CHAPTER II

LITERATURE REVIEWS

MNPs containing *aeg*PNA oligomers will be a promising candidate biomolecular detection probe with high sensitivity and specificity in the future due to their nano size and remarkable physical properties. Three important segments were required to assemble the desired biomolecular probes: 1) peptide nucleic acid, DNA mimic, (*aeg*PNA) oligomers containing natural or non natural bases, 2) functionalized magnetite nanoparticles (MNPs), 3) azalactone, reactive electrophile molecule, as linker molecule. To understand the concept of this exploration, basic information and background of deoxyribonucleic acid (DNA) and peptide nucleic acid (*aeg*PNA), magnetite nanoparticles (MNPs), and azlactone will be discussed in the following.

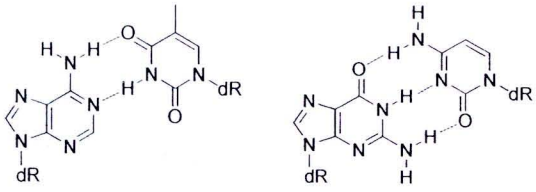
DNA and Peptide Nucleic Acid (*aeg*PNA)

Deoxyribonucleic Acid or DNA [19], repeating unit of nucleotides, contains genetic information which transmitted from one generation to the next. Usually, nucleotides, consisting of the phosphate backbone, are connected by ester linkage at 5' position of 2-deoxyribose sugar and heterocyclic nitrogenous bases (purine and pyrimidine) which linked to 1' position of 2-deoxyribose sugar ring by *N*-glycosidic bond.

Undergo polymerization via enzymatic process; the monomer of nucleotide reacts with other monomers to form dimer and oligomer, respectively, *via* phosphate diester linkage at 3' position of sugar ring. Finally, the assemblies of biopolymers produce the single strand DNA and then the duplex DNA structure is thermodynamically generated which is the most detected form in nature.

The main reason that DNA is usually found in double helix structure contributes from intermolecular hydrogen bonding between complementary bases in the interior structure of DNA. The stability of DNA on the specific paring of each of the four bases with a single partner (adenine (A) pairing with thymine (T) via two hydrogen bonds and

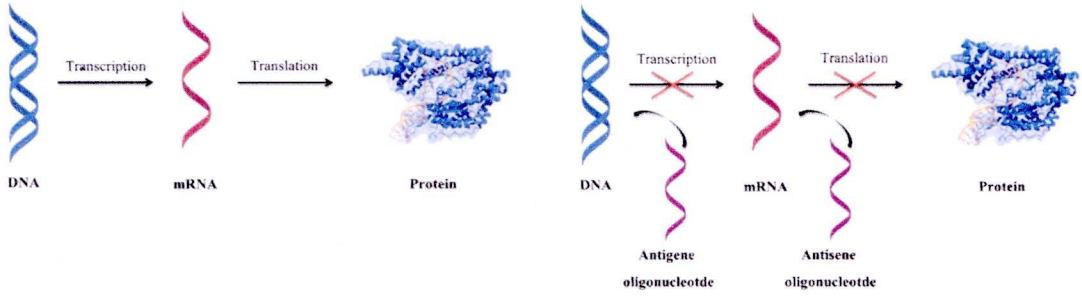
cytosine (C) pairing with guanine (G) via three hydrogen bonds) produces information storage. The specific paring are called "Watson-Crick base pairing rules" (Figure 5) [20]. In addition to the paired bases, base stacking is one of the factors that contribute to DNA stability. In nature, Bases are roughly perpendicular to the helix axis and their π bond are located above and below adjacent aromatic moieties produces π-π interaction which so called a π-π base stacking [21].



dR = 2-deoxyribofuranose

Figure 5 Hydrogen bonding between A : T and G : C base paring [20]

Base-pairing is the most important factors that associated with double helix formation for gene expression in living cells. Normally, the expression of genetic information consists of two processes. The former is transcription and the latter is translation. Toward the development of pharmaceuticals, the oligonucleotides synthesis is the most attractive to then using regular cellular processes. For example, antigene therapy is focused on inhibiting transcription *via* selective binding to DNA (Figure 6) while antisense approach is preventing translation of the gene *via* selective binding to mRNA, thus blocking its translation into a protein (Figure 6). Because of the high specificity involved in the binding of oligonucleotides to their targets, these treatments can apply to cure diseases without destroying the cells [22] by insertion of alien oligonucleotide into cell. However, one disadvantage point is that DNA is easily degraded by nucleases and protease *in vivo* [23]; therefore, modification of DNA was initiated to explore the desired molecule that would appropriate for any applications.



(a) **(b)**

Figure 6 Illustration of gene expression (a) normal gene expression (b) antigene and antisense strategies [22]

Interesting modification of the backbone modification that gave promising candidate for antigene and antisense agent is peptide nucleic acid or PNA. Nielsen and co-workers [23] firstly introduced *aeg*PNA or aminoethylglycine PNA in 1991, a DNA analog, in which each ribose phosphate unit is replaced by an *N*-(2-aminoethyl)-glycine unit that joins by amide bonds as backbone connected with a purine (A and G) or pyrimidine (C and T) nucleobased by methylene carbonyl linkages. PNA are depicted like a peptide, with the *N*-terminus at the first position and the *C*-terminus at the end (Figure 7).

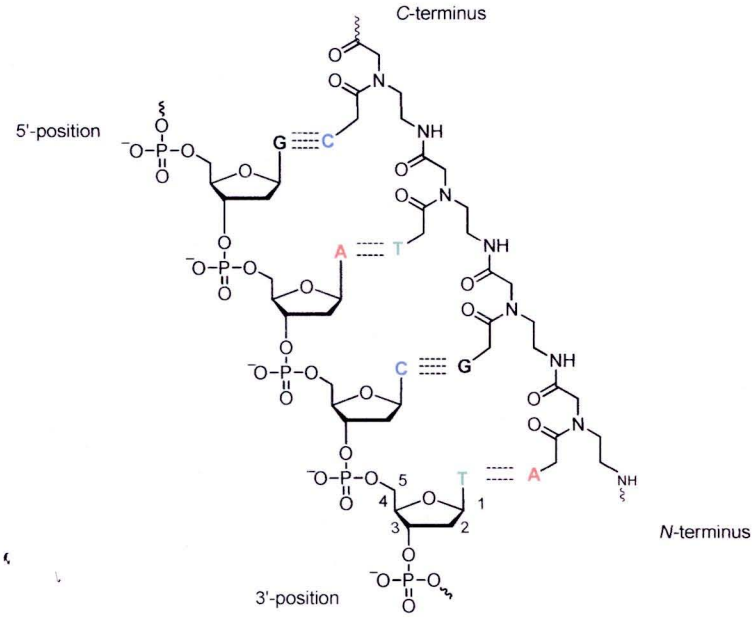


Figure 7 *aeg*PNA structure and Watson-Crick base paring with DNA [23]

The *aeg*PNA can generally hybridize with DNA according to Watson-Crick base pairing (Figure 7) and PNA has a high thermal stability to form double and triple helix complexes in sequence specificity due to lacking of phosphate backbone on *aeg*PNA leading to diminish of an electrostatic force between DNA/mRNA and *aeg*PNA. Furthermore, *aeg*PNA can bind to complementary DNA/mRNA in both antiparallel and parallel orientation. However, antiparallel hybrids are considerably more stable, and the parallel hybrid has been shown to have a thermal stability different formation (Table 1) [24]. Additionally, *aeg*PNA has strong resistance to enzyme nuclease and protease making it become ideally for gene therapy.

Table 1 Thermal stability form duplex helix complexes [24]

Duplex helix complexes	Thermal stability (T_m)
DNA-DNA (antiparallel)	53.3 °C
*aeg*PNA-DNA (antiparallel)	56.1 °C
*aeg*PNA-DNA (parallel)	69.5 °C
DNA-mRNA (antiparallel)	50.6 °C
*aeg*PNA-mRNA (antiparallel)	72.3 °C
*aeg*PNA-mRNA (parallel)	51.2 °C

From these specific properties, *aeg*PNA has become an interesting tool for several applications in biotechnology and medical fields. Currently, the application of *aeg*PNA is divided into 3 major areas: biomolecular devices, antisense-antigene therapy and biomolecular probe as biosensor [23, 24, 25]. The biomolecular probe can integrate with nanotechnology and biology such as nanotube [26], nanowire [27], and nanoparticle probe [28, 29] for DNA sequence analysis which received considerable attention.

Magnetite Nanoparticles (MNPs)

Magnetite particles (MPs) with both microscale and nanoscale are paramagnetic or superparamagnetic particles. With their interesting characteristics, they facilitate purification and detection of biomolecules in a wide range of samples. In particular, their high surface area and superparamagnetic such as magnetite (Fe_3O_4), and maghemite (γ-Fe_2O_3) nanoparticle (MNPs) that has drawn the attention of scientific and technological community due to their unique physical and chemical properties [30] (nanometer size, high surface area, and inert to oxidation reaction). Magnetite is a very promising candidate since its biocompatibility has already been proven. Magnetite (Fe_3O_4), also be written as $FeO.Fe_2O_3$, is consists of wustite (FeO) and hematite (Fe_2O_3) [31]. Several potential applications of magnetite in bionanotechnology have been recently reported such as magnetic resonance imaging contrast agent (MRI) [32], drug delivery [33],

hyperthermia treatment [34], biomolecular (DNA, RNA, proteins and peptide) magnetic separation [35, 36], and medical diagnosis [37] (Figure 8).

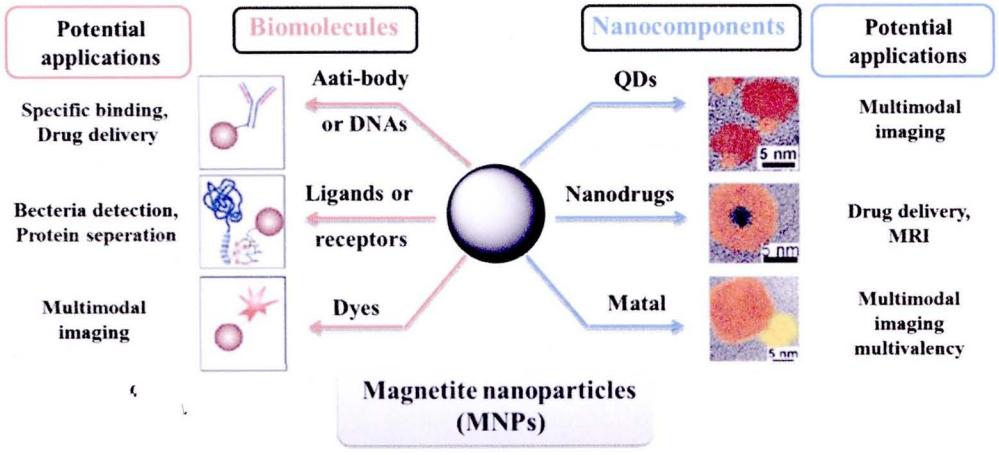


Figure 8 Illustration of two strategies to fabricate multifunctional magnetite nanoparticles and their potential applications [38]

These nanoparticles have many advantages. For example, they can easily synthesized *via* various synthesis methodology such as thermal decomposition [39], microemulsion [40, 41], gas phase deposition [42], and co-precipitation of Fe^{2+} and Fe^{3+} aqueous salt solutions by addition of a base [43, 44] which is widely employed at laboratory scale to synthesize MNPs for biomolecular application.

To make nanoparticle functionalized as design, the surface modification of MNPs is required after the nanosize synthesis in order to stabilize the particle and to enhance its biocompatibility. Surface modification of MNPs mainly uses polymers and organic reagents as coated layer [31, 45]. In addition to enhance stability, their modified surface can provide a platform for incorporating of biological functional molecules, such as amino acid [46], protein [47, 48], and enzyme immobilization [49].

Magnetite Nanoparticles (MNPs) as probe for nucleic acid diagnosis tools

Application of MNPs as diagnostic tools and nucleic acid biosensor has increased tremendously over the past years as demonstrated *via* the large number of publications [50]. Moreover, incorporation of *aeg*PNA with MNPs offers some advantages over DNA such as higher specificity, sensitivity in the detection of DNA sequence and no separation of hybridized and unhybridized probe is required. Employment of *aeg*PNA based assays for DNA sequencing can easily employed several simple techniques as detector such as color differentiation [51, 52, 53], fluorescence [54, 55, 56] and resistance value change [57]. However, MNPs can also have a drawback since non-specific background signals may be generated from the unhybridized probe.

Zhu and co-worker [58] described immobilization of the MNPs with *ss*DNA onto zinc nanoparticle as oligonucleotide label. The target *ss*DNA with the phosphate group at the 5' end was then covalently immobilized to the amino group of MNPs *via* phosphoramidate bond in the presence of 1-ethyl-3-(3-dimeth-ylaminopropyl) carbo diimide (EDAC), (Figure 9). The nanoparticle labeled oligonucleotides probe **(3)** was used to identify the target *ss*DNA immobilized onto MNPs. The hybridization probe was assessed by the dissolution of the zinc sulfide nanoparticles anchored on the hybrids and the indirect determination of the dissolved zinc ions by anodic stripping voltammetry (ASV). It was shown that this probe exhibited good selectivity and high sensitivity and it could effectively discriminate the complementary sequence.

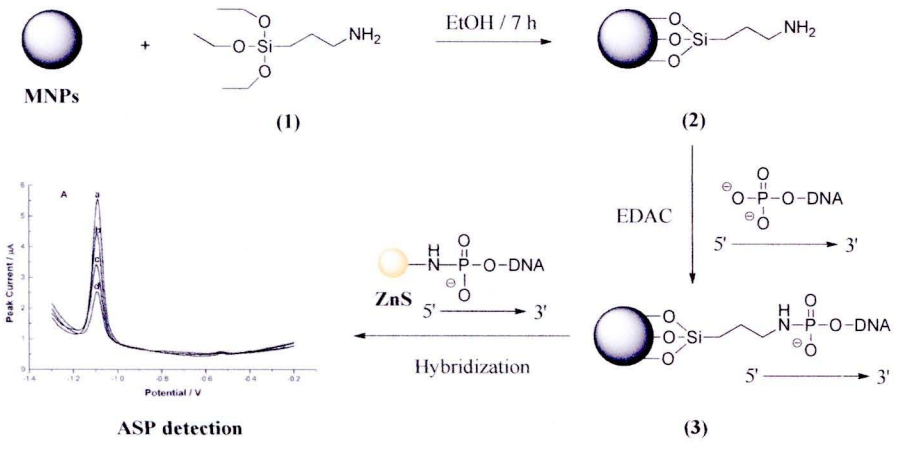


Figure 9 Preparation of DNA-MNPs for DNA target and AVS detection [58]

Furthermore, Stoeva and co-workers [59] investigated a novel method for synthesis of three-layer composite particles with silica core **(7)**, magnetite inner layer, and gold surface with ligands containing either thiol or disulfide group (Figure 10) for attachment of *ss* DNA *via* thiol-disulfide bonds. Then, *ss* DNA probe can hybridize with *ss*DNA target. Significantly, the aggregate structures exhibit sharp melting profiles when heated above the melting temperature of the duplex DNA linkers, which predetermines their utility in high selectivity DNA detection.

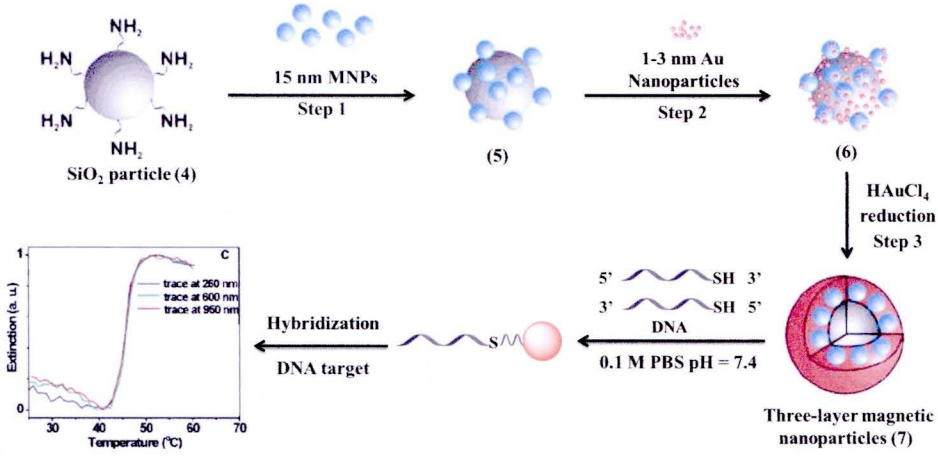


Figure 10 Preparations of the three-layer MNPs, DNA-MNPs probe and hybridization with *ss*DNA target [59]

Employment of *ss*DNA as biomolecular probe is quite simple and good due to Watson-Crick base pairing is remarkably specific. However, probing the mismatch discrimination of the oligonucleotide recognizer by *ss*DNA as a probe is not sufficiently selective; moreover, the oligonucleotide recognizer is susceptible to hydroxylation by endogenous nucleases and protease. Therefore, such *ss*DNA probes might pose problems in practical applications. To rectify those problems, PNA is now widely applied as a probe in biotechnological and medical application. For many purposes, it can be used in place of natural or modified oligonucleotide [60]. Most of applications of PNA probes involve the use of the original Nielsen's *aeg*PNA system since it is the only PNA that is presently commercially available.

From the previous study, Kerman and co-workers [61] demonstrated the use of biotinlated *aeg*PNA probe with steptavidine coated magnetic beads in conjunction with a redox-active intercalator Moedola's bule (MDB), or 7-dimethyl-amino-1,2-benzopheno xazinium salt. The *aeg*PNA:DNA hybrid was separated from the unbound *aeg*PNA by labeling of the DNA samples with biotin. The *aeg*PNA hybrid with ssDNA was monitoring by voltammetric signal of MDB (Figure 11).

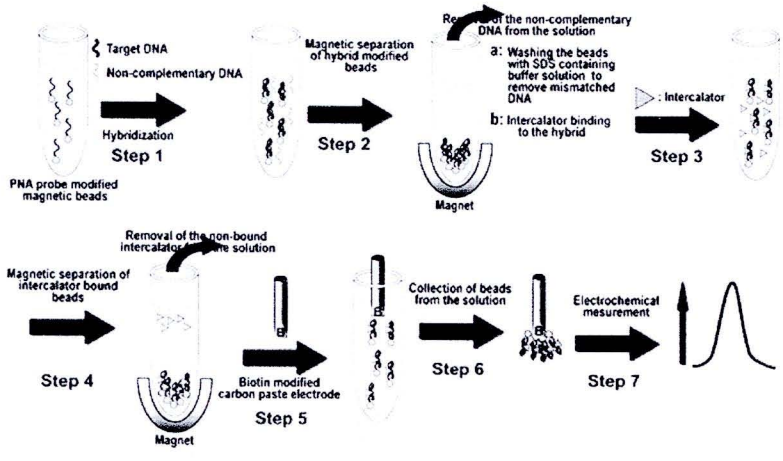


Figure 11 *aeg*PNA modified magnetite bead-based assay for the specific detection of hybridization in connection with an intercalator, Meldola's blue (MDB) [61]

In addition, there are reports on the use of *aeg*PNA in combination with MALDI-TOF mass spectrometry to monitor DNA sequence [62]. The DNA sample was first tagged with biotin and coated onto streptavidin magnetic beads. The captured DNA sample was then hybridized with *aeg*PNA probe. After stringent washing of the *aeg*PNA and DNA bead conjugate to remove the unhybridized *aeg*PNA probe, the beads were directly monitored by MALDI-TOF mass spectrometry. The present of *m/z* of *aeg*PNA indicated that the sequence of DNA and *aeg*PNA are complementary. It should be noted that only molecular mass of *aeg*PNA was observed because it is a peptide molecule which easily ionizes (Figure 12).

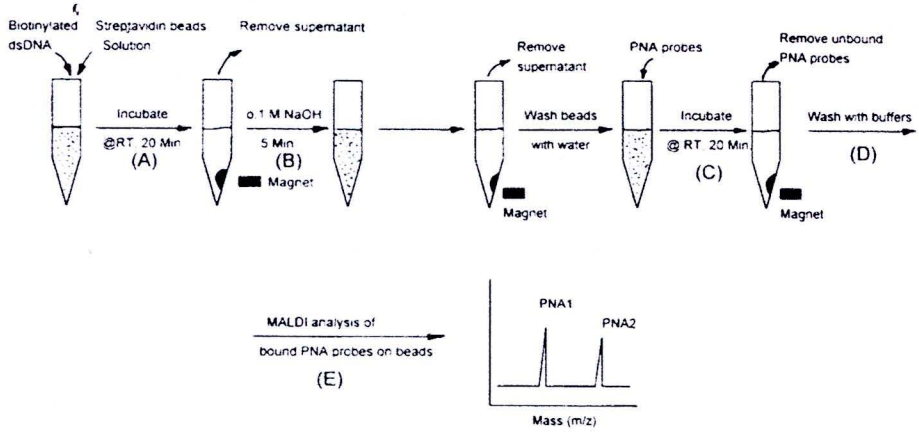


Figure 12 Affinity capture assay for DNA. The detected molecular weight of the *aeg*PNA probe indicates the DNA sample [62]

Furthermore, Wang and co-workers [63] recently mentioned a modified surface of MNPs using 3-(mercaptopropyl)trimethoxysilan **(8)**. Next, *aeg*PNA at *C*-terminus attached onto MNPs surface *via* a thiol-disulfide exchange reaction (Figure 13). The product was immobilized **(10)** and hybridized onto MNPs which monitoring by surface-enhanced raman scattering (SERS) and UV spectroscopy. The SERS, and optical analytical technique offers several important advantage, such as a rapid and non-destructive analytical process, highly compound-specific information for chemical analysis. The raman technique also requires little sample preparation, which allows on-

line analysis and field applications. The results showed that the *aeg*PNA-MNPs probe were able to easily hybridize with the perfect-match *ss*DNA target while they showed no affinity to the non-complementary *ss*DNA.

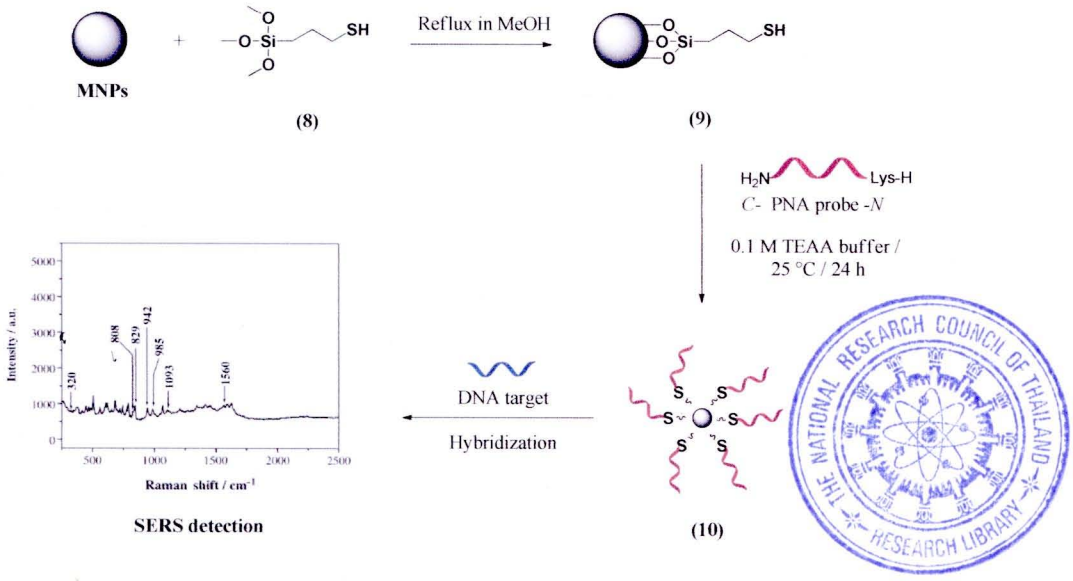


Figure 13 Thiol layer-coated MNPs with PNA as PNA probe and hybridized with perfect-match *ss*DNA were immobilized by SERS [63]

In 2008, Pita and co-workers [64] reported the synthesis of water-soluble gold covered with cobalt ferrite superparamagnetic nanoparticles as well as their use for the development of specific DNA biosensors. The metallic covering stage which were achieved by bonding gold seeds through an affinity and trap strategy (Figure 14). Magnetite nanoparticles are functionalized with a mixture of amino and thiol groups that facilitate the electrostatic attraction and further chemisorption of the gold nanoparticles, respectively. Using these gold nanoparticles as seeds, a complete coating shell is achieved by gold salt-iterative reduction. Thiol-modified *ss aeg*PNA oligomers can be directly immobilized onto Au-magnetic nanoparticles. These PNA-modified, gold-covered magnetite nanoparticles can interact specifically with a DNA target molecule complementary to the attached *aeg*PNA probe. The specific *aeg*PNA hybridized with

DNA is monitored by the detection of an external fluorescent molecule, which intercalates in the *aeg*PNA:DNA double helix complexes. These results suggested that it can use as an improved biosensor for DNA sequence analysis from the modification of nanoparticle. Furthermore, it can be easily separated and less time-consuming procedures.

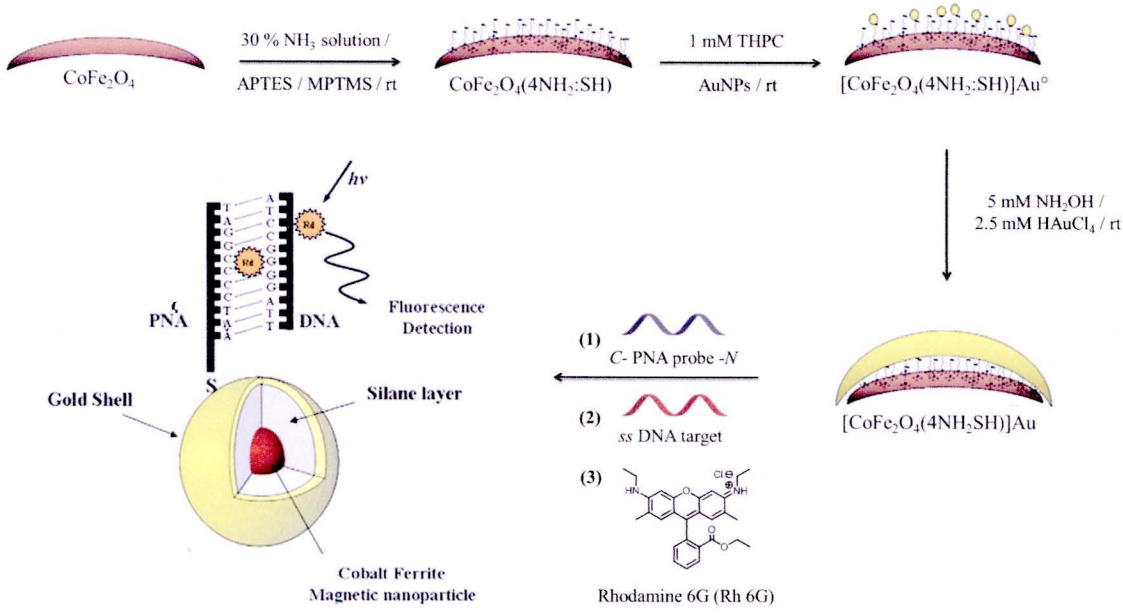
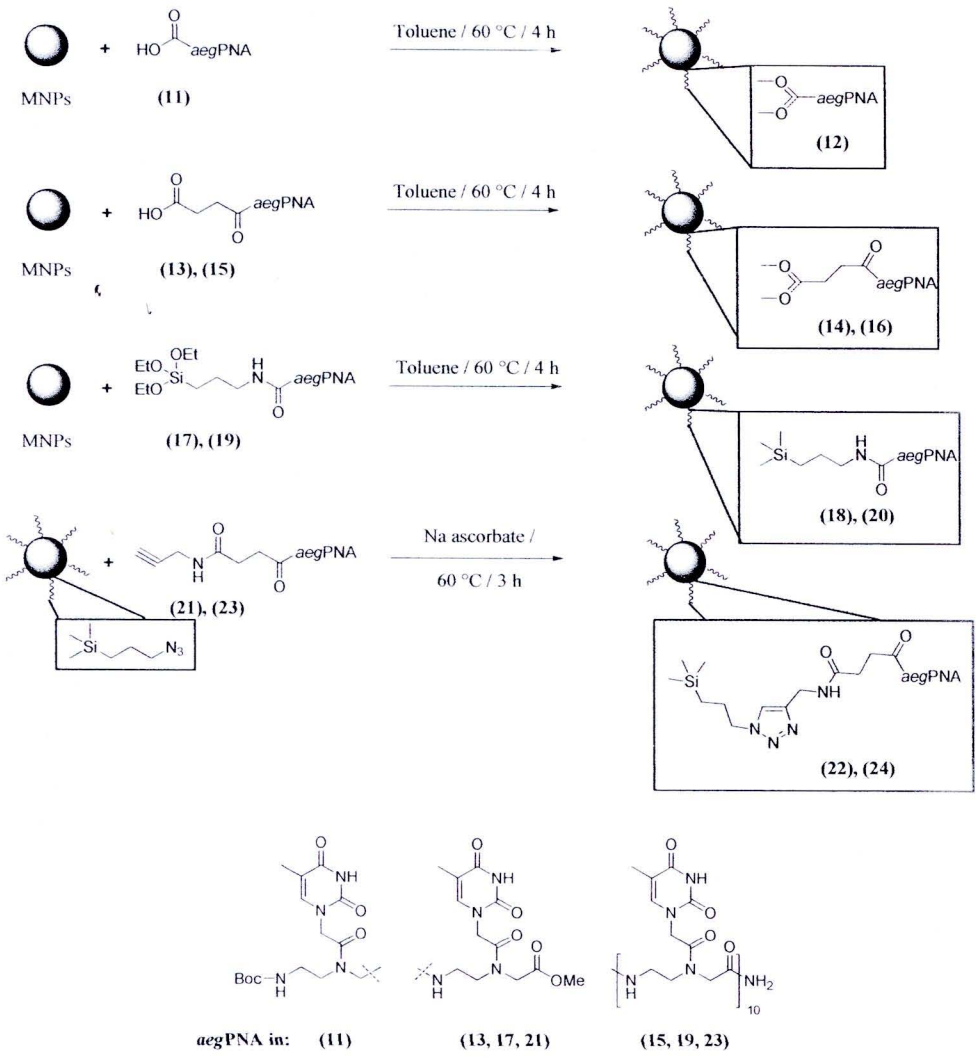


Figure 14 Synthesis of gold shell–cobalt MNPs coated with thiolated PNA for DNA target hybridization and fluorescence detection [64]

Moreover, Prencipe and co-workers [65] established a versatile, effective synthetic platform for the development of monomer and decamer *aeg*PNA-nanoconjugates, (Scheme 3) starting from synthetic *aeg*PNA and nanometer-sized maghemite. The sequence selective *ss*DNA recognition and sequestration ability of the resulting *aeg*PNA-MNPs probe were assessed according to their capability of enhancing the of proton transverse relaxation times (T_2) in aqueous solution under conventional hybridization conditions with complementary *ss*DNA. The resulting *aeg*PNA probe has considerable potential for biomedical application, as it can be easily manipulated and delivered to a desired target by the simple application of a controlled external electromagnetic field. The preliminary results demonstrated that these *aeg*PNA probe

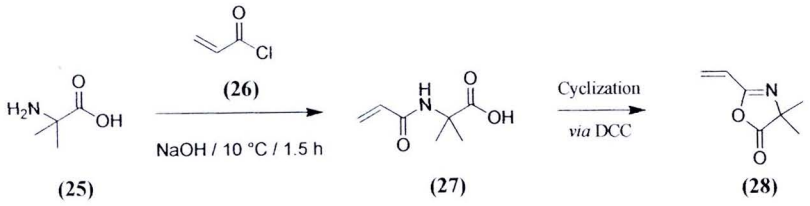
maintained excellent performances in *aeg*PNA and DNA hybridization through the measurement of variation of proton transverse relaxation times (T_2) of water dispersions in the presence of complementary DNA. The major problem concerns the solubility of these *aeg*PNA hybridized with nanometer size under the conditions utilized.



Scheme 3 Preparation of *aeg*PNA-MNPs probe *via* carboxylate and trialkoxy silane group linkers: *aeg*PNA can either indicate PNA monomer or 10 mers [65]

Azlactone compound as electrophilic linker

Azlactone, electrophilic cyclic ester, has attracted the attention of researchers during the last decades. Taylor and co-worker [66] reported the developed a relatively simple synthesis of 2-vinyl-4,4-dimethyl-5-oxazolone, or VDM **(28)** similar with lactone structures using the mixed anhydride method on *N*-acrylol-2-methylalanine (Scheme 4) which easily synthesized. Azlactone heterocyclic compound exhibited electrophilic behavior because of its high reactivity toward nucleophiles including amino ending groups of primary amines, alcohols, thiols, and peptide. Nucleophiles can react with VDM *via* ring-opening reaction [67]. The present of electrophilic ring is a site for polymer modification using a nucleophile connected to a dye [68], chromophore [69], biomolecule groups [70] through a suitable spacer allows the synthesis of functionalized side chain polymer (Figure 15).



Scheme 4 Synthesis of 2-vinyl-4,4-dimethyl-5-oxazolone, or VDM [66]

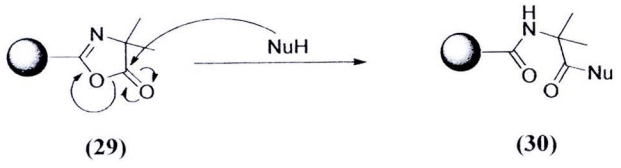


Figure 15 Rign-opening reaction of nucleophiles attached to azlaztone functionalized polymer networks on solid support [71]

From these properties, VDM has become an interesting for design polymeric materials with a wide range of properties for particles, template, mono and multilayered film applications. Frist, Fournier and co-worker [72] presented that synthesis of co-polymerization containing VDM onto a Wang resin as solid supported *via* atom transfer radical polymerization (ATRP) using copper mediated living radical (Figure 16 and 17) then they have been investigated by scavenging process with benzylamine. The results illustrated that the influence of the architecture of the grafted co- polymer, grafted homopolymers and block co-polymers showed good efficiency for removal of benzylamine, although the proximity of the azlactone rings prevents the reaction from being quantitative. The best results in terms of reactivity and efficiency toward benzylamine were observed with grafted statistical copolymers. Such result could be explained by an improvement of the accessibility of the azlactone rings by the presence of styrene units.

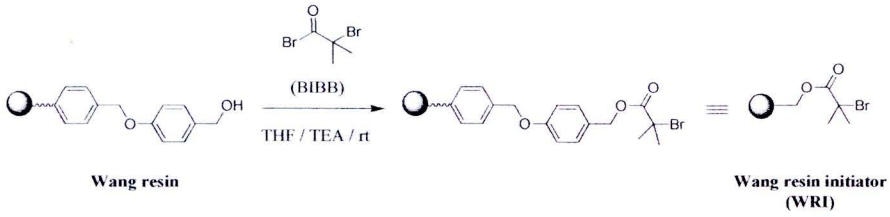


Figure 16 Synthesis of Wang resin initiator for ATRP [72]

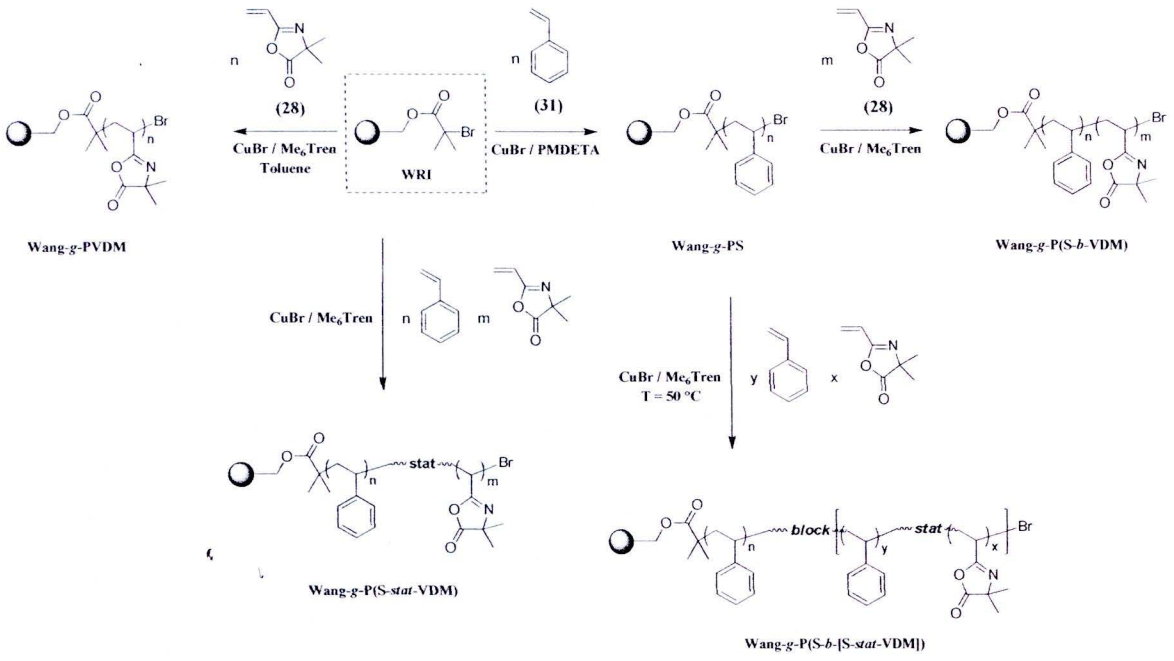


Figure 17 Synthesis of different supported architectures based on VDM for Wang resin initiator [72]

Furthermore, Cullen and co-worker [73] reported the application of VDM brushes onto surface-anchored as templates to covalently immobilize protein with activities close to those of free protein. Specifically PVDMA brushes synthesized *via* ATRP from surface grafted initiators were potential scaffolds for protein immobilization (Figure 18). In order to compare the effectiveness of poly(acrylic acid) (PAA) for enzymes, quantitative data on the effect of brush length, present swelling, kinetics of binding, activity, and its temperature / pH dependence of bound ribonuclease A (RNase A). The results show that PVDMA brushes can bind of RNase A with activities close to those of free emzyme. Si-*g*-PVDMA-RNase A showed similar temperature and pH dependence to that of the native enzyme, indicating no significant changes in the conformation or active site of the protein. The use of PVDMA brushes was extended to other biotechnologically relevant enzymes. The immobilization of DNase I, glucose oxidase, and trypsin results in protein with activities close to or higher than those in native

form. Compared to the NHS activated PAA brushes, PVDMA brushes do not require any pre-activation for binding process.

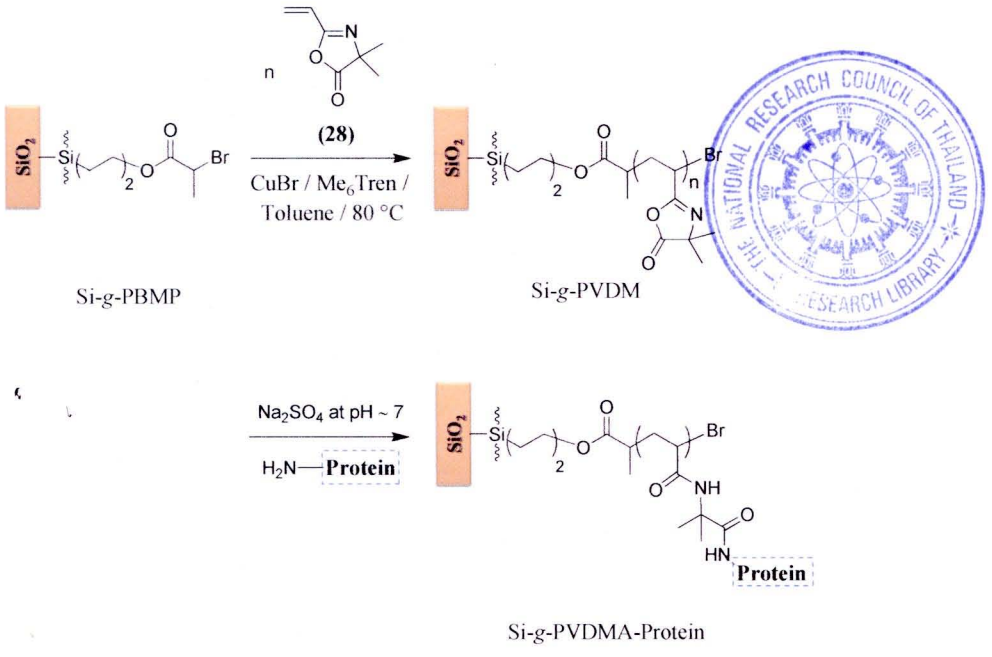


Figure 18 Synthesis of surface-anchored PVDM as templates for the immobilization of protein [73]

In 2002, Fontaine and co-worker [74] investigated the grafting of VDM onto activated poly(propylene) (PP) films and fabrics *via* eletron-beam (EB) techniques. Then, the active film containing VDM was subsequently employed as the anchored to trap sericin, a natural water-soluble protein released from silk degumming (Figure 19), which is thrown as a waste in silk industries. PVDM can be coated on the surface of PP films and fabrics via EB irradiation followed by graft PVDM chains are useful for the immobilization of benzylamine, Jeffamine M600, poly(β-benzl aspartae) ω-benzylamide or sericin as a model of peptidic chain. The results suggested that EB technique is the most suitable ways of modifying the polymer surface of films and fabrics for textile industry due to normal pressure treatment and high efficiency. Furthermore, fabrics could

be used for deproteinization of aqueous solutions as well as in the field of combinatorial chemistry as scavengers or substrates.

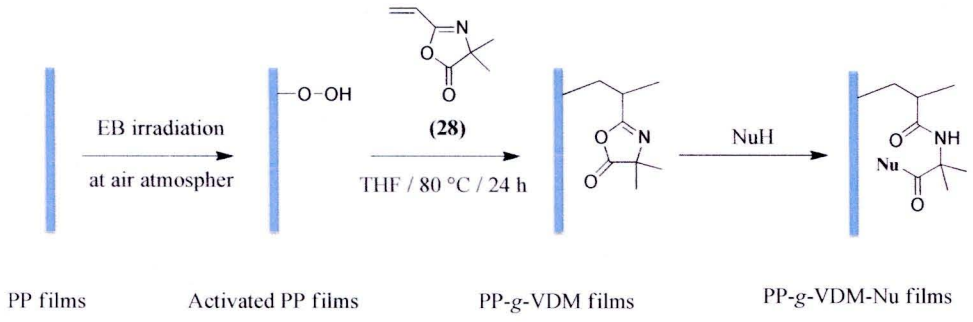


Figure 19 Grafting of VDM onto EB activated PP films and immobilization of nucleophiles [74]

Next, in 2009 Barringer and co-worker [75] described the development of a procedure for a reactive, multilayer polymer scaffold incorporating polymers based on VDM on a silicon wafer that can be attached functionalized by biomolecule containing primary amine. The silicon wafer, a poly(glycidyl methacrylate) (PGMA) base layer, reacted with dianimohexane to produce the interface with primary amines, which can be attached with azlactone moiety of poly(1-vinyl-2-pyrrolidone-*co*-VDM) *via* nucleophilic addition, in order to graft the co-polymer to the substrate. Residual unreacted azlactone ring of polymer can be used to immobilize dansylcadaverine (Figure 20). It was shown that attachment of dansylcadaverine, which generally difficult to attach on any polymeric surface, can easily proceed without a catalyst at room temperature to yield a stable amide linkage complexes.

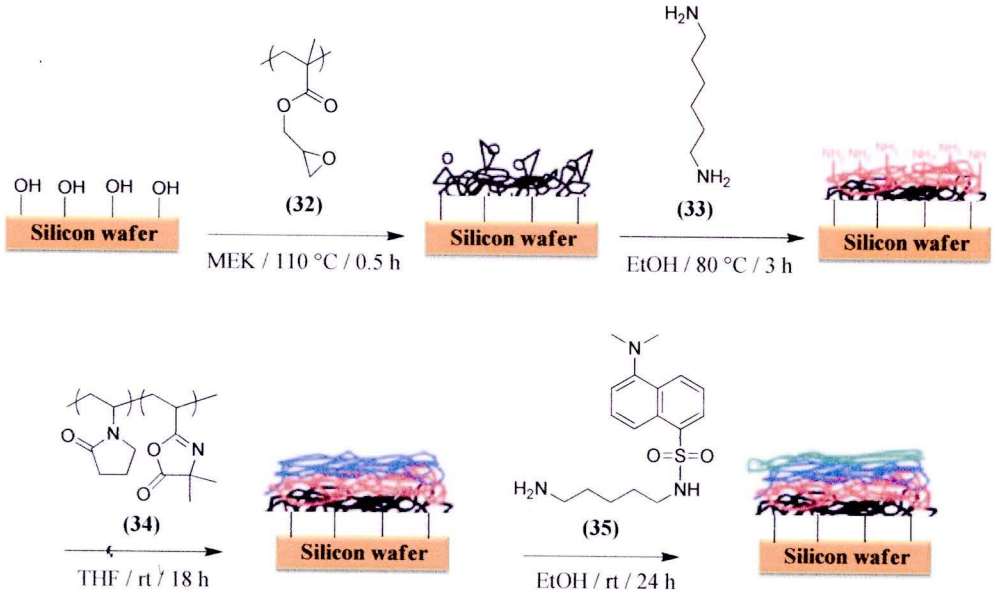


Figure 20 Preparation of the multilayer assembly process [75]