CHAPTER II

THEORY AND LITERATURE REVIEW

2.1 Historic Overview of Tyrosine-derived Polycarbonates [1]

Over the last 25 years, significant efforts have been devoted to the development of polymeric biomaterials. Historically, the vast majority of these efforts were focused on identifying 'off the shelf' polymers that were biologically inert and stable under physiological conditions. These materials were used as permanent prosthesis such as bone and joint replacements, dental devices and cosmetic implants. However, the emerging fields of tissue engineering and the need for advanced drug and gene delivery systems have resulted in an increasing need for resorbable and/or degradable polymers.

The main driving forces for the development of new, resorbable and/or degradable biomaterials are (i) the need of the pharmaceutical industry to develop advanced drug delivery systems for the many new peptide and protein drugs that will become available through biotechnology and genomics, (ii) the need of the medical device industry to develop degradable implants (scaffolds) for tissue regeneration and tissue engineering applications, and (iii) the need to improve the biocompatibility of biosensors and implantable medical devices. This last application calls for new materials with surfaces that prevent scarring and/or protein adsorption at the implant/tissue interface.

Pseudo-poly(amino acid)s have been extensively studied and have found practical, biomedical applications. They were first described in 1984 [2]. Naturally occurring amino acids are linked by non-amide bonds, such as ester, iminocarbonate and carbonate bonds. The resulting polymers contain the same monomeric building blocks as conventional poly(amino acid)s, but do not have a peptide-like backbone structure. For example, tyrosine-derived polycarbonates and tyrosine-derived polyarylates are shown below.

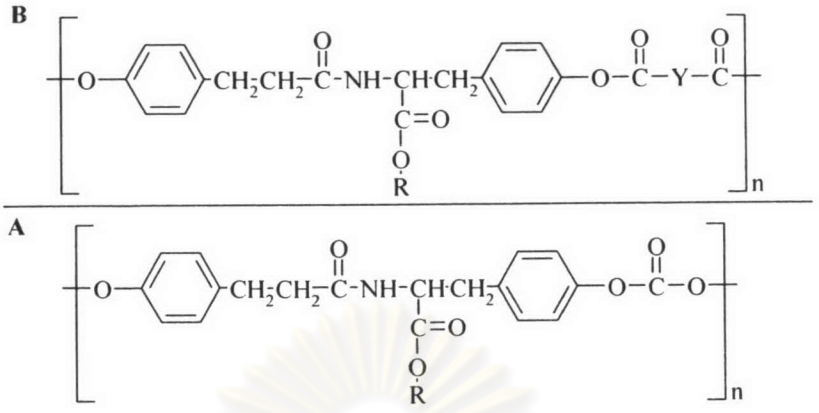


Figure 2.1 Chemical structures of (A) tyrosine-derived polycarbonates, (B) tyrosine-derived polyarylates.

Diphenols, such as Bisphenol A, are frequently used in industry, since their aromatic backbone structures can significantly increase the stiffness and mechanical strength of polymers. However, Bis-phenol A and other industrially used diphenols are cytotoxic and can therefore not be used as monomers in degradable biomaterials. There was a significant need for a non-cytotoxic, diphenolic monomer that could be used as a building block in the design of degradable implant materials. This need was addressed by the development of several tyrosine-based monomers. Tyrosine is the only major, natural nutrient containing an aromatic hydroxyl group. In view of the non-processibility of conventional poly-(L-tyrosine), which cannot be used as an engineering plastic, the development of a tyrosine-based pseudo-poly(amino acid) was envisioned. In this context, derivatives of tyrosine dipeptide can be regarded as diphenols and may be employed as replacements for the industrially used diphenols in the design of medical implant materials (Figure 2.2). This approach led, for the first time, to tyrosine-derived polymers with favorable engineering properties.

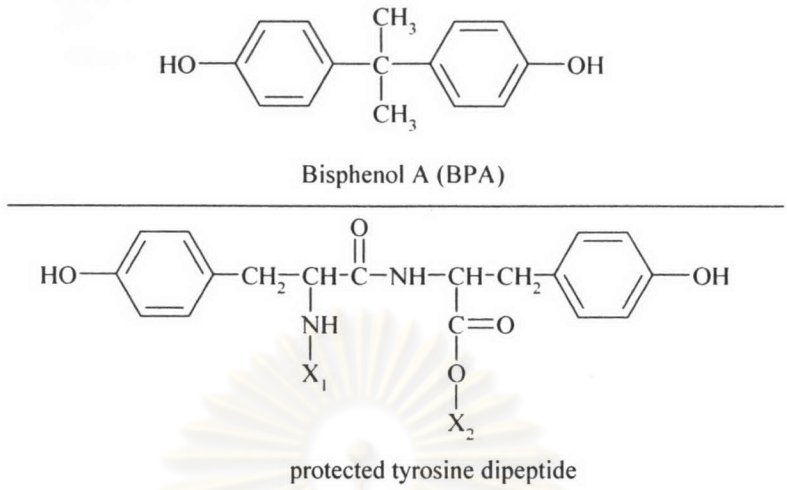


Figure 2.2 Chemical structures of tyrosine dipeptide and Bisphenol A.

In the dipeptide structure, the amino terminal group and the carboxylic acid terminal group are shown with appropriate chemical protecting groups attached (X_1 and X_2). The nature of these protecting groups affects the chemical synthesis of the polymer as well as the final physicomechanical properties of the resulting polymer. The challenge of the early studies was to identify suitable protecting groups that will lead to non-toxic, fully degradable polymers with good engineering properties. The combination of these different properties within one single design proved to be a difficult task and early investigations did not lead to readily processable materials. Later, it was recognized that the number of inter-chain hydrogen bonding sites per monomer unit had to be minimized. These studies led to the replacement of one tyrosine molecule by desaminotyrosine [3-(4'-hydroxyphenyl)propionic acid] and the identification of desaminotyrosyl-tyrosine alkyl esters (Figure 2.3) as fully biocompatible replacements for Bisphenol A and other industrial diphenols in a wide range of polymers. Monomer synthesis from 3-(4'-hydroxy phenyl) propionic acid and tyrosine alkyl esters was accomplished by carbodiimide-mediated coupling reactions, following known procedures of peptide synthesis [3], giving typical yields of 70%. Monomers carrying an ethyl, butyl, hexyl, or octyl ester pendent chain have been investigated extensively. The carboxylic acid terminal group is protected by an alkyl ester which can be regarded as a pendent chain after polymerization. The structure of the alkyl esters is indicated by the following nomenclature convention:

DTE, de-saminotyrosyl-tyrosine ethyl ester; DTB, desaminotyrosyl-tyrosine butyl ester; DTH, desaminotyrosyl-tyrosine hexyl ester; DTO, desaminotyrosyl-tyrosine octyl ester.

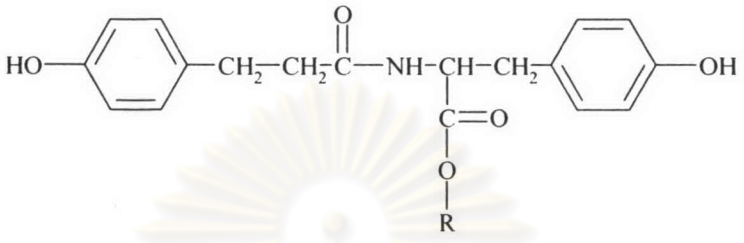


Figure 2.3 Chemical structure of desaminotyrosyl-tyrosine alkyl esters, abbreviated 'DTR'.

Tyrosine-derived polycarbonates are a group of 'homologous' carbonate–amide copolymers differing only in the length of their respective alkyl ester pendent chains. The diphenolic monomers were polymerized using either phosgene or the more easily handled bis(chloromethyl) carbonate tri-phosgene. Polymers with weight-average molecular weights (M_w) of up to 400,000 were obtained [3], although for practical applications, M_w values around 100,000 are usually preferred. Polymer properties, such as glass transition temperature, surface free energy, and mechanical properties, can be easily controlled by varying the length of the alkyl ester pendent chain. Surprisingly, the degradation rate is not a sensitive function of the length of the alkyl ester pendent chain, therefore all poly(DTR carbonate)s can be easily handled under ambient conditions and degrade only slowly under physiological conditions. *In vivo* studies confirmed the absence of enzymatic involvement in the degradation process

Recently, the degradation mechanism was studied in detail by Tangpasuthadol et al. [4,5] utilizing a series of small model compounds that mimic the repeat unit of the polymer, followed by a thorough 3-year degradation study. These results indicated that the backbone carbonate bond is hydrolyzed at a faster rate than the pendent chain ester bond. Only under very acidic conditions (pH $\leq$ 3) did the acid catalyzed hydrolysis of the ester bond become a dominant factor and pendent chain ester

hydrolysis outpaced the rate of hydrolysis of the backbone carbonate bonds. Increasing the length of the pendent chain from ethyl to octyl reduced the rate of hydrolysis of both the ester and carbonate bonds, possibly by hindering the access of water molecules to these bonds. The mechanism of polycarbonate degradation is shown schematically in Figure 2.4. According to this mechanism, the final degradation products *in vitro* are desaminotyrosyl-tyrosine and the alcohol used to protect the carboxylic acid group. *In vivo*, it is reasonable to expect the enzymatic degradation of desaminotyrosyl-tyrosine to desaminotyrosine and L-tyrosine.

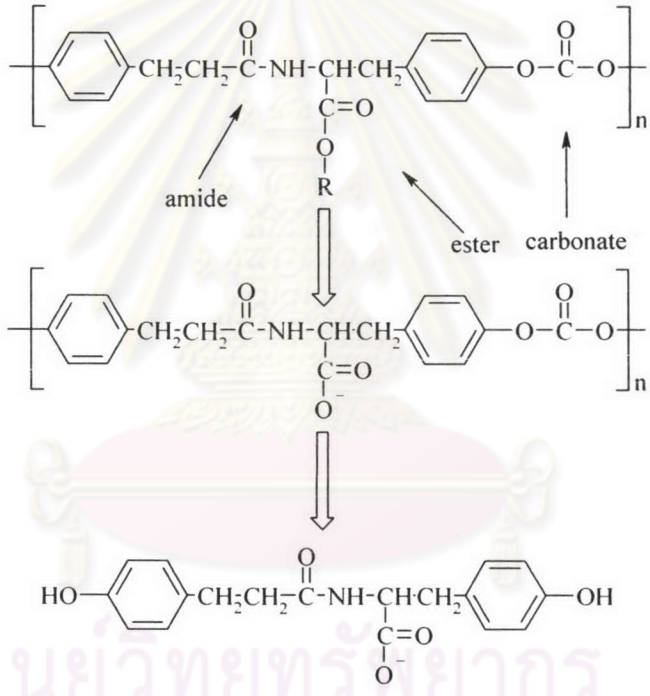


Figure 2.4 Schematic summary of the mechanism of degradation of tyrosine-derived polycarbonates.

The physicomechanical properties and potential applications of tyrosine-derived polycarbonates were studied by Ertel and Kohn. [6] Briefly, the polycarbonates are amorphous polymers. Because of their high hydrophobicity, they do not swell in aqueous media or during the degradation process. Their equilibrium water content is about 2 to 3% and remains below 5% even at advanced stages of degradation. Glass transition temperatures (T_g) range from 52 to 93 ^{o}C and

decomposition temperatures exceed 290 °C, providing a wide temperature window for thermal processing. Thorough evaluations of enthalpy relaxation kinetics determined that storage of polycarbonates at a temperature of T_g -15 °C for only a few hours is sufficient to bring the physical aging process to completion. Even in an unoriented stage (thin solvent cast or compression molded films), tyrosine-derived polycarbonates are characterized by their high mechanical strength (50–70 MPa) and stiffness (1–2 GPa). These values can be further increased by processing conditions that induce molecular orientation.

Table 2.1 Physico-mechanical properties of tyrosine-derived polycarbonates

Polycarbonate derived from	Molecular weight (weight average)	Glass transition temp. (^{0}C)	Decomposition temp. (^{0}C)	Water contact angle (deg)
DTE	176,000	81	290	73
DTB	120,000	66	290	77
DTH	350,000	58	320	86
DTO	450,000	53	300	90

The amino acid L-tyrosine was shown to be a versatile building block for biodegradable and biocompatible polymers. The incorporation of derivatives of tyrosine dipeptide, such as the desaminotyrosyl-tyrosine alkyl esters (DTR), into the backbone of different polymer systems results in versatile polymers with interesting properties. Contrary to most conventional poly(amino acid)s, tyrosine-derived pseudo-poly(amino acid)s exhibit excellent engineering properties and polymer systems can be designed whose members show exceptional strength (polycarbonates). In particular, the tyrosine-derived polycarbonate, poly(DTE carbonate), has been shown to have a high degree of tissue compatibility.

Table 2.2 Cell attachment and proliferation on surfaces of tyrosine-derived polycarbonates [6]

Polymer	1 day attachment (x100 cells cm^{-2})	5 day attachment (x100 cells cm^{-2})
Results for fibroblasts		
poly(DTE carbonate)	46±13	596±100
poly(DTB carbonate)	56±17	410±79
poly(DTH carbonate)	32±10	268±46
Glass (as a control surface)	50±16	555±91
Results for osteoblasts		
poly(DTE carbonate)	34±15	318±93
Glass (as a control surface)	59±22	739±83

While in the past the vast majority of all commercial research involving degradable polymers was limited to the use of poly(lactic acid), poly(glycolic acid) or copolymers thereof, it is obvious that, in the future, a wider range of new materials will be needed. Tyrosine-derived pseudo-poly(amino acid)s represent one of many new 'second generation biomaterials' that will enter into clinical use over the next decade.

2.2 The Cell-adhesive Peptide Arg-Gly-Asp (RGD)

In the early 80s, Pierschbacher discovered that the cell attachment activity could be mimicked by short, immobilized, synthetic peptides containing a short three amino acid sequence, arginine-glycine-aspartic acid (RGD) [7].

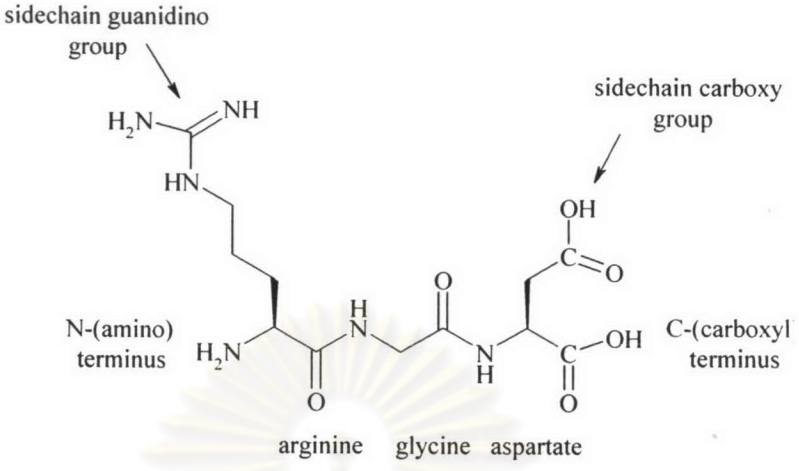


Figure 2.5 Chemical structure of RGD.

The recognition site for many of the integrins that bind to extracellular matrix and platelet adhesion proteins is the tripeptide RGD [8]. The role of the RGD sequence as the recognition site was demonstrated by making progressively smaller fragments of fibronectin and by assaying for the cell attachment-promoting activity in the fragments and in synthetic peptides reproducing the amino acid sequences of such fragments. When coated onto a surface, the fragments and synthetic peptides containing the RGD sequence promote cell attachment, whereas in solution they inhibit the attachment of cells to a surface coated with fibronectin or the peptides themselves. Changes in the peptides as small as the exchange of alanine for the glycine or glutamic acid for the aspartic acid, which constitute the addition of a single methyl or methylene group to the RGD tripeptide, or the replacement of the arginine with a lysine residue, eliminate these activities of the peptides. Conformation of the amino acids is also important; a peptide in which the aspartic acid is in the D-form is inactive. The RGD sequence is also the cell recognition site of a surprising number of other extracellular matrix (ECM) and platelet adhesion proteins. In addition to fibronectin, these include vitronectin, collagens, fibrinogen, von Willebrand factor, osteopontin, bone sialoprotein I, thrombospondin, tenascin, laminin, and entactin.

Integrins are a family of membrane glycoproteins consisting of two subunits, α and β. The structural models for the various integrins are depicted in Figure 2.6. The α subunits are homologous to one another but not to the β subunits, which form

their own homologous group. Both integrin subunits have a large extracellular domain, a trans-membrane segment, and a cytoplasmic tail. The α and β subunits are noncovalently bound to one another, and this association is promoted by divalent cations.

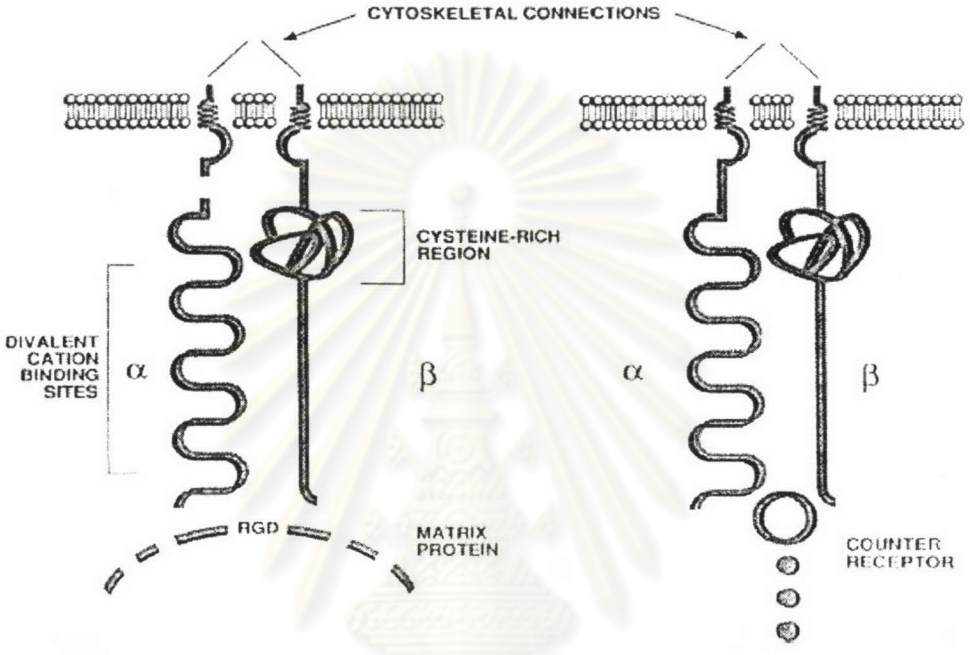


Figure 2.6 Schematic drawing of the general structure of integrin [9]. Two types of integrins are shown, one with an α subunit which is proteolytically processed into two disulfide-linked fragments at a cleavage site (shown as a gap in the structure on the left). Other integrins have subunits that are not processed.

Figure 2.7 lists the commonly accepted ligands for many integrins. The known subunits, the subunit combinations that form the known integrins, and the known ligands for these integrins are shown. Also shown is the RGD specificity of those integrins that bind to this sequence. FN, fibronectin; Fn alt., fibronectin alternatively spliced domain; LM, laminin; VN, vitronectin; Coll, collagen; vWF, von Willebrand factor; FB, fibrinogen; OP, osteopontin; BSP 1, bone sialoprotein 1; ICAM-1, ICAM-2, intercellular adhesion molecules; FX, factor X; BM, basement membrane; C3bi, complement component C3bi.

It is clear that the specificity of ligands is quite complex, for one integrin can bind more than one ECM protein, and in addition, a single ECM protein can bind to more than one integrin. In the latter case, when the binding is RGD dependent, more than one integrin is using the same general binding region on a single ECM protein, the RGD sequence.

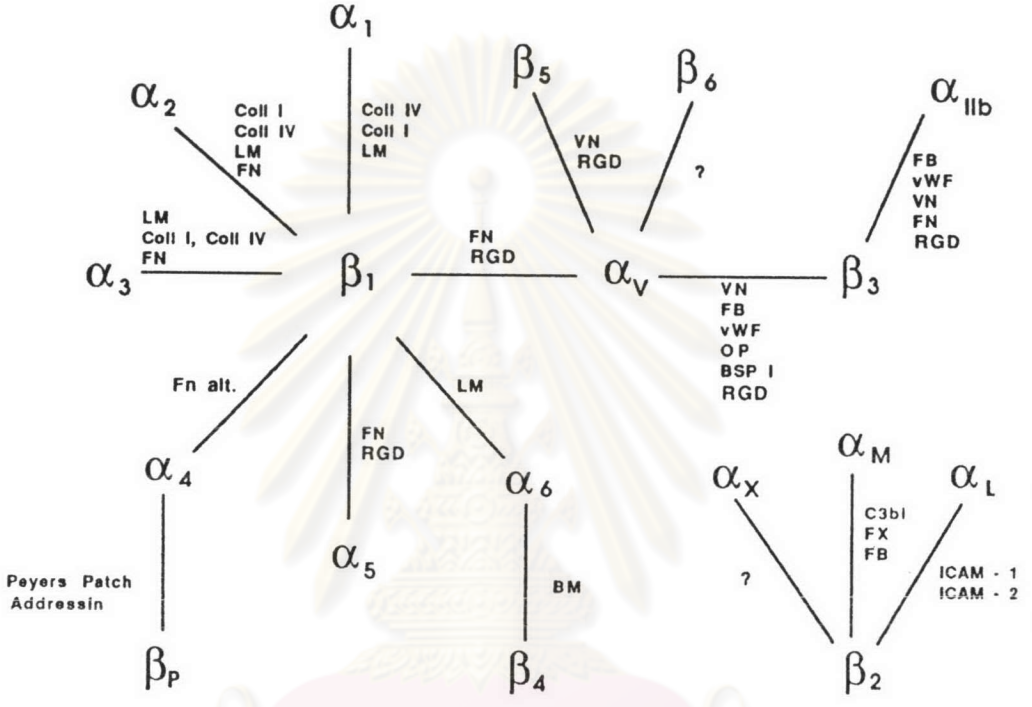


Figure 2.7 Integrin family [10].

Cell adhesion plays an important role in a variety of basic biological processes, including guiding cells into their appropriate locations in the body, providing cell anchorage, and controlling cell proliferation, differentiation, and apoptosis. Adhesion peptides have found important uses as probes for these phenomena. In addition, there are also practical applications for these peptides. Adhesive peptides can be used in two different ways: When attached to a surface, they promote cell attachment, whereas when presented in solution, they prevent attachment that would otherwise occur. Both modes of using the peptide have found applications. Surface-coated RGD peptides are being investigated for improvement of tissue compatibility of various implanted devices.

Other applications being explored include the targeting of the specific integrin ($\alpha_v\beta_3$) in osteoporosis. Osteoclasts attach to bone through this integrin (and possibly some other av integrins), and inhibiting their attachment with peptides prevents bone degradation *in vitro* and *in vivo*. Protein engineering with RGD can have applications in protein targeting and gene therapy with viruses. Advances in the application of RGD and related sequences to various purposes will depend on detailed understanding of integrin-ligand recognition. Much progress has been made recently in this field. Adhesion peptides, RGD in particular, have provided a great deal of information about cell adhesion mechanisms and are serving as a basis for the development of a new group of pharmaceuticals.

2.3 Surface Modification of Biomaterial by Immobilization of Biomolecules

A current trend to enhance biocompatibility consists of chemical modification of the biomaterial surface by the grafting of biologically active molecules such as peptides, proteins, and antibodies. This procedure offers the advantage of improving surface properties with respect to biocompatibility without adverse effect on the bulk properties of the system. Immobilization of such molecules can be achieved by a variety of different techniques that exploit either physical adsorption (through Van der Waals, hydrophobic, or electrostatic forces) or chemical binding. Both approaches have advantages and disadvantages. Physical adsorption processes are generally experimentally simple and often allow retention of the biomolecular activity. However, the adsorption is often reversible, with target molecules being removed by certain buffers or detergents or replaced by other molecules in solution. In contrast, chemical immobilization involves the covalent bonding (or complexation) of the target molecule to the solid phase. This method is experimentally more difficult and often exposes the molecule to a harsher environment. However, the resultant irreversible binding which can be produced with high levels of surface coverage makes this approach more popular, although in some cases chemical binding can alter the conformational structure and active center of the molecule, causing a reduction in activity. Some aspects of the physical adsorption and chemical binding are summarized in Table 2.3.

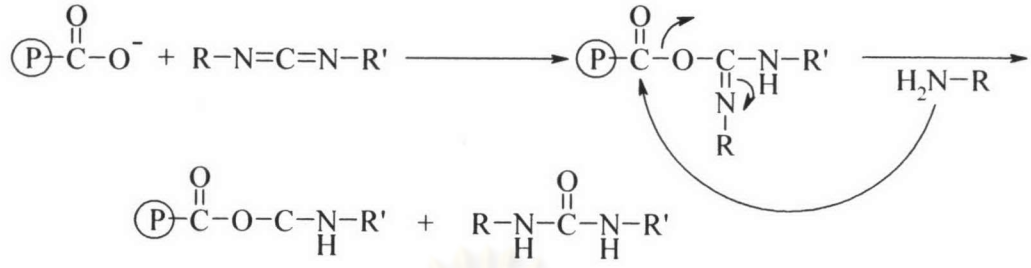
Table 2.3 Nature and properties of physical adsorption and chemical binding.

	Physical adsorption	Chemical binding
Principles	- Van der Waals forces - hydrophobic forces - electrostatic interactions	- covalent bond - complexation - coordination
Advantages	- easy to prepare under mild experimental conditions - adsorption reversible - biological activity retained	- controlled coverage - stable in physiological conditions and for multiple uses
Disadvantages	- not stable under all physiological conditions - one time use - poor reproducibility	- stringent reaction conditions - some biological activity may be lost

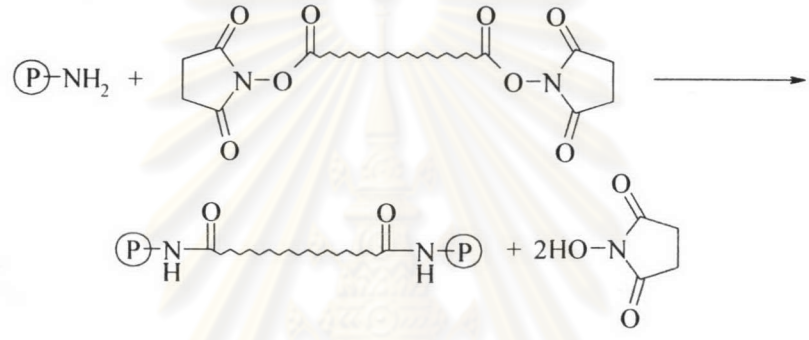
2.3.1 Crosslinking and Conjugation Reactions

The concept of cross-linking and conjugation originally stems from protein and peptide chemistry. Chemical cross-linking involves joining of two molecular components by a covalent bond achieved through the use of cross-linking reagents. The components may be proteins, drugs, nucleic acids, or solid substrates. The chemical cross-linkers are bifunctional reagents containing two reactive functional groups derived from classical chemical modification agents. The reagents are capable of reacting with the side chains of the amino acids of proteins. They may be classified into homobifunctional, heterobifunctional, and zero-length crosslinkers. The zero-length crosslinkers are essentially group-activating reagents which cause the formation of a covalent bond between the components without incorporation of any extrinsic atoms. The homobifunctional reagents consist of two identical functional groups and the heterobifunctional reagents contain two different types of reactive functional moieties. Model reactions for the three kinds of crosslinkers are shown below.

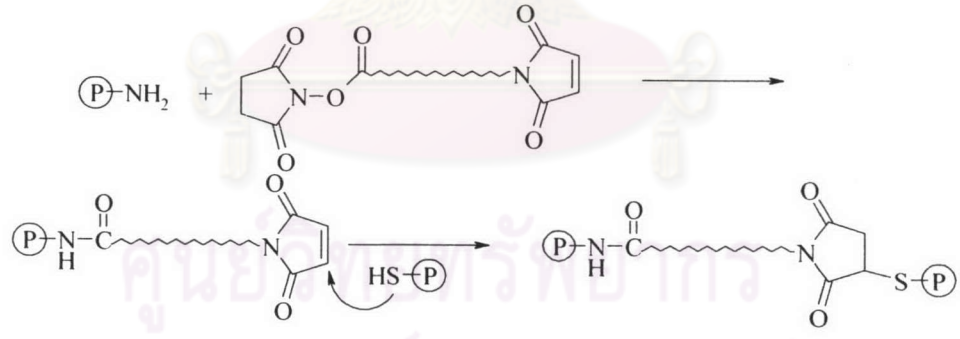
a) Carbodiimides are examples of zero-length crosslinkers



b) Reagents with bifunctional succinimidyl esters are examples of homobifunctional crosslinkers



c) Reagents with succinimidyl ester- and maleimidyl groups are examples of heterobifunctional crosslinkers



2.3.2 Chemical Reactivity of Proteins

Chemical crosslinking and conjugation of proteins and peptides depend on the reactivity of the constituents of proteins as well as the specificity of crosslinkers used. In order to preserve the biological activity of the individual protein, the reaction site on the protein must be those amino acids that are not involved in its biological functions. The biological activity loss of proteins can be caused by disturbances of their secondary and tertiary structures, their surface charges, their hydrophobic and hydrophilic properties, and their native conformations. Thus, only those amino acid

residues that are not situated at the active centers or settings critical to the integrity of the tertiary structures of proteins may be targets for chemical modification. Such amino acids must be located on the surface of the protein and are easily accessible by crosslinkers. It follows, therefore, that the identity of the reactive functional groups on the exterior of a protein is the most important factor that controls its reactivity towards crosslinking reagents. By knowing which functional groups are located at the protein - solvent interface, the protein may be modified without sacrificing its biological activity.

All proteins are composed of amino acids. There are twenty common amino acids with side chains of different sizes, shapes, charges, polarities, and chemical reactivity. These physico-chemical properties determine the precise structure and function of each individual protein. Glycine, alanine, valine, leucine, isoleucine, methionine, and proline have nonpolar aliphatic side chains while phenylalanine and tryptophan have nonpolar aromatic side groups. These hydrophobic amino acids are generally located in the interior of proteins forming the so-called hydrophobic core of many molecules. Other amino acids, arginine, aspartic acid, glutamic acid, cysteine, histidine, lysine, and tyrosine have ionizable side chains. Together with asparagine, glutamine, serine, and threonine which contain non-ionic polar groups, they are usually located on the protein surface where they can interact strongly with the aqueous environment.

The chemical reactivities of peptides and proteins depend on the side chains of their amino acid compositions as well as the free amino and carboxyl groups of the N- and C-terminal residues, respectively. Studies of chemical modification have revealed that only a few of the amino acid side chains are really reactive. Of the twenty amino acids, the alkyl side chains of the hydrophobic residues are chemically inert except the photochemical insertion. Only eight of the hydrophilic side chains are chemically active. These are the guanidinyl group of arginine, the γ - and β-carboxyl groups of glutamic and aspartic acids, respectively, the thiol group of cysteine, the imidazolyl group of histidine, the ε-amino group of lysine, the thiolether moiety of methionine, the indolyl group of tryptophan and the phenolic hydroxyl group of tyrosine (Figure 2.8). Table 2.4 summarizes the various chemical modification reactions of these active side chains. The most important reactions are alkylation and acylation. In

alkylation, an alkyl group is transferred to the nucleophilic atom, whereas in acylation, an acyl group is bonded.

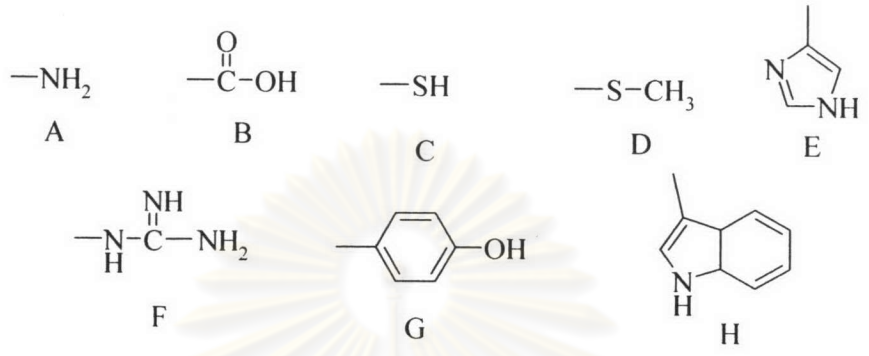


Figure 2.8 Reactive groups of amino acid side chains. Functional groups A to F are the six most reactive entities. G and H are less reactive. (A) amino groups of N-terminal amino acids and -amino groups of lysines; (B) carboxyl groups of aspartic, glutamic acids and C-terminal amino acids; (C) thiol group of cysteine; (D) thioether of methionine; (E) imidazolyl group of histidine; (F) guanidinyl group of arginine; (G) phenolic group of tyrosine; and (H) indolyl group of tryptophan.

Table 2.4 Chemical modification of amino acid side chains

Amino Acid	Side Chain	Alkylation or Arylation	Acylation	Oxidation
cysteine	-CH_2SH	+	+	+
lysine	-NH_2	+	+	-
methionine	-S-CH_3	+	-	+
histidine	imidazolyl	+	+	+
tyrosine	-Ph-OH	+	+	+
tryptophan	indolyl	+	-	+
aspartic and glutamic acids	-COOH	-	+	-
arginine	-NHC(=NH)NH_2	-	-	-

Because protonation decreases the nucleophilicity of a species, the pH of the medium affects the rate of many nucleophilic reactions. The relationship between protonation and the pH depends on the pKa of the nucleophile. Table 2.5 lists the pKas of the reactive groups in free amino groups and in model peptides. Because the pKa is a function of temperature, ionic strength, and microenvironment of the ionizable group, the table reflects only the approximate values of these groups in proteins. Using these values, the ratio of protonated to deprotonated species at a certain pH can be calculated by the Henderson-Hesselbalch equation:

$$pH = pKa + \log\{[A^-]/[AH]\}$$

Table 2.5 pKa of some reactive groups in amino acids and model peptides.

Functional group	Amino acid residue	pKa in free amino acid	pKa in model peptides
α-COOH	C-terminal	1.8-2.6	3.1-3.7
β-COOH	aspartic acid	3.9	4.4-4.6
γ-COOH	glutamic acid	4.3	4.4-4.6
α-NH_3	N-terminal	8.8 – 10.8	7.6 – 8.0
-SH	cysteine	8.3	8.5-8.8
ε-NH_3^+	lysine	10.8	10.0-10.2
-NHC(=NH_2^+)NH_2	arginine	12.5	>12

Thus, at a fixed pH, the most reactive group is usually the one with the lowest pKa. Because of their differences in the pKa values, the degree of protonation of these amino acid side-chain groups at a certain pH provides a basis for differential modification. For example, at neutrality, the amino groups are protonated rendering them unreactive. For a selective reaction with the carboxyl group, the condition of an acidic pH should be selected. At higher pHs, other nucleophiles, particularly the thiol group, will react. As a consequence, it should be obvious that changing the pH also provides means to control the course of a chemical reaction.

2.3.3 Nucleophilic Substitution and Addition Reactions

Most of the protein modification reactions are nucleophilic substitution and addition reactions. For example, the reaction of succinimidyl esters with amino groups is an S_N2 substitution, while that of maleimidyl groups with thiols is a nucleophilic addition. The reaction rate of S_N2 substitution, a bimolecular nucleophilic substitution reaction, depends on the chemical reactivity of the involved species and the steric effects. The chemical reactivity involves the ability of the leaving-group to leave and the nucleophilicity of the attacking group. The easier it is for the leaving group to come off, the faster the reaction will be. Similarly, the greater the nucleophilicity, the more expeditiously the product will be formed. In term of protein modification, the relative chemical reactivity is basically a function of nucleophilicity of the amino acid side chains. On the other hand, the steric effects play a more important role on the surface reactions. Generally, in the bulk chemistry the less the bulky groups around the reactive target-atom, the easier the reaction will be. However on the surface, besides the native properties of molecules, the neighboring molecules also affect the steric hindrance. The denser the packed molecules, the more difficult the reaction will be. Therefore, a densely packed monolayer is not an ideal candidate for S_N2 surface reactions. Nucleophilic additions are accepted as stepwise reactions. They are classified to base- and acid-catalyzed additions. In order to avoid the side-reactions, the specific reaction of maleimidyl groups with thiols is often carried out under weakly acidic conditions.

2.3.4 Immobilization of RGD Peptides on Polymers [11]

In most cases, RGD peptides are linked to polymers via a stable covalent amide bond. This is usually done by reacting an activated surface carboxylic acid group with the nucleophilic N-terminus of the peptide. Carboxylic acid groups can be activated by using a peptide coupling reagent, e.g. 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC, also referred to as water soluble carbodiimide, WSC), dicyclohexyl-carbodiimide (DCC) or carbonyl diimidazole (CDI). Two problems may arise by this coupling method: Firstly there are further reactive functional groups in the RGD peptide (carboxyl groups at the C-terminus and in the aspartic acid side chain and the nucleophilic guanidino group of the arginine

side chain) and secondly the coupling reagent and the activated carboxyl groups can be deactivated quickly by hydrolysis. One possibility to overcome these problems is to block the reactive amino acid side chains with protecting groups and to use other solvents than water (e.g. DMF, dichloromethane or acetone). By this method, protected RGD peptides were successfully linked to polymers [12]. A major drawback of this immobilization strategy is the need of harsh conditions to remove the protecting groups. Even if the more labile Pbf (2,2,4,6,7-pentamethyl-dihydrobenzofurane-5-sulfonyl) protecting group for the arginine side chain is used, deprotection with trifluoroacetic acid (TFA) usually takes as long as 1–3 h. The malodorous deprotection mixtures can be replaced by using triethylsilane (TES) or triisopropylsilane (TIPS) as scavengers. Alternatively, coupling of unprotected RGD peptides in water is possible, employing a two step procedure: First, activation of the surface carboxyl group as an active ester that is less prone to hydrolysis, e.g. *N*-hydroxysuccinimide (NHS) esters, and second coupling of the peptide in water (Figure 2.9). NHS active esters on polymers are usually generated in dichloromethane or DMF, using DCC or EDC for activation of the carboxyl groups and subsequent reaction with NHS [13]. Alternatively, the TSTU reagent (*O*-(*N*-succinimidyl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate) reagent can be used, containing both the activation moiety as well as the NHS group. The half lifetime of NHS active esters at neutral pH ranges from several minutes to hours, depending on the substituents of the α-carbon [14]. NHS active esters that have been dried can be stored for several months [13, 14]. Best conditions for coupling the RGD peptide to the NHS active ester are pH 8–9 in phosphate or sodium bicarbonate buffer. Coupling is usually complete in 1–2 h. More elevated pH values and high concentrations of buffer or salts can reduce the half lifetime of the NHS active ester down to less than 1 min. Following this protocol, there is no need for protecting groups. Because of the two step procedure, the aspartate side chain carboxyl group is not activated for coupling, and due to protonation in water the nucleophilicity of the arginine side chain is nearly abolished. In some cases coupling was performed at lower pH and/or lower temperatures with longer coupling times resulting in higher yields. Also an excess of peptide may increase yields.

Creating NHS active esters in water using NHS and the water soluble coupling reagent EDC is also possible [15], but RGD peptide coupling yields are usually lower. In some cases the NHS active esters were introduced into the polymer by copolymerizing monomers that contain an NHS active ester. Polymers that contain surface amino groups can be treated with succinic anhydride to generate surface carboxyl groups, which can be reacted with RGD peptides as described above. Amino groups can directly be converted into preactivated carboxyl groups by using an excess of bisactivated moieties like disuccinimidyl tartate, disuccinimidyl suberate, ethylene glycol bis(succinimidyl succinate) (EGS) or *N,N'*-disuccinimidyl carbonate (DSC). Similarly bistresyl-PEG was used as a linker, leading to immobilized RGD peptide via amine bonds. Surfaces containing hydroxyl groups can similarly be preactivated with tresyl chloride (Figure 2.9). Alternatively hydroxyl groups can be treated with *N,N'*-disuccinimidyl carbonate or *p*-nitrophenyl chlorocarbonate to achieve activated material that either reacts readily with the peptide under aqueous conditions or can be stored for some time. Released *p*-nitrophenol upon coupling can be detected and quantified by UV absorption. *p*-Nitrophenol and tetramethylurea (from TSTU) are toxic and have to be removed carefully from the surfaces prior to biological applications.

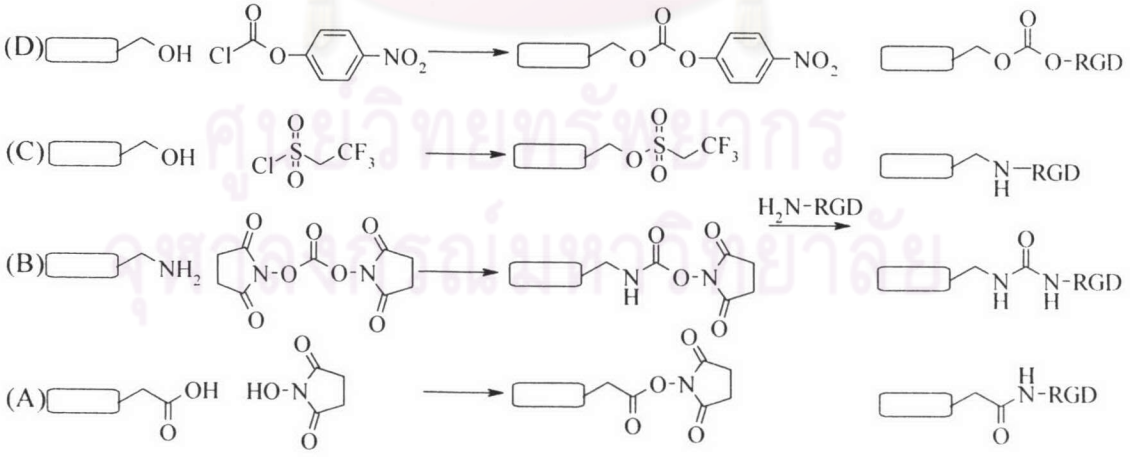


Figure 2.9 RGD peptides react via the *N*-terminus with different groups on polymers: (A) carboxyl groups, preactivated with a carbodiimide and NHS to generate an active ester, (B) amino groups, preactivated with DSC, (C) hydroxyl groups, preactivated as tresylate, (D) hydroxyl groups, preactivated as *p*-nitrophenyl carbonate.

In a more recent approach named chemoselective ligation, selected pairs of functional groups are used to form stable bonds without the need of an activating agent and without interfering with other functional groups. Chemoselective ligation proceeds usually under mild conditions and results in good yields. A bromoacetyl containing RGD cyclopeptide was successfully linked to a thiol functionalized surface and an aminooxy-terminated RGD cyclopeptide reacts readily with aldehyde groups leading to a stable oxime bond (Figure 2.10). Imine bonds resulting from coupling of a surface aldehyde group and the peptide's N-terminus have to be reduced to a more stable amine bond prior to application (''reductive amination''). Thiol (cysteine) bearing RGD peptides can be linked in a Michael addition reaction to acrylic esters or acryl amides in excellent yields [21]. In a similar addition reaction thiol containing RGD peptides were linked to maleinimide functionalized surfaces under mild conditions (Figure 2.10). The latter thiol reactive surface was created by reacting surface amino groups with a heterobifunctional linker, sulfo-SMCC (sulfosuccinimidyl 4-(*N*-maleinimidomethyl)cyclohexane-1-carboxylate). Also benzoquinone as a Michael type bifunctional linker between surface hydroxyl groups and the N-terminal amine was proposed. In some cases RGD peptides are immobilized by radical reactions. An acrylamide tailored RGD cyclopeptide was successfully immobilized on PMMA surfaces using camphorquinone and UV irradiation. Benzophenone as well as aromaticazide functionalized RGD peptides have been used to react similarly.

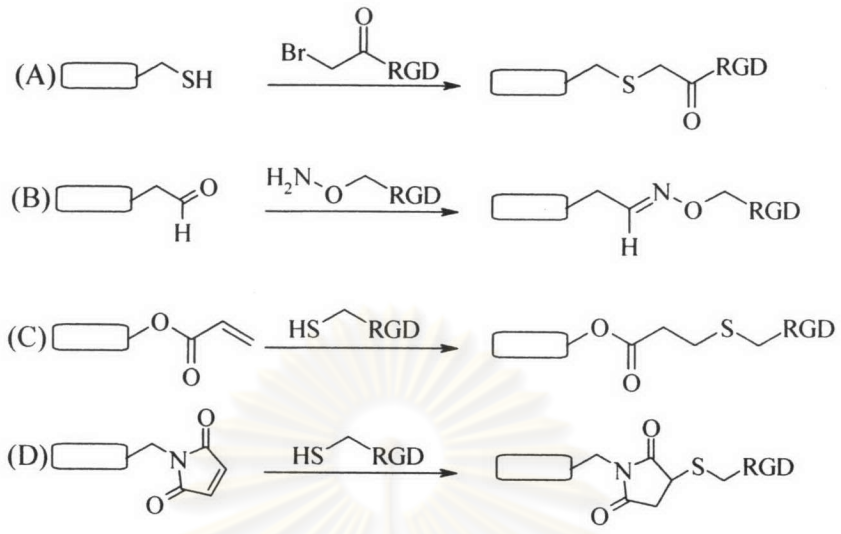


Figure 2.10 Chemoselective ligation of selected pairs of functional groups. (A) thiol and bromoacetyl-RGD. (B) aldehyde and aminooxy-RGD. (C) acrylate and thiol-RGD. (D) maleinimide and thiol-RGD.

In 1998, Xiao and coworkers design a surface modification method for covalent attachment of Arg-Gly-Asp (RGD)-containing peptides on Ti surfaces. The surface modification route is shown in Figure 2.11. Water-vapor-plasma-pretreated titanium surfaces were first activated by (3-aminopropyl)triethoxysilane (APTES), followed by reaction of terminal amines with succinimidyl esters of the crosslinkers, *N*-succinimidyl-6-maleimidylhexanoate, *N*-succimidyl trans-4-(maleimidyl-methyl)cyclohexane-1-carboxylate or *N*-succinimidyl-3-maleimidylpropionate. The final step involved the covalent binding of the thiol-bearing, RGD-containing peptides (glycine-arginine-glycine-aspatic acid-serine-proline-cysteine, GRGDSPC or arginine-glycine-aspatic acid-cysteine, RGDC) through maleimidyl groups. Infrared reflection absorption spectroscopy (IRAS), x-ray photoelectron spectroscopy (XPS) and radiolabelling technique were used for surface characterization. An approximate coverage of 0.2-0.4 peptides/nm^2 was calculated [16].

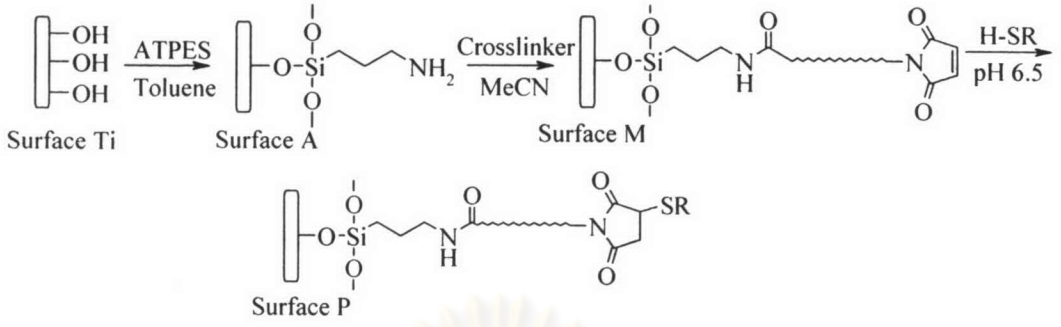


Figure 2.11 Schematic representation of the modification route. Surface Ti: water-vapor plasma-pretreated titanium; Surface A: poly(3-aminopropyl)siloxane pendant surface; Surface M: maleimide-modified surfaces with different alkyl chains; Surface P: peptide- or L-cysteine-modified surfaces; H-SR: L-cysteine, RGDC, GRGDSPC.

In 2001, Quirk and coworkers immobilized GRGDS peptide on the surfaces of poly(lactic acid)(PLA). Direct immobilization is impossible due to the lack of functional groups to support covalent attachment. They demonstrated a method to overcome this problem, by firstly attaching the peptide to poly(L-lysine) (PLL) using NHS/EDCI as a coupling agent to yield PLL-GRGDS. Bovine aortic endothelial cells seeded on the PLA coated with a 0.01% w/v solution of PLL-GRGDS showed a marked increase in spreading over unmodified PLA [12].

In 2002, Davis and coworkers modified silicon surface by RGD peptide attachment. Silicon surfaces coupled with a synthetic RGD peptide, as characterized by x-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM), displayed enhanced fibroblast proliferation and bioactivity. Results demonstrate an almost three-fold greater cell attachment/proliferation on RGD immobilized surfaces compared to unmodified silicon surfaces [17].

In 2004, Yoon and coworkers immobilized glycine-arginine-glycine-aspatic acid-tyrosine, GRGDY onto the surface of highly porous biodegradable polymer scaffolds for enhancing cell adhesion and function. A carboxyl terminal end of poly(D,L-lactic-*co*-glycolic acid) (PLGA) was functionalized with a primary amine group by conjugating hexaethylene glycol-diamine. The PLGA-NH_2 was blended with PLGA in varying ratios to prepare films by solvent casting or to fabricate porous scaffolds by a gas foaming/salt leaching method. Under hydrating conditions, the

activated GRGDY could be directly immobilized to the surface exposed amine groups of the PLGA-NH_2 blend films or scaffolds. For the PLGA blend films, the surface density of GRGDY, surface wettability change, and cell adhesion behaviors were characterized. The extent of cell adhesion was substantially enhanced by increasing the blend ratio of PLGA-NH_2 to PLGA. The level of an alkaline phosphatase activity, measured as a degree of cell differentiation, was also enhanced as a result of the introduction of cell adhesive peptides [18].

2.4 Analysis of RGD-modified Polymers

Analysis of RGD-modified surfaces should answer the question of the presence of RGD peptides (qualitatively) and of the amount of the peptide in a certain area or volume of the polymer (quantitatively). Quantification is not only important to optimize RGD coupling conditions [19], but also to discover the effects of RGD surface density on cell response. Water contact angle analysis is a relatively simple method of characterizing surfaces. Smaller angles indicate an increased hydrophilicity of a solid surface and are usually detected when the zwitterionic RGD moieties are immobilized.

In a more specific approach, amide bonds on solid surfaces can be detected via ATR-FTIR (attenuated total reflection FTIR) at 1650 and 1530 cm^{-1}. If samples can be prepared as thin films, FTIR can be employed. In one case the rate of RGD coupling to a isocyanate functionalized surface was measured by quantifying a disappearing NCO band. X-ray photoelectron spectroscopy (XPS) is used to perform surface elemental analysis. Usually an enrichment of nitrogen and C=O carbons can be detected. By varying the take-off angle, vertical distribution of nitrogen as indicator for the peptide can be examined. If a specific elemental tag like fluorine or iodine is incorporated in the RGD peptide, XPS can be used for quantification [20].

Some more general methods for RGD peptide quantification include amino acid analysis (AAA) and radioassays. In AAA the whole surface is hydrolyzed, and the free amino acids are detected using standard amino acid analyzers [21]. Alternatively the hydrolysate can be quantified as a mixture by using the ninhydrin test and comparing its UV absorption values with calibrated controls.

Employing radioiodination very small amounts (fmol/cm^2) of peptides can be detected. Radioactivity is usually introduced in peptides by oxidative iodination of tyrosine. Thus Na^{125}I is used in combination with immobilized iodogen as oxidant. After purification of the iodinated peptide a certain amount of radioactivity is assigned to a certain amount of spectroscopically quantified peptide [20]. In some applications radioiodinated peptide was diluted with non-modified peptide prior to surface immobilization [21].

By using a gamma counter, surface density gradients of a radioiodinated RGD peptide could be detected. Indirect quantification of immobilized RGD peptide can be achieved by measuring the amount of unreacted soluble peptide, by using HPLC/UV detection, the TNBS (trinitrobenzene sulfonicac id) assay or Ellmans reagent for thiol containing peptides. Indirect quantification, however, leads only to results with reduced reliability. The Sakaguchi assay for detecting arginine groups and the BCA (bicinchonic acid) assay which is usually used for protein quantification [22] require careful calibration when applied for RGD quantification. Determining RGD densities by the amount of attached cells or by an ELISA, using Merck's anticyclo(RGDfK) antibody led only to semiquantitative but nevertheless useful results.

Detection of microdistribution of RGD peptides is a challenging task and has not been solved yet. The methods used so far include AFM or 2D ToF SIMS detection of bigger RGD containing moieties like comb copolymers and latex beads [20,23]. Microdistribution of RGD peptides could possibly be discovered by high resolution XPS or AFM using integrin or RGD antibody coated AFM tips.

2.5 Cell Surface and Cell Adhesion [23, 24]

Adhesive interactions enable individual cells to form and stabilize close contacts in order to maintain higher-order tissue specializations and facilitate information transfer. The study of cell adhesion is largely a study of individual adhesion molecules, which participate in a variety of cellular processes that include:

1. cell-cell recognition
2. maintenance of cell-contacts and tissue integrity
3. cell-signaling, information transfer and differentiation
4. cell-migration

Adhesive mechanisms are grouped into two major categories: cell-cell and cell-extracellular matrix adhesion.

1. Cell-cell adhesion involves "close" interactions (typically within 15-20 nm distances) at the cell-surface that are mediated by transmembrane and/or membrane-associated glycoproteins
2. Many cells interact with their extracellular environments by adhering to a "network" of secreted glycoproteins and other glycoconjugates, termed collectively extracellular matrix (ECM).
3. Multiple types of adhesive mechanisms are available to most cells. Some of these mechanisms are functionally redundant (i.e., compensatory).

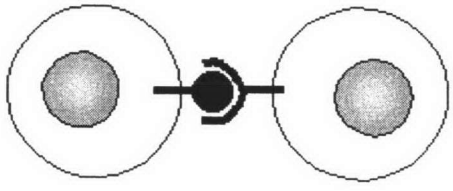
The view of the cell surface has been modified in recent years by the realization that the cytoskeleton is often physically associated with adhesion molecules at the cell surface. Some of these adhesion molecules function as transmembrane linking between the cytoskeleton and the ECM.

The Molecular Basis for Cell Adhesion

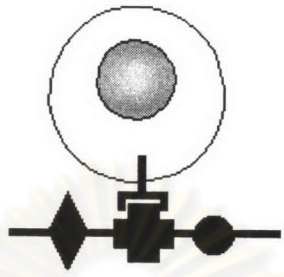
Cells use 4 basic types of molecular interactions to adhere:

1. Homophilic adhesion involves the binding of adhesion molecules on the surface of one cell to the same kind of adhesion molecules on another cell.

2. Heterophilic adhesion involves the binding of adhesion molecules on one cell to adhesion molecules of a different kind on another cell.



3. Cells can also bind to one another indirectly, by adhering to an extracellular "intermediate" (e.g., an ECM linker molecule).



4. Cell-substrate adhesion involves the binding of a cell-surface receptor to a secreted ECM molecule immobilized on the substrate.

Many cell adhesive interactions can be further distinguished by their requirements for divalent cations. For example, some adhesion molecules require Ca^{++} or Mg^{++} for function.

Several families of cell surface receptor molecules function in cell-cell and cell-ECM adhesion. Although structurally distinct, representatives from each of these families share a number of common features. They are all transmembrane proteins, which have large extracellular ligand-binding domains and relatively short cytoplasmic domains. The four most widely studied and functionally important groups of adhesion molecules are the immunoglobulin-superfamily (IgSF), and the cadherin (CAD), selectin, and integrin families.

1. Cadherins require Ca^{++} for adhesive function and are involved in homophilic cell-cell adhesion. Members of the cadherin family are expressed in specific tissues during development where they are believed to play important roles in morphogenesis. Cells expressing different CADs will "sort out" into homotypic (tissue-specific) aggregates under appropriate experimental conditions. E-cadherin (E-CAD) is a critical component of the zonula adherens junction. E-CAD links adjacent epithelial cells together and colocalizes with "bands" of actin filaments that encircle each epithelial cell. Loss of E-CAD is associated with many cancers of epithelial origin.

2. Selectins are adhesion molecules that mediate a variety of heterophilic interactions among leukocytes and endothelial cells that are important in inflammation and

immune response. Selectins are characterized by a single Ca^{++}-dependent lectin domain, which binds to specific carbohydrate groups on adjacent cells.

3. The immunoglobulin superfamily (IgSF) includes a number adhesion receptors characterized by the presence of large extracellular globular-domains held in place by disulfide bonds. IgSF receptors do not require divalent cations for adhesive function. Most, but not all, members of this family are involved in homophilic cell-cell adhesion and are thought to play important roles in embryonic development. I-CAM is a member of the IgSF that is expressed on activated endothelial cells where it is involved in heterophilic cell-cell adhesion. I-CAM binds to integrin counter-receptors expressed on leukocytes (white blood cells).

4. The integrins function as cellular receptors for many ECM glycoproteins. They provide a transmembrane linkage between the ECM and the cytoskeleton. Integrins have several important features and functions:

a. Integrins are heterodimers composed of 2 distinct glycocoprotein subunits termed α and β. The α subunit binds divalent cations (e.g., Ca^{++}), which are essential for integrin ligand binding function.

b. The integrins comprise a large family of closely related receptor complexes. Different $\alpha\beta$ subunit combinations differ in their ligand binding specificities and affinities. A large number of α subunits combine with a relatively smaller number of β subunits to yield multiple receptors. Some integrins specifically recognize only one ligand while others can interact with multiple ligands. Considerable functional "redundancy" is apparent in members of the integrin family.

c. Integrin-dependent adhesion can stimulate cell-signaling pathways important in regulating cell-growth, survival and gene expression

d. Not all integrins are involved in cell-ECM adhesion. Some integrins are involved in heterophilic cell-cell adhesion (e.g., by binding select IgSF receptors)

The ECM also plays an indirect role in cell-cell adhesion. Unlike the close contacts mediated by CAMs, CADs and Selectins, ECM molecules provide an intercellular "scaffold" to which cells adhere.

2.6 Characterization Techniques [9, 25]

2.6.1 Contact Angle Measurements

Contact angle measurements are often used to assess changes in the wetting characteristics of a surface and hence indicate a change in surface energy. The technique is based on the three-phase boundary equilibrium described by Young's equation, (Figure 2.12)

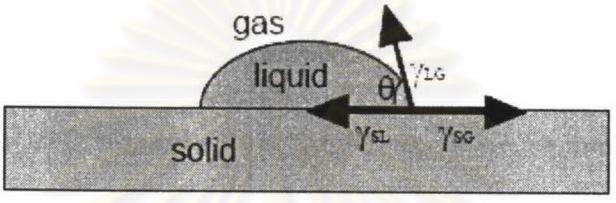


Figure 2.12 Schematic representation of the Young's equation.

$$\gamma_{LG}\cos\theta = \gamma_{SG} - \gamma_{SL}$$

where γ_{ij} is the interfacial tension between the phases i and j, with subscripts L, G, S corresponding to liquid, gas, and solid phase respectively and θ refers to the equilibrium contact angle.

The Young's equation applies for a perfectly homogeneous atomically flat and rigid surface and therefore supposes many simplifications. In the case of real surfaces, the contact angle value is affected by surface roughness, heterogeneity, vapor spreading pressure, and chemical contamination of the wetting liquid. Although the technique to measure contact angles is easy, data interpretation is not straightforward and the nature of different contributions to the surface is a matter of discussion. Generally, one can define the complete wetting, wetting, partial wetting, and nonwetting according to Figure 2.13.

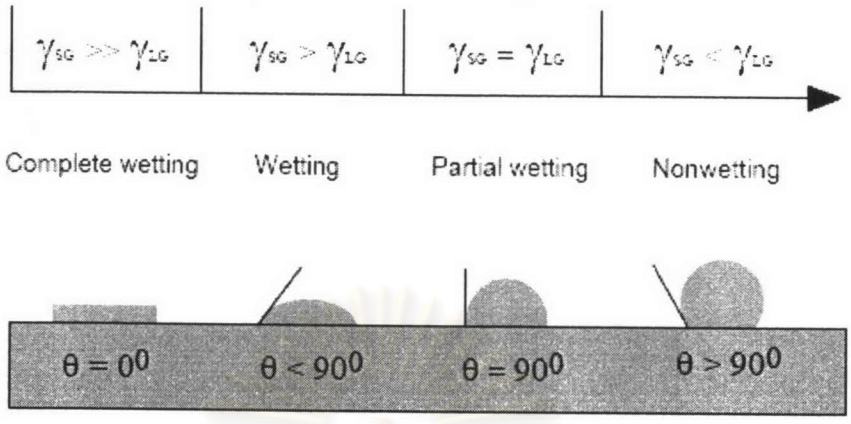


Figure 2.13 Schematic representation of wettability.

2.6.2 X-ray Photoelectron Spectroscopy (XPS)

XPS is an abbreviation for X-ray Photoelectron Spectroscopy. Another name is ESCA which is an acronym for Electron Spectroscopy for Chemical Analysis. In XPS or ESCA, a beam of (monochromatic) X-rays is first produced by electron bombardment of an anode material (Al, Mg, Si). When the X-rays interact with the sample under investigation, they can ionize electrons that are in core levels (such as 1s, 2s, etc.). If the binding energy of the electron in the core hole was E_B, then the kinetic energy of the electron ejected from the surface can be given in the energy diagram (Figure 2.14).

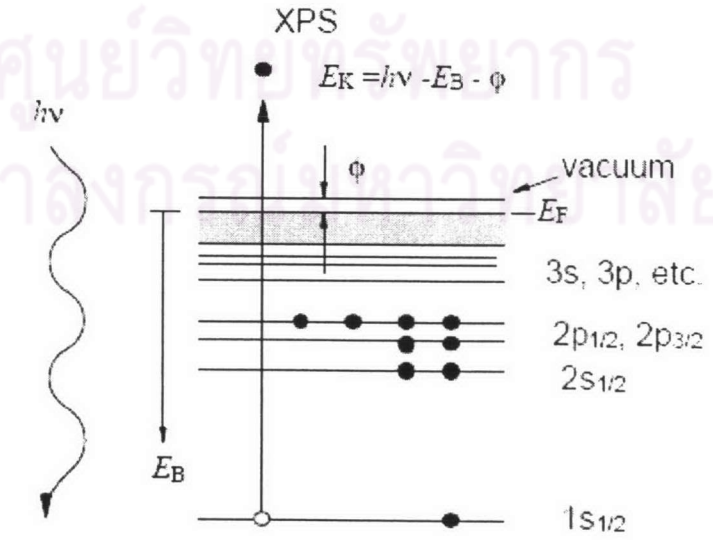


Figure 2.14 Schematic diagram of the x-ray photoelectron emission process.

$$E_K = h - E_B - \phi$$

where E_K is the measured electron kinetic energy, h is the energy of the exciting radiation, E_B is the binding energy of the electron in the solid, and ϕ is the spectrometer work function. Since h is a well-defined quantity, the electron binding energies can be calculated by measuring the kinetic energies of the electrons that are ejected from the sample, using the above equation. The electron energies are measured using an electrostatic energy analyzer such as a "hemispherical analyzer". The analyzer measures the kinetic energy distribution of the emitted electrons. A schematic drawing of the main components of the XPS instrument is shown in Figure 2.15. The main components of the system include the vacuum system, the x-ray sources, the sample stage, and the analyzer. The energy discrimination of the electrons is obtained by sweeping the potential(s) in the lens or by using a grid system in front of the analyzer. The sensitivity of the instrument is dependent on the X-ray source used, the analyzed area, geometrical factors and the efficiencies of the lens, the analyzer and the detector. The energy resolution is due to the inherent width of the X-ray radiation and the resolving power of the spectrometer.

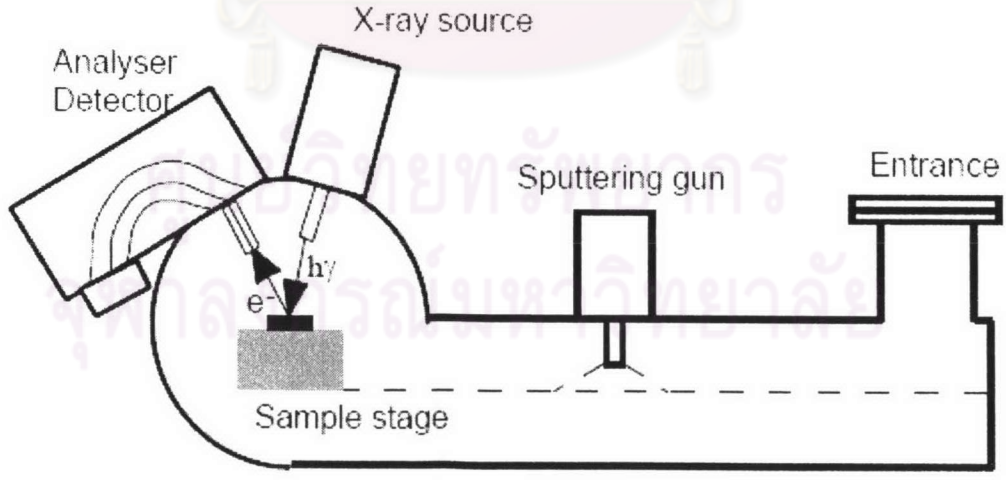


Figure 2.15 Schematic drawing of XPS instrument.

XPS can provide the following information

1. Elemental identification. Because the number of protons increases as we progress through the periodic table, the electron binding energies for a fixed core level (such as the 1s level) will increase monotonically; thus, measuring the electron kinetic energy is equivalent to determining which elements are present on the surface.
2. Oxidation states for any given elements. There will be small shifts in the binding energies due to changes in oxidation states; higher oxidation states generally have higher binding energies, and emit electrons with lower kinetic energies.
3. Quantitative analyses through curve fitting and calculation of atomic concentrations because the photoelectron intensity is directly related to the atomic concentrations of the photoemitting atoms.
4. Depth profiling when combined with ion etching (sputtering) techniques.
5. Images or maps showing the distribution of the elements or their chemical states over the surface. Modern instruments can have a spatial resolution down to a few microns.