APTAMERS IN BIOANALYSIS

Edited by

MARCO MASCINI
University of Florence, Italy

WILEY

A JOHN WILEY & SONS, INC., PUBLICATION

APTAMERS IN BIOANALYSIS

APTAMERS IN BIOANALYSIS

Edited by

MARCO MASCINI
University of Florence, Italy

WILEY

A JOHN WILEY & SONS, INC., PUBLICATION

Published by John Wiley & Sons, Inc., Hoboken, New Jersey.
Published simultaneously in Canada

Library of Congress Cataloging-in-Publication Data:

Mascini, Marco
Aptamers in bioanalysis /
p. cm.
Includes bibliographical references and index.
ISBN: 978-0-470-14830-3 (cloth)

Printed in the United States of America

10 9 8 7 6 5 4 3 2 1

CONTENTS

PREFACE

I am very pleased to act as editor of this book, *Aptamers in Bioanalysis*. Aptamers started as therapeutic agents and in very few years they have become a hot topic in analytical chemistry. In our laboratory we are working on realizing reliable sensors and biosensors, and aptamers appear as optimal components for their assembling. Aptamers appear in this application as a new class of ligands with exceptional binding constants (micromolar to picomolar range). Aptamers are also entering many analytical applications, such as, in the technologies based on separation science (various chromatographic techniques or capillary electrophoresis). Many new exciting analytical problems can be solved with these new compounds.

The increasing presence of contaminants in food, air, or drinking water that are capable of causing intoxication, diseases, or chronic illness has led to the need for analytical systems capable of rapid, and often multianalyte, measurements of complex samples. This need also exists in the medical field where multiparameter diagnostic systems are increasingly required to detect all the well-known and the more recently discovered biomarkers for different diseases. Unfortunately for medical practitioners, disease-related biomarkers are either physiologically present in minute quantities or severely contaminated by nonspecific compounds in a patient's bloodstream or body fluid. Hence, highly sensitive as well as specific recognition elements are required for effective detection of such biomarkers.

When the detection system requires a biomolecular recognition event, antibody-based detection methodologies are still considered the standard assays in environmental, food, and clinical analysis. These assays are well established, and they have been demonstrated to reach the desired sensitivity and selectivity. However, the use

of antibodies in multianalyte detection methods and in the analysis of very complex samples encounter some limitations derived mainly from the nature and synthesis of these protein receptors. In order to circumvent some of these drawbacks, other recognition molecules are being explored as alternatives.

The awareness that nucleic acids, RNA in particular, can assume stable secondary structures and that they can be easily synthesized and functionalized, has opened the door for aptamers in several applications.

The main advantage is overcoming the use of animals or cell lines for the production of the molecules. Moreover, antibodies against molecules that are not immunogenic are difficult to generate. Aptamers, on the contrary, are isolated by in vitro methods that are independent of animals: an in vitro combinatorial library can be generated and exploited against any target. In addition, generation of antibodies in vivo means that the animal immune system selects the sites on the target protein to which the antibodies bind. The in vivo parameters restrict the identification of antibodies that can recognize targets only under physiological conditions limiting the extension to which the antibodies can be functionalized and applied.

Moreover, the aptamer selection process can be manipulated to obtain aptamers that bind to a specific region of the target, with specific binding properties, and in different binding conditions. After selection, aptamers are produced by chemical synthesis and purified to a very high degree eliminating the batch-to-batch variation found when using antibodies. By chemical synthesis, modifications in the aptamer can be introduced enhancing the stability, affinity, and specificity of the molecules. Often the kinetic parameters of an aptamer-target complex can be changed for higher affinity or specificity. Another advantage over antibodies can be seen in the higher temperature stability of aptamers; in fact, antibodies are large proteins sensitive to temperature that can undergo irreversible denaturation. On the contrary, aptamers are very stable and they can recover their native active conformation after denaturation.

The selection process itself, with the amplification step, gives some advantages to aptamers with respect to other "nonnatural" receptors, such as oligopeptides, which cannot be amplified during their selection procedure. Therefore, once again the polymerase chain reaction appears as the magic tool to solve the problem of obtaing highly selective ligands. Our colleagues in genetics departments are working to overcome this issue and in the future we will be happy to obtain from libraries other nice ligands of different nature, like polypeptides or polysaccharides, rather than oligonucleotides!

We now have a new class of biosensors, aptasensors, which use aptamers as highly selective recognition elements. As receptor molecules, aptamers allow widespread applicability to a diverse array of target analytes due to their analyte-impartial synthetic generation process. Aptasensors realized on micro- and nanoscale platforms afford many potential advantages, such as miniaturized construction; rapid, sensitive, and specific detection; high throughput; reduced

costs; and minimized material consumption. Thus, micro- and nanoaptasensors are highly attractive for a broad range of applications, such as proteomics, metabolomics, environmental monitoring, counterterrorism, and clinical diagnostics and therapeutics.

MARCO MASCINI

CONTRIBUTORS

MORITZ K. BEISSENHIRTZ, Analytical Biochemistry, Institute of Biochemistry and Biology, University of Potsdam, Karl-Liebknecht-Strasse 24–25, Bldg. 25, D 14476 Potsdam-Golm, Germany

JEAN-PIERRE DAGUER, Université Victor Segalen Bordeaux II, INSERM U869, Laboratoire ARNA, Bâtiment 3A 1er étage, 146 Rue Léo Saignat, 33 076 Bordeaux Cédex, France

ERIC DAUSSE, Université Victor Segalen Bordeaux II, INSERM U869, Laboratoire ARNA, Bâtiment 3A 1er étage, 146 Rue Léo Saignat, 33 076 Bordeaux Cédex, France

SHAOJUN DONG, State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences Changchun, Jilin 130022, P.R. China

TIBOR HIANIK, Department of Nuclear Physics and Biophysics, Faculty of Mathematics, Physics and Informatics, Comenius University, Mlynská dolina F1, 842 48 Bratislava, Slovakia

KAZUNORI IKEBUKURO, Department of Biotechnology & Life Science, Faculty of Technology, Tokyo University of Agriculture & Technology, 2-24-16 Nakacho, Koganei, Tokyo, 184–8588, Japan

KAGAN KERMAN, Department of Chemistry, University of Saskatchewan, 110 Science Place, Saskatoon, S7N 5C9 Saskatchewan, Canada

SERGEY N. KRYLOV, Department of Chemistry, York University, Toronto, M3J 1P3 Ontario, Canada

Eik Leupold, Leibniz Institute of Molecular Pharmacology, Robert-Rössle-strasse 10, D 13125 Berlin, Germany

Bingling Li, State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, Jilin 130022, P.R. China

Fred Lisdat, University of Applied Sciences Wildau, Biosystems Technology, Bahnhofstrasse 1, D 15745 Wildau, Germany

Marco Mascini, Department of Chemistry, University of Florence, Via della Lastruccia 3, 50019 Sesto Fiorentino, Italy

Linda B. McGown, Department of Chemistry and Chemical Biology, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

Maria Minunni, Department of Chemistry, University of Florence, Via della Lastruccia 3, 50019 Sesto Fiorentino, Italy

Oliver Pänke, Fraunhofer Institute of Biomedical Engineering, Am Mühlenberg 13, D 14476 Potsdam-Golm, Germany

Eric Peyrin, DPM UMR 5063 CNRS/Université de Grenoble, Bât E (C) André Rassat, Domaine Universitaire, 301 avenue de la Chimie, BP 53 38 041 Grenoble Cédex 9, France

Corinne Ravelet, DPM UMR 5063 CNRS/Université de Grenoble, Bât E (C) André Rassat, Domaine Universitaire, 301 avenue de la Chimie, BP 53 38 041 Grenoble Cédex 9, France

Frieder W. Scheller, Analytical Biochemistry, Institute of Biochemistry and Biology, University of Potsdam, Karl-Liebknecht-Strasse 24–25, Bldg. 25, D 14476 Potsdam-Golm, Germany

Koji Sode, Department of Biotechnology and Life Science, Faculty of Technology, Tokyo University of Agriculture & Technology, 2-24-16 Naka-cho, Koganei, Tokyo 184–8588, Japan

Walter Stöcklein, Analytical Biochemistry, Institute of Biochemistry and Biology, University of Potsdam, Karl-Liebknecht-Strasse 24–25, Bldg. 25, D 14476 Potsdam-Golm, Germany

Regina Stoltenburg, Centre for Environmental Research Leipzig-Halle GmbH (UFZ), Environmental and Biotechnology Centre (UBZ), Permoserstr. 15, D-04318 Leipzig, Germany

Beate Strehlitz, Centre for Environmental Research Leipzig-Halle GmbH (UFZ), Environmental and Biotechnology Centre (UBZ), Permoserstr. 15, D-04318 Leipzig, Germany

EIICHI TAMIYA, Nanobiotechnology and Biodevice Lab, Department of Applied Physics. Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565–0871, Japan

SARA TOMBELLI, Department of Chemistry, University of Florence, Via della Lastruccia 3, 50019 Sesto Fiorentino, Italy

JEAN-JACQUES TOULMÉ, Université Victor Segalen Bordeaux II, INSERM U869, Laboratoire ARNA, Bâtiment 3A 1er étage, 146 Rue Léo Saignat, 33 076 Bordeaux Cédex, France

HUI WEI, State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, Jilin 130022, P.R. China

ITAMAR WILLNER, Institute of Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

ULLA WOLLENBERGER, Analytical Biochemistry, Institute of Biochemistry and Biology, University of Potsdam, Karl-Liebknecht-Strasse 24–25, Bldg. 25, D 14476 Potsdam-Golm, Germany

WATARU YOSHIDA, Department of Biotechnology and Life Science, Faculty of Technology, Tokyo University of Agriculture and Technology, 2-24-16 Nakacho, Koganei, Tokyo 184–8588, Japan

MAYA ZAYATS, Institute of Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

PART I

INTRODUCTION

CHAPTER 1

APTAMERS: LIGANDS FOR ALL REASONS

JEAN-JACQUES TOULMÉ, JEAN-PIERRE DAGUER, and ERIC DAUSSE

1.1 INTRODUCTION

Several strategies were described over the last 25 years that make the use of synthetic oligonucleotides in different perspectives of interest for biology and medicine, thanks to the development of very powerful and relatively cheap methods for nucleic acid synthesis on solid support. These strategies generally do not take into account the genetic information borne by the oligonucleotide chain but rather, explore its wide potential of molecular interaction and recognition. The *antisense approach* was the first to be developed (Belikova et al., 1967). It rests on a simple hypothesis: the binding to a target mRNA of a complementary sequence (the antisense sequence), thus leading to the formation of a local double-stranded structure, might interfere with reading the message. This was demonstrated experimentally in the late 1970s by pioneering works of Zamecnik and Stephenson (1978), on the one hand, and Miller et al. (1974), on the other. The progress made in the sequencing of entire genomes offered multiple opportunities for validating this technique, which was used not only against messenger but also against pre-mRNA (Sazani and Kole, 2003) or viral RNA (Toulmé et al., 2001). Very quickly it turned out that regular DNA or RNA oligomers suffered from weaknesses for use in biological media. Numerous chemically modified oligonucleotide analogs were synthesized and evaluated (Wilson and Keefe, 2006). In particular, modifications were introduced for improving resistance to nucleases (Toulmé, 2001). A number of clinical trials have been and are still being carried out (Aboul-Fadl, 2005), but disapointingly, a single antisense

Aptamers in Bioanalysis, Edited by Marco Mascini

oligonucleotide was approved by the U.S. Food and Drug Administration for the treatment of cytomegalovirus–related retinitis (Orr, 2001). More recently, similar modifications were introduced in small interfering RNA (siRNA) that also bind their target RNA through Watson–Crick base pairing (Wilson and Keefe, 2006). siRNAs generally show a higher efficiency than the homologous antisense sequence, due to triggering of the enzymatic destruction of the target transcript by the interference machinery (Scanlon, 2004; Chakraborty, 2007). But both antisense and siRNA suffer from the same types of limitations: target access, specificity of interaction, and cell uptake (Shi and Hoekstra, 2004; Kurreck, 2006).

Antisense oligomers and siRNAs sequences are designed rationally on the basis of Watson–Crick complementarity with the target transcript. During the last 15 years, combinatorial approaches have been developed in both chemistry and biology (Fernandez-Gacio et al., 2003; Frankel et al., 2003; Li and Liu, 2004). In such methods a family of candidates is randomly synthesized. Molecules exhibiting the desired property are then extracted from this pool. There is no prerequisite to the use of such methods: The structure of the target does not need to be known nor does one need to postulate the interactions that will take place between the target and the successful candidate. The interest in a combinatorial approach is related directly to the size of the pool—the molecular diversity—that can be screened. From this point of view, oligonucleotide libraries surpass by several logs the complexity of any other type of library. In vitro selection of oligonucleotides can be undertaken in pools containing up to 10^{15} different candidates (Gold et al., 1995). This is in large part related to the information encoded in the candidate. Consequently, in contrast to any other compound, oligonucleotide candidates from the pool can be amplified and analyzed very easily. Indeed, covalent fusion between an mRNA and the polypeptide it codes for allows the screening of very large libraries (10^{12} to 10^{13} independent fusion candidates) and makes the in vitro selection of proteins by ribosome display a very powerful method (Roberts, 1999).

Pioneering work recognized the interest of either selection or in vitro evolution of nucleic acid mixtures for the identification of protein-binding sites or replication suitability (Mills et al., 1967; Blackwell and Weintraub, 1990). But in 1990, three laboratories independently described a procedure for the identification, within large pools of randomly synthesized molecules, of nucleic acid sequences exhibiting a predetermined property: affinity for a given target (Ellington and Szostak, 1990; Tuerk and Gold, 1990) or enzymatic activity (Robertson and Joyce, 1990). This was achieved through repeated rounds of selection and amplification, thus ensuring directed evolution of the starting pool in response to selection pressure on the population. This method, now known as *SELEX* (systematic evolution of ligands by exponential enrichment) leads to the selection of aptamers [i.e., oligo*mers* able (*apt*) to carry out some function]. Since numerous papers described an entire range of applications for aptamers that take advantage of their wide potential due to both the high strength and the specificity of their interaction with their target (Osborne and Ellington, 1997; Famulok, 1999; Jayasena, 1999; Wilson and Szostak, 1999; Brody and Gold, 2000; Toulmé,

2000). Examples are found in many different fields, and excellent reviews were recently published that cover the use of aptamers for the validation of targets (Toulmé et al., 2001; Blank and Blind, 2005; Bunka and Stockley, 2006), for the design of therapeutic agents (Nimjee et al., 2005), for cancer (Ireson and Kelland, 2006), for infectious diseases (Held et al., 2006; James, 2007), for gene therapy (Fichou and Ferec, 2006; Que-Gewirth and Sullenger, 2007), for the development of analytic tools (Tombelli et al., 2005; Mairal et al., 2007), or for the design of probes for imaging (Pestourie et al., 2005). We describe the SELEX process briefly and review a few points dealing with two wide areas of application for aptamers: regulation and sensing.

1.2 THE POWER OF SELECTION AND APTAMER REFINEMENT

Only a few general points are addressed here. The reader is referred to a recent review by Gopinath (2007) for details on procedures for the selection of aptamers. Like any other combinatorial method, the SELEX methodology first requires synthesis of the library. Compared to other libraries, it is easy to prepare an unbiased pool of DNA sequences, as the coupling efficiency of the A, T, G, or C phosphoramidite is very similar. One could compensate for the slightly different incorporation of nucleotides: a mixture of phosphoramidites in a ratio of 1.5 : 1.25 : 1.15 : 1.0 (A : C : G : U/T) is believed to produce a balanced mixture of sequences (Ho et al., 1996). The diversity of the library is fixed by the length of the random region. One generates 4^n different sequences n nucleotides long. The experimental limit (about 10^{15}) corresponds to the diversity obtained for a random region about 25 nucleotides long. The full theoretical diversity is not covered for pools of candidates with a wider randomized window. For instance, only 1/1000 of the theoretical population will be present in a library of candidates with 30 random nucleotides. However, as the fidelity of the polymerases used in the SELEX process is rather low, each amplification round will generate variants that were not present at the preceding selection step, hence increasing the size of the sequence space explored.

Aptamers are then isolated by an iterative process (typically, 7 to 15 rounds) of binding, partitioning, and amplifying nucleic acid variants (Figure 1.1). The evolution of the population is driven by the selection conditions; the stringency (concentration, incubation time, washes, etc.) is increased progressively from round to round for selecting the candidates exhibiting the highest possible affinity (Gopinath, 2007). Selection is a tedious and time-consuming process when carried out manually. Automated selection was reported about 10 years ago (Cox et al., 1998; Brody and Gold, 2000; Eulberg et al., 2005). Several biotech companies have developed specialized procedures for high-throughput production of aptamers that reduce the isolation time from several months to a few days (Blank and Blind, 2005). An automated microchannel-based platform was recently described (Hybarger et al., 2006). The aptamers generated by automated selection are equivalent to those derived from manual selection, and those isolated against proteins show dissociation constants in the nanomolar range.

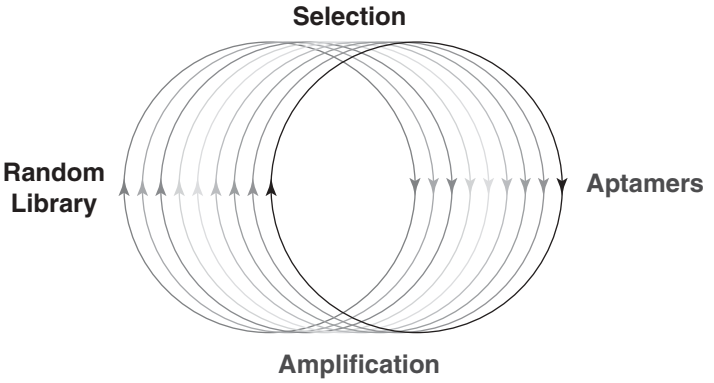

Figure 1.1 Scheme of in vitro selection. (See insert for color representation.)

Intramolecular base pairing defines higher-order structures. Therefore, a nucleic acid library of sequences is actually a library of three-dimensional shapes. Every candidate will display a unique combination of double-stranded helical segments, loops, and bulges. Each nucleotide may contribute hydrogen bonds and electrostatic and van der Waals interactions. The scaffold resulting from the intramolecular folding of oligonucleotides constituting the library offers a three-dimensional potential for interacting with any type of target. The selection process therefore corresponds to the capture of candidates that display a set of interacting groups complementary to that of the target. The association is even optimized through an induced fit mechanism: the aptamer acquires its final shape upon binding to its target (Patel et al., 1997; Hermann and Patel, 2000). This results in both a very strong affinity and a high specificity. For small molecules (e.g., amino acids, nucleosides, dyes, antibiotics), equilibrium dissociation constants in the micromolar range are frequent, whereas K_d values from 10^{-9} to 10^{-12} M^{-1} are typically obtained for proteins (Osborne and Ellington, 1997; Jayasena, 1999).

What makes aptamers ligands of great interest is their exquisite specificity. One of the clearest examples is the aptamer selected against theophylline, a purine derivative used for the treatment of asthma. This aptamer binds with a 10,000-fold-lower affinity to caffeine, another purine analog that differs from theophylline by a single methyl group on the N-7 position (Jenison et al., 1995). This exquisite selectivity was achieved through a careful selection procedure: oligonucleotides bound to the support functionalized with theophylline were first eluted with a solution of caffeine that made if possible to get rid of candidates that did not discriminate between the two purine compounds. A high degree of specificity could be reached even without such a counter-selection step. For example, the pseudoknot aptamer selected by Gold and co-workers against the reverse transcriptase (RT) of the human immunodeficiency virus does not bind to murine or feline RTs (Tuerk et al., 1992). But aptamers have been raised

against these enzymes that display similar affinity (K_d = 5 to 20 nM) and do not bind to the HIV enzyme (Chen and Gold, 1994; Chen et al., 1996). Strikingly, these aptamers are specific inhibitors of their cognate enzyme, indicating that they hinder and very likely bind to the catalytic site, as these enzyme are nucleic acid–binding proteins. Despite the identity of the function ensured by these polymerases, specific aptamers have been selected, suggesting that they do not interact with the conserved residues responsible for the catalytic activity. Similar results were obtained for targets that are not natural ligands of nucleic acids: aptamers raised against human immunoglobulin E (IgE) or against human matrix metalloproteinase 9 do not bind to their murine homolog (Mendonsa and Bowser, 2004; Da Rocha-Gomes et al., unpublished results). The same level of specificity can be reached with nucleic acid targets. It has been demonstrated that an aptamer raised against a hairpin structure and interacting with the loop through the formation of six base pairs was far more specific than the antisense sequence generating the same pattern of Watson–Crick pairing (Darfeuille et al., 2006). Compared to the antisense–sense duplex, the three-dimensional structure of the aptamer–hairpin kissing complex provides addional elements of recognition. Interestingly, in vitro selection was used to identify hybridization probes that discriminate strongly between variants of the human papilloma virus (Brukner et al., 2007). Optimizing the probes rather than the hybridization conditions generated oligonucleotides that show a "relaxed" binding potential (i.e., partial complementarity) but that minimize cross-reactivity. This method might be generalizable to the design of genotyping kits.

It has been possible, however, to select aptamers that cross-react with different molecular species. In toggle SELEX, the target is changed during alternating rounds of selection. This strategy yields aptamers that recognize both human and porcine thrombin and display similar properties toward the two proteins: plasma clot formation and platelet activation (White et al., 2001). This approach is useful when one wants to raise ligands recognizing a class of structurally and chemically related targets.

Due to these properties, aptamers are considered to be "chemical antibodies" and actually rival or sometimes surpass antibodies. Indeed, the ease and the reproducibilty of the synthesis as well as several other properties make aptamers interesting alternatives: it is, for instance, possible to raise aptamers against toxic substances.

1.3 THE CHEMISTRY DRIVES THE SHAPE

The initial random library is always synthesized as DNA, but the starting pool used for selection can be made of RNA candidates. In this case the DNA library is transcribed prior to SELEX. Either RNA or DNA aptamers exhibiting similar properties can be selected against a given target. The sequences selected against the HIV-1 reverse transcriptase constitute a good example. DNA and RNA aptamers are competitive inhibitors of each other, indicating that there is

a major site for aptamer interaction on the surface of the enzyme that drives the selection (Tuerk et al., 1992; Schneider et al., 1995). Importantly, the anti-HIV-1 RT RNA and DNA aptamers have very different sequences and structures. Whereas the RNA aptamer is a pseudoknot, the DNA aptamer is an imperfect hairpin. The DNA version of the RNA pseudoknot does not bind to the HIV-1 RT. It should be remembered that the conformation of DNA and RNA double helices differs. As aptamers are shapes, not surprisingly, changing the chemistry changes—generally weakens or even abolishes—the binding properties. It is therefore of prime importance to define the chemistry of the library prior to performing the selection. The choice of the aptamer chemistry is partly guided by the intended use of the aptamer. DNA aptamers are far cheaper than RNA aptamers. But if post-SELEX modifications have to be introduced, there are more RNA-mimic oligonucleotide derivatives; in addition, RNA aptamers can be expressed inside cells from DNA expression vectors.

Chemically modified aptamers have been developed due to intrinsic limitations of regular RNA and DNA oligomers (Wilson and Keefe, 2006). In particular, it has long been recognized from studies on antisense sequences that unmodified nucleic acids are short-lived species in biological media. The presence of nucleases in serum leads to the rapid digestion of natural oligonucleotides. Numerous modifications have been described by chemists that render nucleic acid resistant to nucleases (Toulmé, 2001; Wilson and Keefe, 2006). These include substitution at the 2′ position (e.g., 2′-*O*-methyl, 2′-fluoro) (Kubik et al., 1997; Prakash and Bhat, 2007), phosphate modification (e.g., phosphorothioate, phosphoramidate, morpholino) (Koizumi, 2007), nucleoside modification (e.g., α anomer, bicyclic sugar) (Orum and Wengel, 2001), or even the use of a polyamide backbone (peptide nucleic acid) (Elayadi and Corey, 2001). However, most of these modifications cannot be introduced during the SELEX process, as the modifed nucleotides are not substrates for polymerases and therefore cannot be used by the relevant enzymes either for generating the initial library or for amplifying the oligomers selected.

2′-Fluoro- or 2′-aminopyrimidine derivatives (Figure 1.2a) are widely used for the production of aptamers in which regular purine residues are incorporated (Aurup et al., 1992; Proske et al., 2002; Rhie et al., 2003). The resulting oligonucleotides show improved resistance to nucleases. Alternatively, the four phosphorothiate triphosphates in which a nonbridging oxygen of the internucleoside linker has been substituted by sulfur can be used in polymerase chain reaction (PCR) amplification (Andréola et al., 2000). Similarly, ribonucleoside boranophosphates have been demonstrated to be incorporated by T7 RNA polymerase (Shaw et al., 2003). This enzyme is also able to polymerize transcripts containing 4′-thiopyrimidines (Figure 1.2a), a modification that increases their stability by about 50-fold relative to unmodified RNA (Kato et al., 2005). It was recently reported that the combination of mutated T7 RNA polymerases, Y639F and Y639F/H784A, allows the efficient incorporation of all four 2′-*O*-methyl nucleotides (Chelliserrykattil and Ellington, 2004; Burmeister et al., 2005, 2006).

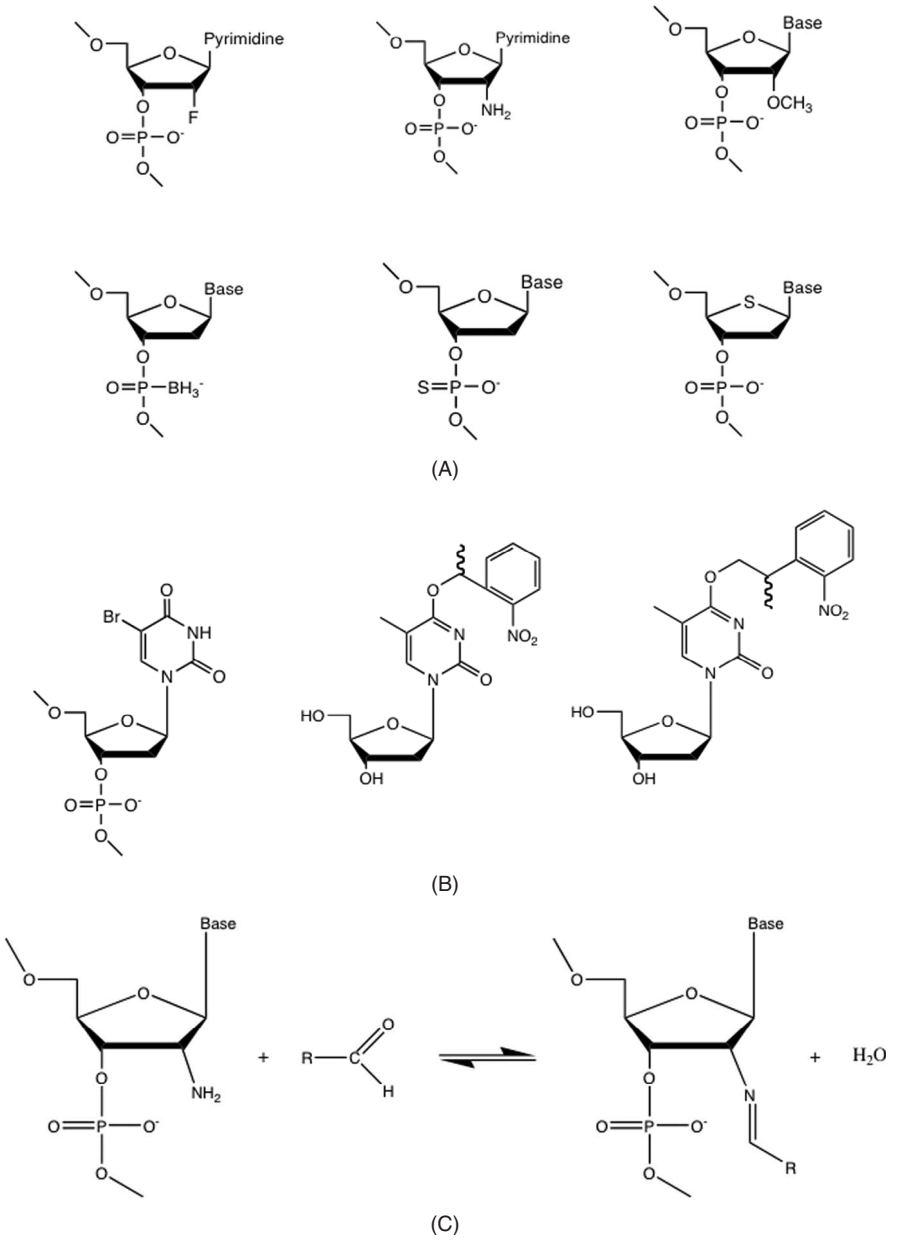

Figure 1.2 Modified nucleotides and nucleosides described in the text. (A) Nucleotides incorporated by polymerases yielding nuclease-resistant oligonucleotides. Top from left to right: 2′-fluoro, 2′-amino, 2′-*O*-methyl. Bottom from left to right: boranophosphate, phosphorothioate, 4′-thio. (B) Photosensitive residues. Left to right: 5-bromo-U, 2-(2-nitrophenyl)ethyl T, 2-(2-nitrophenyl)propyl T. (C) Amino–imino equilibrium used in 2D-SELEX (see the text).

The positions that remain unmodified at the end of the in vitro selection procedure (e.g., the purine residues in a selection carried out with 2′-fluoropyrimidine triphosphates) can be modified post-SELEX for further optimization of the aptamers. A systematic study of the 64 variants of the six-membered apical loop of an anti-TAR aptamer led to the identification of locked nucleic acid/2′-*O*-methyl chimeras fully resistant to nucleases that displayed anti-HIV-1 properties in a cell culture assay (Di Primo et al., 2007). Identification of the few residues that cannot be modified in an RNA aptamer can be carried out by chemical interference, a method used to identify chemical variants of the aptamer originally selected. Such an approach led to the synthesis of a modified anti-HIV-1 reverse transcriptase in which all but two of the positions of the RNA aptamer were substituted by 2′-*O*-methyl residues (Green et al., 1995). This was also the case for the aptamer used for age-related macula degeneration in human beings (Ruckman et al., 1998).

An original approach developed by Klussmann and co-workers relies on L-enantiomers of aptamers (called *spiegelmers*) (Vater and Klussmann, 2003). L-DNA (or L-RNA) is the mirror image of natural D-DNA (or D-RNA). L-nucleic acids are fully resistant to nucleases, but they cannot be processed by polymerases. Therefore, a natural D-aptamer will first be raised against the mirror image of the target of interest. Once identified, the L version of the sequence selected, the spiegelmer, will be synthesized chemically; it will give rise to a complex with the natural target characterized by properties identical to that formed between the D-aptamer and the mirror image of the target. This strategy is restricted to small molecules for which the enantiomer of the target can be synthesized. It has been applied successfully to amino acids, nucleosides, and peptides (Klussmann et al., 1996; Wlotzka et al., 2002). L-Aptamers targeted to calcitonin gene-related peptide binding and to the monocyte chemoattractant protein CCL2 were shown to be efficient in vivo in an animal model (Denekas et al., 2006; Kulkarni et al., 2007).

A new methodology has been described that aims at increasing the molecular diversity of aptamers by a process that rests on the simultaneous use of SELEX and dynamic combinatorial chemistry (Ganesan, 2002), called *2D-SELEX*. The concept has been validated using oligonucleotides that contain unmodified purine nucleosides and 2′-aminopyrimidine nucleosides (Bugaut et al., 2004). Such oligonucleotides are amenable to standard in vitro selection (i.e., they can be amplified). The 2′-amino group can react reversibly with aldehydes, thus generating imines (Figure 1.2c). A random library of 2′-amino oligonucleotides was prepared as usual for selection. Upon mixing with a small library of aldehydes, this generates a dynamic pool of 2′-amino, 2′-imino oligonucleotides (Figure 1.2c). In the presence of the target, the pool will be enriched in such oligonucleotides. Therefore, both the scaffold (the oligonucleotide sequence) and the pendant groups (the 2′-imino substituents) will be selected at once. Following capture, the iminooligomers selected are hydrolyzed. The regenerated amino oligonucleotides are then PCR-amplified and a new round of 2D-SELEX is carried out. At the end of the process the candidates selected are cloned