Chapter 2

Literature Review

2.1. RNA interference

RNAi or RNA interference is the biological process which can inhibit protein expression in cells by interacting with mRNA, with no involvement with DNA or the transcriptional process. It has become an interesting topic in biomedical research and drug development since its discovery.

2.1.1. Discovery of RNAi

RNAi mechanisms was first known as Post-transcriptional gene silencing (PTGS), which is the phenomenon where the accumulation of mRNA in the cytoplasm is reduced but transcription is still detectable. It has been investigated in studies of transgenic delivery of genes into several organisms. PTGS has been reported in plants (known as co-suppression) (Napoli et al. 1990, de Carvalho et al. 1992), fungi (known as quelling) (Romano et al. 1992), and ciliates (Ruiz et al. 1998). However, at that time, the mechanism was unknown. In 1998, Fire and Mello (Fire, Xu et al. 1998) observed that the silencing of specific genes occurred with treatment of double stranded RNA in *Caenorhabditis elegans* (*C. elegans*). This shows that dsRNA can serve as a regulatory factor of the cell which can control gene expression without DNA alteration.

2.1.2. RNAi pathway

As shown in *Figure 2.1,* RNAi mechanism initiates from long double stranded RNAs which can be introduced to the cells as a synthetic RNA, viral RNA, or RNA

transcribed from DNA. In addition, small interfering RNA (siRNA), the intermediates in the RNAi pathway, can also be used to promote the RNAi process. Long dsRNAs can interact with the Dicer, protein with exonuclease activity, resulting in dsRNA cleavage into short dsRNA with 2 nucleotide overhangs at the 3' end. The size of dsRNA is reduced into 21 base pairs and is called siRNA. The 5'end of siRNA normally carries a phosphate, while the 3'end does not. The duplex siRNAs are moved to the RISC (RNA-induced silencing complex), unwinding and one strand activates the RISC. Only one strand (guide strand) is bound to the RISC resulting in site-specific cleavage and silencing of the mRNA message but the other strand (passenger strand) is degraded. As the mRNA is degraded, the siRNA-RISC complex is released to pair with another mRNA target. The complementarity between the siRNA and the target RNA determines the sequence specificity.

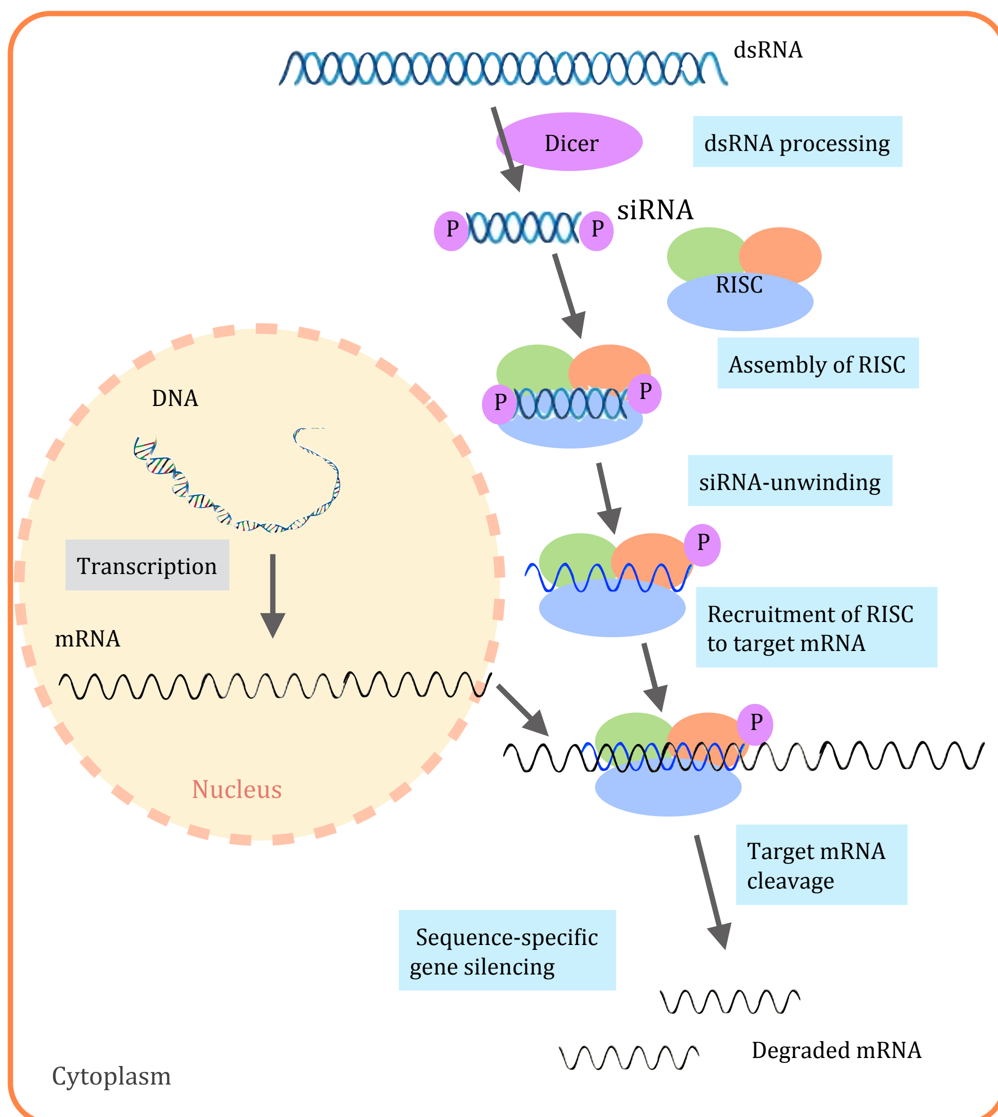

Figure 2.1: RNAi mechanisms. Pathway was modified from (Stevenson 2003).

2.1.3. Protein involved in RNAi process

Dicer

Dicer, a multidomain ribonuclease enzyme, was identified by Bernstein, E in 2001 (Bernstein et al. 2001). Its function is to specifically digest double stranded RNA (Nicholson 1999), which is important to RNAi, as it is responsible for the cleavage of long dsRNA into siRNA. This protein in human has molecular weight of approximately 220 kDa, and appears in "L-shape" on the electron microscopy (Lau et al. 2009, Wang et al. 2009). As shown in *Figure 2.2,* Dicer is composed of a dsRNA-binding domain (dsRBD) and two RNase III domains at its C terminus; and DExH-box RNA helicase-like domain, a domain of unknown function (DUF283), and a Piwi Argonaute Zwille (PAZ) domain at its N terminal (Takeshita et al. 2007). PAZ locates about 65 Å from RNase III domains which matches the length of approximately 25 base pairs of dsRNA (Macrae et al. 2006). PAZ binds to the 3'-end of dsRNA, whereas helicase translocates dsRNA into the ribonuclease domain. Recent study from Lau, PW (2012) (Lau et al. 2012) provides the new structural analysis of Dicer which support the possible interaction of Dicer with the RNA-induced Silencing Complex.

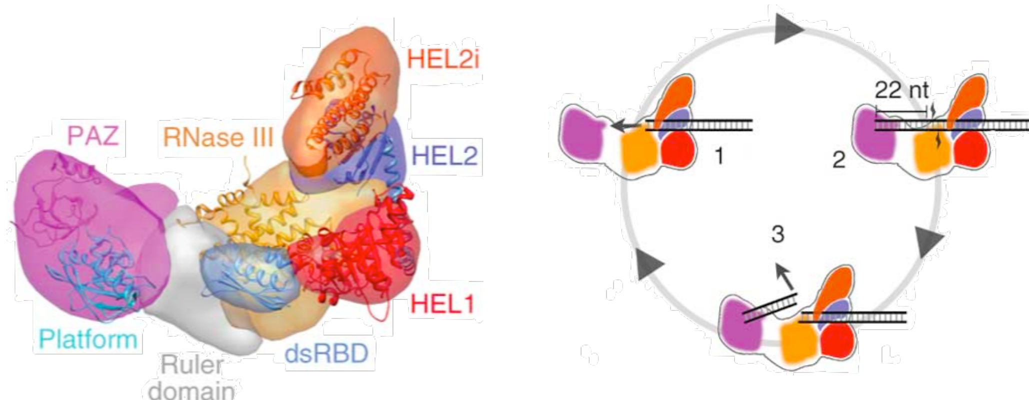

Figure 2.2: The composition of Dicer and its mechanism. The composition of Dicer mapped with the crystal structure (left) and the cleavage processing diagram of Dicer (right), starting from (1) the translocation of dsRNA into the nuclease module, (2) the recognition of dsRNA and cleavage, and (3) the releasing of siRNA product. The diagram obtained from Lau, PW (2012) (Lau, Guiley et al. 2012).

RNA-induced Silencing Complex (RISC)

RNA-induced Silencing Complex (RISC) is the group of proteins involved in silencing process of RNAi. RISC was first investigated as the protein with the sequence-specific nuclease activity, its active form contained a 25-nucleotides RNA (Hammond et al. 2000). RISC can be varied in size, such as 200 kDa (Nykanen et al. 2001) or 500 kDa (Hammond et al. 2001) in Drosophila and 160 kDa (Martinez et al. 2002) or 550 kDa (Hutvagner et al. 2002) in human. The association of siRNA into RISC requires

support from Dicer to transfer the small dsRNA into the cleavage site in Agonaute (Ago) (as shown in *Figure 2.3*).

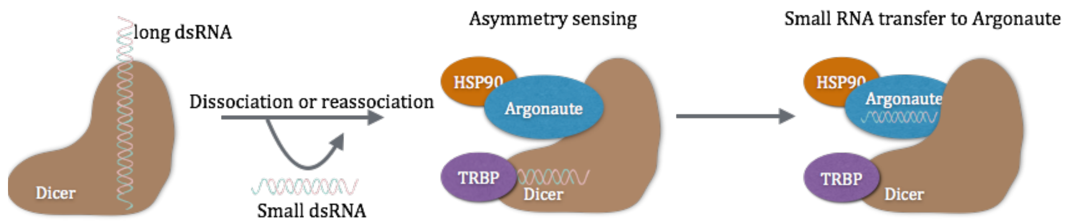

Figure 2.3: A proposed model of two main proteins, Dicer and RISC, and their actions in RNAi process. (redrawn from (Meister 2013)).

The minimal components of RISC in mammalian cells include Dicer, dsRNA-binding protein (TRBP), and Argonaute (Ago) (Gregory et al. 2005). In addition, the Heat shock cognate protein (HSP90) is also required for the conformational change of Ago in order to accommodate the RNA duplex (Johnston et al. 2010). Ago contains four domains, the N-terminal, middle (MID), P-element-induced wimpy testis (PIWI) and Piwi Argonaute Zwille (PAZ) domain (Cerutti et al. 2000) (as shown in *Figure 2.4)*. PIWI domain is the slicer domain which shared similarity to the RNaseH (Song et al. 2004). PAZ domain, also found in Dicer, recognises the dinucleotide overhang at 3′-end (Simon et al. 2011). Ago not only play an important role in RNAi machinery, it is also involved in DNA double strand break repair (Wei et al. 2012) and alternative splicing (Ameyar-Zazoua et al. 2012).

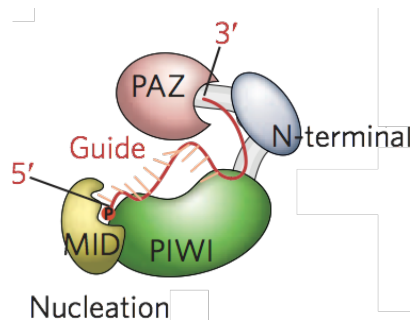

Figure 2.4: The model of the Argonaute with the association of small single-stranded RNA in RNAi machinery (Jinek et al. 2009)**.**

2.1.4. Localisation of RNAi processing

dsRNA is recognised as the foreign nucleotides in the cells, RNA process then has to be performed to the specific sites. Ago was localised in the cellular compartments in cytoplasms which is called the Processing bodies (P-bodies) (Sen et al. 2005). P-bodies was found to have the components with 5' to 3' exonuclease activity (Sheth et al. 2003). Mutation of Ago, which unable to be located in P-bodies, shows loss of

silencing activity (Liu, Rivas, et al. 2005). These indicate the RNAi activity are processed in the P-bodies.

2.1.5. Type of RNAi

RNAi can be introduced to the cells as a synthetic form designed to target the specific genes. Three main types of RNAi used in medical research include small hairpin RNA (shRNA), small interfering RNA (siRNA), and micro RNA (miRNA) as shown in *Figure 2.5*.

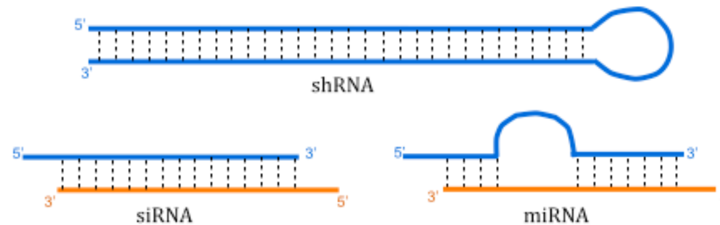

Figure 2.5: Three types of RNAi including shRNA, siRNA and miRNA.

shRNA is a short one stranded RNA which can be formed into the double stranded loop structure according to its complementary sequences. Its length is about 19-29 base pairs including 4 nucleotides to form the loop and 2 nucleotides as a 3'-overhang (Ge et al. 2010). With the hairpin form, shRNA needs to be processed to become siRNA before entering the pathway. The structure of shRNA provides protection from RNase, and helps improve the half-life of RNA in the cells. shRNA can be introduced to the cells as a plasmid DNA designed for expression of complementary sequence RNA.

siRNA is a double stranded RNA with a length of 21-25 nucleotides. siRNA has 5'-phosphate and 3'-hydroxyl terminals with 2-3 overhanged nucleotides at the 3'- end of each strand. This type of RNAi is used extensively in the RNAi studies according to the size. In addition, siRNA is an intermediate in the RNAi pathway, therefore without additional processing required, it can go directly to the pathway to perform its function.

miRNA is a small hairpin loop RNA similar to shRNA apart from the imperfect annealing. Its size is about 21-28 base pairs. miRNA share similar pathway with siRNA but instead of starting from long dsRNA, miRNA originates from pri-miRNA and is processed into pre-miRNA (70 nucleotides) before being cleaved and becoming mature miRNA. miRNA can be found naturally as the non-coding RNA which functions as a gene regulatory pathway. It was initially identified as small temporal RNA (stRNA) (Lee et al. 1993) when it was first discovered.

2.1.6. Functions and applications of RNAi

RNAi is recognised as the viral defense mechanism, especially for RNA virus. This antiviral mechanism was found in plants (Baulcombe 1996, Goodwin et al. 1996), invertebrates (Zambon et al. 2006), and recently in mammalian cells (Li et al. 2013, Maillard et al. 2013). RNAi also plays an important role in post-transcriptional control of gene expression, as microRNA shares some steps in RNAi pathway to inhibit the protein expression by mRNA decay (Pillai et al. 2007). The RNAi mechanism became an attractive tool in bioscience to investigate the function of unknown function genes or understand the relationships between genes. Apart from those, silencing activity of RNAi offers a potential therapeutic approaches (Davidson et al. 2011, Burnett et al. 2012). RNAi therapy provides the specific knockdown of target mRNA in order to block the expression of malfunctional proteins, or to control protein expression in overexpressed genes. This therapeutic concept has been developed in the treatment of cancer (Guo et al. 2013, Uchino et al. 2013), neurogenerative disorders (Boudreau et al. 2010, Chen et al. 2013), and viral infections (Motavaf et al. 2012, Zhou and Rossi 2012). Applications of RNAi for biomedical research and treatments are still in progress. One of the important obstacles for RNAi studies are the lack of suitable system to provide protection and to promote delivery of these RNAi molecules into the target cells.

2.2. Cellular barriers

2.2.1. Nature of mammalian cell membrane

Mammalian cell membranes are composed of large amounts of phospholipids with additional sphingolipids, and glycolipids. The bilayer is formed by the amphiphilic nature of the phospholipids, with the hydrophilic “head” and hydrophobic “tails” of phospholipids. This amphiphilic property leads to the self-assembly and compartmentalisation in aqueous solution as presented in *Figure 2.6.* The self-assembly is beneficial for the cells to separate and protect themselves from the environment, it is also useful to maintain and regulate the chemical balance inside the cells by their semi-permeability.

The cell membrane acts as the cellular barrier for external materials; diffusion across the phospholipid bilayer is restricted for almost all molecules and ions. Small molecules such as ions, amino acids, and sugars can transport through the plasma membrane via integral membrane protein channels. Mammalian cell membranes express a negative charge on their surface (Elul 1967), according to the presence of negatively charged phosphatidylcholine, and other glycerophospholipids which are abundant component of the cell membrane. This results in repelling of materials

with similar charges. However, the lipid membrane is fluidic at physiological temperature, this provides the membrane flexibility and capability to deform with low energy required. This is important parameter for the intracellular uptake of several substances including nutrients, and signaling molecules. This intracellular uptake is known as endocytosis.

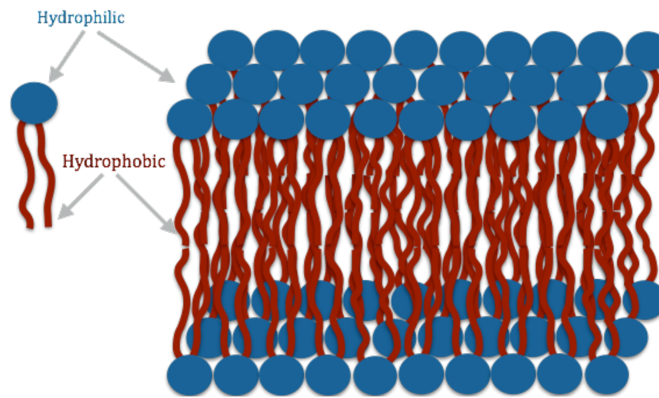

Figure 2.6: Phospholipids and Phospholipid bilayer.

2.2.2. Endocytosis

Endocytosis is the process that brings macromolecules, large particles, small molecules, and even small cells into the eukaryotic cell. This process is crucial for cells in many aspects, such as nutrient uptake, cell adhesion and migration, receptor signaling, pathogen entry, neurotransmission, and drug delivery. Most endocytosis processes have 3 steps in common. The first step is the binding step; particles, nutrients or small molecules have to attach to the cell membrane, causing the receptor to sense the signal (this does not occur in phagocytosis and macropinocytosis), and resulting in the next step. The second step is the deformation of the membrane, after a signal is sent through the membrane, leading to membrane coating, and formation of a membrane invagination. The last step is endocytic pathways, which differ with regard to the size of the endocytic vesicle, the nature of the cargo (ligands, receptors and lipids) and the mechanism of vesicle formation.

According to *Figure 2.7*, endocytosis can be categorised as (i) 'phagocytosis' or cell eating (the uptake of large particles) (Tauber 2003), (ii) 'macropinocytosis' or cell drinking (the uptake of fluid) (Lewis 1931), (iii) 'Clathrin-mediated endocytosis' (Pearse 1976), (iv) 'Caveolae-mediated endocytosis' (Palade 1953, Yamada 1955), and (v) Clathrin-, Caveolae-independent endocytosis. Clathrin- and Caveolae-mediated endocytosis are named after the main proteins on their coated vesicles, which are clathrin (Pearse 1976) and caveolin (Rothberg et al. 1992), respectively. Clathrin is the cytosolic protein which can assemble at the plasma

membrane. This protein is associated with adaptor protein-2 (AP-2) (Schmid 1997) and other adaptors to initiate and drive the membrane deformation to create the vesicle out of the membrane. The size of clathrin vesicle is varied with the maximum diameter of about 200 nm (Cureton et al. 2009). Whereas, caveolae coated vesicles are composed of caveolin protein as a main component but with smaller vesicle size of about 70-80 nm in diameter (Palade et al. 1968).

In general, endocytic vesicles are fused to the endosomal compartment "Early endosomes" followed by transportation to the "multivesicular bodies" (MVB), "Late endosomes", and finally end up in the "Lysosomes" for the degradation of proteins, polysaccharides, lipids, DNA and RNA. These endosomal compartments are different in their protein compositions and ions concentrations (as presented in *Figure 2.8*), which lead to the pH variation in each endosomal compartments. All lysosomal enzymes are acid hydrolases. RNase found in lysosomes are member of the T2 family that has optimum activity at pH 4-5 with very low substrate specificity (Irie 1999, Luhtala et al. 2010).

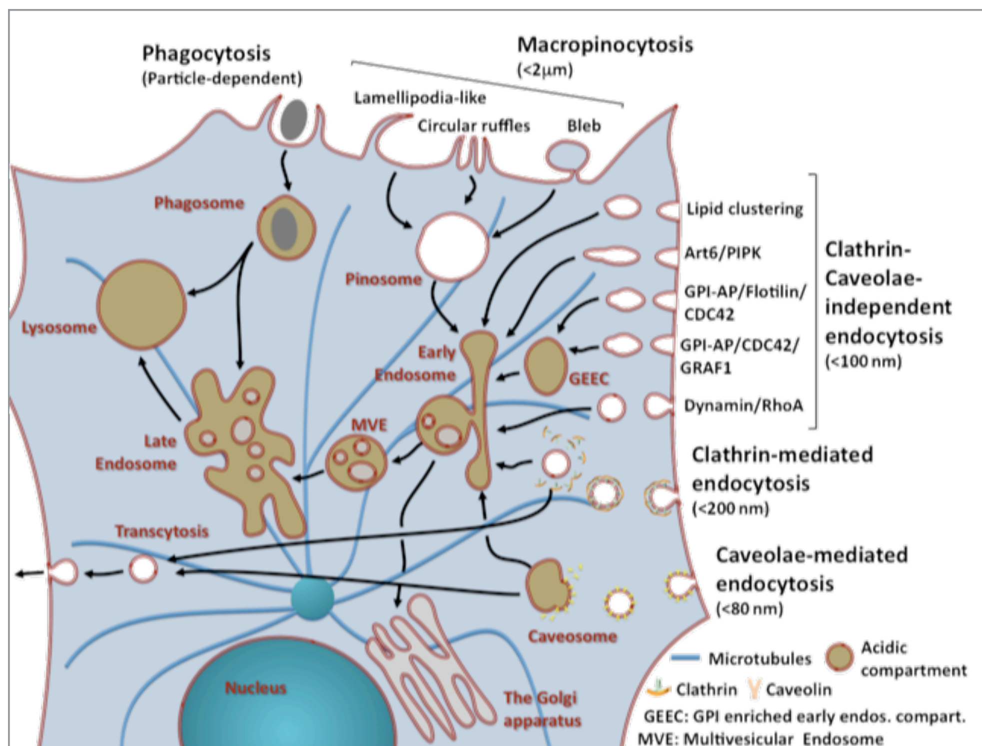

Figure 2.7: The overview of the endocytosis in eukaryotic cells (Canton et al. 2012).

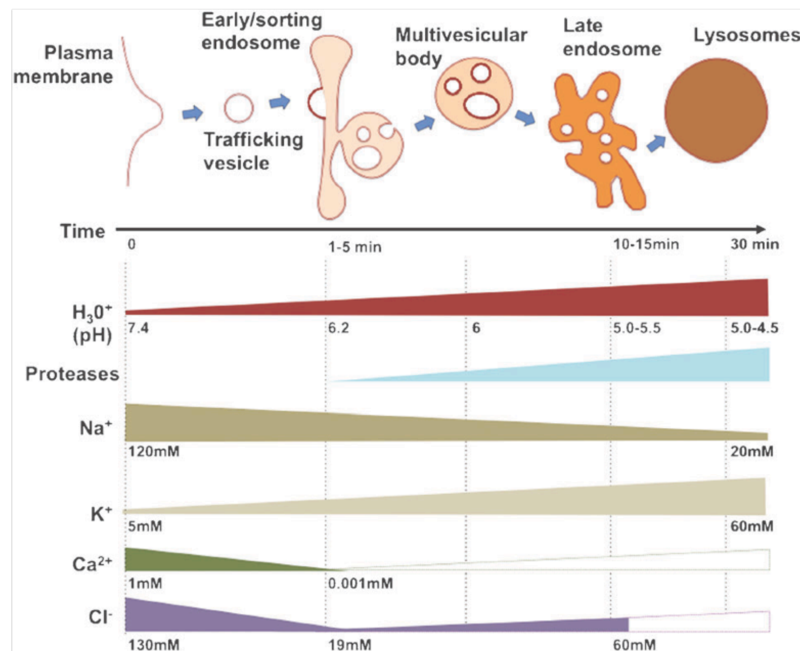

Figure 2.8: Diagram of the condition inside endocytic organelles including the ion concentration, and pH, relative to time (Canton and Battaglia 2012).

2.3. Improvement on RNAi delivery

The RNAi therapeutics requires the system to protect RNAi from both extracellular and intracellular nucleases and provide delivery across cell membrane barriers (*in vitro* perspectives) as well as to overcome the biological barriers and to enhance the ability to reach the targeting cells (*in vivo* aspects).

2.3.1. RNA instability

Direct delivery of naked siRNA results in very low gene-inhibiting effects both *in vitro* and *in vivo*, as it can be effectively degraded by available RNase in the serum and cytoplasm. The half-life reported for unmodified siRNAs in serum was only about 6 minutes *in vivo* (Soutschek et al. 2004). Apart from the enzymatic degradation, RNA is also unstable as the 2'-OH group of RNA is susceptible to the protonation in alkaline conditions (Voet et al. 2011). Previous studies shows that the pK_a of dsRNA is varied according to the neighbor base pairs, the pK_a value range from 6.5-8.1 (Wilcox et al. 2013). Therefore, mild acidic conditions are more suitable for the working conditions in RNA experiments, for example RNA is retained in acidic chloroform (pH 4.5-5.0) in RNA purification techniques (Sambrook et al. 2001). In order to protect siRNA from enzymatic degradation, an improvement of siRNA stability was performed by a variety of chemical modifications, such as backbone modification, 2'-sugar modifications, locked nucleic acid (LNA)

modifications, and nucleobase modification (as shown *Figure 2.9*) (Manoharan 2004).

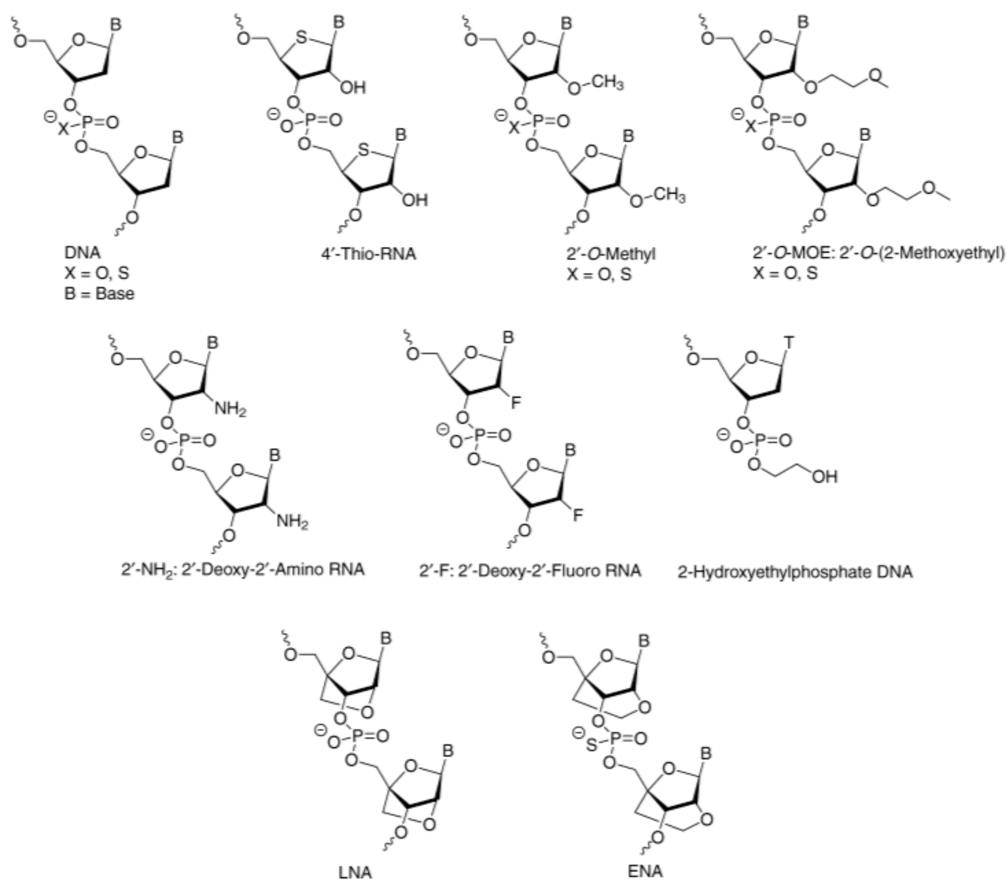

Figure 2.9: Some examples of RNA modification (Manoharan 2004).

However, only enhancement of the siRNA stability is inadequate. The major issue in the application of siRNA is that it is a large molecule (~7 nm in length, ~13 kDa in weight), with a strong negative charge (eg. ~40 negative phosphate charges on the siRNA backbone), thereby it cannot freely cross the cell membrane by itself (because of the nature of cell membrane, as described previously).

2.3.2. Off-targeting

Effects of siRNA study can be in sequence-specific and -nonspecific. Presence of dsRNA can induce the antiviral activation, this mechanism is sequence-independent. Cells transfected with the foreign nucleic acids consider these molecules as the indication of viral infection. Cells response to dsRNA by activation of interferons (Braganca et al. 1998, Judge et al. 2005) through the dsRNA-dependent protein kinase R (PKR) (Williams 1999), and also production of pro-inflammatory cytokines (Sioud 2005) to promote inflammation. Interferons (IFNs) (Isaacs et al. 1957) are

the group of a secreted cytokine which important for the response to invaders such as viruses, bacteria and parasites. They are a key factor in modulating the antiviral response in the cell by induction of IFN-inducible genes or IFN-stimulated genes (ISGs) (Larner et al. 1986). Among those genes, some can be transcribed and translated into the proteins that act as transcription factors, called Interferon Regulatory Factors (IRFs) (Fujita et al. 1988). IFN can also induce the gene involved in exonuclease to degrade the viral genetic materials, membrane bound protein to present antigen to the immune cells, and signaling molecules to activate downstream processes.

Another off-target effect of siRNA is the sequence-dependent by binding to non-target mRNA, resulting in suppression of non-target genes and less efficient knockdown of the target gene. siRNAs as short as 11 nucleotides can cause gene silencing (Jackson et al. 2003a). However, this unwanted effect could be avoided by using bioinformatic tools to selectively design the target-matched siRNA by choosing the appropriate sequence of siRNA and avoiding possible matching with other genes.

2.3.3. Available siRNA delivery system

Physical approaches

Physiological techniques such as electroporation (Kishida et al. 2004, Fyrberg et al. 2010), sonoporation (Saito et al. 2007, Sakai et al. 2009), and microneedles (Singh et al. 2010, Lara et al. 2012, Hickerson et al. 2013), have been studied with generally limited therapeutic purposes. For examples, the use of microneedle to directly apply siRNA into the cells is limited to the specific area where the device can be applied, the usefulness of this technique is restricted to skin disorders. Even though these physical application techniques are advantageous in their simplicity, limitations, such as inability to protect nucleic acids from enzymatic degradation, lack of target specificity and likelihood of cell damage, remain to be considered.

Viral vectors

Viral vectors are the most commonly used carriers for gene transfer because of their high transduction efficiency and ability to transfect cells that are difficult to be transfected by other methods. Viral vectors are designed to integrate siRNA or shRNA in their genomes. Viral vector can provide improvement on the stability and protection of RNA from degradation. After transcription, shRNA or siRNA will enter the RNAi pathway, shRNA can be cleaved by Dicer to become mature siRNA. The most widely used viral vectors are Adenovirus (Shen et al. 2003), Adeno-associated virus (Hommel et al. 2003, Boden et al. 2004), Retrovirus (Barton et al. 2002), and

Lentivirus. Adenoviral vectors can deliver RNAi to both dividing and non-dividing cells, but these viral vectors can not provide integration of RNAi to the host genome. Adeno-associated viral vectors are the most secure vector among those viral vectors, they do not induce a strong immune response (Stilwell et al. 2004). Both Adeno-associated viral vectors and Retroviral vectors provide genome integration, which prolong the expression of RNAi in the cells. However, Retroviral vectors are randomly integrated, this may cause unefficient silencing and toxicity to the host cells. Lentiviral vectors enable transduction into nucleus. Although viral vectors offer high delivery efficiency and effective knockdown activity, safety issues are the major concern. The injection of viral vectors can cause undesirable immune response effects, and it is anticipated that the toxicity of viral vectors will deter their use in humans. A new generation of these viral vectors provide lower risk in biosafety, by genetic engineering to lessen the toxicity, however, the remaining immunogenicity is still the big burden.

Non-viral vectors

Liposome and Lipoplex

Liposomes have been used as a carrier for nucleic acid delivery, they are composed of amphiphillic lipids that allow self-assembly in aqueous solution. Discovered more than 50 years ago by Alec D Bangham (Bangham et al. 1964), they are biocompatible and biodegradable as they are made up of natural lipids. With additional cationic properties, they can bind and form strong electrostatic interactions with the anionic siRNA and other nucleic acids resulting in the “lipoplex”. Study of lipoplex formation and release mechanism have been investigated already in plasmid DNA/lipoplex, but not siRNA/lipoplex. However, the phenomena that occurred in plasmid DNA/ lipoplex are likely to also occur in the siRNA/lipoplex due to their similar anionic characteristic. This binding can protect nucleic acids from degradation, and net cationic charge also facilitates endocytosis due to the negative charge of cellular membranes. Liposomes are the most commonly used system which are composed of either synthetic or natural cationic phospholipids. They are well-known as non-viral gene and drug delivery vehicles for several decades with many advantages including the commercial availability, ease of manipulation, and relatively high transfection efficiency. However, they have been shown to be leaky and unstable over time. In addition, liposome delivery lacks a defined drug release mechanism. It has been partially demonstrated that liposome interactions with plasma components induces membrane destabilisation and subsequent leakage of entrapped solutes into circulation with the helper lipids (Wrobel et al. 1995a, Almofti et al. 2003b). Unbinding between liposomes and nucleic acids occurred by charge neutralisation

through cellular anionic lipids (Xu et al. 1996, Zelphati et al. 1996) (as shown in *Figure 2.10*).

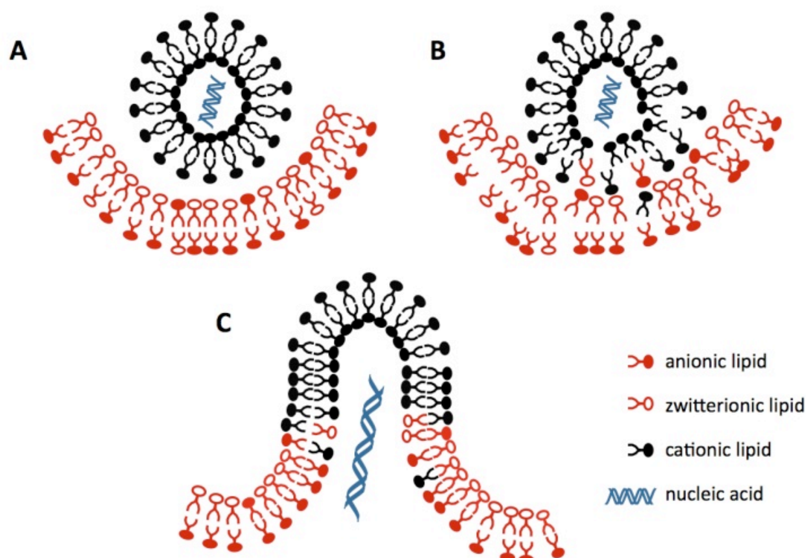

Figure 2.10: Proposed mechanism of nucleic acid/lipoplex releasing by "Fusion" of lipoplex lipid to the lipid membrane. (A) Endocytosis of lipoplex (B) Interaction between anionic cellular lipids and cationic lipoplex lipids, results in deformation of lipoplex by fusion to the cellular membrane. (C) Releasing of nucleic acids to the cytoplam (Liang et al. 2012).

The complex formation of liposome and nucleic acids has been proposed as lamellar, inverted hexagonal forms, depended on the lipid composition and ratio between cationic lipids and nucleic acids (as shown in *Figure 2.11*) (Tresset 2009b). The massive structure of lipid-nucleic acids complex could affect the ability of internalisation (Ross et al. 1999) as the endocytosis is a size-dependent mechanism. Furthermore, use of natural lipids as part of liposome could interfere with the phospholipid membrane composition of the cell.

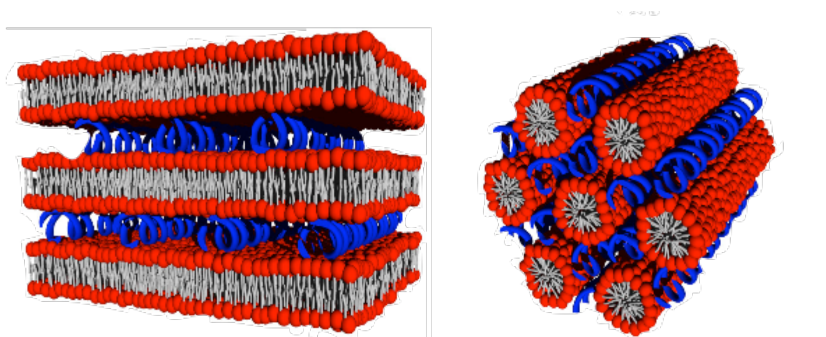

Figure 2.11: Possible complex formation of lipoplex. Complexed lamellar and complexed micellar hexagonal, lipids are in red (headgroup) and grey (hydrocarbon chain), while DNA are in blue (Tresset 2009b).

Moreover, according to the preparation, high amounts of unused RNA molecules could induce a stress response that could alter cell metabolism or affect cell lethality (Barreau et al. 2006). Changes in cellular activities including cytotoxicity

(Nguyen et al. 2007) and inflammatory response (Lonez et al. 2008) have been observed in cells transfected with lipid/nucleic acid complex.

Polymeric nanoparticles

Cationic polymers or cationic-containing block copolymers have been proposed as an option beside lipids. This is because a polymer can be synthesised to obtain desirable properties such as stability, solubility, biocompatibility, and to provide self-assembly. Cationic polymers, such as Polyethylenimines (PEI), chitosan (Howard et al. 2006), and poly(L-lysine) can be used to incorporate siRNA for complex formation according to the electrostatic interaction between anionic siRNA and cationic polymers. In addition to this complexation, polycation can be co-polymerised with other polymers such as poly(etheylene oxide) (PEO) or poly(ethylene glycol) (PEG), poly(d,l-lactide-co-glycolide) (PLGA) and poly[(N-2-hydroxypropyl)methacryla-mide] (PHPMA) or directly conjugated to siRNA. Some examples of polymeric nanoparticles are shown in *Table 2.1.*

Table 2.1: Examples of polymeric nanoparticle siRNA delivery systems.

Type of nanoparticle	**Purpose of investigation**	**Ref.**
PEG conjugated siRNA	VEGF siRNA for anti-angiogenesis	(Kim et al. 2006)
Folate-PEG-distearoylphosphatidylethanolamine nanoparticles	Folate-linked lipid-based nanoparticles for siRNA delivery in KB tumor xenografts	(Yoshizawa et al. 2008)
Liposome-entrapped PEGylated nanoparticles	Multifunctional nanoparticle mediates tumor delivery of siRNA	(Kenny et al. 2011)
PEGylated nanoparticles	Efficient gene silencing in metastatic tumor by siRNA formulated in surface-modified nanoparticles	(Li et al. 2008)
Liposomes-protamine-hyaluronic acid nanoparticles	An efficient and low immunostimulatory nanoparticle formulation for systemic siRNA delivery to the tumor	(Chono et al. 2008)
Calcium phosphate (LCP) nanoparticle (NP)	Biodegradable calcium phosphate nanoparticle with lipid coating for systemic siRNA delivery	(Li et al. 2010)
Chitosan/polyethylenimine nanoparticles	Tumor-homing glycol chitosan/ polyethylenimine nanoparticles for the systemic delivery of siRNA in tumor-bearing mice	(Huh et al. 2010)
PEGylated nanoparticles	pH-sensitive fusogenic peptide nanoparticles mediated gene silencing	(Hatakeyama et al. 2009)

Polymeric carriers with cationic surfaces can interact with the anionic lipid on the cells membrane with the similar pathway as lipoplex, and be internalised via endocytosis. The endosomal release can be enhanced by attachment of special peptides, lipids or by using a stimuli-responsive polymer to induce endosome breakdown. Fusogenic lipids or peptides can be used as an alternative strategy for siRNA release mechanism, the "cell penetrating peptides (CPPs)" which are able to translocate through the cell membranes have been attached to polyplexes to promote the endosomal release (Endoh et al. 2009). pH-responsive polymers such as poly (amidoamine) (Patil et al. 2009), dimethylaminoethyl methacrylate (DMAEMA) with propylacrylic acid (PAA) (Convertine et al. 2009), and 1,4,7-triazanonylimino-bis[N-(oleicyl-cysteinyl-histinyl-1-aminoethyl)propion-amide] (THCO) (Wang et al. 2008) have also been used. The proposed release mechanism of pH-sensitive polymers were presented as shown in *Figure 2.12.* The process is known as "proton sponge" (Behr 1997), it is inducible by low pH in the endosomal compartment. A polymer with pKa close to endosome will be protonated and more protons will be pumped to maintain the low pH condition. This results in high ion concentration and high osmotic pressure, leading to endosome rupture and release of siRNA into cytoplasm.

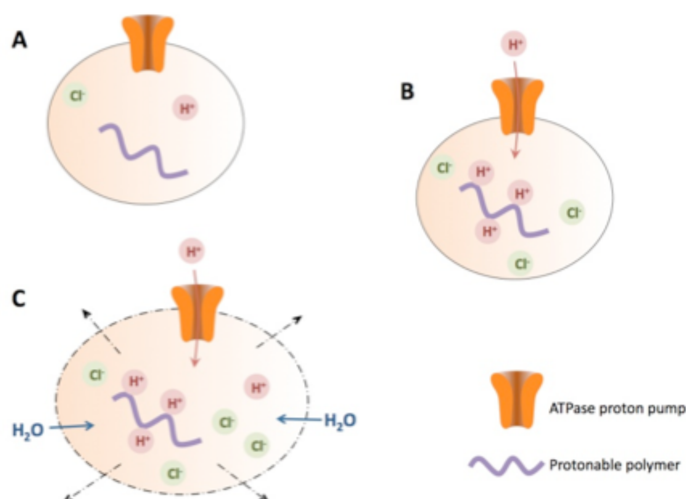

Figure 2.12: Proposed endosomal release mechanism, 'proton sponge' hypotheses. (Liang and Lam 2012).

2.3.4. Progress in RNAi delivery system and the pre-clinical study

RNAi have been studied and developed towards the therapeutics purposes as shown in *Table 2.2.*

Table 2.2: List of current progress in RNAi-based therapeutics in clinical study[1].

Company	Clinical phase	Status	Drug	Target	Disease	Delivery system
Allergan	II	Terminated	AGN211745	VEGFR1	Age-related macular degeneration, choroidal neovascularisation	naked siRNA
Anylum Pharmaceuticals	III	Active	Patisarin	TTR	Transthyretin-mediated amyloidosis	LNP
	II	Active	ALN-TTR02	TTR	Transthyretin-mediated amyloidosis	LNP
	II	Completed	ALN-RSV01	RSV nucleocapsid	Respiratory syncytial virus infections	naked siRNA
	I	Completed	ALN-VSP02	KSP and VEGF	Solid Tumours	LNP
	I	Recruiting	ALN-AT3	antithrombin	Hemophilia and other bleeding disorders	GalNAc-siRNA
	I	Completed	ALN-PCS02	PCSK9	Hypercholesterolaemia	LNP
Arrowhead research	I	Completed	ARC-520	Conserved region of Hepatitis B	Hepatitis B	Dynamic polyconjugates
Calando	I	Active	CALAA-01	RRM	Solid Tumours	Cyclodextrin NP

[1] Information obtained from "Key RNAi Drugs in the Clinic." Retrieved February 24, 2014, from http://www.genomeweb.com/rnai/key-rnai-drugs-clinic and (Kanasty, Dorkin, Vegas, & Anderson, 2013).

Company	Clinical phase	Status	Drug	Target	Disease	Delivery system
Calimmune	I/II	Active	Cal-1	Chemokine receptor 5	HIV	lentiviral vector encoding shRNA
Gradalis	II	Active	FANG	Furin	Colorectal cancer with liver metastases, advanced melanoma, Ovarian cancer, and Ewing's sarcoma.	bi-functional shRNA
	I	Recruiting	pbi-shRNA STMN1	Stathmin-1	metastatic solid tumours	shRNA/fusogenic DOTAP-cholesterol cationic liposome
Marina Biotech	I/II	Recruiting	CEQ508	CTNNB1	Familial adenomatous polyposis	*E.coli*-carrying shRNA
MD Anderson Cancer Center	I	Recruiting	siRNA–EphA2–DOPC	EphA2	Advanced cancers	LNP
Nitto Denko	I	Active	ND-L02-s0201	Heat shock protein 47	Fibrotic disease	vitamin A-coupled LNP
Opko Health	II	Completed	Bevasiranib	VEGF	Diabetic macular oedema, macular degeneration	naked siRNA
Pachyonychia Congenita Project	I	Completed	TD101	K6a (N171K mutation)	Pachyonychia congenita	naked siRNA
Quark Pharmaceuticals	II	Active	QPI-1007	Caspase 2	Non-arteritic Anterior Ischaemic Optic Neuropathy	naked siRNA
	II	Active	PF-655 (PF-04523655)	RTP801	Choroidal neovascularization, diabetic retinopathy, diabetic macular oedema	naked siRNA

Company	Clinical phase	Status	Drug	Target	Disease	Delivery system
	I	Completed	I5NP	p53	Kidney injury, acute renal failure	naked siRNA
	I/II	Recruiting	I5NP	p53	Delayed graft function, complications of kidney transplant	naked siRNA
RXi Pharmaceuticals	II	Active	RXI-109	Connective tissue growth factor	Fibrotic disease	self-delivered RNAi technology
Senesco Technologies	I/II	Recruiting	SNS01-T	eIF5A	B cell cancers	siRNA-pDNA-PEI complex
Silence Therapeutics	I/II	Active	Atu027	Protein Kinase-3	Pancreatic cancer	LNP
Silenseed	I/II	Active	LODER	K-RAS	Pancreatic cancer	polymeric matrix containing siRNA
Sylentis	II	Completed	SYL040012	adrenergic receptor β-2	Ocular hypertension	naked siRNA
	I/II	Active	SYL1001	transient receptor potential cation channel subfamily V member 1	Dry eye syndrome	naked siRNA
Tekmira Pharmaceuticals	I	Active	TKM-Ebola	Zaire strain of Ebola	Ebola-virus infection	LNP
	I/II	Active	TKM-PLK1	Proliferation-associated protein polo-like kinase 1	Gastrointestinal neuroendocrine tumour, Adrenocortical carcinoma	LNP
	I	Terminated	PRO-040201	Apolipoprotein B	Hypercholesterolaemia	LNP

2.4. Block copolymer

Block copolymers offer advantages over other vectors because of their potential to be modified for specific requirement. Properties such as stimuli responsiveness, charge, length and molecular weight are adjustable and controllable.

2.4.1. Block copolymer characteristics

Block copolymers are composed of two (A, B) or more (A, B, C,…) different polymerised monomers which are connected by covalent bonds. One polymerised monomer is referred to one block, so the name diblock, triblock, tetrablock copolymers are copolymers with two, three, and four different blocks, respectively. This work focuses on diblock copolymers. Diblock copolymers can be notated as A_m-b-B_n where A and B are two different polymers, while m and n stand for mean degrees of polymerisation of each polymer. As the property of block copolymers can be optimised, it is useful for the biological study by designing of amphiphilic block copolymers, to have similar character as phospholipids. This amphiphile can be freely soluble in specific solvent and become self-assemble in aqueous solution in various forms as shown in *Figure 2.13*.

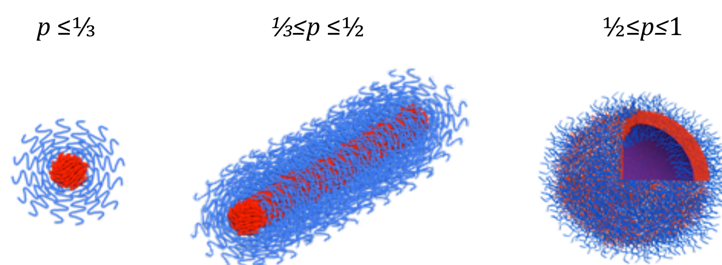

Figure 2.13: Example of amphiphilic block copolymer self-assemble morphology. Spherical micelle (left), Cylindrical micelle (center), and Vesicle or polymersomes (Discher et al. 1999) (right). The hydrophilic regions are shown in blue while the hydrophobic regions are in red.

The important criterion for different forms of amphiphilic block copolymers is the membrane curvature. It can be determined by the molecular packing parameter (p), which is obtained from the following equation,

$$p = \frac{v}{a_0 l}$$

where, v is the molecular volume of hydrophobic block, a_0 is the optimal interfacial area between the hydrophilic and hydrophobic segments, and l is the length of the hydrophobic part (Nagarajan 2002, Smart et al. 2008).

The spherical micelle (p≤⅓) is the sphere-shaped particle that has hydrophobic parts packed in the core and hydrophilic parts face to an aqueous solution. The cylindrical micelle (⅓≤p≤½) is similar to the spherical micelle but the hydrophobic

parts gathered as rod-shaped, resulting in the worm-like structure. The vesicular form (½≤p≤1) is the sac-like structure with block copolymer membranes as its shell, with the hydrophobic layer inside its membrane. The size of the micelle in aqueous solution is about 5-50 nm in diameter; while vesicle forms can range from 50 nm up to a micron. The size of these nanoparticles depends on the type of polymers together with the degree of polymerisation.

Diblock copolymer properties are adjustable by variety of polymer combinations, degree of polymerisation, and conjugation with other molecules, as shown in *Figure 2.14.* This results in size optimisation, stimuli responsiveness, capability to encapsulate a wide range of molecules, and ability to target or interact with other molecules. These properties are important not only for chemistry or materials engineering but also for biomedical application such as drug delivery, coating materials.

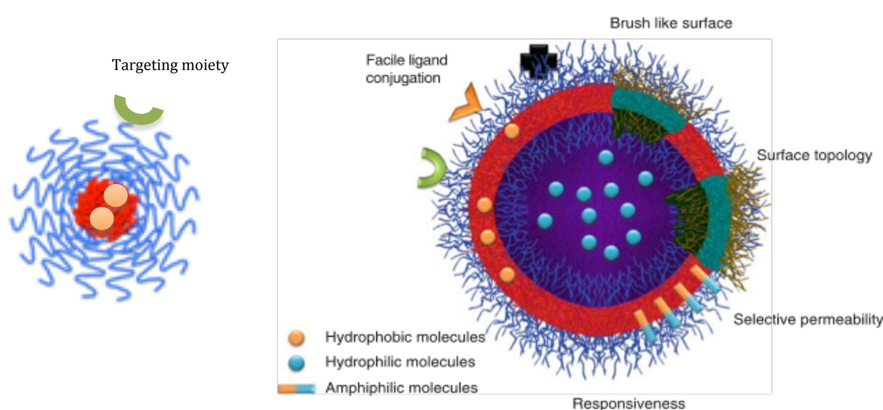

Figure 2.14: Diblock copolymers and their tunable properties for biomedical applications. (a) spherical micelle (adapted from (Kataoka et al. 2001)) and (b) polymeric vesicle (Massignani, Lomas, et al. 2010).

An example of commercially available block copolymer used for drug delivery is Pluronic® (BASF, Wyandotte, MI, USA), the ethylene oxide and propylene oxide based copolymers. This thermal responsive block copolymer has been studied for therapeutic purpose (Kabanov et al. 2002). It can be used to deliver anti-cancer drug (Hosseinzadeh et al. 2012, Abdullah-Al-Nahain et al. 2013), genetic materials (Kabanov et al. 2005), enhancing transfection efficiency (Pluronic® P85) (Astafieva et al. 1996). Another example is used of block copolymers for bioimaging (Tian et al. 2010, Biswas et al. 2011) by conjugating fluorescent dye into the polymer. PEG-poly(lactic acid) (Kim et al. 2009) and poly(1,2-butadiene)-poly(ethylene oxide) polymersome with fibronectin mimetic peptides (Pangburn et al. 2012) have been studied for *in vitro* siRNA delivery.

2.4.2. PMPC-PDPA

Poly (2-methacryloyloxyethyl phosphorylcholine)-co-poly(2-(diisopropylamino) ethyl methacrylate) or PMPC-PDPA has been used as a carrier for various molecules such as dye, hydrophobic drug, small molecules, antibody and DNA. It has the two important properties from two different blocks; biocompatibility and pH responsiveness. PMPC and PDPA structures are shown in *Figure 2.15.*

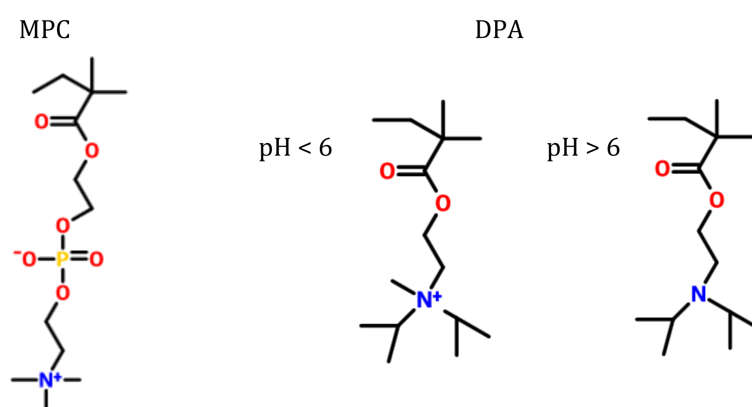

Figure 2.15: Structure of MPC and DPA.

MPC, the monomer of PMPC has been used widely to prepare biocompatible copolymers for contact lense coating with low eye irritation (Lewis 2000). DPA is considered as a weak cationic polyelectrolyte. Its pKa from acid titration studies is about 6.3 (Bories-Azeau et al. 2004) resulting in its pH responsive property. DPA is water soluble at pH lower than pKa but can be deprotonated and become hydrophilic at pH higher than its pKa. MPC can be homopolymerised and prepared as blocked copolymer with several polymers including Poly(2-hydroxyethyl methacrylate) (HEMA) (Madsen et al. 2006), poly(2-dimethylamino)ethyl methacrylate (PDMA) (Yuan et al. 2006), poly(2-hydroxypropyl methacrylate) (PHPMA) (Madsen et al. 2009) and DPA (Ma et al. 2003). MPC copolymerised with DPA has been used for preparing micelles (Ma et al. 2003, Yu et al. 2003) According to the previous study, $PMPC_{30}$-$PDPA_{60}$ is fully solvated at pH 2.3 and forms micellar structures with diameters of 44 nm at pH 7.1-7.3 (Ma et al. 2003). Differences in degree of polymerisation affect polymersome formation, a higher degree of polymerisation of DPA will increase the size of polymersomes. With similar temperature at 20°C and pH 7.4, $PMPC_{25}$-$PDPA_{47}$, $PMPC_{25}$-$PDPA_{77}$, $PMPC_{25}$-$PDPA_{94}$ and $PMPC_{25}$-$PDPA_{147}$ formed polymersomes with diameters of about 50, 100, 180, and 300 nm, respectively.

2.4.3. Advantages over other methods

Self-assemble in aqueous solution

The PDPA part of block copolymer contains a pH-responsive property, this allow self-assembly when the solution pH reaches the polymer pKa. The PDPA homopolymer is solubilised in aqueous solution at pH lower than 6.3 and being weak cationic polyelectrolyte. The tertiary amine group of PDPA is deprotonated at pH above 6.3, resulting in self-assembly or polymersomes formation as PDPA is insoluble (as shown in *Figure 2.16*).

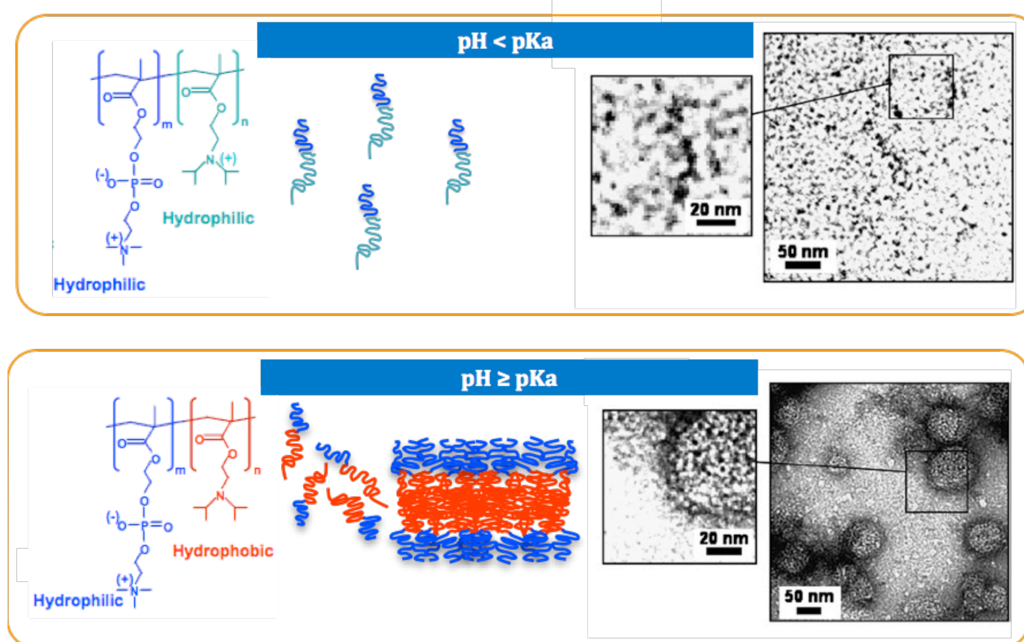

Figure 2.16: Self-assembly and polymersomes formation. Picture was modified from (Lomas, Massignani et al. 2008)

Encapsulation of hydrophilic and hydrophobic molecules

PMPC-PDPA has been reported to use for vesicles formation by using its pH responsive property and it shows the ability to capture dye (Massignani, Canton et al. 2010) including Rhodamine B octadecyl ester perchlorate, phospholipids, BODIPY, and Propidium Iodide; gold nanoparticles (Du et al. 2005); antibody (Canton, Massignani et al. 2013); doxorubicin (Pegoraro et al. 2013), Bovine serum albumin (Wang et al. 2012b) and plasmid DNA (Lomas, Canton et al. 2007, Wang, Chierico et al. 2012b).

Size-control for optimum uptake

Size-control can enhance the cellular uptake of polymersomes. Previous studies showed the impact of particle size of micellar glycocluster nanoparticles (Nakai et al. 2003) and gold nanoparticles on cellular uptake (Chithrani et al. 2006). Both groups

suggested the optimal diameter for particle internalisation in HeLa cells is about 40-50 nm. These studies are correlated with the theoretical study from our group (Chaudhuri et al. 2011) which suggest unequal size distribution on cellular uptake, with desirable diameter of about 40-45 nm. A previous study on cellular uptake of PMPC-PDPA polymersomes in size ranging from 100-400 nm showed the maximum uptake is on 100 nm diameter polymersomes (Massignani et al. 2009).

pH responsiveness and endosomal escape

The pH responsive property of PDPA, PMPC-PDPA enable it to be dissolved in acidic conditions (details in *Figure 2.16*). This property is beneficial for release of encapsulated molecules into the cytoplasm or nucleus. Polymersomes internalisation occurred through endocytosis, polymersomes are trapped inside the endosomal compartments. As mentioned previously (in *Figure 2.8*), the pH inside the endocytic vesicles is varied as a consequence of different ion concentration in each endosomal compartments. This acidic condition induces polymer disassociation due to the PDPA becoming hydrophilic and soluble at low pH. In addition, each polymersome contains large number of PMPC-PDPA unimers (above 5000 unimers per polymersome of 100 nm in diameter) after disassembly, which can generate the osmotic pressure. This can induce transient osmolysis of endosomal membranes. This phenomenon provides releasing of polymers and contents out of endosomes, those molecules are then transferred to the cytosol (the overview of the endosomal escape is shown in *Figure 2.17*). The endosomal escape of PMPC-PDPA was supported by using polymersomes with and without pH-sensitive part to deliver rhodamine dye into fibroblasts, signal of rhodamine dye in the cell with pH-responsive polymer distributed across the cytosol, whereas in non-pH sensitive polymer, the signal was limited and punctuated as shown in *Figure 2.18* (LoPresti et al. 2009). In contrast, PMPC-PDPA micelles contain much lower number of unimers required for micelle formation (10 unimers per micelle in 20 nm in diameter). These micelle can be disassemble according to the pH responsiveness property, however, such amount of unimers (500 times less than polymersomes) does not warrant enough osmotic pressure to achieve the endosomal escape.

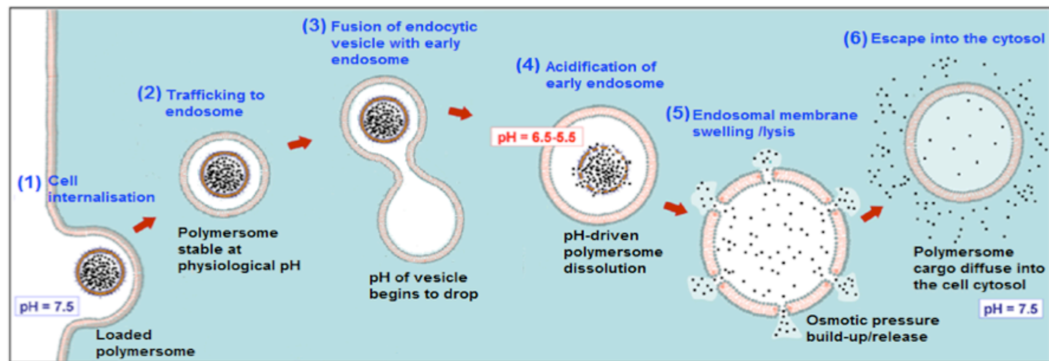

Figure 2.17: The proposed mechanism for endosomal release of PMPC-PDPA polymersomes. The picture was adapted from (Robertson et al. 2014).

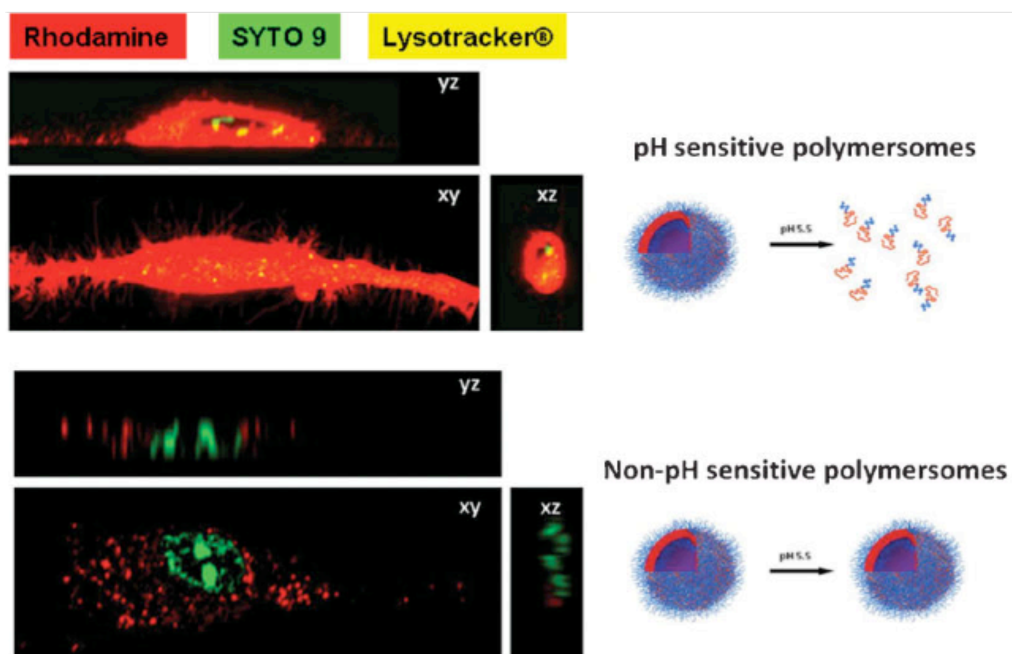

Figure 2.18: Evidence of endosomal escape of polymersomes in human dermal fibroblast. The picture was adapted from (Massignani, LoPresti et al. 2009).