Biosurfaces : A Materials Science and Engineering Perspective

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TISSUE INTERACTION WITH BIOMATERIALS

Aditi Pandey, Anup K. Patel, and Kantesh Balani

Biomaterials Characterization and Processing Laboratory, Department of Materials Science and Engineering, Indian Institute of Technology Kanpur, Kanpur, India

As discussed in Chapter 1, the pivotal role of biomaterials has been visualized – in mimicking a living part, in particular, representing a part of the host system. This chapter guides the reader to the living world resided by the tissue (of the host) and the biomaterials (implant). It describes the interaction between the tissue and the biomaterials. It specifically focuses on cell adhesion and migration on the biomaterial surface, its controlled movement, the extracellular matrix (ECM) being enacted by the biomaterial and eventually concluding with the final stage of biomineralization.

2.1 INTRODUCTION

The developments in the fields of materials science and engineering and the cell and molecular biology have made it possible to gather information regarding the tissue–biomaterial interactions, *in vivo.* Biomaterials being designed nowadays are characterized by a highly engineered and sophisticated or classic architectural build-up, which encourages the biological scientists to take up the challenge of defining various biomaterial interactions and functions (on gene and molecular level) in physiological

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environment on implantation of the biomaterial. Biological tissue is composed of cells and intercellular or interstitial substances (especially ECM and various body fluids). A biomaterial when implanted in the host interacts with the concerned tissue and the tissue-specific cells in its environment. The cascade of the interactions and response a biomaterial follows begins with the material–cell contacts. The cell attachment to the implant follows two main strategies for the cell–material interactions: one is by creating an inert surface that inhibits the cell attachment and proliferation, and the other is by creating a surface that promotes them. The implants constructed to fulfill the former criterion are used in the joint prostheses (as heads and cups) [1], blood-contacting devices (heart valves), smooth bioinert vascular prostheses, vesicles for drug delivery or catheters for hemodialysis [1–5] or intraocular lenses [6, 7]. Those in the latter case are used in bone implants for the formation of osseous tissue [8–10] or skin substitutes made up of polymeric sheet with a feeder layer of fibroblasts that is covered by keratinocytes [11]. The various stages a biomaterial encounters when implanted in the host system have been depicted in this chapter. An implanted biomaterial is known to come in contact with proteins and, in turn, with the cells. The proteins mediate cell adhesive interactions, which are followed by cell migration, inflammation, and elicited immune response. This concept of cell–biomaterial interaction provides a foundation for the synthesis of scaffolds, which can be seeded *in vitro* with cells for the study of these interactions along with their biocompatibility and cytocompatibilty and then finally tested *in vivo* in an animal model, which provides a confirmation for their application in the human system. A scaffold is an artificial structure providing a platform for the growth and support of cells, controlling tissue formation in a way that is analogous to communication and patterning within the cells during embryological development.

A scientific interdisciplinary field emerging in the modern era of today is "tissue engineering," which involves the synthesis of materials (scaffolds) and the analysis of the interaction of scaffold with living cells (aiding in tissue formation or regeneration) for the replacement of organs or tissues in a host system, producing diagnostic or therapeutic effects. This synthesized scaffold first undergoes the *in vitro* analysis, which involves the culturing of cells in a suitable environment supported by desirable cells, growth media, optimum pH, temperature, moisture and CO_2 level, and also the various experiments for the biocompatibility and cytocompatibilty. If the scaffold is found to be cytocompatible and biocompatible, it is ready for the next step of implantation *in vivo* into the host system at the appropriate anatomic location, whereby it again follows the same procedure of cell culture as performed *in vitro*. A deep knowledge of the methodology a cell follows during interaction with a biomaterial becomes an essential need in the study, which is expanded herewith.

Cell–biomaterial interactions follow two different types of route of attachment: (i) favored and mediated by receptors (which attach to proteins or ligands adsorbed on the implant surface from the ECM) and (ii) carried out in the absence of receptors. This chapter throws light on the former (receptor mediated). However, in the latter case (non-receptor mediated), the cells attach to the surface by non-specific interactions carried out by weak bonding (hydrogen, polar or ionic, electrostatic bonding) or with the help of chemical groups attached to the implant surface, thereby without the involvement of the ECM proteins [12, 13]. The cells, if are unable to synthesize their own

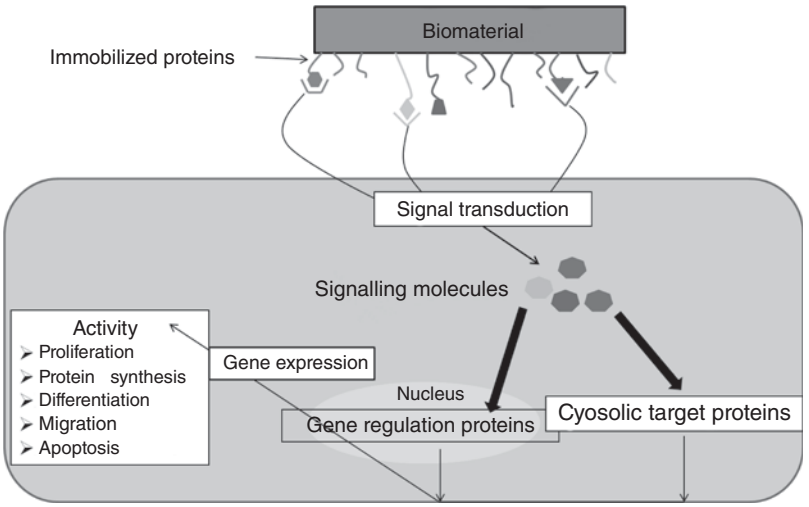

Figure 2.1. Proteins immobilized on the biomaterial surface, which interact with the cell surface receptors, which in turn produce various response. (Adapted from [19].)

ECM molecules or do not posses it on their cell membrane, undergo a phenomenon called *programmed cell death*, better known as *apoptosis* [14–17]. Another type of cell death, known as *necrosis,* occurs due to environmental factors, causing unprogrammed or accidental death.

The major route of cell attachment is that mediated by receptors. The proteins or ligands adsorb on the implant surface from the ECM and are then bound by the cytoskeletal receptor molecules projecting outwards from the cell [18]. Many of the signaling pathways are a result of cell adhesion and interaction between the cell surface receptors and ligands on the material surface. The signaling pathways target specific cytosolic proteins and also gene-regulating proteins for further response such as proliferation, differentiation, migration or apoptosis (Fig. 2.1). The protein-adhered biomaterial surface sends signals to the cells leading to signal transduction, thereby producing signaling molecules, which target the nucleus or the cytosolic proteins and may produce various phenotypic expressions.

The synthesis of a biocompatible material that is not of biological origin is the main strategy undertaken by scientists these days. The reason behind engineering an artificial material is that it is not recognized by the host as foreign and thereby does not elicit immune response, leading to successful application. The cascade of events following implantation begins majorly with protein adsorption on the biomaterial surface onto which the cells adhere by receptor–ligand interaction.

2.2 PROTEIN ADSORPTION AND CELL ADHESION

Protein adsorption can be effectively studied by using either single protein solutions (e.g., in a buffer solution, to study the fundamentals of protein adsorption and the bioreactions elicited by it) or complex multiprotein solutions (e.g., in blood plasma, to study

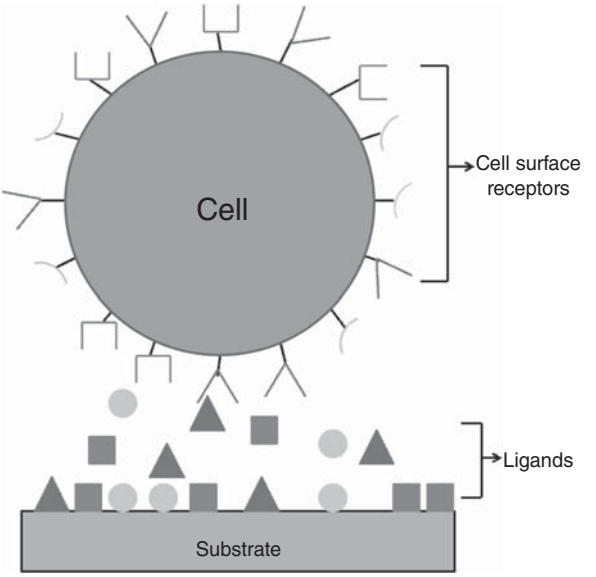

Figure 2.2. The enlarged view of the cell with its receptors and the layer of proteins–ligands adsorbed on the biomaterial surface. (Adapted from [21].)

the response toward an implanted biomaterial) [20]. The adsorption of proteins – the "cell adhesion proteins" to the material intensifies the attachment of cells, which posses receptors (cell-membrane-spanning proteins), binding distinctively to the adhered proteins known as *ligands* (Fig. 2.2).

These membrane-spanning proteins are named as *the integrin proteins*, present on most of the cells. This process of protein pre-adsorption also encourages the cell flattening and spreading on the biomaterial [20].

With respect to single protein solutions, the "monolayer model" of protein adsorption can be elucidated, wherein saturation effect of protein is seen [20, 22]. Saturation effect refers to a protein first being adsorbed to a surface in its rapid initial phase and then reaching a steady state of adsorption. If represented graphically in the adsorption isotherm, the pattern obtained is linear initially followed by saturation. This follows the famous Langmuir adsorption pattern (Fig. 2.3), which is explained by the following equation:

$$\frac{\Gamma}{\Gamma_m} = \frac{KC}{1 + KC}$$

where, Γ is adsorbed protein per unit area, Γ_m is its maximum value for adsorption and C is the protein concentration. K is the equilibrium constant for the reaction process.

However, the Langmuir's isotherm has been questionable for long for its impracticality for the point that it says one protein molecule is bound by only one active site. Therefore, Langmuir–Freunlich isotherm has been studied for the protein adsorption behavior for more than monolayer adsorption. In this case, the second parameter (n) was a constant depending on the type of protein [23–26].

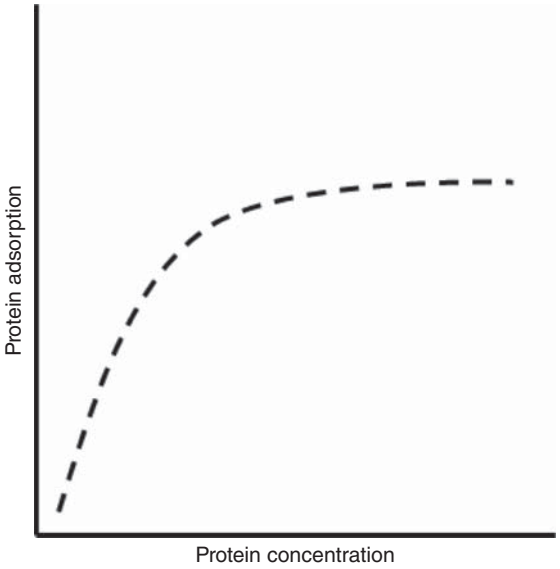

Figure 2.3. Langmuirs's adsorption isotherm.

It is as follows:

$$\frac{\Gamma}{\Gamma_m} = \frac{KC^{1/n}}{1 + KC^{1/n}}$$

It can be noted that K depends both on the protein and the surface, while *n* depends only on the type of protein [24–26].

With regard to complex multiprotein solutions, the adsorption is selective following a competitive phenomenon. Competition between different proteins is observed considering the exposed surface sites for adsorption, as the solid surface can accommodate only a limited number of proteins. This greatly depends on the affinity of the proteins toward the surface. After IgG and albumin, fibrinogen is present in the largest concentration in the blood plasma. Fibrinogen is a protein for which the blood platelets have a receptor and is involved in thrombosis and hemostasis [27]. Fibrinogen has been studied extensively, and it is often proposed that in its adsorbed state, it does not support biocompatibility [28]. A biomaterial (e.g., polyethylene) when exposed to the blood plasma contains fibrinogen as its maximum adsorbed phase. Hemoglobin, a protein present in very minute amount in the plasma, is still adsorbed in similar quantity as the other dominant proteins (mentioned previously) owing to its high affinity. However, albumin, even after its high presence in the plasma, is adsorbed in concentrations typically similar to that of fibrinogen. In this case, it can be inferred that according to the law of mass action, the high albumin concentration in plasma is a factor leading to its adsorption onto the material surface. Therefore, another important factor guiding the competitive adsorption process is the mass concentration of the proteins in their bulk phase [20].

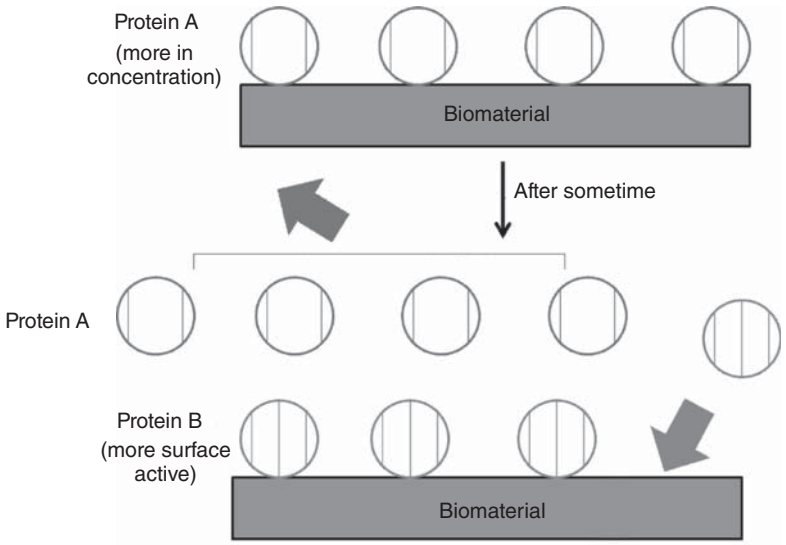

Figure 2.4. Vroman effect explained by proteins A and B, wherein the protein A that was first adsorbed onto the biomaterial surface is replaced by protein B with time. (Adapted from [30].)

The protein adsorption phenomenon cannot be completed without mentioning the "Vroman effect" taking the example of fibrinogen. According to this phenomenon, the initially adsorbed fibrinogen is later displaced by proteins that are more surface active, especially kininogen, a high molecular weight plasma protein. In addition, transitions in the fibrinogen adsorbed make it less displaceable with time [29]. Vroman effect is represented in Fig. 2.4, taking the example of two proteins, A and B. Protein A gets adsorbed to the surface at first, due to its high cytoplasmic concentration, but later, it is replaced by protein B.

Although it has been known that protein adsorption is the major step to initiate the cell–material interactions, on implantation, the bare surface of a biomaterial comes in contact with blood and other body fluids and at first becomes surrounded by water molecules, thereby creating a hydration shell around the material. The extent and interaction pattern of the water molecules depends on the surface properties of the material, which also determines one of the major steps initiating the cell–biomaterial interaction – the protein adsorption. Protein adsorb to hydrophobic surface in a different manner in comparison to the hydrophilic materials. Enthalpic forces form the major driving force in adsorption to hydrophilic substrates, whereas in adsorption to hydrophobic substrates, the entropic forces are involved [31]. Disruption of the hydration shell (dehydration) by the protein adsorption to the surface is a thermodynamically favored process, as it increases the entropy of the system. Therefore, adsorption to hydrophilic surfaces is generally reversible while to hydrophobic surfaces is not. Adsorbed proteins, on hydrophobic surfaces, further get denatured, which also contributes to irreversible adsorption [32]. Hydrophobic surfaces have a tendency to adsorb proteins in comparison to hydrophilic surfaces that tend to resist [33]. During the stage of proteins adsorption, the biomaterial surface, with the view to be recognized

by the host, is covered by a layer of proteins from the ECM, such as fibronectin, laminin, collagen, and vitronectin [15–17, 20, 34, 35]. These proteins are further recognized by cell surface receptors proteins. As mentioned in Chapter 1, proteins are the biomolecules (polymers) made up of peptide sequences constituted by amino acids. These amino acid sequences in a protein account for the adhesion to their respective cell-membrane-bound receptors. Integrins constitute the family of ubiquitous receptor transmembrane proteins on cells, which are made up of one α and one β chain (made up of several subunits). The different combinations of the subunits of these chains possess diverse specificity toward majorly ECM proteins (preferentially) and also to cell surface and other plasma proteins [20, 36, 37]. The integrins recognize a sequence of amino acids Arg-Gly-Asp symbolized as RGD present on different proteins, for instance, fibronectin, vitronectin, and so on. The diagrammatic explanation of integrin binding through its α and β subunits to the RGD sequence of the adhesion protein adsorbed on the biomaterial surface is shown in Fig. 2.5.

A type of integrin $\alpha_2\beta_1$ recognizes the sequence of amino acid Asp-Gly-Glu-Ala (DGEA) on collagen. In addition to the RGD sequence, the integrins can also bind to the nearby amino acids. The amino acid sequences are receptor (integrin) specific, while this is not the same for the receptor molecule, that is if the sequence is absent, it binds to some other peptide or ECM molecule. Therefore, these integrins are sometimes referred to as *most promiscuous receptors* [17, 39–45].

Basically, the response that a biomaterial presents depends on the interaction of protein molecules on material surface, involving both binding in the initial stage and subsequent unfolding. Protein unfolding or denaturing would allow the amino acids lying inside to reach the external environment, which makes them available for external interactions [35]. Owing to the tendency to unfold (to allow further formation of

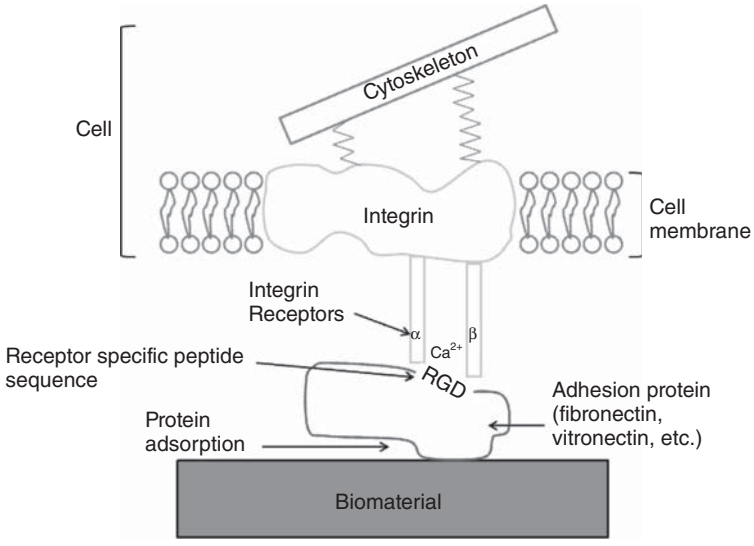

Figure 2.5. The integrin protein interacting by its α and β chain with the RGD sequence of the adhesion protein (such as fibronectin and vitronectin). (Adapted from [38].)

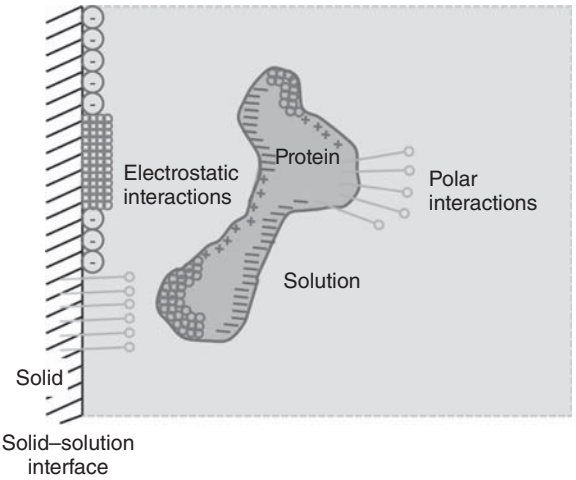

Figure 2.6. The various protein–material interactions. (Adapted from [52].)

bond with surface) and low structural stability of proteins, they may undergo conformational changes [20]. On the basis of this, proteins are named as *soft* (with low thermodynamic stability) and *hard* (high thermodynamic stability) [46]. Soft proteins tend to unfold, while hard proteins are stable toward it. Differential Scanning Calorimetry (DSC) technique has shown that soft proteins tend to lose their structure (which depends on how much soft they are). Protein adsorption to different substrates has been extensively investigated by different techniques that include atomic force microscopy, FT-IR, spectroscopic imaging, ellipsometry, electron microscopy, quartz crystal microbalance, fluorescence probe techniques, sodium dodecyl sulfate gel electrophoresis, and so on [47]. Adsorption of proteins to the biomaterial surface is directed by interactions between the molecular groups present on the surface of the material and those of the protein (hydrogen bonding, electrostatic interactions, van der Waals interactions etc.), which in turn determines the entropic interactions with those adsorbed proteins. Figure 2.6 shows the protein–material interactions. These entropic changes occur due to the protein unfolding because of the release of the bound water from the surface [48, 49]. According to some physiochemical studies, complete denaturation of the adsorbed proteins has been suggested. However, these changes are referred to be more limited by the biological activity of the probes [50]. Therefore, in the adsorbed state, enzymes still own some of their biological activity, which is also a function of the surface loading. The proteins on the material surface reorganize themselves carrying different confirmations, which has been studied by the binding of monoclonal antibody (MAb) directed against its fragment D. This adsorbed phase fibrinogen is shown to be bound to the MAb, while in the solution, it does not [51]. The aforementioned finding suggests that the proteins, in their adsorbed state on the material surface, reorganize themselves to different confirmation, such that they may bind to a molecule to which they do not bind in their solution state (i.e., in which they occur as free state).

The principles of protein adsorption to a material surface can thus be summarized as depending on the properties of the surface, the bulk concentration of the protein, the

different selectivity (for proteins) of different surfaces, biological activity of the adsorbed protein and the post-adsorptive organization of the protein layer.

Having discussed the importance of proteins in initiating the response toward a biomaterial, a familiarity and knowledge of the tissue/cell–biomaterial interactions are needed to elaborately study this response, which is laid down in the rest of the chapter.

2.2.1 Cell Adhesion

After the adhesion of the proteins onto the biomaterial surface, the cells adhere with those adsorbed proteins through their cell surface receptors (receptor-mediated adhesion) and further interact with the neighboring cells (cell–cell interaction). The cell–protein interaction is briefed as follows.

2.2.1.1 Cell–Protein Adhesion. The receptors as described earlier, the integrin proteins, carry out the cell–protein adhesion by recruiting regions called as *focal adhesions* on the cell membrane, which are distinct streak-like or dot-like nano- or micro-domains. At these sites, integrins communicate with several structural and signaling molecules. The focal adhesion sites are represented by membrane-associated cytoskeleton proteins, called as *focal adhesion proteins*, the examples of which include talin, filamin, α-actinin, paxillin, and vinculin (Fig. 2.7) [17, 40–42, 44].

These proteins are capable of linking the integrin receptors with the actin cytoskeleton, which is associated with cellular membranes of organelles, nuclear membranes,

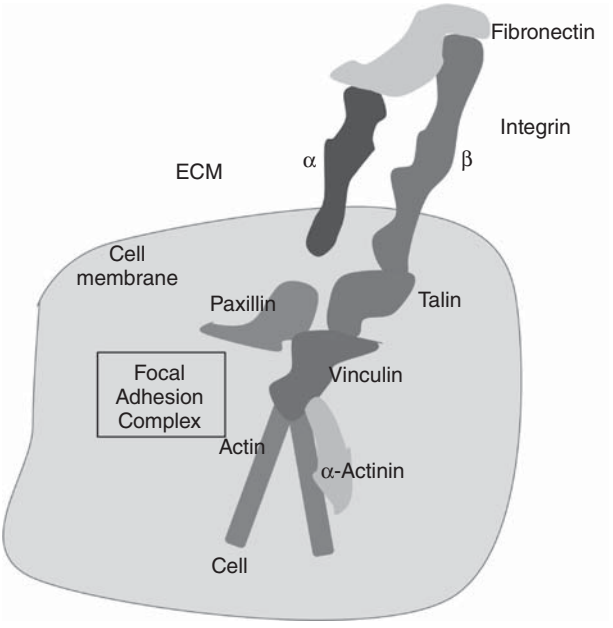

Figure 2.7. Interaction of the ECM proteins with those of the focal adhesion complex. (Adapted from [53].) *(See insert for color representation of this figure.)*

and also different enzymes. Therefore, being associated with the cells, the focal adhesion proteins influence the cell behavior (which includes transport and secretion of molecules) and endocytosis and also perform a decisive role for cell proliferation, differentiation or apoptosis [14, 17, 44, 54, 55]. This receptor-mediated cell adhesion through the ECM molecules is highly dependent on the physical and chemical properties of biomaterial surface, such as wettability, surface roughness and topography, electrical charge, mechanical properties (flexibility or rigidity), porosity, solubility, crystallinity, pH, or the presence of chemical functional groups or certain atoms (amine, oxygen groups, carbon atoms, etc.).

As mentioned earlier, subsequently after the cell–protein interaction, the cells communicate with each other (cell–cell adhesion), the mechanisms of which are described in the following section.

2.2.1.2 Cell–Cell Adhesion. The cell to cell adhesion mechanism is a way through which cells after attachment to the substrate communicates with one other with the help of certain proteins, which are also grouped under the "cell adhesion proteins." *Homophilic interaction* is a term that involves the adhesion molecule binding to same type of protein present on a neighboring cell [37]. The calcium-dependent cadherin proteins form the major of such kind of interactions [56]. In cell to cell adhesion, the target protein (of a receptor) could be a "counter receptor" or a complex carbohydrate, which is linked to a protein in the cell membrane. The homophilic adhesion of cadherin involves the binding of cadherin to another such protein through certain domains specific for cell interaction, the example of which includes a short recognition sequence His-Ala-Val [37]. The cytoplasmic domain of these cadherins binds to catenin, which in turn provides linkage with the actin cytoskeleton. Figure 2.8 represents the cell–cell adhesion interactions, wherein the homophilic cadherin interaction recruits the proteins, p120, β-catenin and α-catenin, which interact with vinculin protein and further vinculin, in turn causing actin–myosin interaction [57].

The homophilic binding of cadherin is followed by the proteins p120, catenins, vinculin, actin, and myosin coming into action. The activity of cadherins is sensitive to the concentration of calcium ions in the cell. The calcium ions make the extracellular domains rigid and enable homophilic interactions. Three calcium ions bind to each pocket, between cadherin extracellular domains, with different affinities [58, 59]. When cells are introduced to chelators such as EDTA, the calcium ions are further no more available to perform their function and lead to a disruption in the conformation, which subjects the cadherins to proteolytic attack.

The two major pathways cells take up to interact and adhere with the substrate (cell–biomaterial) and with each other (cell–cell) have been studied until now. After cell attachment, the next step in the tissue–biomaterial interaction is "cell migration."

2.3 CELL MIGRATION

To elaborate on this, when cells interact with the biomaterials, their ability to move by interacting with the material surface or neighboring cells forms an essential part of tissue

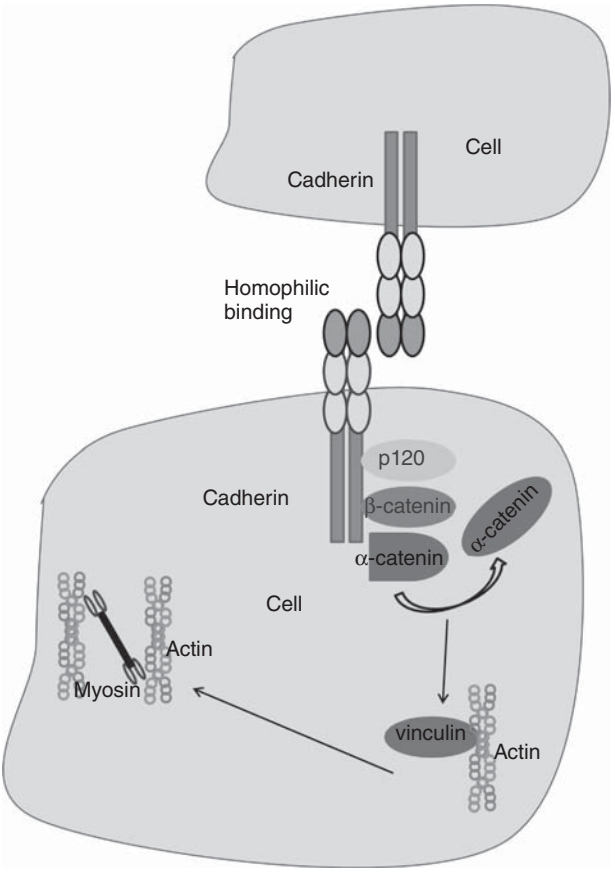

Figure 2.8. Cell–cell interaction and the proteins involved therein. (Adapted from [57].) *(See insert for color representation of this figure.)*

formation or regeneration [18]. Just as migration of cells in a tissue plays a critical role in the development of organs and organisms, cell migration is indeed an important phenomenon in the field of tissue engineering. The movement of cells on substrate requires three structural elements, namely: (i) an ECM ligand on surface, (ii) ligand-specific receptor on cells and (iii) the cytoskeleton inside the cells [60, 61]. Cell migration could be considered a cycle that comprises four major steps enlisted as follows [62]:

I. Lamellipod formation by the extension of the leading margin of the cell over the substratum. Lamellipod formation is characterized at the front of the cell by a thin piece of membrane and cytoplasm.
II. Attachment to the substratum.
III. Contraction or pulling by the new adhesion points formed for anchorage.
IV. Detachment at the rear end of the cell (Fig. 2.9).

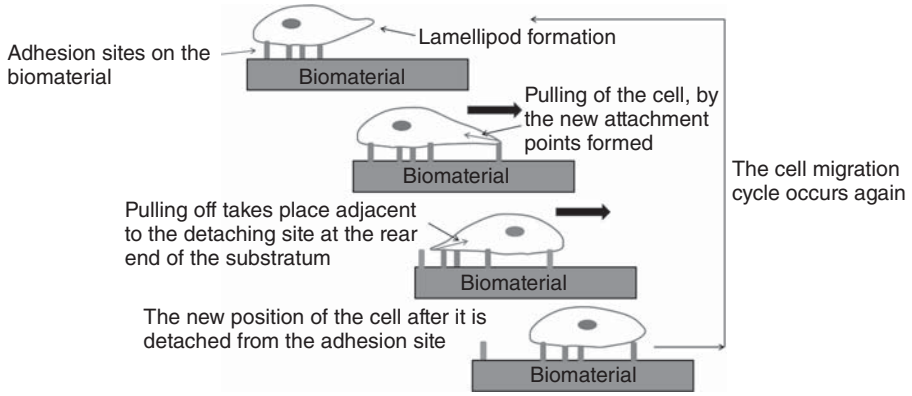

Figure 2.9. Cell migration cycle. (Adapted from [63].)

These processes are carried out in the presence of various extracellular and intracellular molecules. Since in this cellular locomotion process one end of the cell moves forward (spreads) while the other end retracts, this may be considered as a polarized cell [64]. Majorly, this is caused by the polymerization of actin filaments [65]. Amoeboid movement is a type of cell motility involving actin. The lamellar protrusion formed is coupled with polymerization of actin, which involves the factors Arp2/3 complex, capping, and gelsolin protein [62, 65–67]. The modification of cell morphology is controlled by the small GTPases (Rac1, RhoA, and Cdc42) protein family and is often referred to as cell polarization [68]. Integrins help in the anchorage of the cell to the substratum by binding to the ECM molecules present on the outside of the cell, as well as to the actin cytoskeletal filaments inside [69]. The candidates involved in forming linkage between integrin and the actin filaments are most likely to be talin, α-actinin, and vinculin. Studies indicate that the calcium-activated cysteine protease, calpains, promote focal adhesion disassembly, thereby helping in cell migration. The focal adhesion disassembly is brought about by cleavage of the focal adhesion-related proteins. The nucleus and the cell body move into the protrusion, a process called as *traction* [65], involving the actin and myosin (cytoskeletal filaments) cooperation [65]. The rear end is known as *the tail*, comprising the cytoplasm, which is left behind the cell body. Adhesive release at the rear may involve weakening or severing the integrin–cytoskeletal or integrin–ECM linkage, following which the integrins get separated from the actin and continue to be associated with the substrate as "footprints" [70, 71]. After traction, the tail de-adheres and retraction occurs, following which this process of locomotion occurs again [65]. Retraction of the trailing end of cells is mediated by the Rho/ROCK signaling, which has a role in disassembly during detachment of cell [72]. Inhibition of Rho-kinase or MLCK leads to a morphology characterized by impaired rear end detachment [73]. The diagrammatic representation of the cell migratory proteins is shown in Fig. 2.10. Cell migration by cell polarization is regulated by the small GTPases (Rac1, RhoA, and Cdc42) protein family, leading to the lamellipod formation at the front. The forward movement of the cell goes along with the disruption of the focal adhesion (through proteolytic cleavage) at the rear end, which is promoted by the calpain proteins [68].

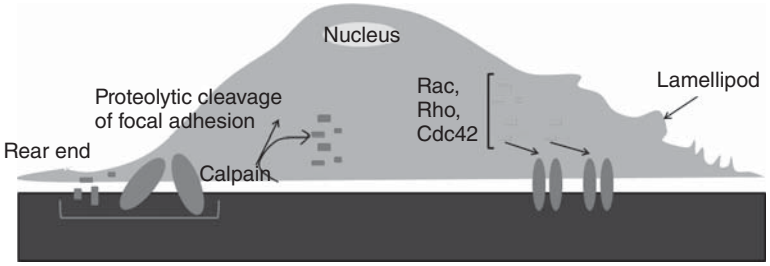

Figure 2.10. Cell migration and the associated calpain involvement. (Adapted from [74].)

A model developed by Lauffenburger et al. demonstrated that the rate of cell migration is a function of attachment strength of the cell with the substrate [75]. This attachment strength may be regulated by (i) the ligand density on the surface, (ii) the expression levels of integrin, and (iii) the binding affinity of the integrin with the ligand [76]. Cell migration was observed maximum when the cell–substrate attachment strength was at intermediate level, as cell detachment is also an integral part of the cell migration process [75].

Specialised types of integrin-mediated adhesions are formed by highly migratory and invasive cells, called as *invadopodia* or *podosomes*. Invadopodia are formed generally in cancerous cells. The architecture of podosomes and invadopodia is defined by a rich actin core, wherein the actin polymerization machinery and the actin regulatory proteins drive membrane protrusions [77]. Fibroblasts that were transformed by the v-Src oncogene have revealed the presence of podosomes [78], and cells of monocytic lineages such as osteoclasts, dendritic cells and macrophages also show podosomes [79].

Migration of a mammalian cell in isotropic environment may be explained by persistent random walk [80, 81]. Cells show persistence of movement by following a relatively straight path over short periods of time. However, if cell position is observed for long time periods, cell movement appears to be similar to Brownian motion showing frequent directional changes. To explain the persistence random walk, two parameters, namely speed, S (displacement of cell per unit time) and persistence time, P (average time between the significant directional changes), are usually defined [82]. The P and S values are dependent on the type of cell and its microenvironment [60].

Persistence random walk in migrating cell population can be interrupted by cell–cell contacts. This phenomenon exhibited by cells is known as *contact inhibition of locomotion*; after such contacts, cells halt and change their travel direction [61]. For example, collision among fibroblasts stops membrane ruffling near the contact areas and result the formation of a quiescent (or dormant) region, whereas remaining areas continue to ruffle. After a period of time, the cells in contact break the adhesion and thereafter move in new directions [61].

The interaction of cells and tissues with material surface is indeed an important phenomenon in promoting new tissue deposition and also for the integration with the ECM. To obtain a desired response, the cell and ECM deposition must be carefully controlled, for which an understanding of the topography, chemistry, and mechanical properties of a scaffold is needed, which shall be discussed in the following section.

2.4 CONTROLLED CELL DEPOSITION

Cell–ECM interactions are governed by the cell adhesion proteins interacting with the cell surface receptors as described earlier. It is essential to understand that a biomaterial mimics the ECM for the cells and interacts with them by sending specific signals. It should be noted that the surface topography, chemistry and mechanical properties of a scaffold have shown significant dependence of the cell behaviors such as adhesion, growth, migration and differentiation. Therefore, the surface of a biomaterial is critically important in determining biomaterial–tissue interactions. This concept has lead to the development of various surface modification techniques. Controlling cell behavior by the synthesis of surface-engineered biomaterials is a critical step in the development of tissue engineering scaffolds. The next section continues with the description of various parameters and approaches in order to develop such engineered scaffolds. Different factors that affect the cell growth are discussed herewith.

2.4.1 Hydrophobicity

Studies have shown that the more hydrophilic a surface is, the more is the cell adhesion [83, 84]. For instance, osteoblast adhesion was found to decrease as the contact angle increased from 0° to 106°, and the fibroblasts were reported to show maximum adhesion between 60° and 80° [85, 86]. 7F2 mouse osteoblasts demonstrated accelerated metabolic activity on hydrophilic surface ($\theta = 24–31°$), and also osteodifferentiation was observed in comparison to their unmodified counterparts ($\theta = 72°$) [87]. The same was demonstrated by neuronal spreading and neurite outgrowth when the hydrophobicity of the material surface was reduced [88, 89]. Surface hydrophobicity can be measured by contact angle a water droplet subtends on a material surface. The contact angle imposed on the material classifies the material into hydrophobic and hydrophilic. If the angle formed is between 60° and 90°, the surface is said to be hydrophilic, and if more than 90°, it is said to be hydrophobic.

2.4.2 Material Chemistry and Surface Charge

The surface charge has recently been described to a great extent with respect to the cell attachment phenomenon. The amount of surface charge can affect cell behavior [90]. Enhanced cell adhesion and proliferation are observed to increase the charge density of pol(styrene-ran-acrylic acid) [91]. It was also reported by many researchers that by incorporating positive and negative ions to the implanted surfaces, improved biocompatibility, high cell affinity and enhanced cell differentiation were observed [92]. Citing the example of HEMA hydrogels, which were incorporated with the positive ions, significantly increased cell attachment and spreading of fibroblasts and osteoblasts in comparison to the negative and the neutral charges [93]. The surface charge can modify cell behavior through chemical functional groups of the polymer material. Polyethylene surfaces were prepared with different chargeable functional groups by using the corona discharge, graft copolymerization and also substitution reactions for the study of their effect on the cell behavior [94]. It was found that the Chinese hamster ovary cells show more adhesion to the grafted surfaces than that to the control polyethylene because

of the grafting of hydrophilic functional groups, which increases the wettability. The polar and positive-charged surfaces (polyethylene grafted with amine group) promoted cell adhesion and spreading, while the negative-charged surfaces (polyethylene grafted with carboxylic acid group) showed poor growth. The neutral amide and hydroxyl group grafted surfaces showed a similar kind of response in a number of cell attachments, but the morphology of the attached cells was quite distinct. Surface charge may also modulate adsorption of proteins to direct the integrin binding, thereby controlling the cell adhesion [36]. It was reported that negative charge incorporation may facilitate protein adsorption, promoting cell adhesion [95]. Surfaces with different chargeable functional groups modulated fibronectin adsorption and also directed integrin binding to control the cell adhesion of MC3T3 osteoblasts to the fibronectin-coated surface following the trend $OH > COOH = NH_2 > CH_3$ [96]. Although the molecular mechanisms behind the modulation of cellular activities dependent on surface charge are still not understood clearly, the findings reveal the important role played by the surface charge in applications of tissue engineering and cell biology [36].

2.4.3 Surface Topography and Roughness

Surface topography and roughness have shown to provide intimations to the cells eliciting cellular response. These include control of cellular adhesion, their morphology, cell death known as *apoptosis* and gene regulation. Therefore, texture modification of the material surface may show dramatic effects on guiding the tissue growth. Modification of the surface by selective attachment of proteins or functional groups can be carried out by techniques such as photolithography. Photolithography involves the use of the photoresist layer, wherein patters are created by light exposure to certain areas, which degrades those specific portions, leaving a bare surface that can be modified by protein or functional group attachment. The photopatterning of proteins (called *contact guidance*) gives rise to substrates possessing certain specific areas for cell adhesion [97]. The material can also be deeply etched to form grooves or pits, which when encountered by cells lead to a change in the shape of cells, and may further help in alignment or elongation along these topographic features. Contact guidance is a term that defines the cell activity directed by a groove in a material surface [97]. This phenomenon is known to prevent epithelial down-growth on the dental implants, which directs the formation of bone along certain areas of an implant. Ordered alignment of cells can also be generated by this technique, which is an important goal in muscles, nerves and blood vessels. According to observations made, the topography and roughness should be in the range 1–10 μm that is relevant to mimicking the biological scale [97]. In accordance with the literature, cell growth on micro-rough surfaces was stimulated toward differentiation, which was displayed by the gene expression when compared to cells grown on smooth surfaces. However, the response that cells show toward the roughness is highly dependent on the cell type. For example, human fetal osteoblast cells (hFOB) when cultured on rough surfaces show an elevated amount of cell spreading and proliferation [98].

Other methods of modifying the surface include laser ablation or wet etching for surface roughening. Chemical patterns for cell substrates can be created by a newer method of surface modification known as *microcontact printing*, wherein an elastomeric

stamp (having bas relief features) is used for the transfer of an "inked" material on a substrate [99].

Several metallic components are used as biomaterials in the orthopaedic field, and knowing that calcium phosphate coatings increase the bone attachment, many techniques have been developed for titanium coating with calcium phosphate. A process known as *ion sputtering* transfers a thin layer of $CaPO_4$.

Furthermore, the chemistry of a surface can be modified by numerous chemical reactions, which react with surface atoms or molecules without coating them with a new layer. These are categorized into two, namely non-specific and specific chemical reactions. Briefing about the non-specific type, a variety of functional groups is present on the surface. Examples include chromic acid oxidation surfaces made of polyethylene and radio frequency glow discharge (RFGD), which is a corona discharge modification of materials in air. In RFGD, materials are treated in oxygen, carbon dioxide, argon, nitrogen, or water vapor plasma, where the metal surfaces are oxidized to a mixture of sub-oxides. However, the specific chemical surface reactions lead to the modification of only one functional group (creating another group with a high yield while allowing some other side reactions) [100].

Silanization is another phenomenon by which a material surface can be modified. It is a cost-effective and straightforward method involving a liquid phase chemical reaction, often used in the modification of hydroxylated surface (Fig. 2.11). Glass, alumina, quartz, germanium, silicon and many other metal oxide surfaces rich in hydroxyl group follow the silanization for their surface modification. Silane reactions are simple and stable owing to their covalent and cross-linked structure [100].

Discussing the self-assembled monolayers (SAMs), these are films formed on the surface spontaneously as highly ordered structures (two-dimensional crystals) on certain substrates [101–105]. Two processes that are important for the formation of SAMs are as follows [103]:

- An adsorption (moderate to strong) of an anchor – a chemical group – to the surface (generally 30–100 kcal/mol).
- The interaction of the alkyl chains by van der Waals forces.

The molecular-level bonding via chemisorption provides a driving force for entire surface coverage, which also assists in removing the contaminants from the reacting biomaterial surface. It must also be noted that closely lying monolayer chains adsorbed on the biomaterial surface also allow crystallization of alkyl groups due to weak van der Waals interaction between the molecular alkyl chains. The ease of formation of SAMs, chemical stability and, in many cases, the possibility of changing the outer most group in the external environment are some of the advantages of SAMs. Most SAMs are based on the assembly of *n*-alkyl chain, but SAMs can be formed from other classes of molecules such as proteins [106], nucleotide bases, porphyrins, and aromatic ring hydrocarbons.

A biomimetic surface modification approach, which controls cell–biomaterial interactions, is the pre-adsorption of the proteins on the implant surface. The RGD sequences found in fibronectin (as described earlier in the chapter) are widely used to deposit on the material surface for mediating cell adhesion. In addition, adsorption of other molecules

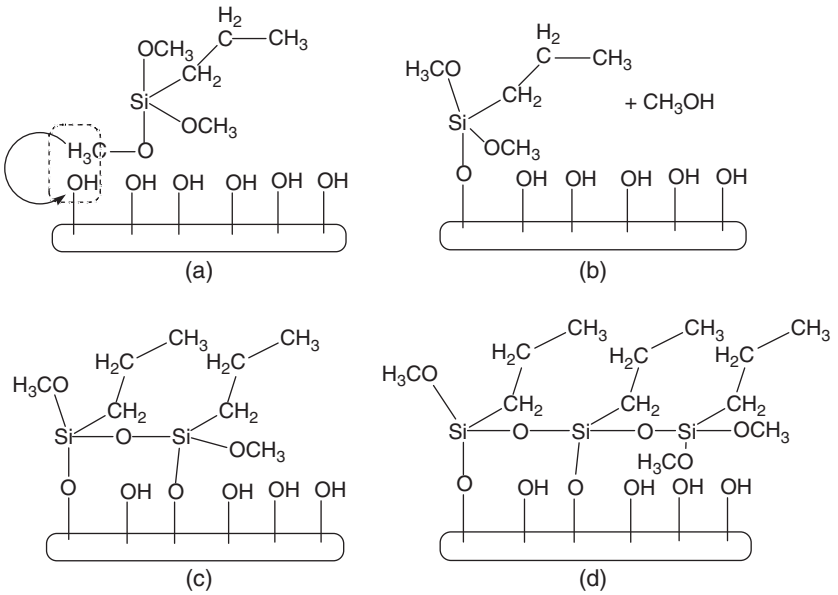

Figure 2.11. The silanization process showing (silane surface modification reaction) (a) the presence of hydroxyl groups on the surface, immersed in non-aqueous solution of n-propyl trimethoxysilane (nPTMS), following with (b) the coupling of methoxy groups of nPTMS with hydroxyl group (releasing methanol) on the surface. (c) Then, methoxy groups of other nPTMS molecules react, one with hydroxyl group, and the other with methoxy group of earlier nPTMS molecule, but it is also possible that (d) another nPTMS molecule reacts only with the methoxy group of nPTMS (and not the surface); thus silane film network is generated on the biomaterial surface. (Adapted from [100].)

such as growth factors can control the tissue biomaterial interactions. Silanization can be used to attach proteins to the biomaterial surface by covalent bonds. Physical adsorption methods such as van der Waals and electrostatic binding can be used to immobilise proteins as well; however, it is least specific and also tends to release the adsorbed proteins.

Surface modification, on the contrary, can also be used to create protein resistant surface, which is needed in applications involving blood contacts such as vascular grafts. For instance, cell adhesion on polyethylene-oxide-treated surfaces was significantly reduced [19]. Following cell adhesion, migration and controlled movement on the biomaterial surface, deposition of an ECM on the material surface facilitating the various tissue–biomaterial interactions is discussed in the next section. It is essential to know how the ECM and its components regulate implant interactions with the host.

2.5 EXTRACELLULAR MATRIX

Tissues and organs have a non-cellular gel-like element, secreted by cells termed as ECM, which acts as a connector for cells and proteins [107]. It provides structural support to individual cell and also acts as a key factor to regulate the cell functions, which

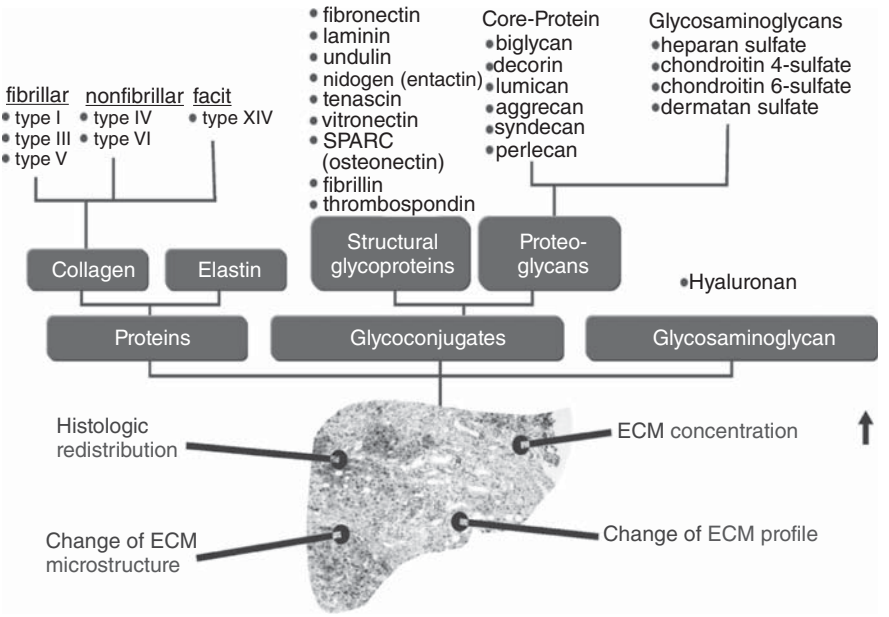

Figure 2.12. Different components of ECM. (Adapted from [108].)

lead to cell fate process such as differentiation with homeostasis and tissue morphogenesis. There are two main constituents of the ECM: (i) proteoglycans (PGs) and (ii) proteins with fibrous structures such as fibronectin, collagen and laminin as shown in Fig. 2.12. The transmembrane receptors known as *integrins* take part in significant roles such as cell signaling and cell-to-ECM attachment.

A. Proteoglycans covered most of the interstitial space of ECM of the tissues as hydrated gel form [109]. These proteoglycans made up by complex structures of carbohydrates known as *glycosaminoglycans* (GAGs) attached with proteins molecules. These protein molecules may attach with different GAGs and vice versa. These proteoglycans play distinctive roles such as fastening, buffering and hydration properties [108].
B. Fibrous proteins: collagen is a plentiful fibrous protein that is found in the body within the ECM, which covers more than 29% of the entire protein groups. It is a main constituent of the ECM and is formed by fibroblasts and epithelial cells. The most important functions of collagen are regulating cell adhesion and proliferation, strengthening to cell attachment and cell migration, and so on [110].

Collagens constitute a large family of 19 related glycoproteins, from collagen I to collagen XIX. These care classified on the basis of triple helix configuration, as well as the order of amino acid involved in these chains. The sequential arrangement of amino acids is highly specific, as every third amino acid faces in the direction of the center of the spiral helical structure, and smallest amino acid such as glycine encloses very

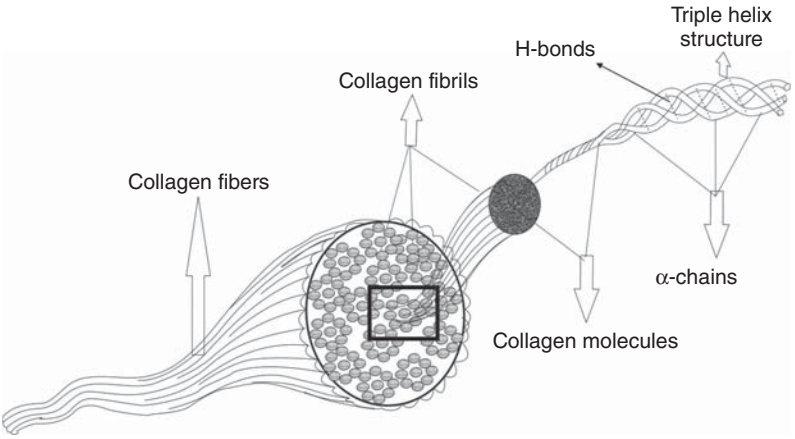

Figure 2.13. Structure of a collagen fiber.

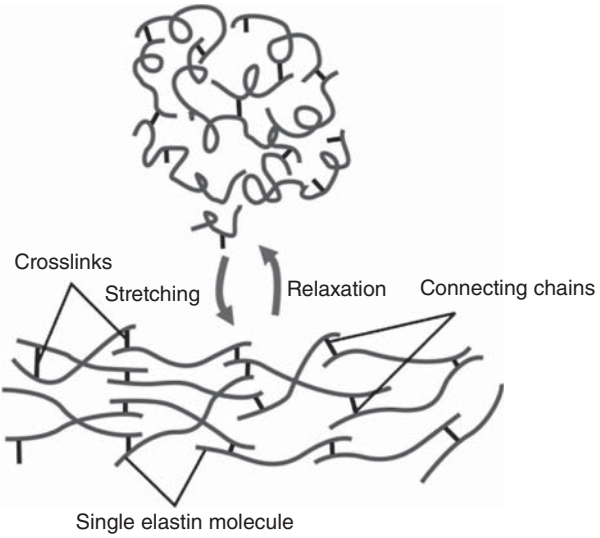

Figure 2.14. Structure of elastin in two conditions showing stretching (below) and relaxation (above).

limited space. However, proline and hydroxyproline amino acids stabilize to the helical configuration through hydrogen bonding as shown in Fig. 2.13.

Elastin, acting as a precursor, is an important amorphous protein also known as *tropoelastin* (as shown in Fig. 2.14), which forms elastic fibers with the help of other ECM components (see Fig. 2.12). These elastin fibers are stabilized by lysyl oxidase enzyme, which covalently cross-links the elastin molecule by eliminating lysine amino acids moieties. The major role of elastin is to help regulate the mechanical properties of ECM.

Fibronectins, another component of fibrous protein found in ECM, are a division of protein created by alternative fusion from a single gene. These are found soluble in nature in blood as well as in ECM in the form of disulphide-bonded fibrils. The major role of fibronectins is to provide the requisite position to cells and keep the specific components of the ECM united together. Laminins are large fibrous proteins found in ECM, which provide a variety of sites for receptors to bind at the cell surface.

ECM is physically integrated with cells and capillaries in functional tissues, as shown in Fig. 2.15. This matrix provides a base where the cell can adhere and proliferate and also bind to other cells collectively, make them more physically strong to provide physical and mechanical support and pass signals and intermingle with others. The cell surfaces have a number of ECM receptors and adhesion molecules, which are responsible for cell–ECM communications. During normal development and in response to the tissue damage, these adhesive communications synchronize with cell surface receptors, nucleus and cytoskeleton. The consequential intracellular communications affect the specific functions such as cell differentiation, proliferation, gene expression and cell mobility [107].

As described in the earlier section, when an implant is inserted into the host, cells start to come from body fluid and accumulate at the surroundings of implant surface and undergo characteristic cell fates process, that is, differentiation, proliferation, migration as well as gene expression, which leads to cell–cell and cell–material communications. Furthermore, cells secrete ECM molecules to fill the gap between the cell–cell as well as cell–material and also provide the structural mechanical support to cells and proteins. The main functions of the ECM during this process are as follows [107]:

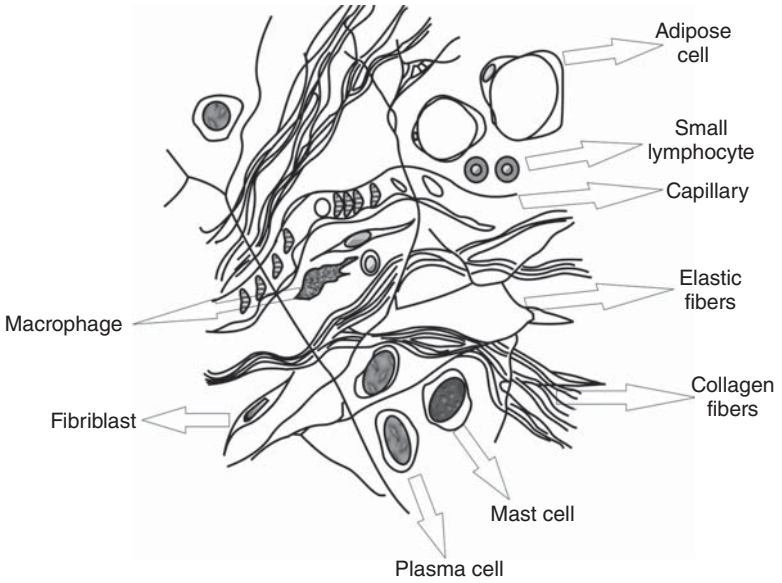

Figure 2.15. Integrated structure of ECM within the functional tissue. (Adapted from [107].)

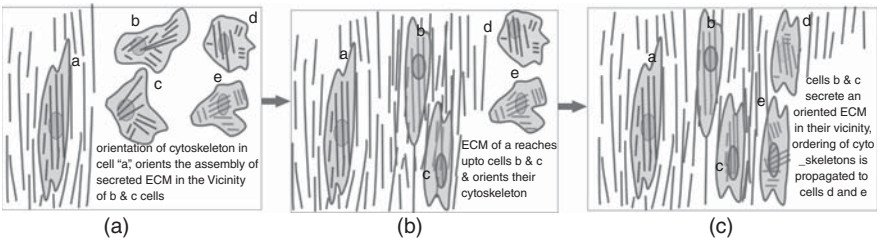

Figure 2.16. Orientation of cytoskeleton in cells and of the assembly of secreted ECM in the vicinity. (Adapted from [113].)

- Control of cell growth.
- Determination of the orientation of cells.
- Preservation of cell differentiation.
- Provision of scaffold to renewal tissues.
- Mechanical support for cell ANCHORAGE.
- Organization of the microenvironment of tissues.
- Storage and arrangement of soluble monitoring molecules.

Therefore, ECM is considered as a main controller of tissue and cell behavior [111, 112]. This key regulating nature comes from correlative studies made during tissue development, differentiation, growth factor and cytokine. This influence, when there are changes, occurs in the composition and distribution of ECM. Secreted ECM orients the cytoskeleton of the cells by preferentially organizing them in its vicinity. Figure 2.16 shows schematically the role of ECM as a key regulator of cell behavior [113].

ECM molecules such collagen, elastin, fibronectin and so on are bioactive in nature and play a significant role in mechanotransduction. These giant molecules mediate interactions of cytoskeleton with coupled integrins and are carried out at cellular level such as cell communication. In this way, ECM acts as a main regulator of cell fate process. [114, 115]. The first section of this chapter described the process of biomineralization, wherein after the ECM deposition, mineral phase is deposited on the biomaterial from the surrounding environment, which is described in the following section.

2.6 BIOMINERALIZATION

Biomineralization process describes the formation of a frame and an interface with the help of a variety of organic giant macromolecules and inorganic mineral phases and also provides their structural understanding. There are several structural morphologies of mineralized tissues, which are reported on the basis of the different mineral structure and giant organic molecules as shown in Figs. 2.17 and 2.18 [116, 117]. In general, biomineralization is defined as "the process by which living organisms secrete inorganic minerals in an organized manner with exceptional physical properties, by virtue of finely controlled microstructure, morphology and hierarchical organization of the minerals and accompanying organic material" [116–118].

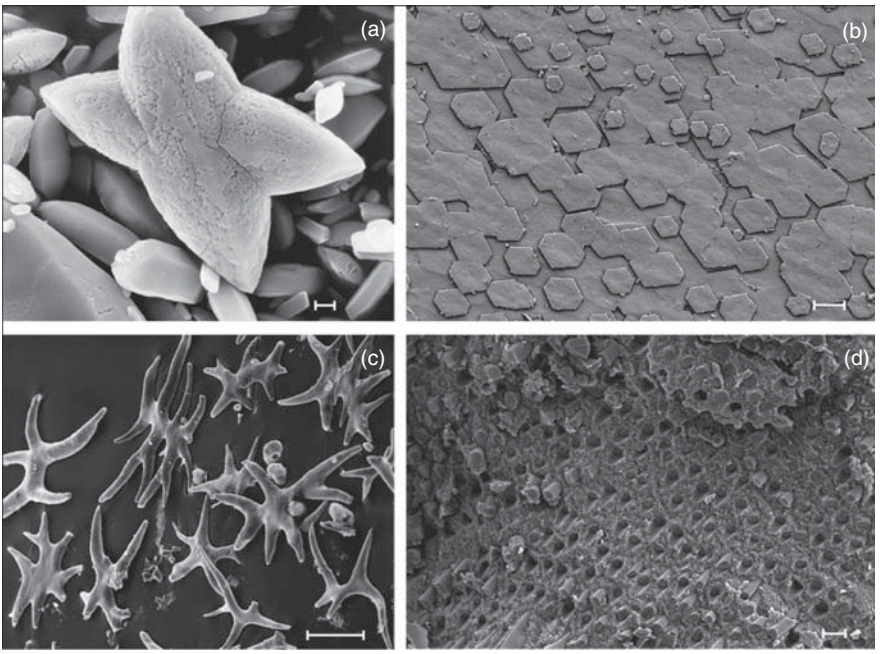

Figure 2.17. Electron micrographs of mineralized tissues (a) statoconia from the bullfrog, scale bar: 1 μm. (b) Aragontic nacre, scale bar: 5 μm. (c) Antler-shaped spicules from the ascidian Pyura sacciformis composed of carbonated apatite, scale bar: 100 μm. (d) Fracture surface of the working stone part of the sea urchin tooth, scale bar: 2 μm. (Reprinted with permission from Elsevier, Ref. [132].)

Biomineralization involves two ways: initially the development of mineral phase ions, which mediate nucleation and deposition of minerals, and furthermore, these mineral phases controlled by living system homogeneously throughout the surrounding [119]. Biomineralization creates heterogeneous accumulations and composites, which are formed by organic/inorganic components with heterogeneous distributions [118].

Living organisms can build and design natural biomaterials themselves, such as bone and teeth [120]. These materials are extremely specific with respect to their functions that leads to motivate chemists, physicists and particularly materialists to study the process of biocomposites formation, microstructure and specific properties [121]. A lot of studies have been performed, and the strategies were developed to build and control the properties of biomaterials similarly to the natural one known as *biomimicking*. These strategies are applied to tune the implant materials for biomedical applications through various bioinspired methods by templating of molecules and surface organizations [116, 122].

Usually, all groups of organisms have the ability to form inorganic minerals with complex form via biological processes, including prokaryotes (e.g., magnetite nanocrystals, which are formed in specific bacteria) and also humans (e.g., hard tissues such as bone). The unique examples of naturally occurring biomineralization found in habitats are “diatoms with structured cell walls” and “eukaryotic algae with single cell wall.”

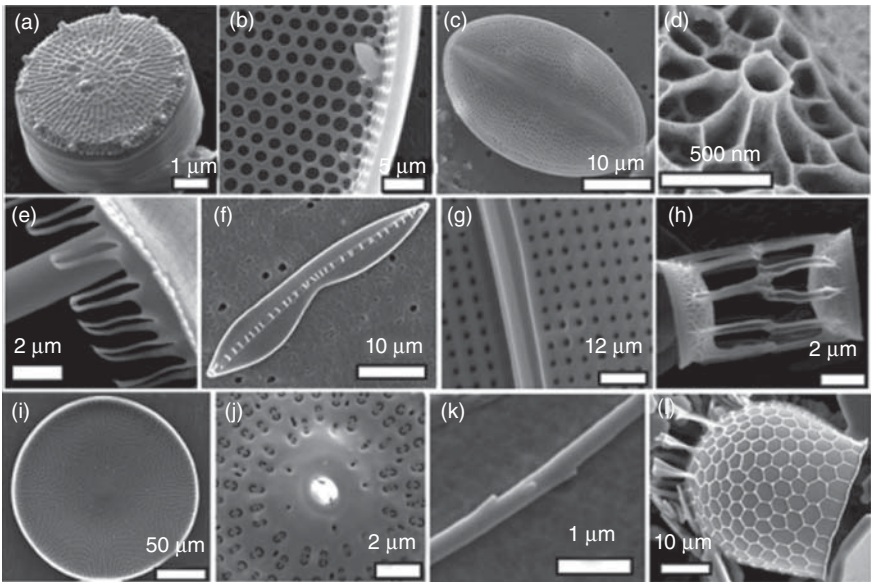

Figure 2.18. Structures of diatom silica biominerals (a) *Thalassiosira pseudonana* (b) close up of *Coscinodiscus wailesii* (c) *Cocconeis* sp. (d) rimoportula from *Thalassiosira weissflogii* (e) corona structure of *Ditylum brightwellii* (f) *Bacilaria paxillifer* (g) close up of pores in *Gyrosigma balticum* (h) *Skeletonema costatum* (i) valve of *C. wailesii* (j) close up of pores in *D. Brightwellii* (k) seta of *Chaetoceros gracilis* and (l) *Stephanopyxis turris*. (Reprinted with permission from ACS, Ref. [123].)

These diatom cells are specifically made up by SiO_2 and are specific in their structural arrangement with patterned pores as shown in Fig. 2.18.

The survival of an organism depends on the abilities to design and construct these biominerals, that is inorganic materials of significant importance, which mostly depends on their shape, size, atomic structure as well as defects and also fabricates hierarchical structural functioning devices [125, 126].

2.6.1 Inorganic Structure of Life

The sudden proliferation in the number and type of shells and micro-skeletons, made up of minerals such as calcium carbonate, calcium phosphate or silica, over half a billion years ago has had far-reaching biological implications on the global scale [116]. The evolution of biomineralization has provided organisms with strong and tough building materials. A tough skeleton can be made solely from an organic biopolymer. The insect cuticle is an example, which consists of a polysaccharide called *a chitin*. The inorganic minerals are hard, brittle and tough, while organic moieties are comparatively soft in nature. The combination of both materials produces inorganic–organic hybrid materials or biocomposites with well-defined mechanical properties. With structural support and mechanical strength, biomineralization has also mediated some other functions such as

motion, protection, gravity sensing as well as optical. The major aim to study biomineralization in the context of bioinorganic chemistry includes the following:

- The structural and compositional characterization.
- Understanding the functional properties of biomaterials.
- Elucidation of the processes through which organic and organization of inorganic minerals-based materials takes place.

There is a variety of biominerals formed and reported; some are listed in the following section.

2.6.2 The Major Groups of Biominerals

According to several studies, scientists report calcium as the main constituent of biominerals as shown in Table 2.1. The calcium-based biominerals cover about 50% of all available minerals [116, 118, 120, 127–129]. There are some minerals given in Table 2.1, those basically formed by controlled as well as induced mineralization methods.

2.6.3 Types of Biomineralization

The biomineralization processes are mainly grouped in two categories on the basis of their biological control:

1. Biologically induced mineralization
2. Organic matrix-mediated mineralization (also known as *biologically controlled mineralization*).

The basic outlines of the types of biomineralization are summarized as follows:

2.6.3.1 Biologically Induced Mineralization. With the interactions between the surrounding environment and metabolism (a process by which energy is produced by nutrients), minerals are precipitated and deposited on the material surfaces and categorized as biological-induced minerals. $CaCO_3$ precipitation in types of green algae is one such example [130, 131].

$$Ca^{2+} + 2HCO_3^- \leftrightarrow CaCO_3 + CO_2 + H_2O$$

In this situation, cell surfaces act as main producing agents for nucleation and growth of new biominerals at the material surface. The metabolism as well as creation of energy into the environment of the body fluid with acid–base redox surroundings specially refers to pH, pCO_2 and formed products [127]. The schematic diagram of biologically induced mineralization is presented in Fig. 2.19.

2.6.3.2 Biologically Controlled Mineralization. In this biomineralization, the nucleation, growth and morphology of deposited minerals are controlled by cellular activities of living system, and the extent of control varies from one species to

TABLE 2.1. A Few Ca-, Mg-, Fe-, Mn-, and P-Based Biominerals

Name	Formula
Carbonates	
Calcite	$CaCO_3$
Vaterite	$CaCO_3$
Monohydrocalcite	$CaCO_3 \cdot H_2O$
Protodolomite	$CaMg(CO_3)_2$
Amorphous calcium carbonate	$CaCO_3 \cdot H_2O$ or $CaCO_3$
Phosphates	
Octacalcium phosphate	$Ca_8H_2(PO_4)_6$
Carbonated-hydroxylapatite (dahllite)	$Ca_5(PO_4,CO_3)_3(OH)$
Whitlockite	$Ca_{18}H_2(Mg,Fe)_2^{+2}(PO4)_{14}$
Struvite	$Mg(NH_4)(PO_4) \cdot 6H_2O$
Vivianite	$Fe_3^{+2}(PO_4)_2 \cdot 8H_2O$
Amorphous calcium pyrophosphate	$Ca_2P_2O_7 \cdot 2H_2O$
Sulfates	
Gypsum	$CaSO_4 \cdot 2H_2O$
Barite	$BaSO_4$
Celestite	$SrSO_4$
Jarosite	$KFe_3^{+3}(SO_4)_2(OH)_6$
Oxides	
Magnetite	Fe_3O_4
Amorphous ilmenite	$Fe^{+2}TiO_3$
Amorphous iron oxide	Fe_2O_3
Amorphous manganese oxide	Mn_3O_4
Hydroxides and hydrous oxides	
Goethite	α-FeOOH
Ferrihydrite	$5Fe_2O_3 \cdot 9H_2$
Todorokite	$(Mn^{+2}CaMg)Mn_3^{+4}O_7 \cdot H_2O$
Birnessite	$Na_4Mn_{14}O_{27} \cdot 9H_2O$
Organic crystals	
Whewellite	$CaC_2O_4 \cdot H_2O$
Glushinskite	$MgC_2O_4 \cdot 4H_2O$
Manganese oxalate (unnamed)	$Mn_2C_2O_4 \cdot 2H_2O$

Source: Courtesy of Mineralogical Society of America, Ref. [127].

another [116]. Usually, minerals, for example, hard tissues such as bone and teeth, are produced in isolated ambient condition. This may occur extra-, inter- or intra-cellularly. These dictate to the site of mineralization corresponds to specific cells. In some specific conditions, this biomineralization initiates at intracellular and later proceed at extracellular [127]. The biomineralization is directed and occurs at particular positions such as at

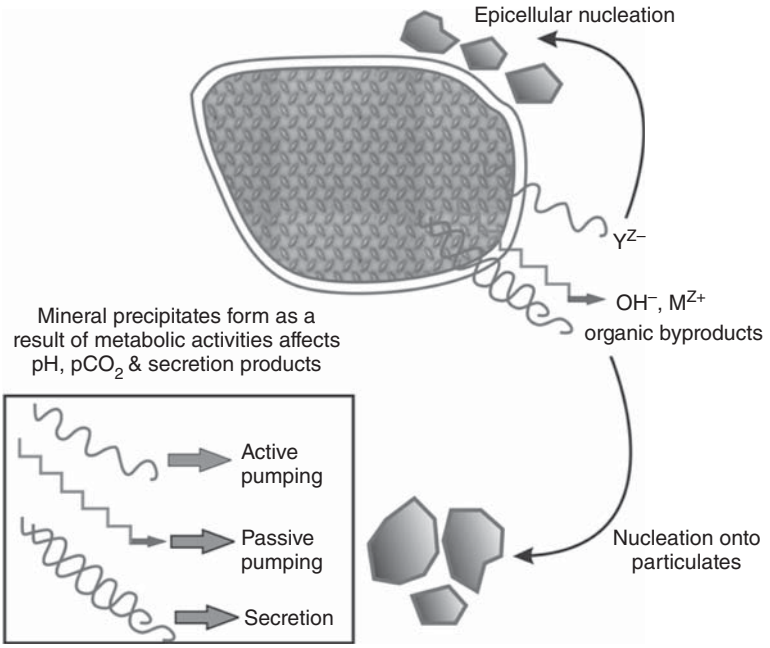

Figure 2.19. Schematic representation of mineralization induced biologically showing different biomineralization sites. (Adapted from [127].)

the walls of cells (epicellular), within the cells (intracellular), at the interface of the cell (intercellular) and at the outside of the cell (extracellular). Biomineralization categories are explained in the following sections.

2.6.3.2.1 Extracellular Mineralization. In this type of mineralization, the cell secretes a variety of macromolecules extacellularly, which act as a source of mineralization. These are composed of polysaccharides with proteins in the form of three-dimensional (3-D) structural arrangement. The structures and compositions of these proteins are citied in order to organize and regulate the functions of secreted biominerals [127, 132]. This may produce at three sites such as at the outer of the cell wall, within the cell wall and at the adjacent surrounding of the tissue. [133]. Produced element can be transport via cell in two ways as follows [134]:

A. Initially, membrane pumped to cations into the adjacent area, which formed a supersaturated fluid [134] that adjusted at significant distance from matrix as shown in Fig. 2.20.
B. Finally, cations may be aggregated at loaded vesicles followed by exported via membrane, mediated and broken by intermediate precursors present at the matrix surface as shown in Fig. 2.21.

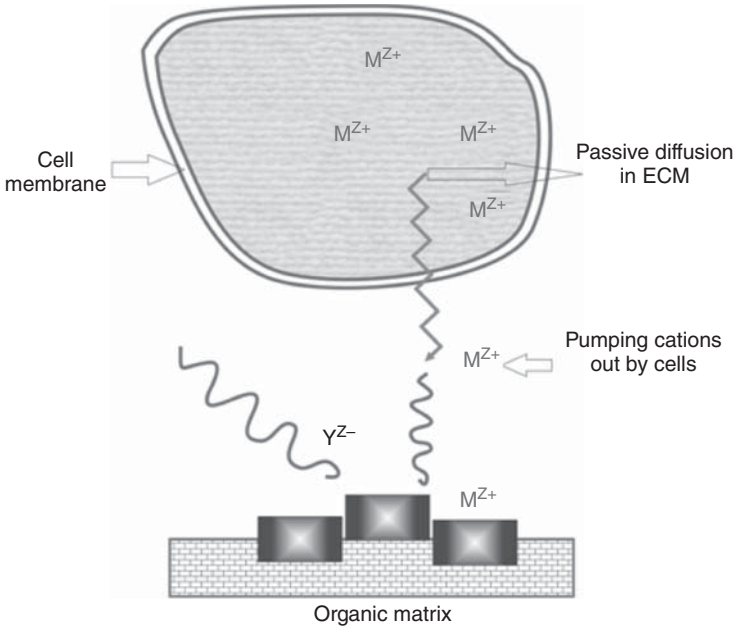

Figure 2.20. Schematic representation of pumping of cations through membrane and their diffusion. (Adapted from [122].)

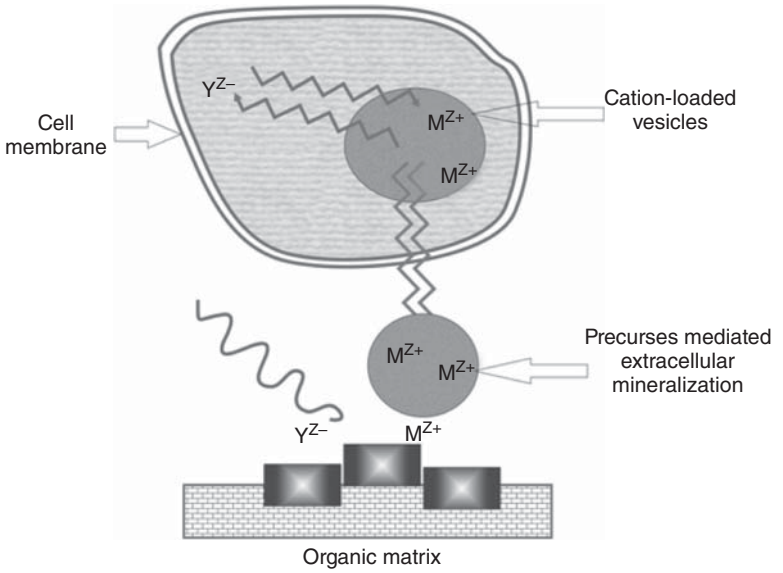

Figure 2.21. Schematic representation of aggregated cations and their final secretion at matrix surface. (Adapted from [122].)

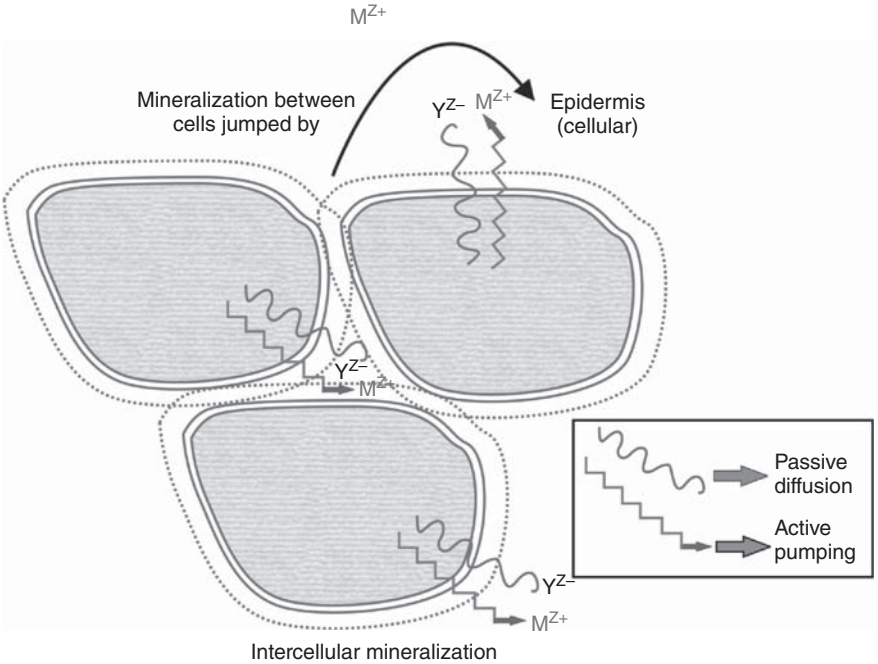

Figure 2.22. Schematic representations of intercellular mineralization. The epithelial cell surfaces direct to the nucleation and growth mechanism of produced minerals with respect to specific orientation. (Adapted from [127].)

2.6.3.2.2 Intercellular Mineralization. Intercellular mineralization occurs in single-celled organisms. Initially, it appears to be extracellular in type [127], and epidermis acts as a separate source of mineral formation, as schematically shown in Fig. 2.22. The epithelial element directs to the formation of mineral phases by control of nucleation and growth mechanism. This mineral formation is found to be specific in nature; for example, calcareous algae produce calcite crystals, which have orientation of *c*-axis perpendicular to the surface of the cell [135, 136].

2.6.3.2.3 Intracellular Mineralization. This kind of mineralization may be produced and controlled by vesicles inside the cells. The composition and morphology of produced minerals are mediated by discrete crystallization surroundings [127, 137]. In this case, the organic moiety acts as a precursor to produce concentration-controlled biominerals. The concentration of trace elements (Si, Mn, Fe, etc.) as well as pCO_2 and pH is controlled by compartment membrane. The schematic in Fig. 2.23 shows the labeled "intracellular mineralization" [127].

There are two ways to transfer the biominerals from membranes: first is the migration of vesicles and exocytosis of mature biominerals, whereas the second one is a fusion of compartment membrane with plasma membrane, which leads to the exposure of premature biominerals.

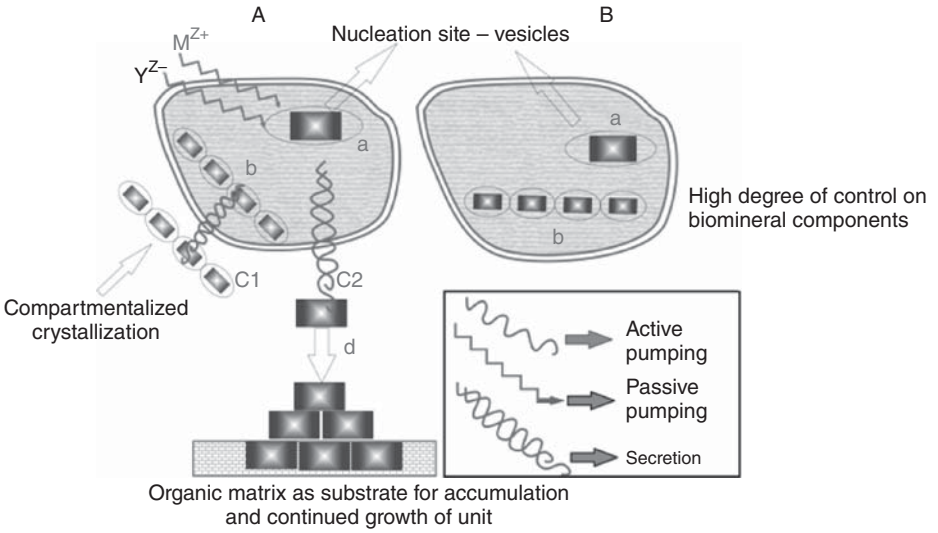

Figure 2.23. Schematic diagram of intracellular mineralization, which shows the compartmentalized crystallization, site of nucleation (vesicles A and B). (Adapted from [127].)

This type of mineralization is found in magnetosome-producing bacteria. These are especially magnetite and gregite euhedral crystal structure and found in the form of chains in the presence of magnetic field [130, 138]. In Fig. 2.23, (A) shows the nucleation of mineral, whereas (a) and (b) represent an intracellular and intracellular surroundings, respectively, similarly C1 and C2 stand for assembled and individual secretions, respectively, and (B) shows the units of biominerals into the cell, whereas (a) and (b) represent a single growth unit and high order of intracellularly organized growth unit, respectively.

2.6.4 Biomineral Types and Functions

About 25 essential mineral elements are necessary for the living system: H, C, O, and N are the major, Mg, P, K, Na, and Ca are the medium and Si, Mn, and Fe are the trace elements of over 60 different biological minerals. Calcium is a special element due to its common constituents of familiar skeletal structure such as bone and shells [133]. Bones, a natural biocomposites, are composed of calcium phosphate and organic collagens fibrils with body fluid where shells are built from calcium carbonate. Some examples of biomineralization are given as follows.

2.6.4.1 The Apatite. The formation of natural apatite minerals on biomaterials surrounding in the body fluid is an essential condition for biomaterial to bind with the host bone. Studies show that there are 12 steps taking place on the cell–materials interface as given in Table 2.2 [139]. Calcium phosphate is a major proportion of bone, which has close similarity to that of naturally occurring apatite due to Ca/P ratio of 1.67 [140]. In order to form the mineral apatite, all essential elements should be present in their appropriate compositions. These elements are Ca^{+2}, P^{+3}, O^{2-}, Cl^{-}, F^{-}, and OH^{-} ions.

TABLE 2.2. Steps Involved in Cell–Material Interactions at the Interface

Stage	Log time (h)	Surface reaction
1	1	Formation of Si-OH bonds and release of $Si(OH)_4$ bioactive glass
2		
3		Polycondensation of SiOH + SiOH = Si-O-Si
4		Adsorption of amorphous Ca + PO_4 + CO_3
5	2	Crystallization of hydroxyl carbonate apatite (HCA)
6	10	Adsorption of biological moieties in HCA layer
7	20	Action of macrophages
8		Attachment of osteoblast stem cells
9	100	Differentiation of stem cells
10		Generation of matrix
11		Crystallization of matrix
12		Proliferation of bone

Source: Courtesy of JWS, Ref. [139].

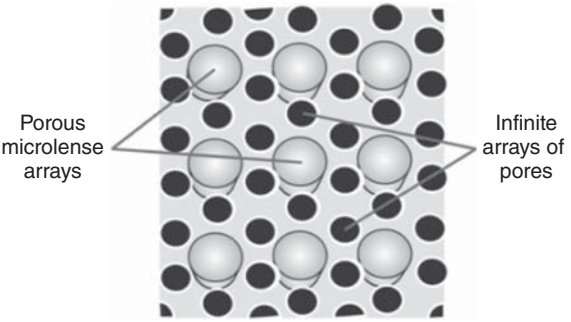

Figure 2.24. Schematic representation of micro-lens with précised array. (Adapted from [141].)

2.6.4.2 Lenses. It is the necessity of microlens arrays, for a wide range of applications, that they should be porous, light weight as well as adjustable in nature with respect to their symmetry, shape and size, which are usually regulated by polarization and wave vectors of beam. The other parameters such as exposure time, intensity and concentration of laser are the main factors to control the pore size. [141]. Calcite is also used as lens in the compound eyes of creatures called *trilobites*. These eyes consist of hexagonally packed arrays of calcite single crystals shown in Fig. 2.24. Single calcite crystal is well known for its ability to doubly refract white light [133].

2.6.4.3 Calcium Carbonate Vaterite and Amorphous Phases. Most of the calcium carbonate in biological system have the structure of calcite or aragonite and varterite, which is least thermodynamically stable. Inner ears of fish contain mainly varterite minerals, whereas the amorphous form of calcium carbonate, which acts as the

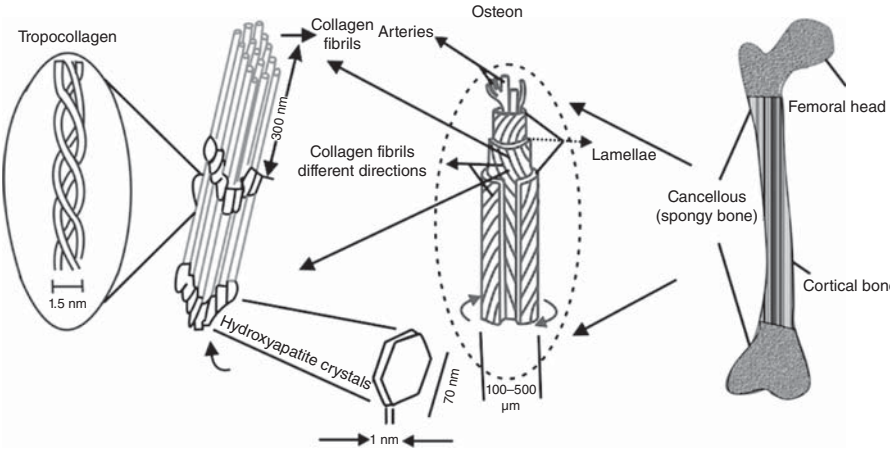

Figure 2.25. Multi-length scale hierarchy in natural bone.

main storage source of calcium, is also present in the leaves of plants in the form of a spindle shape. This is unstable in aqueous environment because of rapid phase transformation [142], while biominerals are stabilized by the adsorption of polysaccharides.

2.6.4.4 Calcium Phosphate. This is the main constituent of bones and teeth, which is found as minerals, in the form of naturally occurring hydroxyapatite (HAP), associated with giant macromolecules, such as a variety of proteins. The chemistry of biological HAP is quite intricate due to the non-stoichiometric character [133]. The simplest composition with essential minerals such as – calcium, magnesium, and phosphate-ion-based carbonated HA, is Ca_{10} $(PO_4, CO_3)_6$ $(OH)_2$ (termed as *dahllite*), most abundant in mammals' bones and teeth [143].

2.6.4.4.1 Bone. Bone is a best example of natural biocomposite with hierarchical structure. The mineralized fibrils with other minerals are main constituents as stated earlier, and the collagen matrix has extremely different mechanical properties [144]. The major components of bone are made from HAP, collagen fibrils with water. In a particular bone, the fractions of crystals and collagens were found to be approximately 70% and 30%, respectively, with other protein molecules in body fluid. The schematic presentation of human natural bone is given in Fig. 2.25. The major part is mineralized collagen fibrils. Four organizations of bones are possible: lamellar bone, fibered bone, bulk dentin and woven type. In reptiles and fish, circumferential lamellar bone is possible. The osteonal bone is of much biomedical significance and most abundant in humans [145].

The bone tissue formation and mineral nucleation carried out by ECM, collagen in the matrix, act as templates for mineral crystals formation [146]. Bone has different shapes and sizes depending on the anatomical locations to survive, protect and give the structural support to the body parts in all possible functions without any negotiation [147]. Bone is a living part of the body. During pregnancy (internal) and under

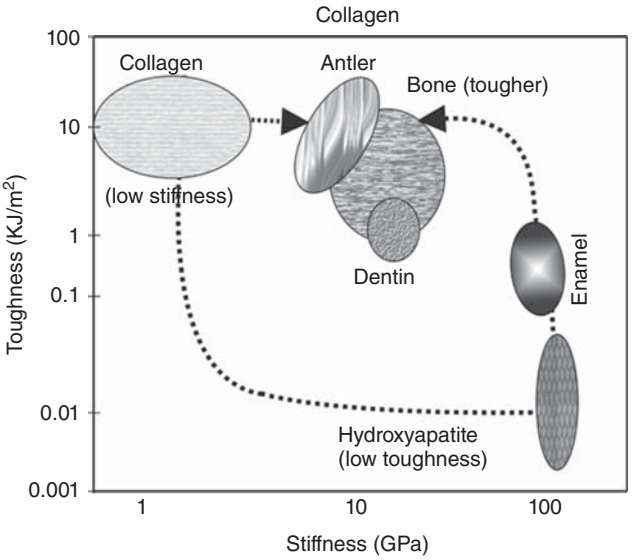

Figure 2.26. Typical values of stiffness and toughness (fracture energy) for tissues mineralized with hydroxyapatite. (Adapted from [144].)

mechanical loading (external stress), bone acts as a living organ by itself and undergoes remodeling, dissolution as well as continuous growth [133]. Bone properties mainly depend on the compositions of HAP and collagen fibrils such as glycoproteins in the matrix. The hybrid components consist of organic and inorganic moieties and possess higher toughness compared to only HAP based as shown in Fig. 2.26. It was found that the fast-moving animals have low mineral and high fiber content, such as deer, and vice versa, for example, whales, which have a high proportion of HAP.

2.6.4.4.2 Teeth. The main parts of tooth are pliant materials, dentin and enamel. The basic block of dentin is mineralized collagen fibril and apatite crystals. These are different types as peritubular and intertubular dentin and enamel [145]. The structure and organization of human teeth with enamel, canine and bone are shown in Fig. 2.27.

Teeth, similarly to bones, are designed and derived to withstand specific mechanical stress. Enamel part is the hardest and most highly mineralized substance in the human body, which contains approximately 95% by weight of HAP crystals and due to specific interweaving long ribbon-like structure is able to sustain stress resistances [143]. It is also possible that with time, well-fledged biominerals may produce highly biomineralized volume fraction of the erupted teeth by successive removal of soft proteins such as enamelin and amelogein, respectively [133]. Dentine contains collagen and is similar to bone. Fluoride ions play an important role in dental health by incorporation into HAP lattice to stabilize it and enhance the stability as well as suppress the degree of solubility of minerals segments. The teeth of fish contain a high level of natural fluoride compared to men, and that is why shark enamel has approximately 1000 times more solubility protection than the human enamel.

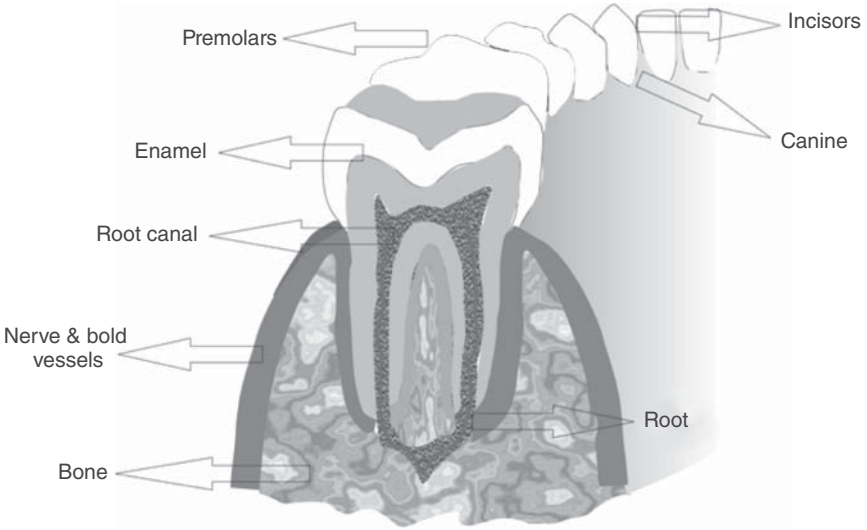

Figure 2.27. The human teeth with outer enamel and bone. (*Courtesy:* http://www.webmd.com/oral-health/picture-of-the-teeth.)

In summary, the interdisciplinary field of "tissue engineering" is continuing to contribute toward the mankind with the success of developing biological substitutes. The synthesized scaffolds follow a methodology before their implantation, which is as follows:

Cell recruitment (the isolation of cells and their expansion for the *in vitro* tissue culture studies of the scaffolds),

Biomaterial interaction – cells obtained from the previous step are cultured on the scaffolds following the steps of:

- Protein and Cell Adhesion – attachment of the proteins with the material and in turn with the cells.
- Cell migration – movement of the cell on the host implant surface.
- Controlled cell proliferation – importance of implant surface (hydrophobicity, surface chemistry and charge), which can control the cell interaction with the surface.

Implantation – on implantation, again the same process of cell adhesion, proliferation and further the formation of the ECM on the material with the final stage of biomineralization.

Cells in connecting tissues are embedded with ECM to give support and bind to cells as well as regulate their shape and behavior. ECM contains different types of proteins, which influence cell spreading proliferation and attachments. It may be concluded that ECM is a fundamental component of all cells and tissues, which performs a variety of functions such as control of cell growth to regulate orientation, cell anchoring, differentiation, and so on. Biomineralization is a process by which the living organism produces a

variety of essential minerals, which are necessary to harden the tissues. There are mainly two types of biomineralizations: (i) biologically induced and (ii) biologically controlled, by which all essential elements are produces into living organisms, for example, apatite (Ca & P) formation in bone and teeth.

QUESTIONS

1. What is tissue engineering? Describe its role in the betterment of mankind.
2. What is "Vroman" effect?
3. Elaborate on the topic "Integrin as an adhesion protein."
4. What are the "focal adhesions"? Explain with examples of the proteins involved in it.
5. How does cadherin help in cell adhesion?
6. How does the biomaterial surface play a critical role in determining "Biomaterial–Tissue Interaction"? Describe the various factors involved.
7. What is ECM? Explain its various functions?
8. What are different types of proteins in ECM and their role?
9. Define the term *biomineralization* and its significance.
10. Explain the various types of biomineralization with neat diagrams.
11. What are different minerals induced by biomineralization? Give any ten examples.
12. Explain how ECM regulates cell and tissue behavior. Support it with a diagram.
13. How biomineralization is important in formation of hard tissues in human life?
14. What are the different parts of teeth. Explain with sketch.

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