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Biomedical Applications of Natural Proteins

An Emerging Era in Biomedical Sciences

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Dedicated to Bharat Ratna
Dr. Bhimrao Ambedkar ji
and Dr. A.P.J. Abdul Kalam ji

Foreword

The advent of next-generation sequencing (NGS) and high-throughput screening (HTS) technologies have revolutionized the approach of biomedical research in understanding human disease conditions. Treatment and cure of diseases involves in-depth investigations into the structure and functional complexity of biological macromolecules, the chief amongst which undoubtedly, are the proteins. The nascent field of Proteomics employs the tools of NGS and HTS (protein microarrays) to understand an estimated 2 million proteins encoded by approximately 25000 genes in the human body which are critically important for carrying out all the relevant metabolic and physiological functions. Although many standard text-books dealing with the fundamentals of protein structure and function are available in the market, Dr. Dhiraj Kumar and Dr. Rajesh Kundapur has diligently put together a volume which explores the biomedically important proteins from a functional viewpoint. I strongly believe that this book will be an excellent companion for both students as well as researchers and will inspire many to take up research related to the topics covered in this book.

November 2014

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Preface

Proteins are the most abundant and functionally diverse molecules in living organisms. Virtually every life process depends on this class of molecules. Proteins like enzymes and hormones direct and regulate metabolism in the body, whereas movement of muscles are permitted by contractile proteins. Collagen protein forms a framework for the deposition of calcium phosphate in bones whereas, heat shock proteins protect cell from different environmental stresses. In the blood stream, proteins such as hemoglobin and albumin, shuttle molecules essential to life, and immunoglobulins fight against infectious bacteria and viruses. In short, it is hard to believe life without proteins. Animal products like milk and silk are sources of economy for humans so, if one organism lacks a protein which is necessary for its growth and development, in order to rescue that organism, a protein from other organism isolated and purified as a whole or expressed in bacterial, yeast system or mammalian system and administered. The Human Genome Project and the different proteomic studies which followed have roughly estimated the total number of proteins present in human body to be at least 2 million.

Biomedical applications of natural proteins: an emerging era in Biomedical sciences provides the reader with an overview that the basic information of protein science from purification up to homogeneity to in the form of life saving drugs. Silk is an ideal biomaterial that can provide functional insights into relationships between molecular biology and polymer chemistry. Silk proteins are obtained from silk during the processing chain of textile production. The important characteristics of silk, particularly its non-toxicity, biodegradability, high thermal stability, minimal inflammatory reaction, excellent water vapour permeability and biocompatibility, suggest that silk can be used as a biomaterial in medical and therapeutic applications. It is widely believed that silk is the only part which is economically important, but recent studies showed that there are number of proteins found in the silkworm which can be successfully used as therapeutic agents. Heat shock proteins (HSPs) a group of proteins usually expressed against different environmental conditions in order to protect the cell from the environmental onslaughts. These HSPs can also be employed as diagnostic or therapeutic targets for different diseases. There are different bioactive proteins with versatile functions.

Interleukins (ILs) are proteins which use variety of signalling pathways to facilitate cellular adaptive mechanisms. Interleukins belong to the class of cytokines produced by variety of tissues and blood cells and plays crucial role in different diseases. The present book discusses Interleukins in general and IL-8 in particular. The protein intake as a dietary source was very well known fact which is very much necessary for the growth and development of the body but excess intake causes health havocs. Synthesis and biomedical applications of self-assembled nanoparticles of silk protein and nanocomposites of gelatin like examples are there which justifies that natural protein can be used for diverse biomedical applications. It is well known fact that infectious agents such as viruses, bacteria and prions, are responsible for spread of infection in humans. Proteins of these source mainly involved in causing a disease and as well as protecting the host from it. Various membrane proteins of microorganisms (virus, bacteria, parasites etc.), prions are responsible for causing different diseases. Proteins like heat shock proteins/chaperons aid in protection from diseases. Considering the drawbacks of synthetic biomaterials, the use of natural proteins as natural biomaterial and their possible application in biomedical science, could be of enormous value.

Biomedical applications of natural proteins: an emerging era in biomedical sciences volume will be of equal interest to pre-doctoral and post-doctoral researchers, as well as graduate and post graduate students studying and conducting research in the interdisciplinary fields of Natural Proteomics, Biomedical Sciences, Nanotechnology and Molecular Biology.

This book will be an excellent companion for both students as well as researchers and will inspire many to take up research related to the topics covered in this book.

Dhiraj Kumar
Rajesh R. Kundapur

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There are no words to convey our emotions and gratitude to our respected parents, Smt. Rita Devi, Shri Jiya Lal and Smt. Prabha R. Kundapur, Shri Raghu S. Kundapur, for their moral support, love, inspiration and blessing to make this dream come true and it was also the inspiration of two little angels of our families, who gave us additional energy to complete this task.

We offer our regards and blessings to all of those who supported us in any respect during the completion of our book.

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Dhiraj Kumar
Rajesh R. Kundapur

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About the Editors

Dr. Dhiraj Kumar obtained his Doctorate degree in Applied Animal Science from Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow, India. He is presently working as Assistant Professor in the Department of Zoology, Guru Ghasidas Vishwavidyalaya (A Central University), Bilaspur (C.G.), India. He was the recipient of the young scientist award for his research contribution in the area of Zoology and he qualified National level tests such as CSIR-UGC-JRF/NET and ICAR-NET. He has published several research papers in National and International journals of repute and filed a patent for his new innovation. Recently he has been offered for Postdoctoral research from esteemed universities of China and Spain. Currently, his research is focused in the area of biomedical science and insect molecular biology.

Dr. Rajesh R. Kundapur obtained his M.Sc. in Biochemistry from Karnatak University, Dharwad, Karnataka, India. To pursue his research career, he later joined National Centre for Cell Science, Pune. With a publication and some years of experience in molecular biology he later moved to Karnataka University for his Ph.D. Dr. Rajesh Kundapur has extensive work experience in the field of Protein Chemistry. He also has national and international publications to his credit. He has been awarded the prestigious Dr. D.S. Kothari Postdoctoral Fellowship and he is presently serving as research consultant at the National AIDS Research Institute, PUNE. Through his research work he has contributed to the field of Silkworm Biology.

Chapter 1
Protein-Based Fibers

Dhandapani Saravanan and T.V. Ayeshvaryaa

Abstract Textile fibers and fabrics are one of the most essential aspects of human life, which often undergo many changes in terms of the fibers and structures of the materials used. Needless to say, natural fibers have always been of interest to many due to the inherent advantages associated with such fibers. Protein fibers such as wool and silk give high levels of comfort due to the unique structure and aesthetic feel. The production of these fibers often fluctuates since it is dependent on agricultural activities. Notwithstanding the demand for protein fibers, regenerated fibers using natural proteins are produced as alternatives for wool and silk, with properties that feel closer to those fibers.

Keywords Exotic fibers · Organic wool · Protein · Regeneration · Silk · Wild silk · Wool

1.1 Introduction

Nature, always, has been a source of various raw materials to meet man's fiber and textile needs. No matter in which climatic zone humans have settled, they have been able to utilize the fibers of the native species to make clothing and buildings. The first fiber reinforced composite material was made with clay and straw to construct walls and houses in Egypt, 3000 years ago. Before the era of synthetic fibers, man relied on plant and animal fibers for clothing, ornaments, and accessories. Life, as it has been realized, cannot exist without polymers. Proteins, with

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large numbers of amino acids combined together with amide linkages, perform a variety of vital roles in plants and animals. Proteins are the bases for wool, silk, and other animal-derived filaments and fibers. Carbohydrates, with repeating units derived from simple sugars, are among the most abundantly available compounds in plants in the form of fibers, including cotton, linen, and other vegetable fibers. Nevertheless, both carbohydrates and proteins are important fiber forming natural polymers.

The properties of natural fibers are limited by the form in which they are available in their natural state and offer less scope for modifications. Fibers such as linen and silk are difficult to isolate from their sources, which make them scarce and expensive. For more than a century, researchers across the world have been investigating the complex structures found in living organisms, with a major focus on the nitrogen-containing substances of high molecular weight known as proteins. Proteins are essential macromolecules in all biological systems and have well-defined three-dimensional structures and play key roles in all metabolic processes. Protein molecules exist in wide ranges, from small to large, highly reactive to inert, low strength to high strength, flexible to rigid arrangement, hydrophilic, lipophilic, etc.

Recently, there have been enormous amounts of research activities on regenerating natural proteins, derived from various sources, into fiber form on account of their unique physical and chemical characteristics. The key research activities include genetic controls and mimicking the preserved sequences of amino acids and the unique properties. In the domestication front, attempts have been made to preserve the original properties of the fibers produced by insects and animals with sustainability measures and less impact onto the environment.

1.2 Market Share of Natural Fibers

The World Apparel Fibre Consumption Survey was undertaken recently by the Economic and Social Development Department of the Food and Agriculture Organization of the United Nations (EST/FAO) along with the International Cotton Advisory Committee (ICAC) to assess the consumption pattern of different textile fibers [1]. It has been observed that, in line with global economic progress, the total textile fiber production peaked in 2007 at 67.7 million tons, which reached another peak production during 2010 at 69.7 million tons of textile fibers after the brief downfalls during 2008 and 2009 (Fig. 1.1). Again, there has been an increase in fiber production in the year 2103 with a total of 85.4 million tons, with the major driving force witnessed by the demand for synthetic (noncellulosic) fiber groups (61.8 %), followed by man-made cellulosic (6.8 %) fibers [2].

Sericulture or silk production, though the quantity is less (<0.2 % share), had a long and secretive history protected by the Chinese tradition. At later stages, the Chinese lost their secret to the Koreans, Japanese, and Indians. According to FAO, the world raw silk production for 2010 was 164,971 tons. Approximately 98 % of

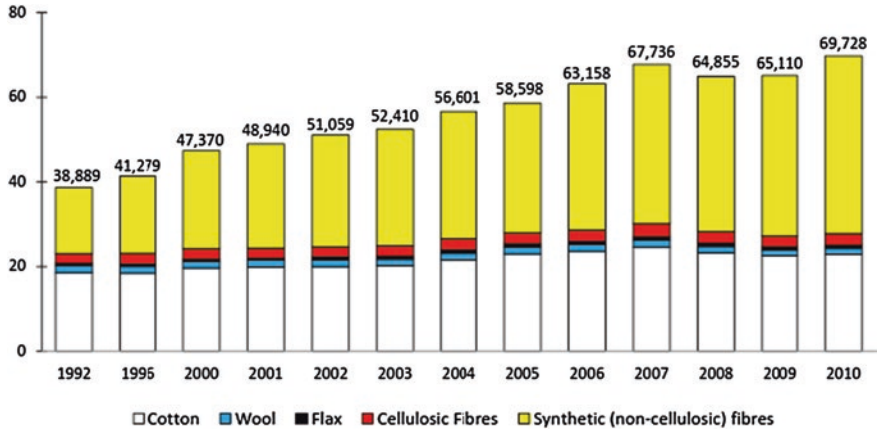

Fig. 1.1 Global consumption of apparel fibers (in 1,000,000 tons) [1]

Table 1.1 Major silk producing countries

Country	Silk production (Tons)
China	126,001
India	19,000
Vietnam	7367
Turkmenistan	4500
Thailand	1600
Brazil	1300
Uzbekistan	1200
Iran	900
Democratic People's Republic of Korea	350
Tajikistan	200
Indonesia	120
Japan	105

the world's production comes from the Asian countries, especially Eastern Asia; China is still the leader in raw silk production [3] followed by India (Table 1.1). Afghanistan, Kyrgyzstan, Turkey, Cambodia, Italy, Lebanon, Bulgaria, Greece, Egypt, Kenya, Botswana, Nigeria, Zambia, Zimbabwe, Bangladesh, Colombia, Nepal, Uganda, Malaysia, Romania, and Bolivia produce silk fibers in the range of 1–50 tons per year [4, 5]. The sericulture industry provides employment to more than 10 million people across the world and silk production has steadily grown during the past three decades. Among the four major types of natural silks available, mulberry silk is the most important and contributes as much as 90 % of total silk production; today, the term "silk" is synonymous with the domesticated mulberry silkworm silk obtained from *Bombyx mori*. Three other important types of

non-mulberry silks include Eri, Tasar, and Muga silks. Besides, many insects also produce silk filaments, which are insignificant in terms of quantity and commercial value.

When one talks about wool, it could well mean the fiber from sheep; however, the term "wool" can be applied to the hair of other mammals including cashmere and mohair from goats, camelids, and angora rabbits.

Sheep domestication started as early as 10,000 years ago and many laws were enforced by the United Kingdom to protect wool domestication and the industry sustenance during the eighteenth century. Today, wool production has assumed global industry status, with Australia, China, Argentina, the United States, and New Zealand serving as the major suppliers of raw wool—but wool is produced worldwide in about 100 countries from organized and unorganized farms.

Global wool production is approximately 1.3 million tons per year, of which 60 % goes into apparel production. Australia is the leading producer of wool (25 % of total clippings), mostly from merino sheep. China is the second largest producer (18 %) of wool, and the largest producer of crossbred wool, followed by United States (17 %) and New Zealand (11 %). Argentina (3 %), Turkey (2 %), Iran (2 %), UK (2 %), India (2 %), Sudan (2 %), and South Africa (1 %) are other minor wool producing countries in the world [6]. However, the wool fiber production, across the world, witnesses a marginal decrease every year for different reasons [7].

In many parts of the world, both wool and silk fabrics are considered to be premium garments and provide esteem value to the consumers of these fibers. The main factors promoting the demand include increase in disposable income, urbanization, increase in the nuclear family concept, easy availability of housing finance, growth of end-use sectors like housing, office space, hospitality, health care, restaurants. Figure 1.2 shows the classification of the different animal or luxury fibers that are widely used in apparel production [8].

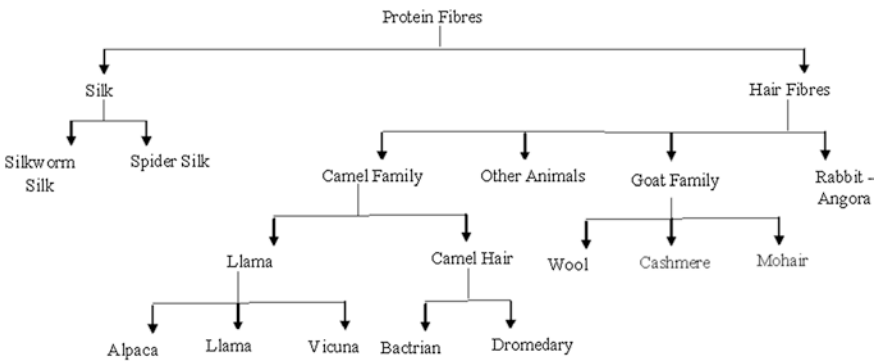

Fig. 1.2 Classification of protein fibers

1.3 Natural Silks

1.3.1 Silkworm Silk

Silk is produced by over 30,000 species of spiders (class *Arachnida*), by many insects particularly of the order *Lepidoptera,* and the most commercially exploited silk moths belong to the family either *Bombycidae* or *Saturniidae*. Silkworm silks are well understood due to their extensive use in the textile industry over the past 5000 years. The cultivation of silkworms for the purpose of producing silk is known as sericulture. Sericulture involves the agricultural practice of growing mulberry leaves, the primary food for the silkworm, raising silkworms for silk production, harvesting silks from the cocoons, and processing of the silk into useful textile materials [9]. Mulberry leaves are renewable and sustainable as the trees produce the leaves year after year; one mature mulberry tree will produce enough foliage for 100 silkworms [10]. Over the decades, silk production has undergone a drastic change, from the highly unorganized to integrated operations.

Silkworms are divided into different categories based on their lifecycle period, (a) univoltine breed (one generation per year), usually found in Europe, (b) bivoltine breed (two generations per year), found in Japan, China, and Korea, and (c) multivoltine breed (up to eight generations per year) usually found in the tropical zones [3]. Such differences occur mainly due to the difference in the climatic conditions that play a crucial role in the laying and hatching of eggs.

The life cycle of *B. mori* runs for 55–60 days, and the organism passes through a series of developmental phases, namely egg, larva, pupa, and moth. Silk production occurs during cocoon formation, around day 26 in the cycle, just before molt to the pupa. Silk is extruded from spinnerets and the filament is drawn into a complex architecture following the movement of the head, numerical figure *eight* (Fig. 1.3). Once, the spinning of the cocoon is complete, the moth emerges after a 10–15 day period of adult development in the cocoon [4].

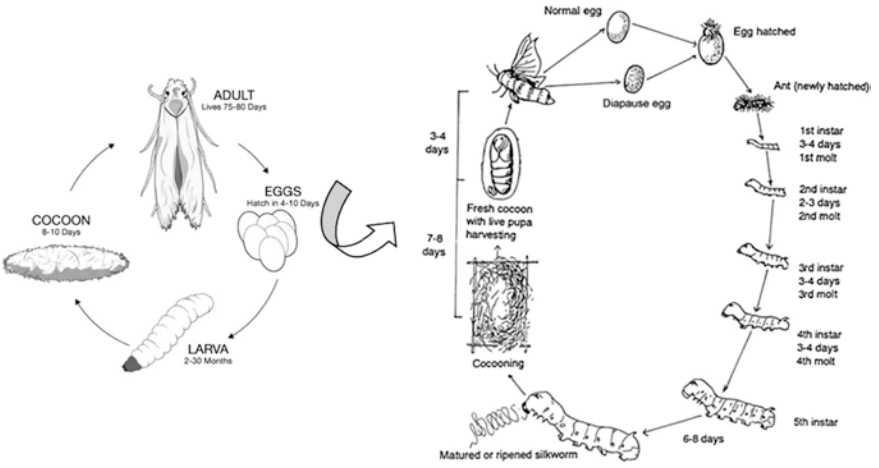

Fig. 1.3 Life cycle of silkworm (*Source* www.thewormlady.ca)

Silkworm cocoons are composed of continuous twin filaments of silk (fibroin), held together, intact, by an adhesive protein known as *sericin*. Sericin is white, greenish, or golden-yellow color depending on the breed of the silkworm. Sericin is a kind of globular protein with predominantly amorphous structure even though it is also subjected to similar stress levels as that of fibroin, while spinning. Sericin is rich in amino acids such as serine, glycine, and aspartic acid totaling to an extent of two-thirds. Sericin proteins are synthesized, secreted, and stored in the middle gland, form a sheath around the fibroin core during spinning of the silk filament with the main function of lowering the shear stress and absorbing the water released from the fibroin during fiber formation and spinning processes [11, 12]. Once the sericin is removed (degumming), the usable silk filaments can be reeled off the cocoon using different reeling methods [13].

Raw silks are tested and graded according to specified and accepted standards, evolved by the erstwhile International Silk Association based on denier (fineness) and other tests. The grades are expressed in the order of 4A, 3A, 2A, A, and B [14].

1.3.1.1 Sericin Fractions and Degumming

In the silk filament, fibroin chains are aligned along the fiber axis, held together by a close network of intermolecular hydrogen bonds and salt linkage/electrovalent bonds formed between the amino acids of adjacent chains. Compared to fibroin, sericin is richer in cystine, which varies between the protein present in the gland and cocoon and, among the species of silkworms. The sericin content of reeled filaments has been found to vary from the outer to inner layers of the cocoon [15]. The density of sericin is around 1.41 g/cc with a very low degree of crystallinity and moisture regain of, closer to, 12 %. In contrast to fibroin, more polar groups are available in sericin of which 60 % are hydroxyl groups, 30 % are acidic groups, and 10 % are alkaline residues, which contributes to the higher moisture regain values than fibroin [16–19].

Besides fibroin (70–80 %) and sericin (20–30 %), raw silk also contains natural impurities like wax (0.4–0.8 %), inorganic matters (0.7 %), carbohydrates (1.2–1.6 %) and pigments (0.2 %). Color/pigments in the domesticated silk appear prior to cocoon formation (yellow, green pigments) or, 1 or 2 days after cocoon formation (brown pigments), mainly decided by dietary conditions of the worms. The sericin content in the silk filament varies among the types of natural silks (Table 1.2) with the highest content existing in mulberry silk, produced by *B. mori* and the lowest in the case of eri silks [20].

Table 1.2 Sericin content of silk fibers

Type of silk	Sericin content (%)
Mulberry	20–30
Tasar	5–15
Muga	7–8
Eri	4–5

Three different fractions of sericin are obtained based on their relative solubility and histological staining, which reveal the existence of three distinct layers of sericin in the gland itself [19, 20]. Recently, four stratified fractions were obtained from sericin identified using differing rates of dissolution in hot water and also based on the UV absorption methods and classified as Sericin A (soluble at pH 4 but insoluble in 75 % alcohol), Sericin B (sparingly soluble in hot water, resists proteolysis), Sericin C (extremely insoluble in common solvents and is unaffected by enzymes), and Sericin D (small fraction, soluble in 75 % alcohol). Table 1.3 gives the composition of the various amino acids present in sericin component and its comparison with other protein fibers. Sericin of tasar silk is reported to contain at least nine kinds of proteins laid down in a wavy form rather than as structured layers. Wild silk fibers have noticeable properties such as high chemical and thermal resistance and high glass transition temperatures.

Fibroin is water insoluble, while sericin is soluble at elevated temperatures and swells in the presence of many chemicals [21]. Degumming is defined as a process involving "the removal of sericin from silk yarns or fabrics, or from silk waste prior to spinning at controlled conditions, intended to have little or no effect on the underlying fibroin" [22]. Addition of surface active agents like soap or nonionic detergents aids the removal of sericin and improves the wetting properties of degummed filaments. Removal of sericin in degumming is a combination of reactions

Table 1.3 Amino acid composition (mole %) of proteins

Amino acid	Sericin I	Sericin II	Sericin III	Sericin IV	Whole sericin	Fibroin	Spider silk
Glycine	13.21	12.81	15.69	11.89	13.9	43.7	37.1
Alanine	4.68	6.69	6.68	9.3	5.9	28.8	21.1
Valine	2.97	2.21	3.21	4.16	2.7	2.2	1.8
Leucine	0.86	0.96	1.27	6.26	1.1	0.5	3.8
Isoleucine	0.59	0.57	0.85	3.50	0.7	0.7	0.9
Serine	34.03	36.64	28.15	12.40	33.4	11.9	4.5
Theronine	10.34	8.48	11.36	7.25	9.7	0.9	1.7
Aspartic Acid	16.94	16.95	16.13	12.64	16.7	1.3	2.5
Glutamic Acid	4.73	3.64	4.09	11.32	4.4	1.0	9.2
Phenylanine	0.45	0.44	0.50	2.83	0.5	0.6	0.7
Tyrosine	2.53	2.43	3.15	2.45	2.6	5.1	–
Lysine	3.28	3.29	2.64	7.11	3.3	0.3	0.5
Histidine	1.25	1.22	1.49	1.87	1.3	0.2	0.5
Arginine	3.20	2.65	3.68	3.93	3.1	0.5	7.6
Proline	0.58	0.63	0.66	2.75	0.6	0.5	4.3
Tryptophan	0.19	0.20	0.25	0.23	0.2	0.3	2.9
Cystine	0.17	0.15	0.12	0.13	0.1	0.2	0.3
Methionine	0.04	0.04	0.04	0.12	0.04	0.1	0.4

Table 1.4 Methods of degumming of silk

Degumming method	Process description
Classical method	Treatment with Marseille soap followed by a boiling off and mild alkaline rinsing
Physical method	Treatment with water at 121 °C under pressure for 2 h
Enzyme method	Treatment with enzyme followed by boiling off with hot water under pressure and mild alkaline solutions
Acid method	Treatment with tartaric, succinic acid, dichloro, and trichloro acetic acids for 30 min at near boiling temperatures
Continuous process	Treatment with powder products containing sodium compounds (phosphate and carbonate) and preferably with a soap additive. Treatment with liquid products containing additionally to sodium salts, anionic surfactants, and chelating agents

such as dispersion, solubilization, and hydrolysis of different sericin polypeptides [23]. Tasar silks are more difficult to degum than mulberry silks because of the presence of more mineral matters, chemically resistant nature, and, perhaps, also due to the complex nature of the proteins [24, 25]. Various methods of degumming are shown in Table 1.4.

Since all the sericin is not removed by physical or water extraction method, addition of nonionic synthetic detergents facilitates the removal of sericin while retaining the strength of the yarns [22, 26]. Treatments at high temperatures lead to a change in morphology in the case of mulberry silk, while it remains unchanged in wild silks.

Alkali formed on the hydrolysis of soap facilitates the degumming of silk fibers and is the widely used degumming process commercially [26, 27]. Neutralized soap solutions have practically no degumming action on silk and, traditionally, olive oil (Marseille's soap) soap is preferred due to its gentle way of degumming the silk.

Sericin is preferentially attacked by the dilute acids, specifically the peptide bonds adjacent to aspartic acid and glutamic acid residues, which vary significantly in the case of sericin and fibroin (Table 1.3). Various acids used for degumming have been categorized into three groups, namely chloroacetic acid, dibasic acid, and hydroxyl acid [28]. In terms of weight loss, all the acids are equally effective; considering weight loss and tenacity together succinic acid gives better results followed by tartaric acid and monochloroacetic acid.

Enzymes are used as alternatives for alkalis, acids, and soaps to preserve the physical properties, uniform removal of sericin, and to reduce the pollution levels [29]. The hydrophilic nature of sericin accounts for the relative ease with which it can be solubilized during degumming, digested by the proteolytic enzymes. Enzymatic hydrolysis of sericin can be achieved using trypsin, chymotrypsin, pepsin, papain, carboxypeptidase, degummases, and luecine amino peptidase. In the case of highly twisted yarns, proteases fail to remove sericin completely; however,

sericin removal can be enhanced by mechanical agitations and ultrasonication [29–32]. Combinations of lipase and protease have been suggested for degumming [12, 33] and to remove the waxy substances from raw silk, and cellulases have also been included in degumming to facilitate the removal of impurities and improve wettability [34].

Degumming imparts softness to silk and better resistance to wear in spite of loss in weight and volume. No changes are observed; physical properties between mulberry and tasar are in terms of bending length, flexural rigidity, and tenacity, while crease recovery decreases after degumming [12, 24, 27, 35–38]. Enzyme degumming results in separation of the twin filaments, evident from microscopic analysis [12]. The dull appearance and stiff handle of raw fabric disappears after degumming and silk becomes shiny, soft, and scroopy.

Many times, the entire silk sericin is not removed from raw silks, but only quantities sufficient to make the silk soft, lustrous, and workable in dyeing and bleaching are removed [28, 31, 37]. *Cuite* silk represents completely degummed silk, *souple* silk is defined as the silk in which sericin is removed to the extent of 10–15 and 2–5 % of sericin from raw silk is removed in the case of *ecru* silk. Partial fixation or complete fixation of sericin using cross-linking agents like cyanuric chloride, triglycidyl isocyanurate, or hexamethylene diisocynate, *N*, *N*-dihydroxy ethylene bisacrylamide, glutaraldehyde [11, 30], prior to degumming, improves the breaking strength of silk. Sericin fixation takes place due to reaction between amino groups in the proteins and cross-linking agent, which result in poor luster and low degree of whiteness and sometimes even discoloration.

Weighting is a textile manufacturing practice and peculiar to silk manufacturing that involves the application of metallic salts to add physical weight to silk fabrics, which in turn increases the selling price of the fabrics. By means of weighting process, the manufacturer can increase the weight of silk fabrics by 3–4 times [34], using solutions rich in tannin, iron, or tin followed by washing.

1.3.1.2 Organic Silk

Silk, like other protein fibers coming from living beings, such as sheep and alpacas, can be easily produced based on organic guidelines as they begin to be approved. Many silk fibers are probably already being produced in an organic environment, especially those produced in smaller villages and rural environments. Nowadays, textile industries are developing programs that either use 100 % organically grown silk, or blend small percentages of organic silk with conventional silk in their products [39, 40].

1.3.1.3 Wild Silks

Silk is not only obtained from *B. mori* alone, there are many species of wild silk caterpillars (Table 1.5) that also produce silk cocoons used in the production of silk fabrics, sometimes called 'wild silks' or 'peace silks', since the caterpillars are allowed to live the life cycle completely, natural lives in the wild environment, without being sacrificed for textile production. Most wild silkworms are multivoltine, which means that they produce cocoons several times during the year rather than just once a year like univoltine.

These wild caterpillars spin silks that are different in texture and color from the domesticated *B. mori* and the wild silk cocoon strands are shorter because they come from cocoons that are already damaged by the moth, while emerging from the cocoon [4]. Wild silk caterpillars secrete a protein with a slightly different structure. Most of these silks are much darker in color than the domesticated silk, probably because of the difference in feed. Wild silks are harder to bleach, and do not take dyes well. They are generally uneven in texture, but when made into fabrics are often more durable than common silks. Wild silks are used principally in velvet, plush, and in heavy or rough cloths such as *pongees* and *shantung* [10].

Tasar Silk

Tasar (Tussah) is copperish, dark beige to light brown in color, coarser sturdier than *B. mori* silk but has good feel and appeal. As the silkworms do not grow in a controlled environment, the moth hatches from the cocoon, which in turn affects the filament length and lustrous nature. Tasar silk is produced by univoltine or bivoltine silkworms, *Antherae amylitta* which mainly thrive on the food plants *Asan* and *Arjun* [40].

India and China are the major producers of wild silks in the world. There are many varieties such as the Chinese Tasar *Antherea pernyi Guerin*, the Indian tasar silkworm *Antheraea mylitte Dury*, and the Japanese tasar silkworm *Antheraea*

Table 1.5 Types of silk fibers [3]

Common name	Scientific name	Country of origin
Mulberry silkworm	*Bombyx mori*	China
Tropical tasar silkworm	*Antheraea mylitta*	India
Oak tasar silkworm	*Antheraea proylei*	India
	Antheraea frithi	
	Antheraea compta	
	Antheraea pernyi	China
	Antheraea yamamai	Japan
Muga silkworm	*Antherea assama*	India
Eri silkworm	*Philosamia ricini*	India

yamamai Querin which is peculiar and produces the green silk filaments [4]. The Chinese and Japanese tasar worms feed on oak leaves and other allied species.

Oak Tasar is a finer variety of tasar produced by the silkworm, *Antheraea proyeli*, which feed on the natural food plants of oak abundantly found in the sub-Himalayan belt of India. China is the major producer of oak tasar in the world from *Antherae apernyi,* followed by India. Rearing of tropical tasar silkworms is carried out in the central and southern plateau of India, which are prone to humid and dense areas like Bihar, Madhya Pradesh, Orissa, West Bengal, Uttar Pradesh, and Maharashtra [14].

Eri Silk

Eri silk also known as Endi or Errandi, a semi-domesticated multivoltine silk, is the product of the domesticated silkworm, *Philosamia ricini* and *Antherea assamensis* that feeds mainly on leaves of castor and *Kessaru* plants. It tops in the non-mulberry silk production and the bulk of eri silk is produced in many parts of India like Assam, and in Bihar, West Bengal, Orissa, and Manipur [41, 42]. Eri culture is a household activity practiced mainly for protein rich pupae, a delicacy for tribals. This golden yellow color silk is a prerogative of India and the pride of Assam state. Eri silk has the look of wool mixed with cotton but the feel and softness of silk. Eri silk can be blended with wool, silk, cotton, ramie, jute, and synthetic fibers for enhanced exploitation. Products like quilts, lining, etc., can be made from eri after degumming and opening. Eri is fast becoming popular in recent years.

Muga Silk

Muga silk is obtained from the semi-domesticated silkworm (*Antherea assamensis*), known for its glossy fine texture, durability, and natural golden amber glow that increases with time and washings. These silkworms feed on the aromatic leaves of *Som* and *Soalu* plants and are reared on trees similar to that of tasar and eri. Muga silk is naturally stain resistant and is never bleached or dyed. In India, almost 40 % of Indian silk are estimated to be woven in handlooms for silk saris produced in places such as Kancheepuram, Kumbakonam, Arni, Mysore, Bangalore, Dharmavaram and Pochampalli, Varanasi and Murshidabad [42].

1.3.1.4 By-Products from Silk Production

Apart from silk fibers and filaments obtained from the silk cocoons, various by-products are also obtained from silk cultivation [10, 41, 43]. Silk fibroin of the silkworm is an ideal biomaterial due to biocompatibility, biodegradation,

nontoxicity, absorption properties, and has been widely used as sutures and for many other medical applications.

The male silk moth tonic is used to nourish the kidney and promote the circulation of blood. Feces of silkworm contains chlorophyll that can be extracted by acetone, further saponified, and treated with copper sulfate and subsequently converted into food additives and medicines to cure diseases against hepatitis virus and gastric ulcer.

Eri pupae are considered as the staple food among many tribes, which enjoys higher calorific value (460 kCal/100 g) than equivalent quantities of cow milk, chicken, sugar, carrots. The pupae oil, with similar properties like vegetable oils, contains more than 70 % of unsaturated fatty acids such as oleic, linoleic, linolenic, and diacids, which are used as the nutrient oils, food, oleo-chemical industries, and as medicine to reduce blood cholesterol and blood pressure. Pupae protein contains more than 36 % of essential amino acids including high nutrients like leucine, which plays a major role in human health. Peptone extracted from pupae can be used as the medium to culture antibiotics.

Silk sericin has potential applications in various fields, which include medicine, pharmaceuticals, and cosmetics [34, 44]. Silk sericin blended with polyvinyl alcohol has the ability to form hydrogel with good mechanical strength. Silk sericin membranes, with their good biocompatibility and adequate flexibility, can be used for wound healing and skin scaffolds and artificial skin and sulfonated sericin show the antithrombotic effect. Sericin grafted with methyl methacrylate or styrene is used in contact lenses. Sericin containing foods can relieve constipation, accelerate mineral absorption, and suppress bowel cancer and also has antitumor activities. In the cosmetic front, sericin is used in skin, hair, and nail cosmetics to reduce wrinkles, aging effects, and retain elasticity of the skin and prevent epidermal water loss.

1.3.2 Spider Silk

Spiders are some of the most diverse organisms on the earth, and they have been present for over 400 million years. Spider silk is soft and fine, but strong and elastic, obtained from certain *Madagascan* species, including *Nephila madagascarensis, Miranda aurentiam,* and *Epeira.* Silk filaments spun by spiders and silkworms are found to possess superior properties than other silk producing insects and more than 2500 orb weaving species exist worldwide [45–48]. Spiders have six or seven sets of glands, each producing different fibers (Table 1.6) and these glands remained undifferentiated, early in the evolution [49–52]. The spinnerets, microscopic tubes originating from glands, are classified into major, minor ampullate, and the term “ampulla” is used to describe the distal part of the secretary zone [52]. Many attempts have been made continuously in the past to harvest and convert spider silk filaments into different fabric forms [45, 49, 53].

Table 1.6 Type of glands, silks and their functions

Silk	Gland	Spinneret used	Function	Amino acid composition
Dragline	Major ampullate	Anterior/median	Orb web frame, radii	Glycine (37 %), Alanine (18 %), small chains (62 %), polar (26 %)
Viscid	Flagelliform	Posterior	Prey capture, sticky spiral	Glycine (44 %), proline (21 %), small side chains (56 %), polar (17 %)
Glue-like	Aggregate	Posterior	Prey capture, attachment	Glycine (14 %), proline (11 %), polar glue (49 %), small side chains (27 %
Minor	Minor ampullate	Anterior/median	Orb web frame	Glycine (43 %), Alanine (37 %), small side chains (85 %), polar (26 %)
Cocoon	Cylindrical	Median/ posterior	Reproduction	Serine (28 %), alanine (24 %), small side chains (61 %), polar (50 %)
Wrapping	Aciniform	Median/ posterior	Wrapping captured prey	Serine (15 %), glycine (13 %), alanine (11 %), small side chains (40 %), polar (47 %)
Attachment	Piriform	Anterior	Attachment	Serine (15 %), side chains (32 %), polar (58 %)

Orb-web spiders invest little energy in searching for prey and majority of the energy is spent on silk synthesis and construction of webs. Figure 1.4 shows various threads and the web constructed by the orb-weaving spiders, *Araneus* and *Nephila* [46].

Dragline, minor ampullate, and viscid silks form the major portions of the orb web and dragline silk, in the form of mooring threads, framework, and pre-tensioned radial threads, together dominates the web architecture [47, 48, 54, 55]. Multi strands of spider silks are laid in the web to withstand adverse conditions and impact created by the fast moving prey. The perfume-coated dragline helps to find their mates, swing from place to place, and store the food and egg. Minor ampullate silk produced by median spinneret is characterized by higher elasticity and low strength and are generally finer than major ampullate silk with better uniformity but uncertain properties [56–58]. Capture threads with glue droplets, a composition that contains 80 % water, and amino acids, glycoprotein, lipids, salts, and low molecular weight compounds to the extent of 20–30 %, ranging from 7 to 29 μ are used for aerodynamic damping of the impact caused by the flying insects [55, 59–61] Capture threads produced by the *flagelliform* glands of *Nephila clavipes* are highly compliant with an aqueous solution that forms sticky droplets,

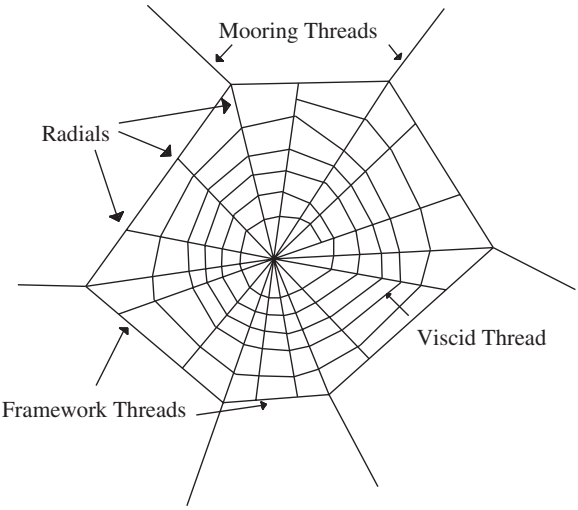

Fig. 1.4 Schematic diagram of spider web

which enhance the damping and harvest water from the air [59]. While stretching, the core filament inside the droplet extends to a level of 200 %, which prevents the rebound of prey after catching [59, 62–64].

Super contraction of dragline silk takes place in the presence of polar solvents and also air at about a relative humidity of 90 % or more and takes up slackness of the webs, and restores shape and tension after prey capture [65–70]. Molecular organization of *Araneus diadematus* silk fibroin is unaffected by exposure to 100 °C for over 50 h [47, 71, 72] and the functional properties are retained up to 180 °C without affecting the initial modulus. Camouflaging, dark and bright patterns over the body of the spiders and the webs, with the reflection patterns that resemble many flowers in UV light, help to attract and trap the insects into the web [73–75].

Though availability of the dragline silk is limited, it is widely used in defense [49, 58, 76, 77] and medical [45, 56, 78, 79] applications. Until World War II, spider silk was used as crossed-hairs in optical devices including microscopes, telescope, and bomb guiding systems [49]. The ability to dissipate energy at very high strain rates makes spider silk suitable for the body armor system and ideal for ballistic protection vests [76, 80]. Early use of spider silk in the form of web, rather than a fiber, includes wound dressing to help blood clot. Spider silk proteins can be used to coat medical implants for better performance. Surgical threads, biomembranes, and scaffolds for tissue engineering are the possible areas of applications in biomedical and biomaterial fields, due to low inflammatory potential of silk proteins and antithrombic nature [81, 82]. Because of the high cost of production, spider silk is not used in the textile industry; however, many attempts are continuously made to regenerate the silk from existing sources [83].

1.3.3 Other Insect Silks

Mussel silk [84] obtained from a bivalve (*Bivalvia* is a class of marine and freshwater mollusks with laterally compressed bodies enclosed by a shell), *Pinna squamosa*, found in the shallow waters along the Italina and Dalmatian shores of the Adriatic. The strong brown filament, or byssus, is secreted by the mussel to anchor itself onto rocks or other surfaces. The byssus is combed and then spun into a silk popularly known as "*fish wool.*"

Coan silk obtained from the larvae of *Pachypasaatus* D., from the Mediterranean regions (southern Italy, Greece, Romania, Turkey, etc.), feed primarily on trees such as pine, juniper, and oak [84]. They spin white cocoons and since ancient times, *coan* silk has been used to make the crimson-dyed apparels worn by the dignitaries of Rome.

Fagara silk is obtained from the giant silk moth *Attacus atlas* L. in the Indo-Australian biogeographic regions, China and Sudan [85]. They spin light-brown cocoons nearly two inches long with peduncles of varying lengths.

Anaphe silk of Southern and Central Africa is produced by silkworms of the genus *Anaphe*: *A. moloneyi Druce*, *A. panda Boisduval*, *A. reticulate Walker*, *A. ambrizia Butler*, *A. carteri Walsingham*, *A. venata Butler*, and *A. infracta Walsingham*. They spin cocoons in communes, all enclosed by a thin layer of silk [86]. The fabric is elastic and stronger than that of mulberry silk, used in velvet and plush.

1.4 Hair Fibers

1.4.1 Wool

For centuries man has looked to wool as the source of warmth feel in clothing and blankets and sheep (*Ovisaries*) were first domesticated more than 5000 years ago. Angora goats, producers of mohair, originated in Asia's Himalayan Mountains and migrated to Turkey. Kashmir goat, whose fine undercoat produces cashmere, got its name from the Kashmir region of India. Camel shave been used for centuries in the deserts of Africa and Asia for transportation as well as wool for production. As the trade routes opened from Asia, many exotic fibers found their way to Europe and the rest of the world. Today, wool production has grown to be a global industry, with Australia, China, Argentina, the United States, and New Zealand serving as the major suppliers of raw wool; however, wool is produced worldwide in about 100 countries. When one talks about wool, one almost and always means the fibers obtained from sheep, although the term "wool" can be applied to the hairs of other mammals including cashmere and mohair from goats, alpaca, camel, and angora from goats and rabbits.

Table 1.7 Classification of wool fibers

Category	Fiber diameter (μ)	Fiber length (mm)
Ultrafine merino	<15.5	65–100
Superfine merino	15.6–18.5	
Fine merino	18.6–20	
Medium merino	20.1–23	
Strong merino	>23	
Fine crossbred	27–31	90–140
Medium crossbred	32–35	150–225
Coarse crossbred	>36	150 onwards
Carpet wools	35–45	

In scientific terms, wool is considered to be a protein known as *keratin*. The quality of wool is determined by its fiber diameter, crimp, yield, color, and staple strength [87]. Wool is also separated into grades based on the measurement of the wool's diameter in microns and also its style (Table 1.7).

Cross section of wool is generally circular and wool is composed of 50 % carbon, 20–25 % oxygen, 16–17 % nitrogen, 7 % hydrogen, and 3–4 % sulfur. A typical wool fiber has a swollen root at the base and a tip with taper. The wool fiber is complex in structure and composed of three tissues, cuticle, cortex, and medulla. The cuticle is formed of hard thin scales, which overlap and protrude for about 1/3 of their length and directed toward the tip. In all cases, free end of scales project outwards and toward tip. They are sometimes described as little "barbs" because of their prickly nature. The outermost layer of this scale is epicuticle and below this are exo and endo cuticles (Fig. 1.5). Both epi and exocuticles contain a high proportion of sulfur with cystine cross-linkages giving them a protection against biological attack and chemical attack, while the endo cuticle is less resistant. Unfortunately, wool fibers also interlock with each other due to the locking of the scales, a phenomenon known as 'felting'. Machine-washable shrink-resistant wool is produced by masking or partially removing the surface scales to reduce the felting properties of the wool fiber and thus make it shrink-resistant [88]. There are intercellular membranes, which act as a cement holding the cuticle for the adjacent tissues. About 90 % of wool fiber is composed of cortical cells. The tensile strength, elastic properties, and the natural color of wool are determined mainly by the nature of these cortical cells. Two distinct tissues exist in cortex known as ortho and paracortex, which lead to differential staining with acetic acid and *Jenus Green*. The distribution of the ortho and para cortical cells is bilateral and each forms approximately a hemi-cylinder. Ortho cortex has a macro-fibrillar structure, whereas in para cortex there are no clearly defined macro-fibrillar boundaries. But micro-fibrils are more closely packed than in ortho cortex. Since the fiber spirals around its crimped form para cortex is found always inside the curvature.

The main difference between wool fibers with silk is the existence of sulfur containing amino acids like cystine and methionine (Table 1.3) and these acids form covalent bonds. Apart from the strongest covalent bond, other bonds like

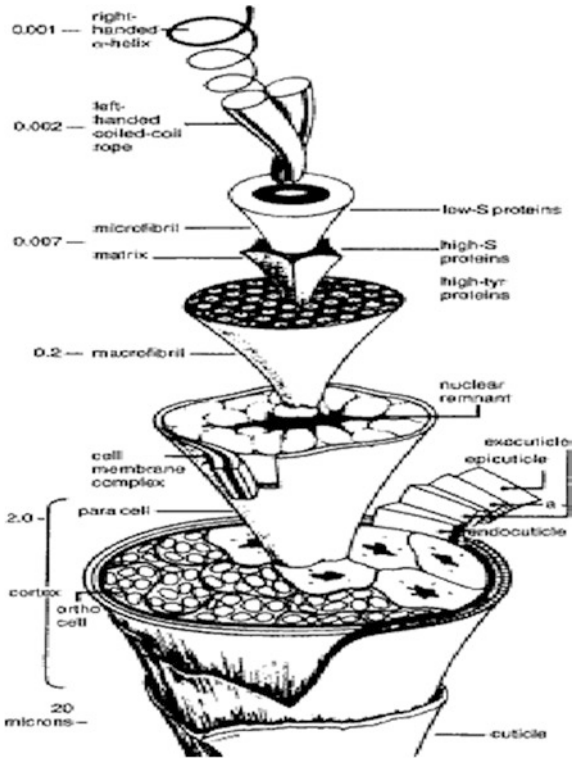

Fig. 1.5 Physical structure of wool fiber

ionic bond, hydrogen bond, and van der Waal forces also exist in wool fibers, which seem to stabilize the alpha-helix structure in the fiber.

Wool fibers have a density of 1.30 gm/cc, which falls in line with cotton (1.54 gm/cc) and silk (1.34 gm/cc). This density value is highly influenced by the voids present in the fibers. Another most important factor that makes fiber more different is its moisture regain value, which is expressed as a percentage ratio of weight of undried fibers to its dried fiber weight. Owing to the presence of more polar groups, wool fibers have the highest moisture regain value (14–19 %) than any natural fibers as well as synthetic and regenerated textile fibers. Wool has the highest differential heat of sorption values at different moisture levels, a unique property that gives comfort to the wearers while moving from hot to cold and cold to hot environments, by releasing or absorbing the heat from the surrounding atmosphere.

The distinct mechanical properties of wool fibers are mainly attributed to the folded molecular structure of keratin. When the fibers are made to extend by force, they go back to their original state, a phenomenon known as elasticity or elastic recovery. At low extension level (1 %) wool fibers have the highest recovery to the extent of 99 %, similar to nylon. Wool fiber possesses many inherent advantages

[89] and some of them include naturally water absorbent, mildew and mold resistant, thermal insulator, fire retardant, water repellent, naturally wrinkle resistant, resistance against static charge, dirt, and dust and nonallergenic nature.

Sheep shearing is the process by which the woolen fleece of a sheep is removed from the skin, carried out during the spring season, usually. New technologies have been developed that use sensitive, robot-controlled arms to do the clipping. After shearing, the wool is separated into four main categories: fleece (which makes up the vast bulk), broken, bellies, and locks. Quality of the fleece is decided based on the technique known as wool classing based on fineness, yield, staple length, staple strength, and sometimes color and comfort factor.

In sorting, the wool fibers are classified into sections of different quality fibers clipped from different parts of the body. The best quality of wool comes from the shoulders and sides of the sheep and is used for clothing; the lesser quality comes from the lower legs and is used to make rugs (Fig. 1.6).

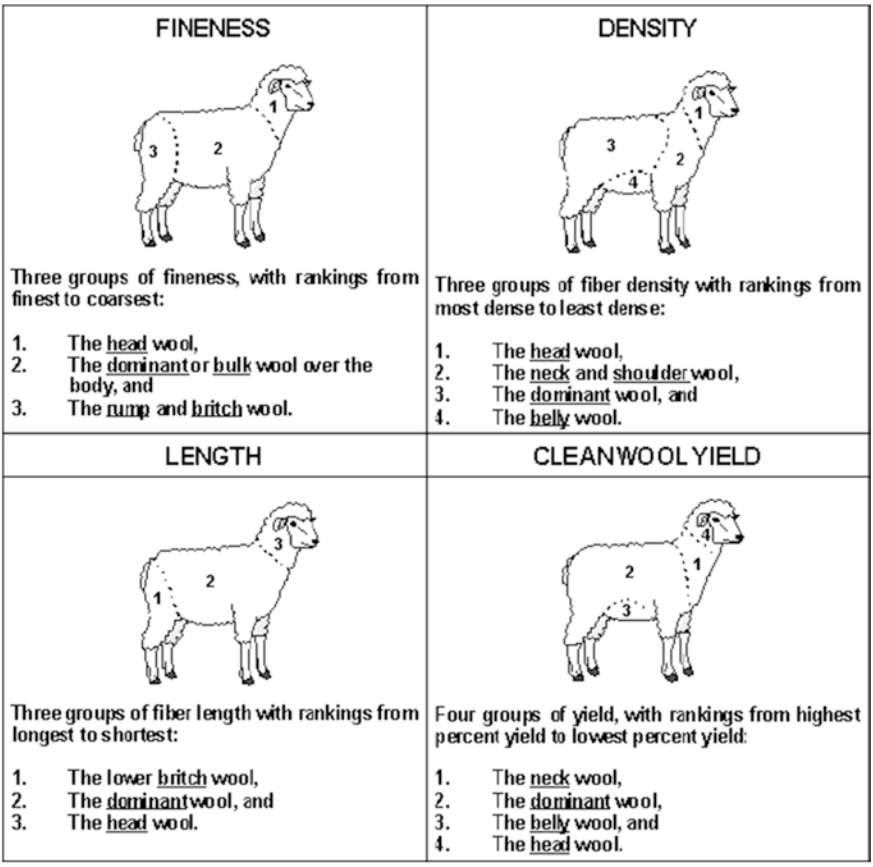

Fig. 1.6 Classification of wool fibers [90]

Besides different classifications, wool fibers are also grouped based on the type of the yarn produced. Worsted is a type of yarn derived from Worstead, a village which was once the manufacturing hub in the English county of Norfolk. Worsted yarns are made from the long-staple pasture wool from sheep breeds. Worsted wool fabric is typically used in the making of tailored garments such as suits, as opposed to woolen yarns which are used for knitted comfort wears. Virgin wool is wool spun for the first time, while shoddy or recycled wool fibers are made by opening the used wool fabrics and re-spinning the recovered fibers.

1.4.2 Wool Scouring and Carbonizing

Wool fibers grow from the follicle, surrounded by the suint and sebaceous glands. Sunit glands protect hairs from sunlight and the latter protects the sheep from mechanical damage through secretion of fatty substances.

The impurities in wool fibers can be classified into two categories: added impurities or adventitious impurities (composed of particles of dried grass, straws, burrs, etc.), removed by carbonizing after scouring and natural impurities that are removed during the scouring operation. Before wool fibers are carded, they are scoured and scouring is also known as *wool washing* that can result in weight loss values as high as 40 %. Various types of scouring include emulsion scouring, suint scouring, solvent scouring, and freeze scouring. The recovered greasy substances from the scouring treatment are refined (lanolin) and used in a variety of consumer products and cosmetics. At the end of scouring, 0.5–0.75 % fat is left out in wool fibers to facilitate spinning operation.

In emulsion scouring, soap solution is recommended for scouring since it is relatively easier to emulsify the wool fats above their melting point (40–45 °C), without damaging the fibers, using soft soaps or potash-olive-oil soaps. Three main functions expected from the soaps are to wet the greasy surface, emulsify the grease and stabilize the emulsified grease, and suspend the dirt [90]. Fatty acids of wool grease absorb the alkali of soap and reduce its value, which necessitates the alkalinity sufficient enough to neutralize the free fatty acids and also to improve soap value. Normally sodium carbonate is added to the extent of 0.25 %.

Wool suint is soluble in water so preliminary steeping helps to remove the suint and also helps to remove heavy dirts. Grease removal is brought in by the use of clear suint solution and fats are emulsified. Wool fats may also be removed using solvents like benzene, carbon tetrachloride, white spirit, or perchloroethylene.

When raw greasy wool is subjected to very low temperature, the fatty substances freeze and become hard and brittle without affecting wool fibers and their structure. After subjecting the fibers at −35 to −45 °C in a freezing chamber, the wool fibers are agitated vigorously so that frozen fats are broken into fine powders and fall away with substantial quantities of vegetable matters. In this method, 30–55 % of actual grease can be removed and due to the absence of chemicals, wool fibers achieve better color, softer than that obtained in the usual scouring process.

Once the wool fibers are properly scoured, they are subjected to carbonization, to remove the vegetable matters of cellulosic nature like seeds, burrs, straw, stem by converting them into friable hydrocellulose, which otherwise would make the spinning process difficult. Fabric carbonizing is preferred in the woolen, while the worsted system prefers carbonizing immediately after scouring, before spinning. Carbonizing process consists of immersing the scoured wool in dilute solution of strong acids (sulfuric/hydrochloride) followed by pre-drying, baking, crushing the carbonized substances, and subsequently neutralizing the residual acids.

1.4.3 Organic Wool

Organic wool is clipped from the sheep raised under natural, healthy, and responsible animal husbandry methods without involving any synthetic or harmful chemicals, which in turn reduce or eliminate the need for most agricultural chemicals and promote healthy soil conditions and waterways [91, 92]. The sheep are grazed on pesticide-free land that is not over-grazed, never sprayed, or dipped as commonly practiced in conventional sheep farming. The raw wool is scoured using biodegradable cleansing agents and the organic wool yarns are not (hazardous) chemically treated during the entire production process, i.e., from farm to finished garment or product.

Organic wool keeps the body warm in winter and comfortable in summer, sensitive to body temperature. Organic wool is naturally resistant to mites, molds, mildews, odors, and is almost entirely nonallergenic. Fortunately, more eco-conscious designers are incorporating sustainable textiles like organic wool and organic cotton into their collections as a reflection of their environmental ethics and as the sustainability measure, in the designs and products. The International Wool Textile Organization (IWTO) has adopted a new organic wool standard (reflecting the Global Organic Textile Standards) that meets the standards set by the EU Eco-label.

1.4.4 Cool Wool

Cool Wool was a successful campaign organized by The Woolmark Company in the 1980s [93]. In 2012 Cool Wool was relaunched to meet the demands of the geographical regions with high temperature climates such as the Gulf States, India, and Turkey. *Cool Wool* garments are manufactured from lightweight merino wool fabrics with a maximum areal density of the fabric at 190 g/m^2, and a maximum mean wool fiber fineness of 22.5 μ. The most influencing fashion designers introduced merino wool fabrics in the Spring Summer Collections 2014 with a wide variety of superfine knits, inspired by the concept of *Cool Wool*, for luxury and quality, particularly among the younger generation.

1.4.5 Exotic Hair Fibers

Besides sheep wool, many hair fibers are extracted from the animals (Fig. 1.7) that normally live in high altitude regions. In such regions, the animals have hairy skins to protect themselves from the adverse conditions. Also, these animals have two different coats of fibers, outer and inner or coarse and fine (Table 1.8), to protect themselves from the sun, rain, dust, and insulation against extreme cold conditions. However, yield of the fibers obtained from these animals is extremely low compared to that of sheep. Camels [94], yak [94], bison [95], alpaca [96], angora goat [97] and rabbit [98], cashmere [99], and llama [100] are some of the animals that produce hairy fibers (Fig. 1.7). The fibers are pulled or clipped annually, except angora rabbits, during the spring season. The South American camelid group comprises the alpaca, llama, vicuna, and guanaco. Apart from the angora rabbit, all these animals live in areas where the climatic conditions are harsh. Alpaca and vicuna have single coat of fibers, like sheep. Since these fibers are not available in plenty, the products produced using these fibers are sold in high premium markets; a piece of scarf made of vicuna fibers can cost as much as 1500 US$.

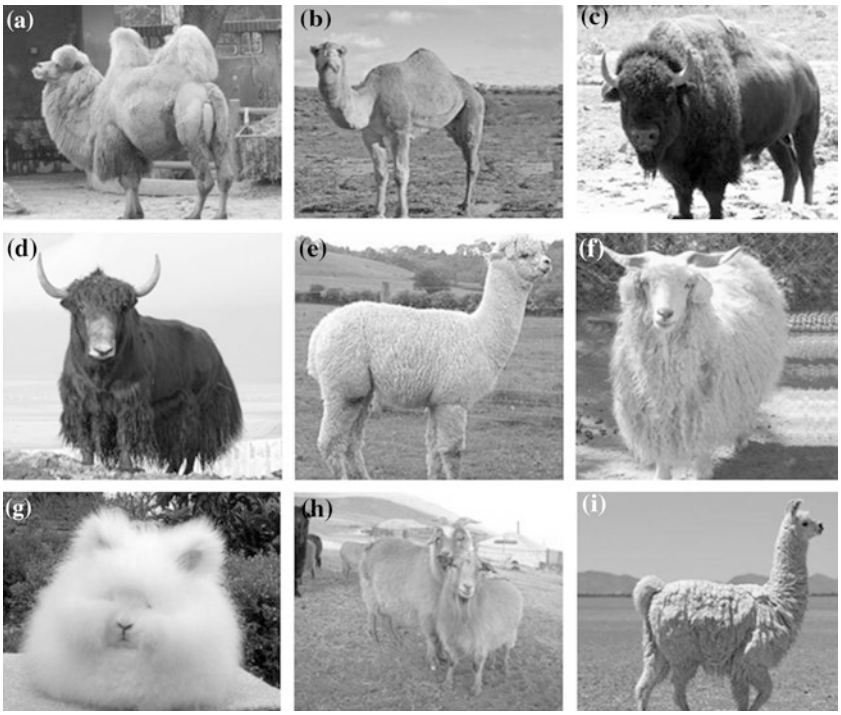

Fig. 1.7 Animals producing exotic hair fibers. **a** Bacterian camel. **b** Dromedary camel. **c** Yak. **d** Bison. **e** Alpaca. **f** Angora goat. **g** Angora rabbit. **h** Cashmere. **i** Llama

Table 1.8 Properties of exotic hair fibers

Type of fiber	Fineness (μ)	Length (mm)	Fiber yield per animal per year (kg)
Alpaca	20–36	200–550	3.0–5.0
Angora-Rabbit	14	60	0.4–0.8
Cashmere	12.5–19	35–50	0.1–0.2
Llama	19–38	80–250	2.0–5.0
Camel Hair	18–24	36–40	3.5–5.0
Yak	15–20	35–50	0.1
Vicuna	12–13	–	0.1–0.5
Bison	18.5–60.0	–	–

1.4.5.1 Alpaca

Alpaca are the domesticated species, *Suri alpaca* and the *Huacaya alpaca,* of South American camelid and resemble small llama in appearance. Approximately 80 % of alpaca population is present in Peru. It provides more warmth than sheep wool and is lighter in weight. Most alpaca fiber is white, but it also comes in various shades of brown and black. The fiber of the *suri* is the longest and is fine and silky, between 20 and 34 μ in diameter and 8–12 cm in length.

1.4.5.2 Angora

Angora wool or Angora fiber refers to the down coat produced by the Angora rabbit. Angora is known for its soft, thin fibers of around 12–16 μ for quality fiber and easy to feel. Angora fibers come in white, black, and various shades of brown, sheared once in 3 months. French hairs are longer and spikier and contain guard hair that is hollow and does not take up dyes. The second type of hair is less spiky and is used to make softer yarns that are sometimes considered as alternatives to cashmere. China is the world's leading producer of Angora rabbit hair, contributing almost 90 % of world production followed by Chile, the second largest producer.

1.4.5.3 Cashmere Wool

It is a special wool obtained from the Cashmere goat. Cashmere is characterized by its luxuriously soft fibers, with high napability and loft. It is used to provide natural lightweight thermal insulation (warmth) without bulk. Fibers are highly adaptable and easily constructed into fine or thick yarns, and light to heavyweight fabrics.

1.4.5.4 Llama

The llama, a camelid, is a domesticated two-coated animal. Llama fleece varies from 0 to 20 % guard hair [101]. The fibers of llamas and alpacas have a tubular structure with medullation that is structurally different from the solid or corticated fibers of sheep and other hairs. The degree of medullation decreases with fiber fineness, which essentially means an interrupted medullation in the fine llama and alpaca fiber or none at all. Llama fibers are predominantly used in knitwear and suiting cloth.

1.4.5.5 Camel Hair

Camel hairs are collected by shearing and by combing during the molting season, a period that ranges from 6 to 8 weeks. Hairs are sorted according to shade and age of the animals and, the color varies from reddish to light brown; white is the most valuable one but rare to find. Fabrics made from camel hair are usually left in the natural state or dyed to a darker shade. The inner down fibers are used to make fine overcoats and sportswear. The outer coat hairs are used to make felt for Mongolian yurts or tents and for herdsmen's winter coats.

1.4.5.6 Yak

The yak belongs to the hoofed *bovidae* (*Bosgrunniens* and *Bosmutus*) family. Yaks are widely reared in the mountainous regions of the Tibet Plateau for milk and meat production. The outer hair is separated from the inner down hair. The color of yak hair varies from black to piebald and some very rare white. The inner down hair of the calf has a diameter of 15–17 μ and is 4–5 cm in length. Adult down is 18–20 μ in diameter and 3–3.5 cm in length. The yak fibers are used locally for weaving hut coverings, blankets, mats, and sacks.

1.4.5.7 Bison

The coat of the bison contains two different types of fibers [102]. The main coat is made up of coarse fibers called guard hairs, and the downy undercoat. This undercoat is shed annually and consists of fine, soft fibers which provide warmth and protection to the animal from harsh winter conditions.

1.4.5.8 Mohair

Angora goats produce beautiful, luxurious, and incredibly durable fiber called mohair. It is both durable and resilient, has high luster, and the fibers are sheared

twice a year. Angora goats acquired the name from the ancient Turkish city Ankara, where they originated. Presently, South Africa produces nearly 60 % of the total world production of mohair fibers. Fibers have smooth cuticular scales on the surface that reduce the felting tendency of the fibers with relatively lower prickliness. Mohair from young goats, *kid mohair*, is used in knitwear and the stronger 'fine hair' types are used in coating and rug manufacture.

1.5 Regenerated Protein Fibers

In the year 1665, Robert Hook of Hooke's Law fame proposed a remarkable suggestion that one day "there might be a way found out to make an artificial glutinous composition much resembling that substance out of which silk worm draws his clew…. and drawing out into small wires for use" [103] since then, it took more than a couple of centuries for man to produce a commercially successful man-made filament. The Courtauld family, whose business was silk weaving, first produced man-made viscose rayon in Britain. In 1905, the first factory for producing the fibers came in Coventry. The first patent on the viscose process was granted to Cross and Bevan in England in 1893 [104]. That paved the way for further developments in the field of man-made fiber production with the development of many regenerated cellulosic and protein fibers including casein, groundnut, soybean, spider silk, and corn proteins. Interestingly, regenerated protein fibers have been attempted as a measure to provide an alternative fiber to wool fibers, whose price increases year after year.

Azlon is the generic name given to manufactured fibers composed of regenerated natural proteins [105]. Azlon is produced by dissolving proteins like casein from milk, soybean protein, and zein from corn in a dilute alkaline solution and extruding the solution using a coagulating bath. The first commercial protein fiber was produced from casein by Antonio Ferretti in Italy in 1935. An American factory made similar commercial staple fibers from casein and sold it under the trade name of *Aralac*. In 1939, USDA facilitated the discovery of the glutinous protein and termed as 'Zein' which was found adaptable for fiber manufacturing. On many occasions, regenerated proteins are produced using copolymer routes also and, with the advent of electro spinning, these fibers have found new attraction with fineness as low as 5 nm [106]. Developments in the solution spinning have led to increase in the production rates of fiber production to the extent of 1000 m/min [105]. Many of the regenerated protein fibers have similar properties (Table 1.9), in terms of density, moisture regain, and mechanical properties. Soybean fibers are produced commercially and are gaining a sizeable market share gradually in these years and more literature is available on soybean fibers [106].

Table 1.9 Properties of regenerated protein fibres

Property	Casein	Vicara	Ardil
Density (g/cc)	1.3	1.25	1.31
Moisture regain (%)	14	10	13–15
Tenacity (g/d)—conditioned	1.0–1.1	1.2–1.5	0.7–0.9
Tenacity (g/d)—wet	0.5–0.6	0.6–0.8	0.4–0.6
Elongation (%)—conditioned	50–60	25–35	40–60
Elongation (%)—wet	60–70	30–45	50–70
Elastic recovery	High	High	Good

1.5.1 Zein Fibers

Zein fibers were commercially sold as Vicara from 1948 to 1957. *Vicara* means 'sustained application' or 'sustained thinking'. *Zein* molecules need to be uncoiled from the typical globule before they can be spun into fiber. *Zein* fibers have also been produced by electro spinning from acetic acid, aqueous methanol, ethanol, and isopropyl alcohol [107], which follows ribbon configurations with circular cross-section, predominantly.

Fibers are smooth with a circular cross section. Tenacity of the fibers is low (Table 1.9), which necessitates the blending of *vicara* with other fibers like wool, rayon, and acetate fibers. With standard moisture regain values at 10 %, it shows similar behavior as that of silk fibers, and also supports higher dye uptake than silk fibers. *Vicara* is resistant to mildew, insects, sunlight, and temperatures up to 140 °C.

1.5.2 Ardil and Sarelon

Economically, peanut-protein fiber is perhaps in a better position than its related products to compete with other proteins and their products. In 1937, David Trail in England reported the production of peanut protein-urea solutions into an acid coagulating bath to form a new fiber, *Ardil* or *Sarelon*. Shortage in the production and availability of wool fibers, during World War II motivated many manufacturers to invest more in *Ardil* fiber production. *Ardil* was developed by the Imperial Chemical Industry (ICI) in the mid-1940s, which subsequently stopped production in 1957 due to a sudden drop in the price of wool fibers, after the World War.

The steps followed in separating the protein from solvent-extracted peanut meal include preparation of a water-meal mixture using a suitable solvent, clarification of the mixture by screening and filtering, precipitation and separation of proteins followed by drying. Whenever required, the protein is dissolved in the solvent and extruded into a coagulation bath (Fig. 1.8) that contains sulfuric acid and sodium

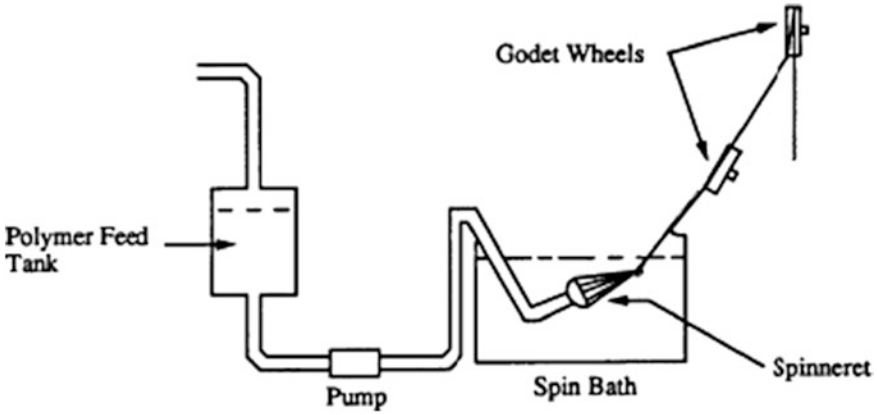

Fig. 1.8 Wet spinning for regenerated fibers

sulfate to produce a continuous filament. After the plastic-like fiber has been formed in the solution, it is withdrawn in a filament form, stretched (drawn), and reacted with other chemicals, such as sodium chloride and formaldehyde [108, 109].

Peanut fibers are cream color in their natural state and have about 80 % of the strength of woo and can be stretched without breaking similar to wool. It has a high degree of dimensional stability and does not shrink appreciably in hot water.

1.5.3 Casein

Milk fiber was first introduced in 1930 in Italy and America to compete with wool fibers under the brand name *Cyarn*, *Aralac*, *Lanatil*, and *Merinova*. During World War I, when the Germans were looking out for some newer sources of fabrics, it led to the discovery and production of protein fibers derived from milk protein [108].

Milk is skimmed to remove the cream and then heated to 40 °C followed by the addition of acid to coagulate the proteins which separate as a curd-like precipitate. The precipitated protein, casein, is washed to remove acids and salts. 100 kg of milk yields 3 kg of casein and it might result in the same amount of fiber in the spinning process. The casein solution is extruded into a coagulation bath that consists of water (100 parts), sulfuric acid (2 parts), formaldehyde (5 parts, optional), and glucose (20 parts). Extruded fibers are drawn, dried, and often cross-linked (optionally) using formaldehyde and then cut into the staple fiber form. It is totally eco-friendly and considered as the green product. Casein fibers contain 17 amino acids and are naturally antibacterial. The milk protein contains the natural humectant factor which can help to maintain the skin moisture. The major usages of milk fibers include T-Shirts, underwear, sportswear, ladies outerwear, and sweaters.

1.6 Conclusion

Since the 1950s, the manufacture and use of synthetic fibers have increased significantly and offer stiff competition to the gamut of natural fibers. However, natural fibers offer a healthy, fashionable, and responsible choice to consumers where the possibilities of inculcating sustainability focus are much better compared to synthetic fibers, which largely rely on petroleum distillation products that are depleting in nature. In recent years, natural cellulosic and protein fibers are largely used in various technical textile segments, whose market potentials are increasing year after year. On realizing the potential of natural fibers on human life, the United Nations General Assembly declared 2009 as the *International Year of Natural Fibres* to enhance the focus on natural fibers and to enhance the sustainable cultivation and production practices. Today, many textile and garment labeling systems are available to demonstrate the eco-friendliness and sustainability of natural fibers, which are not yet completely understood by many synthetic fiber industries. It is of economic importance to agriculture that the agricultural wastes and surplus proteins produced are used in effective ways to enhance the lifestyle of humans and also to provide a measure of income generation. Regenerated cellulosic and protein fibers are expected to offer stiff competition to synthetic fibers and make the presence of these fibers felt in the coming years.

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Chapter 2
Biomedical Applications of Heat Shock Proteins

Rajesh R. Kundapur and Dhiraj Kumar

Abstract Heat shock proteins (HSPs) constitute a heterogeneous group of molecules which are phylogenetically conserved and initially known for their role in proper folding of nascent or misfolded proteins. Their expression is increased due to different stresses. Initially discovered due to heat stress, HSPs came beyond their role as molecular chaperones. Now there is evidence that HSPs perform functions more than what they were believed to earlier. There are reports that show that HSPs are actively involved in cell signaling and act as anti-inflammatory and pro-inflammatory agents in normal and in different ailments. Normally, HSPs are found inside the cell but they are also found secreted into the extracellular milieu and displayed onto the cell surface to represent different cellular conditions. While present on the cell surface they also carry processed peptides of the respective cell. Their solubility nature evokes most of the immune modulatory effects on different cell types. This versatile nature of HSPs can be employed for different biomedical applications. HSPs, both as diagnostic markers as well as drug targets in the field of autoimmune diseases, different cancers, are discussed here. Few non-biomedical but commercially important applications are also discussed in brief.

Keywords HSPs · Chaperones · Autoimmune diseases · Cancer · Immune modulators · Chaperonotherapy

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2.1 Introduction

An accidental increase in the incubator temperature caused puffing of polytene chromosomes in Drosophila. This phenomenon was observed by Ritossa [1] and interestingly, the response is the same as other stressors. At that time none thought that a mere accidental discovery would become a stepping stone in the field of stress biology and introduce the fascinating group of proteins called heat shock proteins to the world of biology. The puffing of chromosome was nothing but transcription of selected genes. The behavior of cells exposed to different stresses is generally known as heat shock response and candidate proteins overexpressed are referred to as heat shock proteins (HSPs). The term "heat shock protein" was coined by Tissieres et al. [2] as these proteins increase in synthesis due to sudden increases in temperature. HSPs and heat shock response were thoroughly reviewed by our group [3] with special reference to silkworm *Bombyx mori*. The term chaperone also exists side-by-side but many reviews cleared the ambiguity because by nature, all HSPs are not chaperones and all chaperones are not HSPs. HSPs are classified based on structural similarities, but other than protecting the cells from environmental insults they also perform vital functions that are necessary for the development and proper maintenance of the cell (Table 2.1). Although HSPs are responsible for many events taking place inside the cell, like other therapeutic proteins, they are not directly included in the therapeutic protein list as HSP screening is necessary for diagnosis of different diseases, among them the most important is different malignant states. Kaigorodova and Bogatyuk recently emphasized the role of HSPs as prognostic markers of cancer disease. They also mentioned that the tumor cells make use of HSPs in order to escape from apoptosis, which signifies the role of HSPs and their inhibition as an effective method to control cancer development [4]. The three roles of HSPs, such as chaperone and protecting cells from stress, antigen display on the cell surface, and secretion from the cell, makes them one of the highly versatile proteins. This chapter gives a brief idea about applications of HSPs in the field of Cancer biology, autoimmune diseases to name but a few, and also concludes with a few commercial applications.

Table 2.1 Heat shock proteins and their biomedical applications

Heat shock proteins	Biomedical applications
HSP90	Presents peptides to T lymphocytes, inhibition can control cancer growth [5]
HSP70	As a cytokine inducer or inhibitor [6]
HSP60	Monocyte and dendritic cell stimulator [7], insecticide [8]
HSP27	Anti-inflammatory protein [9]
HSP10	Early pregnancy factor [10]

2.1.1 In the Treatment of Autoimmune Diseases

Based on their molecular weight, HSPs are classified as HSP100, HSP 90, HSP70, HSP60, HSP40, and small HSPs. Their structure conserved from bacteria to man and high immunogenicity make them attractive targets for investigation in the area of autoimmunity. Most studies indicate that recognition of peptides derived from HSPs by the immune system can have an anti-inflammatory effect and down-regulate the chronic state of inflammation via modulation of cytokine secretion. It has been demonstrated that in juvenile rheumatoid arthritis (JIA) peptides derived from bacterial and human HSP40 modulate autoimmune inflammation [11]. There are several clinical implications related to HSPs and their role in different autoimmune diseases. One potential clinical application would be to exploit the presence of HSP antibodies for screening at-risk patients to detect significant autoimmune diseases. HSPs from bacterial origin act as antigens in host response against pathogen. As HSPs are phylogenetically conserved, they in turn promote autoimmunity [12]. Structural domains are conserved between the HSPs of pathogen and host, which is the potential source for autoimmune diseases. Although HSPs are highly conserved the immune response to few but not all HSPs is reported. Atherosclerosis is one such case where HSPs have been shown to play a major role. Under normal conditions, vascular endothelial cell surface is devoid of HSP60, whereas in atherosclerosis, HSP60 is translocated to the cytoplasm and then to the vascular endothelial cell surface. On the vascular endothelial cell surface HSP60 induces the expression of E-selectin, VCAM-1, ICAM-1, and IL-6 within the endothelial cell which contributes to the HSP60-directed autoimmune pathogenesis of atherosclerosis [13, 14]. Similarly, expression of HSP10 [15], HSP27 [16], HSP70 [17], and HSP90 [18] was found to be significantly increased in atherosclerosis, which suggests the possibility of use of different HSPs for screening, diagnosis, and prognosis in the treatment of atherosclerosis.

2.1.2 Fight Against Cancer

As mentioned earlier tumor is very clever by nature as it makes use of the peculiar property of HSPs to thrive against the defense mechanism of the human body, i.e., apoptosis. Apoptosis or programmed cell death is mainly responsible for maintaining body shapes during development; it destroys auto antibody producing, pathogen infested and damaged DNA containing cells. Members of the HSP family when associated with tumor are found to inhibit apoptosis and promote cancer growth by promoting tumor cell proliferation. In this regard HSP90 stands first because it has many client proteins with which it interacts, such as epidermal growth factor receptor, Bcr-Abl fusion proteins, mutant p53, hypoxia inducible factor 1α, and matrix metalloproteinase 2, which all are involved in different cancer signaling pathways. Researchers believe and it is proven that inhibition of HSPs, especially HSP90, could lead to both caspase-dependent and independent apoptosis of cancer cells [19, 20]. There are

several inhibitors available for different HSPs [21–23], but the success story talks only about HSP90 so far. Although HSP90 exists in almost all living organisms, it is typically and highly expressed and activated in cancer cells [24]. HSP90 inhibition by geldanamycin showed decrease in the growth of cancerous tissue since the availability of a number of drugs such as radicicol, 17-allylamino-17-demethoxygeldanomycin (17-AAG) [25, 26]. Yet the main concern with these anticancer drugs is drug toxicity, which can be answered in the near future. Rohde and group showed that the other major HSPs, i.e., HSP70 family members are equally potent in controlling the growth of cancer cells when inhibited [27]. Inhibitors for other HSPs and their role in cancer progression need to appear in the literature in the near future.

Another feature of HSPs that can be used as a weapon to fight cancer is their ability to display tumor antigenic determinants or polypeptides on immunogenic cell surface. These HSPs along with tumor polypeptide can be used for development of vaccine to elicit antitumor immunity [28, 29]. So far, HSP-based vaccines have been prepared from HSP70, Gp96, HSP90, HSP110, and Grp170 and show promising results [30]. Different biochemical methods have been proposed for successful isolation of HSP-antigen complexes from different tumors [28, 31–34].

An interesting property of HSPs worth mention, which can be of biomedical importance, is their secretion into the extracellular milieu. It was Tytell and colleagues who reported the transfer of HSP70, HSc70, and HSP100 from adjacent glial cells into the squid giant axon for the first time [35]; followed by Hightower and Guidon [36], who reported that HSPs are also released from cultured rat embryo cells. Since then a number of studies have reported the release of different HSPs from different cell types and also explain the reason and mechanism behind the release of HSPs [37]. Concurrently, HSPs were released from both necrotic cells, tumor cells, and from intact unstressed cells under basal conditions, using a defined secretion mechanism [38, 39]. There are reports of participation of HSP60 in extracellular molecular interactions and cell signaling and also in key intracellular pathways of some types of tumor cells; the idea of using HSP60 in anticancer therapy (chaperonotherapy) is being investigated. HSP60 could be used either as an anticancer agent alone or in combination with tumor antigens, or as target for anti-chaperone compounds.

The advancement in the field of proteomics characterized HSP expression in different types of cancers and suggests that these HSP expressions be either used as biomarkers or targets for cancer therapy. The proteomic study also helped in designing novel inhibitors for different HSPs. The studies reveal that HSP overexpression in cancer cells provides resistance to many anticancer compounds and chemotherapeutics. Hence, it is now necessary to include HSP inhibitor along with anticancer regime.

2.1.3 HSPs Are Immune Modulators

A number of experimental data suggests that intracellular or extracellular HSPs perform immune modulatory functions by interacting with innate and adaptive immune cells. This is achieved by binding of a variety of cellular receptors to play

an important role in health and diseases. HSP10 is a potent inhibitor of inflammation in multiple sclerosis and experimental allergic encephalomyelitis shown by studies carried on animal models of the respective diseases [40, 41]. HSP27 is known to activate monocytes to produce IL-10 which is an anti-inflammatory and immunoinhibitory cytokine [9]. The serum analysis of patients with several diseases showed increase in HSP27 suggesting a role in immune modulation. HSP27 is even capable of altering the expression of surface receptors on monocytes. HSP70 also induces the production of anti-inflammatory cytokine IL-10 and inhibits the production of TNF-α [6]. HSP90 is actively involved in innate immune response as it is a part of LPS receptor complex and its homolog gp96 is mainly involved in folding of Toll-like receptors. Immune reactions to bacterial HSP60 (GroEL) is so prominent in some instances that they should be regarded as the characteristic sign of infection, for example, chlamydial HSP60 was detected in atherosclerotic lesions [42]. The CD14 receptor is well known for its binding to LPS and other bacterial products. Interestingly, it was found that HSP60 could interact with CD14 receptor and activates innate immune response by activating mononuclear cells [43]. Human HSP60 could activate TNF-a, IL-6, IL-12, IL-15, and NO which is the same as classical bacterial inflammation agent LPS [44, 45]. From these findings it is evident that HSP60 acts as an endogenous danger signal. TLR2 and TLR4 are the receptors of HSP60, HSP70, and gp96 (similar to cytoplasmic HSP90). The latest in the list is the study of efficacy of HSP-based vaccine against pneumococcal carriage, lung colonization, and sepsis in mouse models. The immunization elicitated by use of HSPs includes ClpP (HSP100/Clp peptidase subunit), DnaJ (HSP40), and GroEL (HSP60), to protect against using different serotypes of Streptococcus pneumonia [46].

2.1.4 Early Pregnancy Factor (HSP10)

HSP10, also called chaperonin 10 (Cpn10), is a eukaryotic homolog of the bacterial GroES protein, which serves as a co-chaperone for HSP60 (GroEL) in protein folding and assembly processes. HSP10 is usually found localized in mitochondria [47]. After years of study and equally followed by controversies, it is now a well-accepted fact that HSP10 is an early pregnancy factor (EPF) acting as a secreted hormone or cytokine to inhibit immune responses in pregnancy. EPF appears in the maternal serum within 24 h after fertilization hence the name early pregnancy factor. It has been detected in different animal species such as mouse [48], sheep [49], pig [50], horse [51], and cow [49, 52]. Detectable quantities of EPF were found to be present in serum [53], amniotic fluid [54], and fetal serum [55] of pregnant women. The growing fetus and mother contain different sets of major histocompatibility antigens, so the fetus is allograft to mother; at this stage HSP10 comes to the rescue [10]. In this context, HSP10 can be used as a biomarker for detection of occurrence of fertilization, predicting the prognosis of pregnancy, and also as a marker to evaluate embryo quality which is necessary to measure the success of in vitro fertilization and embryo transfer treatment.

2.1.5 Nonmedical, but Commercially Important Applications

Induction of HSP expression not only develops resistance to heat shock but also helps in increase in other commercial characters such as silk in Silkworm (*Bombyx mori*). Pre-exposure to higher temperature has been reported to increase silk production by virtue of expression of HSPs in the silkworm larvae [56].

Recently, Ray [57] designed heat-shock-resistant surfaces using immobilized α-Crystallin. α-Crystallin is a multimeric protein that belongs to a group of small HSPs The α-Crystallin grafted surfaces could also lead to the design of potent heat-shock-resistant surfaces that can find wide applications in storage and shipping of protein-based biopharmaceuticals.

In another interesting study, Yoshida [8] showed that the neurotoxin present in *Enterobacter aerogenes,* which is a salivary symbiont of insect predator known as Antilon, is nothing but HSP60. A single residue change in a protein makes it to be a potent insecticide.

2.2 Conclusion

The three roles of HSPs, such as chaperone and protecting cells from stress, antigen display on the cell surface, and secretion from the cell, make them one of the highly versatile proteins. This versatile nature answered most of the questions that arose in the molecular biologist's mind decades ago. Because HSPs are phylogenetically conserved, their role in autoimmune diseases is obvious. HSPs are overexpressed by different tumors and are also released into the extracellular milieu, which makes them wonderful therapeutic targets and diagnostic markers. The involvement of HSPs in diverse biological and immunological processes could be potentially used in many therapeutic applications. The induction of different HSP syntheses by novel pharmaceuticals or crude plant preparations or by other means might boost the organism's ability to withstand potentially damaging environmental insults, could be used as a novel strategy for intervention in cardiovascular and retinal injuries, as well as in organ preservation and transplantation, to name but a few. Based on previous findings HSPs have the potential to be used in the development of immune system-based therapeutics that are targeted against diseases such as cancer and autoimmune diseases. Although a large number of data are published in high impact journals, the challenge still exists before the scientific community to evaluate the benefits and risks associated with such applications of HSPs. There are many other HSPs whose biomedical applications are yet to be explored.

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Chapter 3
Biomedical Applications of Silkworm Pupae Proteins

Dhiraj Kumar, Param Dev and R.Venkatesh Kumar

Abstract Silkworm is a biologically important and unique insect which engineer a structure called cocoon. Apart from extraction of silk fiber from cocoons, this complex fibrous protein membranous shell ensures the successful metamorphosis of the silkworm larvae to pupae and finally to silk moth. The pupae of mulberry and non-mulberry silkworms have been in consideration as new available source of high quality protein that contains all the essential amino acids required for human health. In recent years, research has been focused on various biomedical applications of silkworm pupae proteins. Pupae proteins are efficiently worked in wound dressings, hepatoprotective and antiapoptotic activity, antigenotoxicity, regulation of blood glucose and lipids, anticancer agent, etc. Therefore, silkworm pupae could be utilized as food supplement and its enormous proteins open the new dimension for biomedical science.

Keywords Silkworm pupae · Proteins · Diseases · Biomedical applications

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3.1 Introduction

Among the economically beneficial insects, silkworms are an incredibly significant animal model for researchers next to Drosophila, belonging to the order Lepidoptera of phylum Arthropoda. India is the only country which hosts all five known commercially exploited types of natural silkworms (Mulberry, tropical tasar, temperate tasar, eri, and muga) along with several of their wild relatives. During metamorphosis all five types of silkworms pass from egg, larva, pupa, and adult stages to complete their life cycle, therefore they are known as holometabolic insects. Silkworms are the only identified insects that provide food, fiber, and biomedical significance. After spinning of silk (cocoon) at larval stage they convert into pupa inside the cocoon shell. Silkworm pupae are obtained after the extraction procedure of silk thread and are not used commercially as an edible insect in India except in the northeastern states. They consume silkworm pupa in their diet regularly due to its rich protein content and several medicinal properties.

Silkworm pupae protein has been considered to be a new available source of high-quality protein that contains all the amino acids needed by the human body. Nevertheless, it is not popular among consumers, where silkworm pupae are an interesting optional product. Silkworm pupae are used as animal feed, organic fertilizer, food material, and traditional medicine in a few Asian countries, namely Korea, China, Thailand, Japan, and India [1–5]. Silkworm pupae are the optional food product and people began its commercialization in different food and biomedical industry to fulfill the nutritional requirements of hidden hungers and treatment of various diseases of mankind.

3.2 Nutritional Value of Silkworm Pupae

Silkworm pupae contain 55.60 % of total protein and 32.2 % lipid content by dry weight. The silkworm pupae protein is boosted with high level of essential amino acids, namely methionine, valine, and phenylalanine. The contents of essential amino acids of silkworm pupae protein fulfill the requirements of FAO/WHO/UNU as they suggested in 2007. Aside from the high protein content it is also a chief source of omega-3 fatty acids, particularly α-linolenic, linoleic acid, DHA, and EPA.

Silkworm pupae per 100 g sample contain various biochemicals such as 55 g protein, 8.5 g fat, 6 g fiber, 25.43 g carbohydrates, and 389.60 (Kcal/100 g) energy contents. Silkworm pupae also contain diverse mineral compositions (mg/100 g) such as 102.31 mg calcium, 1826.59 mg potassium, 287.96 mg magnesium, 1369.94 mg phosphorus, 274.57 mg sodium, 9.54 mg iron, 17.75 mg zinc, 2.08 mg manganese and copper, and 0.08 mg selenium to complete the mineral requirement in a healthy diet. Additionally, it also comprises a number of vitamins such as Vitamin A (273.99 μg), Vitamin E (51.45 IU/kg), Vitamin C (<5.78 mg), Vitamin B1 (1.91 mg), Vitamin B2 (5.43 mg), Vitamin B3 (15.20 mg), Vitamin B5 (12.49 mg), Vitamin B7 (144.51 μg), Vitamin B9 (0.41 mg), and Vitamin B12 (0.5 mg/100 g).

Table 3.1 Amino acid composition in different types of silkworm pupae

Amino acid composition (g/100 g)	Mulberry silkworm pupae	Tasar silkworm pupae	Eri silkworm pupae
Aspartic acid	10.9	6.41	9.89
Threonine	5.4	4.64	4.75
Serine	4.7	4.64	5.25
Glutamic acid	14.9	12.7	12.9
Proline	4.0	–	6.46
Glycine	4.6	4.42	4.94
Alanine	5.5	6.26	6.14
Cystine	1.4	1.5	0.53
Valine	5.6	6.63	5.36
Methionine	4.6	1.47	2.31
Isoleucine	5.7	7.95	4.42
Leucine	8.3	3.24	6.63
Tyrosine	5.4	2.06	6.40
Phenylalanine	5.1	8.1	5.24
Histidine	2.5	2.94	2.67
Lysine	7.5	4.54	6.54
Arginine	6.8	12.2	4.41
Total amino acids	102	89.8	94.8
Total essential amino acids	51.5	43.1	44.9

Source Roa [8], Zhou et al. [9, 10], Longvah et al. [11]

In the fat bodies of larvae, pupae, and moths of silkworm 138, 217 and 86 expression of protein profiles were determined, respectively, of which 12 were shared by the three stages. 92, 150, and 45 specific proteins were identified in the larval, pupal, and moth stages, respectively, in which 17, 68, and 9 had very important functional annotations. Numerous ribosomal proteins (L4, L5, L23, P2, S3, S10, S11, and S15A) are established in fat bodies of silkworm pupae, while only three (L14, S7, and S20) were found in larval and moth fat bodies. Furthermore, 23 metabolic enzymes are also present in the pupal stage of silkworm, whereas only four and two metabolic enzymes are known in the larval and moth stages [6]. Recently, it has been documented that silkworm pupae have excellent antioxidant potential to scavenge free radicals and good antityrosinase activity and also high levels of palmitic acid, oleic acid, stearic acid, linoleic acid, and palmitoleic acid in profiles of fatty acids [7]. Amino acids composition in different types of silkworms pupae are shown in Table 3.1.

3.3 Biomedical Applications of Silkworm Pupae and Their Protein

3.3.1 Antiapoptotic Activity of 30 kDa Lipoproteins

Silkworm pupa fat body and hemolymph are abundant in 30 kDa family of lipoproteins (LP1-5) and are low molecular weight lipoproteins. It was confirmed that the silkworm protein 30Kc6 is one of the members of the 30K family proteins that transport lipids and inhibit cell apoptosis in the insect and mammalian cells [12–15]. However, effects of 30Kc6 on cell apoptosis of human vascular endothelial cell (HUVEC) and the underlying mechanism are largely unknown.

In vivo data of Wei [16] demonstrated that oral feeding of the silkworm protein 30Kc6 dramatically improved the conditions of atherosclerotic rabbits by decreasing serum levels of total triglyceride (TG), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), and total cholesterol (TC). Furthermore, 30Kc6 alleviated the extent of lesions in aorta and liver in atherosclerotic rabbits. These data are not only helpful in understanding the antiapoptotic mechanism of the 30K family proteins, but also provide important information about prevention and treatment of human cardiovascular diseases.

3.3.2 Antioxidant, Antigenotoxic, and Hepatoprotective Properties

Silkworm pupae contain vitamin B2, which can be important to avoid the serious effects of vitamin B2 deficiency [17]. Further, fermented silkworm powder has a protective effect in alcohol-induced hepatotoxicity in a rat model [18]. Further, it is reported that silkworm pupae contain 45–55 % protein content on a dry matter basis, which can significantly increase the hemoglobin and serum total protein in rats, producing protective effects on the liver in carbon tetrachloride-induced rat hepatic injury [19, 20]. Meetali [7] also evaluated the antioxidant and antigenotoxic effects of Muga silkworm pupa, therefore, it is recommended that silkworm pupae can be utilized as natural antioxidants in various food products.

3.3.3 Role as Bioreactor

Researchers report that recombinant proteins have been identified in *Bombyx mori* cells or silkworm larvae, apart from pupae. Alternatively, Jian [21] selected silkworm pupae to express the protein of interest using *B. mori* nucleopolyhedrovirus (BmNPV). They studied the expression, purification, and characterization of human granulocyte macrophage colony stimulating factor (GM-CSF) using silkworm pupae (*B. mori*) and concluded that silkworm pupae could be more suitable for expression of heterologous proteins as a bioreactor.

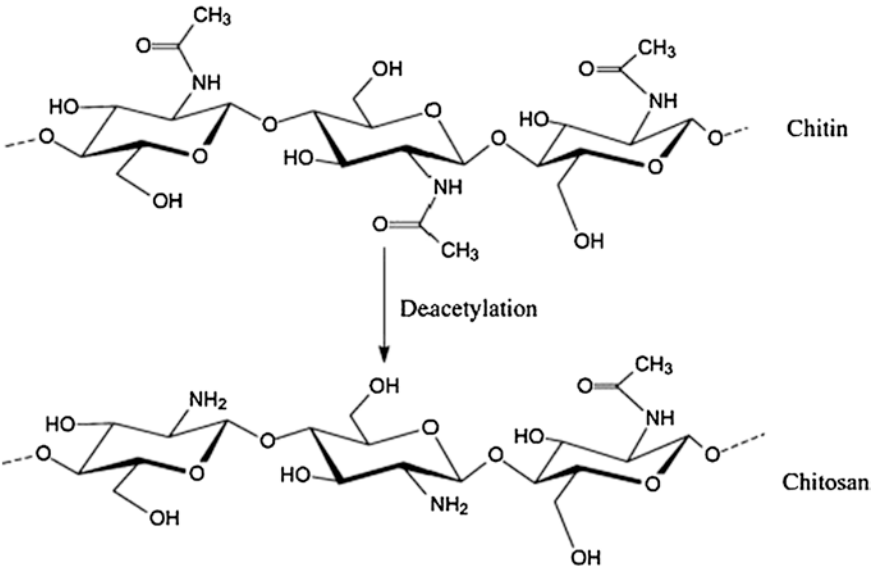

Fig. 3.1 Mechanism of chitosan production from chitin

3.3.4 Silkworm Pupae Chrysalides and Its Role in Pharmaceutical Industries

Silkworm pupae exoskeleton and internal organs such as spiracle and tracheae are lined by chitin. Nevertheless, the chrysalides of the silkworm pupae are an alternative source of chitin and consequently of chitosan [22].

β-1,4-N-acetyl-D-glucosamine (chitosan) is a derivative of chitin after deacetylation (Fig. 3.1). Importantly, chitosan is a biodegradable cationic biopolymer and could assist in reduction of pollutants in residual waters by adsorption and chelating with heavy metallic ions and can also act in coagulation of colloidal particles and silkworm pupae chrysalides having anticancerous property [23–26]. The fatty acids of odd numbers contained in the silkworm chrysalis oil also has high antitumor activity. Despite this, chitin and chitosan are being exploited in a variety of biomedical applications, including drug delivery, tissue engineering, tissue scaffolds, and wound dressings. The polycationic properties of chitosan are being developed for use in biosensors by immobilizing enzymes, in wound dressings to induce cell migration and proliferation at the wound site, and in tissue engineering as a scaffold [27].

Chitin has a great role in silkworm. Chitin has been used to prepare affinity chromatography column to isolate lectins and determine their structure [28]. Chitin and 6-O-carboxymethyl chitin activate peritoneal macrophages in vivo, suppress the growth of tumor cells in mice, and stimulate nonspecific host resistance against *Escherichia coli* infection.

Marguerite [29] studied the possible applications of chitin and chitosan. Austin [30] and Hirano [31] for the first time processed chitin in the form of films and fibers from silkworm pupae skin. However, the major development of chitin film and fiber is in pharmaceutical and medical applications as wound dressing material [32, 33] and controlled drug release [34]. In addition, an interesting application is composite bone filling material, which forms a self-hardening paste for tissue regeneration in treatment of periodontal bony defects [35] and its oligomers have been claimed as anticancer drugs. Wattanathron [36] identified that silkworm pupae protect against Alzheimer's disease. Biological properties of chitosan are biocompatibility, hemostatic, bacteriostatic, fungistatic, spermicidal, and anticholestermis.

3.4 Other Significant Biomedical Applications of Silkworm Pupae

Oiled and de-oiled silkworm pupae contain high-quality and quantity of protein. There are various pharmacological functions in the human body also recorded.

3.4.1 Regulation of Blood Lipids

Pupal oil can effectively reduce triglycerides, prevent and treat fatty livers [37], protect the liver after consumption of alcohols, improve the blood quality and the environment within the blood vessel, effectively soften the blood vessels, lower blood pressure, and prevent arteriosclerosis and thrombosis.

3.4.2 Reduction of Blood Glucose

Pupal oil enables the prostaglandins to maintain balance with effects of preventing prostate diseases, improving the functions of insulin-producing beta cells, restoring the fatty acid desaturase activity of cells in diabetic patients and has marked hypoglycemic effect free from reoccurrence [38].

3.4.3 Improvement of Physical Fitness

The natural steroids contained in the oil can improve fertility and enhance sexual function; the unsaturated fatty acids in the oil which cannot be synthesized by

humans can enhance the flexibility of immune cell membrane, increase the vitality of the immune cells, so that the barriers to human health are more robust, and the occurrence of sub-health and disease is effectively prevented.

3.4.4 Replenishment of Brain Power and Enhancement of Intelligence

The metabolites EPA and DHA, commonly known as Brain Gold, can promote the synthesis of nucleic acid and monoamine neurotransmitters in the brain and effectively enhance the mental memory.

3.4.5 Skin Care

The α-linolenic acid and other active substances contained in pupal oil can join the synthesis of human tissue cell membranes, effectively prevent and mitigate symptoms like wrinkles, pigmentation, sallow skins, and premature aging of modern women. At the same time it can improve the body superoxide dismutase activity and decrease free radical with good antiaging effects.

3.5 Future Prospective and Conclusion

Silkworm pupae are natural enormous byproducts of the silk industry with excellent applications in the field of biomedical science and the pharmaceutical industry. The proteins extracted or identified from silkworm pupae expressed great medicinal value to cure different deadly diseases. Most importantly, functions of pupal protein include drug delivery, tissue engineering, tissue scaffolds, wound dressings, regulation of blood lipids and glucose, antiapoptotic, antioxidant, antigenotoxic, hepatoprotective activity, bioreactor, and as anticancer agent.

Silkworms can be used as future animal models since they have been exploited for commercial importance in the past few years for production of recombinant proteins. Concomitantly, the above study will generate valuable information about proteomics of silkworm pupae and in future potential pupal proteins should be investigated through metagenomics, which could be exploited commercially by biomedical, pharmaceutical, biotech, and probiotic industries for the benefit of human health.

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Chapter 4
Studies on Indian Eri Silk Electrospun Fibroin Scaffold for Biomedical Applications

Muthumanickkam Andiappan and Subramanian Sundaramoorthy

Abstract Silk is a natural biopolymer, which mainly consists of two proteins, fibroin and sericin. Silk has better biocompatibility and human blood compatibility than other natural biopolymers. Numerous studies have been conducted on application of cultivated mulberry silk (*Bombyx mori*) fibroin for biomedical applications and research still continues. A type of wild silk, eri (*Samia cynthia ricini*) has less amount of sericin, which induces inflammatory reaction than mulberry silk. Studies on application of Eri silk for biomedical application is limited. This paper discusses the development of electrospun fibrous mat from eri silk fibroin and physical, chemical, and biological characterization of the mat for the properties required for using them as biomaterials. The nanofibers had the average diameter in the range of 400–500 nm. Eri silk was found to possess higher thermal stability than mulberry silk. The cell viability percentage of eri silk fibroin was found to be higher than that of mulberry, and the cell attachment, binding, and spreading on the eri silk fibroin scaffold was superior compared to the mulberry silk fibroin scaffold. The eri silk fibroin scaffold coated with hydroxyapatite enhanced the bone cell attachment and cell growth compared to that of pure silk fibroin.

Keywords Eri silk · Fibroin protein · Tissue engineering · Biomaterial

4.1 Introduction

Tissue loss or organ failure, resulting from traumatic or nontraumatic destruction, require surgical treatments to repair, replace, maintain, or augment the functions of the affected tissue or organ, using some additional functional component, that

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facilitates an improved life to patients. Tissue engineering is an emerging field in biomedical methodology, to support and hasten the regeneration of defective and damaged tissues, based on the natural healing potential of cells derived from a variety of patients [1]. Tissue engineering follows the principles of cell transplantation, materials science, and bioengineering toward the development of biological substitutes that would restore and maintain normal functioning of the human body [2]. The scaffold used for tissue engineering application should mimic the function of the natural extracellular matrix. The primary functions of the scaffold are: (i) to provide an adhesion substrate for the cell, facilitating the localization and delivery of cells when they are implanted; (ii) to provide temporary mechanical support to the newly developed tissue and maintain a 3D structure, and (iii) to support the development of new tissues with suitable functions [3].

4.2 Structure of the Scaffold

The scaffolds are mainly synthesized from biopolymers, using different techniques, such as solvent casting and particulate leaching, gas foaming, fiber bonding, phase separation, melt molding, emulsion freeze drying, and solution casting and freeze drying. However, these systems have inherent limitations, which offer very insufficient pore size and porosity to the scaffold. The scaffold in different structures such as films, braids, and knits has been used for tissue engineering [4]. The nanofibrous mat has many favorable characteristics, such as high porosity, a wide range of pore size distribution, and high surface area-to-volume ratio, which are favorable parameters for cell attachment, growth, and proliferation [5, 6]. The electrospinning process produces highly porous nonwoven fabric consisting of ultrafine fibers. Electrospinning offers a good opportunity for manipulating the structural and mechanical properties [7] and also enabling the use of different polymers' fibrous assembly as a scaffold for cell culture.

4.3 Material for the Scaffold

Many synthetic biodegradable polymers, such as poly L-lactic acid (PLLA) [8], polyglycolic acid (PGA), polycaprolactone (PCL), copolymers of the above polymers, and other synthetic polymers have been used for developing scaffolds in tissue engineering [9]. They have certain disadvantages such as high rate of degradation under physiological conditions, low mechanical strength, less biocompatibility, and prohibition of the addition of the cell growth factor due to the solvents used for preparation of scaffold [10, 11]. These problems have been overcome by natural biodegradable biopolymers, such as collagen, gelatin, chitosan, and silk fibroin [12].

4.3.1 Silk

Silk is popularly known in the textile industry for its luster and mechanical properties [13]. Silk was discovered in China around 2700 B.C. Silk is traditionally produced by sericulture. This ancient art was practiced in China, Korea, and Japan since the fourth century, and in the sixth century this technique reached Europe via the Silk Route [14]. Silk is now produced across Asia and Europe, although the main sources are China, India, and Japan. Silk has been of interest for over 5000 years not only for its properties of texture, tenacity, and dyeing, but also for its use in cosmetics, creams, lotions, makeup, powders, bath preparations, and pharmaceuticals [15]. Silks are generally defined as protein polymers that are spun into fibers by some *Lepidoptera* larvae such as silkworms, spiders, scorpions, mites, and flies [16]. Silk, a natural filament produced by the silkworm *Bombyx mori* has been used traditionally in the form of filaments in textiles for thousands of years. This silk contains a fibrous protein termed fibroin (both heavy and light chains) that forms the thread core and glue-like proteins termed sericin that surround the fibroin filament to reinforce them together. The fibroin is a highly insoluble protein containing up to 90 % of the amino acids glycine, alanine, and serine, leading to antiparallel β-pleated sheet formation in the filament [17]. Silk has been used as a textile material for a long time. After the removal of sericin from silk fibroin, it has been considered as the starting raw material for non-textile applications, especially in the biomedical, cosmetic, and biotechnological fields, such as surgical sutures, wound cover materials, controlled drug release carriers, tissue engineering scaffolds and repair materials for skins, bones, ligaments, etc. [16]. Silk fibroin has more mechanical strength than other synthetic biomaterials and it has higher combined strength and toughness due to the presence of an antiparallel β-sheet structure. The most extensively used silk is obtained from different types of silkworms such as mulberry silk (*Bombyx mori*), and non-mulberry silks such as Eri (*Attacusricinii*), muga (*Antheraeassama*), and tasar (*Antheraeperni*). The mulberry silk belongs to the *Bombycidae* family, whereas the non-mulberry silks belongs to *Saturniidae*. Silk fibroin causes low inflammatory response to the host tissues, compared to collagen. Silk fibroin has good oxygen and vapor permeability, and high tensile strength with better flexibility than other natural polymers [16, 18]. Silk fibroin possesses tripeptide linkage [Arg (R)-Gly (G)-Asp (D)] (Argnine, glycine and aspartic acid), which supports cell attachment and spreading during cell culture [19].

4.3.2 Eri Silk

Eri silk belongs to the family *Saturnidae* and species *ricini*. The most widely domesticated eri silkworm is *Samia cynthia ricini* [20]. *Samia cynthia ricini* (Family: *Lepidoptera*:*Saturniidae*), the Indian eri silkworm, contributes significantly to

the production of commercial silk, and is widely distributed in the Brahmaputra river valley in northeastern India [21]. Eri silkworm (*Samia ricini*) is a traditional source of food in northeast India, where it is grown primarily for silk and food uses [22]. The eri silkworm is polyphagous in nature and feeds on leaves of several food plants [23], but 'Castor plant' (*Ricinuscommunis*) is the most important host plant. The leaves of plants like kesseroo (*Heteropanaxfragrans*), 'Gomari' (*Gmelinaarborea*), 'Gulancha' (*Tinosporacordifolia*), etc., are also used [24, 25]. The study shows that the castor plant feed yields more silk than other plant feeds. Eri silkworm is multivoltine in character, and can be reared indoors. It is generally hardy and not susceptible to diseases.

4.3.3 Composition of Eri Silk

The eri silk filament is extruded from the silkworm; it consists of two portions, fibroin and sericin. Sericin is glue-like gum that covers the core filament. Both fibroin and sericin are produced by very large flattened cells lining a pair of long tubular silk glands.

The silk gland is divided into three regions, the thin and flexuous posterior part, and the wider middle and anterior parts. The silk fibroins are synthesized in the posterior part of the silk gland, and then transported down the lumen into the middle part of the silk gland, in which it is stored in a concentrated state as a weak gel before spinning. It has been pointed out that the silk press part is important in the process of fiber formation from the liquid silk fibroin [26]. Eri silk is mainly made up of fibroin, sericin, and fat. Sericin is a non-filament which is dissolved in water, whereas the fibroin is a filament covered by sericin. The percentage of chemical composition of eri silk is listed in Table 4.1.

4.3.4 Physical Properties of Eri Silk

Eri cocoons are usually white in color; however, brick red color cocoons are also available. Eri silk cocoons cannot be reeled as they are made up of entangled layers, and are therefore spun like cotton into yarn. The reeling process does

Table 4.1 Chemical composition of eri silk

Component	Percentage (%)
Fibroin	72.2
Sericin	5–11.9 %
Fat	1.3
Moisture	14.6

Eri silk contains low amount of sericin of 4.96 % compared to 10 % in mulberry silk, 8.62 % in tasar silk, and 7.88 % in muga silk [25]

not involve the killing of silk moth, as it is an open-mouthed cocoon [27]. The fineness of eri silk ranges from 14 to 16 μ. The average filament length of eri silk is approximately 450 m. Eri silk is durable and strong with a typical texture. Eri silk is similar to cotton and has a unique aesthetic appeal. It appears like wool mixed with cotton and the softness of silk. Each eri cocoon weighs about 1–5 g with a shell weight of 0.2–0.7 g. The denier (d) of the filament is 2.2–2.5 d, with a tenacity of 3–3.5 g/d. Eri silk has an elongation at break percentage of up to 20–22. It has excellent thermal properties, and can be substituted for wool. The moisture retention capacity is 11 %. Eri silk is more crystalline than any other non-mulberry silks [28]; also, eri silk has a higher elasticity, strong durability and immunity against disease and insects.

4.3.5 Chemical Properties of Eri Silk Fibroin

Eri silk fibroin consists mainly of repeated similar sequences (about 100 times) of alternative appearances of the polyalanine $(Ala)^{12-13}$ region and the Gly-rich region [29–31]. Eri silk is a protein containing two major amino acid residues, alanine and glycine. *Samia cynthia ricini* is a wild silkworm and the amino acid composition of the silk fibroin is different from that of the silk fibroin of the domesticated silkworm *Bombyx mori*. The sum of Gly and Ala residues in Eri silk is 82 % which is similar to mulberry silk, but the relative composition of Ala and Gly is reversed [32]. The primary structure of eri silk fibroin is composed of alternate blocks of polyalanine and glycine-rich regions. The alanine is dominant in the crystalline region of the silk fibroin, whereas the glycine (Gly) motif provides elasticity to the amorphous region of the silk fibroin. The eri silk fibroin is in the form of α-helix and random coil structure (silk I structure) in the silkworm gland as shown in Fig. 4.1 and the silk II structure is present in the spun silk filament after spinning, which is attributed to the β-sheet in the silk fibroin [32, 33]. Three major polypeptides are found in eri silk with different molecular weights of 97, 45, and 66 kDa. The molecules of 66 kDa represent sericin, whereas 97 and 45 kDa indicate the presence of polypeptides, which are connected by a disulfide bond in the silk fibroin. The eri silk fibroin aqueous solution contains 70 % alanine in a helix structure, and the rest of the alanine is in the form of a random coil structure. The silk liquid transition from α-helix to β-structure by a thermal or mechanical method was observed by Asakura et al. [32] and Nakazawa et al. [34].

4.3.6 Eri Silk Fibroin as Biomaterial

Mulberry and tasar silk fibroins, in various forms, have been widely used for tissue engineering and other medical applications. However, muga fibroin is used only in the form of nonwoven film, foam, and sponge for tissue engineering applications.

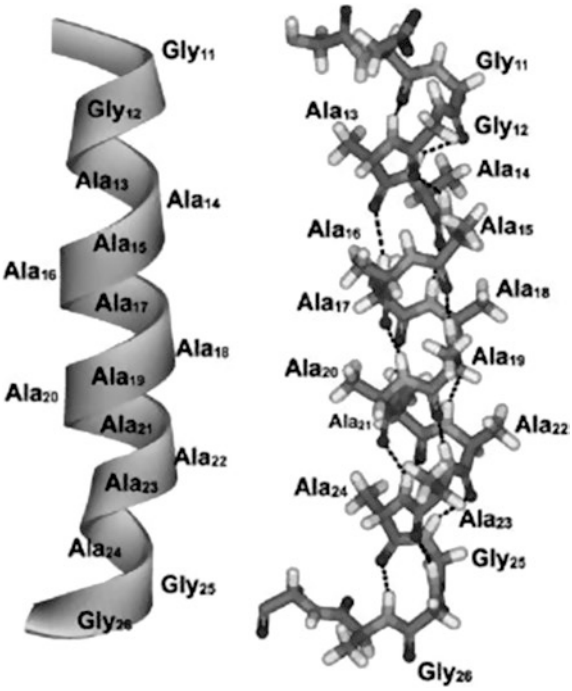

Fig. 4.1 α-Helix structure [29]

The application of eri silk (*Samia cynthia ricini*) fibroin as a biomaterial is limited compared to other silk fibroins, as it is a rare species available only in certain regions of Asian countries. The sericin content in silk is one of the factors which induce an inflammatory response in the tissue. The eri silk contains lower amount of sericin than mulberry silk and other wild silks. The eri silk is a wild silkworm and the amino acid composition of its silk fibroin is different from that of the silk fibroin of the domesticated silkworm, mulberry. The sum of glycine (Gly) and alanine (Ala) residues is 82 %, which is similar to mulberry silk, but the relative composition of Ala and Gly is reversed [35]. Eri silk fibroin contains a higher amount of moisture than mulberry silk, which enhances cell attachment and spreading. The eri silk fibroin has higher amount of hydrophilic and positive charged amino acid, which improves cell viability and cell attachment [36]. Generally all wild silks have higher tensile strength than mulberry silk.

Non-mulberry silkworms such as *Philosamia ricini* (eri silk) are hardy, resistant to environmental stress, and less susceptible to diseases compared to *bombyx mori* and other mulberry silkworms due to difference in the biochemical composition and immunochemical characteristics of fibroin heavy [37]. The low density of all non-mulberry silks has higher proportion bulkier groups in the silk fibroin and their structure has higher amorphous content [38, 39]. The non-mulberry silks (eri silk) basically contain more amounts of hydrophilic groups, amino acids, and possess higher molecular weight [38, 39]. Hence, eri silk may be a better material for being used as scaffold for tissue engineering applications.

4.4 Production and Characterization of Electrospun Eri Silk Fibroin Scaffold

Silk fibroin scaffolds were used in various forms such as film, foam, sponge, hydrogel, and electrospun mat for biomedical applications [40]. However, the silk fibroin scaffold has the problem of curling and shrinking, when treated with the solutions used for tissue culture. Amiraliyan et al. [41] and Li et al. [42] treated the electrospun nanofibrous mat of mulberry silk (B*ombyx mori*) with methanol and ethanol, to improve its structural stability and crystallinity. Muthumanickkam et al. [43] treated the eri silk fibroin electrospun scaffold with ethanol to prevent the problem of curling and shrinking during tissue culture.

4.4.1 Preparation Eri Silk Fibroin Scaffold

The eri silk was degummed with sodium carbonate solution to remove the sericin from the silk filament. The degummed silk (silk fibroin) was dissolved in trifluoroacetic acid. The fibroin solution was used to make fibrous mat using electrospinning method. Then it was treated with ethanol at room temperature for 30 min to improve the dimensional stability.

4.4.2 Characterization of Eri Silk Fibroin Scaffold

The degradation study of scaffold was carried out by immersing it in PBS solution with protease enzyme. The degradation rate at different periods was studied by measuring weight loss. FTIR spectroscopy showed that the amide I band intensity had decreased due to increase in degradation time, and the amide II band intensity had completely disappeared [44], due to breakage of the tripeptide bond in the eri silk fibroin. TGA showed that enzymatic treated eri silk scaffold started to decompose at 100 °C, whereas the raw eri silk fibroin scaffold started to decompose at 350 °C, due to breakage of peptide bond in the eri silk fibroin by the protease enzyme. SEM was taken before and after the degradation of surface of the eri silk fibroin scaffold by the protease enzyme. The images suggested that the fibroin was degraded through a surface erosion process, i.e., individual layers of the silk fiber had fragmented from the exposed surface area, which could lead to complete degradation.

The scaffold pretreated with ethanol was not structurally damaged by the PBS, whereas in the scaffold directly treated with PBS, the surface was structurally deformed. Weight loss of the scaffold was observed for 30 days of enzymatic degradation; the weight loss had increased with increase in incubation time. The porosity of the scaffold decreased after the ethanol and PBS treatment due to

shrinkage of the scaffold. The enzymatic degradation of the eri silk fibroin scaffold was lesser than that of the raw eri silk fibroin. The enzymatic degradation caused a reduction in the tensile stress of 27.2 %, due to breakage of the peptide bond in the eri silk fibroin.

4.5 Comparison of Scaffolds Produced from Eri Silk and Mulberry Silk Fibroins

Mulberry silk is being widely used as biomaterial; Mutumanickkam et al. [43] compared the electrospun nanofibrous scaffolds produced from eri silk and mulberry silk fibroins for physical and biological characteristics.

4.5.1 Preparation of Eri Silk and Mulberry Silk Fibroin Scaffolds

The scaffold was produced from eri silk fibroin by electrospinning method as mentioned in Sect. 4.2. The mulberry silk fibroin scaffold was also prepared under the same conditions as used for eri silk.

4.5.2 Characterization of Eri Silk and Mulberry Silk Fibroin Scaffolds

The eri silk and mulberry silk fibroin scaffolds were produced by the electrospinning method, and the majority of the fibers had diameter of range 401–500 nm. The SEM image of the scaffold is shown in Fig. 4.2. The thermal stability of the eri silk fibroin scaffold was found to be higher than that of mulberry silk fibroin scaffold. The ethanol treatment of scaffold increased the crystallinity percentage and crystal size of both the eri and mulberry silk fibroin scaffolds. The α-helix structure of the untreated nanofibrous scaffold changed to a β-sheet structure [45], due to ethanol treatment of the scaffold. The tenacity was found to be higher for eri silk scaffold than that of mulberry scaffold.

The blood compatibility of scaffold was evaluated using hemolysis test method. The hemolysis percent of the eri silk scaffold was less than mulberry silk scaffold; however, both the scaffolds had hemolysis percent less than the hemolysis value of 5 %, which indicate that both the scaffolds have good biocompatibility. The platelet adhesion of the scaffolds was observed by SEM. The platelet adhesion on the surface of eri silk fibroin scaffold was lesser than that on the mulberry scaffold [46]. The rat L6 fibroblast cells attachment on the scaffolds was observed by SEM. The cell viability percentage of eri silk was higher than that of mulberry, and cell

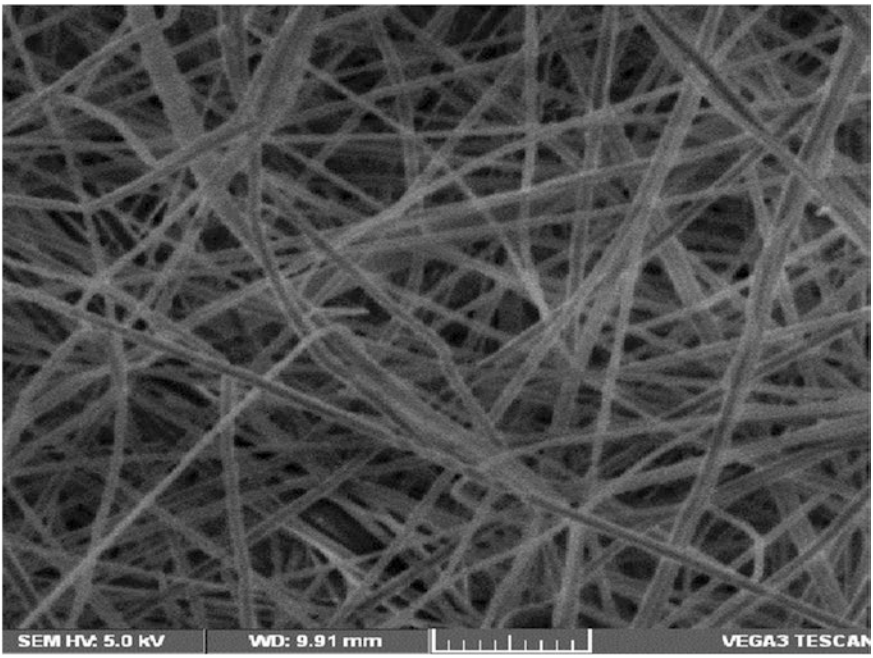

Fig. 4.2 SEM image of eri silk fibroin scaffold

attachment, binding, and spreading in the eri silk fibroin scaffold was superior compared to the mulberry silk fibroin scaffold. Hence, it was concluded that eri silk fibroin scaffolds, which show better performance compared to those of mulberry silk, can be used for tissue engineering applications.

4.6 Electrospun Scaffold Produced from Eri Silk Fibroin Loaded with Amoxicillin

Muthumanickkam [47] produced scaffold from eri silk fibroin loaded with amoxicillin by electrospinning method. NIH 3T3 and myoblast L6 cells line were used to evaluate the biocompatibility and cytotoxicity of eri silk fibroin. The drug loaded scaffolds were characterized using SEM, TGA, and FTIR spectrometer.

4.6.1 Preparation Amoxicillin Loaded Eri Silk Scaffold

The eri silk was immersed in sodium carbonate solution, boiling at 75 °C and at a pH level of 8.5–9.0, for 30 min to remove sericin from the silk fiber. Then, the

eri silk fibroin solution was prepared by dissolving in a mixture of 60:40 (v/v) containing 99 % conc. tri fluoro acetic acid and chloroform for 10 min. The silk fibroin to solvent ratio (w/vol.) was optimized such that the fiber could be formed without spraying or beads formation during electrospinning. The optimum silk fibroin to solvent ratio was found to be 13 % (w/v). Electrospinning was carried out at a voltage of 20 kV with a constant flow rate of 0.5 ml/h using a syringe pump. The distance between syringe and collection drum was kept at 15 cm. To produce the drug loaded fibrous mat, amoxicillin and silk fibroin were dissolved in the mixture of 60:40 (vol./vol.) 99 % conc. tri fluoro acetic acid and chloroform for 10 min. A preliminary study on antibacterial property of drug loaded eri silk fibroin scaffold showed that the zone of inhibition was formed at a minimum of 50 % (w/w) ratio of drug in silk fibroin. Hence drug loaded fibrous scaffolds were produced at two drug to silk fibroin ratios viz., 50 and 100 % (w/w).

4.6.2 Characterization of Amoxicillin Loaded Eri Silk Scaffold

FTIR spectra showed the presence of amoxicillin loaded in the eri silk fibroin scaffold. The amide I band absorption at 1655 cm^{-1} was found in the pure eri silk fibroin scaffold, whereas the absorption band was obtained at 1665 cm^{-1} for 100 % drug loaded scaffold and pure amoxicillin. The amoxicillin drug loaded eri silk fibroin scaffold commenced to decompose at 195 °C, which might be due to the presence of amoxicillin in the eri silk fibroin scaffold. The antibacterial activity of the drug loaded scaffold was assessed by the Agar diffusion method. The area of zone inhibition increased with increase in the percentage of the amoxicillin drug presence in the scaffold. The rate of drug release in the 50 and 100 % drug loaded scaffold was initially rapid and later, it was gradual. The release at the beginning was rapid due to the direct contact of the drug loaded fiber with the PBS. Thereafter, the release was gradual, due to swelling of the fiber present in the scaffold.

4.7 Electrospun Eri Silk Fibroin Scaffold Coated with Hydroxy Apatite for Bone Tissue Engineering

Natural bone is a complex inorganic and organic nanocomposite material, in which about 70 wt% of hydroxyapatite (Hap) nanocrystals and about 30 wt% of collagen fibrils are well organized into a hierarchical architecture over several length scales [48]. Bioactive ceramics, such as hydroxyapatite (Hap) and bioglass are widely used as bone substitute materials because they can bond directly to living bone [49]. Hydroxyapatite possesses higher mechanical strength and better

stability. Synthetic polymeric materials and hydroxyapatite have been used for tissue engineering during the past two decades. Regenerated silk fibroin obtained from *Bombyx mori* silk is the most extensively characterized silk fibroin with outstanding biocompatible properties, and its composite with hydroxyapatite has high osteoconductivity [50–53]. In earlier studies, regenerated *Bombyx mori* silk fibroin and *Antheraea pernyi* silk fibroin were used to synthesize hydroxyapatite mineralized fibroin for bone tissue engineering. Muthumanickkam et al. [54] synthesized the eri silk fibroin (ESF) nano fiber scaffold by the electrospinning method, and the developed hydroxyapatite eri silk fibroin (Hap-ESF) scaffold by alternate soaking in $CaCl_2$ and Na_2HPO_4.

4.7.1 Preparation of Composite of Eri Silk Fibroin and Hydroxyapatite

The eri silk fibroin scaffold was produced by electrospinning method as discussed in Sect. 4.2. The scaffold was immersed in 0.5 M of calcium chloride solution in a tris buffer for 12 h at a pH of 10.4. The scaffold was rinsed with distilled water and subsequently immersed in 0.5 M of Na_2HPO_4 solution in a tris buffer for 12 h at a pH of 10.4. The scaffold was again rinsed with distilled water. The above-mentioned steps were repeated thrice.

4.7.2 Characterization of Composite of Eri Silk Fibroin and Hydroxyapatite

FTIR spectra showed amide I band absorption at 1658 cm^{-1} and amide II band absorption at 1524–1530 cm^{-1} in the *Hap*-eri silk fibroin scaffold, as well as the raw eri silk fibroin scaffold. The intensity of the amide peaks of the *Hap-ESF* scaffold was less compared to that of the pure ESF, which is due to the formation of the bond between the calcium ions and C=O. The thermal stability of *Hap*-coated eri silk fibroin was higher than raw eri silk fibroin scaffold due to the presence of the inorganic hydroxyapatite salt. The *ESF-Hap* coated eri silk fibroin showed a strong intensity peak at 31.40° (2θ) due to the presence of hydroxyapatite in the eri silk fibroin. The crystal size of the *ESF-Hap* scaffold was higher than that of the raw eri silk fibroin scaffold, due to the hydroxyapatite deposited in the eri silk fibroin scaffold. The tensile stress of the *ESF-Hap* coated scaffold was lesser compared to that of raw eri silk fibroin scaffold, because of the increase in stiffness in silk scaffold, due to *Hap* coating. The percentage of the water uptake increased in the *Hap-ESF* scaffold compared with the raw eri silk fibroin scaffold. The hemolytic percentage of the *ESF-Hap* was lesser than that of the raw eri silk fibroin scaffold, due to the presence of hydroxyapatite, which is hydrophilic in nature.

4.8 Conclusion

The platelet adhesion on the surface of the *ESF-Hap* coated scaffold was lesser than that of the raw eri silk fibroin scaffold due to the presence of hydroxyapatite in the coated scaffold, which increased the hydrophilic nature of the coated scaffold. Fluorescent images showed the *hMsc* cell attachment and spreading on the eri silk fibroin scaffold and on the *ESF-Hap* coated scaffold. The cell density of the cell attachment on the Hap-*ESF* coated scaffold and the raw eri silk increased with increase in incubation time. The Hap-*ESF* scaffold showed higher cell viability than the *ESF* scaffold, as the Hap enhanced the hydrophilicity in the scaffold

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Chapter 5
Self-assembled Nanoparticles Prepared from Tasar *Antherea mylitta* Silk Sericin

Shyam Kumar Vootla, Cho Chong Su and Syed Iqbal Masanakatti

Abstract Silk sericin is the gum-like protein surrounding the fibroin in silk fibers, and generally it is removed during the fiber processing to produce fabrics. Like other silk proteins, the silk of tasar silkworms is also composed of sericin and fibroin. In this study tasar silk sericin was extracted and was confirmed to be a 70 KDa protein by SDS PAGE. The use of sericin in the field of biomedical applications has increased their use for various purposes, the extracted silk sericin was pegylated with activated PEG (MW 5000) and the conjugated product was characterized by FT-IR, ^{1}H NMR, CD, DSC, XRD, and AF TEM and self-assembled nanoparticles were prepared.

Keywords Silk naoparticles · *Antherea mylitta* · Sericin · Activated PEG

5.1 Introduction

Silks are fibrous proteins spun by a variety of species including silkworms and spiders. Silk protein consists of two proteins, fibroin and sericin, and sericin should be removed before textile application. The removed sericin is treated as waste, and in the case of *Bombyx mori* silk, about 150,000 tons of sericin is abandoned every

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year. Generally, silkworm silk must be degummed for biomedical applications in order to remove the immunogenic sericin coating, but in this study we propose a novel approach of using silk sericin as drug carrier to specific regions in the body where we can avoid immunogenic problems like in the GI tract [1]. Nature produces enormous biopolymers for specific application but only a part of them has been identified and even less is utilized [2]. Our research goal is characterization of the wild silkworm sericin and further application of this biopolymer in the biomedical fields [3]. We expect that the wild silkworm sericin could be a new candidate polymer in biomedical application. Bioactive molecules can be attached to sericin by covalent bonding in order to form the bioconjugate [4]. Sericin nanoparticles will be prepared by the attachment of polyethylene glycol (PEG) [5]. The sericin nanoparticles will be applied to cosmetic application for protecting active ingredients like retinol.

5.2 Materials and Methods

5.2.1 Materials

2-O-[Methoxy(polyethylene glycol)]-4,6-dichloro-s-triazine (actPEG, MW 5000) was purchased from Sigma (St. Louis, MO). All other chemicals were reagent-grade products obtained commercially.

5.2.2 Preparation of TSS

Tasar Silk Sericin (TSS) was extracted using 1 % NaCl, the silk from tasar cocoons was peeled into pieces, silk was placed in 1 % sodium chloride by shaking overnight. The solution was dialyzed for 2 days (8000 MW cut off), followed by ethanol precipitation.

5.2.3 SDS Page

The extracted protein was analyzed using SDS PAGE 8 % gels to know the molecular weight of the extracted protein using standard protocol.

5.2.4 Preparation of Sericin–PEG Conjugate

actPEG (135.37 mg) was added to 12 ml of 0.10 % (w/v) SS aqueous solution containing 0.1 M sodium borate (pH 9.4) at 4 ℃ [6]. The mixture was then reacted at 4 ℃ overnight. Subsequently, the solution was dialyzed against distilled water using dialysis membrane (MW CO 12,000) for 2 days.

5.2.5 ^{1}H NMR Measurement

^{1}H NMR spectra were measured at 25C on AVANCE 600 spectrometer

5.2.6 FT-IR Measurement

Samples of TSS, PEG, TSS-PEG mixture and TSS-PEG conjugate prepared with KBr pellet were measured on a Thermo FT-IR spectrophotometer

5.2.7 Circular Dichroism (CD) Measurement

CD Spectra of the TSS protein and PEG conjugated protein were measured using a Jasco J-815 spectropolarimeter equipped with a quartz cell having a path length of 10 mm at room temperature.

5.2.8 Differential Scanning Calorimeter (DSC) Measurement

DSC measurements were measured using Universal V3.6C TA instrument at a heating rate of 10 ℃ min-1

5.2.9 Amino Acid Analysis

SS and sericin–PEG conjugate were hydrolyzed under vacuum in 6 N HCl at 110 ℃ for 20 h. The hydrolyzed samples were dried in a rotary evaporator at 40 ℃,

and dissolved in 0.02 N HCl, and applied to an amino acid analyzer after filtering using a Pharmacia Biotech. System Biochrom 20 Plus type amino acid analyzer.

5.2.10 Preparation of TSS Nanoparticles

10 mg of TSS-PEG conjugate was dissolved in 2 ml ethanol, the solution was dialyzed against distilled water using a dialysis membrane (MW CO 12,000) for 2 days.

5.2.11 Particle Size Measurement

Particle sizes were measured using ELS-8000 (Otsuka Electronics)

5.2.12 Transmission Electron Microscope

The nanoparticles were observed using TEM (JEM 1010, JEOL, Japan. One drop of nanoparticles was placed on a copper grid and negatively stained with 1 % uranyl acetate solution for 30 s. The grid was allowed to dry for 10 min and examined

5.3 Results and Discussion

5.3.1 Conformation of Sericin PEG Conjugate

550 mg of the product, sericin–PEG, was obtained from 50 mg of starting material, SS. Assuming that 100 % of SS was recovered, the weight of the product was 8.4-fold higher than that of the starting material. Therefore, sericin–PEG is composed of PEG and SS at the weight ratio of 7.4:1 and contains 11.9 % SS. If the average molecular weights of the amino acid units constituting SS and actPEG are 113 and 5000, respectively, the mole ratio of amino acid residue to PEG is calculated to be 6[=(1/113)/(7.4/5000)]. This result suggests that 16.7 mol% of amino acid residues in SS reacted with PEG [7].

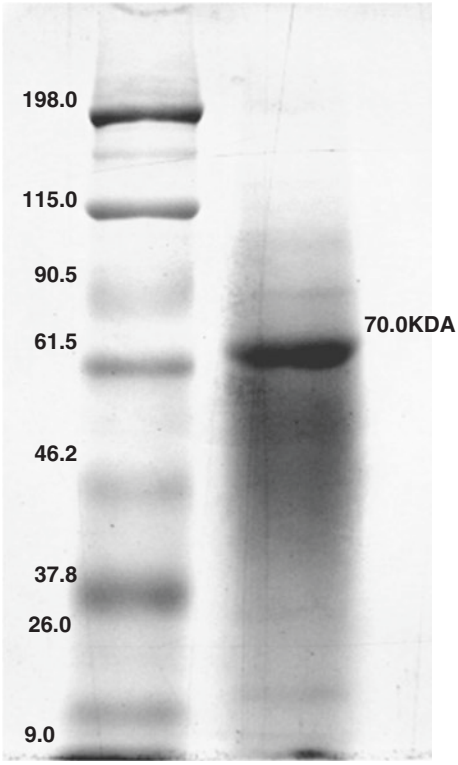

Fig. 5.1 Tasar silk sericin protein on 8 % SDS PAGE gels, lane 1 marker lane 2 tasar sericin (70 KDa)

Electrophoretic separation of the protein extracted by the above-metioned protocol was determined to be a 70 KDa protein as analyzed in 8 % SDS PAGE gels (Fig. 5.1)

Figure 5.2 shows ^{1}H NMR spectrum of sericin–PEG conjugate. The results indicate that the proton peaks at 6.68 and 6.96 ppm of the tyrosine residue in SS shifted downfield to 7.09 and 7.26 ppm, respectively, which suggests a change in the molecular environment of the tyrosine residue caused by modification; that is, the shift is the result of the shielding effect of the triazine ring on the tyrosine residue [8]. Due to the electron-withdrawing effects of the PEG-triazine ring, the valence electron density around the protons attached to the carbon decreased. From the result of NMR, it can be said that the tyrosine residues of the sericin reacted with actPEG. Cyanuric chloride reacted with nucleophilic groups such as amino, imino, and hydroxyl groups [9]. Therefore, the amino group of the lysine residue and the imidazole group of the histidine residue in sericin could react with

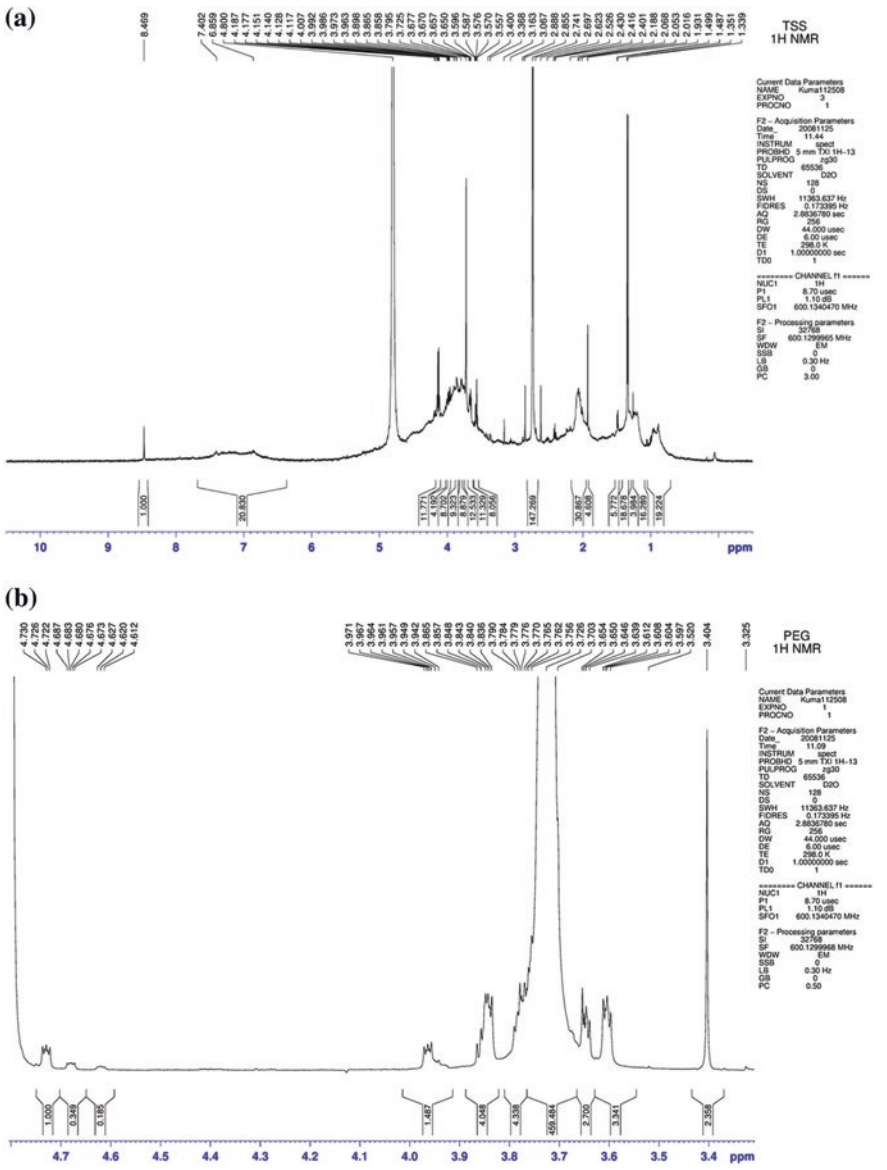

Fig. 5.2 ^{1}H NMR spectra of Tasar Silk sericin (**a**) Activated PEG (**b**) Tasar sericin—PEG conjugate (**c**)

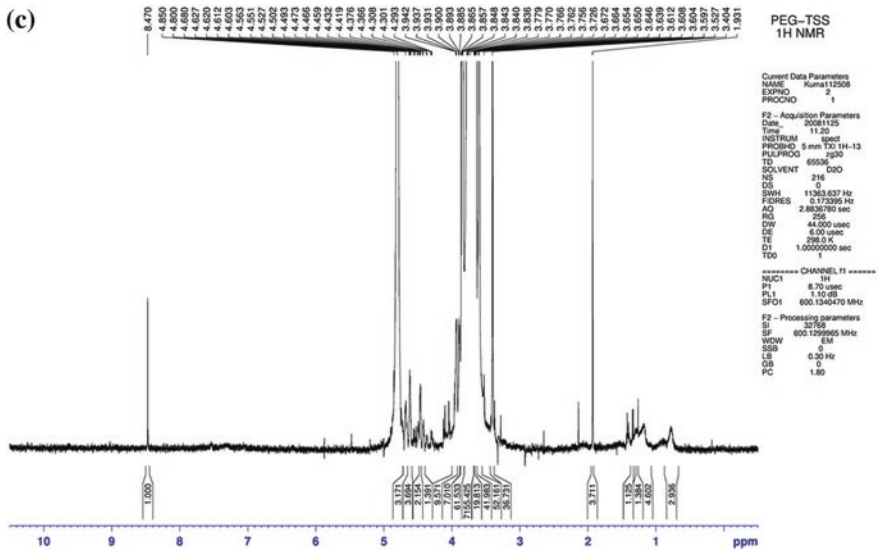

Fig. 5.2 (continued)

actPEG [10, 11]. However, the peaks of these residues could not be detected by NMR measurement due to their very low contents in SS. Amino acid analysis revealed that the contents of serine residues in SS and sericin–PEG were 40.5 and 27.9 %, respectively, an indication that serine residues in SS reacted with PEG, although other residues in SS did not change much after modification.

5.3.2 Conformation of Sericin in Sericin PEG

Figure 5.3 shows IR spectra of SS, sericin–PEG, PEG, and sericin/PEG mixture. In the spectrum of sericin–PEG, two new bands appeared at 2887.2 and 1110.9 cm^{-1} compared with the spectrum of SS. These new peaks were assigned to –CH2– stretching [13] and C–O–C stretching [14], respectively, which indicate that PEG chains are introduced into SS. SS exhibited absorption bands at 1665 (amide I), 1535 (amide II), and 669 cm^{-1} (amide V), which are characteristics of random-coil conformation, whereas sericin–PEG exhibited absorption bands at 1653.8 (amide I), 1546.6 (amide II), and 842.7 (amide V), which are characteristics of sheet structure. The absorption bands of amides I, II, and V of SS in sericin/PEG mixture appeared at 1646, 1530, and 669 cm^{-1}, respectively. The results suggest that the coexistence of PEG molecule in SS caused the conformational change from random coil to β-sheet (Fig. 5.4).

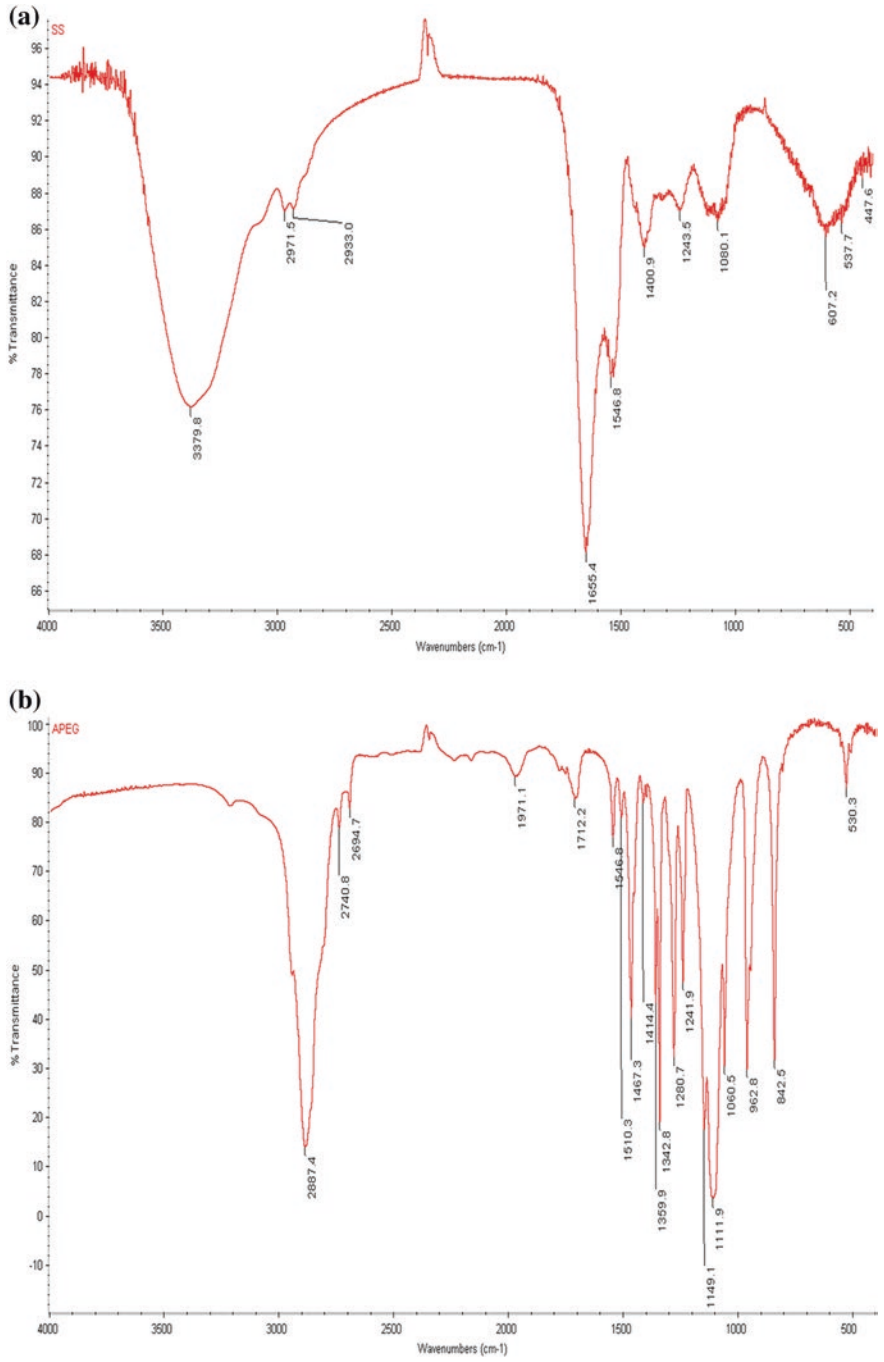

Fig. 5.3 FT-IR spectra of **a** Tasar sericin, **b** Activated PEG, **c** Tasar sericin PEG mixture, and **d** Tasar sericin–PEG conjugate

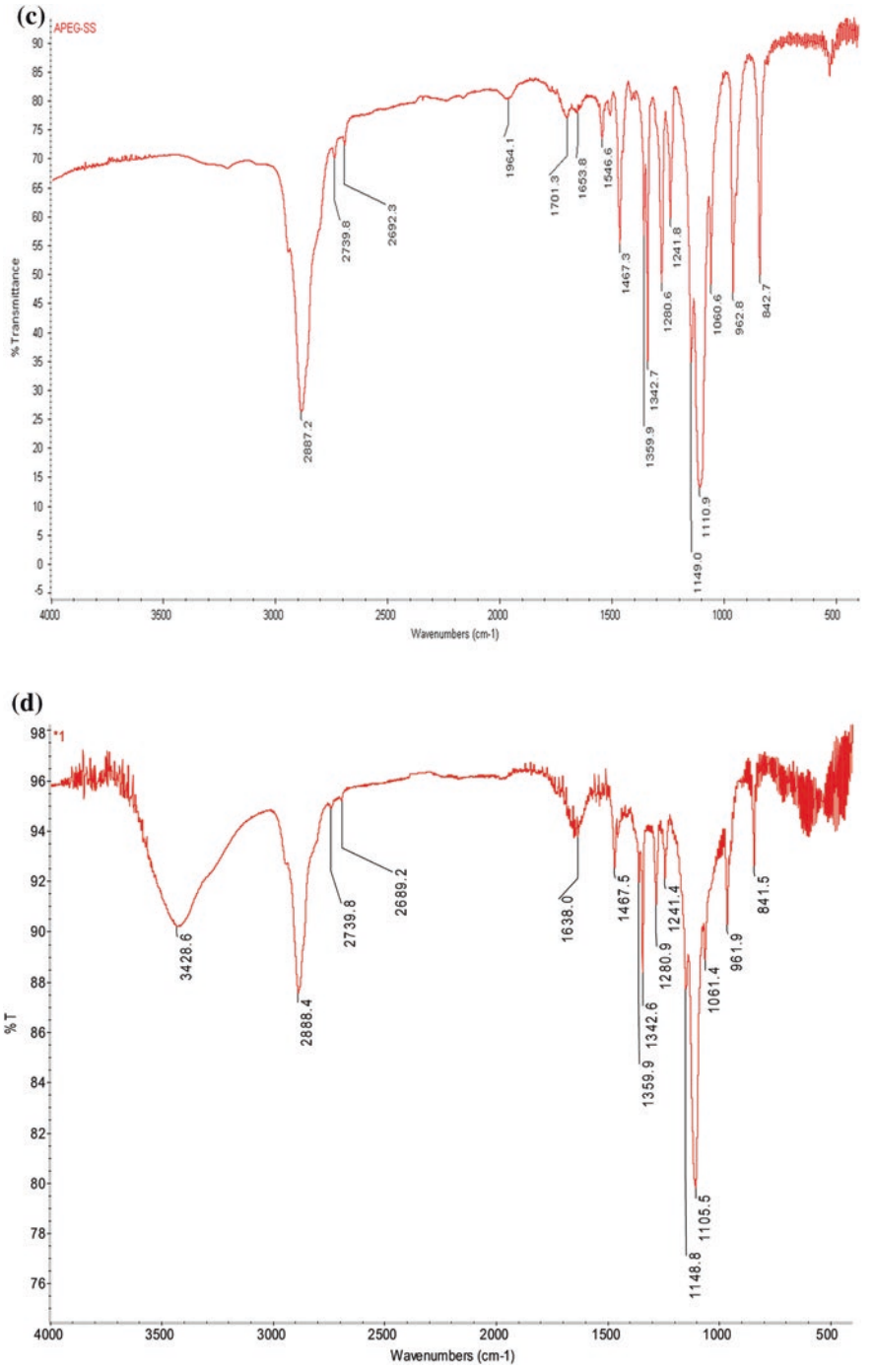

Fig. 5.3 (continued)

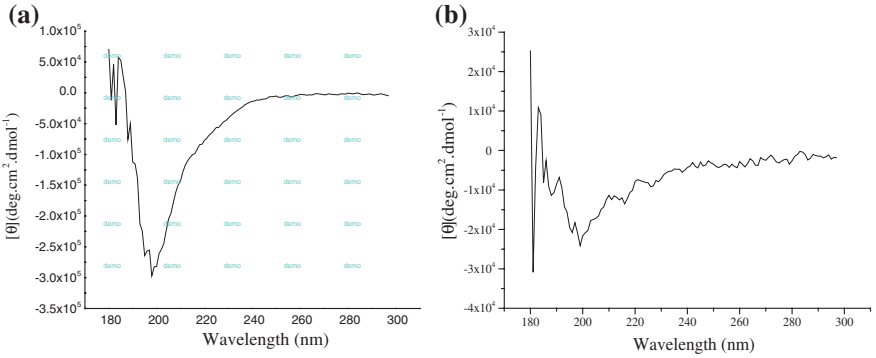

Fig. 5.4 CD spectra of tasar sericin and tasar sericin–PEG conjugate. **a** Tasar sericin. **b** Tasar–PEG conjugate

5.3.3 Thermal Properties of Sericin–PEG

Figure 5.5 shows DSC curves of PEG, SS, and sericin–PEG. Decomposition of SS began to occur at 200–220 ℃, whereas two endothermic peaks were observed at 52.4 and 196 ℃ in sericin–PEG. The melting point of PEG was 56–59 ℃ as already reported [6]. Thus, the endothermic peak of sericin–PEG at 52.4 ℃ is considered to be due to the melting of PEG in sericin–PEG, probably caused by a decrease in the crystallinity PEG after conjugation with sericin. Thermal decomposition temperature of SS (198 ℃) shifted to 196 ℃ in sericin–PEG. In addition, the exothermic peak, which appeared at 162 ℃ for sericin–PEG, can be attributed to the transition from random coil to β-structure of tasar sericin.

5.3.4 Preparation of Nanoparticles

Particle sizes of the sericin–PEG nanoparticles with a mean diameter of 204.3 nm prepared by the diafiltration method are shown in Table 5.1. The self-assembled polymeric nanoparticles were prepared from the sericin–PEG conjugate consisting of sericin and PEG as the hydrophobic and hydrophilic parts, respectively. Figure 5.6a, b show SEM and TEM photographs of sericin–PEG nanoparticles, respectively. Shapes of the nanoparticles were almost spherical, and the sizes ranged about 200–400 nm in diameter (Fig. 5.6).

CD spectra of SS and sericin–PEG in aqueous solution are shown in Fig. 5.4. CD spectrum of SS showed a peak at 200 nm trough, an indication of a random coil conformation. On the contrary, the spectrum of sericin–PEG in aqueous solution exhibited a negative peak at 200 nm and a negative extreme at 220 nm, which were characteristic of β-sheet structure containing a random coil conformation [6, 14]. The results suggest that conformational change of SS in sericin–PEG occurred from random coil to β-structure after the introduction of PEG.

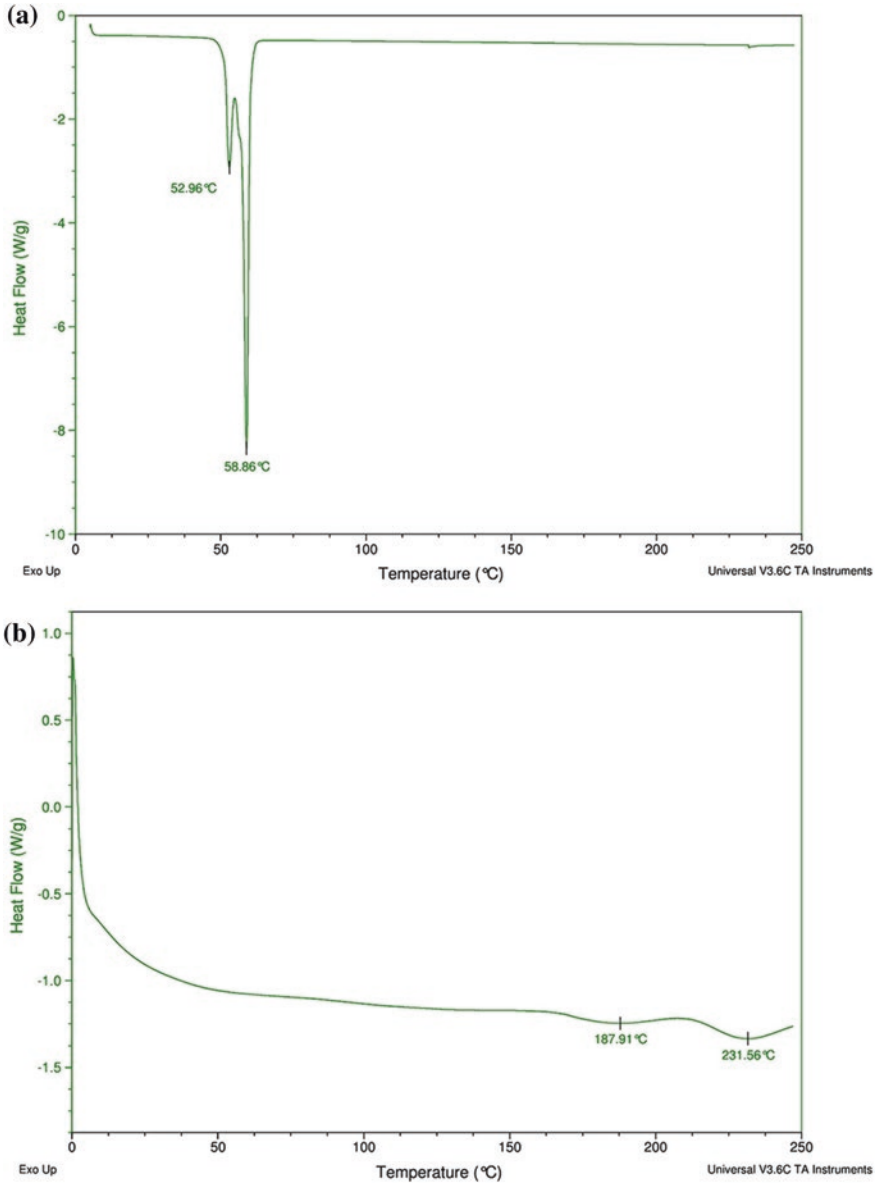

Fig. 5.5 DSC thermogram of activated PEG (**a**) Tasar sericin (**b**) Sericin–PEG conjugate (**c**)

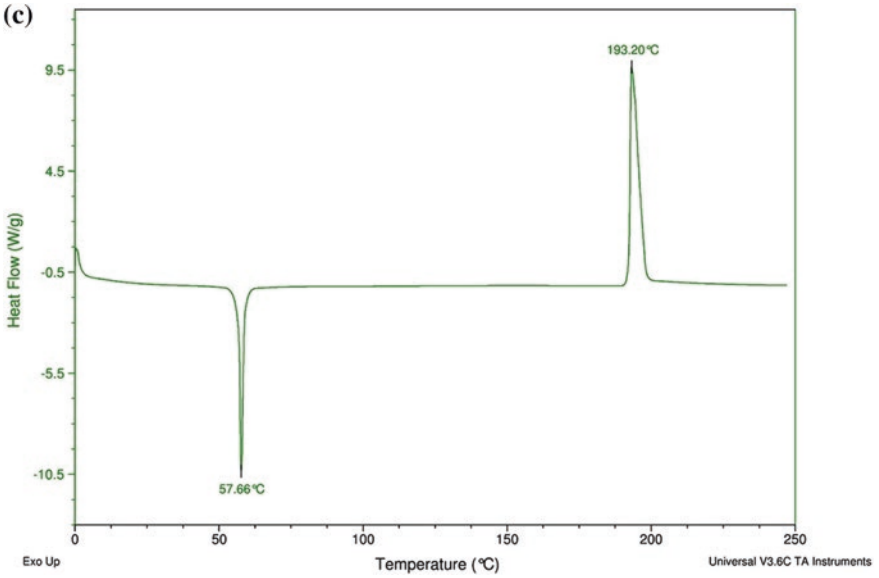

Fig. 5.5 (continued)

Table 5.1 Average size distribution of self-assembled nanoparticles

Sample	Particle size (nm)		
	Intensity	Number	Weight
Sericin + Activated PEG congjugate	204.3 ± 41	152.6 ± 32	172 ± 36

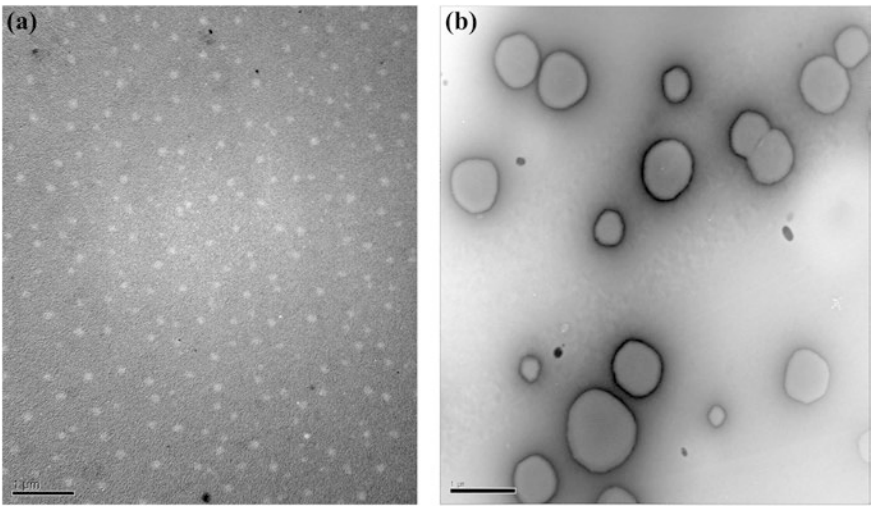

Fig. 5.6 TEM photographs of tasar sericin–PEG nanoparticles. **a** Tasar nanoparticles TEM. **b** Tasar nanoparticles AF TEM

5.4 Conclusion

The sericin–PEG conjugate was prepared by reacting actPEG with sericin. Aliphatic and aromatic hydroxyl groups of the serine and tyrosine residues in SS as the reaction sites were clarified through amino acid analysis and ^{1}H NMR measurement, respectively. From IR and CD measurements, the introduction of PEG chains into SS was found to induce the conformational change from random coil to β-sheet. DSC thermogram of sericin–PEG suggests that SS chains in sericin–PEG affected the crystallization of PEG.

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Chapter 6
Bioactive Proteins: Source, Synthesis, and Applications

Nangue Arlette Vyry Wouatsa

Abstract This chapter gives a brief description of bioactive proteins, their various sources, their main mode of synthesis, and their applications. Since the use of bioactive peptide hormone insulin for the treatment of *Diabetes mellitus*, the search and development of medically important proteins has expanded tremendously. Nowadays, protein-based therapeutics is a booming and emerging era in the biomedical sector. This is mainly attributed to the high market value but also to the efficacy of protein-based therapeutics. Among the top selling anticancer drugs, three are monoclonal antibodies, viz, bevacizumab, trastuzumab, and rituximab. Bioactive proteins used as therapeutics have always been extracted mostly from plants and higher animals but recent studies show some uncommon sources such as leech saliva, snake venom, frog skin, and marine sponges. These uncommon sources of bioactive proteins are some examples of the recent advances in bioactive proteins research which are summarized in this chapter. Many of the proteins discovered from these new sources hold the premise for further development into drugs, antibodies, vaccines, etc.

Keywords Bioactive protein · Therapeutics · Biomedical · Bevacizumab · Trastuzumab · Rituximab

6.1 Introduction

In all living cells, DNA stores the genetic information which codes for the amino acid sequences of proteins. These proteins account for 50 % of the dry weight of all living organisms, namely human, plants, animals, microorganisms,

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etc. [1, 2]. Equipped with various functional groups, proteins are involved in many cellular functions/events. They are responsible for: the shape and stability of cells and tissues; the transport of ions, metabolites, oxygen, and CO_2 across biological membranes; the storage and catalysis as well as protection and defense [3]. Owing to their vast array of properties, in-depth studies of their mechanisms of action led to the discovery of their therapeutics benefits which were not thought of. Studies of protein structure and function helped in the discovery of the human peptide hormone insulin, which was then genetically reproduced in *E. coli* and used as the main drug for treatment of *Diabetes mellitus* [4]. Following the example of recombinant insulin, the active search for protein-based therapeutics led to the development of new biotech companies. Till date, protein-based therapeutics and derivatives constitute a booming sector in the pharmaceutical industry. According to Dimitrov [5], there are more than 100 therapeutic proteins approved for clinical use in the European Union and the USA, and their market accounted for USD 108 billion in 2010. Moreover, three of the top selling anticancer drugs with sales of more than USD 6 billion are monoclonal antibodies, viz, bevacizumab, trastuzumab, and rituximab [6, 7]. This trend is expected to increase in the future mostly based on the fact that several proteins have not yet been fully investigated or even discovered. Thus, in view of the biological and medical relevance of proteins, this chapter describes briefly the state of knowledge on bioactive proteins, their various sources, their synthesis, and applications. Attempt has been made to provide readers with current information available on the subject.

6.2 Sources of Bioactive Proteins

Bioactive proteins can be defined as proteins with a therapeutic or biological property. Accordingly, bioactive proteins can be termed as biopharmaceuticals. A biopharmaceutical is a protein or nucleic acid-based pharmaceutical substance used for therapeutic or diagnosis purpose, which is produced by other means than direct extraction from native source [8]. A look at the literature reveals several sources of bioactive proteins including plants, animals, and marine sponges.

6.2.1 Bioactive Proteins from Plant Sources

Garcia et al. [9] and Kannan et al. [10] recently published two reviews listing several bioactive proteins and peptides from edible plants possessing antihypertensive, antioxidant, hypocholesterolemic, antithrombotic, and immunostimulating activities. Some of these plants include chickpea, pea, soybean, and lentils which contain lectins, protease inhibitors (such as trypsin and chymotrypsin inhibitors), and the non-antinutritional component, angiotensin I-converting enzyme (ACE)

inhibitor. Lectins are well known to reduce certain forms of cancer, activate innate defense mechanisms, and manage obesity, while protease inhibitors possess strong anti-inflammatory properties [11]. Besides these plant-derived bioactive proteins, mushrooms constitute an excellent source of proteins such as lectins, fungal immunomodulatory proteins (FIP), ribosome inactivating proteins (RIP), ribonucleases, laccases, etc., with interesting biological activities, viz., antitumor, antiviral, antimicrobial, antioxidative, and immunomodulatory activities [12]. Recently, a novel antitumor protein of 18.5 kDa purified from the edible mushroom *Pholiota nameko* (PNAP) exhibited significant antioxidant activity, but also showed antitumor activity against cancer cell lines such as MCF7 and Hela cells by inducing apoptosis of cancer cells [13].

6.2.2 Bioactive Proteins from Animal Sources

The well-known bioactive proteins in this category are milk-derived proteins which have been mostly characterized from the whey. These whey-derived proteins, namely alpha-lactalbumin, beta lacto-globulin, lactoferrin, lactoperoxidase, and growth factors exert a wide range of bioactivities affecting the cardiovascular, immune, and nervous systems [14, 15]. Compared to plants, animals have been mostly associated with the good quality of their proteins. Being a source of proteins of choice, several animal-derived proteins have been investigated and their therapeutic potential discovered. Human Insulin (the polymer of two peptide chains viz., chains A and chains B of 21 and 30 residues, respectively) is the prime example of animal peptide hormone used as drug.

Table 6.1 Some pharmaceutically active proteins originally isolated from animal sources (derived from Walsh [8])

Pharmaceutical protein	Animal source of isolation	Affections treated
Insulin (polypeptide of 51 AA)	Porcine/bovine pancreatic tissue	*Diabetes mellitus*
Glucagon (peptide of 29 AA)	Porcine/bovine pancreatic tissue	Insulin-induced hypoglycemia
FSH (heterodimeric glycoproteins of around 28 kDa)	Urine of postmenopausal women	Subfertility/infertility
HCG (heterodimeric glycoproteins of around 28 kDa)	Urine of pregnant women	Subfertility/infertility
Peptide hormones (e.g., gonadorelin, oxytocin, vasopressin)	Pituitary gland	Various
Human serum albumin	Human plasma/placenta	Plasma volume expander
HGH (polypeptide hormone of 191 AA)	Human anterior pituitary	Idiopathic short stature, Turner's syndrome, and chronic renal failure.

kDa kilodalton, *AA* Amino Acid, *FSH* Follicle Stimulating Hormone, *HGH* Human Growth Hormone, *HCG* Human Chorionic Gonadotrophin

Insulin, which is synthesized in the B cells of the pancreas, reduces the blood sugar level by promoting glycolysis, glycogen synthesis, and conversion of glucose into fatty acids. By contrast, it inhibits gluconeogenesis and glycogen degradation [3]. Because of its main role in carbohydrate metabolism, insulin is currently produced using several recombinant DNA-technologies for treatment of *Diabetes mellitus*. Following the example of insulin, other pharmaceutical proteins originating from animal sources have also been developed. Some of them are listed in Table 6.1.

Besides the proteins listed in Table 6.1, the search for proteins of interest is still ongoing. A look at recently published works on the subject shows uncommon sources of bioactive proteins, viz., frogs, snakes, sharks cartilages, Chinese tree shrew, among others. Some of these newly discovered proteins are regrouped in Table 6.2.

Table 6.2 Some newly reported bioactive proteins from animal sources

Animal source	Protein	Biological activities	References
Lamprey (*Lampetra japonica*) buccal gland	Protein extract	Anticoagulant, thrombolytic, local anesthetic, and immunosuppressant	[16]
Shark (*Isurus oxyrinchus*) cartilage	AR10 protein fraction	Immunostimulatory effect on the NK (Natural Killer) cells cytotoxicity	[17]
Leech (*Macrobdella decora*) saliva	Saratin, bdellin, destabilase, hirudin, decorsin, antistatin, eglin, serine protease inhibitors, lectoxin-like c-type lectins, ficolin, disintegrins, and histidine-rich proteins	Various	[18]
Chinese tree shrew (*Tupaia belangeri chinensis*)	TC26RFa peptide	Analgesic activity comparable to that of morphine, good anti-inflammatory agent	[19]
Snakes venoms			
Crotalus durissus cumanensis	L- amino acid oxidase (CdcLAAO)	Antimicrobial activity against *S. aureus* and *A. baumanni*	[20]
Bothrops pirajai	P-I class metalloproteinase (BpirMP)	Fibrinolytic and thrombolytic activities	[21]
Bothrops atrox	Metalloproteinase Batroxase	Fibrinolytic and thrombolytic activities	[22]
Frog skin			
Hyla japonica	Analgesin-HJ and Analgesin-HJ(I5T)	Analgesic activities	[23]
Odorrana graham	AH90	Wound healing activity	[24]
Clinotarsus curtipes	Brevinin1CTcu1 (B1CTcu1) to brevinin1CTcu5 (B1CTcu5)	Antibacterial activity	[25]

6.2.3 Bioactive Proteins from Marine Sponges

Although marine biodiversity is very important for its multiple benefits, viz., foods, climate, recreational activity, marine biodiversity represents an untapped source of novel and chemically diverse medicines. Several studies on marine organisms have already reported numerous compounds of biological significance, most of them being non-peptide compounds [26]. With regard to bioactive marine peptides/proteins, Kumar et al. [27] isolated antibacterial proteins of various molecular weights (20–120 kDa) from the marine sponge *Spongosorites halichondriodes* (Dendy 1905) collected in the Mumbai coastal area. Their work was preceded by Boobathy et al. [28], who had already described the isolation of bioactive proteins from the marine sponge, *Callyspongia diffusa*, also collected in Mumbai coast. The crude protein extract inhibited the growth of *Vibrio cholera* and showed three well-defined bands of 19.5, 39.0, and 66.2 kDa when analyzed with SDS-PAGE.

6.3 Synthesis of Bioactive Proteins

Translation of mRNA into amino acids sequence is the process by which proteins are synthesized in the cells. This synthesis is mainly performed on ribosomes. However, for a large-scale application of proteins of interest, this main mode of synthesis is not suitable for extraction of bioactive proteins, many of which are produced in small amounts from their native source [2]. Efforts were then made to produce the desired proteins using other means. Currently, the different methods of protein synthesis come into two groups: direct chemical synthesis and recombinant DNA technologies [1].

6.3.1 Direct Chemical Synthesis

The direct chemical synthesis of proteins is done by automated solid-phase methods. The main principle of these methods as described by Berg et al. [1] relies on the synthesis of polypeptide chains with their carboxyl end linked to an insoluble support, mostly a resin. Basically, the α-amino group of the desired peptide sequence is protected with *tert*-butyloxycarbonyl (*t*-Boc) and its α-carboxyl-terminal amino acid is then attached to the solid support (Fig. 6.1). Next, the amino group is deprotected and the subsequent amino acid residue (in the protected *t*-Boc form) and dicyclohexylcarbodiimide (DCC), the coupling agent, are added together. DCC will activate the α-carboxyl-terminal of the incoming amino acid which will attack the free amino group of the growing peptide

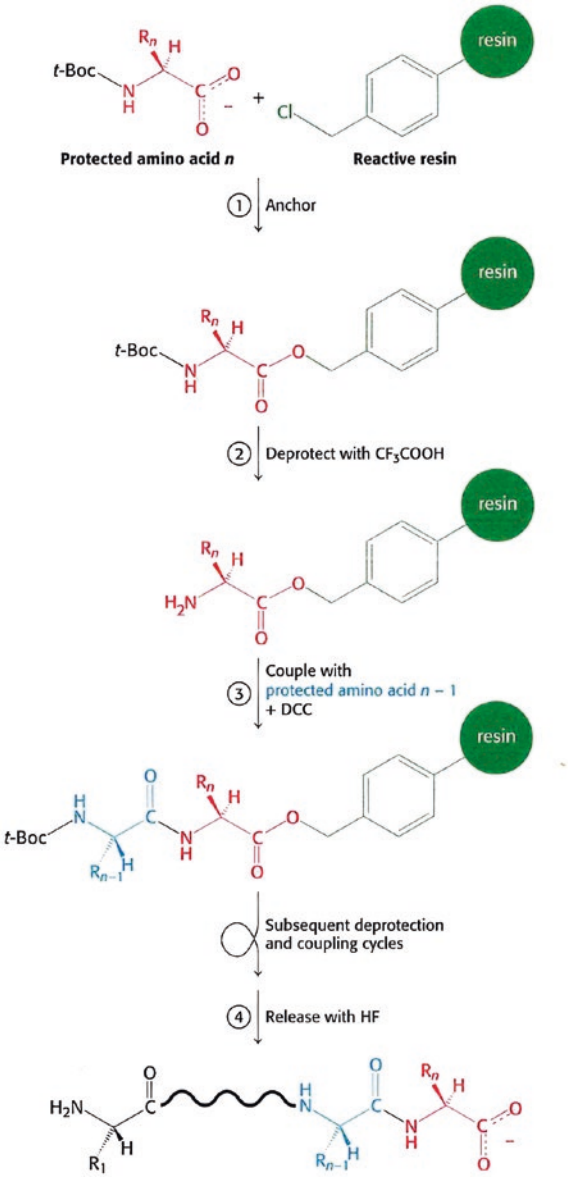

Fig. 6.1 Representation of solid-phase synthesis of peptide [1]

chain leading to a peptide bond. Additional amino acids are linked in the same sequence of reactions and at the end of the synthesis, the peptide is released from the beads by adding hydrofluoric acid (HF), which cleaves the carboxyl ester anchor without disrupting peptide bonds.

6.3.2 Recombinant DNA Technologies

Recombinant DNA technologies also called genetic engineering create novel protein results of site-directed mutagenesis, which are performed either by deletion, insertion, or substitution. In mutation by substitution, only a single base is replaced with an oligonucleotide encoding the new amino acid, whereas in mutation by insertion, specific plasmids are designed to insert a DNA cassette containing the desired mutation [1, 2]. Although developed in 1970, Recombinant DNA technologies have evolved over the years and still constitute a current approach of production of novel genes and proteins. Notwithstanding the fact that the preferred host for the genetic engineering is Gram-negative bacteria *E. coli*, other organisms are also selected as expression systems of recombinant biopharmaceuticals [29]. These include yeasts [30], human/animal cells [31], transgenic animals [32], and plant expressions systems [33–36]. Of these, the latter are gaining more attention nowadays because of their numerous advantages, viz.:

- Low cost and high-level transgene expression;
- Multi-gene engineering in single transformation event and transgene containment by maternal inheritance;
- Lack of gene silencing, undesirable foreign DNA, position, and pleiotropic effects [33, 34].

Moreover, among plant-based systems, chloroplast-engineered technology is proving to be a good platform for successful production of vaccines and therapeutic proteins and therefore comes as a much cheaper and potential alternative to bacterial expression systems [34].

6.4 Applications

Bioactive proteins find applications mainly as therapeutics owing to their biological properties, but they can also be developed into functional foods or used as tools to study the biological, biochemical, and biophysical characteristics of medically important proteins. All these applications have been made possible with the advent of recombinant DNA techniques. Indeed, with recombinant DNA techniques, the antigenic proteins at the surface of any infectious agent (virus, bacteria, etc.) can be safely produced without the risk of infection and used as vaccine. Examples of protein-derived vaccines produced using recombinant techniques include: Hepatitis B Vaccine sold under the commercial name Engerix or Recombivax HB [4], Human Papilloma Virus, and Anti Rhesus Immunoglobulin G vaccines. Besides vaccines, recombinant techniques provided other protein therapeutics such as drugs, antibodies, and enzymes.

Protein therapeutics are exploited not only for their healing effect but also for their other functional uses such as immunity boosters. The applications of protein

therapeutics include cancer, metabolic, cardiovascular, gastrointestinal, renal, respiratory, and infectious diseases to cite a few. Protein therapeutics are categorized into four groups according to the activity, viz., enzymatic or regulatory activity; diseases target agents, vaccines, and diagnosis [5]. Examples of well-known protein therapeutics with their trade names sold for their enzymatic or regulatory activities include insulin (Novolin), interleukin (Neumega), Follicule Stimulating Hormone (Follistin). With regard to vaccines and diseases target, several protein therapeutics developed till date are monoclonal antibodies, the vast majority of which are used for the target and treatment of cancer [6, 7, 37–39]. In fact, in August 2011, 12 approved monoclonal antibodies were available on the market for their clinical use against cancer [6], namely

- Rituximab, Ibritumomab tiuxetan, Tositumomab, and ^{131}**I**-Tositumomab which are used against Non-Hodgkin's lymphoma;
- Trastuzumab prescribed for breast cancer;
- Alemtuzumab indicated for chronic myeloid leukemia;
- Cetuximab, Bevacizumab, and Panitumumab employed for the treatment of colorectal cancer;
- Catumaxomab used against malignant ascites;
- Ofatumumab for chronic lymphocytic leukemia;
- Ipilimumab against metastatic melanoma and;
- Brentuximab vedotin indicated for Hodgkin lymphoma and systemic anaplastic large cell lymphoma [6].

The applications of bioactive proteins are countless taking into account the vast possibilities offered by genetic engineering techniques. Other than traditional therapeutics, medically important proteins can be engineered in transgenic foods or animals, thus opening the way for functional foods with dual purposes, nutritional and medicinal. In line with this, Wakasa and Takaiwa [36] recently developed transgenic rice seeds accumulating high level of recombinant protein using a callus-specific selection system, CSP (callus-specific promoter). The mutated gene rice acetolactate synthase (mALS) was highly expressed in the transgenic rice seeds. This protocol described by Wakasa and Takaiwa [36] can be fully exploited and/or adapted with other plants/crops to yield transgenic plants accumulating good amounts of proteins of interest.

6.5 Conclusion

The development of protein-based therapeutics is increasing mostly due to the faster clinical development and FDA approval time compared to small drugs; the higher market profitability and high body tolerance which render them less prone to elicit immune responses. In this chapter, several sources of bioactive proteins were cited, viz., plants, animals, marine sponges but these are likely not the only sources of bioactive proteins per se. Proteins are present in all living organisms,

thus, there is still a vast number of putative bioactive proteins which have not been discovered and these include proteins from marine species. Taking the example of marine sponges, two pioneer works in India show that marine sponges may contain bioactive proteins. Although these works are still at early stages, they show however promising features for in-depth research in that direction. Future areas of research also include the search and development of protein-based therapeutics for treatment of infectious diseases, particularly AIDS, tuberculosis, malaria, and the optimization of protein-based therapeutics with improved properties (stability, shelf-life, delivery, and adsorption).

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Chapter 7
Role of Protein Interleukin 8 (IL-8) in Human Life

Richa Sharma, Namrita Lall and Navneet Kishore

Abstract Cytokines are small- to medium-sized active proteins which mediate a vast array of biological functions. The cytokines are grouped into families based on the structure of their receptors. Some findings suggest that although there are many cytokines, the receptors for these cytokines fall into relatively small number of families. Interleukin-8 (IL-8) is a chemo-attractant cytokine produced by a variety of tissues and blood cells. It is also involved with diseases associated primarily with neutrophil influx. IL 8 regulates transcriptional activation after treatment of producer cells with stimuli, encoded by a single mRNA transcript and is produced as a 99 amino acid non-glycosylated peptide. While regulation at post-transcriptional level, the IL 8 gene containing motifs rich in AT repetitive sequence are responsible for destabilization of IL 8 protein. Conversely, IL 8 plays a crucial role in a variety of inflammatory diseases such as allergy, arthritis, periodontitis, bronchiolitis, cystic fibrosis, alcoholic hepatitis, asthma, psoriasis, palmoplantar pustulosis, acne vulgaris, dermatitis, and many more. However, this chapter briefly describes the role of IL 8 in the human living system.

Keywords Cytokines · Interleukin-8 · Inflammation · Skin disorders

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7.1 Introduction

The chemical messengers of the immune system that help the cells of immune system to communicate with one another are termed as cytokines. These chemical entities are made of proteins that are released by various immune cells and may act upon other cells to harmonize the regulation of immune system. Originally, the term lymphokine was used to identify a family of hormone-like protein substances secreted by antigen-stimulated T lymphocytes. These chemical entities played a crucial role in the immune system as they control chemotaxis of neutrophils, maturation of T-cells, antibody production of B-cells, etc. After it was known that various cell types, including monocytes, neurons, neuroglial cells, endothelial cells, etc., secrete similar substances, terms like monokines, cytokines, and interleukins were used. Cytokine is a more appropriate name for a class of soluble proteins (molecular weight less than 100,000 daltons or 100 kDa) that are released by a cell to send messages that are delivered to the same cell (autocrine), an adjacent cell (paracrine), or a distant cell (endocrine) [1]. The historical nomenclature of cytokines was based on cell from which it originated. However, the interleukin naming based on assigning sequential number to a factor is a rational method and is now widely known and accepted. Also, cytokines are grouped into families and have now settled by classifying them based on the structure of their receptor. Fitzgerald et al. [2] explained the six major families of cytokine receptors as shown in Table 7.1. These cytokines are involved in reproduction, growth and development, normal homeostatic regulation, response to injury and repair, blood clotting, and host resistance (immunity and tolerance). Many different types of cells can produce the same cytokine, and a single cytokine may act on a wide variety of target cells. In addition, it can be said that there are no circumstances in which cytokines are produced individually. Rather they are produced together with other cytokines in patterns characteristic of a particular stimulus or disease [1].

7.2 Interleukin-8 (IL-8)

Interleukin 8 is a chemo-attractant cytokine produced by various tissues and blood cells. IL-8 was first identified as soluble factor in supernatants of monocytes after stimulation by endotoxins in the year 1986–1987. Later, it was recognized as a prototype of chemotactic cytokines which are mostly associated with tissue derived inflammatory functions. Based on its function, IL-8 is variously known as human leukocyte protein, T cell chemotactic factor, and monocyte derived neutrophil chemotactic factor. The main role of bioactivity of IL-8 involves its effects on activation of polymorphonuclear neutrophils, specifically by chemotaxis action [3].

7.3 Structure of IL-8 Protein

The molecular weight of IL-8 is around 8,000. It is produced as a dimeric protein of two identical subunits (8–10 kD each) from a 99 amino acid non-glycosylated

Table 7.1 Classification of cytokine families according to receptor structures

Family	Key receptor feature	Members	Shared function	Cytokine structure
Hematopoietin receptors (type I)	Cytososlic box 1/2	IL-6R, G-CSFR, gp130, IL-12R	T/B cell activation	4 α-helical bundles
	WSXWS sequence extracellularly	LIFR, IL-2Rβ, IL-2Rγ, IL-4R, IL-3Rα, IL-7Rα, IL-9R, GM-CSFR, EpoR, prolactin R, GH-R, Tpo-R; β-chain for IL-3R, GM-CSFR and IL-5R	Hematopoiesis	
Interferon receptors (type II)	Cytosolic box 1/2	IFNα/βR, IFN-Rα/β	Antiviral (not IL-10)	4 α-helical bundles
	Fibronectin domains extracellularly	IL-10R		
TNF receptors	Cytosolic death domain	P55 TNFR, p75 TNRR (no death domain) LTβR, NGFR, CD40, CD30, CD27, 4-1BB, OX-40, TRAMP (DR3), TRAILR (DR4)	Pro-inflammatory	Jelly roll motif
	Four Cys-rich regions extracellularly			
IL-1/Toll-like receptors	Cytosolic Toll/IL-1R (TIR) domain Ig domains (IL-1R subgroup) or leucine-rich repeats (TLR subgroup) extracellularly	IL-1RI, IL-1RII (no TIR), IL-1RAcP, IL-18R α/β chains, T1/ST2, IL-1Rrp2, TIGGIR-1, IL-1RAPL, SIGIRR TLR-1-TLR-10	Pro-inflammatory	β-trefoil
Tyrosine kinase receptors	Cytosolic tyrosine kinase domain	M-CSFR, EGFR, TGF, IGFs, FGFs	Growth factors	β-sheet
Chemokine receptors	7 transmembrane spanning regions	IL-8, MCPs, RANTES, Eotaxin	Chemotactic	Triple-stranded antiparallel β-sheet in Greek motif

peptide rich in cysteine residues. The protein is identified as heparin binding protein. Also known as neutrophil activation factor or protein (NAP-1), the protein of IL-8 belongs to the superfamily involved in cell-specific chemotaxis, mediation of cell growth, and inflammatory [3]. Each subunit comprises of secondary structure elements of long C-terminal helix extending from residues 56–72 and a triple-stranded antiparallel β-sheet. The placement of helix and triple-stranded β-sheet within each subunit is steadied by various hydrophobic interactions.

The disulfide bridge is right-handed and extends between Cys-7 and Cys-34, while the one which extends between Cys-9 and Cys-50 is left handed. The disulfide bridges formed from these cysteine residues are essential for the activity of IL 8. The N-terminal end comprise of signal sequence, which may be cleaved enzymatically upon release from producer cell [4]. The N-terminus comprised of a series of turns at residues Gln-8/Cys-9, Ile-10, Lys-11 and See-14 and makes a long loop extending from Gln-8 to His-18 follows the helical turn by residues 19–22 which leads to strand 1. Strands 1 and 2 are connected by a loop at Ser-30 and Ala-35. Hydrogen bonding exists between the amides Gly-31 and Cys-34; Cys-34 and Gly-31. Strand 3 leads to the helix from residues 52–55, which are further stabilized by hydrogen bonds. Along with the mentioned bonds, various other coordinating forces are involved to retain the protein structure. Baggiolini et al. [5] also reported considerable homology of IL-8 peptide from platelet alpha granules such as platelet basic protein (PBP), connective tissue activating peptide III, and platelet factor 4. It was also reported that IL-8 possesses structural and biological similarities with a recently discovered macrophage inflammatory protein 2 (MIP-2).

7.4 Transcription and Translation of IL-8 Protein

The IL-8 gene is located on 4q12-21 chromosome covering 5.25 kbp of DNA. It comprises four exons and three introns which are located in cluster with other C-X-C chemokines on the same DNA. It is encoded by a single mRNA transcript of 1.8 kb [4]. The 5′ flanking region of IL-8 gene is regulator of various known elements and serves as binding sites for factors like NFκB, NF-IL-6, AP-1, AP-2, AP-3, interferon regulatory factor-1, and glucocorticoid response elements. Various stimuli such as bacterial endotoxins, reactive oxygen intermediated, free nitrogen species, IL-1α, IL-1β, and TNF-α trigger the transcriptional activation from producer cells. The 3′ flanking region of IL-8 gene is responsible for post-transcriptional regulation. The gene contains repetitive ATTTA motifs which may be responsible for destabilization of various cytokine mRNAs.

Holmes et al. [6] isolated a complementary IL-8 receptor form the human neutrophils. It was found that the amino acid sequence depicts the receptor as a member of superfamily of receptors that coupled to G proteins (guanine nucleotide binding proteins) and may transduce signals for the IL-8 family of pro-inflammatory cytokines.

Wu et al. [7] studied the G protein-coupled signal transduction pathways for IL-8. The pathways by which IL-8 activates inositide-specific phospholipase C (PLC) were examined by co-expression of different components of guanosine triphosphate binding protein (G protein) pathway in COS-7 cells. It was found that IL-8 receptor interacted with endogenous toxin sensitive G proteins or with recombinant G protein to release free beta gamma subunits that could then specifically activate the beta 2 isoform of PLC. The findings of Wu et al. [7] suggested that the signal transduction pathways of IL-8 are limited to specific G proteins. It involves activation of protein kinase C.

7.5 Essential Role of IL-8 in Inflammation

Microbial infection, autoimmune reactions, and tissue injuries caused by various factors lead to abnormal expressions of redness, heat, swelling, or pain. These signs are the consequences of inflammation. Inflammation is a sequential event of infiltration of leukocytes as a result of various chemotactic factors which may act as leukocyte attractants and are produced at the site of infection/injury. These chemotactic mediators trigger the inflammatory reactions by inducing vasodilation which facilitates diapedesis of defense cells (Fig. 7.1).

IL-8 acts as leukocyte chemotactic activating cytokine and plays a vital role in inducing inflammation. It has also been found to demonstrate chemotactic activities for T lymphocytes and basophils along with neutrophils. Along with chemotactic properties, IL-8 is reported to increase the expression of Mac-1 and CR-1

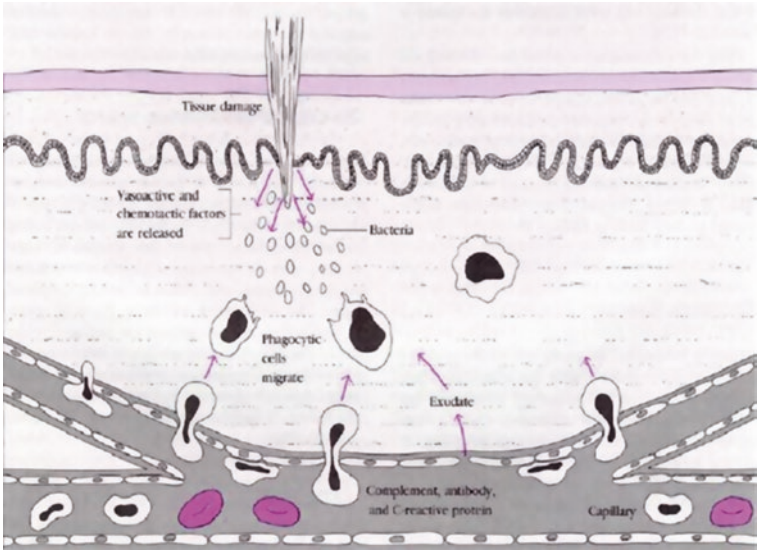

Fig. 7.1 Events showing role of chemotactic factor (IL-8) factor in inflammation

integrins, which enhances the capability of recognizing and binding to various molecules of invading microbes by stimulating the release lysosomal enzymes from neutrophils [8].

7.6 IL-8 in the Living Cells

Previous studies have shown that the biological properties of IL-8 are very similar to chemotactic peptides C5a and fMet-Leu-Phe. IL-8 is responsible for eliciting full pattern of responses that occur in stimulated neutrophils. There are some properties listing as follows:

7.6.1 Respiratory Burst, Neutrophils, and IL-8

The functional responses of IL-8 are initiated by binding of protagonist to its receptor and then can be discontinued when the protagonist-receptor complex dissociates. The example of respiratory burst complements the above statement. Respiratory burst is a characteristic property of phagocytosis by neutrophils, which is essential for killing the invading microorganisms. At the site of infection, several messenger molecules (IL-8) are generated that attracts neutrophils and directs their migration toward invading microbes (chemotaxis). It results from the association and activation of NADPH oxidase. Neutrophil activation occurs by binding of protagonist to its receptor. The ligation of chemo-attractant receptors by fMet-Leu-Phe and IL-8 leads to neutrophil activation and initiates a series of functional responses inducing the respiratory burst. Following the activation of neutrophils, several proteins are assembled to form the respiratory burst enzyme. Covalent modification by kinases and phosphatases, interactions with cofactors, lipids, and nucleotides are recognized as an important element of assembly process. By activation of phosphatidylinositol-specific phospholipase C, Ca^{2+} ions are released from the intracellular organelle which leads to a transient increase in the concentration in the cytosol. The influx of Ca^{2+} ions in the cytosol favors the neutrophil response. This series of process can be prevented by an antagonist. As mentioned above, respiratory burst is induced by fMet-Leu-Phe and can be terminated by addition of butoxycarbonyl derivative which acts as antagonistic. The respiratory burst as induced by IL-8 is also reported to be inhibited by staurosporine and wortmannin (fungal metabolite) which may act by blocking certain protagonist responses by interfering with calcium-independent pathway. When antagonistic is trapped by antibody, the same effect can be observed. Also, all the IL-8 mediated responses can be prevented by pretreatment of the cells by *Bordetella pertussis* toxin [5, 9].

Waltz et al. [10] examined the changes in cytosolic free calcium and respiratory burst on human neutrophils and monocytes by stimulating them with neutrophil

activating peptide-1 (NAP-1), also known as IL-8. The researchers found that NAP-1 induced a rapid and transient rise of cytosolic free calcium and respiratory burst in neutrophils. Similar results were observed for monocytes. However, on stimulation with concanavalin A, the duration and rate respiratory burst was enhanced several fold. Waltz et al. [10] further concluded that the increase in neutrophil activation by stimulating agents may contribute to the formation of oxygen derived radical by NAP-1/IL-8.

7.6.2 IL-8 Is Not Species Specific

Researchers have studied the in vivo effects of IL-8 mediated responses in various laboratory animals. Injection into the skin of rabbits results in plasma exudation and further massive neutrophil infiltration. Similar results were also reported on intradermal injection of IL-8 in rats, mice, guinea pigs, and dogs. The resistance of IL-8 to inactivation of plasma peptides and its low grade of degradation by proteases favor its long duration of action in various biological systems [5]. This property of IL-8 and other cytokines has allowed researchers to determine more precisely the role of cytokines individually. The use of transgenic mice which overexpressed certain cytokines, or mice deficient in cytokines, their receptor or signaling proteins activated by them, have given new scope to investigators to study and understand the role of cytokines in vivo [2].

7.6.3 IL-8 May Be Produced by Many Cells

Various researchers have reported the production of IL-8 by a variety of cells on suitable stimulation. Interestingly it was found that different cells such as blood monocytes, alveolar macrophages, endothelial cells, fibroblasts, epithelial cells, hepatoma cells, and human leukemia monocytic cells have shown to elicit in vitro and in vivo IL-8 responses after appropriate stimulation [5, 11].

7.7 Role of IL-8 in Various Diseases Including Skin Disorders

The clinical signs of inflammation include redness, heat, and swelling which are due to vascular alterations in the area of injury. This inflammation is long-lasting and causes significant and serious tissue destruction which leads to various skin disorders. A number of factors are responsible for inflammatory response including interleukin-8 [12].

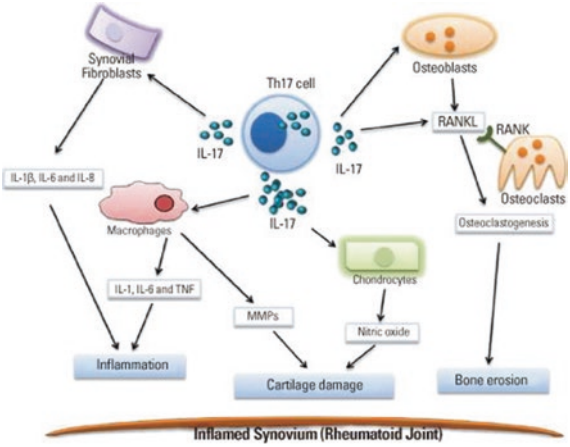

Fig. 7.2 Synergistic role of IL-8, IL-6 and IL-17 in the inflammation of Rheumatoid Arthritis

7.7.1 IL-8 and Rheumatoid Arthritis (RA)

IL-8 along with IL-6 cytokines is produced to act as major instigators of RA joint inflammation. Studies have shown that disruption of IL-8 gene by gene knockout leads to protection against arthritis in animal models, confirming their perpetuation as pro-inflammatory cytokine in the joint inflammation of RA. Earlier studies have also reported TNF-α and IL-1β as a major inducer of IL-6 along with IL-8 in RA synovium. In a study conducted by [13], using mice and human joint explants revealed the synergistic and additive function of IL-17 along with IL-8 and IL-6 in inducing inflammation in RA (Fig. 7.2). The synergistic action of IL-17, IL-8, and IL-6 appears to involve activation of phosphatidylinositol 3-kinase, Akt, and NF-κB in fibroblast-like synoviocytes (FLS) as the signaling pathways [13, 14]. In RA and other inflammatory joint diseases, IL-8 could bring about accumulation of neutrophils, which are considered a major source of cartilage-degrading enzymes.

7.7.2 IL-8 and Asthma

Asthma is characterized by reversible airway obstruction, bronchial hyperactivity along with inflammation. The respiratory epithelium is important as it is the first tissue that comes in contact to inhaled allergen and thus triggers the release of cytokines and other mediators. IL-8 is involved with neutrophil chemotaxis and activation is produced by respiratory epithelium. Also, recent in vitro studies have suggested the chemotaxis and activation of eosinophils by IL-8. A study by Yamanotto et al. [15] found that IL-8 is produced by airway epithelial cells to activate neutrophils and eosinophils in the airway. In particular, neutrophils release neutrophil elastase, which induces the expression of IL-8 gene in airway epithelial cells which further cause the release of IL-8. It can therefore be speculated that

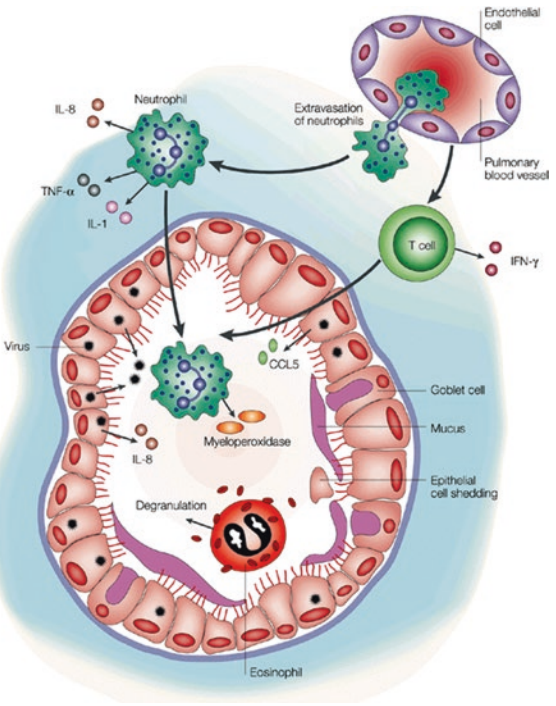

Fig. 7.3 Inflammatory events in asthma and role of IL-8

IL-8 plays an important role in the inflammatory cycle of asthma thereby recruiting neutrophils and eosinophils (Fig. 7.3).

7.7.3 IL-8 and Periodontal Disease

Periodontal disease is caused by anaerobic periodontopathogens, namely *Prevotella intermedia*, *Porphyromonas gingivalis*, *Bacteroides forsythus,* and *Campylobacter rectus*. The metabolites from these bacteria and related molecules indirectly stimulate monocytes to release IL-8 along with other cytokines such as IL-1 and TNF-α. The release of these inflammatory mediators activates the process in endothelial and other periodontal cells.

IL-8 involved in the initiation and progression of periodontal disease causes activation of neutrophils which may lead to degradation of connective tissue constituents by neutrophil enzymes (Fig. 7.4). In a study conducted by [16], a significant positive correlation between the levels of IL-8 and vascular endothelial growth factor (VEGF) was found and it was speculated that IL-8 may also take part in inflammatory angiogenesis.

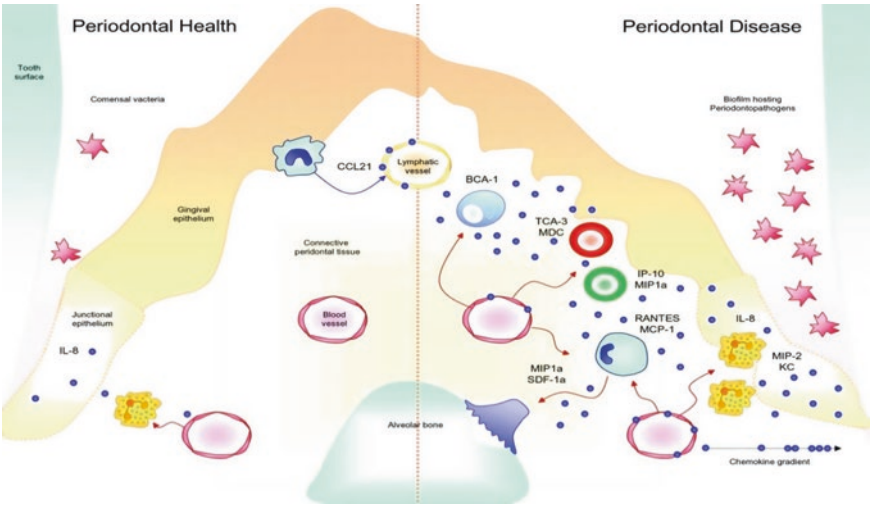

Fig. 7.4 Inflammatory events and role of IL-8 in periodontal disease

7.7.4 IL-8 Role in Dermatitis

In a study conducted by [17], the inflammatory events responsible for leucocyte trafficking after topical application of poison ivy/oak extract were determined. The cutaneous inflammation was accompanied by simultaneous appearance of many clinical and immunopathological parameters that lead to contact dermatitis. After epicuticular application of allergen (poison ivy/oak extract), the cellular events induced in human skin included morphological changes in endothelial cells, which preceded the migration of lymphocytes into the dermis and later into the epidermis.

Therefore, such cellular alterations stimulate resident cells, keratinocytes, endothelial cells, and dermal dendrocytes to produce molecular mediators such as IL-8 and TNF-α where IL-8 acts as T cell chemotactic factor (Fig. 7.5). Keratinocyte IL-8 expression in such contact dermatitis is correlated well with the migration of T lymphocytes toward the epidermis. The findings of this study stating in vivo correlation involved keratinocyte TNF-α expression followed by IL-8 release resembles that found in vitro and supports the notion that keratinocytes-derived IL-8 is an important T cell chemotaxin both in vivo and in vitro. The role of IL-8 in acne vulgaris can be well explained by in vitro studies conducted by various researchers. Recently, Sharma [18] reported how the inflammatory events trigger as a result of *Propionibacterium acnes* infection. The in vitro co-culture of *P. acnes* with human macrophage cell line (U937) caused the release of pro-inflammatory cytokines like IL-8, TNF-α and leads to the pathogenesis of acne vulgaris.

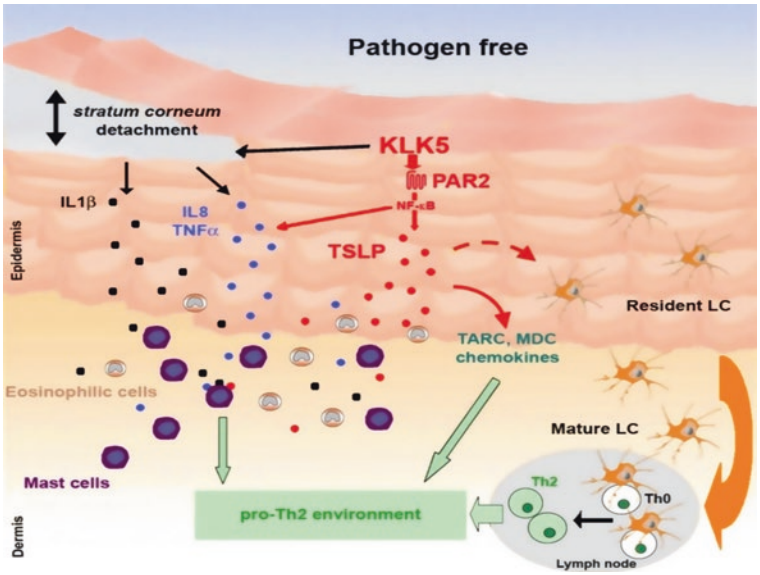

Fig. 7.5 Essential role of IL-8 in skin disorders

Apart from above stated disease, the involvement of IL-8 in other health and disorders is suggested by various researchers. For example, IL 8 is responsible for neutrophil infiltration around myocardial infarction areas and therefore contributes to the reperfusion damage accompanying with inflammatory cell products. The further information on pathophysiological functions of IL-8 can be explored by inducing the production of this mediator in normal and diseased tissues. Form extensive research it can now be said that on turbulences in tissue homeostasis, it is widely expressed in response to IL-1 and TNF and causes neutrophil infiltration. The important role in IL-8 dependent neutrophil emigration is presumably played by venular endothelium. On stimulation with IL-1 or TNF, endothelial cells express adhesion proteins that promote their interaction with circulating neutrophils, and release IL-8 as a stimulus of migration. Neutrophil accumulation in alveolar spaces is an important pathogenic event idiopathic pulmonary fibrosis which leads to lung parenchymal cell injury and further breakdown of interstitial structures. The adult respiratory distress syndrome is characterized by extensive tissue damage due to degrading activity of elastase and protease released from neutrophils. Under physiological conditions, IL-8 may also drive steady emigration of neutrophils from blood to the tissue compartment and thus appear to be particularly suited for the focal recruitment of neutrophils at the inflamed sites [5].

Understanding the emerging potential role of IL-8 in inflammation can help researchers to explore and innovate new approaches in the design of anti-inflammatory agents that may act as antagonistics of this inflammatory mediator.

7.8 Conclusion

This review provides an important insight into the essentials of IL-8 in the human system. Not only does it lead to inflammation, it also helps evoke immune responses by recruiting the defense cells of the body.

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Chapter 8
Importance of Natural Proteins in Infectious Diseases

Prabhat Ranjan, Asmita Dey, Vinod Praveen Sharma and Neeraj Kumar Tiwari

Abstract Proteins are important biomolecules, extensively involved in almost all biological processes. A number of proteins are also implicated in infectious diseases. Bacterial proteins used in adhesion to host epithelium, bacterial toxins, and viral membrane glycoproteins are some of the proteins involved in infectious diseases. Even components of the host innate immune system like Toll-like receptors and Nod-like receptors and adaptive immune components like immunoglobulins aiding in defense against pathogens are important biological proteins. Chaperones like acid and heat shock proteins provide protection from high temperatures, metabolic poisons, and other stressful conditions. Several natural and artificial proteins are components of vaccines, a key strategy to control fatal diseases, lacking empirical treatment. It is necessary to investigate these proteins, to develop new biomedical tools and technologies, aiding in eradication of various diseases. Thus, further research should be carried out in this field, for saving and improving quality of human lives.

Keywords Toll-like receptors · Major histocompatibility complex · Immune response

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8.1 Introduction

An infectious disease is a disease that is caused by the invasion of a host by agents whose activities harm the host's tissues (that is, they cause disease) and can be transmitted to other individuals (that is, they are infectious). Infectious diseases, also known as transmissible diseases or communicable diseases, comprise clinically evident illness (i.e., characteristic medical signs and/or symptoms of disease) resulting from the infection, presence, and growth of pathogenic biological agents in an individual host organism. Infections are caused by infectious agents such as viruses, and prions, microorganisms such as bacteria. Proteins might be involved in causing a disease as well as protecting the host from it. Various membrane proteins of microorganisms (virus, bacteria, parasites, etc.), prions are responsible for causing different diseases. Proteins like heat shock proteins/chaperons aid in causing as well as protecting from diseases. Proteins might also be involved in playing solely protective roles against infections like various cell surface receptor proteins (Toll-like receptors, Nod-like receptors, etc.), which trigger protective immune responses. Nowadays, various antimicrobial peptides or antibiotics are known to kill microorganisms or inhibit their growth, thus aiding in treatment of diseases.

Thus, proteins, one of the most incredible biomolecules, are extensively involved in almost all biological mechanisms, especially those implicated in pathophysiology of infectious diseases. The understanding of this topic is essential for developing different biomedical tools, for combating various diseases, thus, improving the quality of human life.

8.2 Bacterial Proteins

A number of bacterial proteins are involved in its pathogenesis and virulence. Bacterial infections are usually initiated by adherence of the microbe to a specific epithelial surface of the host, otherwise the organism will get removed: (1) Fimbrial adhesions involved in mediating attachment of some bacteria to mammalian cell surfaces, e.g., *Neisseria gonorrhoeae* use pilli to adhere to the mucous membrane of urethra and can resist the flushing action of urine [1, 2]. (2) Nonfimbrial adhesion including the filamentous hemagglutinin of *Bordetella pertussia* helping in attachment to respiratory epithelium, a mannose-resistant hemagglutinin produced by *Salmonella typhimurium* and a fibrillar hemagglutinin from *Helecobacter pylori* [3, 4]. Other adhesions like Protein F produced by *Streptococcus pyogenes*, aiding in attachment to pharyngeal epithelium [5]. Some extracellular bacterial proteins considered invasins like hyaluronidase, produced by *Streptococci, Staphylococci,* and *Clostridia*, which degrades hyaluronic acid of connective tissue [6, 7], collagenase produced by *Clostridium sp*, which dissolves collagen framework of muscles [8], neuraminidase produced by *Vibrio cholerae* and *Shigella dysenteriae*, which degrades neuraminic acid of intestinal

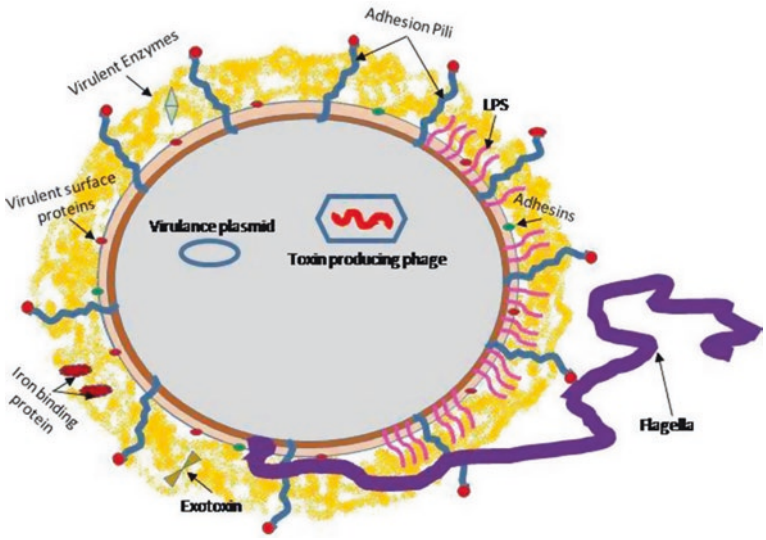

Fig. 8.1 Structure of bacteria

mucosa [9]. Other extracellular proteins like invasive enzymes, e.g., coagulase, contributes to the formation of fibrin walls around staphylococcal lesions [10]; exotoxins (proteins released extracellularly), like neurotoxin (Tetanus toxin, by *Clostridium tetani*, Botulinum toxin by *Clostridium botulinum*) [11] and cytotoxins (Diphtheria toxin produced by *Corynebacterium diptheriae*) [12, 13], also known as A-B toxins (consisting of 2 subunits: one binds to cell surface receptor and the other is transferred into the cell to damage the cell) [14], cytolytic toxins (attacking cell constituents causing lysis) like hemolysins produced by *Bordetella pertussis*, inducing apoptosis of host cells, super antigen toxins (e.g., superantigen, sized 22KDa produced by 5–25 % of *Staphylococcus aureus* isolates, causing toxic shock syndrome (TSS) by stimulating the release of large amounts of interleukin-1, interleukin-2 and tumor necrosis factor, etc.) [15]. Enterotoxins (exotoxins that act on the small intestine, generally causing massive secretion of fluid into the intestinal lumen, leading to vomiting and diarrhea) produced by *Vibrio cholerae, E. coli* O157:H7. Endotoxins (generally cell-bound toxins released only when cells are lysed) produced by most Gram-negative bacteria are generally non-proteinaceous, lipopolysaccharide in nature [16] (Fig. 8.1).

8.3 Viral Proteins

Viral glycoproteins, made up of carbohydrates and proteins mainly help in adherence of viruses to host cell surfaces and internalization of viral components. The

addition of sugar chains or glycosylation of the protein surface-components, can happen either at asparagine, and is termed *N*-glycosylation, or at hydroxylysine, hydroxyproline, serine, or threonine, and is termed *O*-glycosylation. Glycosylation is often present in proteins that are at least in part located in extracellular space. The sugar group can assist in protein folding or improve its stability. Glycoproteins also aid in immune cell recognition. Viral glycoproteins are composed of three parts: External/Ecto-domain, which interacts with host; Transmembrane segment, which spans the viral envelope membrane, typically α-helix; and Endo-domain, the internal part. Ecto-domain can be of two classes: Class I, found in Orthomyxoviruses, Paramyxoviruses, Retroviruses, Filoviruses and Coronaviruses; Class II, found in Flaviviruses and Alphaviruses. Other types of ecto-domains that do not fit in class I and II are Glycoprotein B (gB) of Herpes Simplex Virus (HSV) and Glycoprotein G of Vesicular Stomatitis Virus (VSV) [17–20].

Glycoproteins on the surface of viruses are anchored in the lipid bilayer of the envelope by means of hydrophobic bonds, and only about 30 amino acids penetrate into the virus. These transmembrane proteins have large external domains and small cytoplasmic domains. Viral glycoproteins are oligomers that are associated with each other to form tetramers, etc. There are often the spikes seen on the virus surface. Some glycoproteins such as the influenza hemagglutinin are anchored at both ends, thus forming a loop. In such cases a signal sequence at

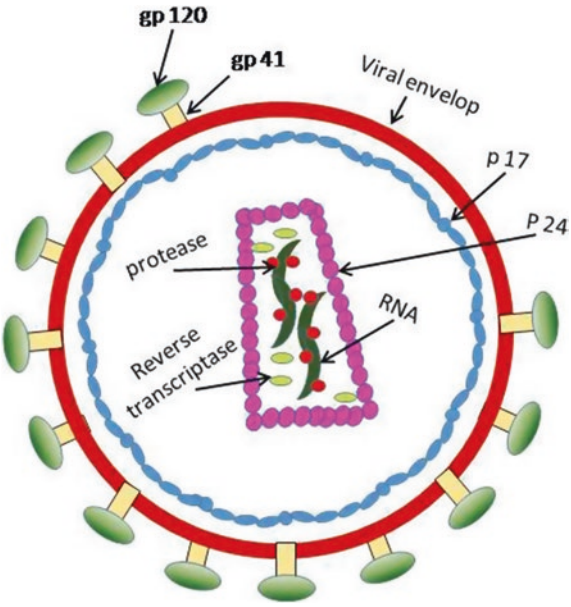

Fig. 8.2 Structure of human immunodeficiency virus (HIV)

the amino end has been removed. There are two types of glycoproteins: External glycoprotein anchored in the envelope by a single transmembrane domain, and a short internal tail. These proteins are usually the major antigens of the virus and involved in functions such as hemagglutination, receptor binding, and membrane fusion. The other classes are channel proteins, which are mostly hydrophobic proteins that form a protein lined channel through the envelope. This protein alters permeability of the membrane (e.g., ion channel). Such proteins are important in modifying the internal environment of the virus [21–24] (Fig. 8.2).

8.4 Toll-Like Receptors

The first line of defense against pathogenic microorganisms is innate immunity, and its activation is initiated by the recognition of microbial structures by pattern recognition receptors (PRRs). The first and most studied class of PRRs is that of a proteinaceous receptor named Toll-like receptors (TLRs), named after the Toll receptor of *Drosophila melanogaster*. Ten human TLRs have been identified, from TLR 1 through 10, with important roles in host defense against bacteria, viruses, and fungi [25, 26]. They recognize a large array of pathogen- associated molecular patterns (PAMPs), including peptidoglycan, lipoproteins, lipopeptides, phenol-soluble modulin, lipoteichoic acid, lipoarabinomannan, atypical lipopolysaccharides (LPSs), porins, flagellin, heat shock proteins (HSP), lycoinositol phospholipids, glycolipids, zymosan, also nucleic acids like double-stranded RNA of viruses, etc. [27, 28]. TLR family members are characterized structurally by the presence of a leucine-rich repeat (LRR) domain in their extracellular domain and a TIR (Toll/interleukin-1 receptor homology) domain in their intracellular region [29]. Expression of TLRs is modulated by a variety of factors such as microbial invasion, microbial components, and cytokines.

There are two distinct signaling pathways of TLRs: Myd88-dependent and Myd88-independent pathways. The MyD88-dependent pathway signals via MyD88, IRAK, and TRAF6 and leads to NF-κB activation. The activity of NF-κB (Nuclear Factor Kappa-light-chain-Enhancer of Activated B cells) is regulated by association with I-κB, which sequesters NF-κB in the cytoplasm until phosphorylated on serine residues by the I-κB kinase (IKK) complex. This phosphorylation leads to the dissociation and nuclear translocation of NF-κB. NF-κB is a transcription factor involved in upregulation of genes responsible for both the innate and adaptive immune responses, e.g., genes involved in T cell development, maturation, and proliferation, cell apoptosis, etc. Similarly, in the MyD88-independent pathway an adaptor molecule named TIR domain-containing adaptor protein (TIRAP)/MyD88- adaptor-like (Mal) is involved (Fig. 8.3).

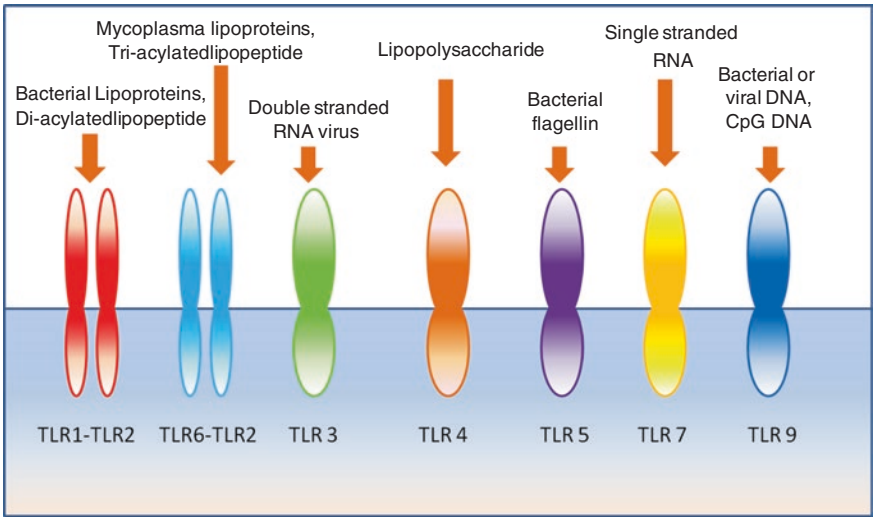

Fig. 8.3 Toll-like receptors

8.5 Nod-Like Receptors

Nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) are a group of evolutionarily conserved intracellular proteinaceous PRRs that play a vital role in innate immunity and host physiology, in both plants and animals [30, 31]. In humans there are 22 known NLRs, and the association of mutations and single nucleotide polymorphisms (SNPs) in their genes with human diseases reflect their vital role in host defense. NLRs also play important roles in reproduction and embryonic development. The characteristic feature of NLRs is a central NOD (or NACHT) domain, required for oligomerization, an *N*-terminal homotypic protein–protein interaction domain and a *C*-terminal series of leucine-rich repeats (LRRs) involved in agonist sensing or ligand binding [32].

Mammalian NLRs are subdivided into four subfamilies based on the variation in their *N*-terminal domain: NLRA or Class II transactivator (CIITA) contains an acid transactivation domain, NLRBs or neuronal apoptosis inhibitor proteins (NAIPs) possess a baculovirus inhibitor of apoptosis protein repeat (BIR), NLRCs have a caspase-recruitment domain (CARD), and NLRPs a pyrin domain (PYD). NLRX1 contains a CARD-related X effector domain. Among the NLRs, NLRP1, NLRP3, NLRP6, NLRP7, NLRP12, NLRC4, and NAIP have been reported to operate via inflammasomes. Other NLRs such as NOD1, NOD2, NLRP10, NLRX1, NLRC5, and CIITA do not directly engage the inflammatory caspases, but instead activate nuclear factor-kB (NF-kB), mitogen-activated protein kinases (MAPKs), and interferon (IFN) regulatory factors (IRFs) to stimulate innate immunity [32].

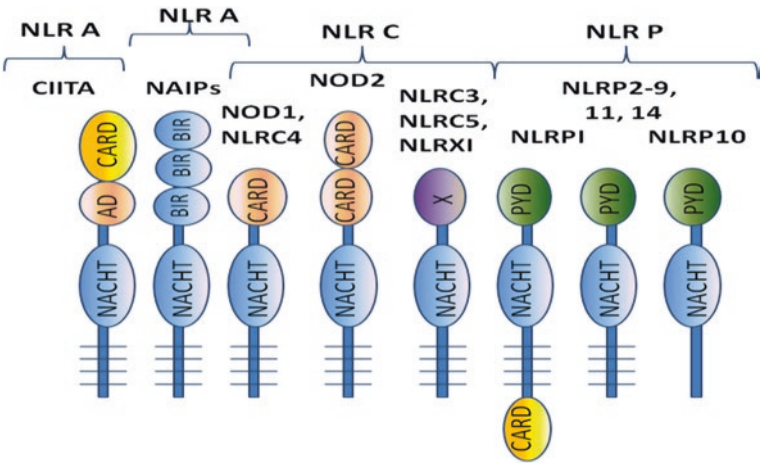

Fig. 8.4 Nod-like receptors

NOD-like receptors have been described as master regulators of innate immunity. NLRs are essential in recognition of microbial- and pathogen-associated molecular patterns (MAMPs and PAMPs), and have the ability to initiate and support robust immune responses through the formation of inflammasomes and the activation of NF-kB, IRF, and MAPK pathways. Functions such as the enhancement of MHC transcription and presentation implicate NLRs in adaptive immunity, and their roles in reproduction indicate a broader responsibility of this gene family than previously suspected. The potency of NLRs in inducing immune defenses is vital for the host, but can also provide serious problems when dysregulation or malfunction occurs (Fig. 8.4).

8.6 Heat Shock Protein/Chaperones

The concept of "protein interaction" is generally used to describe the physical contact between proteins and their interacting partners. However, protein interactions do not always have to be physical [33]. Protein interaction networks are useful resources in the abstraction of basic science knowledge and in the development of biomedical applications. Therefore, protein interaction networks can elucidate the molecular basis of disease, which in turn can inform methods for prevention, diagnosis, and treatment [34].

Microorganisms frequently change the pH of their own habitat by producing acidic or basic metabolic waste products. If the external pH decreases to 4.5 or lower, chaperones such as acid shock proteins and heat shock proteins are synthesized. Chaperones were first discovered because they dramatically increased in concentration when cells were exposed to high temperatures, metabolic poisons,

and other stressful conditions. Thus many chaperones are often called heat shock proteins or stress proteins. Heat shock proteins influence infectious disease processes in a number of diverse ways: they are involved in the propagation of prions, replication and morphogenesis of viruses, and resistance of parasites to chemotherapy. Several heat shock proteins function as intracellular chaperones for other proteins. They play an important role in protein-protein interactions such as folding and assisting in establishment of proper protein conformation (shape) and prevention of unwanted protein aggregation. By helping to stabilize partially unfolded proteins, HSPs aid in transporting proteins across membranes within the cell [35]. These proteins also appear to be important mediators of bacteria-host interactions and inflammation, the latter via interactions with cell surface molecules and structures such as Toll-like receptors and lipid rafts. Heat shock proteins can be expressed on the surface of infected cells, and this is likely to provide a target for the innate immune response. Elevated levels of circulating HSP are present in infectious diseases and these proteins might therefore regulate inflammatory responses to pathogenic challenge on a systemic basis [36]. For many years it was believed that polypeptides would spontaneously fold into their final native shape, either as they were synthesized by ribosomes or shortly after completion of protein synthesis. Although the amino acid sequence of a polypeptide does determine its final conformation, it is now clear that special helper proteins aid the newly formed or nascent polypeptide in folding to its proper shape [37]. These proteins, called molecular chaperones, recognize only unfolded polypeptides or partly denatured proteins and do not bind to normal, functional proteins. Their role is essential because the cytoplasmic matrix is filled with nascent polypeptide chains and proteins. Under such conditions it is quite likely that new polypeptide chains often will fold improperly and aggregate to form nonfunctional complexes. Molecular chaperones are key players of the homeostasis of the proteome network and suppress incorrect folding and may reverse any incorrect folding that has already taken place [38]. Therefore, their expression and the activity are tightly regulated at both the transcriptional and posttranslational level at various age-related diseases (e.g., degenerative diseases and cancer) [39].

Chaperones and ATP-dependent proteases collectively engaged in the first line of defense of mitochondrial protein quality control (mtPQC) system, and this way it monitors the removal of oxidatively damaged polypeptides. For example, ‘Lon protease’ governs the removal of oxidized proteins in yeast and mammalian mitochondria [40].

8.7 Transmembrane Protein and Cellular Junctions

Many transmembrane proteins are also involved in the protection from infectious diseases. For example, interferon-induced transmembrane protein-3 (IFITM3) is a virus restriction factor mediating cellular resistance to influenza viruses and other viruses that enter cells via the acidic endosome [41]. Transmembrane mucins

and their *O*-glycans on the glycocalyx provide the transcellular barrier, a second layer of protection. Cell surface glycans bind carbohydrate-binding proteins that respond to extrinsic signals and modulates pathogenic responses. Apart from maintaining the homeostasis, it also restricts drug targeting of epithelial cells [42]. Some of the members of gap junction (GJ) family proteins like intercellular channels, which connect the cytoplasm of neighboring cells and hemichannels that connect the intra- and extracellular milieu, are also known to participate in physiologic and pathologic processes including electrical conduction, inflammation, immune system activation, tissue repair/remodeling, and response to bacterial and viral infections. However, little is known about the role of GJ channels in parasite infection.

8.8 Protection from Infectious Diseases

A number of proteins take part in protection of human body from external harmful agents. Keratin protein is a component of skin which is considered the first line of defense. This innate immunity component serves to prevent microorganisms from entering the body. Lysozyme, an enzyme present in tears and saliva, and thus, another protein also confer protection to the body. Likewise, almost all the elements of our immune system, e.g., Immunoglobulins (antibodies), various cell surface proteins (T and B cell receptors, Major histocompatibility complex, CD4, CD8, CD3, etc.), the several adaptor proteins and enzymes, involved in signal transduction pathways, are all of protein origin and crucial for combating against external infections.

Since peptides play a crucial role in the fundamental physiological and biochemical functions of life, they have for decades now attracted much attention for their potential therapeutic use. Compared with small chemical entity drugs, peptide-based drugs possess certain favorable characteristics, including:

- Higher potency: Peptide-based drugs generally are very active on their target receptor, which translates into a high effect at a low dose;
- Higher selectivity: Peptides can very tightly fit to their receptors, which make them much more selective than smaller molecules. This means that peptides tend to bind only to their target receptor and therefore are less likely to be associated with serious adverse side effects;
- Naturally occurring biologics—better safety: Peptides are naturally degraded in the blood stream by circulating enzymes to their component amino acids. As these are natural biological products, peptide drugs are also associated with less accumulation in body tissue and fewer toxicity findings also owing to their low doses.

There are a few challenges associated with the use of peptides like they are generally short-lived, cannot be administered orally and have low product stability.

Various peptide/glycopeptides drugs are administered: Vancomycin, Vasopressin, Insulin (recombinant), Nesiritide, Ceruletide, Bentiromide, Exenatide, etc.

Vaccination, composed of several natural and artificial proteins, is a key strategy for the control of various infectious diseases. Many pathogens, such as *Streptococcus pneumoniae*, *Haemophilus influenzae* type b (Hib), and *Neisseria meningitidis* produce on their surfaces dense and complex glycan structures, which represent an optimal target for eliciting carbohydrate specific antibodies able to confer protection against those bacteria. The traditional mechanism of action of glycoconjugates has considered peptides generated from the carrier protein to be responsible for T cell help recruitment. Progress of synthetic and biosynthetic methods for the preparation of glycoconjugates gives new insights for the design of improved carbohydrate-peptide conjugate vaccines [43]. Similarly, *Staphylococcus aureus* is a prominent cause of human infections worldwide and is notorious for its ability to acquire resistance to antibiotics. Methicillin-resistant *S. aureus* (MRSA), in particular, is endemic in hospitals and is the most frequent cause of community-associated bacterial infections in the United States. *S. aureus* produces numerous molecules that can potentially promote immune evasion, including protein A (SpA), an immunoglobulin (Ig)-binding protein present on the bacterial surface and freely secreted into the extracellular environment. This finding provides the foundation to develop a vaccine that prevents severe *S. aureus* infections [44].

8.9 Prion

Stanley B. Prusiner coined the term prion in 1982. It is basically derived from the words protein and infectious [45]. The protein that prions are made of (PrP) is found throughout the body, even in healthy people and animals. However, PrP found in infectious material has a different structure and is resistant to proteases, the enzymes in the body that can normally break down proteins. The normal form of the protein is called PrP^{C}, while the infectious form is called PrP^{Sc}—the *C* refers to 'cellular' or 'common' PrP, while the *Sc* refers to 'scrapie', the prototypic prion disease, occurring in sheep [46]. A prion in the Scrapie form (PrP^{Sc}) is an infectious agent composed of protein in a misfolded form [47]. So, they are not considered living organisms but may propagate by transmitting a misfolded protein state. If a prion enters a healthy organism, it induces existing, properly folded proteins to convert into the disease-associated, prion form; the prion acts as a template to guide the misfolding of more proteins into prion form. These newly formed prions can then go on to convert more proteins themselves; this triggers a chain reaction that produces large amounts of the prion form [48]. This altered structure is extremely stable and accumulates in infected tissue, causing tissue damage and cell death [49]. All long-term hematopoietic stem cells express PrP on their cell membrane and those hematopoietic tissues with PrP-null stem cells exhibit increased sensitivity to cell depletion [50].

A naturally occurring disinfectant exists within common lichens and might actually be able to stop prions in the wild. Christopher Johnson and his team describe experiments with lichens, symbiotic collections of algae, fungus, and bacteria that casual observers might mistake for moss. Three common species of lichens, the team has found, exude an enzyme that breaks down the prion. They showed that these lichen extracts efficiently degrade disease-associated prion protein (PrPTSE), the probable etiological agent of the transmissible spongiform encephalopathies (TSEs), and suggest that some lichens could have potential to inactivate TSE infectivity on the landscape or be a source for agents to degrade prions [51].

8.10 Conclusion

Proteins are one of the most important biomolecules, involved in any biological process of all living beings, playing useful as well as harmful roles in their survival. It is necessary to investigate these proteins, and to develop new biomedical tools and technologies, which will play a key role in evasion of various diseases, thus, saving and improving quality of lives. Further research is ongoing and should be carried out to explore this area of utmost concern.

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Chapter 9
Adverse Effect in Human Beings Associated with Excess Dietary Protein Intake

Bhanu Pratap Singh Gautam, Manjul Gondwal and Navneet Kishore

Abstract Proteins are the vital macronutrients for the growth and maintenance in human body. Based on the recommended dietary allowance (RDA) for protein is differs person to person according to their body requirements. In common there are three significant measures of protein intake, absolute intake, intake related to body weight and intake as a fraction of total energy. However, high protein consumption diets above the current RDA could be cause disorders on human health. Excessive protein in the body adversely affects the bone, also causes hyperinsulinemia, hyperammonemia, hyperaminoacidemia, nausea, diarrhea, and even death. Moreover, the search for benefits, causes, and role of proteins in the biological system is ongoing worldwide and cited in many literature reports. However, the present article focused on the basic evidence about the protein-rich diets, excess protein intake, and various disorders fashioned in the human body by excess protein intake. This chapter may help to aware many populaces about their protein intake.

Keywords Requisite of protein · Protein-rich diets · Excess protein intake · Adverse effects

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9.1 Introduction

Protein molecules are the backbones of the biological system and they exist in all the living organisms on the earth. These protein molecules are the key for most of the complex functions in the organisms which make their life possible by playing a role in biochemical reactions within the cells. Proteins are longer chains of amino acid units (greater than 100) which are linked by peptide bonds. Proteins are the essential macronutrients needed by the human body for growth and maintenance and considered as universal in cellular chemistry for carrying out most of the biochemical reactions in the cells. According to the Recommended Daily Allowance (RDA), the essential requirement of proteins by every human is defined as the minimum level of protein necessary to maintain short-term nitrogen balance under controlled energy intake conditions. The people intake protein from animals like fish, meat, eggs, poultry and dairy products whereas from the plants are mainly through legumes, nuts, and grains. The current recommended dietary allowance (RDA) for protein is 1.0, 1.5, and 0.8 g/kg body weight per day for children, youngsters, and adults, respectively [1]. However, high-protein diets are promoted intensively by the nutritional supplements industry and they are considered to be "the gold standard" by many athletes (especially bodybuilders) for muscle development and/or body fat loss [1–4]. On the other hand, several scientists claim that the overuse of protein supplements or high dietary protein intake could cause disorders to human health [5–7].

It is a matter of much controversy over the advantages and disadvantages of various quantities of protein utilization and the metabolic fate of the amino acid content of various proteins, mainly due to the limited amounts of data pertaining to protein metabolism and amino acid kinetics in humans. RDA level assumes the primary use of amino acids as substrates for synthesis of body proteins; however, there are evidences that additional metabolic roles for some amino acids require plasma and intracellular levels above minimum needs for protein synthesis [8]. As protein foods related with saturated fat are generally expensive. Combining US protein and fat recommendations (average total energy intake of 820 kcal/d) and the average US energy intake of approximately 2100 kcal/d [9], it is possible to estimate by default that the carbohydrate intake current nutrition policy recommends at 1280 kcal/d (320 g/d), which produces a carbohydrate, protein intake ratio of >3.5 [8]. The aim of this chapter is to decide the potential health dangers due to high protein intake obtained from diet or nutritional supplements based on the human studies present in the literature.

For carbohydrate eating, no minimum RDA has been established; the minimum daily carbohydrate requirement for tissues can be determined at approximately 100–200 g glucose/d [10], giving a dietary intake ratio of approximately 1.5 for the minimum metabolic needs for carbohydrate to protein. This is evidence that high carbohydrate diets may increase blood triglycerides [11], reduce fat oxidation [12], and reduce satiety [13]. Some evidence for an increased protein intake in the Western diet is due to the less development of agriculture and is well known to suppress appetite and food intake in humans [14].

9.2 Structure of Proteins

The proteins are large molecular complex architecture. The protein and peptide are linear and adapt a variety of specific folded three-dimensional patterns and conformation (Fig. 9.1). Among the three macronutrients (carbohydrate, fat, and protein), protein has the most suppressing effect on food intake. In addition, dietary protein has been shown to induce higher satiating and thermogenic effects and greater weight loss than carbohydrates [15, 16].

Structure of a protein is directly related to its function, so that anything that severely disrupts the shape will also disrupt the function.

There are four types of protein structure.

1. Primary structure
2. Secondary structure
3. Tertiary structure
4. Quaternary structure

For such "open" structures there is no theoretical limit on their repeat number, since incremental addition of repeats is not sterically impeded. These rod-like or superhelical structures present an extensive solvent-accessible surface that is well suited to binding large substrates such as proteins and nucleic acids. By contrast, duplication of repeats in a superhelix with a small pitch results in a closed barrel-like structure, with a relatively small surface area available for ligand interactions with smaller ligands(e.g., *Kelch* in Fig. 9.2).

Tandomly repetitive structures often occur in regular arrangements, either in linear arrays (e.g., *Iafp* in Fig. 9.1) or as a super helix with repeats arranged about a common axis (eg., *HEAT* in Fig. 9.2).

These assemblies are likely to present different advantages than the open structures of rods and super helices. They are compact and stable, with

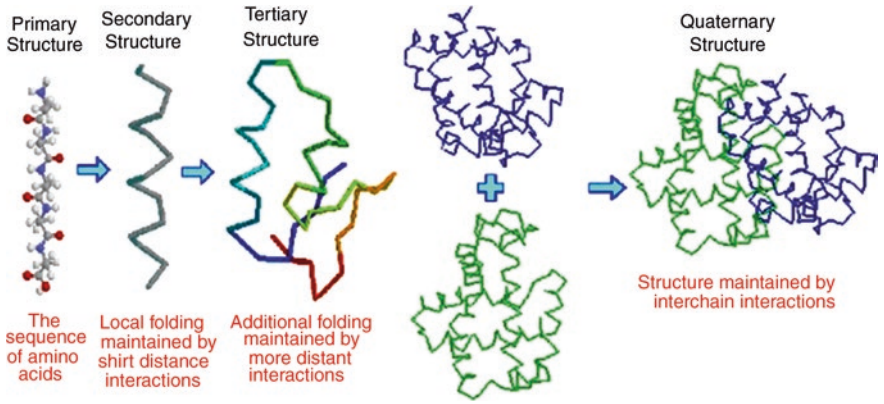

Fig. 9.1 Structure of the protein

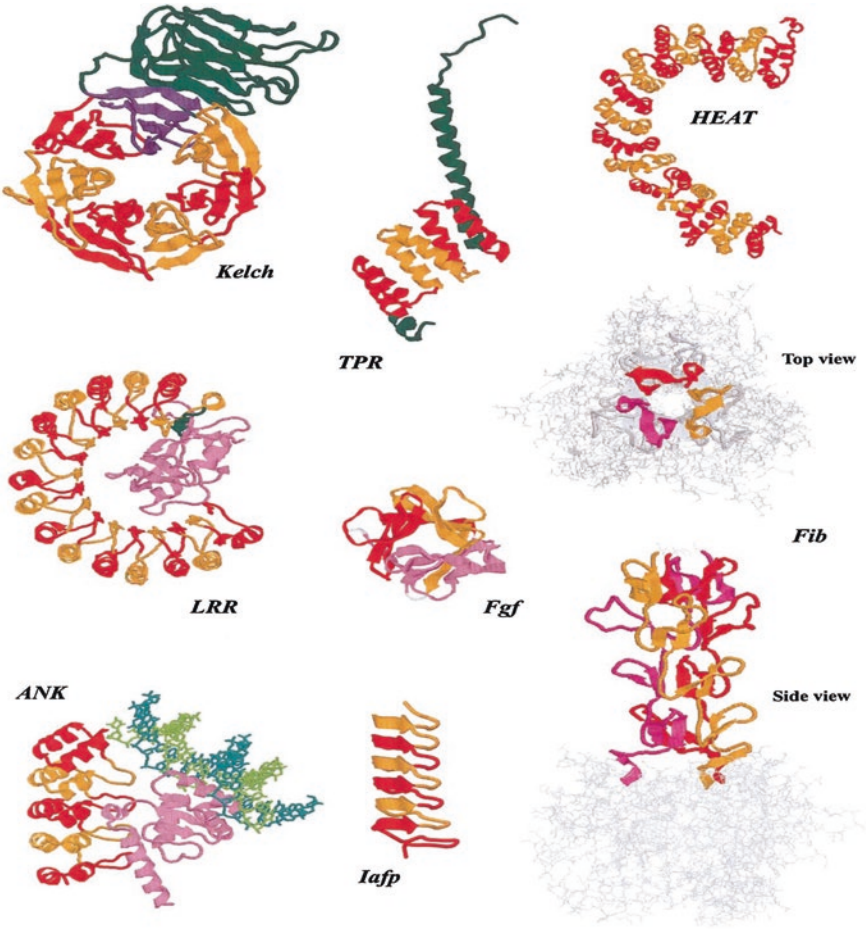

Fig. 9.2 Showing the *kelch*, *TPR*, *LRR*, *ANK,* and *lafp* of protein [55]

opportunities for small ligands to be bound either along the internal axis of the barrel or on the axis at the barrel's periphery. Fixation of a repeat doubling and sequence similarities among repeats may erode quickly. Thus equivalent HEAT repeats in invertebrate and mammalian orthologues average only 13 %sequence identity [17]. These light similarities imply that the functional constraints on individual repeats are relatively weak, when compared to the constraints forced on the repeat assembly as a whole. By contrast, a function that is exacting on the structure of repeats, such as those in the ice-binding b-sheet domain of insect antifreeze proteins [18], results in repeats being highly similar in sequence.

9.3 Classification of Proteins

On the bases of following, the proteins are classified as

1. On the basis of their functions (Table 9.1)
2. Posttranslational modification (Table 9.2)
3. Location in the living cell (Table 9.3)

9.4 Need of Top Protein Intake

An individual consuming a diet containing 35 % energy as protein appears to be consuming a dangerously excessive level of protein. However, if the total dietary energy intake is 8000 kJ/d, this equates to 165 g protein per day. For an 80 kg person this would be equivalent to (2.1 g kg^{-1} d^{-1}), well below the maximal level. Even for a 60 kg individual (2.7 g kg^{-1} d^{-1}) it is below the maximal safe level. There are three principal ways in which protein intake can be quoted:

(1) As the amount consumed per kilogram of body weight per day.
(2) As the absolute amount consumed in grams per day.
(3) As a percentage contribution to daily energy intake based on its energy content of 17 kJ/g.

Table 9.1 Classification of protein on the basis of their functions

S. No.	Functions of proteins	
1.	Antibodies	Interferon, Fibrin
2.	Enzymes	DNA and RNA polymerase
3.	Hormones	Endorphine and enkephalin
4.	Motor proteins	Actin, Myosin
5.	Receptors	Transmembrane proteins
6.	Transport proteins	Cytochrome C, Albumin, Hemoglobin
7.	Structural proteins	Collagen, Elastin
8.	Storage proteins	Egg albumin, milk casein

Table 9.2 Classification of protein on the basis of their posttranslational modification and location in the living cell

S. No.	a. Posttranslational modification	b. Location in the living cell
1.	Native protein	Membrane proteins
2.	Cleaved protein	Internal proteins
3.	Prions	External proteins
4.	Protein with disulfide bonds	Virus proteins
5.	Protein complexes	
6.	Chemically modified proteins	
7.	Glycoprotein	

A more convenient and practical approach, which may still provide beneficial outcomes is a 25 % protein energy diet. Any such diet with high protein intake should also contain a wide range of whole grain cereals, fresh vegetables, and fruits, rich in micronutrients and potassium alkali salts needed to reduce the potential renal acid load and subsequent urinary calcium loss that can occur due to the acidic nature of protein-rich diets. Care should be taken at this level of protein intake as other nutrient-rich foods may be displaced from the diet, leading to micronutrient deficiencies. High-protein diets are actually low in carbohydrate (25–90 g/d) and contain large amounts of total fat (50–60 %), and saturated animal fat (30–50 g/d) [19, 20]. A more convenient and practical approach, which may still provide beneficial outcomes is a 25 % protein energy diet, which would provide 118 g protein on an 8000 kJ/d diet at 1.5 g kg^{-1} d^{-1} for an 80 kg individual. These fad diets should not be incorrectly compared with the recommendations of this article, nor to the studies cited in this article which are neither high in fat nor low in carbohydrate [8, 21–24], particularly in light of recent minimum carbohydrate recommendations of 130 g/d [25]. Numerous studies have also shown improvements in blood lipid profiles on diets with increased protein intakes. O'Dea et al. [26] showed a marked improvement in carbohydrate and lipid metabolism in diabetic Australian Aborigines after temporary reversion to a traditional hunter–gather lifestyle, where energy derived from protein reached 54 % [27].

9.5 Protein Metabolism

Advances in understanding protein metabolism have been made in the last few years with the dual tracer methodology for assessing differences between exogenous and endogenous amino acid contribution to the protein pool, enhancing our comprehension of amino acid absorption kinetics. The application of radioactive and stable isotopes for the measurement of gluconeogenesis using mass isotopomer distribution analysis (MIDA) have also furthered our understanding the role of amino acids play postprandially [28]. However, the role of dietary protein and amino acids in modulating insulin and glucagon secretion are less clear, as is an understanding of what fraction of amino acid load contributes to structural or functional protein needs, oxidation, gluconeogenesis, or a combination of all three. Although protein digestibility has been established for milk, pea, whey, casein, and free amino acids derived from enteral protein, less is known about specific absorption rates of protein-based foods such as meat, chicken, fish, and legumes. The practical implications for appreciative this information is exemplified by a novice bodybuilder who may consume 250–400 g of protein isolate on a daily basis [29, 30]. To develop better understanding of amino acid kinetics, the initial part of this chapter will examine protein metabolism, focusing on the quantification of maximal protein consumption using available data on maximal rates of urea synthesis, and amino acid absorption rates and suggest using this data, a possible upper limit (measured in grams per day) for the rate of amino acid metabolism (Table 9.3).

Table 9.3 Degradation pathway of proteins and peptides [16]

S. No.	Degradation pathways and physical instability	Mechanism
1.	Adsorption	Amphiphilic natures of protein cause adsorption at various interfaces like air-water and air solid
2.	Denaturation	Nonproteolytic modification of a unique structure of a native protein that affects definite change in physical, chemical, and biological properties. Several examples of denaturizing agents are urea, alcohol, acetic acid, sodium dodecyl sulfate, polyethylene glycol
3.	Aggregation and precipitation	The denatured, unfolded protein may rearrange in such a manner that hydrophobic aminoacid residue of various molecules associate together to form the aggregates. If aggregation is on macroscopic scale, precipitation occurs
4.	Oxidation and Reduction	Oxidation occurs during isolation, synthesis, and storage of proteins. Temperature, pH, trace amount of metal ion and buffers influence these reactions. Glucagon is an exception as it retains biological activity even after oxidation
5.	Deamidation	The hydrolysis of the side chain amide linkage of an amino acid residue leading to the formation of a free carboxylic acid
6.	Proteolysis	It may occur on exposing the proteins to harsh conditions like prolonged exposure to extreme of pH or high temperature or proteolytic enzyme
7.	Disulfide exchange	A peptide chain with more than one disulfide can enter into this reaction and thereby change in conformation
8.	B-elimination	It proceeds through a carbanion intermediate. Protein residues susceptible to it under alkaline conditions include Cys, Lys, Phe, Sre, and Thr
9.	Racemization	It is alteration of L-amino acids to D, L-mixtures. Racemization forms peptide bonds that are sensitive to proteolytic enzymes

9.6 Adverse Effects of High-Protein Diet

The potential adverse effects of protein overconsumption are given as

9.6.1 Bone Disorders

High-protein diet produces a large amount of acid in body fluids [31]. The kidneys respond to this dietary acid challenge with net acid excretion and, at the same time as, the skeleton supplies buffer by active resorption of bone resulting in extreme calcium loss [31]. Moreover, in a study where protein intake was varied from

47 g/day (low protein diet) to 95 g/day (medium protein diet) and to 142 g/day (high-protein diet), the urinary calcium increased considerably with each increase in protein (168, 240, and 301 mg, resp.) [32]. Another study on subjects consuming diets containing 48 g protein daily to 142 g showed that urinary calcium doubled, while the calcium balance became negative [33]. In addition, the effect of dietary protein on markers of bone turnover has been evaluated [34]. In this study, the subjects were on a well-balanced diet for 2 weeks which was followed by 4 days of experimental diet containing one of three levels of protein (low, medium, or high).

Moreover, acid loading directly inhibits renal calcium reabsorption leading to hypercalciuria in combination with the excessive bone loss [3, 35]. In a metabolic study, an increase in protein intake from about 47 to 112 g caused an increase in urinary calcium and a decrease in calcium retention. The data indicated that protein-induced hypercalciuria was due to an elevation in glomerular filtration rate and a lower fractional renal tubular reabsorption of calcium, the latter of which caused by the increased acid loads on the renal tubular cells [36].

Urinary calcium excretion was significantly higher, and urinary N-telopeptide excretion (indicator of bone resorption) was significantly greater during the high-protein intake than during the low protein intake. Data suggested that at high levels of dietary protein, at least a portion of the increase in urinary calcium reflected increased bone resorption [34]. In a potential study, protein was associated with an increased risk of forearm fracture for women who consumed more than 95 g per day compared with those who consumed less than 68 g per day. Women who consumed five or more servings of red meat per week also had a significantly increased risk of forearm fracture compared with women who ate red meat less than once per week [37]. Additionally, subjects on a low-carbohydrate high-protein (LCHP) diet for 6 weeks had increased urinary calcium levels, decreased calcium balance, and decreased serum osteocalcin concentrations [38]. In a potential study, protein was associated with an increased risk of forearm fracture for women who consumed more than 9 5 g per day compared with those who consumed less than 68 g per day. Women who consumed five or more servings of red meat per week also had a significantly increased risk of forearm fracture compared with women who ate red meat less than once per week [37].

Furthermore, the effect of high-protein diets on the excretion of calcium in urine was evaluated in normal persons and patients with nephrolithiasis. All subjects were given diets containing 0.5 g protein/kg/day, while, during the experimental phase, each person received an additional 1.5 g protein/kg/day. There was a steady increase in urinary calcium with the high-protein diet averaging 88 % above control in the normal and 82 %in the patients [39]. In addition, it has been shown that increasing the protein intake from 48 to 141 g daily caused a highly important elevation in urinary calcium, the mean daily values being 175 and 338 mg, respectively [40]. In another study, the association of animal protein-rich diet to calcium metabolism was investigated during a 12-day dietary period. An increase in urinary calcium excretion was found indicative of the animal protein-induced calciuric response which could be a risk factor for the development of

osteoporosis [467]. Moreover, dietary excess (2 g/kg/day) in animal protein for 1 week led to major changes in urinary calcium excretion rates [41].

Furthermore, in a motivating study the effects on urinary calcium levels of increasing dietary protein from 50 to 150 g protein were compared with those of increasing the sulfur amino acids to suggest the amounts present in the 150 g protein diet. The increase in protein intake caused urinary calcium to double, while sulfur amino acids added to the low protein diet also caused urinary calcium to increase [42]. Mainly, it has been shown that the use of high calcium diets is unlikely to prevent the negative calcium balance and likely bone loss induced by the consumption of high-protein diets (protein-induced hypercalciuria) [43]. In this experiment (a 95-day metabolic study), subjects received formula diets supplying 12 g nitrogen or 36 g nitrogen, and about 1400 mg calcium per day. Usually, calcium balances was 37 mg/day on the 12 g nitrogen diet, and considerably lower at 137 mg/day in subjects consuming the high-protein diet [43]. In addition, a potential study showed that a high ratio of dietary animal to vegetable protein increases the rate of bone loss and the risk of fracture in post-menopausal women. Animal foods provide mostly acid precursors, whereas a protein in vegetable food is accompanied by base precursors not found in animal foods. Imbalance between dietary acid and base precursors leads to a chronic net dietary acid load that may have adverse consequences on bone. An increase in vegetable protein intake and a decrease in animal protein intake may decrease bone loss and the risk of hip fracture [44].

9.6.2 Renal Function Disorders

Extreme intakes of proteins are important risk factors for kidney stones [3]. Protein ingestion increases renal acid excretion, and acid loads, in turn, may be buffered in part by bone, which releases calcium to be excreted by the kidney. This protein-induced hypercalciuria could lead to the formation of calcium kidney stones [35]. In addition, animal protein is also the major dietary source of purines, the precursors of uric acid. Extreme intake of animal protein is therefore related with hyperuricosuria, a condition present in some uric acid stone formers [5]. Uric acid solubility is largely determined by the urinary pH. As the pH falls below 5.5–6.0, the solubility of uric acid decreases, and uric acid precipitates, even if hyperuricosuria is not present [5]. An attractive study on the effects of protein overload on stone-forming propensity showed that consumption of high-protein diet for 6 weeks delivers a marked acid load to the kidney and increases the risk for stone formation (urinary citrate levels decreased, and urinary saturation of undissociated uric acid increased) [38]. The decreased ability of urines to inhibit the agglomeration of calcium oxalate crystals could provide a possible physicochemical explanation for the adverse effects of high-protein diet on renal stone formation [41]. Additionally, it has been indicated that high-protein intake could cause increased glomerular filtration rate and decreased fractional renal tubular

reabsorption of calcium and urinary sodium [42]. Furthermore, in a study of three 12-day dietary periods during which the diet of the subjects contained vegetable protein, vegetable and egg protein, or animal protein, it was found that the animal protein-rich diet was associated with the highest excretion of undissociated uric acid due to the reduction in urinary pH [45]. Moreover, citrate excretion was reduced because of the acid load, and urinary crystallization studies revealed that the animal protein diet conferred an increased risk for uric acid stones [45]. In another study it was shown that a high-protein intake induced changes in urinary uric acid and citrate excretion rates and a decrease in the ability of urines to inhibit calcium oxalate monohydrate crystal agglomeration [41].

In another study, healthy subjects with a history of renal stones fed on a low (LPD) and a high (HPD) animal protein diet; after 2 weeks it was found that high dietary intake of purine-rich animal protein had an impact on urinary urate excretion and super saturation in renal stone disease [46]. There was an increase in urinary urate, urinary acid excretion, ammonium ion excretion, and uric acid super saturation and a fall in urine pH on HPD. The risk of forming uric acid or ammonium urate crystals or stones in the urine was increased on a high-protein diet [46]. Besides, in a prospective cohort study it was investigated whether protein intake influences the rate of renal function change over an 11-year period. The results showed that high total protein intake, particularly high intake of nondairy animal protein, may accelerate renal function decline in women with mild renal insufficiency [47]. In addition, a study about the short-term effect of increasing the dietary consumption of animal protein on the urinary risk factors for stone formation showed increased levels of urinary calcium and oxalate. The accompanying increase in dietary purine caused an increase in the excretion of uric acid. Generally, relative chance of forming stones, calculated from a combination of the risk factors, was markedly increased (250 %) during the period of high animal protein ingestion [48].

9.6.3 Risk of Increased Cancer

Up to 80 % of breast, bowel, and prostate cancers are known to dietary practices, and international comparisons show positive relations with high-meat diet [6]. The association, though, seems to have been more continuously found for red meat or processed meat and colorectal cancer [49]. It should be noticed that red meat is the main dietary source of saturated fat, which has been related with breast and colorectal cancers [30]. Moreover, NH_3 and N-nitroso compounds (NOC) formed from residues by bacteria in the large bowel are probably also important. NH_3 is a promotor of large bowel tumors chemically induced by NOC, and some of the chromosomal mutations found in human colorectal cancer are consistent with the effects of NOC and heterocyclic amines [6]. In a cohort study, subjects who were free of diagnosed cancer completed a validated food frequency survey and provided detailed information on other lifestyle and health-related factors. An

elevated risk of colon cancer was related with red meat intake [50]. Men who ate beef, pork, or lamb as a main dish five or more times per week had an elevated relative risk compared to men eating these foods less than once per month. The association with red meat was not confounded appreciably by other dietary factors, physical activity, body mass, alcohol intake, cigarette smoking, or aspirin use [50]. Furthermore, in a prospective study subjects without a history of cancer, inflammatory bowel disease, or familiar polyposis completed a dietary questionnaire [51]. After adjustment for total energy intake, animal fat was positively associated with the risk of colon cancer. The relative risk of colon cancer in subjects who ate beef, pork, or lamb as a main dish everyday was increased, as compared with those reporting consumption less than once a month [51]. In an interesting study, the overall dataset derived from an integrated series of case-control studies included histologically confirmed neoplasm; controls were patients admitted to hospital for acute, non-neoplastic conditions unrelated to long-term modifications in diet [52]. The multivariate odds ratios (ORs) for the highest tertile of red meat intake ($\geq$7 times/week) compared with the lowest ($\leq$3 times/week) were 1.6 for stomach, 1.9 for colon, 1.7 for rectal, 1.6 for pancreatic, 1.6 for bladder, 1.2 for breast, 1.5 for endometrial, and 1.3 for ovarian cancers. Thus, reducing red meat intake might lower the risk for several common neoplasms [52].

9.6.4 Liver Function Disorders

In addition, high-protein/high-meat diet could cause disorders of liver function and precipitated progression of coronary artery disease. Hyperalbuminemia and elevated transaminases have been associated with high-protein diet [53]. Individuals on high protein supplements developed intermittent abdominal pain, transient elevations in transaminases, and hyperalbuminemia without there being any identifiable cause. The symptoms and abnormalities on the laboratory tests resolved after the high-protein intake was discontinued [53].

9.6.5 Coronary Artery Diseases

In a case-control study, subjects (treatment group/TG) were studied for 1 year by using myocardial perfusion imaging (MPI), echocardiography (ECHO), and serial blood work [54]. MPI and ECHO were performed at the beginning and end of the study for each individual. The TG studied modified their dietary intake as instructed. Additional subjects (high-protein group/HPG) elected a different dietary regimen consisting of a "high-protein" diet [54]. Subjects in the TG demonstrated a reduction in each of the independent variables studied with falling off in both the extent and severity of coronary artery disease (CAD) as quantitatively measured by MPI. Individuals in the HPG showed degeneration of

their independent variables. These results would suggest that high-protein diets may precipitate progression of CAD through increases in lipid deposition and inflammatory and coagulation pathways [54].

9.7 Conclusions

Although the fact that short-term high-protein diet could be required in several pathological conditions (malnutrition, sarcopenia, etc.), it is evident that "too much of a good thing" in diet could be useless or even harmful for healthy individuals. Many adults or even adolescents (especially athletes or body builders) self-prescribe protein supplements and fail to see the risks of using them, mainly due to unwise beliefs in their performance-enhancing abilities. Individuals who follow these diets are therefore at risk. Extra protein is not used efficiently by the body and may oblige a metabolic saddle on the bones, kidneys, and liver. In addition, high-protein/high-meat diets may also be associated with increased risk for coronary heart disease due to intakes of saturated fat and cholesterol or even cancer. Strategy for diet should remain closely to what has been clinically proved, and by this standard there is presently no basis to suggest high protein/high-meat intake above the suggested dietary allowance for healthy adults.

The amounts of protein consumed by humans vary over a wide range. When dietary nitrogen and essential amino acid intakes are above the requirement levels, healthy individuals appear to adapt well to highly variable dietary protein intakes, because frank signs or symptoms of amino acid excess are observed seldom, if at all, under usual dietary conditions. Thus, definition of tolerable ranges of amino acid intake in healthy people will require approaches that identify deviations from normal physiological and biochemical adaptive processes at the subclinical level.

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Chapter 10
Gelatin Nanocomposites (GNCs): An Efficient Drug Delivery System

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Abstract The literature reported that natural proteins are being widely used as potential carriers for site-specific drug delivery as they bear nontoxic and biocompatible behavior. Modified proteins have shown also their potent role in gene delivery, cell culture, and tissue engineering. Gelatin is one of the most versatile widely used proteins in pharmaceutics because of biocompatibility, biodegradability, low cost, and other applications. These advantages led to its application in the synthesis of nanoparticles to deliver drugs and genes in the last few decades. Impact of gelatin binding to various drugs has been investigated for controlled release applications by various research groups. Various parameters like cross-linking density and isoelectric point can be used to tune the optimization of gelatin degradation and drug delivery kinetics. At present, gelatin nanocomposites play a crucial role in various aspects of biology and medical sciences. Various cross-linkers used to improve the physicochemical behavior of gelatin nanocomposites (GNCs) have been reported. Further, physicochemical behaviors of GNCs

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including drug loading, release, particle size, zeta-potential, cytotoxicity, cellular uptake, and stability are explained. Various groups explained the applications of GNCs in delivery of drugs and genes as well as their in vivo pharmacological performances. Our emphasis is to study the interaction of various factors of biological macromolecules in the extracellular matrix which regulate the function of bioactive molecules. In light of the importance of GNCs, gelatin has proven to be a good biomaterial for the controlled release of several biologically active molecules. Although, research is still continued to improve the role of gelatin to release drugs/genes through the use of composite scaffolds and gelatin modification.

Keywords Gelatin · Modified biopolymers · Biodegradation · Drug delivery

10.1 Introduction

Gelatin is a well-known structural natural protein used in the daily life in the scientific and technological areas for the preparation of a great variety of composite materials. In spite of its abundance and common applications, gelatin presents itself with a mixed character between a protein as it is derived from collagen, and a synthetic linear polymer with random spatial arrangement above certain temperature [1–4]. Gelatin has numerous applications like biomedicine, biocompatibility, and biodegradability are decisive and the reinforcement of gelatin matrix by assembling to inorganic or hybrid nanoparticles (NPs) is also required to improve its mechanical stability. Alternative treatments such as chemical crosslinking may also contribute to reduce water swelling and enhance the mechanical properties as well as thermal stability [5–10]. Incorporation of inorganic solids into the proteinous matrix allows the tailoring in terms of the mechanical and functional properties of the resulting gelatin-based composites. Different strategies have been proposed by various groups to tune the functional properties of gelatin like selection of inorganic solids, grafting of suitable functional groups to the gelatin hybrids, and combination of additional polymers or fillers in ternary composites. Using these approaches, advanced functional materials of increasing complexity were developed and opening the way for a wide range of applications of the gelatin-based nanocomposites [11–15].

10.2 Properties of Gelatin and Its Composites

10.2.1 Composition

Gelatin is a mixture of peptides and proteins produced by partial hydrolysis of collagen extracted from the skin, bones, and connective tissues of domestic animals. Pharma grades of gelatin are usually obtained from beef bones, although some beef bone gelatin is used by the food industry [6–10].

10.2.2 Properties

Natural molecular bonds between individual collagen strands are broken down to a form that rearranges more easily. Gelatin melts when heated and solidifies when cooled again. In the presence of water, it forms a semi-solid gel.

10.2.3 Salvation Properties

Gelatin forms a viscous solution in water, and sets to a gel on cooling. Further, its chemical composition resembles to that of its parent collagen. Gelatin is soluble in many polar solvents and shows viscoelastic flow and streaming birefringence.

10.2.4 Thermal Properties

Gelatin gels exist over a small temperature range, wherein the upper limit being the melting point of the gel depends on gelatin grade and the lower limit is the freezing point at which ice crystallizes [16–21].

10.2.5 Mechanical Properties

The literature revealed that gelatin gels are sensitive to temperature and time. The viscosity of the aqueous gelatin increases with concentration and when kept on cooling (≈4 °C). Further, viscosity and other mechanical properties can also be tuned by mixing other inorganic/organic material to it.

10.3 Applications of Various Organic, Inorganic, and Polymeric Systems for Drug Delivery (Fig. 10.1)

10.3.1 Metal Nanoparticles (NPs) Containing Biomolecules

Applications of metal nanoparticles (NPs) have been liberated by the use of nanobioconjugates after the discovery of immune gold labeling. Metal NPs have been used in various biomedical applications including drug delivery (vehicle for delivering drugs, proteins, peptides, plasmids, DNAs, etc.), detection, diagnosis, and therapy. Metal NPs (gold or silver) have optical and electronic properties derived based on size and composition. These nanomaterials have found important

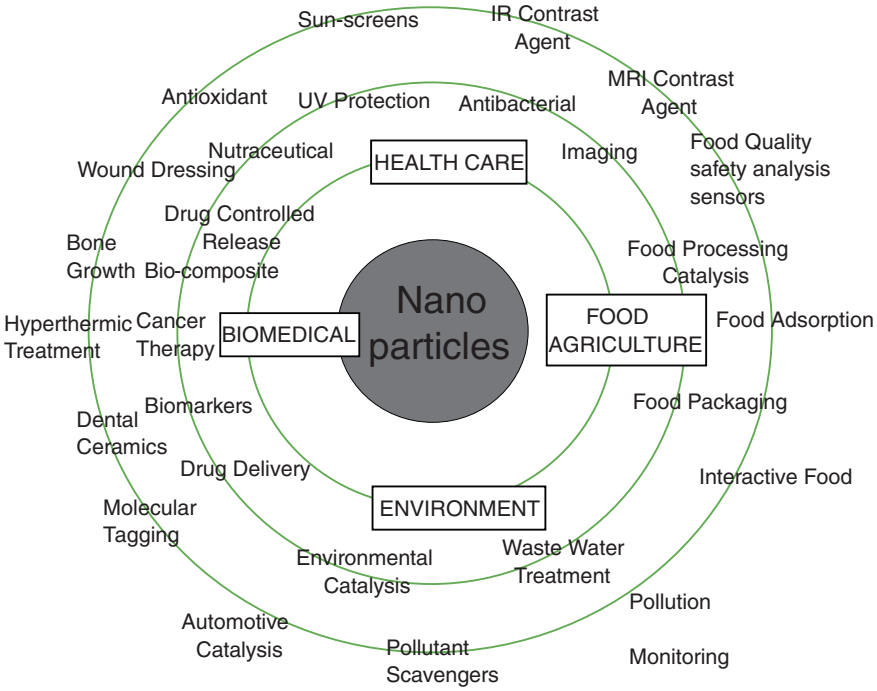

Fig. 10.1 Various applications of nanoparticles in various fields of sciences

applications as chemical sensors, when coupled to affinity ligands. Au NPs conjugated with specific oligonucleotides can sense complementary DNA strands, detectable by color changes [22–25].

10.3.2 Core Metal Nanoparticle

Au nanoparticles can be readily functionalized with probe molecules such as antibodies, enzymes, nucleotides, etc. These hybrid nanostructures are the active elements of a number of biosensor assays, drug and gene delivery systems, laser confocal microscopy diagnostic tools, and other biomaterial-based imaging systems. Silver (Ag) has been known since ancient times as a very effective antimicrobial agent. Ag NPs have been routinely used to prevent the attack of a broad spectrum of microorganisms on prostheses, catheters, vascular grafts, and human skin, reduce infection in burn treatment, arthroplasty, etc. [22–25].

10.3.3 Magnetic Metal Nanoparticles (NPs)

Currently, magnetic nanoparticles (MNPs) have attracted the researchers due to their unique magnetic property. They have the ability to function at the cellular and molecular level of biological interactions making them an attractive platform as contrast agents for magnetic resonance imaging (MRI) and for drug delivery. Recent advances in the field of nanotechnology, the ability to specifically tailor the features and properties of magnetic NPs for these biomedical applications has been improved. However, the safety and efficacy of using magnetic nanoparticles is debatable among scientists. Various researchers worked on the relationship between biocompatibility and surface chemistry of gelatin and magnetic NPs-based composites. The results show that the biocompatibility of gelatin nanocomposites is dependent on both the cell type and the nanoparticles' surface chemistry [26–30].

10.3.4 Metal-Oxide Nanoparticles

Metal-oxide nanoparticles such as titanium oxide (TiO_2) and zinc oxide (ZnO) serve many functions in various polymeric materials. Traditionally, they have been used as pigments to enhance the appearance and improve the durability of polymeric products and usually they have been considered to be inert. Magnetic NPs exhibit unique physical and chemical properties due to their limited size and high density of corner or edge surface sites [31–33].

10.3.5 Effect of Size of NPs

The size and shape of metal nanoparticles influence the properties of gelatin. It comprises the structural characteristics like the lattice symmetry and cell parameters. Metal oxides are usually robust and stable systems with well-defined crystallographic structures. However, the growing importance of surface free energy and stress with decreasing particle size must be considered. Changes in thermodynamic stability associated with size can induce modification of structural transformations and sometimes cause the nanoparticle to disappear due to interactions with its surrounding environment and high surface free energy. In order to display mechanical or structural stability, a nanoparticle must have a low surface free energy. As a consequence of this requirement, phases that have low stability in bulk materials can become very stable in nanostructures. This structural phenomenon has been detected in TiO_2, VO_x, Al_2O_3, or MoO_x oxides [29, 30, 34, 35].

10.4 C-Based Giant Like Molecules (Fullerenes, CNT, Graphene) (Fig. 10.2)

Carbon nanotubes and buckyball clusters belong to the fullerenes (composed of carbon). Carbon nanotubes (CNTs) are carbon coaxial graphite sheets of < 100 nm rolled up into cylinders. They can be classified in two categories based on their structure (i) single-walled carbon nanotubes (SWNT) and (ii) multi-walled carbon nanotubes (MWNT). They have been applied in biology as biosensors for detecting protein, DNA, diagnostics, and carriers. These types of NPs are insoluble in several solvents, provoking toxicity problems and some health concerns. However, they can be chemically modified to make them water soluble and can be functionalized so that they can be linked to active molecules such as nucleic acids, proteins, and therapeutic agents. They have unique electronic, structural, and thermal characteristics that make them appropriate vehicles for drug delivery systems [36–40] (Fig. 10.3).

Liu et al. used single-walled carbon nanotubes (SWNT) chemically functionalized with PEG-paclitaxel (SWNT-PEG-PTX) in a xenograft breast cancer mouse model. They also observed that higher tumor uptake of PTX and higher ratios of tumor to normal-organ PTX uptake for SWNT-PEG-PTX compared to taxol and PEG-PTX. They also showed effective in vivo delivery of SWNT-PEG-PTX with higher tumor suppression efficacy and minimum side effects than taxol. Due to their physicochemical properties, carbon nanotubes have additional applications in computer, aerospace, electronics, and other industries [41–44].

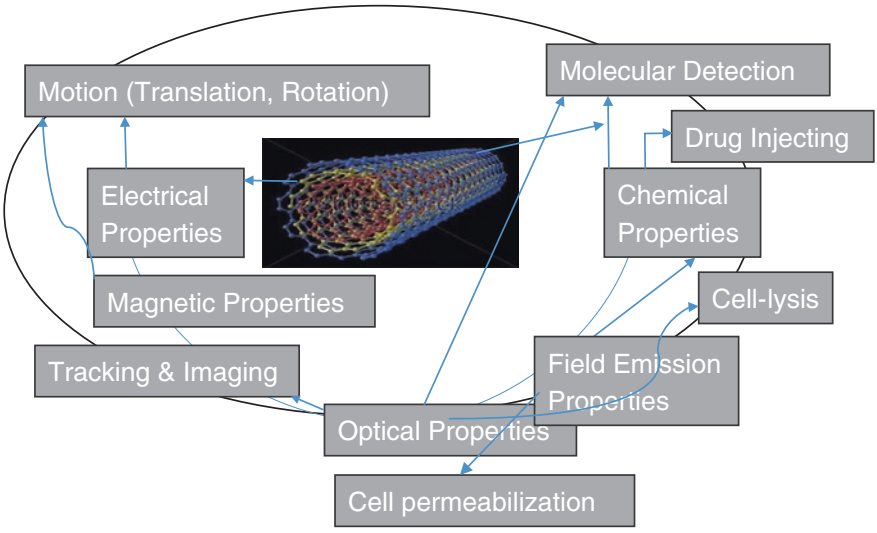

Fig. 10.2 Use of CNT in various fields along with their properties

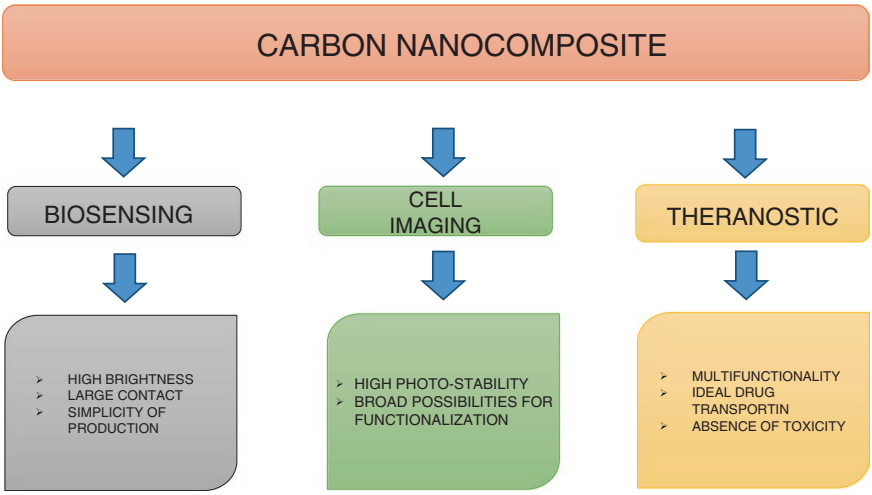

Fig. 10.3 Organic particles in various fields

Buckyball fullerenes have been tested in vitro as carriers for conventional anticancer agents (i.e., fullerene-paclitaxel conjugates) and nucleic acids. However, there is striking evidence that fullerenes can cause oxidative damage to cellular membranes, and cause toxicity. The in vivo efficacy and safety of fullerenes require further studies [42–45].

10.5 Organic Nanoparticles

10.5.1 Polymeric Nanoparticles (PNPs) (Fig. 10.4)

PNPs are colloidal solid particles prepared from biodegradable polymers such as chitosan and collagen or nonbiodegradable polymers such as poly(lactic acid) (PLA) and poly(lactic co-glycolic acid) (PLGA). Their small size (50–300 nm) allows them to penetrate capillaries and to be taken up by the cells and increases the accumulation of the drug at the target site. Majority of these compounds are formulated through a spontaneous self-assembly process using block polymers of two or more polymeric chains with different hydrophilicity. They are considered promising nanocarriers for drug delivery because they can improve the specificity to the target site of action by changing their physicochemical properties and pharmacokinetics. The stability of PLGA NPs can be further improved by coating them with PEG [3, 46–48].

Danhier et al. used paclitaxel-loaded PEG-PLGA-based NPs grafted with RGD peptide and found that the target NPs reduced tumor growth more efficiently, and prolonged survival times of mice, compared with nontargeted nanoparticles [3, 46–48].

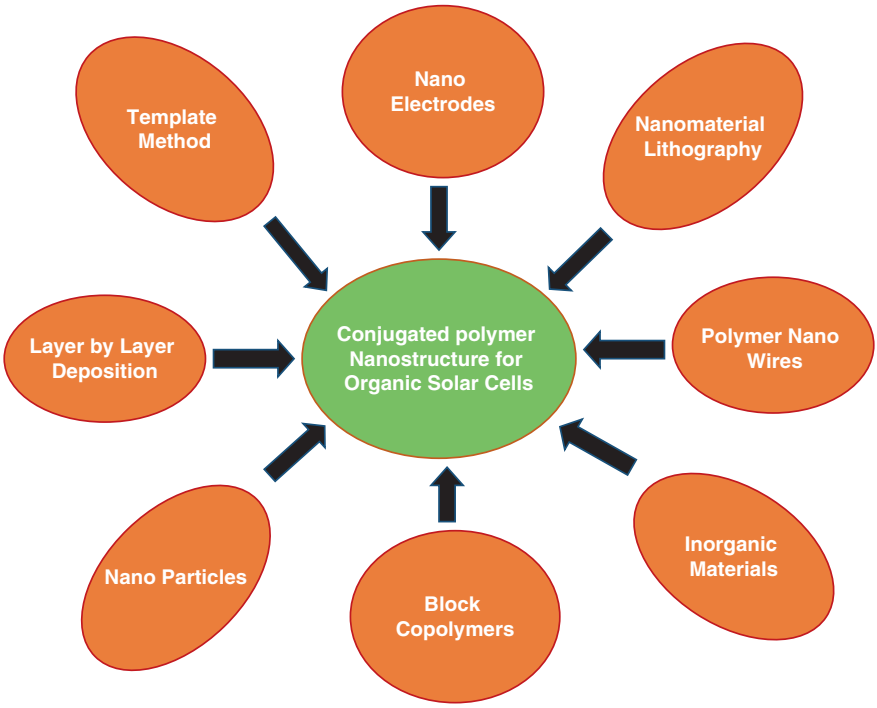

Fig. 10.4 Polymeric nanoparticles in various fields

A very promising polymeric nanoparticle is the chitosan-based nanoparticles. Chitosan is a natural polymer and it is obtained by the partial N-deacetylation of chitin. It is the second most abundant polysaccharide in nature. Doxorubicin (DOX)-loaded chitosan nanoparticles, and DOX-loaded anti-human growth factor receptor 2 (Her2)-surface modified chitosan nanoparticles have been reported. A modified PLGA nanoparticle containing chitosan through physical adsorption and chemical binding methods has been described. However, more in vivo studies are needed to demonstrate the efficacy and safety of PLGA and chitosan nanoparticles as drug carriers [3, 47, 48].

10.5.2 Polymeric Micelles

Polymeric micelles are made up by amphiphilic block copolymers such as poly (ethylene oxide)-poly(β-benzyl-L-aspartate) and poly(*N*-isopropylacrylamide)-polystyrene. Micelles of less than 100 nm assembled with a hydrophobic core and hydrophilic shell are used as drug carriers. The small size of micelles, allows the specific accumulation in the pathologic tissue. Their hydrophobic core and

hydrophilic shell make micelles potent nanocarriers for poorly water soluble anticancer drugs, including paclitaxel and docetaxel. One particular feature of micelles is that the amount of drug released can be controlled by an external stimulus like pH, temperature, ultrasound, or certain enzymes.

Kagaya et al. reported the determination of gene delivery efficacy to vascular lesions using cyclic RGD (cRGD)-PEG-polyplex micelles. They found that cRGD-PEG-polyplex micelles achieved significantly more efficient gene expression and cellular uptake compared with ligand-free PEG-micelles in vitro. Micelles are normally formulated with biocompatible and biodegradable materials which makes them excellent nanocarrier systems. The targeting ability of polymeric micelles is limited due to their low drug incorporation stability and low drug loading [49, 50].

10.5.3 Dendrimers

Dendrimers differ from traditional polymers in the sense that they are highly branched synthetic polymers made of macromolecules such as poly (*N*-isopropylacrylamide)-polystyrene and poly(ethylene oxide)-poly(β-benzyl-L-aspartate) with an inner core diameter of less than 15 nm. Dendrimers possess perfect nanoarchitecture composed of three different parts: (i) focal core, (ii) repetitive units of several interior layers, and (iii) multiple peripheral functional groups. Dendrimers are synthesized from branched monomers in a stepwise manner. It is possible to control several molecular properties including shape, size, dimension, and polarity. Dendrimers offer enormous capacity for solubilization of hydrophobic compounds and can be modified with guest molecules. Therefore, dendrimers have shown enormous potential as anticancer drug delivery systems [44, 51, 52].

Choi et al. reported the synthesis of dendrimers conjugated with fluorescein (FITC) and folic acid (FA) for biomedical application like imaging and therapeutic purposes. They linked dendrimers with complementary DNA oligonucleotides to produce clusters to target cancer cells overexpressing high-affinity folate receptors. Few preclinical or clinical studies of dendrimers as drug carriers is currently available. Thus, it is not possible to make any conclusion about the safety efficacy of dendrimers for human use [53, 54].

10.5.4 Polymeric Nanofibers

Polymeric nanofiber explains fibers with diameters from 1 to 100 nm closely matching the size scale of extracellular matrix (ECM) fibers. Polymeric fibers are derived from inorganic (titanium, silicon or aluminum oxides) or organic

(polyvinyl alcohol, gelatin, poly(N-isopropylacrylamide, polycaprolactone, or polyurethane) materials. There are three techniques for the synthesis of nanofibers, electrospinning, phase separation, and self-assembly; however, the most commonly used is electrospinning. Due to large surface area, low density, high pore volume, and tight pore size of nanofibres, various properties like voltage, capillary collector distance, and polymer flow rate can be tuned. Further, the surface tension and viscoelasticity of nanofibers in solution can also be modified. Nanofibers are being used in medical (tissue engineering), filtration, barriers, wipes, personal care, composite, insulation, garments, and energy storage. They have also been used as drug delivery systems [2, 47, 48, 55].

Tseng and coworkers used biodegradable nanofibers to successfully deliver vancomycin, an antibiotic, to the brain tissue of rats and reduce the toxicity associated with parenteral antibiotic treatment. However, there are very few examples using polymeric nanofibers as cancer drug carriers [2, 47, 48, 55].

10.6 Quantum Dots (Fig. 10.5)

Quantum dots (QD) are small in size (2–10 nm) and colloidal fluorescent semiconductor nanocrystals composed from 10 to 50 atoms of groups II–IV or III–V. They usually consist of a metalloid crystalline core and a shell, which is used to protect the core and renders the QD available for in vivo applications.

The size and shape of QD can be controlled precisely. One of the most valuable properties of QD is their fluorescent behavior, which make them optimal fluorophores for biomedical imaging. Fluorescent QD can be conjugated with bioactive

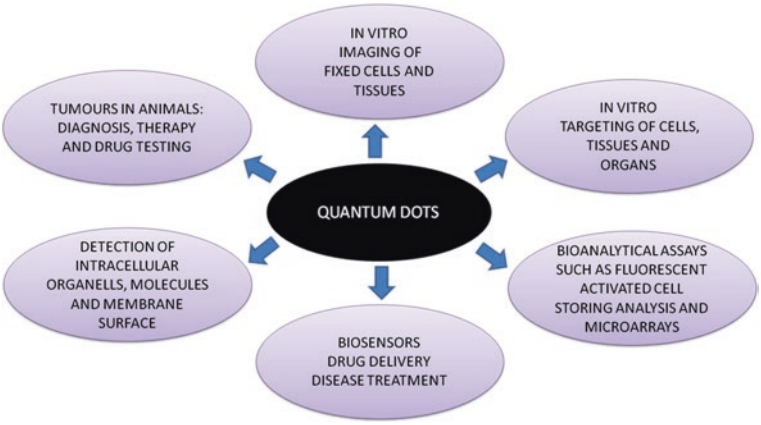

Fig. 10.5 Various applications of quantum dots

moieties or specific ligands (e.g., receptor ligands and antibodies). QD are stable for months without degradation or alteration. QD are mostly used as highly sensitive and multicontrast imaging agents for detection and diagnosis of cancer in vivo. QD are also used in transistors, solar cells, and quantum computing. Nevertheless, because they are composed of hazardous heavy metals, it is important to be cautious about their toxicity [5, 56].

10.7 Gelatin Nanoparticles (Fig. 10.6)

In the last decade, gelatin is extensively used in food and medical products. It is attractive for controlled drugs release due to its nontoxic, biodegradable, bioactive, and inexpensive properties. It is a polyampholyte and bears both cationic and anionic parts along with the hydrophilic group. Various biological applications of gelatin NPs including drug delivery were reported. It is known that mechanical properties, swelling behavior, and thermal properties depend significantly on the crosslinking degree of gelatin. Gelatin NPs can be prepared by desolvation/coacervation or emulsion method. Desolvation/coacervation processes wherein a homogeneous solution of charged macromolecules undergoes liquid–liquid phase separation and results in a polymer-rich dense phase at the bottom and a clear supernatant. The addition of natural salt or alcohol normally promotes coacervation and the control of turbidity that resulted in desired nanoparticles. Many encapsulants have been successfully encapsulated into gelatin nanoparticles (Table 10.1), [16, 18, 36, 39, 57–59].

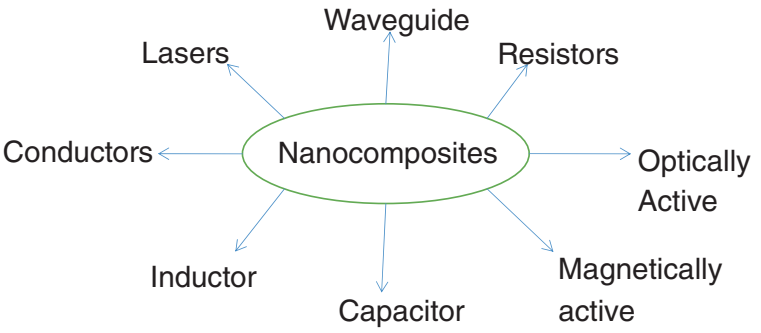

Fig. 10.6 Nanocomposites and their applications

Table 10.1 Encapsulation, synthesis and therapeutics, and mechanism of gelatin diffusion

Encapsulant	Encapsulation efficiency (%)	Synthesis method	Therapeutic improvement	In vitro
Paclitaxel	33–78	Desolvation	Biological activity of Paclitaxel is retained	Paclitaxel-loaded NPs were active against human RT4 bladder transitional cancerous cell
Didanosine	72.5	Double desolvation technique	Slow drug release up to 24 h	Higher accumulation of didanosine in brain
Chloroquine phosphate	15–19	Solvent evaporation method	Reduced side effect	
Insulin	72.8	Ionotropic gelation method	Oral absorption and oral bioactivity was increased	Nanoparticle adhere to intestinal epithelium and internalized by intestinal mucosa
Sulfamethoxazole	39	Solvent evaporation	Slow release up to 10 h	

10.8 Superiority of Gelatin Over Other Polymeric Materials

In last few years, researchers have focused on the various aspects of gelatin within composite materials for the controlled delivery of therapeutics and genes. Composites synergistically combine two or more materials in order to produce a new system with new properties unique to either material alone, like release kinetics, mechanical properties etc. As a mark of its versatility, gelatin has been demonstrated to be a useful component in composites featuring materials ranging from ceramics to natural and synthetic polymers [16, 18, 36, 39, 57–59].

Composites of gelatin and ceramics are used in order to deliver drugs and promote bone regeneration. Most challenging clinical complications occurring due to bone defects are wound infection and nonunion. Two most explored types of drugs can be delivered by ceramic/gelatin composite systems. Hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ (HA) is a naturally-occurring mineral and comprises the inorganic component of bone matrix. A simple two-phase composite, porous HA can be coated with gelatin in order to increase mechanical properties. Increase in mechanical properties is proportional to the concentration of gelatin and it was to hypothesize that gelatin increases HA toughness by bridging material cracks. More advanced fabrication techniques have been employed, so HA NPs can be precipitated within gelatin networks to form scaffolds capable of drug delivery. Compared with HA microparticles without gelatin, cells seeded on composite microparticles experienced greater proliferation. It proves that gelatin–HA composite could potentially be used as an carrier to deliver cell [8, 16, 60–62].

Calcium phosphate cements (CPCs) are mixtures of calcium orthophosphates and can be injected to fill craniofacial and orthopedic defects. CPCs have also been used as carriers for the delivery of drug via incorporating in the liquid or solid phase. CPCs have had great success in biomedicine due to inherent osteoconductivity and ease of material delivery, lack of biodegradation, and drug release. Habraken et al. developed degradable gelatin microparticles into CPCs in order to generate porosity degradation to tissue integration. Subsequently, the embedded gelatin microparticles within this composite system were used not only as paragons but also to deliver drug [63, 64].

Collagen is the building block of the extracellular matrix and therefore are the attractive materials for drug delivery and tissue engineering. These materials have been polymers synthesized by human cells like collagen, hyaluronan, and naturally-derived polymers obtained by other organisms like chitin and silk. Composites of gelatin and other naturally-occurring polymers offer good biocompatibility and enable biomimetic strategies for drug delivery [64, 65].

Hyaluronan has the ability to act as a binding agent for molecules and create interest in making it an attractive biomaterial for drug delivery and tissue engineering. However, the hydrophilicity of hyaluronan can inhibit protein adsorption and prevent cell attachment. Therefore, gelatin–hyaluronan composites have been explored in order to take advantage of the strengths of both materials. Gelatin and hyaluronan have been blended simply by electrospinning to prepare biodegradable sheets with tunable surface tensions that may be used as drug delivery carrier [14, 15, 43, 66].

Literature revealed that chitosan has a high charge density and good biocompatibility like hyaluronan. Gelatin and chitosan are used to prepare polyionic complexes and therefore composite preparation drug release kinetics and degradation can be altered by the amount of gelatin incorporation. The release kinetics can be controlled by varying the Ph. It was observed that on decreasing pH, release of drug increases. Gelatin chitosan sponges have been reported as carriers for drug release to cure wound. Silk is a popular protein obtained from insects and spiders. In recent years, silks and silk-derivatives were used in tissue engineering due to their light weight and tough behavior. Silk was also exposed to reinforce gelatin scaffolds, tensile, and bending strength [14, 15, 43, 66].

Mandal et al. synthesized gelatin–silk nanocomposites which can be used to load and deliver water soluble drugs. Gelatin–silk composites were prepared to enhance the strength of silk and favor the biodegradation profile and drug-loading capability of gelatin. One of the major advantages of synthetic polymers in drug delivery is their modification to prepare suitable material. However, without considering the specific design, many synthetic polymers inherently have demerits like cell-recognition sequences, binding sites, eliciting inflammatory response, and foreign body reaction. Therefore, modification of gelatin in combination with synthetic polymers has been used to prepare highly customizable platforms for drug delivery [20, 67].

Poly(lactic *co*-glycolic acid) (PLGA) is one of the most potent synthetic polymers, which has applications in the field of drug delivery and tissue engineering.

Composite of PLGA and gelatin have been used for controlled delivery and can be prepared via electrospinning techniques. Drug delivery through encapsulation took place within electrospun fibers and it sounds interesting due to the high surface-to-volume ratio. In general, incorporation of gelatin into PLGA composite makes it available for further mechanisms to control the release kinetics and also used to enhance the cell biocompatibility. Cross-linked oligo(poly(ethylene glycol) fumarate) (OPF) is a PEG-based hydrogel with cleavable fumarate groups allowing for biodegradation. OPF has been proven to support controlled drug release, cell encapsulation, and tissue regeneration. However, a great enhancement in the field of drug delivery has been achieved on using OPF hydrogels on the inclusion upon embedded gelatin microparticles. Such composite systems were used for the controlled delivery of biomolecules and others as like DNA, minerals such as HA, for in vitro and in vivo models. A huge research in the area of gelatin–OPF composite system used for controlled drug delivery is still ongoing and has great promises for the generation of complex tissues [46, 54, 68].

Poly(propylene fumarate) (PPF) is one of the synthetic polymer that carries fumarate anion and biodegradable ester bonds which makes it interesting. However, PPF has greater hydrophobicity, stiffness, and strength. The positive aspects of PPF as a substrate alone and when combined with the gelatin microparticles for delivery of drug have been synergized. Gelatin–PPF composites can be tuned and modified to release multiple growth factors at different kinetics which makes them for temporal control over drug delivery. It results in successful bone regeneration in chosen animal models [69–72].

10.9 Gelatin Nanocomposites

Nanocomposite (NC) is a multiphase solid material system, formed by the combination of two or more components that includes polymers as matrices and nanostructured materials in one dimension. In the last few decades, interests in biopolymer-based nanocomposites have been increased due to their biodegradability, biocompatibility, and improved physical and mechanical properties. Further, remarkable antimicrobial functions obtained in certain nanocomposites have a gray area in the application of nanocomposite films in various academic and research applications like biomedical and food packaging areas. Combination of nanostructures and biomaterials provide a platform to academician and researchers to find new nanobiotechnology areas. Metal oxides, such as ZnO, MgO, and TiO_2 are used extensively to construct functional coatings and bionanocomposites because of their stability under harsh processing conditions. Further, these materials also show the antimicrobial, antifungal, antistatic, and UV-blocking properties [1, 25, 73–79].

Use of gelatin as an organic additive in composites with inorganic nanohybrids has gained interest tremendously because of the bioadhesive and biodegradable properties. Thus, several experts have concentrated their research on gelatin films.

Mammalian gelatin films showed excellent mechanical properties in comparison with other types of gelatin films. At present, researchers have focused on the use of marine gelatin sources as alternatives to mammalian gelatins, such as those from fish. Marine gelatin sources are not related to the risk of bovine spongiform encephalopathy [1, 25, 73–79].

10.10 Gelatin Nanocomposites in Drug Delivery

Gelatin when used as a matrix in mineralization has shown a lot of interest in the field of tissue engineering. Composite of gelatin with poly(ethylene glycol), chitosan, and poly(d,l-lactide) have proven for potential pharmaceutical applications.

Rouhi et al. has reported the nanocomposite fish prepared by adding ZnO nanorods (NRs) as fillers and the nanocomposites can be used in drug delivery. The impact of ZnO NR fillers has been investigated to study the mechanical, optical, and electrical properties of fish gelatin bionanocomposite films. It was observed that an increase in Young's modulus and tensile strength of nanocomposites incorporated with 5 % ZnO NRs compared with unfilled gelatin-based films [15, 51, 66, 80]. Gaihre et al. reported gelatin-coated magnetic iron oxide nanoparticles as carrier systems for loading and release of drugs. Magnetic iron oxide nanoparticles (IOPs) were coated with gelatin A and B and the drug-loading efficiency was investigated using doxorubicin (DXR) via various techniques. The DXR-loaded particles are sensitive to pH and showed response to drug release at pH 4 which was high as compared to pH 7.4. Han et al. reported the synthesis of amphiphilic copolymer nanoparticles based on gelatin as drug carriers for the delivery of doxorubicin. Doxorubicin was incorporated into polymeric nanoparticles by double emulsion or nanoprecipitation method. DOX-loaded nanoparticles showed rapid and frequent release at pH 5.0 than at pH 7.4 buffer. They also demonstrated that DOX-loaded copolymer nanoparticles showed comparable anticancer efficacy with the free drug in vitro and in vivo [1, 7, 64]. Cheng et al. reported gelatin-encapsulated iron oxide nanoparticles for platinum (IV) prodrug delivery, enzyme-stimulated release, and also in MRI. They encapsulated Fe_3O_4 nanoparticles in gelatin and demonstrated applications in multifunctional drug delivery system for disease therapy, MR imaging, and fluorescence sensors [17, 81].

Lee et al. reported biocompatible gelatin nanoparticles for tumor-targeted delivery of polymerized siRNA in tumor-bearing mice. The prepared composite presented efficient siRNA delivery in red fluorescence protein expressing melanoma cells (RFP/B16F10) to downregulate target gene expression. The psi-tGel NPs have great potential for systemic siRNA delivery system for cancer therapy, based on their characteristics of low toxicity, tumor accumulation, and effective siRNA delivery [82].

Xiaoyan et al. reported the preparation of chitosan–gelatin scaffold containing tetrandrine-loaded nanoaggregates and its controlled release behavior. They showed that the Ted-loaded nanospheres could be embedded within Cs–Gel

scaffolds and no initial burst release could be observed in the release patterns [5, 10, 13, 63].

Patel et al. reported hyaluronidase enzyme core-5-fluorouracil-loaded chitosan-PEG-gelatin polymeric nanocomposites for controlled drug delivery. These nanoparticles were proven to be potential carriers for targeted and controlled drug delivery to cancer cells [10, 13]. Tran et al. reported enhanced solubility and modified release of poorly water soluble drugs via self-assembled gelatin–oleic acid (GO) nanoparticles. They have compared the drug and drug-loaded nanoparticles in terms of solubility and found drug-loaded NPs more efficient. Furthermore, the release profiles of the model drugs were modified in a controlled manner and the current self-assembled GO nanoparticles can provide a versatile potential in drug delivery and tumor targeting [2, 55, 65, 83]. An et al. reported the synthesis of a biocompatible gelatin-functionalized graphene nanosheets (CNs) and its application for drug delivery. They showed that the prepared nanohybrids system offers a novel formulation that combines gelatin and graphene for biomedical applications. Therefore, the gelatin-GNS with good stability and biocompatibility can be selected as an ideal drug carrier to be applied in biomedicine studies [24, 39].

10.11 Conclusion

Gelatin is a natural biodegradable polymer which exhibits excellent biocompatibility, plasticity, and adhesiveness and is widely applied in tissue engineering. Gelatin NP–composite system often used for various biomedical applications revealed the combined benefits of activity, bioactivity of system, and the morphological features of gelatin. The combination of nanostructures and biomaterials provide gray area for researchers to find new nanobiotechnology areas. Nanorods (NRs) and nanoparticles combined with biomolecules are used for various applications in biomolecular sensors, bioactuators, drug delivery and medicines, such as in photodynamic anticancer therapy. Biodegradable nature of composite containing gelatin may also lead to various specific applications in biomedical.

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