CHAPTER 1

INTRODUCTION

1.1. Ribozymes

The 1989 Nobel Prize winning discovery of a catalytic ribonucleic acid (RNA), termed ribozyme, by Sidney Altman and Thomas Cech has reconstructed the theory about RNA that was merely regarded as a messenger to guide translation in DNA central dogma. The revelation narrated the capability of RNA structure to undergo intricate folding and act as a biocatalyst in specific biochemical reactions despite the lack of diverse functional groups like those of protein enzymes. Over 30 years later, high-resolution analyses of the ribozyme crystal structures have identified several classes of the catalytic RNA that are varied in size (Müller et al., 2016). Their roles within the biological system are mainly thought to revolve around genetic control whereby the ribozymes carry out RNA cleavage, splicing and dehydration synthesis reaction in ribosomes (Liu, Cao, & Lu, 2009).

Some of the naturally occurring ribozymes that are dispersed among both prokaryotes and eukaryotes include Hammerhead, Hepatitis Delta Virus (HDV), and twister ribozymes which catalyse a site-specific RNA cleavage at an active site within the catalytic core, coordinated through Watson-Crick base-pairing interactions and their tertiary structures (Doherty & Doudna, 2001)(Jimenez, Polanco, & Lupták, 2015). The hammerhead ribozyme of plant viroid origin, for instance, is comprised of three short folded helices (called stems) organised in a Y shape with an exposed GUC active site (Figure 1). The ribozyme cleaves RNA into functional unit-length fragments during the unidirectional replication of the circular genome (rolling circle replication) to permit translation.

The self-cleaving process commonly ensues based on an acid-base catalysis by exploiting nucleobase residues as the general acid or base (Wilson, Liu & Lilley, 2016). The RNA cleavage reaction at the active sites involves a nucleophilic attack by a O2’ atom on an adjacent phosphodiester bond in the RNA backbone, producing RNA products with 2’, 3’-cyclic phosphate and 5’-hydroxyl group; an outcome of proton extraction by a basic nucleobase from the 2’-hydroxyl

nucleophile and proton donation to the leaving 5'-hydroxyl group by the acidic base (Jimenez, Polanco, & Lupták, 2015)(Wilson, Liu & Lilley, 2016). Another mechanism of the ribozyme self-cleavage is associated with its nature as a metalloenzyme that requires a metal cofactor for activation (Doherty & Doudna, 2001). For instance, in Hammerhead ribozyme catalysis, divalent ions such as Mg^{2+} have been shown to directly drive the deprotonation of nucleophilic oxygen, stimulating the cleavage reaction.

Despite the detailed illustration of ribozymes structures and the characterizations of their modes of catalysis, limited information is known in regard to the precise significance of ribozymes. Moreover, with recent findings of new natural ribozyme classes (Weinberg et al., 2015), there still exists untapped potential for research on their biological functions.

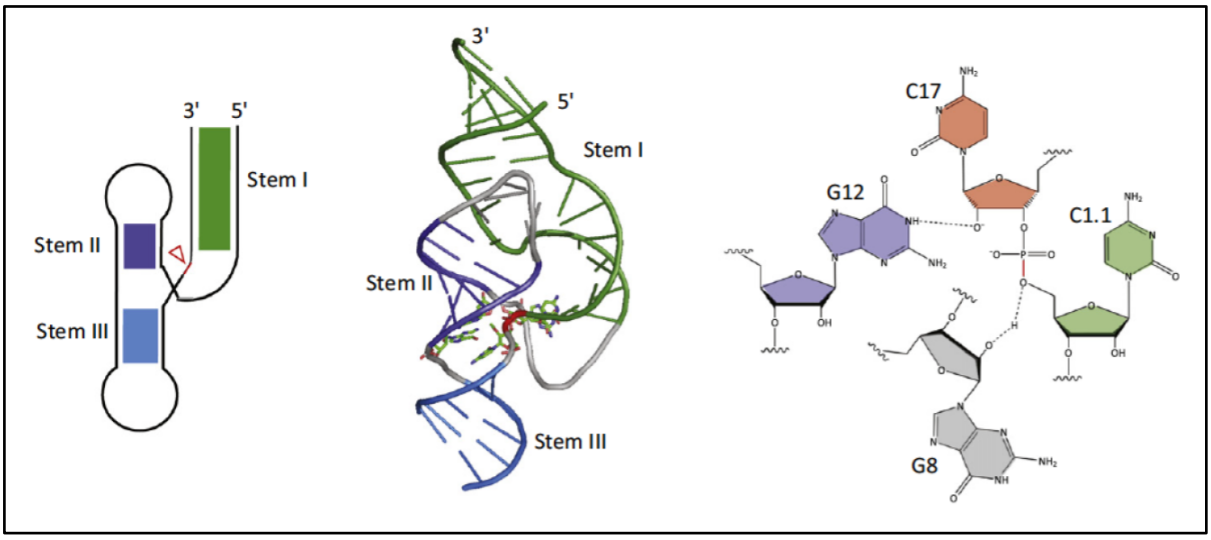

Figure 1. Structure of Hammerhead ribozyme by Jimenez, R. M., Polanco, J. A., & Lupták, A. (2015). Chemistry and Biology of Self-Cleaving Ribozymes. *Trends in Biochemical Sciences,40*(11), 648-661. The Hammerhead ribozyme comprises of three helices, stem I, II and III stabilised in the Y shape structure. Red arrowhead denotes the cleavage site. In the presence of divalent ions, guanine residue, G12 abstracts a proton from the oxygen atom of cytosine residue C17. Deprotonated C17 then induces a nucleophilic attack on the adjacent phosphate of C1.1 while G8 donates hydrogen to the leaving oxyanion group resulting in the release of 2', 3'-cyclic phosphate and 5'-hydroxyl RNA products.

1.2. Hammerhead ribozyme as a molecular tool

Extensive understandings on the architectures and catalytic activity of Hammerhead ribozyme have carved a course of research that is directed towards its application as tools in molecular biology research. Chemically synthesised ribozyme could be tailored to target specific RNA nucleotides

(Rossi, Castanotto, & Bertrand, 1996). While still preserving the catalytic core of the ribozyme, specific sequence modifications were invoked on the helices sequences so that the designed ribozyme hybridizes target RNA upon which the cleavage reaction would result in overall reduction of target gene expression (Khan & Lal, 2003).

The prospect of using ribozyme-driven gene control to contain viral diseases (without available vaccines) has been investigated in mammalian cell lines (Khan & Lal, 2003). Catalytic hammerhead motifs combined with conserved regions of the viral genome such as Long Terminal Repeats (LTR) of HIV-1 (Human Immunodeficiency Virus-1) and anti-HCV (Hepatitis C Virus) ribozyme that cleaves 5' untranslated region of HCV genome have been revealed to significantly reduce viral replication, accentuating the applicability of synthetic ribozyme in reducing viral load in infected patients (Macejak et al., 2001)(Nordstrom, 2005). Moreover, recent development of synthetic single-stranded oligonucleotide sensors, coupled with Hammerhead ribozymes have been effective in creating an allosteric regulatory tool that is capable of measuring specific responses upon binding to the ligands (Vinkenborg, Karnowski & Famulok, 2011). In the effort to characterise a molecular machinery, the resultant "aptazymes" are useful for evaluating the concentration of certain molecules and protein expression levels upon induction by an external stimulus (Vinkenborg, Karnowski & Famulok, 2011).

Nevertheless, complications still prevail in the *in vitro* and *in vivo* application of synthetic ribozymes. The synthesis is thus far constrained to small ribozymes such as hammerhead and hairpin ribozyme that are less stringent to modifications of their stem sequences. Other limitations include the determination of the selective nucleotides on target RNA that is not always attainable, miscellaneous chemical optimizations that are necessary to stabilise the synthetic ribozyme complex and the synthetic ribozyme delivery system that is confined to the usage of plasmids and retroviral vectors.

1.3. *Hammerhead ribozyme for the validation of RNase J1 activity*

In this study, an *in vivo* Hammerhead ribozyme system is established in *E. coli* by integrating the wild-type Hammerhead sequences fused ahead of a dual-reporter gene, EGFP-hDHFR, into the genome of BL21(DE3) cells. EGFP produces fluorescent protein that is readily detectable using fluorescence spectroscopy while hDHFR confers resistance to anti-folate inhibitor. The system is intended to make use of the Hammerhead ribozyme for the validation of RNase J1 activity of *Bacillus subtilis*; a ribonuclease that degrades 5'-hydroxyl-tagged RNA products of the ribozyme self-cleavage reaction.

RNase J1 plays a vital role in the regulation of *glmS* gene expression that is involved in the production of cell wall precursors, glucosamine-6-phosphate (GlcN6P), in *B. subtilis*. *glmS* encodes for glucosamine-6-phosphate synthetase (GlmS) and its mRNA is equipped with the GlcN6P-sensing ribozyme domain in which upon binding to the metabolite, a self-cleavage reaction is induced, producing *glmS* mRNA with 5'-hydroxyl. RNase J1 degrades these transcripts, thereby inhibiting *glmS* translation.

Within the established Hammerhead ribozyme cell system, the gene encoding for RNase J1 is integrated at a different locus of the *E. coli* chromosomal DNA, behind an arabinose-inducible promoter. As Hammerhead ribozyme cleavage generates 5'OH-EGFP-hDHFR mRNA, under conditional RNase J1 expression influenced by arabinose concentration, degradation of the reporter transcripts by RNase J1 is evaluated through a proposed fluorescence assay that measures the expression of EGFP in the cells.

1.4. *Objective of the study*

Validation of RNase J1 activity in BL21(DE3) cell system by utilising Hammerhead ribozyme fused with EGFP-hDHFR reporter integrated into the *E. coli* genome.