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Synthetic antibody: Prospects in aquaculture biosecurity

ChouMin Chong[a], ChenFei Low[b,*]

[a] Laboratory of Marine Biotechnology, Institute of Bioscience, Universiti Putra Malaysia, 43400, UPM Serdang, Selangor, Malaysia
[b] Institute of Systems Biology, Universiti Kebangsaan Malaysia, 43600, UKM Bangi, Selangor, Malaysia

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ABSTRACT

The emerging technology of aptamers that is also known as synthetic antibodies is rivalling antibodies research in the recent years. The unique yet important features of aptamers are advancing antibodies in diverse applications, which include disease diagnosis, prophylactic and therapeutic. The versatility of aptamer has further extended its application to function as gene expression modulator, known as synthetic riboswitches. This report reviewed and discussed the applications of aptamers technology in the biosecurity of aquaculture, the promising developments in biosensor detection for disease diagnosis as well as prophylactic and therapeutic measurements. The application of aptamers technology in immunophenotyping study of aquatic animal is highlighted. Lastly, the future perspective of aptamers in the management of aquatic animal health is discussed, special emphasis on the potential application of aptamers as synthetic riboswitches to enhance host immunity, as well as the growth performance.

1. Synthetic antibody: a technology of aptamers

The aptamers technology that is also known as "synthetic antibodies", is rivalling antibodies in both diagnostic and therapeutic applications of existing as well as emerging diseases [1]. Aptamers, formerly known as nucleic acid ligands were first described in 1980s from the discovery of virus-encoded small-structured RNAs, which possesses high binding affinity and specificity to the viral or host proteins [2]. It can be classified into nucleic acid aptamers and peptide aptamers. Nucleic acid aptamers consist of short strands of DNA or RNA oligonucleotides whilst the peptide aptamers are engineered synthetic or artificial proteins. Nucleic acids are known to rearrange and form discrete three-dimensional conformation that is known as ribozymes in nature, which mediates gene expression, cellular communications and peptide synthesis. The nucleic acid aptamers can be obtained from Systematic Evolution of Ligands by EXponential enrichment (SELEX) process [3,4]. SELEX is an iterative *in vitro* process of selection and amplification of oligonucleotides from its large combinatorial libraries, which contain huge number of randomized oligonucleotides. Randomized sequence of oligonucleotides fold into unique and stable three-dimensional structure with distinctive ligand-binding site, which possess affinity toward specific target. The different types of functional secondary motifs of aptamers include stem and loop hairpin [5], pseudoknot [6], G-quadruplex [7], bulge [8], asymmetric internal loop [8], and symmetric internal loop [8]. To-date, the establishment of SELEX has led to the isolation of a myriad of target-specific aptamers for a wide range of applications. These applications include disease diagnosis, therapeutic and drug delivery, biomarker discovery, target validation, as well as molecular imaging.

SELEX is a repetitive cyclic process to generate aptamers that are recognizing the desired target. Cell-SELEX is a modified SELEX in which whole cells are used as target. The SELEX and cell-SELEX involve multiple steps. First, a large population library of oligonucleotides (typically up to 10^{15} unique molecules) is synthesized by flanking a randomized region with fixed primer regions that are required in PCR amplification. Cellular target molecules (peptides, protein, macromolecules, vitamins etc.) are immobilized on a solid support like agarose, sepharose, or magnetic beads. The oligonucleotide pool is incubated with the target molecules. In SELEX, functional oligonucleotides that are bound to the target are separated from the pool of unbound sequences using physical approaches, such as affinity chromatography, capillary electrophoresis, filter binding, gel electrophoresis or washing. Whereas for cell-SELEX, the separation can be performed through centrifugation, washing or FACS. The bound functional oligonucleotides are eluted by heating and then recovered by DNA extraction procedures. The recovered molecules are subject to amplification process, either by the process of standard PCR or RT-PCR in the case of RNA aptamers. The amplified oligonucleotides display the enriched library that is followed by the subsequent selection cycles. For cell-SELEX, a counter-selection (negative selection) is required to

* Corresponding author. Institute of Systems Biology, Universiti Kebangsaan Malaysia, 43600, UKM Bangi, Selangor, Malaysia.
E-mail address: low@ukm.edu.my (C. Low).

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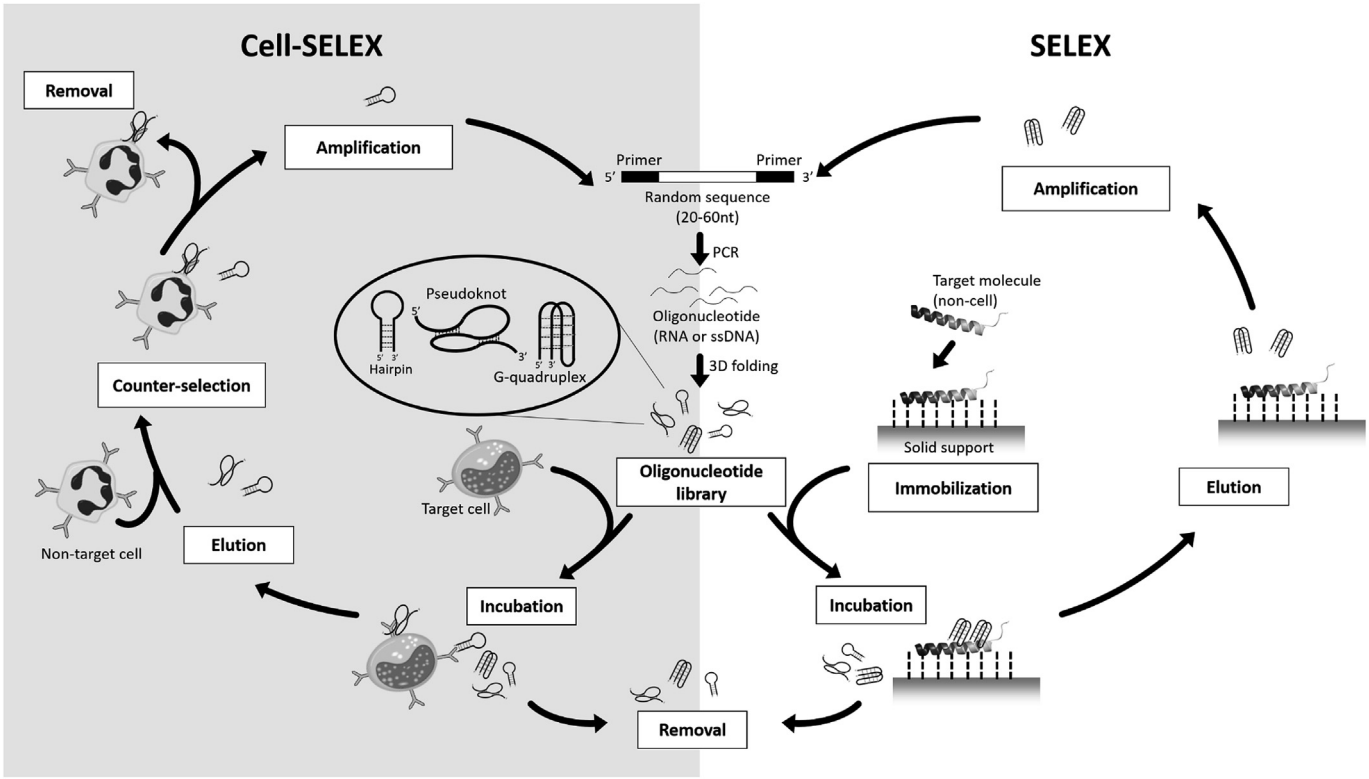

Fig. 1. Schematic representation of the SELEX and Cell-SELEX procedures.

remove unspecific aptamers using non-target cells. The entire SELEX or cell-SELEX process is repeated multiple times, until the pool is dominated by specific binding oligonucleotides of high affinity (see Fig. 1). In the final selection round, cloning and characterization of the specific aptamers are carried out.

1.1. Advantages of aptamers

Production of the appropriate immunotherapeutic antibody is a very laborious and costly process. There are several issues face by the production of immunotherapeutic antibody, such as complication of the development process, bioavailability, contamination of hybridoma, highly immunogenic and low production yield [9,10]. Aptamers is rivalling antibodies due to its unique advantages in production and chemical properties that overcome the aforementioned issues. Aptamers can be synthesized relatively inexpensive and fast, no batch-to-batch variability, and non-immunogenic [8,11]. The production of aptamers is scalable through chemical synthesis in a controlled process that is less likely to be contaminated by microorganisms, vying the *in vivo* biological production of antibodies that is hardly to scale up and prone to viral or bacterial contamination, which subsequently deteriorate the characteristics and quality of the antibodies produced [12]. By adopting simple annealing steps of heating and cooling in a suitable buffer, the unfolded aptamers due to prolonged storage at room temperature, can rearrange into a functional condition. This reversible denaturation feature has reduced the necessity of the cold-chain system that is required for the shipping and storage of antibodies [13].

Other unique yet important feature of aptamers is that, it can be selected for specific target or against cell-surface marker without a prior knowledge of the marker [14]. The generation of aptamers recognizing unknown surface marker on the target cells is possible by adopting Cell-SELEX, which is a modified approach of SELEX that using whole cells as a target [15–18]. This feature of aptamers selection is favorable for biomarker discovery, and potentially leads to the discovery of novel surface protein that is expressed by specific cell type of interest. For instances, in the characterization of different population of immune cells, also known as immunophenotyping, characterization of different tumor or cancer cells markers [18,19], and surface markers of virus-infected cells that are applicable in disease diagnosis [14]. The small size of therapeutic aptamers that is generally less than 100 base nucleotides, make up to the full molecular size of less than 30 kDa as compared to an average of 150 kDa for a full-size monoclonal antibody. In addition to the small size of aptamers, the non-immunogenic property of aptamers greatly contributes to its feasibility as therapeutic agent, allowing the entry of aptamers into cellular compartments without provoking host immune response [20]. Moreover, functional groups can be conjugated orthogonally to the aptamers during synthesis, for instances the conjugation of dyes for detection purposes, and drugs for targeted delivery and treatment. Taken together with the target-specific characteristic of aptamers, they are fitted to serve as a vehicle for targeted drug delivery therapy as in tumor or cancer therapy, increasing the efficacy of the therapy and in addition, reducing the toxicities of the drugs to the non-target healthy cells [21–24].

1.2. Limitations of aptamers and the resolving strategies

The foremost limitation of aptamers without modification is the high susceptibility to degradation. The unmodified nucleic acid or peptide aptamers are prone to nuclease or protease activities respectively. Although the small size of aptamers molecule is favor in therapeutic, but it also renders to renal filtration therefore possesses a shorter systemic half-life. These limitations can be resolved by chemical modifications and conjugations. Nucleotide sugar or phosphodiester bonds can be chemically modified to enhance aptamers resistance against nucleases activities [12,25]. Common phosphodiester linkage modifications include the substitution with methylphosphonate or phosphorothioate analog [26]. Besides, modification of the aptamers using D- and L-stereo-isoform of deoxyoligonucleotides has retained the aptamers binding affinity and further reduces its susceptibility to degradation in the biological system [27]. The modified L-aptamers that is

also known as Spiegelmer binds to natural target due to the fundamental of reciprocal chiral substrate specificity [28]. Spiegelmer against the gonadotropin releasing hormone has been reported [28,29], and many others have been clinically evaluated [30,31]. In order to prolong systemic half-life of the aptamers, conjugation of aptamers to polyethylene glycol or cholesterol has been reported a success [12,20]. Furthermore, the conjugation of polyethylene glycol facilitates the distribution of aptamers to tissues of highly perfused organs in particular [20]. On the whole, limitations of aptamers can be surmounted with various strategies through modifications or conjugations, which has been comprehensively reviewed in recent reports [26,27,31–33], and thus will not be discussed.

A brief insight of aptamers for fisheries research that emphasized on the technicality of aptamer selection has recently been reviewed [34]. Hence, current review comprehensively discusses the insight of nucleic acid aptamers for fisheries research that revolves in the application for disease diagnosis, prophylactic/therapeutic aptamers, as well as the prospects of aptamers application in immunophenotyping research, and the potential application of synthetic riboswitches technology in aquaculture.

2. Biosensor detection for disease diagnosis

Aptamer-based biosensor has received very much attention for the past 20 years, denoted by the exponential increased in the number of research publications since 1998 [35]. Biosensors possess operational advantages that include fast detection; portability and user-friendly; low cost and ease of mass production for the application in detection and diagnosis of diseases [35–39]. Aptamers that are small in size, chemically stable and low in production cost have rivaled traditional antibodies as the transducer-linked molecular recognition element in biosensing. The unprecedented advantages in high sensitivity and selectivity of aptamers have significantly improved the performance of aptamer-based biosensors that employed electrochemical, optical or mass-sensitive analytical techniques [36]. Biosensors for the detection of pathogens, or specific pathogen-related molecules have been established, for instances the HIV [40], hepatitis C virus [41], and flu viruses [42]. The advancement of aptamer-based biosensors are also being developed for the detection and diagnosis of non-infectious diseases, which include cancers [43,44], and myocardial infarction [45].

Specificity and sensitivity of selected aptamers towards its target molecules/cells are among the important characteristics of aptamers to be used as probes in pathogen detection for disease diagnosis. Enzyme-linked aptamer sorbent assay (ELASA) was designed to replace the immunoglobulin used in ELISA for rapid and sensitive detection in disease diagnosis. Although the detection specificity and sensitivity of ELASA compare to ELISA were claimed to be similar [46], but some aptamers were reported to be possessing higher target-binding affinity compared to antibody [47,48], thus suggesting that the configuration of ELASA could possesses higher sensitivity than that of ELISA. The configurations of ELISA and ELASA have been illustrated in Fig. 2, and the significant features of ELASA in comparison to ELISA are emphasized.

The application of aptamers technology in the detection of Singapore grouper iridovirus-infected cells was recently been reported [14]. Novel DNA aptamers were first being developed and characterized to specifically target SGIV-infected cells *in vitro* and *in vivo*. Given the fact that modification of cellular membrane occurs during virus infection [49], probes that target the altered cellular membrane markers of the virus-infected cells can be developed for diagnosis. Cell-SELEX allows the development of novel DNA aptamers that target SGIV-infected cells without the prior knowledge of the altered cellular membrane markers. Although bioassay revealed that one of the cellular membrane marker was trypsin-resistant, but the identity of this cell membrane component has not been investigated in the same study. Knowledge of the altered cellular membrane markers could potentially leads to the understanding of host responses to virus infection, which is a particular interest in virology that urges an investigation. Following the development and characterization of aptamers that specifically targeting SGIV-infected cells, an aptamer-based enzyme-linked apta-sorbent assay was developed [50]. It was claimed to be an assay that is rapid, highly specific and stable, which is widely applicable for the detection of SGIV infection in grouper aquaculture. The similar was approached in the development of aptamer-coat protein-aptamer sandwich enzyme-linked apta-sorbent assay, for the rapid and sensitive detection of redspotted grouper nervous necrosis virus (RGNNV) infection [51]. Different from the previous approach that uses aptamers to detect the SGIV-infected cells, this sandwich enzyme-linked apta-sorbent assay uses aptamer that specifically recognizes RGNNV particle as the bioreceptor, in order to detect the presence of RGNNV in the sample. Despite of its high specificity in recognizing RGNNV, these aptamers also possess antiviral property. The potential of antiviral properties of virus-specific aptamers are reviewed as follow.

3. Prophylactic and therapeutic properties of aptamers

The unique features of aptamers known to be target-specific, low immunogenicity and non-toxic denote its prophylaxis and/or therapeutic potential in human maladies. The therapeutic aptamers are similar to small molecules therapy and monoclonal/polyclonal antibodies. Although the small size of aptamers that is comparable to small molecules therapy that facilitate systemic clearance [10], but the target specificity of aptamers signifies its functional advantage over small molecules therapy. The designated target specificity of aptamers thereby decreases off-target effect and toxicity that are often observed in small molecules therapy [52]. Target specificity of aptamers is similar to antibodies, in which they bind targets with high affinity in a specific orientation through their unique 3-dimensional molecular structure [12,52]. In general, the *in vitro* synthesis of aptamers through SELEX is faster, produces homogenous aptamers population, and reduced immunogenicity by chemical modification on the aptamers. While the *in vivo* production of polyclonal antibodies resulted in heterogenous population, the subsequent selection of monoclonal antibody is laborious, and most importantly is that these natural antibodies are immunogenic. Modifications of nucleic acid aptamers not only reduce its immunogenicity, but also meant to increase its thermodynamic stability. Tremendous research has been conducted to improve *in vivo* stability and functionality of aptamers as therapeutic agent, and these techniques of modification has been extensively reviewed during the last decade [27,53].

Nonetheless, the prophylactic and therapeutic properties of aptamers for aquatic animals are less well studied. Though, first report of RNA aptamers that were produced by *Rhodovulum sulfidophilum* exhibited significant antivirus properties against viral hemorrhagic septicemia virus (VHSV) [54,55] and hirame rhabdovirus [56]. *In vitro* assessment of the antivirus properties of these RNA aptamers demonstrated significant reduction of cytopathic effect (CPE) in virus-infected cells. But the experimental infection was conducted using RNA aptamers-treated virus particle, suggesting that the mechanism of antivirus by the blocking of virus attachment to the host cells. Nevertheless, subsequent *in vivo* assessment observed a significant decrease in the cumulative mortality of VHSV-infected Japanese flounder to 10% at 10 days post-infection, compared to 90% of cumulative mortality in the non-treated control group [55]. Recently, the first antiviral DNA aptamers targeting Singapore grouper iridovirus [57] and grouper nervous necrosis virus [58] were characterized. *In vivo* assessment of the antiviral properties of these DNA aptamers against both Singapore grouper iridovirus and grouper nervous necrosis virus shown significant reduction of cumulative mortality in virus-infected fish. Similar antiviral properties were also observed in the inhibition of soft-shelled turtle iridovirus by antiviral DNA aptamers [59]. However, the experimental infection was conducted using the virus isolate that was pre-treated with aptamers. This approach anticipates the efficiency and efficacy of

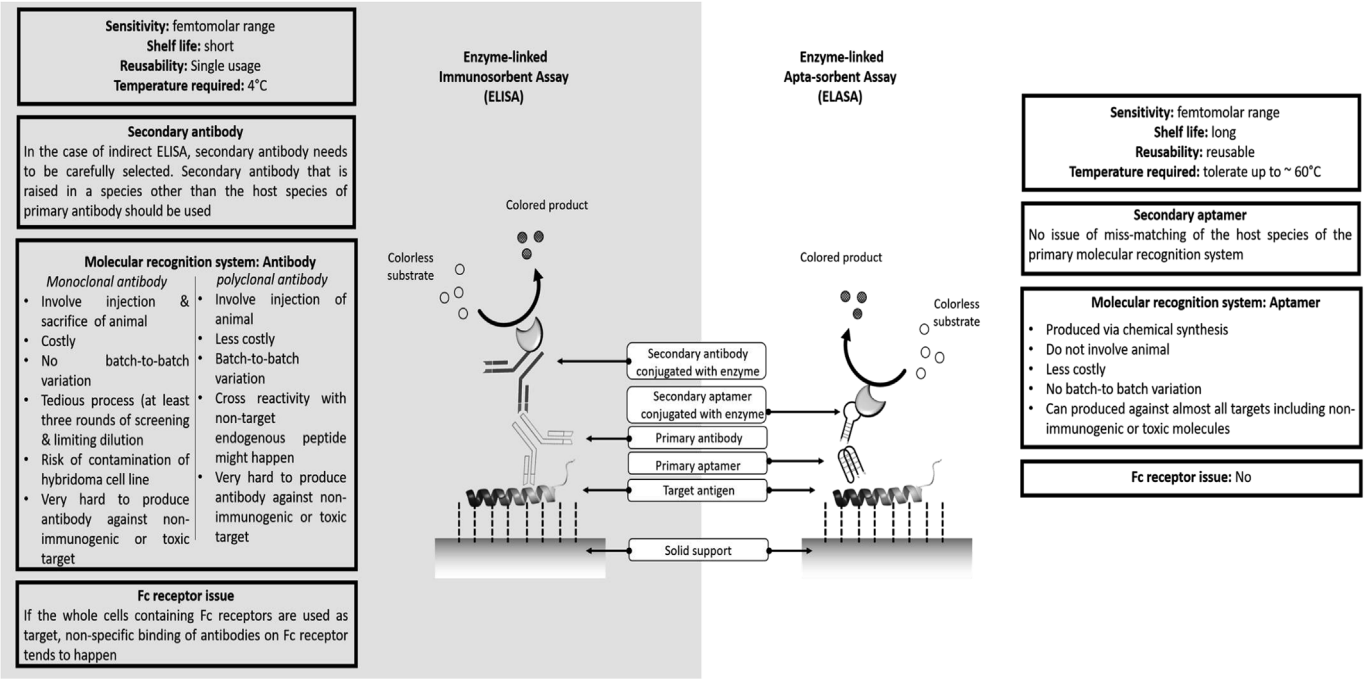

Fig. 2. Comparison between the configurations of ELISA and ELASA, and the significant features in the newly developed ELASA credit to the advantages of aptamers.

aptamers as potential prophylactic agent through the prevention of virus attachment to the host as previously suggested, but the therapeutic properties of aptamers were not assessed. In order to assess therapeutic properties of aptamers, the experimental infection shall be conducted using infectious viral particle, and then followed by the treatment of therapeutic aptamers, instead of using aptamers-treated virus isolate for the experimental infection.

The ability of aptamers to translocate into cellular cytoplasm allows the blocking of virion assembly by targeting the virus coat protein. Alternatively, it can inhibits the RNA dependent RNase polymerase activity to arrest virus replication. These are the potential therapeutic strategies that can be applied in managing viral diseases in aquaculture. The former potential therapeutic strategy was demonstrated in a study to inhibit Grass Carp Reovirus (GCRV) infection using DNA aptamers that targeting the virus capsid protein [60]. DNA aptamers were developed to target one of the important viral outer capsid proteins, known as the VP7. The therapeutic DNA aptamers were then being evaluated in three different experimental assessments: (i) the aptamers and the virus were added to the permissive cells simultaneously; (ii) the aptamers were added to the permissive cells 1 h before viral infection and; (iii) the aptamers were added to the permissive cells 1 h after viral infection. The therapeutic DNA aptamers recorded different degree of *in vitro* inhibition of GCRV infection from these independent experimental assessments, thus they represent the suitable aptamer candidates for the development of anti-GCRV agent. Extensive references that reporting the *in vitro* efficiency of aptamers against several human pathogens are available. For example, RNA aptamers targeting the HIV nucleocapsid protein inhibits the viral genomic RNA packaging, subsequently arresting the production of virion is observed [61]. Similar strategy has been applied to interfere hepatitis B virion assembly by targeting the virus core protein using peptide aptamers [62]. Aptamers that target viral enzymes, particularly polymerases have been reported to inhibit virus replication in an unprecedented efficiency [63–66]. The potential of aptamer-based therapeutic in combating human viral diseases has been extensively studied for the past years, and the progress development were recently reviewed [67]. Thus, it can be assured that the aptamers technology is applicable in disease surveillance and to improve aquatic animal health. Although the prophylactic and therapeutic aptamers research in diseases of aquatic animals are still very much in its infancy, more extensive and comprehensive research should be encouraged to develop and investigate these prophylactic and/or therapeutic aptamers.

4. Application of aptamers technology in aquatic animal diseases study: immunophenotyping

Advancement of the aptamers selection strategy enables the isolation of aptamers to target cells, where prior knowledge of the target molecules on cell surface are not mandatory [2,68], allowing the laborious isolation and purification of the complex markers or targets to be dispensable. Further integration of counter-selection protocol into Cell-SELEX has differentiated the selected aptamers to target specific cell types [2,69], producing cell-specific aptamers that are useful in down-stream applications for instances, cell-type identification, cell count and sorting using flow cytometry. The feasibility of aptamers as versatile probes in the application of flow cytometry has been demonstrated [70]. As a promising alternative to polyclonal and monoclonal antibodies, aptamers have been adopted to recognize desired cell surface receptors. Among these cell surface antigens, cluster of differentiation (CD) molecules are the most popular target proteins for aptamers [11]. In 1982, the nomenclature of CD was coined by the Human Leukocyte Differentiation Antigens (HLDA; and later renamed as Human Cell Differentiation Molecules, HCDM) Workshop as a protocol to identify, characterize and investigate leukocyte surface proteins [71,72]. These CD molecules are being used as targets for immunophenotyping of leukocytes [71]. The generation of RNA aptamers against rat CD4 molecule has been demonstrated [73] and, in the same year of 1998, RNA aptamers against human CD4 were characterized [74]. The 2′—F-pyrimidine-RNA aptamers were used to stain human $CD4^+$ cell surface for the application of flow cytometry analysis.

Despite the impressive impetus discovery of immunoregulatory peptides in different fish species, the functional immunology knowledge of these peptides remains obscure. This is mainly due to the lack of specific cellular marker for leucocytes populations and subpopulations. These specific cellular markers are indispensable in order to contemplate the downstream cellular events of the activation of immune

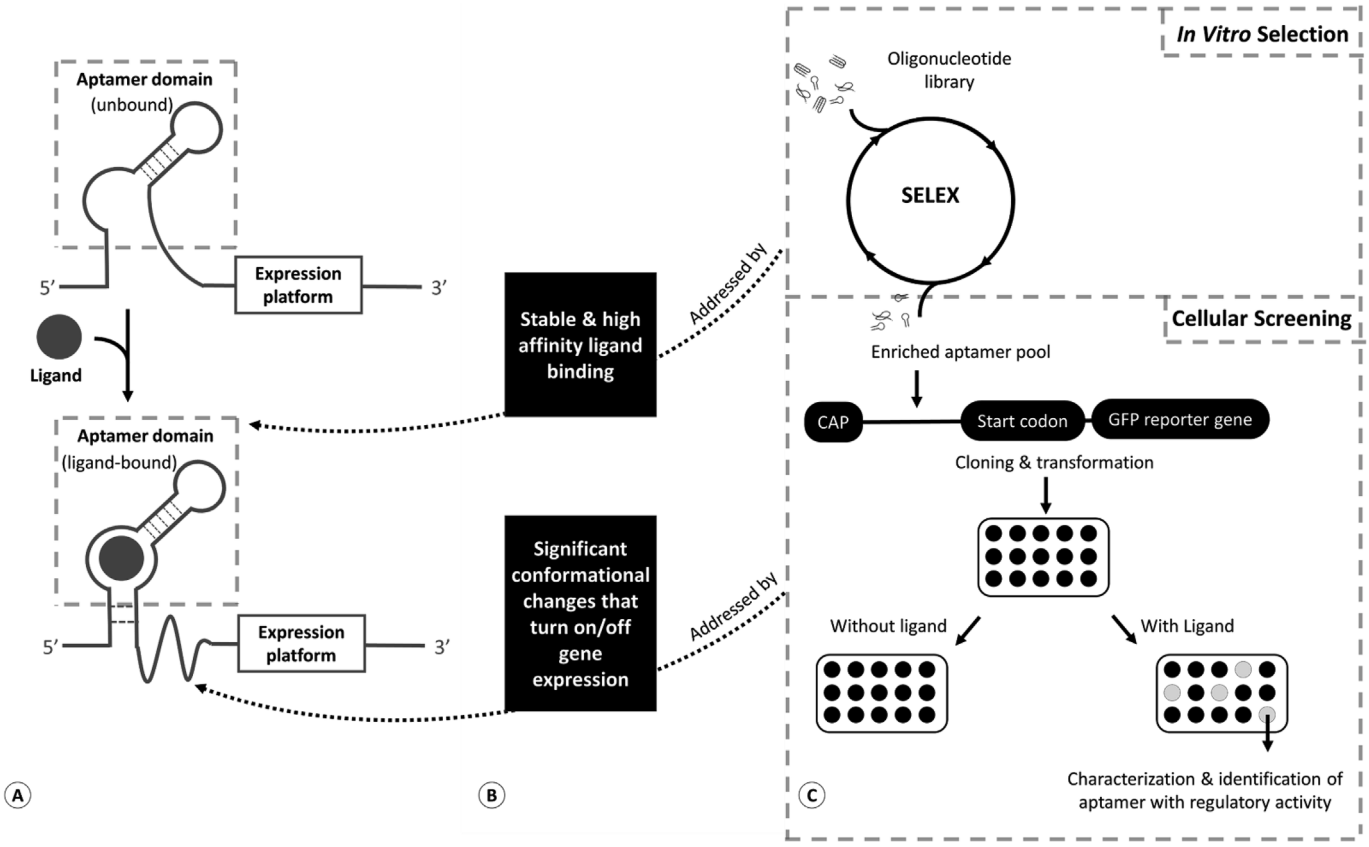

Fig. 3. Synthetic riboswitches are genetic control elements that consist of an aptamer domain and an expression platform. (A) Binding of small molecule ligand to the aptamer domain changes the RNA structure, which converted into a change in gene expression through the expression platform. (B) Functional aptamers as synthetic riboswitches have to possess two properties, which are (i) high ligand binding affinity; and (ii) significant conformational changes that are stable upon ligand binding. (C) High ligand binding affinity is addressed through the *in vitro* SELEX protocol. However, conformational changes of aptamers upon ligand binding cannot be addressed through *in vitro* selection. *In vivo*/cellular screening step is thus required in order to identify and characterize the functional aptamers as synthetic riboswitches with regulatory activity.

responses [75]. The technology of aptamer is well established, where the development of anti-CD aptamers would provide a huge leap in immunophenotyping research [11]. Nevertheless, none of the aptamer was developed for aquatic animal, particularly in fish immunology study. A myriad of cellular markers and determinants in fish immunology has been putatively identified [75,76]. They are the potential targets in aptamers development and characterization, which would enable aforementioned study to track the cellular events of the activation and regulation of immune responses.

5. Future perspective of aptamers in aquatic animal health

The application of aptamers technology in disease surveillance of aquatic animals are yet to be established. However, new therapeutic innovations and applications of aptamers to neutralize lethal viruses have recently been reviewed [77]. It focuses on the pathogens that cause lethal diseases in human, which include HIV, influenza, Dengue, and the newly emerging viral threats of West Nile Virus, Bourbon virus, and MERS. Aptamers were proposed to provide a last line of defense as passive immunity [77,78], in the absence of effective vaccines against these viruses, until the patients developed active immunity. In the case of most viral infection in cultured fish and shrimp/prawn species, the infection in respective fingerling and post-larvae stages often resulted in 90–100% mortality, but the adult fish and shrimp/prawn appeared to be resistant. Therefore, it can be suggested that aptamers could provide a defense against these pathogens during the susceptible stages of fingerling and post-larvae, until the adult stage where hosts have developed their immune responses.

The advance application of aptamers technology has evolved to regulate targeted gene expression systems through synthetic riboswitches [79,80]. It was developed based on the important role of RNAs, which include the noncoding RNA factors [81] and natural RNA aptamers [82] in the regulation of gene expression. Initially, the natural riboswitches were discovered in bacteria as intracellular sensory of vitamin derivatives [83–85]. In the subsequent years throughout the last decade, riboswitches were found to exert regulatory function in controlling translation, transcription, stability of RNA, splicing and ribozyme activity [86,87]. The technicalities of functional synthetic riboswitches are illustrated as in Fig. 3. The comprehensive overview of riboswitches and synthetic riboswitches have been extensively reviewed elsewhere [79,86–88], thus will not be addressed in this review.

While the proof-of-concept has demonstrated that in despite to the inhibition of viral replication by synthetic riboswitches in eukaryotic DNA and RNA viruses [86,89,90], the functionality of synthetic riboswitches in regulating bacterial gene expression [91], and to engineer cellular function that regulate cell growth [92] have also been demonstrated. Although this system is predominantly demonstrated in bacteria and yeast model, it has been described to control mammalian T-cell proliferation in the past years [93]. Clonal expansion of T cells is crucial during T-cell activation in immune response. The success of synthetic riboswitches in controlling T-cell proliferation preconvinces the potential application of synthetic riboswitches in (i) enhancing immunity and resistant against pathogens; or (ii) enhancing the growth performance of the cultured aquatic animal species through the manipulation of growth regulation. The enhancement in growth performance is of economic importance, which reduces the rearing and

maintenance costs of the aquaculture industry. Thus, extensive research of synthetic riboswitches approach in aquatic animals are instigated.

Nevertheless, there are limitations and constraints that need to be addressed for *in vivo* applications of aptamers in aquatic animal, which include the *in vivo* stability of aptamers, delivery method (aquatic environment), and the industrial scale production of aptamers. But these limitations and constraints can be resolved through chemical modification to enhance the *in vivo* stability of aptamers [26,27,31]. Nonetheless, the stability of aptamers to be delivered in aquatic environment has yet to be assessed thus far, but the advantage of aptamers that can be chemically modified while retaining its biological activity promises a great potential for its application in aquatic environment. Even though enormous applications of aptamers have been reported throughout the past decades since its discovery, least development was established for the application in aquatic animal. But the most extensive reports were recorded in the study of the application of aptamers in the detection of *vibrios*, which is one of the important zoonotic pathogen that causes major threat in mariculture [94–97]. The future of aptamers technology in the advancement of disease surveillance and aquatic animal health management is predicted. Other than the aptamer applications in disease diagnosis, prophylaxis and therapeutics, the potential of aptamer technology as a research tool, gene expression modulators, biosensors/biochips, molecular mimicry, and riboswitches applications in aquaculture are mainly in its infancy. Thus the development and application of this technology in aquaculture for the coming years are foreseen.

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