriptase), and an integral RNA (451 bp), hTR (human telomerase RNA).[15] Telomerase shares many properties of ''conventional'' reverse transcriptases but also exhibits some unique features. For example, telomerase's enzymatic activity is sensitive to ribonuclease and protease treatment, implicating both RNA and protein involvement in its enzymatic activity. Fig. 1 shows the model of human telomerase activity. First, telomerase binds to the single-stranded 3′ end of a chromosome (or a synthetic DNA oligonucleotide *in vitro*) through the RNA template. The 3′ end of the DNA forms a hybrid with the RNA template. Telomerase then catalyses the extension of the 3′ end to the end of the template region of the RNA molecule. After six nucleotides (GGTTAG) are sequentially added to the telomere or the primer, the telomerase enzyme complex translocates along the newly synthesised strand and extends the telomere further. A translocation step repositions the new 3′ end of the DNA strand within the template for a second round of telomere synthesis. Only one extension cycle is illustrated in this cartoon, but telomerase adds multiple repeats by moving to new binding sites along the newly synthesised strand.

Four years after the discovery of telomerase in *Tetrahymena* extracts, Morin identified a similar enzymatic activity in tumour-derived human HeLa cells.[5] Since then, the relationship between telomerase activity and tumours has attracted considerable scientific attention. Studies have indicated that telomerase activity is nearly universal in human cancer cell lines and is present in 85–90% of primary tumours.[16] However, telomerase activity is not detectable in most somatic cells, with the exception of some adult pluripotent stem cells that proliferate to renew tissues. Confirmation of the relationship between telomerase activity and cancer has prompted scientists to explore telomerase as a plausible target for cancer diagnosis and therapy.

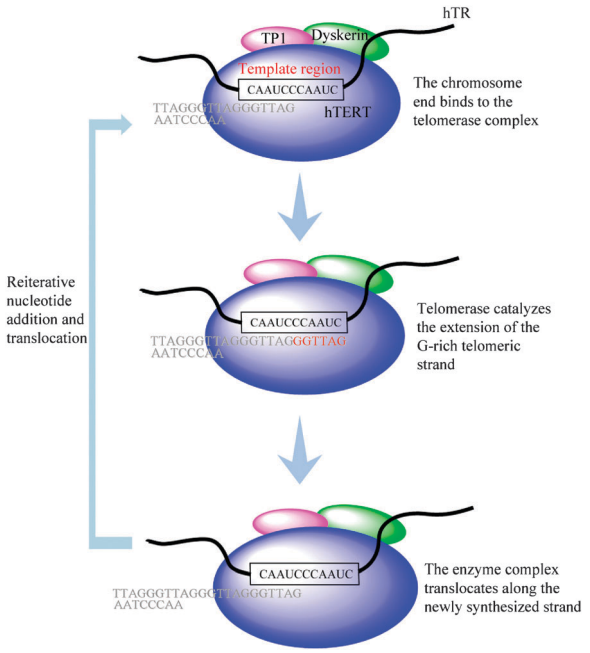

Fig. 1 A simplified model showing the mechanism of telomere extension by telomerase. Steps include the binding of the telomerase complex to the chromosomal end (or primer for *in vitro* telomerase assays), telomere extension catalysed by telomerase, and the translocation of telomerase for the next catalytic cycle.

3. Amplification-based methods for detecting telomerase activity

Based on the pioneering work of Blackburn and Greider, Morin firmly established the existence of telomerase in humans by analysing telomerase activity from tumour-derived human HeLa cells using a primer extension assay.[5] However, this assay had limited sensitivity for detecting telomerase. More sensitive techniques for identifying telomerase were required to rigorously establish telomerase expression patterns. In 1994, a telomere repeat amplification protocol (TRAP) assay was developed that could detect telomerase activity from small tissue biopsies that contained just a few cancer cells.[6] Briefly, a synthetic non-telomeric sequence was used as the substrate; the substrate was then elongated by the telomerase present in a sample, and the elongation products were then amplified by PCR. The original TRAP assay was time-consuming, not easily quantitated and subject to PCR-related artefacts. Additionally, TRAP methods require special attention to account for impurities in the telomerase lysate that may inhibit the TRAP reaction. To a certain extent, these problems have been solved by redesigning the primers, adding internal controls, and adopting end-point detection modes.[17–19] However, this type of analysis could be made more easy to use; there is still a need for a telomerase assay that is more flexible and more easily and quickly performed at low cost. In this section, we focus on the latest TRAP-based studies.

3.1 Advances in TRAP assay design

Understanding how human telomerase proceeds in telomere extension will help us understand the biochemical mechanisms of the telomerase reaction and the molecular mechanisms of potential telomerase inhibitors. Because of the staggered annealing of the primers, the products obtained from the original TRAP method may be longer or shorter than the actual telomerase extension products. Szatmari and Aradi developed a modified TRAP assay that can accurately reproduce the telomerase extension products length, thus indicating the enzyme's processivity.[20] This assay employed one forward primer (MTS) and two reverse primers (RPC3g and RP) for amplification. The forward primer (MTS) was used as the telomerase substrate. The reverse primer, RPC3g, contained a 20 nt tag sequence at its 5′-end, two dinucleotides (GG) at its 3′-end, and three telomeric repeats in the middle. In the first and second PCR cycles, a lower annealing temperature helped ensure that the telomerase products were extended only when the RPC3g primer hybridised with the telomerase products at the correct terminal position. The extended products were subjected to subsequent amplification cycles with an increased annealing temperature using the MTS and RP primer pairs. The amplified products directly reflected the size distribution of the telomerase extension products.

The single-stranded G-rich overhang of the 3′ end of human telomeres functions as the substrate for telomerase extension and can form a G-quadruplex structure in certain ionic environments.[21] Quadruplex ligands can specifically bind to and stabilise quadruplex-prone sequences that disrupt the hybridisation between the overhang and the RNA template of telomerase, thus impairing telomerase activity. G-quadruplex ligand-based telomerase inhibition may be feasible for further cancer management. TRAP and its modified versions have been widely used for measuring G-quadruplex ligand-based telomerase inhibition. Recent work established that the original TRAP is completely unsuitable for detecting telomerase inhibition by quadruplex ligands[22] because the G-quadruplex ligands inhibit not only telomerase activity but also PCR amplification.[22] It is difficult to distinguish the inhibitory effects of G-quadruplexes on Taq polymerase *versus* telomerase. Such problems can be solved by employing a three-step TRAP procedure, such as the one developed by Reed *et al.*[23] In this assay, an additional step removes the G-quadruplex ligand prior to PCR amplification. Although more complicated, this assay has enabled reliable and reproducible measurement of telomerase activity and telomerase inhibitor screening.

The traditional TRAP assay also has less-than-satisfactory specificity. Specifically, primer-dimers are difficult to eradicate, and impurities from high concentrations of cell lysates and tissue samples usually inhibit not only the activity of Taq polymerase but also telomerase. Nanotechnology can help solve some technological and scientific problems encountered by biologists. The specificity and efficiency of TRAP were greatly improved by the use of gold nanoparticles (AuNPs) to develop an AuNP-assisted TRAP method by Xiao *et al.*[24] This method is similar to the traditional TRAP assay but replaces the TS primer with a AuNP-modified TS primer (see Fig. 2A). AuNP-modified TS primers significantly improved the selectivity of the TRAP method. Amplification of telomerase products with this strategy significantly reduced primer-dimer products. Furthermore, telomerase activity was easily detected from 50 telomerase-positive cells, even when mixed with 5000 telomerase-negative somatic cells (Fig. 2B). Although the mechanism by which AuNP-assisted TRAP enhances specificity and efficiency is not entirely clear,

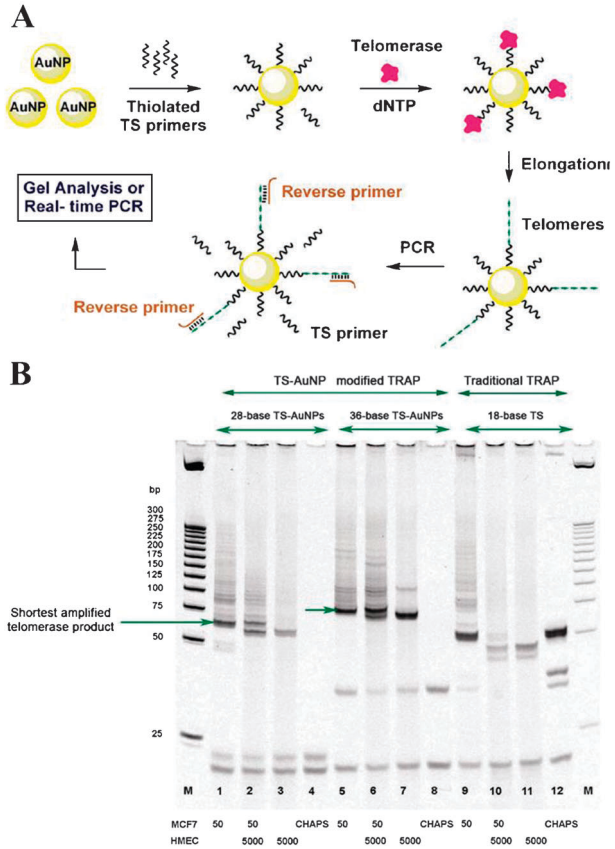

Fig. 2 AuNP-modified primer-based TRAP assay. (A) TS primer covalently attaching to AuNPs through Au–S bond linkage. The resulting AuNP-modified primer serves as the substrate for telomerase extension. The extended products are then amplified on the AuNPs using a reverse primer. (B) Comparative analysis of the products from an AuNP-based TRAP assay and the traditional TRAP method using PAGE. Lanes 1 to 8 show that both 28-base and 36-base TS-AuNPs worked well for detecting telomerase activity from 50 MCF7 cells and 50 MCF7 cells doped with the extract of 5000 HMEC cells. By contrast, the traditional TRAP assay only detected weak telomerase activity from 50 MCF7 cells and was not capable of detecting 50 MCF7 cells doped with the extract of 5000 HMEC cells (Lanes 9 to 12). Reprinted from ref. 24 with permission by the American Chemical Society.

strong adsorption of proteins from the lysate to the AuNPs, which also have a very high ratio of surface area to volume, may play a key role.

3.2 Capillary electrophoresis-TRAP

TRAP assay has become the most frequently used tool for telomerase analysis due its ultra-high sensitivity. However, separating the products by gel electrophoresis and evaluating them by phosphorimagery or densitometry make conventional TRAP laborious and time-consuming. Capillary electrophoresis (CE) is a preferable alternative that can effectively replace gel electrophoresis in TRAP analysis. Compared to gel electrophoresis, CE not only provides much higher throughput and sensitivity but also offers greater reproducibility and full method automation. Atha and co-workers were the first to report CE analysis of human lung cancer cell telomerase activity with laser-induced fluorescence (LIF) detection.[25] CE-TRAP readily detected only 5 to 15 cells, indicating a very high sensitivity. Moreover, this modified assay was complete in only 2 h. In addition to its excellent reproducibility, the CE method was also more accurate than the slab-gel method (CV = 20% *vs.* 35%).[26] Using a single-photon detection-based CE instrument, Kabotyanski *et al.* showed that CE–LIF had a 100- to 1000-fold increased sensitivity over real-time TRAP.[27] Zhelev *et al.* developed a high-throughput telomerase assay by combining a modified TRAP assay with microchip CE.[28] This analysis of telomerase activity requires only approximately 2 min and 1 μL samples. Significantly, due to its super-high resolution, this method can separate the TRAP 6 bp ladder products, enabling the comparison of telomerase activity in samples with small differences in cell concentration.

3.3 Quantitative TRAP detection

An important improvement in telomerase measurement was the development of a real-time quantitative TRAP (Q-TRAP) assay. The advantages of this assay include single-step detection and quantification, increased throughput, and reduced carry-over contamination. The first Q-TRAP assay was based on the ability of SYBR Green I to bind to double-stranded amplicons and emit a fluorescent signal.[29] SYBR Green I-based methods are convenient and cost-effective and can detect telomerase activity in single-cell samples.[30] However, this assay suffers from non-specific amplification because SYBR Green I is a non-sequence-specific fluorescent intercalating agent. Non-specific amplification and primer-dimer complexation may lead to a strong background fluorescent signal. This issue can be partially solved by optimising the primer concentration and annealing temperature.[31] Fajkus and Elmore *et al.* have developed more reliable Q-TRAP for detecting telomerase activity in cultured cells and clinical samples that is based on a fluorescence-quenched hairpin primer and co-amplification of an internal control.[32,33] The use of two spectrally distinct fluorophores to label hairpin primers allows simultaneous detection of the telomerase products and the internal control. However, in this assay fluorescent signals strictly depend on the primer specificity. Further elimination of false-negative results has been achieved by other Q-TRAP technologies that involve the use of molecular beacon probes for targeting the amplified products.[34]

Q-TRAP is still limited in its ability to detect telomerase activity from rare tumour cells in body fluids, such as whole-blood samples. One strategy to overcome this limitation is to enrich the cell of interest before telomerase analysis. Xu *et al.* designed a microfilter purification chip to concentrate rare circulating tumour cells before Q-TRAP detection.[35] To capture cancer cells from blood samples, a parylene-C slot microfilter was constructed based on etching technology, with an array of 30 401 slot openings. To enrich circulating tumour cells based on size exclusion, the slot widths were designed to be smaller than the cancer cell diameter because circulating tumour cells are larger and less deformable than normal blood cells. Whole-blood samples containing cancer cells can be driven to the slot microfiltration area by adjusting the pressure from a nitrogen tank. The processing of 1 mL of whole blood can be completed in less than 5 minutes with 90% capture efficiency, 90% cell viability, and 200-fold sample enrichment. Single-cell analysis of

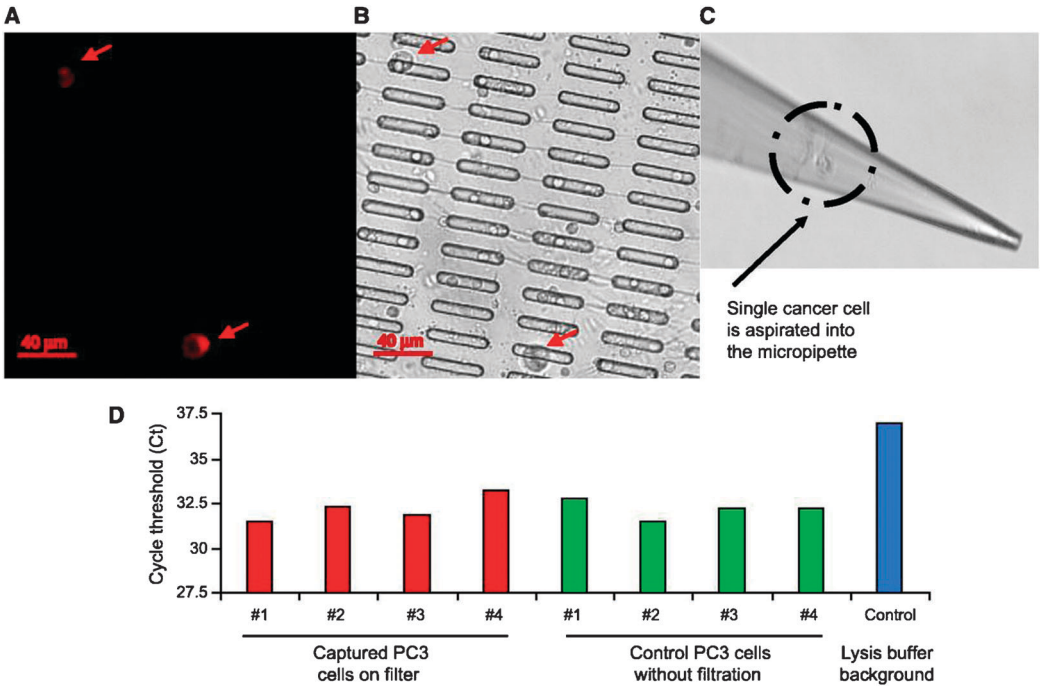

Fig. 3 Analysis of telomerase activity from single live cancer cells based on the microfilter technique. (A) The red spot showing the stained captured cells. (B) Bright-field image of the captured cells. (C) Recovery of a single cell by micropipette. (D) Single-cell telomerase activity assay using qPCR-TRAP. Reprinted by permission from the American Association for Cancer Research.[35]

live-captured cells can be reliably executed by combining the high efficiency of the capture platform with the highly sensitive Q-TRAP detection, as shown in Fig. 3. For example, PC3 cancer cells captured from whole blood on microfilters were identified by immunofluorescent staining (PE-conjugated anti-CD49 antibody) or matched bright-field imaging and then recovered by a micropipette and subjected to Q-TRAP analysis. The Ct values of the Q-TRAP data in Fig. 3D show significantly elevated telomerase activity relative to the negative controls. In another example, cancer cells were isolated and enriched by immunomagnetic separation using monoclonal antibodies against cancer cell surface antigens. Q-TRAP analysis detected telomerase activity in exfoliated cells from the urine of 56 patients with 100% assay sensitivity.[36]

3.4 Other biosensors

Over the past few years, various types of biosensors for post-TRAP detection have been developed to enhance sensitivity and avoid the time-consuming and hazardous gel electrophoresis manipulations.[37] Using the specific dsDNA-binding fluorescent dye PicoGreen to quantify TRAP products, Francis and Friedman developed a label-free, high-throughput fluorescent assay for analysing telomerase activity and its inhibitors.[38] Prior to amplification, a magnetic washing step was used to scavenge Taq polymerase inhibitors. The washing step and the PCR amplification were executed in the same 96-well streptavidin-coated PCR plate. Thus, parallel analysis of 96 compounds for potential telomerase inhibition could be assessed in 3–5 h. A hybridisation protection assay (HPA) has been developed as an effective alternative to gel electrophoresis and other traditional methods for highly sensitive detection of telomerase activity.[39] TRAP-HPA uses an acridinium ester-labelled probe that can be hydrolysed when hybridised to the TRAP products, permitting the homogeneous determination of amplification products in a few minutes. TRAP-HPA was 1000-fold more sensitive than the conventional TRAP assay.

Because telomeric repeats contain half the amount of guanine bases, a label-free electrochemical assay based on the guanine oxidation signal was developed to measure telomerase.[40] In this assay, telomeric repeat-containing PCR products were immobilised onto carbon–lead surfaces by wet adsorption. Guanine oxidation was measured by using differential pulse voltammetry (DPV). Telomerase activity from cell extracts containing as low as 100 ng μL^{-1} protein could be detected. Compared to electrochemical techniques, the major advantage of electrochemiluminescence (ECL) analysis is the absence of a background signal. Recently, we developed a novel approach that combines a modified TRAP assay and a magnetic bead-based ECL platform for the ultrasensitive detection of telomerase activity.[41] In this strategy, the telomerase extension products were amplified using a biotinylated forward primer and a tris(2,2-bipyridine)ruthenium(II)-labelled reverse primer. The amplified products were linked to the surface of streptavidin-coated magnetic beads through the biotin–streptavidin interaction. The TRAP products were quantitated by measuring the ECL signal generated from the electrochemical reaction of tris(2,2-bipyridine)ruthenium(II) and tripropylamine. This assay is highly sensitive because it can determine telomerase activity from single cells.

4. Direct analysis of telomerase activity

Direct in this case means the direct detection of telomerase activity without the need of enzymatic amplification of telomerase

extension products prior to analysis. The tremendous recent progress in developing scientific instrumentation and probe techniques has enabled the development of a number of direct detection methods for telomerase activity. Currently, there are a handful of reports that demonstrate direct detection of telomerase activity with sensitivities comparable or slightly lower than amplification-based methods. In this section, we focus on some of the latest developments aimed at avoiding the problems associated with amplification-based TRAP methods.

4.1 Telomerase purification

The first direct primer extension method used a telomeric primer as the substrate to allow telomerase to synthesise telomeric repeats onto the primers. After electrophoretic separation, telomerase activity was directly visualised upon extension of a primer and could be quantified by measuring the total amount of incorporated radioactive nucleotides.[5] Primer extension method is a powerful tool for telomerase activity measurement because it can reliably characterise the mechanistic details of telomerase function, such as processivity. However, the assay requires large amounts of starting materials to obtain meaningful results because of the low telomerase concentration in telomerase-positive cancer cells. To address this problem, Cohen *et al.* developed a two-step purification technique for telomerase before activity analysis.[4,42] As shown in Fig. 4, when an antibody generated against the peptide antigen ARPAEEATSLEGALSGTRH of the telomerase protein (hTERT amino acids 276 to 294) was incubated with the cell lysate, the telomerase in the cell lysate was bound by the antibody, and the complex was further immobilised onto protein G agarose beads. The bound telomerase was dissociated from the immobilised antibody by competitive removal by excess antigenic peptide. The second step of the purification was based on the binding between telomerase and its substrate. A synthetic telomerase substrate, 5′-biotin-CTAGACCTGTCATCA$(TTAGGG)_3$-3′, was immobilised onto neutravidin beads to provide the affinity reagent. The binding between telomerase and the immobilised DNA substrate was very stable ($t_{1/2} \geq 10$ hours), thus allowing the capture of >90% of the immunopurified telomerase. Upon the addition of dTTP and dATP, telomerase then catalysed the addition of TTA to the substrates. Because substrates ending in TTA display the weakest binding ($t_{1/2} < 5$ min), telomerase was eluted rapidly. Using this two-step enrichment, telomerase was

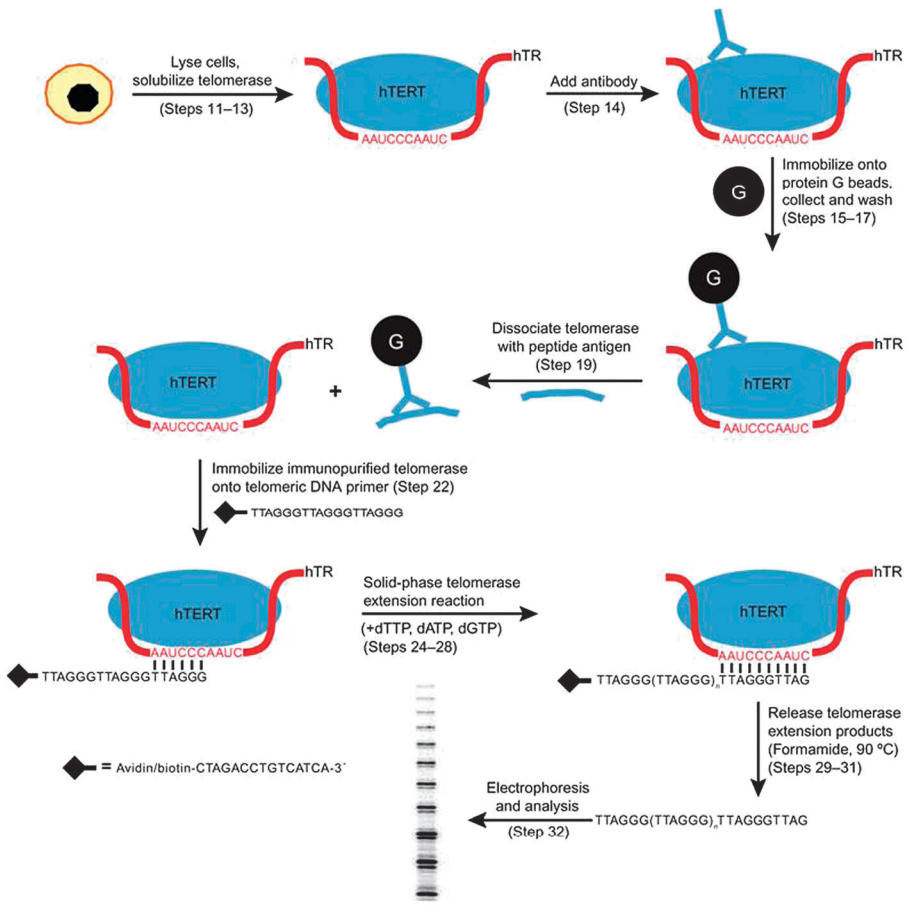

Fig. 4 Two-step purification technique for measuring telomerase activity. In the first step of purification, telomerase extracts are incubated with an antibody against a peptide antigen of telomerase protein and captured by protein G beads. After immunoprecipitating telomerase, telomerase is released from the antibody by adding excess peptide antigens. In the second step of purification, the resultant telomerase is incubated with $(TTAGGG)_3$-modified beads. After the collection of telomerase and the addition of dNTPs to the beads, the immobilised telomeric DNA is extended, resulting in the subsequent release of telomerase because of changes in the binding affinity. Telomerase activity is evaluated by electrophoretic analysis. Reprinted from ref. 42 with permission by Nature Publishing Group.

purified $\sim 10^8$-fold from the cultured cells. Using the electrophoretic separation and radioactivity detection, the 6 bp telomerase ladder products were clearly observed from 10^6 cells.

4.2 Optical detection

Although laborious and sample-intensive gel electrophoresis detection continues to be widely used for measuring telomerase activity, some higher throughput and less sample-intensive luminescent assay systems have been successfully developed in recent years. Kha *et al.* conducted a proof-of-concept experiment with a clinically used chemiluminescence (CL) detection technique for screening telomerase activity and inhibition.[43] The assay includes telomerase extension of a biotinylated TS primer, hybridisation of the telomerase-extended TS primer and a digoxigenin-labelled telomere antisense DNA probe, capture of the hybrid complexes by streptavidin-coated plates, and CL detection with an alkaline phosphatase–antidigoxigenin system. The authors also showed that their sensors could detect approximately 37 500 telomerase-positive cells under sub-optimal conditions. With a commercially available automated robotic system, this assay could be extended to a high-throughput screening format.

Despite the fact that the above described method is well established for high-throughput telomerase analysis, its application for telomerase studies has been limited by issues such as low sensitivity. Recently, two novel approaches have adapted the fluorescent molecular beacon (MBs) strategy to enzymatic-assisted signal amplification to improve the assay's sensitivity.[44,45] MBs are dually labelled with a fluorophore and a quencher. MB can self-assemble to form a stem-loop structure that holds the fluorophore in close proximity to the quencher, resulting in very weak fluorescence. The stem-loop structure can be opened when a complementary target sequence hybridises with it, separating the fluorophore and quencher and restoring the fluorescence. In both assays, the MB was designed to be complementary to telomeric sequences at its loop region. In the presence of telomerase, the TS primer was extended, generating a target. Using an isothermal circular strand-displacement polymerisation reaction[44] or exonuclease III-aided target recycling,[45] one target sequence catalyses many cycles of conversions, leading to an accumulation of optical signals. Both strategies led to significantly enhanced signal gain compared to the traditional MB detection method, with a detection limit of 4 cultured HeLa cells and 30 MCF-7 cells, respectively.

Single-stranded G-rich telomere sequences can form four-stranded quadruplex structures through chemical ligand-based stabilisation under physiologically relevant ionic conditions. This phenomenon is emerging as a novel strategy for cancer therapy, opening up a new avenue for cancer research. Moreover, the unique quadruplex structures can also bind to ligands and assemble into active peroxidase-mimicking DNAzymes. These peroxidase-mimicking DNAzymes catalyse the oxidisation of several organic probes under H_2O_2-rich conditions. These properties enable the use of the telomeric G-quadruplex structures as a simple and cheap means for biomolecule sensing. The first example of this strategy used for detecting telomerase activity is shown in Fig. 5.[46] Multi-TTAGGG repeats were added to the non-telomeric primer by telomerase.

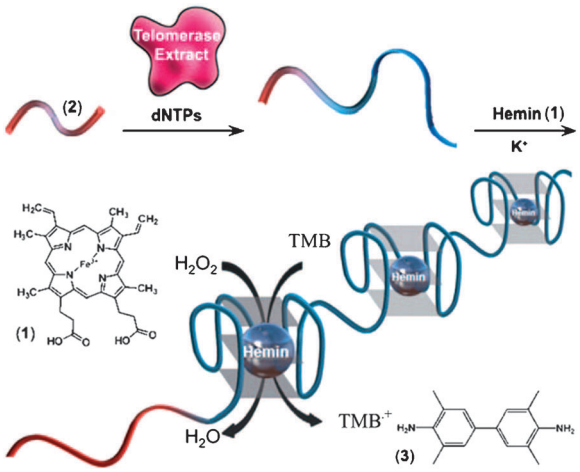

Fig. 5 Analysis of telomerase activity through self-assembly of the telomeric extension products into hemin–G-quadruplex DNAzyme units. Reprinted from ref. 46 with permission by Wiley-VCH.

The resulting telomere products were incubated with hemin under an acidic environment to allow the assembly of a hemin–G-quadruplex complex. DNAzyme-based catalytic reactions occurred when substrates (TMB and H_2O_2) were added. Telomerase activity was determined by measuring absorbance changes or visual detection by the naked eye. The absorbance intensity was relative to the cell extract concentrations and had a reported detection limit of ~ 200 cells μL^{-1}. Telomerase inhibition experiments have also been performed by analysing two known telomerase inhibitors (BIBR 1532 and IX). Recent work improved the assay sensitivity up to 8-fold by adding a 'boosting agent' (DOTASQ) of quadruplex-mediated DNAzyme.[47] As shown by the authors, the freshly synthesised telomeric fragment used for forming the G-quadruplex was also digested into short fragments for more efficient DNAzyme reactions. Another example of a telomerase-based DNAzymes detection strategy generates chemiluminescence (CL) in the presence of luminol and H_2O_2. Using this simple method, the telomerase activity from 100 cultured cancer cells could be detected.[48]

Optical assessment of the telomerase activity will be very valuable in enabling us to understand the biochemical workings of the telomerase reaction and, specifically, to observe the enzymatic processivity. Most of these assays measure the total luminescence or absorption intensity, thereby obscuring enzymatic processivity information. A single-molecule fluorescence two-color coincidence detection technique capable of determining not only the total telomerase activity but also the enzymatic processivity was recently described by Ren *et al.*[49] As shown in Fig. 6, in this method, a three-repeat primer substrate $(TTAGGG)_3$, pre-labelled with Alexa-488, was incubated with telomerase. Cy5-dATPs were incorporated into the primer by telomerase, leading to the co-localisation of the Alexa-488 and Cy5 dyes. Both of the spectrally distinct fluorophores were then simultaneously excited by two overlapping laser beams. Fluorescence was observed from single molecules in the subfemtoliter detection volume using a sub-nanomolar primer concentration. Telomerase activity could be measured by comparing the fluorescence intensity of

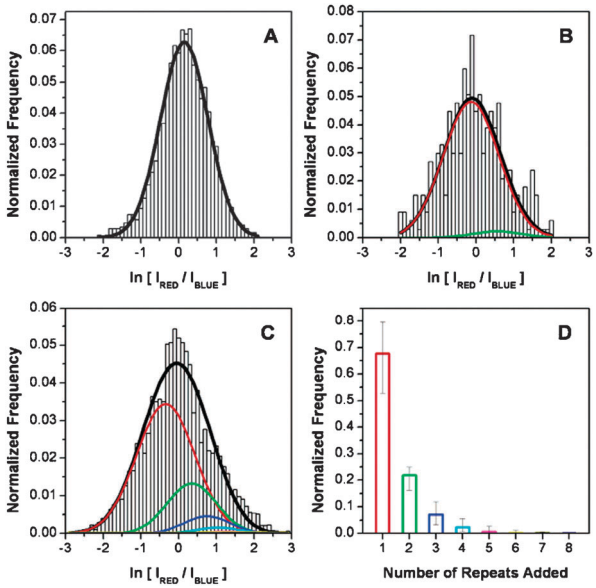

Fig. 6 Single-molecule analysis of telomerase activity and processivity. (A) The coincident events (2991) from the analysis of the single-repeat extension model (Cy5-Alexa-488) were fitted according to a single parameter $\sqrt{K}$, which is defined as 2.52. (B) Analysis of 140 pM mutant telomerase using single-molecule fluorescence analysis. Coincident events (333) were fitted by the processivity parameter at $X = 0.04$. (C) Analysis of 700 pM wild-type telomerase using single-molecule fluorescence analysis. Coincident events (4718) were fitted by a single-variable parameter, the processivity, $X = 0.32 \pm 0.13$. (D) Telomerase extension product populations obtained from (C). $\sqrt{K}$ is defined as 2.5 for both analyses of mutant telomerase and wild-type telomerase. Reprinted from ref. 49 with permission by the American Chemical Society.

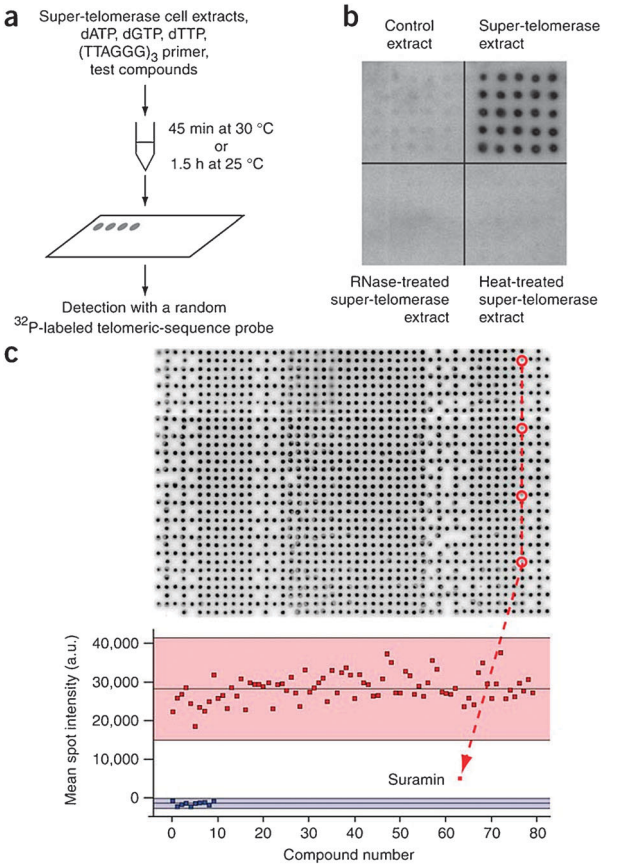

Fig. 7 Analysis of telomerase activity and modulators using Telospot. (a) Schematic illustration showing the principle of Telospot. First, the super-telomerase extracts were incubated with a primer oligonucleotide in the presence of dATP, dGTP, dTTP, and the test compound. Next, a fraction of the reaction was spotted in a macroarray format on the nylon membrane and hybridised with radiolabelled probes. (b) Autoradiography analysis of telomerase activity from mock-transfected (top left), super-telomerase (top right), heat-treated super-telomerase (bottom right) and RNase-treated super-telomerase (bottom left) extracts. (c) Screening of telomerase modulators using Telospot. Membranes hybridised with the telomeric probe were scanned with a PhosphorImager. The distance between two spots is approximately 4.5 mm. The control inhibitor (suramin) spots are circled. The bottom figure shows the scatter plot of the mean spot intensities for each compound and the control (suramin), with a ± 3 s.d. threshold, as shown in the shaded blue and red areas. The z factor was 0.5 for this subset of data. Reprinted from ref. 51 with permission by Nature Publishing Group.

Alexa-488 coupled to the DNA primer to the intensity of the Cy5-dATP substrates incorporated into the primer during the telomerase reaction. The concentration and number of telomeric repeats can be reasonably estimated by statistical analysis of the fluorescent molecule distribution ratios. The data show that the processivity values obtained from this assay closely agree with those observed from the direct primer extension assay. Single-molecule analysis of telomerase activity provides valuable insight into telomerase reaction kinetics. Using this technique, the same group has established that the catalytically functional human telomerase creates a stable hTERT : hTR : substrate interaction in a 1 : 1 : 1 absolute stoichiometry.[50]

4.3 Array and biosensor chip

High-throughput measurement of telomerase activity is important for discovering and characterising telomerase inhibitors. The direct primer extension assay offers a highly reliable alternative to TRAP-based methods, which are limited by the potential risk of false-positive and -negative results and primer dimer issues. Recently, Lingner and co-workers developed a microarray detection technique named Telospot for measuring telomerase activity and screening small-molecule inhibitors (Fig. 7).[51] In this assay, a sufficient quantity of telomerase was produced by over-expressing telomerase components in the cell. Telomeric $(TTAGGG)_3$ primers and dNTPs were extended by telomerase, and the resulting extension products were transferred to a nylon membrane with a spot array and hybridised with radiolabelled telomeric probes. Following hybridisation, the sample membrane was scanned by a PhosphorImager. Total detected radioactivity was proportional to telomerase activity. Telospot can be implemented manually to execute low-throughput analysis of telomerase activity. It can also be adapted to automatic high-throughput formats. To demonstrate the possibility of automating this assay, 1040 drugs were screened in the macroarray with an automatic liquid handler. Three compounds (acriflavinium hydrochloride, suramin, and mitoxantrone hydrochloride) were found to inhibit telomerase (Fig. 7C). Enzymatic processivity should not be affected in this assay because it uses natural nucleotides rather than radioactive nucleotides, thus increasing the reliability of Telospot relative to other radioactive nucleotide-incorporating methods.

Telomerase activity can also be detected in a biosensor chip using surface plasmon resonance (SPR).[52] SPR is a surface-sensitive optical technique that has been widely applied to characterise bio-molecular interactions, such as DNA hybridisation, antibody–antigen binding, and protein–DNA interactions. In this design, telomeric primers were attached to the chip surface by an avidin–biotin linkage. Telomerase activity was measured by the increase in the refractive index signals of the chip film substrate due to the telomerisation reaction. The reported limit of detection of tumour cells was 1 tumour cell per 1000 background cells.[52] In addition to its rapidity and simplicity, this method provides valuable insight into the kinetics of the telomerase reaction with real-time monitoring. In a separate example, Sharon and co-workers demonstrated that AuNP-functionalised antisense telomeric probes could amplify the formation of telomerase-extended telomeres[53] and enhance the SPR signals for telomerase activity analysis, with a sensitivity of 18 cells.

Another emerging array method for ultrasensitive telomerase activity detection is the use of silicon nanowires to create field effect transistor devices (Fig. 8). Silicon nanowire field-effect sensors coated with TS primers have been used for the real-time, label-free, highly sensitive detection of telomerase activity. Silicon nanowires act as signal transducers based on a change in conductance upon primer extension on the nanowire surface.

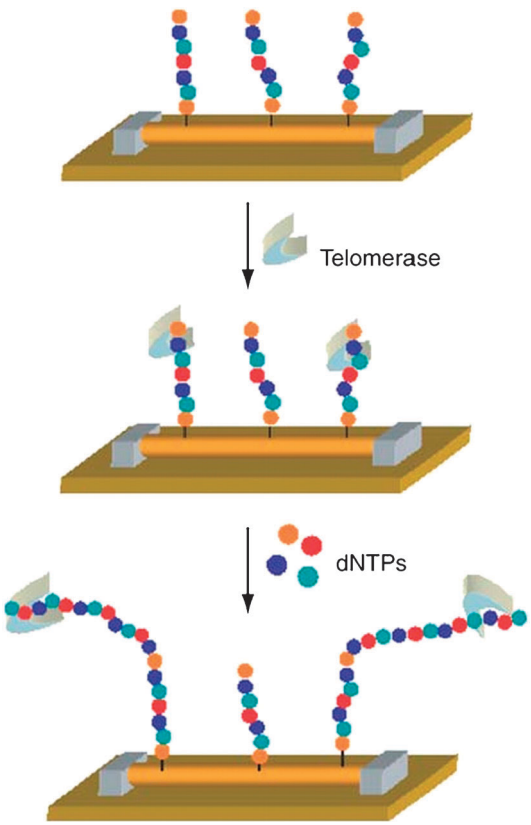

Fig. 8 Detection of telomerase activity with label-free field-effect sensors. Non-telomeric TS primer was linked with a silicon nanowire array. In the presence of telomerase and dNTPs, telomerase catalyzes the addition of TTAGGG repeats to the TS primer. The resulting elongation of the TS primer in silicon nanowire produces an increase of conductance signal. Reprinted from ref. 54 with permission by Nature Publishing Group.

The array formats used in this study will be beneficial for large-scale screening of telomerase inhibitors.[54]

4.4 Electrochemical strategies

In addition to optical techniques, there have been recent inventive electrochemical-based designs for telomerase activity sensing. Electrochemical sensing strategies, with their high sensitivity, low instrumentation costs, and compatibility with micro-manufacturing technology, are attractive alternatives to optical-based methods for biomolecule detection. There are three exemplary electrochemical strategies—based on enzyme labelling, interactive–electroactive binding, and label-free detection—that have recently been adapted for direct telomerase analysis.

Alkaline phosphatase has been used as the electrochemical label, as first demonstrated by Willner and colleagues. Upon hybridisation with the telomerase extension products, the biotinylated antisense telomeric probes were bound to avidin–alkaline phosphatase. Avidin–alkaline phosphatase catalysed the oxidative hydrolysis of 5-bromo-4-chloro-3-indolyl phosphate into insoluble precipitation products on the electrode. The changes in electrode resistances upon biocatalysed precipitation were measured to quantify the telomerase extension products, and thus, telomerase activity.[55] This approach allowed the detection of telomerase activity from 1000 HeLa cancer cells.

G-rich telomeric sequences tend to assemble into tetraplex structures at high concentrations of KCl. Sato and co-workers used ferrocenylnaphthalene diimide, which behaves as a tetraplex DNA binder, to directly determine the telomerase activity.[56] Circular dichroism and quartz crystal microbalance experiments indicated that three ferrocenylnaphthalene diimides bound to a tetraplex structure. DPV gave an electrochemical signal due to the oxidation of the ferrocenylnaphthalene diimide that was bound to the telomeric tetraplex structure on the electrode. This method accurately detected telomerase activity in a linear fashion for a range of 40 to 140 cells μL^{-1}. Li *et al.* developed an electrochemical method based on the binding of $[Ru(NH_3)_6]^{3+}$ to DNA strands to analyse telomerase activity. A highly efficient probe designed to hybridise with telomerase products was constructed by co-labelling AuNPs with telomeric capture DNA and signal DNA. The approach is based on the chrono-coulometric interrogation of $[Ru(NH_3)_6]^{3+}$, which quantitatively binds to AuNP-loaded DNA *via* electrostatic interactions. The authors showed that their sensors could detect telomerase activity from as few as 10 cultured cancer cells.[57]

Electrochemical detection *via* guanine oxidation signals is an attractive approach for measuring telomerase activity because it does not require an electroactive binder or a chemically modified probe.[58] Oxidation signals from the natural electro-activity of guanine residues present in telomerase products can be directly measured. The oxidation signals of guanine residues were studied by DPV. However, because the TS primer contains some guanine residues, this sensor also exhibits a high background signal. The detection limit was estimated to be approximately 3000 HeLa cells. Another label-free electrochemical method for telomerase activity is based on the electrochemical impedance spectroscopy (EIS) technique.[59] A thiolated DNA primer was immobilised onto the Au surface. $[Fe(CN)_6]^{3-/4-}$ was employed as the signal reporter in the supporting electrolyte solution.

The telomerisation reaction generated a longer TTAGGG repeat single-stranded DNA and showed an increased resistance in the transfer of $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ electrons on the Au electrode surface. The resistance increased linearly with the telomerase content. The dynamic range was found to be 10^3 to 10^5 HeLa cancer cells, with a detection limit of 10^3 HeLa cancer cells. Very recently, Sato and Takenaka have implemented a label-free electrochemical method for quantifying telomerase extended DNA by chronocoulometry (CC) with hexaammineruthenium(III) chloride (RuHex).[60] In this assay, telomerase products that resulted from the analysis of extracts of as small as 5–1000 HeLa cells on the TS primer-immobilized electrodes were quantified successfully. Specially, this assay is suitable for quick screening of drug candidates which inhibit telomerase. Based on the analysis of 10 potential telomerase inhibition compounds, the authors firstly showed two possible mechanisms of telomerase inhibition paths: binding of inhibitors to telomerase and binding of inhibitors to the quadruplex generated by telomerase.

4.5 Micro- and nano-materials-assisted detection

Micro- and nanostructured materials, such as AuNPs,[61–63] magnetic microparticles (MMPs),[64–66] and quantum dots (QDs),[53,67] are advantageous for detecting telomerase activity because they can reduce the cost and number of detection steps and potentially improve the reliability, sensitivity, and accuracy of telomerase detection. These materials often exhibit optical, electronic, and magnetic properties that cannot be achieved by the bulk material. Recent research has made tremendous progress in developing new telomerase assays based on nanobiotechnology and direct telomerase extension.

Telomerase assays that provide PCR-like sensitivity without actually using PCR techniques would be very promising. In most cases, direct telomerase assays are limited by their relatively low sensitivity. A recent exception is the development of a biobarcode amplification method, established by Mirkin and co-workers, for the ultrasensitive detection of telomerase activity and inhibition.[61] As outlined in Fig. 9, AuNP probes are functionalised with TS primers that are recognised by telomerase. Additionally, MMPs are conjugated to a capture sequence complementary to the telomerase-elongated products. After telomerisation, the target is captured in a MMP–telomerase products–AuNP sandwich complex, which is then separated from unreacted AuNP probes *via* an applied magnetic field. Next, both the elongated and unmodified oligonucleotide strands are released into solution by I_2 dissolution. After hybridisation with another AuNP-based probe and silver staining, the light scattering measured from array spots complementary to the elongated and unmodified oligonucleotide strands amplified the detection of telomerase activity and inhibition of as few as 10 HeLa cells. Possible limitations of the biobarcode assay include the need for sophisticated instruments and lengthy experimental procedures, which limit its practical application. We have developed a direct telomerase assay using magnetic beads as the capture carriers and ECL nanoprobes as the labels with an *in situ* detection format.[62] In this assay, ECL nanoprobes were synthesised by functionalising AuNPs with both a telomere capture probe and an

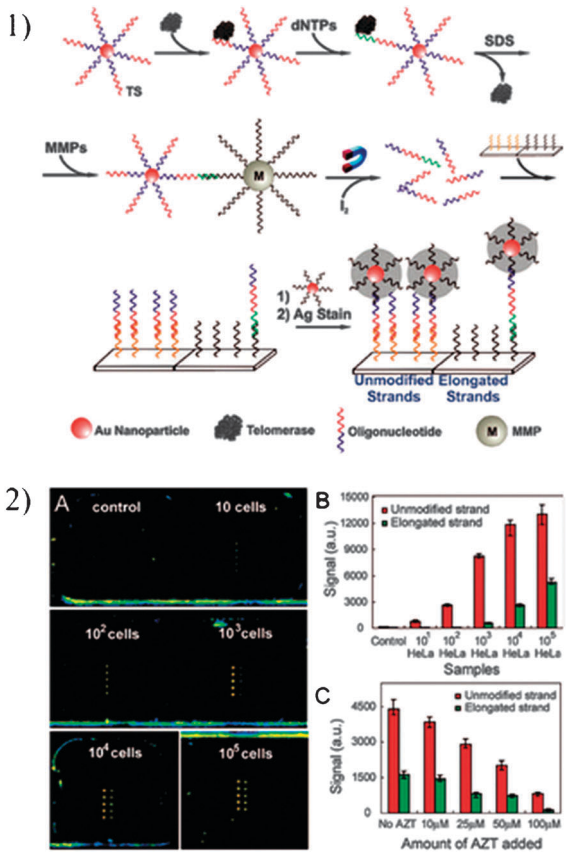

Fig. 9 (1) Analysis of telomerase activity using polyvalent oligonucleotide–AuNP probes. Steps include probe development of AuNPs, telomerase reaction, magnetic separation, DNA strands elution, and scanometric detection. (2A) Scanometric detection of telomerase activity from 10, 102, 103, 104, and 105 HeLa cells. Control signals were detected from 10^5 normal human fibroblast cells. Unmodified (left) and elongated strands (right) were simultaneously detected in each well. (2B) Bar graphs of the scanometric signals from (2A). (2C) Bar graphs of a telomerase inhibitor (3′-azido-3′-deoxythymidine) measurement. Reprinted from ref. 61 with permission by the American Chemical Society.

ECL signal probe. Biotinylated TS primers were extended by telomerase extracted from HeLa cells. The biotinylated telomerase products were then immobilised onto streptavidin-coated magnetic beads after washing, hybridisation, and capture onto the electrode surface. ECL photons were detected in this magnetic platform. Employing ECL nanoprobes resulted in a 100-fold enhanced sensitivity compared to a linear telomere antisense ECL probe. Telomerase levels from as few as 500 cultured cancer cells are easily detectable in this system. This system combines the advantages of magnetic separation of trace amounts of telomerase products and the AuNP amplification features of ECL detection. Moreover, this assay simplifies the analytical procedure by eliminating the release of the DNA probes from the target–nanoparticle complex and the repeated hybridisation of the probes for quantification.

Recently, a novel visual method for measuring telomerase activity was introduced by Wang *et al.*[63] In their method (Fig. 10), 5′-thiol-modified TS primers were immobilised onto the surface of AuNPs through the Au–S linkage. Telomerisation creates long G-quadruplex structures that stick to AuNPs

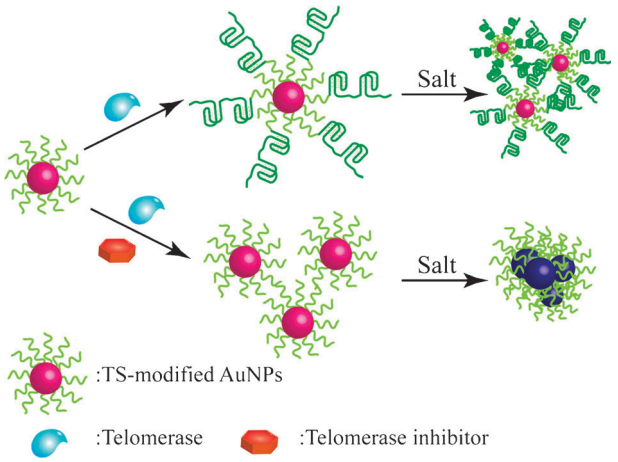

Fig. 10 Telomerase activity detection and profiling using TS primer-modified AuNPs. Reprinted from ref. 63 with permission by Wiley-VCH.

and build a thick barrier on their surface. This barrier results in repulsion between the G-quadruplex DNA-bound GNPs, which is indicated by a negative tendency to change from red to blue by salt-induced aggregation. When no telomerase is present, the primers in modified AuNPs are not extended, and the aggregation of the AuNPs would increase. Detection of the telomerase activity could thus be achieved by colorimetric analysis using the naked eye. Furthermore, quantitative analysis could be executed by spectral measurement. The authors reported an impressive sensitivity (1 HeLa cell μL^{-1}) for detecting telomerase activity.

Magnetic nanoparticles offer an attractive alternative for detecting biomolecules. A magnetic resonance-based technology, magnetic relaxation switch (MRS), has been developed for detecting telomerase activity.[65] Superparamagnetic nanoparticles (approximately 45 nm in diameter) were labelled with two antisense telomeric probes. The nanoprobes were then hybridised with telomerase extension products, causing the nanoprobes to switch from a dispersed state to a clustered (or assembled) state, which causes a concomitant change in the spin–spin relaxation time (T2) of the solution's water protons in the presence of a magnetic field. The change in amplitude of the T2 relaxation time depends linearly upon analyte concentration. Synthetic 30-bp telomeric repeats were detectable to approximately 100 attomoles in test tubes and 10 attomoles in 384 well plates. The telomerase activity determined by this method for cultured cells, tissues, and primary tumour samples correlated highly with the photometric-based ELISA method ($r^2 = 0.94$).

Until now, telomerase activity has not been validated as a practical cancer biomarker, possibly because of the lack of accurate assays that can accommodate clinical standardisation. Developing reference materials for telomerase assays may be an effective solution.[68] The simultaneous detection of telomerase protein levels would help determine the clinical relevance of telomerase activity measurements. In an extended version of the MRS assay, telomerase activity and telomerase protein quantification were simultaneously detected (Fig. 11).[66] Telomerase protein was detected by magnetic nanosensors labelled with anti-telomerase antibodies. Parallel measurements of protein levels and telomerase activity within the same cell lines indicated an incomplete correlation of telomerase protein expression with telomerase activity levels. These results concluded that measuring telomerase activity alone may not be sufficient to obtain accurate information about the telomerase status in a given tumour.

Willner's group also demonstrated a QD-based luminescent quenching method using electron transfer for label-free and homogeneous telomerase activity detection. In their approach, TS primers were conjugated to CdSe/ZnS QDs. In the presence of telomerase and dNTPs, G-rich telomeres were synthesised. An optical readout signal can be acquired from the electron transfer quenching of the QDs by the stacking of hemin on the self-organised G-quadruplexes found on the telomeres[53] or by the intercalation of doxorubicin into the duplex structure based on the hybridisation of complementary strand to the telomere repeat units.[67] This assay can detect telomerase activity from as few as several hundred cancer cells.

5. Current challenges and future perspectives

Tremendous progress has been made over the past two decades toward creating assays for detecting telomerase activity. However, the lack of available assays to measure telomerase in

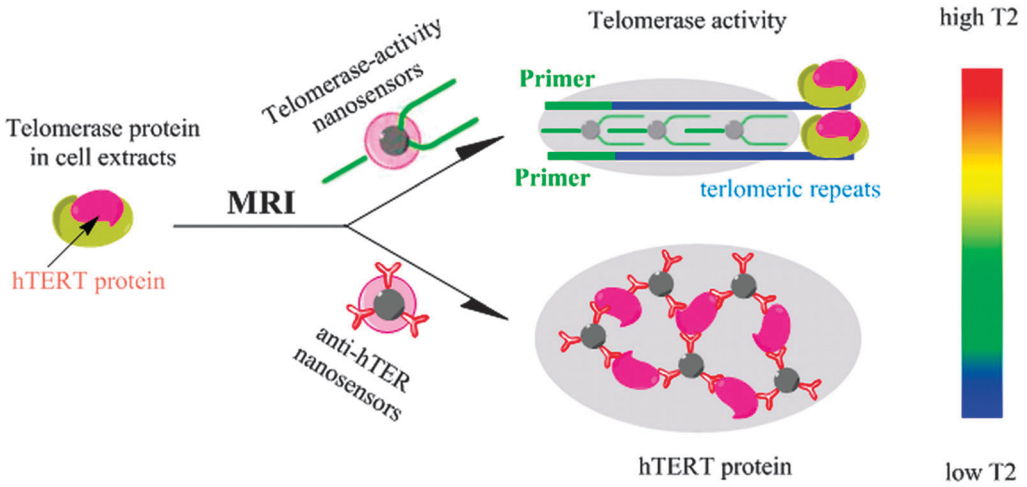

Fig. 11 Integrated magnetic nanosensor for parallel measurement of telomerase protein concentration and telomerase activity.

a sufficiently reliable, quantitative, and sensitive way continues to be a strong driving force in telomerase research. Several challenges remain for scientists to improve or develop more robust telomerase assays for fundamental biochemical research and clinical investigation. In this section, we will briefly discuss some of the key challenges in the field and present some ideas on the future directions.

5.1 Sensitivity

One of the most important criteria for evaluating an assay method is the sensitivity. The lack of highly sensitive analytical methods for measuring telomerase activity has long plagued scientists because even telomerase-positive cancer cells have only approximately one hundred telomerase molecules.[4] The invention of the TRAP method was a milestone for analysing telomerase activity because it made detection of telomerase activity become a routine laboratory tool.[6] However, as discussed in the previous section, the TRAP method has some serious shortcomings. Additionally, the TRAP method is not appropriate for evaluating quadruplex ligand telomerase inhibitors.[22] While the direct primer extension method is not subject to this drawback, it suffers from low sensitivity. In the last decade, extensive research has sought to address this problem, including the development of telomerase enrichment technologies, the use of signal amplification strategies,[44,45] the development of elaborately designed nanoprobes,[61–63,65–67] and adoption of highly sensitive optical detection techniques.[49,50] Some of these strategies have acquired extremely low detection limits, as low as several cells, which represents a sensitivity close to the conventional TRAP method. However, the most recent telomerase literature indicates that TRAP is still the most commonly used experimental research technique. One possible reason for this is the technical barrier for scientists in various disciplines that impedes or delays the transfer of these technologies to other scientists. The other critical challenge is that the sensitivity of the primer extension-based assay is still insufficient to meet these research needs. Most of these primer extension assays are merely proof-of-concept trials. Sensitivity is only achieved with high-purity cultured cancer cells. Few methods can perform as well with clinical tissue samples or body fluid samples, so each method's sensitivity should be further evaluated in complex biological samples.[69]

Although some methods have reported impressive sensitivity, critical problems remain unresolved. For example, in nanoprobe-based technologies, reproducibility is a problem because the amplification factors usually vary from case to case. Thus, synthesis of more uniform nanoparticles and the use of more normative labelling procedures may be the solution. Special precautions should also be taken to ensure the stability of these nanoprobes. For example, some nanomaterials based probes possess relatively poor salt stability, and some routinely used agents, such as mercaptoethanol, can disrupt labelled probes by displacing the alkylthiol-capped oligonucleotides from the AuNPs. For optical signal amplification methods, the focus should be on controlling the enzyme reaction and the adoption of more reasonable signal reading and data processing methods. Finally, the development of more sensitive optical apparatuses and diagnostic tools may also improve the primer extension assay.

5.2 Sample handling

New techniques should be developed for handling cells, tissues, or body fluid samples to increase efficiency, automation potential, and throughput. One basic challenge associated with potential clinical applications is the complex and variable constitution of the samples matrix. For example, samples may contain both cancer cells and adjacent noncancerous cells, and target cells collected from tissues or bodily fluids, such as blood, urine, or saliva, may be contaminated by a large number of carbohydrates and telomerase-negative cells. These non-specific impurities result in matrix effects and have demonstrated inhibitory effects on telomerase activity. The results of a measurement may be seriously affected by matrix effects. Moreover, the selective collection of the target cells of interest is necessary to fulfil the specificity criteria for identifying telomerase as a biomarker because noncancerous cells, such as stem cells and proliferative cells, also express telomerase. Careful consideration is necessary to determine the best methods for sample collection. The recent microfilter array strategy has shown single-cell capture efficiency. However, microfilters are prone to clogging by viscous substances in biological samples. Moreover, special precautions should be taken to prevent cell retention on the slot walls. Microfluidic or so-called micro total analysis systems (μ-TAS) can process and concentrate samples from a relatively high volume and may offer a robust means to automate and miniaturise cancer cell preparation.[70] Isolating cells with paramagnetic beads also offers some advantages, such as a large capture surface area, a ready release of cells after washing, and the potential for automation. Combining immunomagnetic isolation and the microfluidic system may be a future direction for studying telomerase. To further reduce the manual procedure, further work should also focus on the development of microfluidic devices to perform capture and lysis of a cell and the potential for integration with downstream microfluidic amplification and detection methods.

5.3 Reliability of measurement

The reliability of telomerase measurement depends on the precise evaluation of telomerase activity. However, an external standard control experiment that can quantitatively compare and assess telomerase activity has not yet been widely adopted. Furthermore, producing the large amounts of purified telomerase needed for external standard experiments is rather expensive. Future bioengineering progress toward a highly efficient expression and reconstitution system for telomerase will enable external standard control experiments to be executed in routine laboratory studies and clinical diagnoses. Particular attention should also be focused on screening and assessing multiparameter measurements for reliably assessing telomerase activity, which would help define clinical applications. To maintain precision and to minimise assay variability, a candidate reference material, such as the RNA component of human telomerase or the telomerase reverse transcriptase content, could be simultaneously detected with the telomerase activity to help interpret whether the observed changes in telomerase activity are the result of mRNA or protein expression changes because physical changes in both RNA and protein components of human telomerase may contribute to the results bias. Precise evaluation

of telomerase activity will also likely benefit from some emerging techniques. One example is digital PCR,[71] which offers a highly sensitive and precise method for the absolute quantification of specific gene expression. For digital PCR, DNA samples are divided into more than a million small pieces such that the majority of pieces are, ideally, either empty or contain only a single molecule, which is individually amplified by PCR. Data processing by Poisson's distribution of partitioning amplification can estimate an absolute target sequence quantity. Thus, adopting or modifying digital PCR techniques may offer a unique approach to real-time quantitative TRAP for the precise measurement of telomerase activity.

6. Conclusions

Although tremendous efforts have been made in the past two decades toward creating a telomerase assay, use of telomerase as a reliable clinical biomarker for cancer remains a challenge. Analytical methods for measuring telomerase are urgently needed for fundamental biochemical research and clinical diagnosis. Undoubtedly, highly interdisciplinary collaborations would enable more rapid advances in this field. Although some outstanding obstacles still exist, the field of telomerase measurement is still an extremely active research field that is highly important.

Acknowledgements

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