===== REVIEW ARTICLE =====

Rolling Circle Amplification as a Universal Method for the Analysis of a Wide Range of Biological Targets[1]

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Abstract—Detection and quantification of biotargets are important analytical tasks, which are solved using a wide range of various methods. In recent years, methods based on the isothermal amplification of nucleic acids (NAs) have been extensively developed. Among them, a special place is occupied by rolling circle amplification (RCA), which is used not only for the detection of a specific NA but also for the analysis of other biomolecules, and is also a versatile platform for the development of highly sensitive methods and convenient diagnostic devices. The present review reveals a number of methodical aspects of RCA-mediated analysis; in particular, the data on its key molecular participants are presented, the methods for increasing the efficiency and productivity of RCA are described, and different variants of reporter systems are briefly characterized. Differences in the techniques of RCA-mediated analysis of biotargets of various types are shown. Some examples of using different RCA variants for the solution of specific diagnostic problems are given.

INTRODUCTION

The amplification of nucleic acids (NAs) in vitro is the basis of all modern methods in molecular diagnostics of various diseases and the analysis of food products, biological traces, and environmental objects. Of the variety of amplification methods, the polymerase chain reaction (PCR) has been most widely used [1]; however, the necessity of cyclic changes in the temperature of the reaction medium determines the dependence of the PCR on expensive equipment, which limits its applicability, particularly, under field conditions. An alternative to PCR is isothermal methods, such as strand displacement amplification (SDA), rolling circle amplification (RCA), loop-mediated isothermal amplification (LMA), and others [2, 3]. In these methods, polymerases with strand displacement activity are used, which provide the denaturation of double-stranded NAs during polymerization; this eliminates the need for the cycling of the denaturation, annealing, and extension stages, and for performing the reaction; a thermostat is sufficient.

Among isothermal methods, RCA occupies a special place: this is the only reaction that makes it possible to synthesize NA products with the desired (artificially preset) nucleotide sequence. A characteristic feature of RCA is the use of circular DNA templates; the appropriate design of these templates makes possible a combination with other enzymatic reactions and various reporter systems, the performance of bioanalysis in solution, on the surface of solid materials or in living cells, and the conversion into high-performance formats. In contrast to other amplification methods, RCA can be used not only as a technique for the amplification and subsequent detection of the molecules of an analyte, NA, but also as a tool for obtaining functionally active NAs that mediate the detection of other biotargets. The versatility of RCA motivated the development of a great variety of bioanalytical techniques, which manifested itself in numerous publications and served as the basis for creating portable microfluidic, electrochemical, and biosensor diagnostic devices [4–7]. Despite its great potential, RCA has found practical application primarily in molecular diagnostics, governing a high sensitivity and specificity of detection of bioanalytes such as NAs and proteins. In the review, the molecular bases of the RCA method are presented, and its application for the analysis of biotargets of different chemical origin is shown.
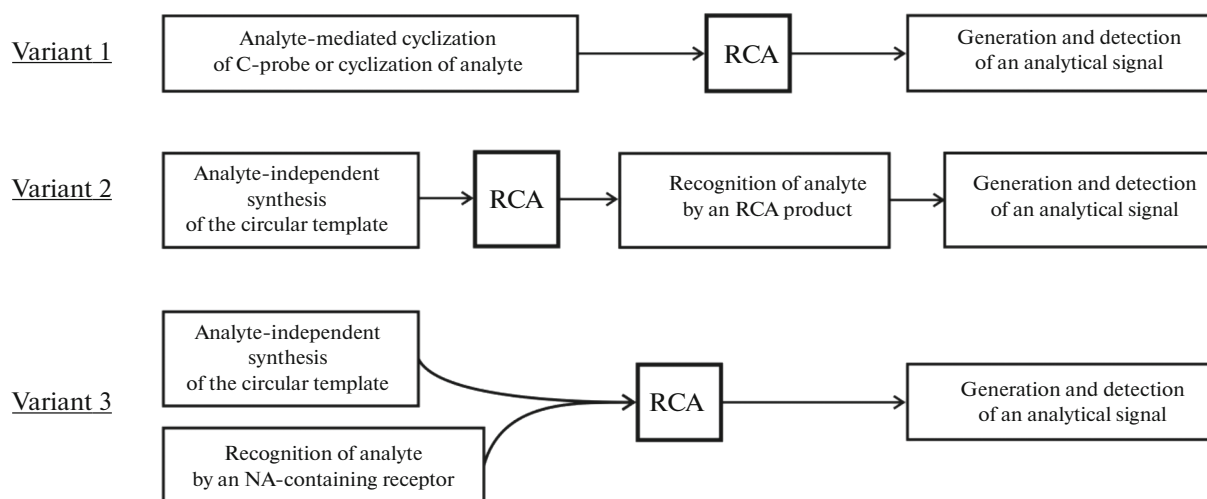

Fig. 1. Variants of including the rolling circle amplification as a separate stage into the protocol of a bioanalytical study.

MOLECULAR BASES
OF ROLLING CIRCLE AMPLIFICATION

Rolling circle amplification is the reaction of the synthesis of NAs on a circular template (CT). RCA as a method for increasing the amount of the analyte is used not only for NAs; in other cases, the reaction is combined with the recognition of a biotarget by a specific receptor. Depending on the nature of the analyte (NAs, protein, small organic molecule), the features of a sample being analyzed (origin, analytical purity, the presence of other simultaneously released substances, and others), and the instruments used, RCA as a separate, independent, stage can occupy different places in the bioanalytical protocol. There are three variants of using this amplification reaction in the analysis (Fig. 1).

Variant 1, which is used in the analysis of NAs, involves the RCA as a stage of the amplification of either directly a target or the CT obtained with its participation. According to variant 2, the analyte is recognized using the RCA products (RCAPs) that form functionally active secondary structures (FSS); the reaction is carried out using the preliminarily synthesized CT. This way, which provides an increase in the concentration of the analytical agent, is versatile and is implemented for a wide range of biotargets. According to variant 3, the analyte is recognized by a receptor bound to an NA, which, as a rule, acts as a primer for RCA, or as an NA capture probe. This way is most commonly used in solid-phase analysis technologies, when an analyte is fixed on the substrate, and the reactions of synthesis, cleavage, or hybridization of NAs are carried out on the surface of the solid phase.

The specificity of the RCA assay is determined by the type of the analyte and the technology of its detection. On the whole, a higher specificity is provided by approaches in which the RCA reaction leads to an increase in the concentration of the analytical agent and/or an enhancement of the analytical signal. Many of the currently available methods of RCA assay show high sensitivity with detection limits on average in the range of femtomolar (for NAs) and picomolar (for proteins) concentrations.

Key components of reaction systems for RCA. The key participants of RCA are a single-stranded CT, at least one primer annealing to the CT (starting primer), and polymerase with the strand displacement activity (Fig. 2) [8]. The CT can contain several different oligonucleotide motifs responsible, e.g., for binding to a target (I) and a primer (II) and for the formation of FSS by the RCA product (III).

After the annealing of the primer to the CT, the synthesis of its complementary copy begins, which continues until polymerase reaches the 5'-end of the primer. Then, the enzyme displaces the strand that interferes with it and continues the synthesis along the circle, making many turns and producing a long single-stranded RCA product (RCAP) whose length can be tens, hundreds, and even thousands of tandem repeats, depending on the processivity of the enzyme [8, 9].

RCA can be performed not only with circular DNA templates but also with RNA templates [9]. In the latter case, the reaction is more often called the rolling circle transcription (RCT); in essence, this is another variant of RCA, since it is characterized by the same basal features and parameters as RCA with the use of DNA templates. The only significant difference is the application of RNA-dependent polymerases, and that the initiation of transcription can require the presence of promoter sequences, which poses limitations upon the design of the CT and primers.

As a rule, CTs are synthesized from oligonucleotide precursors called C-probes (padlock probes) by their
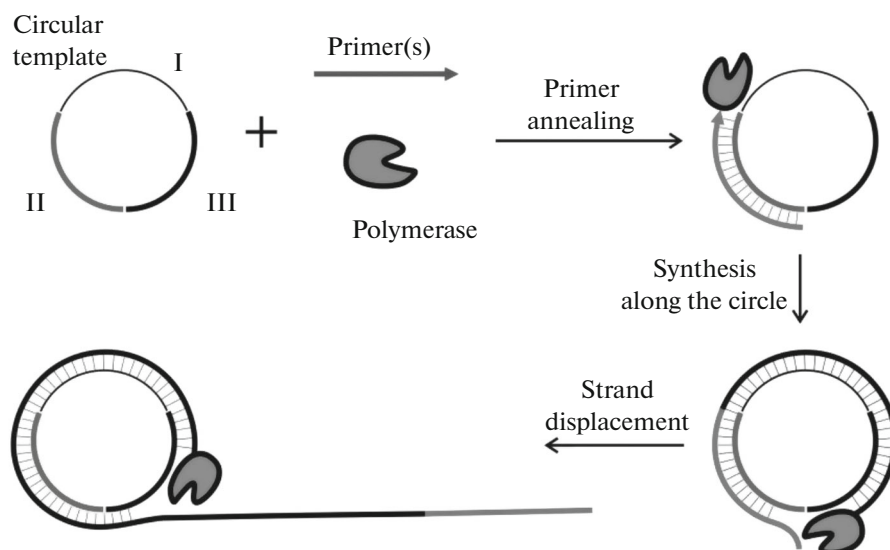
Fig. 2. The key components of the reaction system and the course of RCA. For a circular template, motifs responsible for the binding to the target (I) and to the primer (II), and for the formation of FSSs by an RCA product (III) are denoted by different zones.

cyclization with the use of DNA or RNA ligases or by chemical ligation. As C-probes, linear DNAs or RNAs ~40−60 nt long are commonly used; however, there are data on the closure of both short (from 13 nt on chemical ligation and from 25 nt on enzymatic ligation) and relatively long (up to 105 nt) oligonucleotides [9]. The cyclization of NAs can be performed by template and nontemplate methods. The first variant, which is schematically shown in Fig. 3a, involves the annealing of a C-probe to a so-called supporting template (splint); in this case, at both ends there are 10−20-nt long nucleotide sequences complementary to the splint [10]. Shorter annealing regions (in total <15 nt) reduce the stability of the resulting duplex structure, whereas long regions increase the efficiency of the intermolecular interaction, which results in the formation of linear products [11]. The annealed ends of the C-probe are designed so that they are "butted" to each other and form a nick. In the analysis of an NA target, the NA being detected acts often as a splint.

The template-dependent synthesis of the CT is most often carried out using T4 DNA ligase; however, in the analysis of RNA, ligases with a lower substrate specificity, for example, Chlorella virus DNA ligase (commercial ligase SplintR) or T4 RNA ligases, are used [12, 13]. Nontemplate ligation does not require the presence of a splint; it is performed using T4 RNA ligases or CircLigase (Fig. 3b). Although, nontemplate ligation gives a low yield of the target product, this method is indispensable in studies of NA samples of unknown composition and in the analysis of short-stranded NAs, e.g., fragmented DNA or small RNAs. In addition, the methods are known in which the yield of the CT during ligation is increased by using molecular crowding agents, such as polyethylene glycol [14, 15].

In some cases, the cyclization of NAs by chemical methods is a more convenient alternative to ligases; however, it requires the use of additional reagents or modified C-probes. The first methods of closing the oligonucleotides consisted in the condensation of the 5'-terminal phosphate with 3'-hydroxy group by the action of carbodiimide or bromcyane [16] and of 5'-terminal azidothymidine with 3'-phosphorothioate [17], or using the 5'-O-tosyl group [18]. Later, approaches based on the methods of click chemistry [19], first of all, azide−alkyne cycloaddition, were proposed [20].

The size of the CT significantly affects the efficiency of RCA. Small NA circles (<40 nt) are apparently little suitable for this reaction [14], although there are data on their possible use for amplification [9]. It was shown for linear double-stranded CTs whose size does not exceed one persistent length of DNA (~51 nm or ~160 bp for double-stranded DNA in the B-form [21]) that the efficiency of RCA correlates with the number of turns of the double helix: for circles having the integer number of turns, the amplification efficiency is the least, whereas for circles with the noninteger number of turns, it is the highest [22]. For CTs close in size to plasmids, the RCA efficiency was substantially lower [23].

The nucleotide sequence of the CT can be preset so that its individual regions are annealed within the circle, forming loop-like secondary structures or dumbbells, which promote an increase in the efficiency of C-probe cyclization and determine the presence of double-stranded fragments in RCAPs.

Depending on the system for detecting the results, used after RCA, sequences providing the formation of FSS (aptamers, DNAzymes, G-quadruplexes, loops,
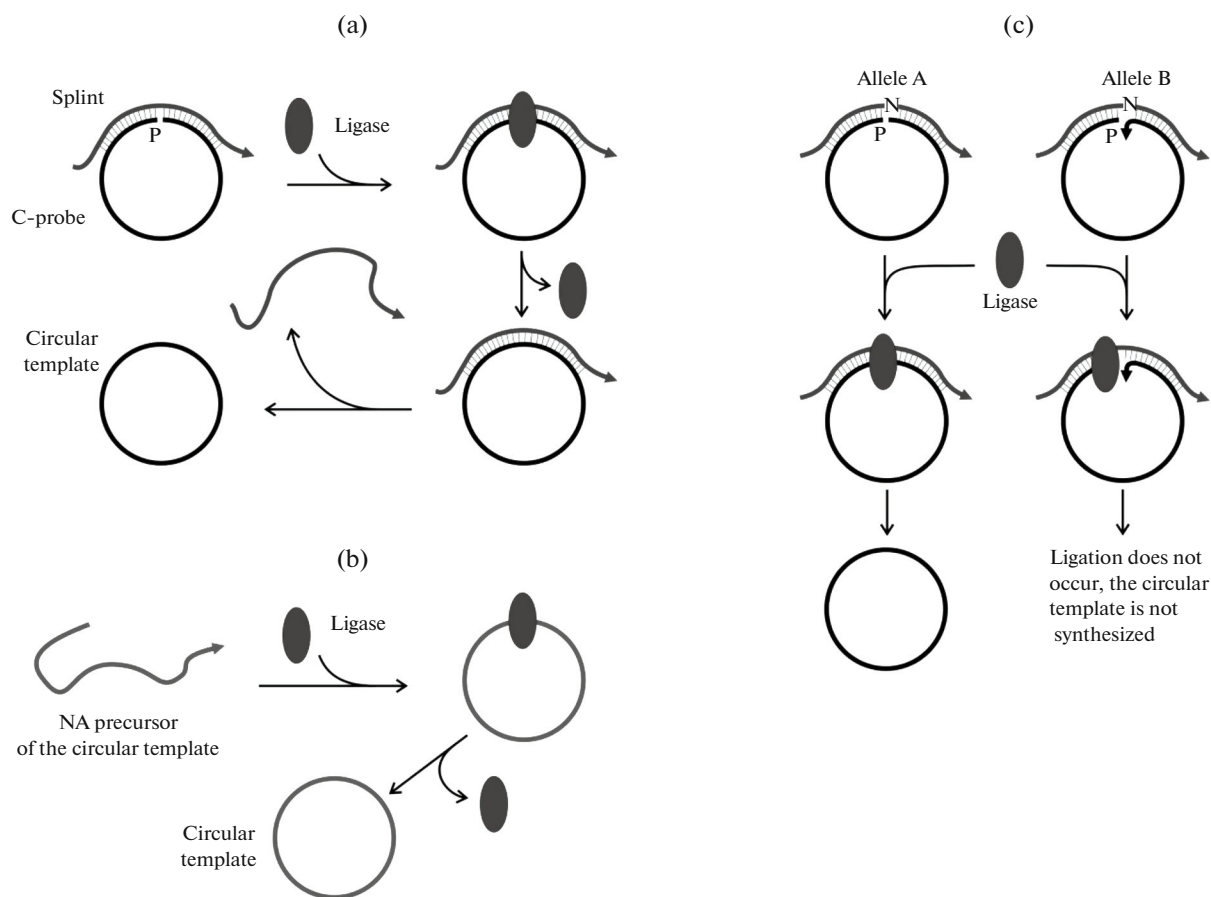
Fig. 3. A scheme of the closure of the C-probe with the splint (a) and without it (b) and the discrimination of polymorphic nucleotides using allele-specific C-probes in the analysis of single nucleotide polymorphism (c).

etc.) or carrying the endonuclease recognition sites, the sites for the annealing of specific hybridization probes, or homonucleotide motifs for binding to metal nanoparticles can be included into the structure of the C-probe. It was also found that CTs with a high content of dA and dC nucleotides increase the efficiency of circle amplification [24].

RCA occurs only by the action of polymerases with strand displacement activity. In this case, the enzyme provides the divergence of NA strands and catalyzes the synthesis of a new strand by displacing the old strand when moving along the template. The choice of these polymerases is quite wide, but the most commonly used are DNA polymerases Bst, Bsm, and Vent without 5'–3'-exonuclease activity, phi29, T7 RNA polymerases, and others. The DNA polymerase phi29 exhibits an extremely high processivity, but it has a too low activity optimum and inactivation temperatures (30 and 65°C, respectively). The large fragment of DNA polymerase I from *Geobacillus stearothermophilus* (Bst exo−) is a relatively thermostable (temperature optimum 60–65°C) and processive enzyme. Its mutant forms are available (e.g., Bst 2.0, Bst 2.0 Warm Start, Bst 3.0 manufactured by New England Biolabs,

United States, https://international.neb.com), which show a higher resistance to inhibitors and ensure a higher amplification rate. The Bst 3.0 variant also exhibits reverse transcriptase activity. Vent exo- is thermostable, has a high temperature maximum of activity (75°C), and possesses processivity comparable with that of Taq DNA polymerase. Phage T7 RNA polymerase is used for RCA, but it requires the presence of promoter sequences or a template with increased content of pyrimidine bases. At the same time, an insignificant change in the content of magnesium ions and ribonucleotide triphosphates varies its substrate specificity [25]. The use of reverse transcriptases for RCA was also reported [26]. Unfortunately, a significant drawback of the most popular DNA polymerase Bst exo- is that, along with the synthesis of a specific product, it can lead to the formation of nonspecific products [27, 28]. It is likely that other thermostable polymerases with strand displacement activity have a similar property (unpublished data). This feature may lead to false results or the misinterpretation of results. Thus, Yang et al. [29] proposed a new mechanism of amplification, the so-called jump-like RCA to explain the generation of products typical for
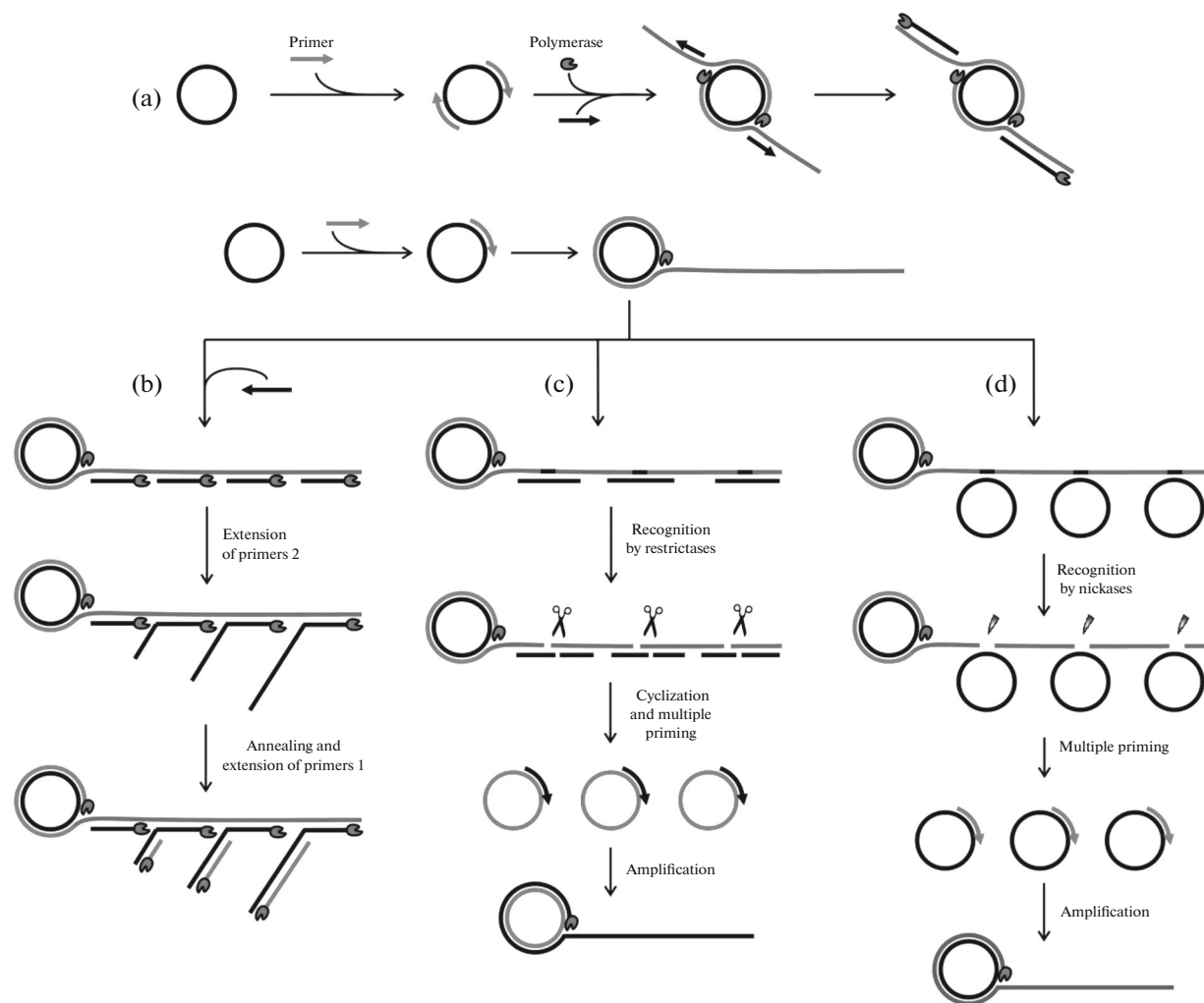
Fig. 4. Schemes of different variants of RCA with several primers: multiprimer RCA (a), ramification (b), circle-to-circle amplification (c), and RCA with primer generation (d).

RCA but formed in the absence of the CT. Earlier, several methods have been proposed to prevent side reactions of amplification that proceed by the action of Bst exo- DNA polymerase [30−32].

Methods for increasing the efficiency and productivity of RCA. Initially, the RCA was considered as a reaction of extension of a single primer that yields only one long single-stranded product. In this case, the reaction is called linear RCA, and the accumulation of products is arithmetic, since the number of templates that initiate the amplification remains constant. The sensitivity of the approaches based on linear RCA is low and often does not afford the diagnostically significant detection of a bioanalyte. To increase the efficiency of the reaction, more than one primer is used. A variant with simultaneous annealing of several primers to the CT received the name multiprimer RCA (Fig. 4a); its use is justified for relatively large CTs (e.g., plasmids) to obtain their copies for subsequent sequencing [33]. With the use of a second primer, which anneals to the single-stranded product of the extension of the first primer, the factor of amplicon multiplication increases, and the reaction begins to acquire an exponential course due to continuous displacement of synthesized strands, multiple annealing, and elongation of both primers (Fig. 4b). This RCA variant, which is called hyperbranched RCA or ramification [34], has found the most extensive use. In 2004, circle-to-circle amplification (C2CA) was developed [35] and later improved [36], which includes repeated cycles of CT replication, cleavage of RCAPs by restrictases, and the generation of novel CTs and their replication (Fig. 4c). Murakami et al. proposed primer generation−RCA [37], which is based on the cleavage of RCAP by nickase and the use of an additional CT (Fig. 4d). The introduction of nicks into RCAP leads to an increase in the number of 3'-ends capable of being elongated by polymerase [38].

The term most often used for all variants of RCA with several priming sequences is exponential RCA.

To increase the specificity and productivity of the RCA assay and to broaden the spectrum of instrumental methods for recording its results, variants of amplification on a solid surface (solid-phase RCA) were developed. For this purpose, one of the NA components of the reaction system: a primer, a splint, or a DNA probe for the target recognition is attached to a support. Depending on the method of analysis, biomolecules are immobilized on the surface of glass (microchip format [39]), polymeric materials (membranes [40] and microchannels [5, 41]), and noble metals and graphite (electrochemical biosensors [6, 7] and surface plasmon resonance spectroscopy [42]). The chemistry of immobilization depends on the material of the support; as a rule, the fixation of biomolecules is accomplished through the formation of covalent bonds during the interaction of the functional groups or surface atoms with the molecule being immobilized (COOH-, NH_2-, HS-, CHO-groups, surface metal atoms, and others) or through affine interaction (biotin−streptavidin, digoxigenin−antibody, antigen−antibody systems, and others). To increase the loading, the surface is functionalized by dendrimeric structures [43].

RCA in the microchip format offers a parallel analysis of multiple samples, which exceeds in performance multiplex assays in micro test tubes [39]. The immobilization of NA components of reaction systems on the surface of magnetic microparticles makes it possible, by using an external magnet, either to selectively isolate the analyte under test and/or transport it to the reaction zone, or to transport RCAP to the zone of the recording of the analytical signal [44]; upon fixation on conducting surfaces, it enables the use of electrochemical methods of analysis [4−6]. Technologies involving solid-phase RCA are characterized by wide variety, and most of them are complex cascade processes that combine the stages of biotarget recognition, competitive annealing of NA structures, production of the CT, RCA, generation of the analytical signal, and others (Fig. 5).

Due to steric hindrances, enzymatic processes at the surface proceed with a lower efficiency; therefore, a novel strategy has been proposed recently to increase the amplicon multiplication factor, which combines the RCA with the technology of so-called DNA walkers. For this, two or more types of DNA probes, of which one recognizes the NA under study, and the others are involved in the generation of the analytical signal, are immobilized on the support. After the NA is fixed by probe no. 1, a cascade of repeated rounds of denaturation−annealing of NA structures and enzymatic reactions is triggered on the surface, leading to the synthesis of RCAPs [45−47].

Detection of the results of RCA assay. The compatibility of RCA with various reporter systems makes it possible to detect the results using a wide range of instrumental methods at the endpoint and in real time. Among the first methods of registration of RCAPs were gel electrophoresis and the use of intercalating dyes and fluorescent hybridization probes, including those that function owing to fluorescence resonance energy transfer (FRET). A change-over of RCA to the solid-phase format and the use of micro- and nano-size particles (magnetic particles, quantum dots, metal nanoparticles, and others) made possible the application of electrochemical, spectroscopic, acoustic, and optical methods. Table 1 gives the most commonly used reporter systems and the principles of their functioning.

As in most amplification methods, the measurement of fluorescence is the most popular way for recording the results of the RCA assay. A fluorescent signal is obtained using various techniques; the most commonly used are the addition of intercalating dyes (SYBR Green I, thioflavin T, porphyrins, etc.) and fluorescent hybridization probes into the reaction mixture or the oxidation of a fluorogenic substrate (Table 1). Unfortunately, although the use of intercalating dyes is a very convenient variant of detecting the RCAPs, it features a low specificity. More reliable results are obtained using the fluorescent probes, oligonucleotides containing at least one fluorophore (an organic dye or a quantum dot) in the molecule, as well as quenchers/acceptors of light radiation. The probes can have different structures: there are simple probes (labeled with one fluorophore), hairpin-shaped probes (molecular beacons), which are cleaved by endonucleases or catalytically active RCAPs (DNAzymes), composite probes, etc. Another convenient technique is the addition of fluorescent nucleoside triphosphates to the reaction mixture, which provide the synthesis of fluorescently labeled amplification products [58−61]; however, it should be taken into account that, in some cases, the use of these substrates can lead to a decrease in the efficiency of the reaction or its complete inhibition [61, 62]. Of some interest, in terms of the reporter system used, is the work of Chen et al. [63], who proposed special "inverted" C-probes with poly-dA motifs, which lead to the formation of RCAPs with multiple dT_{40} repeats. The latter form a strong complex with copper nanoparticles, which is characterized by a high fluorescence quantum yield with a maximum at 580−600 nm and a large Stokes shift ($\Delta\lambda \sim 150$ nm). In technologies involving the measurement of a luminescent signal, RCAPs form DNAzymes or G-quadruplexes, which, in the presence of appropriate cofactors (metal cations, heme, dNTP, and others), acquire the peroxidase activity and catalyze the oxidation of fluorogenic substrates, e.g., luminol, accompanied by the emission of light radiation [52−57].

The detection of RCA results from a change in the solution color often does not require the use of expensive equipment since it can be accomplished visually.
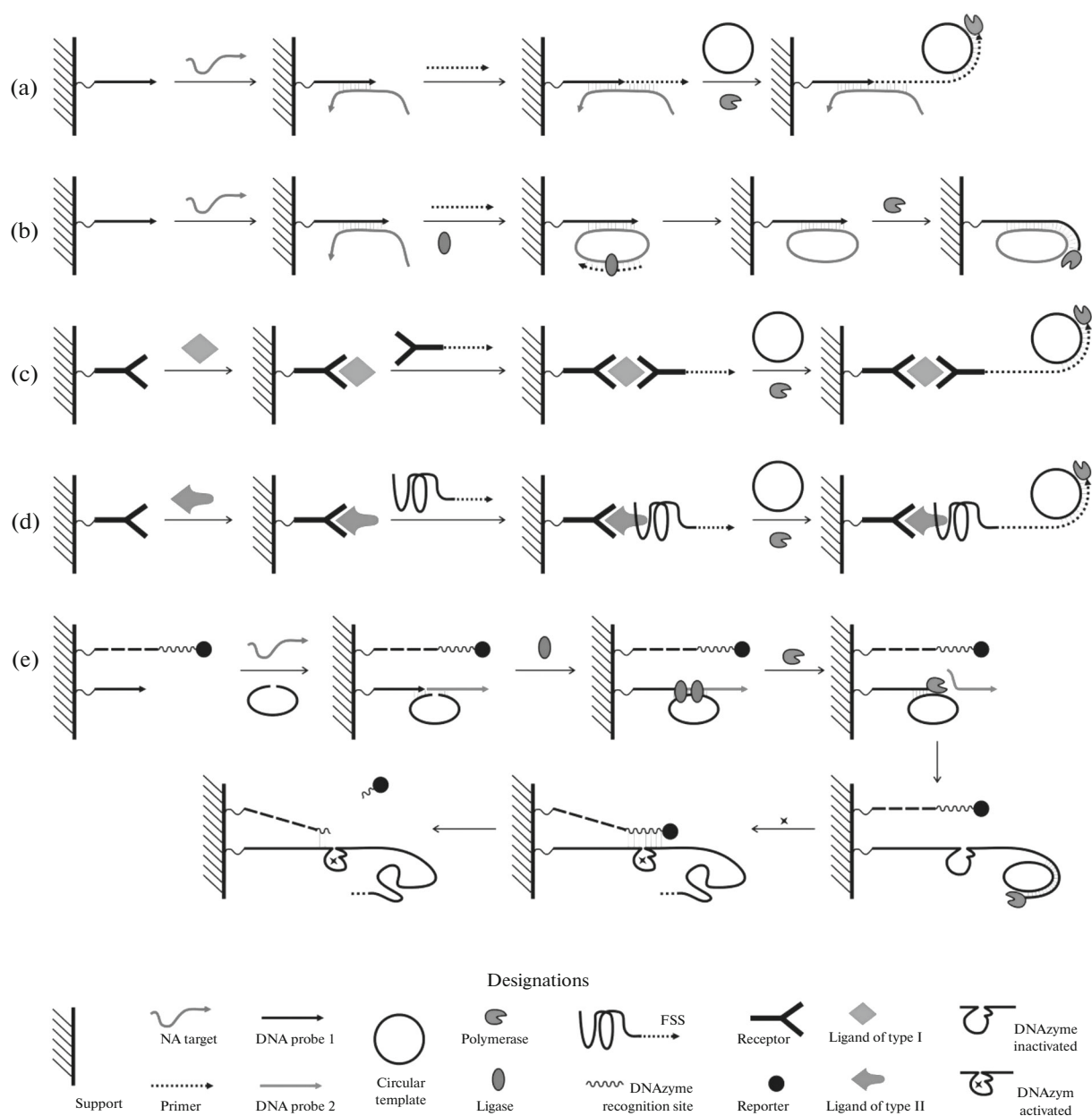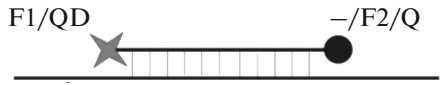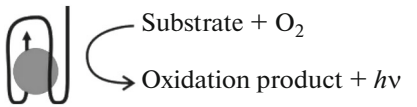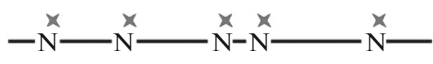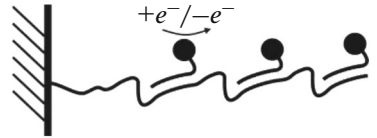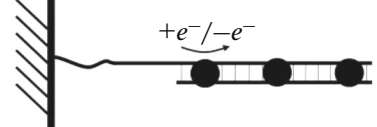
Fig. 5. The most commonly used variants of solid-phase RCA: (a) capture of an NA target by the immobilized DNA probe, target-mediated ligation of the probe with the primer, and the initiation of RCA; (b) capture of an NA target by the immobilized DNA probe, the cyclization of the NA target, and the initiation of RCA; (c) recognition of a target other than NA by an immobilized receptor, formation of a sandwich complex with a conjugate of the receptor with the primer, and the initiation of RCA; (d) recognition of a biotarget other than NA by the immobilized receptor and FSS, which carries the primer, and initiation of RCA; (e) variant of the combination of RCA with the "DNA walker" technology [45].

In colorimetric methods, the oxidation of chromogenic substrates: 2,2'-azino-bis(3-ethylbenzothiazolin-6-sulfonic acid) (ABTS), 3,3'-diaminobenzidine (DAB), 3,3',5,5'-tetramethylbenzidine (TMB), and *o*-phenylenediamine (OPD) to stained products has found the greatest application (Table 1). Colorimetric hybridization probes based on nanoparticles of metals, primarily gold (GNP), were developed (Table 1). The tandem origin of the RCA prod-

uct causes the approach of metal nanoparticles during the annealing of these probes to it; in the case of GNP-based probes, the color of the solution changes from red (purpl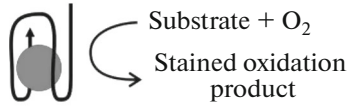e), characteristic of spatially separated GNP, to bluish, typical for GNP aggregates. The stability of GNP-based probes during the amplification analysis is a quite important problem; earlier, we proposed a method for increasing their stability [71]. Along with the use of GNP in the production of colo-

Table 1. The ways of the generation of an analytical signal, widely used in the RCA assay

Type of signal	Reporter	Principle of functioning	Reference
(1) Fluorescent	(a) Intercalating dyes		[48, 49]
	(b) Fluorescent hybridization probes	F1/QD −/F2/Q	[50, 51]
	(c) Fluorogenic substrate	Substrate + O_2 → Oxidation product + $h\nu$	[52−57]
	(d) Fluorescent nucleotides	—N—N—N-N——N—	[58−62]
	(e) Metal nanoparticles and metal complexes		[63]
(2) Colorimetric	(a) Chromogenic substrate	Substrate + O_2 → Stained oxidation product	[64−66]
	(b) Colorimetric hybridization probes	Red → Aggregation → Blue	[67]
(3) Electrochemical	(a) Hybridization probes with an electroactive or a conductive label	$+e^-/-e^-$	[45, 46, 68, 69]
	(b) NA-binding of electroactive particles	$+e^-/-e^-$	[45, 70]

rimetric probes, another advantage of GNP is the possibility of their use for the delivery of oligonucleotides, e.g., RCA primers, to cells [72].

Most electrochemical detection techniques rely on the use of either hybridization probes carrying an electroactive (methylene blue, ferrocene, etc.) or a conductive label (metal nanoparticles), or electroactive particles (heme, metal nanoparticle, methylene blue, etc.) that noncovalently bind to NAs. Electrochemical approaches became the basis for the development of various portable biosensor devices, which are mainly used in medicinal diagnostics [6].

In recent years, the types of analytical signals nontraditional in biochemical investigations have found application in RCA assay. Thus, a new direction is the use of the methodologies of magnetooptics [44, 73] and acoustics [74]. According to Garbarino et al. [73], an NA target being analyzed is fixed on the surface of magnetic microparticles through their capture by special DNA probes, the RCA is carried out, and then, the course of the enzymatic reaction is analyzed from changes in the optical properties of the solution in the magnetic field. Yang et al. developed an acoustic analyzer in which a bacterial DNA being examined is fixed on magnetic microparticles using capture DNA probes after which the RCA is initiated [74]. Since DNA molecules absorb sound radiation in the terahertz range worse than water molecules, RCA products cause substantial absorption of terahertz radiation after the successful completion of the reaction, which can be recorded by acoustic spectroscopy.

AREAS OF APPLICATION OF ROLLING CIRCLE AMPLIFICATION

Initially, RCA was mainly used for the detection of specific nucleotide sequences; however, with the development and emergence of novel molecular tools, it has transformed into a convenient method for studying more complex bioobjects, including in vivo, and began to provide highly sensitive identification of the analytes of various types.

Analysis of nucleic acids. RCA was first applied in genotyping, in particular, in studies of single nucleotide polymorphism (SNP) [75, 76]. For this purpose, allele-specific C-probes are designed whose cyclization by discriminatory ligation is possible only if the 3'-terminal nucleotide is completely complementary to the template DNA (Fig. 3c). Later, SNP typing using RCA was converted into the efficient chip [77] and multiplex [78] formats, and approaches were proposed that combine the allele-specific ligation and RCA with new methods for generating an analytical signal [50].

The greatest number of earlier works on RCA were devoted to the detection of DNAs of various organisms [4, 5, 7]. The improvement of the RCA methodology caused the emergence of new unique methods of DNA analysis. For example, two calorimetric methods for determining the genetic material of the plant pathogen *Phytophthora infestans* [67] and the bacterium *Staphylococcus aureus* [79] were described. The former is based on the use of RCA triggered by the CRISPR/Cas9 system and functionalized GNPs as hybridization probes. A characteristic feature of the second method is a multilevel system of target recognition and generation of an analytical signal, which involves the use of biotin-labeled primers, digoxigenin-labeled probes, conjugates of GNP with antidigoxigenin and peroxidase, and the oxidation of TMB with peroxidase in streptavidin-functionalized microplates.

With the development of RCA, more attention has also been given to the identification of genetic material of various viruses containing single-stranded DNA or RNA, which are convenient as a splint in the cyclization of specific C-probes. At present, the RCA-mediated detection of viruses is implemented both in solution (test tubes) and microfluidic devices [80] and in the format of electrochemical biosensors [81]; it is combined with other enzymatic reactions, e.g., reverse-transcription PCR followed by SDA [82].

In recent years, the emphasis in the application of RCA has shifted toward the analysis of short NAs, primarily, small and noncoding RNAs. Ning et al. described a method for the detection of small RNAs with known length and sequence using RCT in the presence of SuperScript IV reverse transcriptase, *E. coli* RNA polymerase, and Nb.BbvCI nickase [83]. Ciftci et al. proposed a method for the detection of hypervariable RNA viruses, which is based on the use

of C-probes with degenerate 3'-terminal nucleotides [84]. For unambiguous RNA isoform differentiation, special variants of the C-probe, iLock-probes, were proposed [85].

Most of the works devoted to the application of RCA in the analysis of ribonucleic acids concern the quantification of microRNAs, which act as an important marker in the diagnosis of various pathological states [86]. Advances in this area are presented in recent reviews [87−89]. The first work concerned with the detection of microRNA using RCA was published in 2006 [90]. In 2011, another approach called miR-ID was proposed, which consisted in the cyclization of microRNA by nontemplate ligation followed by RT-RCT [26]. An unconventional method for microRNA analysis was described by Zhao et al.; according to the method, RCA is initiated by a special DNA-triplex structure with a TA·T motif [51]. In most cases, microRNA acts as a splint during the cyclization of the C-probe; it is often used simultaneously as a starting primer. To illustrate the diversity of available approaches, Table 2 presents only a few unique technologies of recent years in which unconventional methods of generating an analytical signal in the RCA assay of microRNA have been implemented. Most of the technologies make it possible to detect femtomolar concentrations of microRNA; in addition, many provide the analysis of polymorphic variants of microRNA within one family [92].

In very recent years, the RCA method has been used for the analysis of circular RNAs, which, similar to microRNAs, play an important role in the regulation of gene function [93]. The targets of this type are detected by performing the RCT directly with an RNA sample isolated from the biomaterial, since there is no need to carry out the cyclization of the NA or to additionally introduce it. When performing RCT with reverse transcriptase, synthesized transcripts are detected either by RT-PCR or gel electrophoresis [94].

When developing new methods of RCA assay, the researchers also paid attention to CRISPR/Cas systems of genome editing. Qiu et al. were the first to use the CRISPR/Cas9 system for the detection of microRNA [95]. Later, variants of exponential RCA were proposed in which microRNA acts as a primer for the RCT, which leads to the production of single-stranded RNA with repeats cleaved by Cas12a nuclease; as a result, new primer sequences are generated [96]. The CRISPR/Cas system was applied not only for the detection of specific NA targets but also to assess the formation of nonspecific RCA products, when used as an internal negative control in diagnostic test systems [97].

A significant achievement was the use of RCA for the analysis of NA in vivo, which is particularly timely for RNA molecules [98, 99]. Duckworth et al. proposed a method for the multiplex RNA profiling and assessment of the expression of surface proteins for a

single cell, which made it possible to study the phenotypic and functional heterogeneity of cell populations [100]. The approach described in their work involves the stage of complex formation with lanthanide ions (for detection by mass cytometry) or fluorophores (for detection by flow cytometry). Zhang et al. were the first to perform in vivo detection of microRNA [101]; they developed biodegradable metal organic nanoparticles for the delivery of DNA polymerase phi29 and specific CT into live cells and for the conductance of RCA in them.

Analysis of proteins and small molecules. Proteins rank the second in popularity after NAs among biomolecules detected using RCA approaches; the latter often include the enzyme immunoassay (EIA) techniques. RCA itself acts in the case of proteins as a tool that mediates their recognition and/or the generation of an analytical signal. As a rule, RCA is preceded by the recognition of a protein target by a receptor conjugated with a primer or a DNA probe; this process is often combined with the selective extraction of the target from the sample and/or its fixation onto a solid carrier. The first technology for the detection of proteins by RCA was the approach called by the authors immuno-RCA [102]. According to this approach, the oligonucleotide primer that triggers RCA on a special CT is preliminarily conjugated with an antibody. After the termination of RCA, the amplification product remains bound to the antibody and can be detected by EIA, e.g., after fixation in wells of a standard plate (as shown in Figs. 5c, 5d). As in the case of NA, a large number of unique methodical works devoted to the detection and highly sensitive identification of proteins using RCA have appeared in recent years (Table 3).

In the case of proteins, the analysis in vivo is confined so far only to the detection of membrane proteins. For example, Liu et al. described a method for the visualization of MUC1 using a complex of three oligonucleotides, one of which formed an aptamer to the protein and was responsible for the localization of this complex on the cell surface, the second initiated RCA, and the third was labeled with the fluorophore Cy5 and ensured the generation of an analytical signal [110].

RCA has found application also for the analysis of relatively small organic molecules: nucleoside triphosphates, medicinal and narcotic substances, toxins, and pesticides. Thus, Qiu et al. proposed a method for the identification of dNTP in cell extracts with a detection limit of several pM [111]. Lin et al. described a method for the quantitative estimation of heparin in blood plasma with a detection limit of 0.83 mM, which is based on the heparin-induced displacement of primers triggering the RCA from the complex with protamine [112]. Aptamer-mediated methods for the detection of toxins in various objects (e.g., ochratoxin A in beer [113] and urine [114]), of pharmaceutical agents [115], and phosphoroorganic pesticides [116] were developed.

Detection of exosomes and single cells. By the detection of exosomes and individual cells is meant a selective analysis of their specific types or populations using RCA approaches. The method is based on the recognition of surface biomolecules, mainly protein receptors specific to the object under study, which is accompanied by the generation of an analytical signal. The recognition is carried out by aptameric FSSs; the tandem nature of their arrangement in the chain of RCAPs can provide both the fixation of one RCAP molecule at several points of the surface of the same cell or vesicle and the interaction of one RCAP molecule with several cells or vesicles (Fig. 6). This feature makes possible the formation of a 3D scaffold from NA chains, which is capable of capturing and holding the test object. The formation of a 3D structure is often accompanied by conglomeration, which leads to the gelation of the reaction mass or precipitation [117].

The isolation and subsequent analysis of exosomes have become to be of interest owing to their involvement in intercellular communication processes. Exosomes have a significant potential as biomarkers for the diagnosis of various pathological conditions, e.g., cancer [118, 119]. The most popular target for the interaction with aptamers on the surface of exosomes is the membrane protein CD63, and an analytical signal is generated using various reporter systems such as GNP with fluorescent probes attached to them [120], electroactive complexes of G-quadruplexes with the heme [121], oxidation of ABTS by horseradish peroxidase incapsulated into nanoparticles [66], etc. For increasing the specificity of analysis of exosomes, a method was proposed that uses two DNA probes carrying a cholesterol residue and an aptamer to CD63 at 5'-ends, respectively [122].

Despite their impressive size, cells have also become an object of the application of RCA. Thus, Yao et al. described a method for the selective isolation of bone marrow mesenchymal stem cells circulating in human blood [117]. It involves the use of RCA for producing two types of RCAPs, one of which forms aptamers to the APLP protein, and the second contains regions complementary to the first strand. The incubation of a mixture of RCAPs and blood results in the formation of a three-dimensional network of DNA strands in which stem cells are fixed; the resulting conglomerate easily precipitates and is then isolated. Jiang et al. carried out the capture of the cells of the pathogenic strain *E. coli* O157:H7 by plugging the liquid microchannel flow [41]. A highly sensitive spectrofluorimetric method for the detection of circulating tumor cells was proposed, which was tested on a model of human breast carcinoma [123]. Another, electrochemical method for the detection of circulating tumor cells relies on the capture of tumor cells by an RCA product biotinylated through the incorporation of biotin-labeled dA nucleotides during the synthesis and fixation on a streptavidin-modulated electrode [124].

Table 2. Examples of works on microRNA analysis, which describe unique methods for the generation of an analytical signal

Reference	Role of microRNA	Principle of technology	Detection limit, M
[48]	Primer	MicroRNA primes RCA on a dumbbell C-probe; the product of linear RCA forms G-quadruplexes into which it intercalates thioflavin T, providing the generation of a fluorescent signal	4.9×10^{-15}
[49]	Initiation of cleavage of DNA duplexes and generation of primers	MicroRNA molecules anneal to the RCA product obtained with CT no. 1, and the resulting double-stranded structures are cleaved by a duplex-specific nuclease to fragments that further prime RCA with CT no. 2. A new RCA product forms G-quadruplexes the binding of which to a complex of protoporphyrin IX and Zn^{2+} ions causes the generation of a fluorescent signal	1×10^{-15}
[53]	Splint and primer	MicroRNA ensures the closure of the C-probe and primes RCA, the product of which is cleaved by restrictase into fragments that give single G-quadruplexes, which bind to the heme. The resulting complexes catalyze the oxidation of o-phenylenediamine to a stained product, which causes fluorescence quenching of quantum dots based on MoS_2	4.6×10^{-15}
[54]	Splint	In the presence of microRNA, the C-probe is closed to form a circle, which, in the course of RCA, provides the formation of FSS, the trimer G3, which acquires, after the binding to the heme, the properties of peroxidase. The analytical signal (color change) is generated due to the oxidation of ABTS catalyzed by the indicated peroxidase mimetic	3.7×10^{-14}
[59]	Splint and primer	MicroRNA ensures the closure of the C-probe and primes RCA in the presence of naphthalimide-modified dUTP triphosphate, providing the generation of the fluorescent amplification product	3.58×10^{-15}
[68]	Triggering of a cascade of enzymatic reactions	MicroRNAs from exosomes bind to a special LNA-probe immobilized on magnetic microparticles, displacing oligonucleotides from the complex with it, which then hybridize with probes fixed to a golden electrode and prime RCA on its surface. The RCA product forms G-quadruplexes, which bind the electroactive dye methylene blue, providing the generation of an electrochemical signal	2.75×10^{-15}
[91]	Primer	MicroRNA is captured by the CT, which is held on the surface of streptavidin-modified magnetic particles by a probe partially complementary to it. MicroRNA primes RCA; the amplification product is detected by the intercalating dye SGI	1×10^{-13}
[92]	Splint	MicroRNA is fixed to the surface of microfluid channels using a capture DNA probe and mediates subsequent splint ligation of the adaptor, which acts as a primer for RCA. The cleavage of RCA product by nickase leads to the formation of new molecules that initiate amplification. The results are detected using the intercalating dye SGI	$<1 \times 10^{-20}$

The RCA assay is also used for the identification of various pathogenic microorganisms. Franch et al. developed an approach for identifying mycobacteria, the causative agents of tuberculosis, which involves the detection of the enzymatic activity of topoisomerase IA encoded by mycobacteria [125]. By an example of methicillin-resistant *Staphylococcus aureus*, a technology for the identification of antibiotic-resistant microorganisms was proposed, which is based on the formation of specific aptamers in the course of RCA and involves an enhancement of the analytical signal through the combination with the CRISPR-Cas12a system [126]. Methods were developed for the detection of fungal pathogens, for example, the members of the genus *Fonsecaea* occurring in Brazil [127], and unicellular algae [128].

Table 3. Examples of works on the analysis of proteins, which describe unique ways for the generation of an analytical signal

Reference	Target	Principle of technology	Detection limit
[60]	Alkyladenine DNA glycosylase (AAG) and uracil-DNA glycosylase (UDG)	A bifunctional biotinylated double-stranded DNA probe containing hypoxanthine and uracil at the opposite strands is exposed to alkyladenine DNA glycosylase (AAG) and uracil-DNA glycosylase (UDG), respectively, and is then cleaved by APE1 to form two primers that initiate RCA. The amplification in the presence of labeled nucleotides Cy3-dCTP (for the identification of AAG) and Cy5-dGTP (for the identification of UDG) provides the generation of a fluorescent signal. The immobilization of RCA products on streptavidin-modified magnetic particles provides their selective isolation from the solution, and subsequent cleavage by exonuclease makes it possible to quantify the corresponding enzymes by the fluorescence level	6.1×10^{-9} unit/mL of AAG, 1.5×10^{-9} unit/mL of UDG
[64]	Antibodies to hepatitis C virus (HCV)	Two special DNA probes are conjugated with the HCV antigen. In the presence of the antibody to HCV, the probes come close together and initiate the SDA reaction as a result of which a primer initiating the RCA is produced. The RCA product forms G-quadruplexes with peroxidase activity, which catalyze the oxidation of 3,3',5,5'-tetramethylbenzidine to a colored product	1.0×10^{-12} M
[70]	Thrombin	A graphene electrode is modified by GNP to which then special RCA-initiating DNA probes are attached. As a result of amplification, aptamers to thrombin form, the binding of which on the surface of the electrode changes its electrochemical potential	3.5×10^{-14} M
[103]	T4 DNA ligase and polynucleotide kinase	Polynucleotide kinase and ligase catalyze the phosphorylation and cyclization of the C-probe, respectively. Their content in a sample is estimated from the RCA product the efficiency of production of which correlates with the content of these enzymes in the sample	3.4×10^{-4} unit/mL of ligase, 3.8×10^{-4} unit/mL of polynucleotide kinase
[104]	NF-κB p50	The binding of the protein to a specific hair-pin DNA probe triggers a cascade of enzymatic conversions, which involves the reactions of SDA, RCA, and nickase cleavage and leads, in the case of firm binding, to the formation of G-quadruplexes the interaction of which with thioflavin T provides the generation of a fluorescent signal	1.0×10^{-13} M
[105]	Transcription factors	The binding of proteins to a specific hair-pin DNA probe prevents its cleavage with nickase, and by ensuring its integrity, makes it possible to perform further the closure of the probe and subsequent RCA, resulting in the formation of G-quadruplexes. The interaction of the latter with *N*-methyl-mesoporphyrin IX causes the generation of a fluorescent signal	88×10^{-12} M

Table 3. (Contd.)

Reference	Target	Principle of technology	Detection limit
[106]	Prostate-specific antigen (PSA)	PSA mediates the binding of magnetic particles to GNP that carry primers for RCA whose product hybridizes with probes conjugated with invertase. The enzyme converts sucrose to glucose the level of which is measured by a personal glucometer	0.1 pg/mL
[107]	DNA-methyl transferase 1 and uracil-DNA glyco-sylase (UDG)	A specific DNA probe carrying the methyltrans-ferase recognition site and uracil is subjected to DNA methyl transferase 1 and UDG followed by treatment with BssHII and Endo IV endonucle-ases. As a result of cleavage, a short single-stranded DNA is released, which initiates RCA, the product of which forms G-quadruplexes; the intercalation of thioflavin T in them causes the appearance of a fluorescent signal	0.009 unit/mL of methyl transferase, 0.003 unit/mL of UDG
[108]	Dam-methyltransferase	A special DNA probe with the methyltransferase site is methylated by the action of the enzyme; its subsequent cleavage by DpnI leads to the forma-tion of primers that initiate RCA. The RCA prod-uct is detected using the SGI dye	1.8 unit/mL
[109]	Telomerase	In the presence of active telomerase, a special primer is extended by five nucleotides (GGGTT), which is sufficient for its further participation as a splint in the cyclization of the C-probe and initia-tion of RCA. The level of RCAP production is detected using molecular beacons	–

Other applications of RCA. Along with the analysis of above-described biotargets, RCA is also applied to detect analytes of nonbiological origin. Thus, the methods for the detection of heavy metal ions in the environmental objects were proposed, which are based on the cleavage of fluorescent probes by the action of
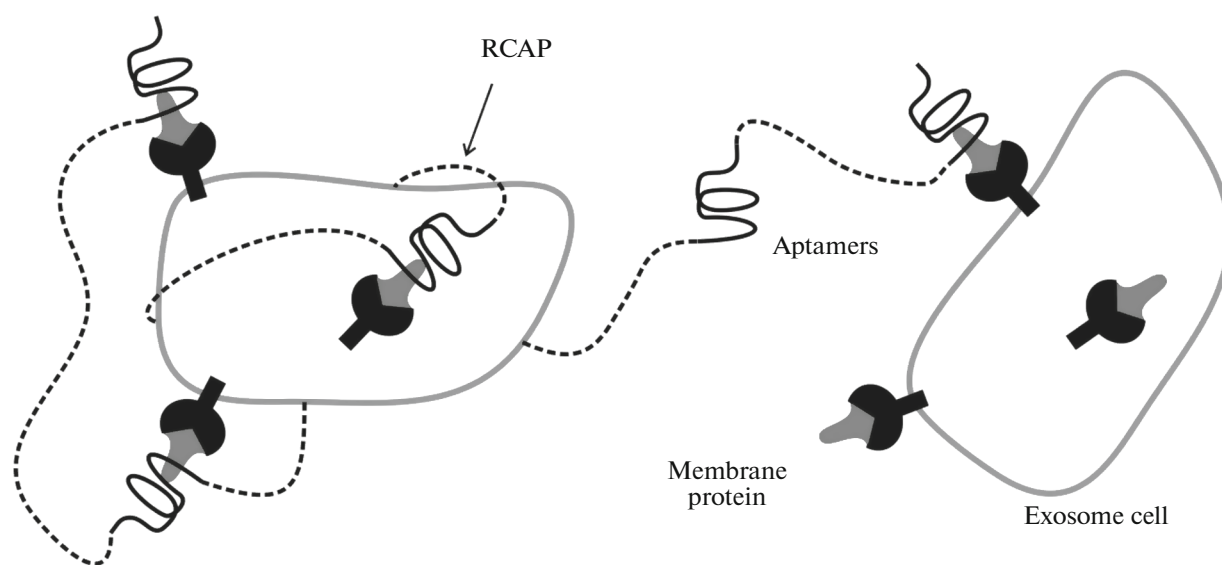

Fig. 6. Recognition of cells or exosomes by an RCA product through the interaction of aptamers with receptors on their surface.

DNAzymes that are contained in the structure of RCAPs and are activated in the presence of these cations or due to complex formation with NA [129]. In addition to analytical applications, RCA is used for the high-performance amplification of large CTs during the creation of libraries for sequencing [33] and for the genetic analysis of bacterial symbionts by the generation of products of the amplification of their specific plasmids during RCA [130]. As in the case of plasmids, the amplification and subsequent sequencing of the mitochondrial genome [131] and circular viral genomes are possible [132]. RCA is a more convenient alternative to PCR for the production of large amounts of candidate sequences during the selection of aptamers using the SELEX strategy [133]. The possibility to construct circular templates with the preset nucleotide sequence makes it possible to obtain RCAPs that provide the formation of self-assembling DNA nanostructures, which is of interest for bionanotechnology [4], in particular, for the targeted delivery of drugs and the systems of RNA interference and genome editing [134−136].

CONCLUSIONS

The data presented in the review demonstrate that rolling circle amplification is a versatile tool, which, when included in a new analytical method or technology, can ensure the analysis of biotargets of various chemical nature: nucleic acids, proteins, enzymes, antibiotics, toxins, etc. RCA-mediated approaches are applicable for both the qualitative detection of analytes and their quantification; they feature a high sensitivity with detection limits up to concentrations of the order of 10^{-15} M. The high specificity and sensitivity of the RCA assay make it possible to detect analytes in complex media, i.e., without isolation in the pure form.

A great number of recent publications on RCA-mediated analysis indicate an increasing interest in, and a significant practical potential of, the RCA reaction; however, the share of studies on its application for the quantification of bioanalytes in real samples is not large enough. In addition, as distinct from other isothermal amplification methods [137−139], RCA has not yet found application in the molecular diagnosis of the SARS-CoV-2 coronavirus. Apparently, further studies are needed to improve and optimize the corresponding RCA-mediated techniques, to test them on clinical material, to simplify, if possible, some technologies and methods, and to search for, or create novel molecular tools that broaden the potential of RCA. Undoubtedly, during the development of diagnostic test systems, the required accuracy and specificity of analysis should be provided, which will rule out both false-positive and false-negative results.

COMPLIANCE WITH ETHICAL STANDARDS

The article does not contain any studies involving human or animal participants, performed by any of the authors.

Conflict of Interests

The authors declare that they have no conflict of interest.

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Translated by S. Sidorova