Rolling Circle Amplification

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Rolling circle amplification (RCA) is an isothermal enzymatic process in which a short nucleic acid primer is amplified to form a long single-stranded nucleic acid using a circular template and special nucleic acid polymerases. Furthermore, this approach can be further engineered into a device for point-of-need monitoring of environmental pollutants.

Keywords: rolling circle amplification ; environmental monitoring ; heavy metals ; organic molecules ; microorganisms

1. Introduction

In recent years, the discharge of contaminants from industrial and agricultural activities and urban wastewater has caused serious contamination of the aqueous system, posing a great potential threat to human health and aquatic life. These contaminants can be divided into three categories: (i) inorganic chemical substances, (ii) organic pollutants and (iii) microorganisms. These substances can cause adverse effects on the environment [1][2][3][4], for example, the disruption of hormones and the endocrine system and the induction of cytotoxicity and/or genotoxicity and carcinogenesis [5][6]. The variable composition of pollutants and their location in aqueous environments over time have resulted in increasing focus on new technologies that use cheap and real-time strategies to monitor pollutants. Most of these strategies are based on laboratory platforms, such as inductively coupled plasma mass spectrometry (ICP-MS) for the detection of heavy metal ions, liquid chromatography-tandem mass spectrometry (LC-MS) for the detection of small organic chemicals or their metabolites, and polymerase chain reaction (PCR) for the detection of nucleic acids and genetic information, which require preprocessing and frequent data sampling, which means that they are both expensive and slow. These aspects highlight the need to develop a new strategy that is more sensitive, portable, and efficient for on-site detection of pollutants composed of multiple substances [7][8][9][10].

Recently, rolling circle amplification (RCA)-based analytical methods have received increasing attention in environmental monitoring. RCA is an uncomplicated and efficient isothermal enzymatic process using unique DNA and RNA polymerases to produce long single-stranded DNA (ssDNA) and RNA [11][12]. In RCA, the polymerase will spontaneously and continuously add nucleotides to the primers that bind to the circular template, generating long ssDNA with tandem repeats of tens to hundreds of orders of magnitude. Unlike PCR, which requires a thermal cycler and thermostable DNA polymerase. RCA can be in solution, on a solid support, or in a complex biological environment at a constant temperature (room temperature to 37 °C). The ability of RCA to grow a long DNA chain on a solid support or inside a cell from one molecular binding event enables the detection of targets at a single molecule level [13][14][15]. In addition, an RCA product comprising repeating cyclic sequences complementary to template DNA can be customized by template design. By designing the template, the customizable DNA product includes functional sequences, including aptamers, DNAzymes, spacer domains, and restriction endonuclease sites. Of course, by hybridizing the RCA product with a complementary nucleic acid linked to a functional part including biotin [16][17], fluorophores [18][19], antibodies [20], and nanoparticles [21][22][23][24], it is easy to synthesize a multifunctional material with a variety of properties, including biorecognition and biosensing. Collectively, the properties of high-efficiency isothermal amplification, single-molecule sensitivity, versatility of structure and composition, and multivalences make RCA a powerful tool in aqueous environments [25][26][27]. Currently, RCA has been extensively studied to develop sensitive methods for detecting DNA, RNA, DNA methylation, single nucleotide polymorphisms, small molecules, proteins, and cells. In addition to diagnosis, RCA has also been proven to be effective for cell-free cloning and sequencing [28][29], in situ genotyping and genome-wide analysis of cells and tissues [30][31][32][33][34]. Recently, RCA has received widespread attention for its use in the production of DNA nanostructures such as origami, nanoribbons, nanotubes, DNA nanoscaffolds, and DNA metamaterials for periodic nanocomponents [11][35][36][37][38]. Importantly, these materials have high prospects in a wide range of applications, including environmental monitoring, drug delivery, and in vivo imaging of manufacturing electronic circuits, including DNA-based materials.

2. Advantages and Disadvantages of the RCA Assay

2.1. Fundamentals of RCA

The RCA reaction typically requires four components: (1) DNA polymerase (e.g., Phi29 DNA polymerase), which includes an appropriate buffer; (2) a short nucleic acid primer; (3) a circular DNA template; and (4) deoxynucleotide triphosphate (dNTP) (monomer or structural unit of RCA product) [11][39][40][41]. In polymerases, Phi29 DNA polymerase is most commonly used because of its excellent capability and continuous strand displacement synthesis capability. Phi29 can handle topological constraints, four-way cross connections, and multiple circular DNA template complexes [39][42][43]. For RCA primers, both RNA and DNA (usually the "target" molecule to be detected) can achieve this goal. Indeed, the target DNA and RNA can be used to connect the first template mediated as a padlock probe (PLP) using RCA reaction circular template cyclizing [44][45]. The circular DNA template (usually 15–200 nucleotides (NT) in length) is a component that can be enzymatically or chemically synthesized through intramolecular phosphate and hydroxyl end groups. Most commonly, the template is a circular DNA template mediated by enzymatic ligation (e.g., T4 DNA ligase) or the use of a special DNA ligase enzyme with a template-free connection to a synthetic CircLigase [15][46]. By designing primers and circular templates, RCA product length, sequence, composition, structure and rigidity may be appropriately adjusted, thereby becoming a highly versatile RCA technique (summarized in **Table 1**).

Table 1. Comparison of RCA, PCR and real-time PCR for the detection of DNA.

Features	Conventional PCR Assay	Real Time-PCR Assay	RCA Assay
Sensitivity	Sensitive	Highly sensitive	Highly sensitive
Specificity	Specific	Specific	Specific
Temperature conditions	Thermal cycle	Thermal cycle	Isothermal
Inhibition by biological samples	Yes	Yes	No
Instruments required	Thermocycler	Thermocycler	Not required
Post-assay analysis	Required	Required	Generally not required
Amplicon detection methods	Gel electrophoresis	Real-time detection/amplification graph	Gel electrophoresis, Turbidity measurement by visual inspection or using a real-time turbidimeter; dye-based visual detection
Qualitative detection	Yes	Yes	Yes
Quantitative detection	No	Yes	Semi-quantitative
Portability	Partially	Yes	Yes
Overall assay time	3–5 h	2.5–4 h	1–1.5 h
Cost effectiveness	Less expensive	Expensive	Less expensive

2.2. Exponential RCA Amplification

One of the powerful functions of RCA is the ability to design a circular template so that the signal generated by a single binding event is exponentially amplified [47][48][49]. Using a plurality of primers hybridizing to the same ring can lead to amplification of a plurality of events, thereby producing a plurality of RCA products [50][51] (**Figure 1**). The number of primers that one circular template can accommodate depends on the length of the primers and the circle. Another method for exponential amplification of RCA uses a so-called hyperbranched RCA (HRCA) (branched or amplification) method, in which the RCA product used as a template for the second and third groups is further expanded using primers [52][53][54][55]. Note that a primer can be integrated into the hyperbranched RCA method to increase the sensitivity, especially when the target is detected at low abundance [56]. Additionally, restriction enzyme digestion followed by enzymatic ligation template-mediated, linear RCA products may be converted to a variety of cyclic products [57][58]. A second set of primers may then be used to incorporate these new cyclic products for further amplification. This "circle to circle amplification" restriction

digestion process, cyclization and amplification may be repeated for additional amplification. Finally, after hybridization with a second set of circles, the RCA product may be treated with nicking enzyme to generate a plurality of primers. The hybridized primer/circular template product obtained from the nickase reaction can be directly used for the next cycle of RCA amplification.

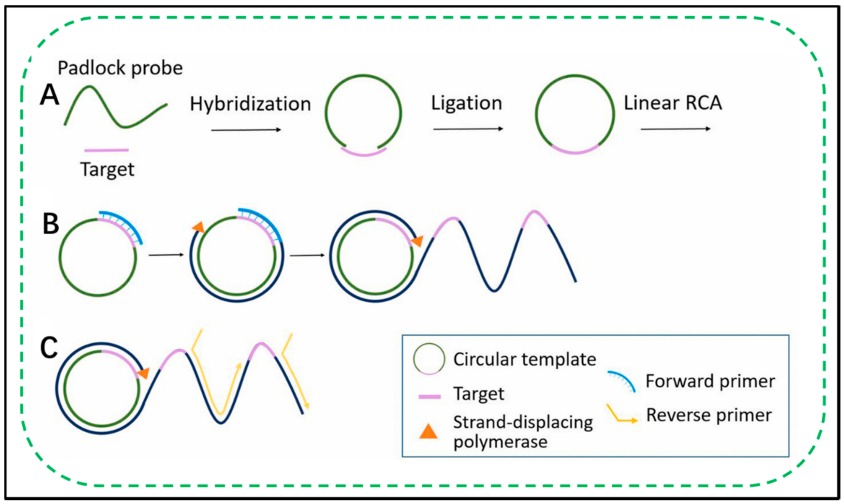

Figure 1. Schematics of RCA mechanisms. (**A**) Ligation RCA. (**B**) Linear RCA. (**C**) Hyperbranched RCA. Adapted with permission from ref. [50].

2.3. Detection of the RCA Product

A variety of signal reading technologies can be used to monitor and detect RCA processes and products. The most common RCA product analysis was carried out by gel electrophoresis. Furthermore, during RCA fluorophore dNTP coupling, fluorescent dye incorporated into the RCA product, bound by a fluorophore, or a complementary strand, can be easily observed using fluorescence-based techniques, including fluorescence spectroscopy, microscopy and flow cytometry [26][59][60]. Combining RCA with molecules such as modified AuNPs, quantum dots, or magnetic beads, it is easy to achieve visualization of RCA products [18][22][61][62]. For instance, RCA products can trigger the assembly of AuNPs for colorimetric and spectral visualization. RCA products can also be combined with magnetic beads to generate diffraction signals.

An electrochemical signal can also be generated by QD hybridization of the RCA products followed by dissolution to achieve high sensitivity [63]. Another method of detecting an electrochemical signal generated by an RCA product comprises inserting the DNA into an organic molecule (e.g., methylene blue), which is inserted into the RCA product [64]. Molecular beacons [65][66] and DNA-intercalating dyes (e.g., SYBR green [67][68]) have also been widely used to detect RCA products, which is also important since real-time monitoring of the reaction in the absence of RCA DNA products results in minimal background fluorescence. In addition to the fluorescence signal, there are some intercalating dyes, such as 3,3-diethylthiadicarbocyanine iodide (DiSC2(5)), that, when combined with the duplex between DNA and peptide nucleic acid (PNA), can be used to produce a colour change from blue to purple [69]. Then, the colour change signal can achieve the goals of the naked eye for detection, which is particularly suitable for real-time diagnostic applications. Furthermore, HRP (horseradish peroxidase) was immobilized on RCA products through biotin modification of DNA to realize visual detection based on colorimetry [70]. Interestingly, the DNAzyme sequence that mimics HRP can catalyse the oxidation of 2,20-azidobis (3-ethylbenzothiazolin-6-sulfonic acid) and generate a blue-green colorimetric signal. It can also be incorporated into RCA products [71]. This “dual-amplification system” (i.e., the RCA and has multiple converting enzyme DNA) enables real-time supersensitive colorimetric detection of target molecules. Finally, the RCA product can be detected by bioluminescence. In this case, the RCA reaction generates a large amount of pyrophosphate that can be used as an adenyl transferase substrate to produce ATP. Then, firefly luciferase ATP acts as a cofactor to produce a bioluminescent signal [72][73].

RCA can not only achieve signal amplification of target nucleic acids through amplification but also has flexible and diverse visualization methods; therefore, it has great potential for application in nucleic acid detection. The advantages of RCA include the following: (1) high sensitivity: RCA has strong amplification ability, the efficiency of exponential RCA can reach 10^9 fold, and it has the potential to detect single copies; (2) high sequence specificity: it can distinguish single nucleotide polymorphisms; (3) the amplified product can be directly used for sequencing after phosphorylation treatment; (4) high throughput: RCA can form a closed circular sequence on the target, ensuring that the signal generated by RCA is concentrated at one point, thereby achieving in situ amplification and slide amplification. However, there are still some

shortcomings in the development of the RCA method: (1) the padlock probe is often close to 100 bp, and therefore, the synthesis cost is relatively high; (2) background interference is a problem during signal detection.

3. Conclusions and Perspective

The quality of the water environment is closely related to human health, food, energy and the economy. As mentioned earlier, RCA-based analysis technology is a reliable alternative for detecting various targets to track environmental pollutants. Due to its simplicity and high selectivity, different types of environmental pollutants can be monitored, and an increasing number of RCA-based analytical methods have been established. As a simple, efficient and temperature-free nucleic acid amplification tool, RCA has now become a powerful tool in the field of environmental monitoring. In particular, when RCAs and functional nucleic acids, including aptamers and DNA enzymes, as well as other assay platforms, PCR, ELISA, microfluidics, surface plasmon resonance (SPR), and nanoparticles, are integrated into ultrasensitive detection of various targets, including nucleic acids, proteins, small molecules, viruses, and cells. From the point of view of materials science, RCA project versatility makes it an exciting tool for the preparation of DNA building blocks and the construction of highly ordered nanostructures and new materials that may have practical applications in biosensing and environmental monitoring.

Furthermore, based on the synthesis of multivalent ligand binding variable RCA, many other multivalent ligand systems are difficult to achieve, including the number of ligands, density, type, and spatial organization, and they represent a new chemical biology tool in environmental monitoring. Despite the many advantages of the RCA system, there are still some challenges to overcome in practical applications. First, preparing a large number of high-purity circular templates may be an unavoidable challenge. For example, in enzymatic ligation methods, in addition to circular DNA, linear multimer byproducts can sometimes be formed. This problem can be partially solved by using low concentrations of DNA in the ligation reaction. Then, the circular DNA product can be purified from the linear byproduct by gel electrophoresis or exonuclease treatment, which only degrades noncircular linear DNA molecules. In addition, it is known that the enzymatic ligation process is effective for relatively large DNA substrates but may not be suitable for making small DNA loops (~30 nt). This may be due to an insufficient number of enzyme binding sites and/or is caused by the induced strain after restriction enzyme digestion. The closed loop of short oligonucleotides. However, this defect can be solved by chemically circularizing DNA oligonucleotides. This method can generate both small (~14 nt) and large circular templates (415 nt) with very good yields (up to 85%) of circular DNA molecules. In addition, other challenges in the practical application of RCA systems include mass production, purification, and storage because RCA products tend to aggregate for a long time due to non-specific intermolecular and intramolecular cross-linking. In addition, due to the large molecular weight of RCA products, nonspecific binding may occur when used in complex water environments such as wastewater. These problems can be minimized by fine-tuning the parameters, including RCA product length, order, composition, and stiffness. For instance, the authors found that the incorporation of polyT (rather than random sequence) spacers between the aptamer domains of RCA products can reduce nonspecific interactions between the multivalent aptamer system and various targets. Finally, computer-aided methods can be used to design RCA sequences and short chains to construct predictable DNA nanostructures to minimize unwanted nonspecific interactions. The analysis method based on RCA is an innovative approach for the simple and rapid detection of various targets in environmental monitoring and can even be applied to on-site detection. This method will open up a new direction for environmental pollution assessment, drug abuse trend assessment, public health assessment, and other fields.

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