Applications and future of aptamers that achieve rapid-onset anticoagulation

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ABSTRACT

In this short Perspective, we discuss the history of, and recent progress toward, the development of aptamers that can serve as rapid onset anticoagulants during cardiopulmonary bypass (CPB), extracorporeal membrane oxygenation (ECMO), and catheter-based diagnostic and interventional procedures, several million of which are performed each year worldwide. Aptamer anticoagulants provide potent and antidote-controllable anticoagulation and have low immunogenicity. New methods of aptamer isolation and engineering have not only improved the quality of aptamers, but also accelerated their development. Unfortunately, no aptamer identified to date can produce an anticoagulant effect as potent as that produced by unfractionated heparin (UFH), the standard anticoagulant for CPB. We have suggested several possible strategies to amplify the anticoagulant potency of existing aptamer anticoagulants.

BACKGROUND

Aptamers are short single-strand DNA or RNA oligonucleotides that bind target molecules with high affinity and specificity (Ellington and Szostak 1990; Sullenger et al. 1990; Tuerk and Gold 1990), each of which depends on an aptamer's capacity to fold into a unique three-dimensional structure (Rusconi et al. 2002; Becker et al. 2010). Russo Krauss et al. have described aptamers as "a kind of nucleic acid version of antibodies with improved properties" (Russo Krauss et al. 2013). Though natural aptamers exist (Sullenger et al. 1990), most aptamers identified to date have been identified and isolated by investigators using the in vitro technique termed systematic evolution of ligands by exponential enrichment (SELEX) (Ellington and Szostak 1990; Tuerk and Gold 1990; Elbashir et al. 2001). In brief, a library of randomly generated oligonucleotides is incubated with the target molecule. Sequences that bind the target are then separated from the unbound sequences via methods such as elution or filtration, and are amplified by PCR. The resulting library of higher affinity sequences is then reincubated with the target. The process is iterated until only sequences with extremely high affinity and specificity dominate the pool. From that pool, individual aptamers are identified via sequencing and undergo further post-SELEX engineering. Since the isolation of the first aptamers in 1990 (Ellington and Szostak 1990; Tuerk and Gold 1990), thousands of aptamers targeting different proteins, small molecules, metal ions, and specific cell lines have been isolated for biosensing, diagnostic, and therapeutic purposes. Among those applications, the identification of aptamers that can achieve rapid-onset anticoagulation was one of the earliest and one that has been intensively explored (Chabata et al. 2018).

Cardiovascular disease is the leading cause of death globally. Millions of cardiac surgical and percutaneous cardiac interventions including cardiopulmonary bypass (CPB), extracorporeal membrane oxygenation (ECMO), percutaneous coronary intervention (PCI), and transcatheter valve replacement (TVR) are performed to treat cardiovascular disease every year (Tsao et al. 2022). These interventions inevitably trigger coagulation, a cascading process involving more than 20 proteins resulting in activation of a protease, thrombin, that catalyzes the cleavage of fibrinogen and formation of fibrin fibers which leads to blood clotting. Therefore, potent anticoagulation is essential during and after those procedures to prevent thrombus formation.

The first line anticoagulant used in cardiac operations is unfractionated heparin (UFH). UFH is a heterogeneous mixture of glycosaminoglycan chains obtained from porcine intestine or bovine lung. UFH's principal

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Article is online at http://www.rnajournal.org/cgi/doi/10.1261/rna.079503.122. Freely available online through the *RNA* Open Access option.

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anticoagulant mechanism becomes initiated when UFH chains bind, and thereby significantly augment the inhibitory activity of, antithrombin (AT), a constitutive circulating serine protease inhibitor (serpin). UFH-AT complexes inactivate several key procoagulant serine proteases, including thrombin (the ultimate procoagulant protease) factors (F)Xa and FIXa. Since each AT molecule forms an irreversible and inactive complex with each protease molecule it inactivates, AT functions as a "suicide" serpin. UFH prevents thrombus formation during CPB. However, it "is not an ideal anticoagulant" (Edmunds 1998) for several reasons. UFH-AT complexes do not effectively inhibit clot-bound thrombin (Weitz et al. 1990). During CPB, UFH permits the continuous generation of thrombin (Edmunds 1998) and the continuous depletion of multiple upstream coagulation factors and AT, which exerts anti-inflammatory, as well as anticoagulant, effects. Thrombin's proinflammatory, as well as procoagulant, effects contribute to CPB-induced systemic inflammation (Popovic et al. 2012). In addition, UFH per se functions as an agonist for platelets (Khuri et al. 1995), neutrophils, and complement, thereby directly contributing to CPB-induced systemic inflammation. Due to its immunogenicity, UFH also can trigger heparin-induced thrombocytopenia (HIT), a potentially life-threatening immune-mediated disorder, in 0.1% to 7% of patients (Morgan et al. 2020). UFH's antidote, protamine, produces an anaphylactic response of varying intensities in 0.6% to 10% of patients.

The drawbacks of UFH have prompted the identification and study of several nonimmunogenic rapid-onset anticoagulants that directly and rapidly bind and inhibit procoagulant proteases. For example, bivalirudin is a recombinant 20-amino acid (aa) synthetic analog of the 65-aa direct and specific bivalent thrombin inhibitor peptide hirudin, the most potent nature-derived direct thrombin inhibitor. Bivalirudin's carboxyl terminus and amino terminus simultaneously binds thrombin's exosite I and catalytic site, respectively, and thereby achieves a potent anticoagulant effect ($K_i = 2$ nM). Consequent to protease cleavage and renal clearance, bivalirudin has a short in vivo half-life of ∼25 min, and thus reduces the need of an antidote. The direct inhibition mechanism of bivalirudin has been reported to yield more consistent anticoagulation with lower rates of bleeding or thromboembolic complications compared with UFH (Lee and Ansell 2011). The low molecular weight of bivalirudin allows it to inhibit clot-bound thrombin. The Society of Thoracic Surgeons/Society of Cardiovascular Anesthesiologists/American Society of Extracorporeal Technology have given bivalirudin a Class IIa recommendation for HIT patients who require CPB (Shore-Lesserson et al. 2018). However, because of bivalirudin's short half-life, those authors recommend that if it is used for CPB, (i) specific measures should be undertaken to prevent stagnation of blood within the CPB circuit, and (ii) stagnant operative field blood should be either discarded or processed with cell salvage. In addition, bivalirudin targets thrombin, the last step of the coagulation cascade; overactivation of thrombin and other upstream coagulation proteins cannot be prevented. As a consequence, a high dose is required, which leads to higher costs (Vasquez et al. 2002).

Small molecule direct inhibitors have also been developed against key coagulation proteases for anticoagulation applications. Argatroban, an L-arginine derivative, reversibly binds and inhibits thrombin's catalytic site ($K_i = 6$ nM). Case reports have described its use for CPB anticoagulation (Furukawa et al. 2001; Green et al. 2021), but to date no multicenter trial has been conducted. Argatroban undergoes predominantly hepatic clearance and has an in vivo half-life of 39 to 51 min (Koster et al. 2007). Because of its clearance mechanism, argatroban may offer an advantage over bivalirudin in HIT patients with renal insufficiency who require CPB. Unfortunately, no reversing agent is available for argatroban. Recently, direct oral anticoagulants (DOACs) such as dabigatran, which binds and inhibits thrombin's catalytic site, and rivaroxaban, apixaban, and edoxaban, which bind and inhibit FXa's catalytic site (Chen et al. 2020), have received FDA approval. Their oral availability makes them ideal for preventative antithrombosis therapy and post-surgical thromboprophylaxis. However, no intravenous formulation of a DOAC is available, DOACs cannot achieve rapid-onset anticoagulation, and none has sufficient potency to prevent clot formation during CPB. (Chen et al. 2020). Andexanet alfa, a FXa-mimicking antidote with no catalytic activity, has received FDA approval for reversing the anticoagulant effects of apixaban and rivaroxaban (Siegal et al. 2015). However, that antidote, like FXa, binds AT and UFH-AT complexes. Thereby, it interferes with UFH's principal anticoagulant mechanism (Watson et al. 2019). Hence, andexanet alfa should not be given to any patient who might require UFH during the first 24 h following its proposed use.

Compared to existing surgical anticoagulants, aptamers have important advantages that seem to make them very good therapeutics candidates for such clinical indications. Aptamers directly bind to their targets and inhibit protein–protein interaction between different coagulation factors and cofactors (Fig. 1; Buddai et al. 2010; Sullenger et al. 2012); therefore, they do not rely on activating antithrombin. Compared with bivalirudin and DOACs, aptamers have no limit on the target of choice and can be isolated to inhibit any coagulation factors and even cofactors that do not have a distinct active center. Meanwhile, aptamers' low immunogenicity also reduces the risk of antibody-mediated adverse effects such as HIT. More importantly, the aptamer's activity can be effectively reversed by hybridizing with an antisense oligonucleotide "antidote." So far, more than a dozen aptamers have been isolated to inhibit coagulation proteases (e.g., thrombin, FXa, FIXa, FVIIa, FXIIa, FXIa), protease cofactors (FV and FVIII), and

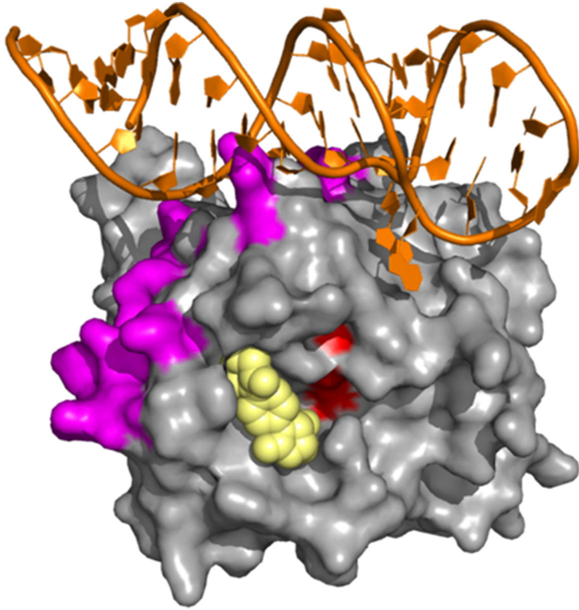

FIGURE 1. Inhibitory mechanism of anticoagulant aptamers. The X-ray crystal structure (PDBID: 5vof) of factor Xa (gray) in complex with an aptamer, 11f7t (orange), and a DOAC, rivaroxaban (yellow). 11f7t's binding site overlaps with factor Xa's putative cofactor Va-binding exosite (magenta; thus it engenders potent anticoagulant activity by blocking prothrombinase assembly rather than blocking the protease's catalytic center [red] or activating antithrombin). On the other hand, the anticoagulant effect of rivaroxaban comes from its direct inhibition of factor Xa's catalytic site.

modulators (e.g., vWF, TFPI), and have shown a variety of strengths as anticoagulants (Bock et al. 1992; Tasset et al. 1997; Rusconi et al. 2000, 2002; White et al. 2001; Buddai et al. 2010; Woodruff et al. 2013, 2017; Burrell et al. 2017). To date, a thrombin aptamer and a factor IXa aptamer have been tested in clinical trials to substitute UFH in surgeries/intervention such as PCI and CABG (Dyke et al. 2006; Povsic et al. 2014; Woodruff and Sullenger 2015). The comparison between aptamer and other types of anticoagulants is summarized in Table 1.

A BRIEF HISTORY OF APTAMER ANTICOAGULANTS

The history of using aptamers to inhibit coagulation proteins can track back to the 1990s. In 1992, Bock et al. published the first study suggesting that aptamers could serve as rapid-onset anticoagulants. Those investigators used SELEX to identify a DNA aptamer, subsequently named HD1, that targeted thrombin ($K_d = 34$ nM) (Bock et al. 1992; Kretz et al. 2006). Thereby, thrombin became the first SELEX target that has no known physiologic interaction with nucleic acid. Subsequent investigations identified additional thrombin aptamers, including HD22 (Tasset et al. 1997), Toggle-25 (White et al. 2001), NU172 ($K_d = 0.1$ nM) (Wagner-Whyte et al. 2007), and R9D-14T ($K_d = 1$ nM) (Bompiani et al. 2012).

HD1, NU172, and R9D-14T each bind thrombin exosite I and produce a significant anticoagulant effect by inhibiting thrombin-catalyzed fibrinogen cleavage (Bock et al. 1992; Griffin et al. 1993; DeAnda et al. 1994; Kretz et al. 2006, 2010; Wagner-Whyte et al. 2007; Bompiani et al. 2012). HD1 and R9D-14T were also reported to bind prothrombin's proexosite I (K_ds = 86 nM and 10 nM, respectively), thereby also inhibiting the cleavage of prothrombin by prothrombinase, a molecular complex comprising FXa binding its cofactor, FVa, on a phospholipid membrane. Both HD1 and NU172 are comprised of unmodified DNA, which has a short half-life in blood (Griffin et al. 1993). Hence, a high concentration and a continuous infusion of HD1 and NU172 were required to maintain anticoagulation during studies of canine CPB (DeAnda et al. 1994) and porcine CPB (Wagner-Whyte et al. 2007), respectively. Both HD22 and Toggle-25 bind thrombin exosite II and thereby inhibit thrombin-catalyzed activation of FV and FVIII. However, neither aptamer binds or inhibits exosite I, nor effectively inhibits thrombin-catalyzed cleavage of fibrinogen. As a result, each produces only a modest anticoagulant effect (Derszniak et al. 2019). Although unsuitable for rapid-onset anticoagulation, those aptamers can be used as probes to track the presence of thrombin in formed thrombi.

Investigators have also identified and studied over a dozen aptamers that target the upstream procoagulant proteases FIXa, FXa, FVIIa, FXIIa, and FXIa, the procoagulant glycoprotein von Willebrand factor (vWF), the procoagulant cofactor FVa/FV, and the FXII and high molecular weight kininogen (HK) activator kallikrein (Rusconi et al. 2000, 2002; Gilbert et al. 2007; Buddai et al. 2010; Woodruff et al. 2013, 2017; Burrell et al. 2017; Soule et al. 2022). The rationale for identifying those aptamers is at least threefold. (i) Inhibiting one or more of upstream proteins may be a necessary part of an overall anticoagulation strategy potent enough to effect CPB anticoagulation. (ii) Since most upstream zymogens and procofactors circulate at concentrations that are less than 10% of the circulating concentration of prothrombin, the aptamer concentrations needed to inhibit the respective proteases and cofactors may be significantly lower than the aptamer concentration needed to inhibit thrombin. (iii) Although an aptamer that inhibits an upstream procoagulant protein may, by itself, not affect anticoagulation potent enough for CPB, it may affect anticoagulation potent enough to facilitate procedures that require less intense anticoagulation than CPB requires.

For example, we developed a modified RNA aptamer, 11f7t, that inhibits FXa binding to its cofactor, FVa, and prohibits FXa-mediated thrombin activation (Buddai et al. 2010). As a result, the aptamer delivered potent anticoagulation in vitro at submicromolar concentration.

TABLE 1. Comparison of different types of anticoagulants

	UFH	Bivalirundin	Direct oral inhibitors	Aptamers
Chemistry	Polysaccharides (12–15 kDa)	Recombinant peptide (2.2 kDa)	Small molecules (0.5 kDa)	Nucleic acids (10–20 kDa)
Mechanism	Activating ATIII	Inhibit both thrombin active site and an exosite	Inhibit catalytic centers of coagulation protease (e.g., IIa and Xa)	Inhibit interactions between different coagulation factors/cofactors
Advantage	• Fast acting • Potent • Cost effective • Protamine reversible	• Fast acting • Potent	• Orally available	• Antidote reversible/ • Does not deplete innate coagulation capacity • Generalizable to different targets
Disadvantage	• Common bleeding disorder • Heparin-induced thrombocytopenia (HIT) 0.1%–7% • Protamine toxicity	• Costly • Nonreversible	• Nonreversible • Not potent enough	• Activity limited by not inhibiting catalytical site • Require high dose, costly

Coagulation factors further upstream of thrombin and FX in the coagulation cascade, such as FIXa and FXIa, are also promising targets because inhibition of these proteins does not entirely block all anticoagulation pathways, which may reduce the risk of bleeding. For example, Rusconi et al. identified the 34-nt aptamer 9.3t, which binds FIXa/FIX, the ultimate procoagulant protease/zymogen of the intrinsic coagulation pathway. That aptamer inhibits both the cleavage of FX by intrinsic tenase (Rusconi et al. 2002), a molecular complex comprising FIXa binding its cofactor FVIIIa on a phospholipid membrane, and the cleavage of FIX by the tissue factor (TF)-FVIIa complex. Pegnivacogin, a modification of 9.3t covalently linked to polyethylene glycol (PEG), does not produce an anticoagulant effect potent enough for CPB anticoagulation (Bel et al. 2016). However, pegnivacogin did provide effective anticoagulation for PCI in over 2000 patients, and pegnivacogin's oligonucleotide antidote, anivamersen, reversed pegnivacogin's anticoagulant effects following completion of the PCIs in those patients (Povsic et al. 2014; Lincoff et al. 2016). A recent study showed that pegnivacogin, renamed DTRI-178, provided thromboprevention comparable to that provided by UFH in neonatal piglets subjected to a 12-h period of veno-arterial ECMO (Reed et al. 2022). The piglets anticoagulated with DTRI-178 also had significantly less bleeding and required significantly less transfused blood to maintain hemodynamic stability than the piglets anticoagulated with UFH. Recently, we have also isolated an FV/FVa binding aptamer that inhibits the formation of the FVa/FXa complex on the cell membrane surface and delivers high anticoagulant activity (Soule et al. 2022). As the circulation concentration of cofactors is typically a few times lower than their protease counterparts, the ability to generate inhibitory aptamer targeting protein cofactors without identified active sites greatly expands the scope of anticoagulant development.

One of the most unique features of aptamers as anticoagulants or general therapeutic agents is their ability to be reversed by oligonucleotide antidotes. As aptamers need to fold into specific tertiary structures to bind to their target, the introduction of an oligo that complements the aptamer can readily destroy such structure and reverse the aptamer's activity (Rusconi et al. 2002). The success of this concept has made aptamers extremely controllable after administration and dramatically reduces the risk of anticoagulant-mediated complications, which was demonstrated in clinical trials of the REG1 system targeting FIXa (Povsic et al. 2014). A similar concept was later extended to develop nonnucleic acid antidotes, where nucleic acid-binding cationic polymers that nonspecifically bind to the negatively charged aptamer backbone, such as protamine sulfate, were used as a universal antidote of different aptamers (Oney et al. 2009). A table summarizing the aptamers mentioned in the section is provided below (Table 2).

CHALLENGES AND PROGRESS

Identifying an aptamer-based anticoagulation strategy that can achieve anticoagulation potent enough to

TABLE 2. Summary of rapid onset anticoagulant aptamers

Name	Target	DNA or RNA	Modifications	Sequence (5′–3′)	Inhibition mechanism	Clinical Stage	Reference
HD1	Thrombin	DNA	Native DNA	GGTTGGTG TGGTTGG	Inhibits exosite I	Clinical	Bock et al. (1992)
NU172	Thrombin	DNA	Native DNA	CGCCTAGG TTGGGTAG GGTGGTGGCG	Inhibits exosite I	Clinical	Wagner-Whyte et al. (2007)
HD22	Thrombin	DNA	Native DNA	AGTCCGTGG TAGGGCAGG TTGGGGTGACT	Inhibits exosite II	In vivo	Tasset et al. (1997)
TOG25t	Thrombin	RNA	2′F RNA 3′ inverted T	GGGAACAA AGCUGAAGU ACUUACCC	Inhibits exosite II	In vivo	White et al. (2001)
11f7t	Factor Xa	RNA	2′F RNA 3′ inverted T	GAGAGCCCCA GCGAGAUAAUAC UUGGCCCCGCUCUU	Inhibits FVa (cofactor) binding	Ex vivo circuit	Buddai et al. (2010)
DTRI-178 (pegnivacogin)	Factor IXa	RNA	2′F/2′ OMe RNA PEGylation	GUGGACUAUACC GCGUAAUGC UGCCUCCAC	Inhibits FX (substrate) binding	Clinical	Dyke et al. (2006)
T18.3	Factor V/Va	RNA	2′F RNA 3′ inverted T	GGACUUUGGAUA ACCUCACCGCA AUGGCGGCU UGUCAGACG ACUCGCUGAG GAUCCGAG	Inhibits FVa/FXa assembly on membrane	In vivo	Soule et al. (2022)

facilitate safe CPB remains a major challenge. Some strategies have been explored to attempt to overcome this challenge. One strategy to improve the inhibitory activity of aptamers is to inhibit multiple sites of the target protein simultaneously. For example, a few bivalent anti-thrombin aptamers were developed by covalently linking an exosite I-binding aptamer and an exosite II-binding aptamer, and they showed significantly higher anticoagulation activity compared to a single exosite-binding aptamer (Muller et al. 2008; Ahmad et al. 2012). We have also observed that combinatorial use of FXa aptamer with FXa-inhibiting DOACs showed improved anticoagulation efficacy, demonstrating potency that rivals UFH (Gunaratne et al. 2018). Our recent study of combinatorial use of FXa aptamer and UFH, or FVa aptamer and LMWH, also demonstrated significantly improved anticoagulation potency (Chabata et al. 2022; Soule et al. 2022). Besides improving aptamer activity toward a single target, other approaches have been explored to inhibit multiple coagulation proteins simultaneously. We have previously shown that the combined presence of 11f7t and R9D-14T can provide more potent anticoagulation than the lone presence of either aptamer (Bompiani et al. 2014). The binding and the anticoagulant potency of each of those aptamers were also maintained when they were covalently conjugated. A single oligonucleotide could reverse the anticoagulant activity of each aptamer conjugated in that hybrid molecule (Soule et al. 2016).

Unmodified DNA and RNA are degraded rapidly in vivo by various nucleases in blood. Meanwhile, the molecular weight of fully truncated aptamers is typically around 10 kDa, and thus the aptamer is rapidly excreted following filtration through the kidney. A study of an unmodified DNA thrombin aptamer showed an in vivo half-life below 5 min, therefore necessitating high doses to be continuously infused during the surgery. With the development of combinatorial chemistry, these problems have been largely solved. 2′ modification of ribose with fluorine and O-methyl groups has shown to improve the nuclease resistance of aptamers significantly (Kratschmer and Levy 2017). End modification, such as inverted dT or covalently ligating the 3′ and 5′ termini of aptamers further improves their chemical stability (Esposito et al. 2014; Riccardi et al. 2020). Meanwhile, PEGylation of nucleic acid therapeutics becomes routine to increase the molecular weight to prevent kidney excretion.

One potential and historically overlooked problem is the immunogenicity of PEG. It has been reported that anti-PEG antibodies exist in a significant percentage of the population, and animal studies have shown that immunity to PEG can lead to attenuated activity of PEG-modified aptamers, if not worse, approximately one percent of the patients who received the PEGylated version of factor IXa experienced a serious allergic reaction to the PEG moiety (Ganson et al. 2016; Povsic et al. 2016; Moreno et al. 2019). Therefore, the use of PEG in future aptamer

formulations should be done with caution. Possible ways to circumvent the imunogenecity of PEG include the development of more biological inert polymers than PEG that have a molecular weight larger than 30 kDa to prevent rapid kidney clearance (Ozer et al. 2022). From our previous experience with preclinical aptamer development, aptamers isolated against human proteins cross-react to various extents with the homologous proteins from animals such as mice, rats, rabbits, pigs, or dogs. Therefore, some aptamers, even with exceptional activity in vitro, face difficulty in preclinical development due to their poor species cross-reactivity. To overcome this barrier, we developed a selection strategy termed "toggle SELEX," which alternatingly challenges the selection library with the target from human or animal sources (White et al. 2001). Such a technique greatly improves the probability that the final aptamer has species cross-reactivity.

As we have seen over the past decades, using aptamers as therapeutic agents to control blood clotting in cardiac surgeries has progressed from concept to large clinical trials and late-stage clinical testing. Although anticoagulant aptamers have so far been unable to replace UFH in some of the most thrombogenic surgical settings such as CPB, they have already shown great potential in many procedures that require less potency, with fewer adverse effects compared to heparin. For example, despite rare allergic reactions to PEG (~0.6% of patients), over 2000 patients with acute coronary syndrome have been successfully anticoagulated using the factor IXa aptamer during cardiac catheterization (Povsic et al. 2014; Lincoff et al. 2016). Moreover, this clinical progress directly demonstrated that anticoagulant aptamer-reversal is rapid and reliable in patients. This impressive translational success clearly bodes well for the therapeutic anticoagulant aptamer field. This clinical experience taken together with new technologies in aptamer isolation, formulation and modification could result in therapeutic aptamers that are more potent, more stable, less immunogenic and even more controllable in the near future. We therefore foresee the expanded use of therapeutic anticoagulant aptamers in cardiac surgeries including during CPB soon. We have summarized the recent progress in developing anticoagulant aptamers in Table 3.

Finally, the technologies used in isolating and developing an aptamer-based anticoagulant can be easily translated into other clinically related applications. For example, the working mechanism of aptamer-based inhibitor via interfering macromolecular interaction makes aptamers attractive inhibitors against various protein enzymes, receptors, and even ligands without defined active sites (e.g., cytokines and cofactors) for the treatment of cancer, cardiovascular diseases, infectious diseases and other diseases. Multivalent aptamers can be generated to further improve their bioactivity. Aptamers binding to nonfunctional domains of proteins can also be isolated as imaging probes to locate their target in vivo. Importantly, the antidote controllable feature of aptamers allows the activity of aptamers to be rapidly reversible in the body, which greatly benefits both therapeutic and imaging applications. In addition, the chemical engineering techniques to improve in vivo stability of aptamer anticoagulants can also be used in developing other aptamer-based therapeutics.

TABLE 3. Summary of strategies to improve aptamer-based anticoagulants

Strategy	Improvement
Generating bivalent/multivalent aptamer	Increase potency and in vivo half-life
Combined use of aptamer and other anticoagulants	Increase potency
Chemically modified aptamer	Increase potency
Conjugation with polymers	Increase in vivo half-life
Toggle SELEX	Increase species cross-reactivity

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health (NIH) grants 5P01HL139420-03 (to B.A.S.) and American Heart Association 23POST1018721 (to H.Y.).

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