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CHAPTER I.2.17 SURFACE-IMMOBILIZED BIOMOLECULES

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INTRODUCTION

Biomolecules such as enzymes, antibodies, affinity proteins, cell receptor ligands, and drugs of all kinds have been immobilized on and within biomaterial surfaces for a wide range of therapeutic, diagnostic, tissue regeneration, separation, and bioprocess applications. Immobilization of heparin on polymer surfaces is one of the earliest examples of a surface-modified, biologically functional biomaterial (Gott et al., 1963). Living cells may also be combined with biomaterials, especially when their surfaces contain cell adhesion peptides or proteins, and the fields of cell culture, artificial organs, and tissue engineering include important examples of cell–surface interactions. These "hybrid" combinations of natural and synthetic materials confer "biological functionality" to the synthetic biomaterial. Many sections and chapters in this textbook cover various aspects of this topic, including adsorption of proteins and adhesion of cells and bacteria on biomaterial surfaces, non-fouling surfaces, cell culture, tissue engineering, artificial organs, drug delivery, and others; this chapter will focus on the methodology involving physical adsorption and chemical immobilization of biomolecules on biomaterial surfaces, especially for applications requiring bioactivity of the immobilized biomolecule.

Among the different classes of biomaterials that could be biologically modified, synthetic polymers are especially interesting because their surfaces may contain reactive groups such as –OH, –COOH or NH_2 groups, or they may be readily modified with other reactive groups such as azide, alkyne, and SH groups. All of these groups can be used to covalently link biomolecules.

Another advantage of polymers as supports for biomolecules is that the polymers may be fabricated in many forms, including films, membranes, tubes, fibers, fabrics, particles, capsules, and porous structures. Furthermore, macromolecular structures can also vary substantially. The latter can include homopolymers, random, alternating, block, and graft copolymers, hyperbranched (comb-like) and star-shaped structures (see Chapter I.2.2 on Polymers).

Living anionic polymerization techniques, along with newer methods of living free-radical polymerizations, now provide fine control of molecular weights with narrow distributions. The molecular forms of solid polymers include non-cross-linked chains that are insoluble at physiologic conditions, cross-linked networks, physical blends, and interpenetrating networks (IPNs) (e.g., Piskin and Hoffman, 1986; see also Chapter I.2.2). "Smart" polymers are sharply responsive in solubility behavior to stimuli, such as temperature, pH, and salt concentration (see Chapter I.2.11 on "Smart" Polymers).

For surfaces of metals, metal oxides, inorganic glasses or ceramics, biological functionality can sometimes be added via a chemically immobilized or physically adsorbed polymeric or surfactant adlayer, or by use of techniques such as plasma gas discharge, corona discharge in air or ozone to modify polymer surface compositions with functional groups (see also Chapter I.2.12). Several researchers have applied mussel adhesive chemistry based on self-condensation of dopamine to form tight bonding layers of polydopamine on a variety of surfaces, including metals, metal oxides, and glasses (Lee et al., 2007; Ku et al., 2010). The amine groups in these polymers may be further functionalized with biomolecules.

PATTERNED SURFACE COMPOSITIONS

(See also Chapter I.2.13.)

Biomaterial surfaces may also be functionalized in geometric patterns (Bernard et al., 1998; Blawas and Reichert, 1998; James et al., 1998; Ito, 1999; Kane et al., 1999; Folch and Toner, 2000). Sometimes the patterned surfaces will have regions that are non-binding to proteins (so-called "non-fouling" compositions) while others may contain covalently-linked cell receptor ligands (Neff et al., 1999; Alsberg et al., 2002; Csucs et al., 2003; VandeVondele et al., 2003), or may have physically adsorbed cell adhesion proteins (McDevitt et al., 2002; Ostuni et al., 2003). A huge industry has also evolved based on "biochips" that contain microarrays of immobilized, single-stranded DNA (for genomic assays) or peptides or proteins (for proteomic assays) (Houseman and Mrksich, 2002; Lee and Mrksich, 2002). The majority of these microarrays utilize inorganic silica chips rather than polymer substrates directly, but it is possible to incorporate functionality through chemical modification with silane chemistries (Puleo, 1997) or adsorption of a polymeric adlayer (Scotchford et al., 2003; Winkelmann et al., 2003).

A variety of methods have been used for the production of these patterned biochips, including photo-initiated synthesis through patterned masks (Ellman and Gallop, 1998; Folch and Toner, 2000), microfluidic fluid exposure (Ismagilov et al., 2001), and protection with adhesive organic protecting layers that are lifted off after exposure to the biomolecular treatment (Jackman et al., 1999).

IMMOBILIZED BIOMOLECULES AND THEIR USES

Many different biologically functional molecules can be chemically or physically immobilized on polymeric supports (Table I.2.17.1) (Laskin, 1985; Tomlinson and Davis, 1986). Examples of applications of these immobilized biological species are listed in Table I.2.17.2. When hydrophilic, molecularly cross-linked or entangled solids are water-swollen above about 15–25% water content, they become hydrogels and biomolecules may be immobilized on the outer gel surface, as well as within the water-containing regions ("meshes") of the swollen polymer gel network. It can be seen that there are many diverse uses of such biofunctional systems in both the medical and biotechnology fields. For example, a number of immobilized enzyme supports and reactor systems (Table I.2.17.3) have been developed for therapeutic uses in the clinic (Table I.2.17.4) (De Myttenaere et al., 1967; Kolff, 1979; Sparks et al., 1969; Chang, 1972; Nose et al., 1983; Schmer et al., 1981; Callegaro and Denri, 1983; Nose et al., 1984; Lavin et al., 1985; Sung et al., 1986). Advantages and disadvantages of immobilized enzymes are listed in Table I.2.17.5.

TABLE I.2.17.1 Examples of Biologically Active Molecules that may be Immobilized on or within Polymeric Biomaterials

Proteins/Peptides
Enzymes
Antibodies
Antigens
Cell adhesion molecules
"Blocking" proteins
Saccharides
Sugars
Oligosaccharides
Polysaccharides
Lipids
Fatty acids
Phospholipids
Glycolipids
Other
Conjugates or mixtures of the above
Drugs
Antithrombogenic agents
Anticancer agents
Antibiotics
Contraceptives
Drug antagonists
Peptide, protein drugs
Ligands
Hormone receptors
Cell surface receptor ligands (peptides, saccharides)
Avidin, biotin
Nucleic Acids, Nucleotides
Single or double-stranded
DNA, RNA (e.g., antisense oliogonucleotides)

IMMOBILIZED CELL LIGANDS AND CELLS

Cell interactions with foreign materials are usually mediated by a biological intermediate, such as adsorbed proteins, as described in Chapter II.1.2. An approach using biologically-functionalized materials can be much more direct, by adsorbing or covalently grafting ligands for cell–surface adhesion receptors, such as integrins, to the material surface (Lutolf and Hubbell, 2005; Patterson et al., 2010) (Table I.2.17.6).

This has been accomplished with peptides grafted randomly over a material surface (Massia and Hubbell,

TABLE I.2.17.2 Application of Immobilized Biomolecules and Cells

Enzymes	Bioreactors (industrial, biomedical) Bioseparations Biosensors Diagnostic assays Biocompatible surfaces
Antibodies, peptides, and other affinity molecules	Biosensors Diagnostic assays Affinity separations Targeted drug delivery Cell culture
Drugs	Thrombo-resistant surfaces Drug delivery systems
Lipids	Thrombo-resistant surfaces Albuminated surfaces
Nucleic acid derivatives and nucleotides	DNA probes Gene therapy
Cells	Bioreactors (industrial) Bioartificial organs Biosensors

TABLE I.2.17.3 Bioreactors, Supports and Designs

"Artificial cell" suspensions

(microcapsules, RBC ghosts, liposomes, reverse micelles [w/o] microspheres)

Biologic Supports

(membranes and tubes of collagen, fibrin ± glycosaminoglycans)

Synthetic Supports

(porous or asymmetric hollow fibres, particulates, parallel plate devices)

TABLE I.2.17.4 Examples of Immobilized Enzymes in Therapeutic Bioreactors

Medical Application	Substrate	Substrate Action
Cancer Treatment		
l-Asparaginase	Asparagine	Cancer cell nutrient
l-Glutaminase	Glutamine	Cancer cell nutrient
l-Arginase	Arginine	Cancer cell nutrient
l-Phenylalanine	Phenylalanine	Toxin lyase
Indole-3-alkane α hydroxylase	Tryptophan	Cancer cell nutrient
Cytosine deaminase	5-Fluorocytosine	Toxin
Liver Failure (Detoxification)		
Bilirubin oxidase	Bilirubin	Toxin
UDP-Gluceronyl transferase	Phenolics	Toxin
Other		
Heparinase	Heparin	Anticoagulant
Urease	Urea	Toxin

TABLE I.2.17.5 Some Advantages and Disadvantages of Immobilized Enzymes

Advantages

Enhanced stability
Can modify enzyme microenvironment
Can separate and reuse enzyme
Enzyme-free product
Lower cost, higher purity product
No immunogenic response (therapeutics)

Disadvantages

Difficult to sterilize
Fouling by other biomolecules
Mass transfer resistances (substrate in and product out)
Adverse biological responses of enzyme support surfaces (*in vivo* or *ex vivo*)
Greater potential for product inhibition

TABLE I.2.17.6 Selected* Peptide Adhesion Domains in Cell Adhesion Proteins

Fibronectin	RGDS LDV REDV
Vitronectin	RGDS
Laminin A chain	LRGDN IKVAV
Laminin B1 chain	YIGSR
Laminin B2 chain	RNIAEIIKDA
Collagen I	RGDT DGEA
Thrombospondin	RGD

*A large number of receptor-binding peptide domains have been identified in numerous adhesion proteins that are able to recapitulate some of the binding character of the entire protein. Only a small selection of widely studied peptides is listed here.

1991), as well as with peptides presented in a pre-clustered manner (Irvine et al., 2001). The latter has important advantages. Cells normally cluster their adhesion receptors into nanoscopic assemblies referred to as "focal contacts" (Geiger et al., 2009), in which both adhesion ligands and receptors are co-clustered. This clustering plays an important role in both cell adhesion mechanics (Ward and Hammer, 1993), and cell signaling (Maheshwari et al., 2000; Geiger et al., 2009). In addition to peptides, saccharides have also been grafted to polymer surfaces to confer biological functionality (Griffith and Lopina, 1998; Chang and Hammer, 2000).

Nanotechnology methods of ligand immobilization have been used extensively to achieve very precise control of ligand immobilization density, ligand clustering, and arrangement, and even ligand exposure from a previously hidden state or hiding after an exposed state. For example, nano-patterning methods have been used to show that cells are capable of sensing and responding to clustering on the length scale of 70 nm of the RGD peptide, which binds to integrins such as $\alpha_v\beta_3$ (Huang et al., 2009). At longer length scales, the cell is unable to sense clustering. Methods of formation of self-assembling monolayers, including stimulus-sensitive self-assembling monolayers, have been used to conduct investigations on the migration- and differentiation-inducing influences of different cell adhesion molecules, and also to identify new receptor–ligand biomolecular pairs (Mrksich, 2009).

Specific biomolecules can be immobilized in order to control cellular interactions; one important class of such functionalizations is the polypeptide growth factor. Such molecules can be immobilized and retain their ability to provide biological cues that signal specific cellular behavior, such as support of liver-specific function of hepatocytes (Kuhl and Griffith-Cima, 1996), induction of neurite extension in neurons (Sakiyama-Elbert et al., 2001), induction of angiogenesis (Zisch et al., 2001) or the differentiation of mesenchymal stem cells into bone-forming osteoblasts (Lutolf et al., 2003a,b; Martino et al., 2009). It is important to understand that these effects are not merely induced by the mechanics of adhesion, i.e., what matters are the details of which adhesion ligand is displayed, and thus which adhesion receptor is ligated and what downstream signaling is activated. For example, stimulation of mesenchymal stem cells with a ligand for integrin $\alpha_5\beta_1$ stimulated osteogenesis, whereas stimulation with a ligand for integrin $\alpha_v\beta_3$ did not. Other molecules may be immobilized that can take part in enzymatic reactions at the surface. McClung et al. (2001, 2003) have immobilized lysines, whose ε-amino groups may interact with pre-adsorbed tissue plasminogen activator (tPA) during coagulation, to enhance fibrin clot dissolution at that surface.

The above paragraphs deal with biomaterial surfaces as though they are two-dimensional. In some devices, this is indeed the case; however, biomaterials have been developed to display their surfaces in three-dimensional situations, even with triggering by cellular remodeling. For example, biomaterial gels have been developed, consisting of cross-linked PEG chains, where the cross-linker is sensitive to proteases, such as plasmin or matrix metalloproteinases, that are activated by cells as they migrate (Lutolf et al., 2003a,b). In this way, as cells migrate in a material and remodel it, they can expose new surfaces and be stimulated by those new surfaces. Using such materials as tools, a number of regenerative medical applications have been targeted, where the biomaterial surface displays both adhesion ligands and polypeptide growth factors (Lutolf and Hubbell, 2005). Such materials, through careful selection of adhesion ligand composition, can control very specific biological processes, such as embryonic stem cell self-renewal (Lee et al., 2010). Materials have been developed that allow for very precise control of the adhesion ligand display and cross-linked nature of the three-dimensional material, e.g., through locally-controlled photochemical manipulation (Kloxin et al., 2009, 2010).

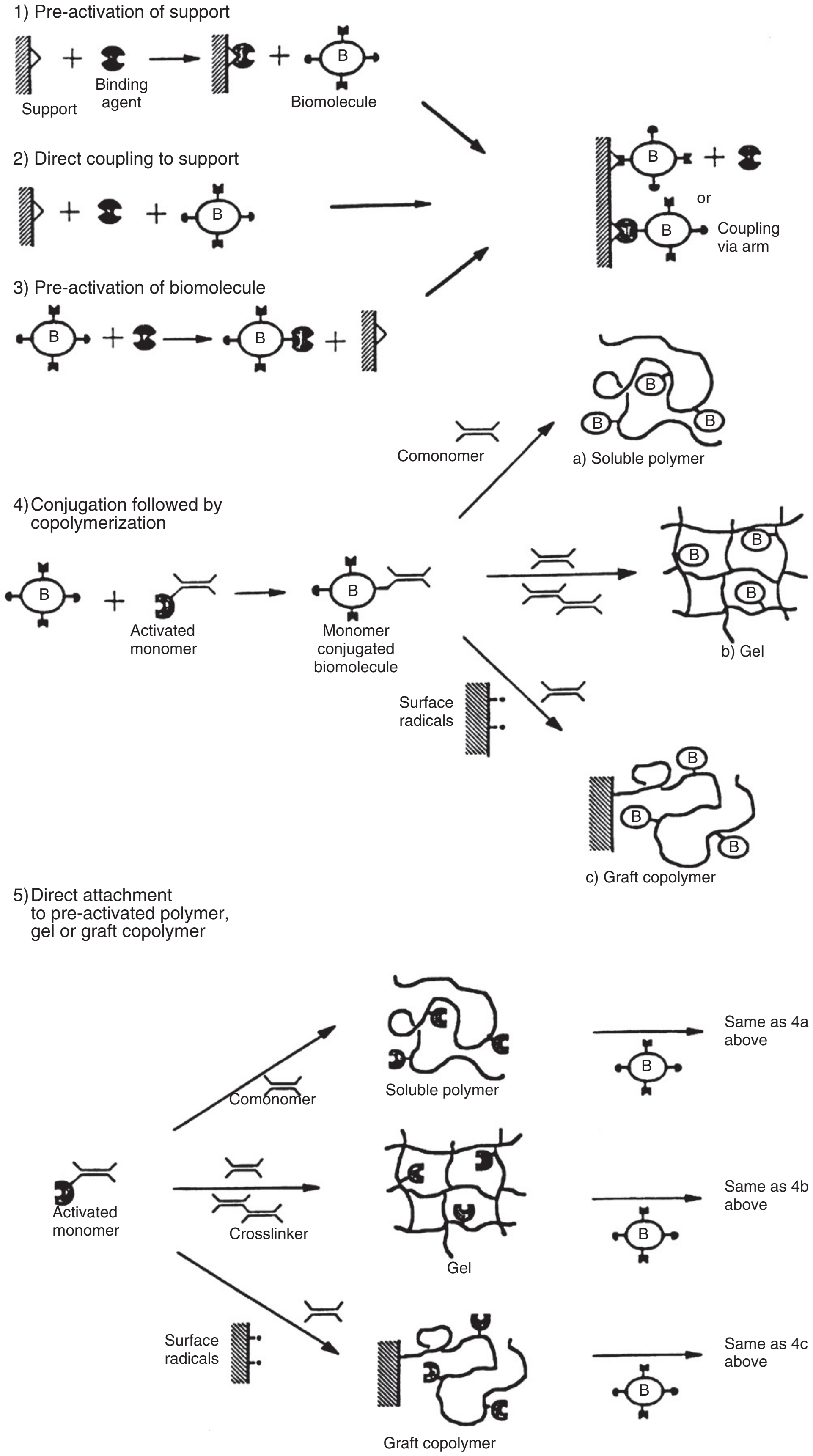
IMMOBILIZATION METHODS

There are three major methods for immobilizing biomolecules: physical adsorption; physical “entrapment;” and covalent attachment (Stark, 1971; Zaborsky, 1973; Dunlap, 1974). Physical adsorption includes: (1) van der Waals interactions; (2) electrostatic interactions; and (3) affinity recognition. Once adsorbed, the molecules may be further cross-linked to each other. Physical “entrapment” systems include: (1) microcapsules; (2) hydrogels; and (3) physical mixtures such as matrix drug delivery systems. Covalent attachment includes: (1) soluble polymer conjugates; (2) conjugates on solid surfaces; or (3) conjugates within hydrogels.

It is clear that the first two are physically based, while the third is based on covalent or “chemical” attachment to the support molecules. However, sometimes the physical attachment process may involve pairs of molecules with very strong affinity interactions, verging on covalent force levels, such as biotin with streptavidin. Thus, it is important to note that the term “immobilization” can refer to a short-term, long-term or “permanent” localization of the biomolecule on or within a support. In the case of a drug delivery system, the immobilized drug is supposed to be released from the support, either over a short period or over a longer-term, while an immobilized enzyme or cell adhesion peptide or protein in an artificial organ is designed to remain attached to or entrapped within the support over the duration of use. Either physical or chemical immobilization can lead to relatively long-term or “permanent” retention on or within a solid support, especially if the immobilized biomolecule is large.

If the polymer support is biodegradable, then the chemically immobilized biomolecule will be released as the matrix erodes or degrades away. Many researchers have chemically immobilized cell adhesion peptides such as RGD onto biodegradable matrices such as PLGA for use as tissue engineering scaffolds. The cells will have the time to bind and regenerate tissue if the support is slowly degrading. If the support degrades more rapidly than the cells’ ability to reach and bind to the adhesion peptides, then the matrix will not function in the intended way. The immobilized biomolecule may also be susceptible to enzymatic degradation *in vivo*, and this remains an interesting aspect that has received relatively little attention.

A large and diverse group of methods have been developed for covalent binding of biomolecules to soluble or solid polymeric supports (Weetall, 1975; Carr and Bowers, 1980; Dean et al., 1985; Gombotz and Hoffman, 1986; Shoemaker et al., 1987; Park and Hoffman, 1990; Yang et al., 1990; Schense and Hubbell, 1999; Lutolf et al., 2003b). Many of these methods are schematically illustrated in Figure I.2.17.1. The same biomolecule may be immobilized by many different methods; specific



Note : Ⓑ may be immobilized with or without a "tether" arm in any of the above

FIGURE I.2.17.1 Schematic cartoons showing various methods for covalent biomolecule immobilization.

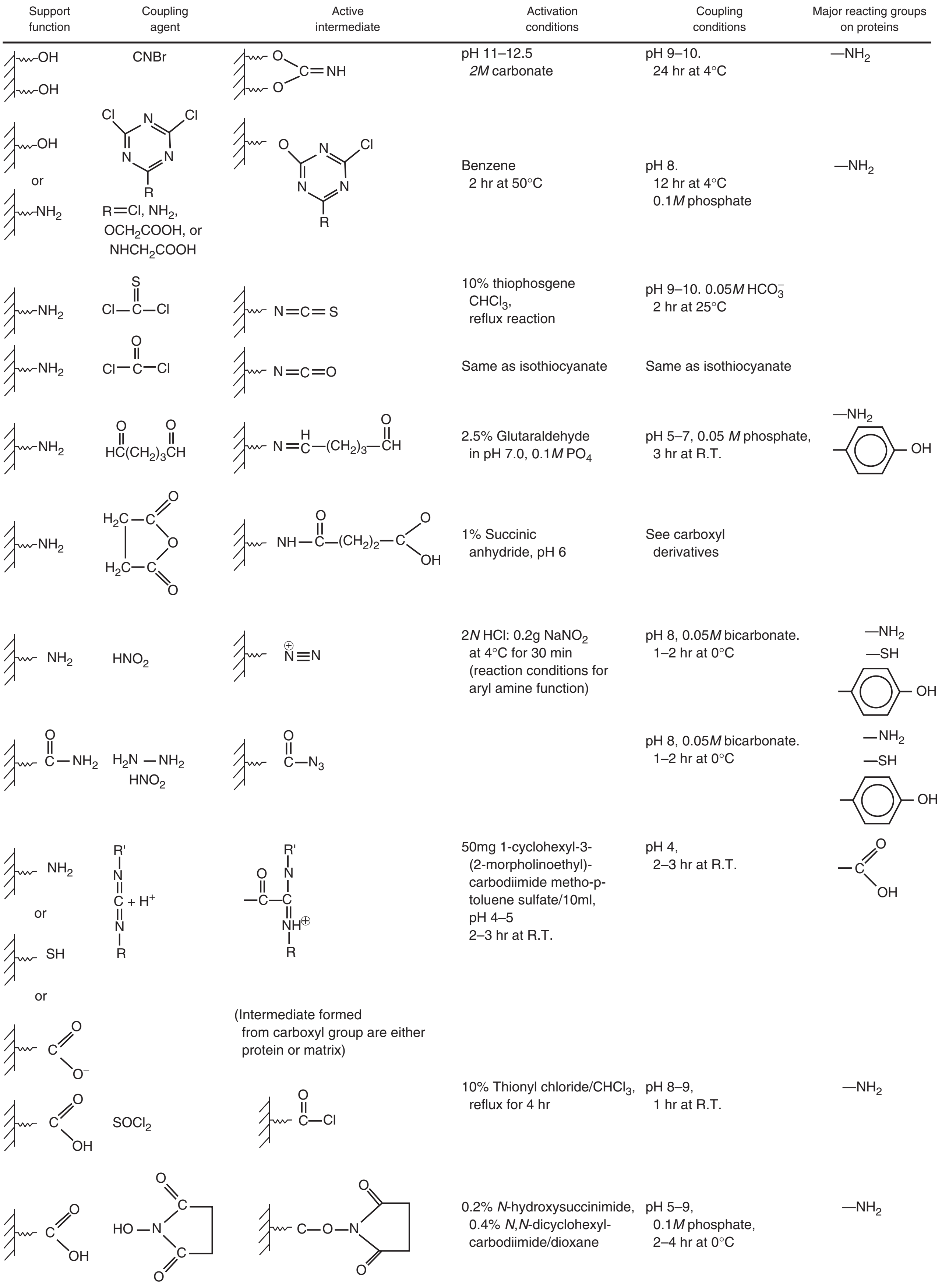


FIGURE I.2.17.2 Examples of various chemical methods used to bond biomolecules directly to reactive supports. (Carr and Bowers, 1980.)

examples of the most common chemical reactions utilized are shown in Figure I.2.17.2. This figure does not include some important reactions, such as thiol-maleimide, thiol-vinyl sulfone, amino-vinyl (Michael additions), and azide-alkyne (click) conjugation reactions. The reader is referred to Hermansson (2008) for many useful details on numerous conjugation chemistries.

For covalent binding to an inert solid polymer surface such as polyethylene or silicone rubber, the surface must first be chemically modified to provide reactive groups for the subsequent immobilization step. If the "inert" polymer support does not contain such groups, then it is necessary to modify it in order to permit covalent immobilization of biomolecules to the surface. A wide number of solid surface modification techniques have been used, including ionizing radiation-initiated graft copolymerization, plasma gas discharge, photochemical grafting, chemical modification (e.g., ozone grafting), and chemical derivatization (Hoffman et al., 1972, 1986; Gombotz and Hoffman, 1986, 1987; Hoffman, 1987, 1988) (see also Chapter I.2.12 on surface modification techniques).

A covalently-immobilized biomolecule may also be attached via a spacer group, sometimes called an "arm" or a "tether" (Cuatrecasas and Anfinsen, 1971; Hoffman et al., 1972; Hoffman, 1987). One of the most popular tethers is a PEG molecule that has been derivatized with different reactive end groups (e.g., Kim and Feijen, 1985), and several companies currently offer a variety of homo- or hetero-bifunctional PEGs with end group chemistries such as N-hydroxysuccinimide (NHS), maleimide, pyridyl disulfide, thiol, vinyl sulfone, alkyne, and azide (the last two are for click chemical attachments). Such spacers can provide greater steric freedom, and thus greater specific activity for the immobilized biomolecule. The spacer arm may also be either hydrolytically or enzymatically degradable, and therefore will release the immobilized biomolecule as it degrades (Kopecek, 1977; Hern and Hubbell, 1998).

Inert surfaces, whether polymeric, metal or ceramic, can also be functionalized through modification of a polymeric adlayer. Such physically adsorbed or chemisorbed polymers can be bound to the surface via electrostatic interactions (VandeVondele et al., 2003), hydrophobic interactions (Neff et al., 1999) or specific chemical interactions, such as that between gold and sulfur atoms (Harder et al., 1998; Bearinger et al., 2003). Metal or ceramic surfaces may also be derivatized with functional groups using silane chemistry, such as with functionalized triethoxysilanes (Massia and Hubbell, 1991; Puleo, 1997) or dopamine polymer chemistry, as described above. Plasma gas discharge has been used to deposit polymeric amino groups for conjugation of hyaluronic acid to a metal surface (Verheye et al., 2000).

As noted earlier, hydrophobic interactions have been used to functionalize hydrophobic surfaces, utilizing

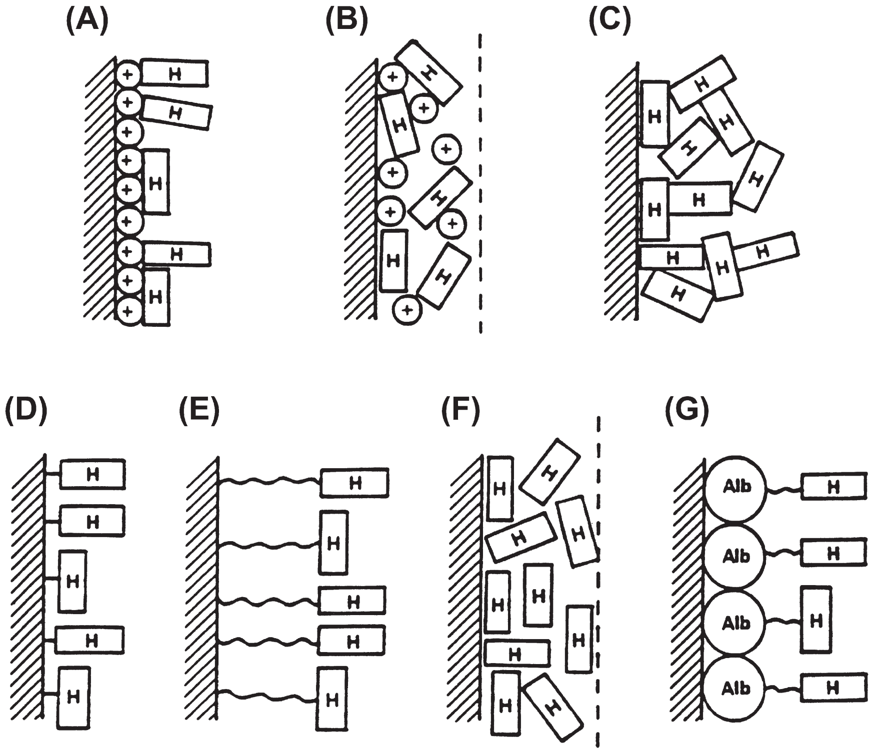


FIGURE I.2.17.3 Various methods for heparinization of surfaces: (A) heparin bound ionically on a positively charged surface; (B) heparin ionically complexed to a cationic polymer, physically coated on a surface; (C) heparin physically coated and self-cross-linked on a surface; (D) heparin covalently linked to a surface; (E) heparin covalently immobilized via spacer arms; (F) heparin dispersed into a hydrophobic polymer; (G) heparin–albumin conjugate immobilized on a surface. (Kim and Feijen, 1985.)

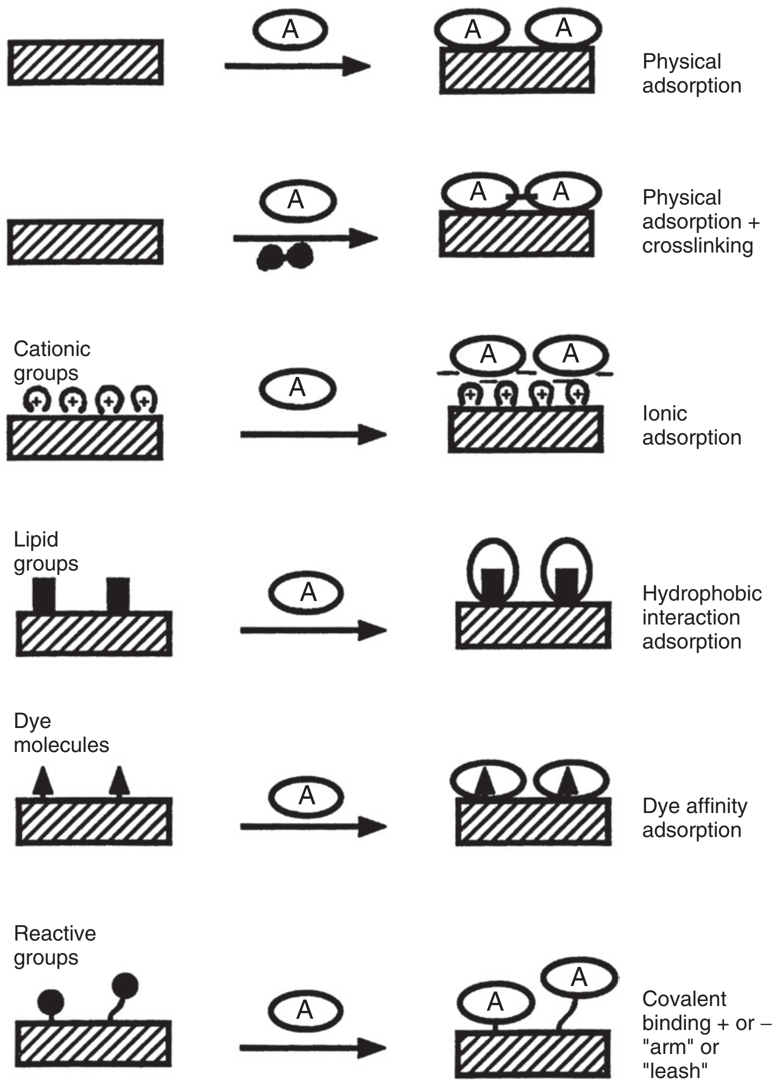


FIGURE I.2.17.4 Schematic of various ways that albumin may be immobilized on a surface. Albumin is often used as a "passivating" protein, to minimize adsorption of other proteins to a surface.

biomolecules such as ligands attached to hydrophobic sequences (e.g., Ista et al., 1999; Nath and Chilkoti, 2003). Surfaces with hydrophobic gradients have also been prepared for this purpose (Detrait et al., 1999). An interesting surface-active product was developed several years ago that was designed to convert a hydrophobic surface to a cell adhesion surface by hydrophobic adsorption; it had an RGD cell adhesion peptide coupled at one end to a hydrophobic peptide sequence.

Sometimes more than one biomolecule may be immobilized to the same support. For example, a soluble polymer designed to "target" a drug molecule may have separately conjugated to it a targeting moiety such as an antibody, along with the drug molecule, which may be attached to the polymer backbone via a biodegradable spacer group (Ringsdorf, 1975; Kopecek, 1977; Goldberg, 1983). For some nucleic acid drugs, sometimes a "nuclear localization signal" or NLS is added to enhance intracellular delivery to the nucleus (e.g., Nair et al., 2003). In another example, the wells in an immunodiagnostic microtiter plate will usually be coated first with an antibody, and then with albumin or casein (to block nonspecific adsorption during the assay); each is physically adsorbed to the well surface. In the case of affinity chromatography supports, the affinity ligand may be covalently coupled to the solid packing, and a "blocking" protein such as albumin or casein may be similarly added to block nonspecific adsorption to the support.

It is evident that there are many different ways in which the same biomolecule can be immobilized to a polymeric support. Heparin and albumin are two common biomolecules that have been immobilized by a number of widely differing methods. These are illustrated schematically in Figures I.2.17.3 and I.2.17.4.

Some of the major features of the different immobilization techniques are compared and contrasted in Table I.2.17.7. The important molecular criteria for successful immobilization of a biomolecule are that a large fraction of the available biomolecules should be immobilized, and a large fraction of those immobilized biomolecules should retain an acceptable level of bioactivity over an economically and/or clinically appropriate time period.

TABLE I.2.17.7 Biomolecule Immobilization Methods

Method	Physical and Electrostatic Adsorption	Cross-linking (After Physical Adsorption)	Entrapment	Covalent Binding
Ease:	High	Moderate	Moderate to low	Low
Loading level possible:	Low (unless high S/V)	Low (unless high S/V)	High	(Depends on S/V and site density)
Leakage (loss):	Relatively high (sens. to ΔpH salts)	Relatively low	Low to none[a]	Low to none
Cost:	Low	Low to moderate	Moderate	High

[a] Except for drug delivery systems.

CONCLUSIONS

It can be seen that there is a wide and diverse range of materials and methods available for immobilization of biomolecules and cells on or within biomaterial supports. Combined with the great variety of possible biomedical and biotechnological applications, this represents a very exciting and fertile field for applied research in biomaterials.

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CHAPTER I.2.18 BIOMIMETIC MATERIALS

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INTRODUCTION: WHAT ARE BIOMIMETIC MATERIALS?

Biomimetic materials are designed to replicate one or more attributes of a material produced by a living organism. This attempt at a definition highlights a shared characteristic of *biomimetic materials* and *biomaterials*, since successful biomaterials serve to either: (1) restore a natural function where the original material is absent or unable to perform properly; or (2) sustain an environment that is optimally conducive to processes such as cell culture, tissue growth, biomolecular assays, and biotechnology-based manufacturing.

How Can Biomaterials Science Benefit From Biomimicry?

In both biomaterials science and the science of biomimetic materials, there is an underlying premise that the desirable characteristics of a natural material can be replicated successfully. However, biomimicry is not just about replicating the useful *properties* of a natural material (human or other animal tissue) in a surrogate product. Biomimicry also draws upon Nature's lessons in the chemistry, processing, and structure of materials. Biomimicry therefore can provide guidance for every step in the procedure by which molecules are selected and manipulated during the design and fabrication of a biomaterial. This chapter highlights biomimicry as a source of inspiration for design and modification of biomaterials and biomedical devices.

Biomimicry recognizes that the functional materials produced by living organisms (and the natural processes by which such materials are made) have evolved over a very long period of time and that, by definition, are biocompatible. If a natural material does an excellent job (and a better job than an existing synthetic counterpart), then mimicry of that material should be considered. In this context, "excellent job" and "better job" are qualified with reference to a product having superior properties, which may include such attributes as being inexpensive or robust or environmentally friendly, or simply being unobtrusive.

It is necessary to take care about what we understand by a *natural material*. Since humans are themselves an integral part of Nature, we have to recognize that human technology and the materials that it creates can be regarded in principle as just further examples of what Nature can achieve. To avoid possible ambiguity, we will consider the term "natural material" to refer to materials that are produced *metabolically* by living organisms.

A CLASSIFICATION OF BIOMIMETIC MATERIALS

Within the context of (bio)materials, it can be helpful to recognize – and convenient to distinguish between – various classes of biomimicry, based on what type of natural attribute is being mimicked. The use of parentheses in "(bio)materials" here signifies that Nature's lessons for materials science can be widely applicable, and are not restricted specifically to biomaterials.

Functional biomimicry occurs when Nature provides the inspiration for one or more physical and/or chemical *properties* of a (bio)material. According to our preceding definition of a biomimetic material, there is some element of functional biomimicry in every successful biomaterial.