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Polymeric Nanoparticles for Bovine Mastitis Treatment

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Dedicated to…
My Parents
For their support throughout my education
and inculcating the sense of responsibility
and independence
My Brothers, Sisters
and
My Wife
For their love and support

Preface

The alarming rise of antibiotic-resistant bacteria has drawn widespread attention. Both bacterial prevalence and antibiotic resistance are on the rise, making bacteria a major public health concern. Antimicrobial resistance poses a serious risk to public health and the ability to combat diseases, and subsequent generations will not be equipped to accept this. One of the biggest threats to the well-being of humans and animals globally is antimicrobial resistance (AMR). Antimicrobial resistance is an evolutionary trait that has helped bacteria survive in a dynamic world. The curative effectiveness of antimicrobial therapies for human and veterinary illnesses is also diminished because bacteria use their incremental mechanism to respond toward the selection pressure caused by antibiotic therapy. New, potentially more effective antibacterial agents have been developed with the use of nanotechnology. Polymeric nanoparticles in particular have been the subject of much study because of their dual role as a drug nanocarrier and an antibacterial agent. The use of polymeric nanoparticles as antibacterial agents represents a fundamental shift in every facet of veterinary practice.

The benefits of polymer-based nanoparticles such as improved biological compatibility, biodegradability, and elimination from human bodies, must be carefully weighed against the current limitations of existing technologies. In particular, the production process is made more difficult by the dimension and size distribution of polymeric nanoparticles. In this strategy, scientists explore novel methods of mass-producing polymeric nanoparticles for use in a broad range of high-value products. Biodegradable, biocompatible, highly active, stable, and little poisonous, Ch-coated polymeric silver and gold nanoparticles are a new class of bioactive hybrid materials in medicine.

The most contemporary edition of this volume covers the evidence and investigation of polymers doped with metallic, metallic oxides, and bimetallic nanoparticles including cutting-edge abrasive advances. There is a discussion of the benefits and drawbacks of various production processes, as well as characterizations, the prevalence of bovine mastitis, antibiotic-resistant bacteria, and reasons behind the establishment of resistance, and the evidential therapy for resistant etiologies making

use of polymeric nanomaterials. The hope of the authors of "Polymeric Nanoparticles for Bovine Mastitis Treatment" is that this monograph will serve as a valuable resource for scientists who are intrigued in exploring the potential of nanomaterials as potential antimicrobial placebos.

Multan, Pakistan — Ali Haider
Lahore, Pakistan — Muhammad Ikram
Lahore, Pakistan — Iram Shahzadi
Multan, Pakistan — Muhammad Asif Raza

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All praises to "ALLAH," the Almighty, most Gracious, the most Merciful and the Sustainer of the worlds, who sent us "MUHAMMAD (PBUH)" as a blessing for whole universe and the best teacher with the ultimate source of wisdom "HOLY QURAN."

Foremost thanks to ALLAH Almighty who blessed me with good health to accomplish my book. I feel great pleasure in expressing my heartiest gratitude and deep sense of obligation to my elder brother and mentor, **Dr. Muhammad Ikram**, Assistant Professor, Department of Physics, for his able guidance, keen interest, skilled advice, constant encouragement, valuable suggestions, and painstaking supervision throughout the course of my study, research work, and completion of this book.

Last but not least, I must acknowledge my thanks to my **loving parents**, my **brothers (Dr. Muhammad Imran, Muhammad Irfan, and Dr. Junaid Haider)**, my sister **(Dr. Anum Shahzadi)**, and my wife **(Mrs. Sehrish Kiran)** for the motivation to take up this program of studies, financial support and their hands in prayers for my success, and great patience and helpfulness throughout the fairly long period of training. Finally, as is customary, the errors that remain are mine alone.

Ali Haider

Contents

Abbreviations

2D	Two dimensional
Å	Angstrom
AFM	Atomic force microscopy
AgS	Silver sulfide
Al_2O_3	Alumina
ATP	Adenosine triphosphate
ATR	Attenuated total reflectance
Au	Gold
ß	Beta
BPD	Pyrene, 1,10-bis(1-Pyrene) decane
BSA	Bovine serum albumin
C_2H_2	Ethyne
C_6H_6	Benzene
CaO	Calcium oxide
CdS	Cadmium sulfide
Ch	Chitosan
CNS	Coagulase-negative *Staphylococci*
CNTs	Carbon nanotubes
CO_2	Carbon dioxide
CPS	Capsular polysaccharides
CPS	Coagulase-positive *Staphylococci*
CPs	Conjugate polymers
Cu	Copper
CuO	Copper oxide
CuS	Copper sulfide
DD	Disulfide
DHF	Dihydro folate
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DSC	Differential scanning calorimetry
DWNTs	Double-walled nanotubes

E. streps	Environmental *streps*
EGF	Epidermal growth factor
EM	Electron microscopy
FCS	Fluorescence correlation spectroscopy
FDA	Food and drug administration
FeS	Ferrous sulfide
FESEM	Field emission scanning electron microscopy
FFF	Field flow fractionation
FTIR	Fourier transform infrared spectroscopy
GAS	Gas antisolvent system
HA	Hydroxyapatite
I.U.	International unit
IL	Interleukin
IMI	Intramammary infection
IMM	Intramammary medication
IR	Infrared
K	Scherrer's constant
L	Lymphocytes
LEDs	Light-emitting diodes
LPS	Lipopolysaccharide
LTA	Lipoteichoic acids
MATE	Multidrug and toxic compound extrusion
MBCs	Minimum bactericidal concentration
MDR	Multidrug-resistant
MFP	Membrane fusion protein
MFS	Major facilitator superfamily
MgO	Magnesium oxide
MICs	Minimum inhibitory concentrations
MMA	Methyl methacrylate
Mn	Manganese
MOFs	Metal organic frameworks
MPS	Mononuclear phagocyte system
MRI	Magnetic resonance imaging
MRSA	Methicillin-resistant *S. aureus*
MS	Mass spectrometry
MWNTs	Multiwalled nanotubes
NaCl	Sodium chloride
NH_3	Ammonia
nm	Nanometer
NMC	National Mastitis Council
NMR	Nuclear magnetic resonance
NPN	N-phenyl-1-naphthylamine
NPs	Nanoparticles
OM	Outer membrane
OMP	Outer membrane protein

P2VP	Poly(2-vinyl pyridine)
PABA	P-aminobenzoic acid
PBPs	Penicillin-binding proteins
Pc	Critical pressure
PCL	Polycaprolactone
PEDOT	Polyethylenedioxythiophene
PEG's	Polyethylene glycol
PEO-PPO	Polymers of ethylene oxide and propylene oxide
PET	Positron emission tomography
PG	Peptidoglycan
pH	Potential of hydrogen
Phe	Phenylalanine
pKa	Acid strength
PLA	Poly(lactic acid)
PLGA	Poly(lactic-co-glycolic acid)
PLGA	Poly(lactide-co-glycolide)
PMMA	Polymethyl methacrylate
PNPs	Polymeric nanoparticles
PTA	Particle tracking analysis
PVP	Polyvinylpyrrolidone
QRDR	Quinolone-resistance-determining region
R&D	Research and development
RESS	Rapid expansion of supercritical solutions
RNA	Ribonucleic acid
RND	Resistance-nodulation-cell division
ROS	Reactive oxygen species
RS	Raman spectroscopy
SAS	Supercritical antisolvent process
SCC	Somatic cell count
$scCO_2$	Supercritical carbon dioxide
SCF	Supercritical fluid
SEM	Scanning electron microscopy
SERS	Surface-enhanced Raman scattering
SiO_2	Silicon dioxide
SMR	Small multidrug resistance
SPECT	Single-photon emission computed tomography
STM	Scanning tunneling microscopy
SWNTs	Single-walled nanotubes
TA	Teichoic acid
TB	Tuberculosis
Tc	Critical temperature
TC	Teat canal
TCJ	Tricyanovinyljulolidene
TEM	Transmission electron microscopy
tg	Glass transition temperature

THF	Tetrahydrofuran
TiO_2	Titanium oxide
TNF-α	Tumor necrosis factor alpha
TRPS	Tunable resistive pulse sensing
US$	United States Dollar
UV-Vis.	Ultraviolet-visible
VRE	Vancomycin-resistant enterococci
VRSA	Vancomycin-resistant *S. aureus*
XPS	X-ray photoelectron spectroscopy
XRD	X-ray powder diffraction
Zn	Zinc
ZnO	Zinc oxide
ZrO_2	Zirconia

List of Figures

List of Tables

Chapter 1
Introduction

Abstract Polymers have evolved through an intricate and fascinating history by mankind's imagination and inventiveness. These materials encompassed poly (carbonate, phenylene oxide, sulfones, imides, aromatic amides) and other elevated-temperature inflexible materials. Scientists have discovered new materials with a diverse set of qualities, from bendable plastics to durable, lightweight composites. The excellent functionalization of polymer PNPs owing to alcohols, amines, and thiols groups may ameliorate the toxicology, dissolution, availability, drug binding to plasma and receptor, and cellular absorption issues. In recent years, biodegradable co and hybrid polymers, especially di, tri, multi, or radial block copolymers have widely been used in polymeric nanoparticles as well as to entrap active components, wherein micelles, capsules, fibers, spheroids colloids, dendrimers, nanoparticle-incorporated polymer matrices, etc. are multifunctionalized polymers nanocarriers. Physicochemical traits (size, framework, and molecular makeup) and environmental conditions (pH and heat) may influence degradation processes for compostable PNPs. Thus, polymer stability, use of numerous solvents during preparation, surfactants, and stabilizers to control size, morphology, and surface properties of nanoparticles, proper drug loading via encapsulation, adsorption, and convalescence, and so on are all important aspects of polymeric nanoparticle development. PNPs may be injected systematically by an array of ways, particularly oral, nasal, transdermal, parenteral, pulmonary, and ocular routes, among others. Modifying a nanoparticle's surface is essential for maintaining its stability and avoiding aggregation that are modified by adhesion, predominantly with thiol groups, disulfide, amines, nitriles, and carboxyl groups. Chemical viability of organo-sulfur in forming an effective covalent interaction to noble metals is largely responsible for its development and use in surface modification.

Keywords Polymers · PNPs · Physicochemical · Colloids · Dendrimers · Nanoparticles

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1.1 History of Polymers

The word "polymer" signifies "multiple parts." (Greek poly, means "numerous," and meros, "parts"). Polymers are macromolecules composed of many small molecules (monomers) that can be coupled together to create long chains with molar masses spanning from thousands to millions. Later nuanced shift in this definition, polymer science is considered as relatively recent field of study that focuses on polymers, organic and manufactured materials, rubber compounds, adhesive products, sealants, etc. which are now extremely widespread. In the form of lubricants, tars, resins, and gums, humans utilized polymers' versatility for millennia. However, the modern polymer industry did not begin to develop until the industrial revolution. J. J. Berzelius, a Swedish chemist, first coined the term "polymer." He believed that benzene (C_6H_6) was a polymer of ethyne (C_2H_2). Controversy surrounded its inception in the 1920s, and its eventual mainstream acceptance is often attributed to Nobel Prize recipient H. Staudinger in 1953 [1].

Polymers have evolved through an intricate and fascinating history by mankind's imagination and inventiveness. At some point in the 1830s, Charles Goodyear figured out to transform organic gooey latex to suitable elastomer by applying temperature and pressure using vulcanization process [2]. Christian F. Schonbein made cellulose nitrate by reacting cellulose with nitric acid in 1847, which was utilized as first synthetic thermoplastic, celluloid in commercial applications during the 1860s. Ancient Egyptians utilized natural resins for adhesives and coatings [3], whereas Greeks and Romans use them as waterproof fabrics. The Mayans made balls and impenetrable garments from natural rubber for hundreds of years; nevertheless, the attributes of these natural polymers were not fully recognized till the nineteenth century, when scientists started to investigate their chemical structures [4]. Leo Baekeland found his initial synthesized polymeric material, Bakelite in 1907 by heating a mixture of phenol and formaldehyde [5, 6]. In the 1930s, scientists at DuPont in the USA developed a wide range of novel polymers including synthetic rubber or Teflon whereas Nylon creation by Wallace H. Carothers in 1935 [7] and polyethylene by Paul Hogan and Robert Banks in 1953 [8] are noteworthy advances in polymer science. Several organic items, like Hevea rubber, were in limited availability in Second World War, prompting an uptick of efforts to manufacture new polymeric materials, notably synthetic rubber as their demand skyrocketed. There have been numerous advancements in polymer science in the years following Second World War. Once the first plastic bottles were made in 1947 [9], plastic was rapidly adopted as the material of choice for everything from toys to automobiles to food containers.

A variety of high-performance engineering plastics polymers were developed in the 1960s and 1970s, allowing them to compete successfully with metals and other conventional materials in the automobile and aerospace industries. These materials encompassed poly (carbonate, phenylene oxide, sulfones, imides, aromatic amides) and other elevated-temperature inflexible materials. Scientists have discovered new materials with a diverse set of qualities, from bendable plastics to durable, lightweight

composites. The usage of polymers in several fields, including textiles, medical implants, as well as electronics makes them one of the century's most consequential discoveries. The emergence of cutting-edge substances has opened up new avenues for research and development in fields as diverse as medicine, engineering, and art. About 80% of organic chemical output goes toward making synthetic polymers like plastics, textile fibers, and synthetic rubbers. Polymeric materials' manufacture and assembly have grown into substantial global enterprises considering their widespread use in modern life [7].

1.2 Types of Polymers

Over the years, scientists have synthesized thousands of unique polymers, yet this count is undoubtedly anticipated to increase. On basis of their processing characteristics or mechanism of polymerization, all polymers can be effectively categorized into the following enumeration (Fig. 1.2).

1.2.1 On Basis of Source of Origin

Polymers from living organisms are referred as natural polymers. These substances are known as plant and animal polymers. Examples include cellulose, silk, wool, RNA, DNA, and natural rubber. Semisynthetic fibers are derived by simple chemical treatment of organic fibers that subsequently acquire improved physical attributes such as durability and elastic modulus. Acetate, cuprammonium, and viscose rayon are examples. The fibers obtained in laboratory by polymerizing basic chemical molecules are manufactured fibers, e.g., nylon, polyethylene, polystyrene, synthetic rubber, Teflon, and Orion.

1.2.2 On Basis of Polymer Structure

Polymers are perceived according to chemical framework with aspects of their chain arrangement like entire chemical profile and arrangement of monomers which have a significant impact on characteristics of polymers. Homochain (polyalkylenes) have entirely of carbon atoms across their framework that can be subdivided further based on whether their backbone contains single or double bonds usually which possess high tensile strength and melting point. Polystyrene and polyolefins (such polyethylene and propylene) along with polycarbonate are examples. Heterochain polymers are classified by sorts of atoms and structural groups such as carbonyl, amide, or ester that make up polymer‘s framework. Polysiloxanes constitute a significant subgroup of –Si–O– core alongside methyl or similar substitution compounds affixed to silicon.

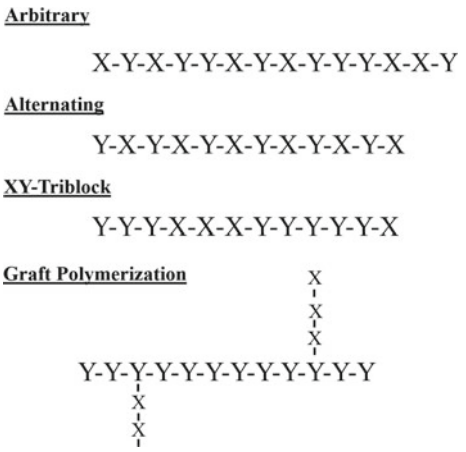

Fig. 1.1 Possible copolymers structures with X and Y repeating units

It is often feasible to generate polymers with unique and desired features by merging two or three varied monomeric units in polymerization. Copolymers are polymers with two distinct repeating elements in their chains, while three chemically distinct repeating units result in terpolymer. For example, XY-block copolymers may be made by the first synthesizing a lengthy block of single monomer (X) preceded by opposite monomeric unit (Y). The central Y block of XYX-triblock copolymers is flanked by X blocks on both extremities as demonstrated in Fig. 1.1. The thermoplastic elastomer polystyrene-blockpolybutadiene-block-polystyrene is a commercially significant XYX-triblock copolymer. It is also feasible to construct graft copolymers by assembling a monomer over preexisting polymer of different subunits such as elastomers and high-impact polystyrene and acrylonitrile butadiene styrene resin.

1.2.3 On Basis of Polymerization Mechanism

Wallace Carothers, an innovator who worked at DuPont from 1928 till his tragic death in 1937, is credited with developing method that categorizes polymers as addition or condensation. Addition polymers include polystyrene, formed by subsequently adding subunits. Some other polymers classified as addition polymers are really ring-opening polymers, formed when a cyclic monomer is subjected to steric hindrance and so cannot undergo addition polymerization with example of trioxane to form polyoxymethylene. Condensation polymers are made by removing water, alcohol, or NH_3 from two monomers with ester and amide linkages. It is possible for a polycondensation process to include monomers, oligomers, or intermediates of higher molecular mass along with functional (carboxyl or hydroxyl) units. The condensation polymerization of adipic acid with hexamethylenediamine to yield nylon-6,6 is a good illustration of this process [7].

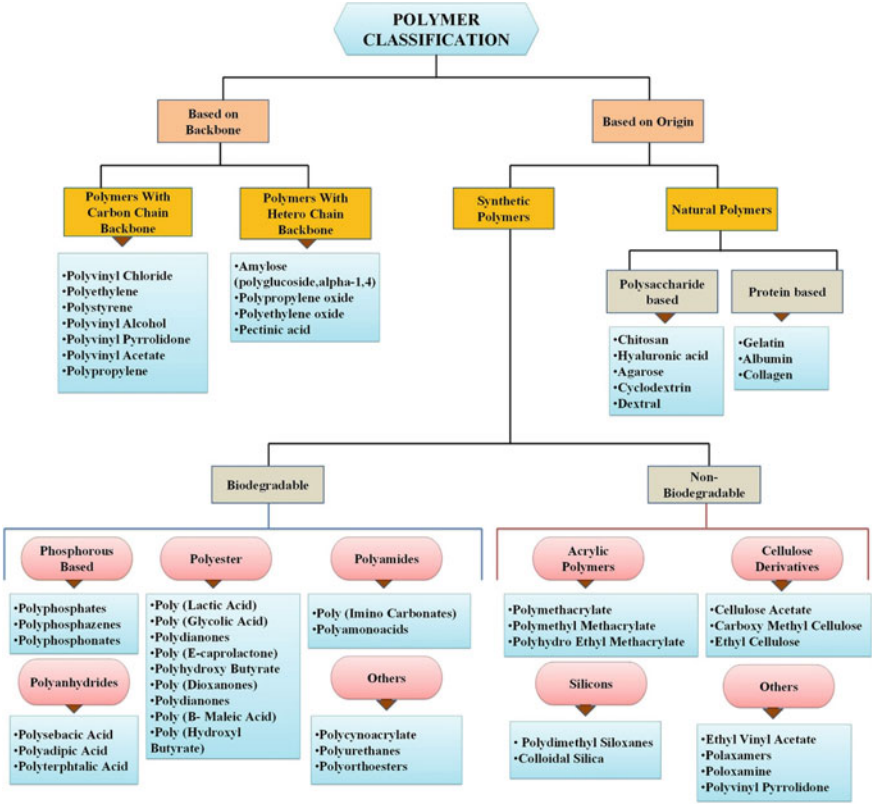

Fig. 1.2 Polymers classification. Reproduced with permission from Ref. [11] Copyright 2019, Elsevier

1.2.4 On Basis of Molecular Dynamics

The mechanical properties of polymeric materials notably tensile strength, tenacity, resilience, and thermal processing behavior are influenced by intermolecular effects such as van der Waals forces and hydrogen bonds. Thermoplastics are polymers that can be modified by heat for processing into a desired shape as heat and pressure can reuse thermoplastic waste. Polystyrene is an important commercial thermoplastic type. In contrast, thermosets are polymers in which the constituent chain units have been chemically bonded through covalent bonds, either at the time of polymerization or thereafter by chemical or thermal processing. After assembly, these networks are impervious to thermal softening, deformation by force, and chemical assault. These characteristics make thermosets suitable for use in composites, varnishes, and adhesives. Fiberglass is trade name of fiber-reinforced plastics formed with epoxy

resin, phenol–formaldehyde resins, and unsaturated polyesters. Examples of thermosets in manufacturing of fiberglass encompass epoxy resin, phenol–formaldehyde resins, and unsaturated polyester-based material.

1.2.5 Based on Growth Polymerization

According to polymerization kinetics, all polymerization mechanisms have been assigned into step growth or chain growth. A few noteworthy exceptions to generalizations that, most condensation polymers develop through steps growth and majority of addition polymers grow via chain development. Substantial molecular count polymer is created initially in chain-growth polymerization near the reactive end of developing chain, and polymerization yield, or percentage of monomer turned into polymer grows significantly. In step-growth phenomenon, the formation of larger polymer (which may contain more than one functional group) occurs near the end of process. It proceeds by forming dimer, trimer, tetramer, etc., such as Dacron. U [10].

1.3 History of Polymeric Nanoparticles

The first reports on the synthesis of polymeric nanoparticles were published in between the years 1960 and 1970, making polymeric nanoparticles history relatively recent when compared to other nanoparticles. Micelles were the first polymeric nanoparticles developed for therapeutic applications [12, 13] through polymerization techniques. In 1972, the emulsion polymerization fabrication of polystyrene nanoparticles was reported [14]. Since then, nevertheless, research into polymeric nanoparticles has expanded significantly, making it a major focus of nanotechnology. From the 1980s, scientists began exploring PNPs applications in drug delivery owing to their capacity to encapsulate as well as protect pharmaceuticals from degradation in the body. One of the first polymers employed for fabrication of nanoparticles via solvent evaporation process is poly(lactide-co-glycolide) (PLGA), which is still commonly used today. Yolles and colleagues reported in 1985 PLGA nanoparticles use for delivery of chemotherapeutic drugs. An uptick in polymeric nanoparticle manufacturing may be traced back to the 1990s, when novel nanoparticle manufacturing techniques including emulsion and microemulsion were developed. Polylactic acid and polyethylene glycol-coated nanoparticles through coacervation are two examples of newly developed polymers that have helped propel the field forward [15, 16].

1.4 Characteristics of Polymeric Nanoparticles

Polymeric nanoparticles comprise a variety of polymers or copolymers, vary in size from 10 to 1000 nm, and are frequently employed material for developing nanoparticle-based drug carriers to entrap or adsorb active compounds [10]. Nanolevel size, surface electrical charge, interface situation, improved dissolution, strong entrapped capability, drug loading potential, and nature are only some polymeric nanoparticles (PNPs) traits that will affect their efficacy and effectiveness in applications [17, 18]. New possibilities to engage with intricate cellular and biological surroundings will be possible by surface functionalization [19] that can impart focused target properties or sensitive stimuli responses [18, 20]. The excellent functionalization of polymer PNPs owing to alcohols, amines, and thiols groups may ameliorate the toxicology, dissolution, availability, drug binding to plasma and receptor, and cellular absorption issues [21]. The fact that polymeric nanoparticles may be produced from components already present in medical equipment and other uses lends credence to the idea that they are biocompatible mainly polylactic coglycolic acid routinely employed in polymeric nanoparticles production is licensed by FDA in medical fields [22].

Furthermore, high surface area and compact dimension of polymeric nanoparticles along with subtherapeutic pharmacological responses, antienzymatic degradation shielding, mucoadhesive nature, longer circulation time [23], and considerable sustainability can improve drug release in controlled way with less toxicity that can be achieved through a diffusion, erosion, and chemical or enzymatic degradation of polymer matrix [18, 24]. Polymers eccentric materials may be spun, dipped, film-cast, or imprinted to make thin films. However, vast majority of pure polymers have inadequate thermal, mechanical, and electronic attributes compared with porcelain and metallic particles. Diverse polymeric categories especially adapted, composite, homo or hetero polymers are insufficient to compensate for many imposingly demanded characteristics [25]. So, owing to the quantum effect at the nanoscale, NPs exhibit properties that set them apart from the bulk form, such as enhanced solubility, changes in crystal structure stability, enhanced environmental chemical adsorption, particle enlargement/aggregation, and intense reactivity [26].

1.5 Development of Polymeric Nanoparticles

Polymeric nanoparticles (1–1000 nm) are biodegradable tiny fragments that may have active compounds encapsulated inside or surface-adsorbed onto the polymeric core to increase their efficacy. The development of polymeric nanoparticles entails a multidisciplinary approach, encompassing polymer chemistry, nanotechnology, and pharmacology knowledge. The term “nanoparticle” pertains to nanocapsules (reservoir system) or nanospheres (matrix system) having distinct morphologies [27–29].

Nanocapsules have viscous core where pharmaceutical substance is typically dissipated and a polymeric exterior regulates drug's release. Nanospheres persistent polymeric system allows therapeutics to be encapsulated as well adsorb to their surface [27, 30]. In recent years, biodegradable co and hybrid polymers, especially di, tri, multi, or radial block copolymers have widely been used in polymeric nanoparticles as well as to entrap active components, wherein micelles, capsules, fibers, spheroids colloids, dendrimers, nanoparticle-incorporated polymer matrices, etc. are multifunctionalized polymers nanocarriers. Moreover, the polymer nanocarrier must be facile to synthesize and devoid of impurities [31]. PNPs experienced modest development relative to other forms of NPs, but research into nanotechnology is now trending as there are now 76 medicines on the market or in clinical trials, with liposome formulations accounting for 55.26%, inorganic NPs for 21% (16 products), and others (polymers and proteins) making up the remaining 13.8% of the market [32], with overall market of USD $293.1 billion for nanomedicine products by 2022 [33].

Nanoproducts are in great demand since their therapeutic effectiveness much exceeds that of conventional medication delivery systems. Polymeric nanoparticles are desirable for industrial scale production considering the ease and cost-effective manufacturing processes such as emulsion, solvent evaporation, and nanoprecipitation [34, 35]. Polymeric nanoparticle research has made great strides in recent decades, with advent of novel materials and methods for their production. For instance, fabrication of polypeptide-based nanoparticles for drug delivery by means of self-assembly [36] or microfluidics [37]. This resulted in more effective and targeted drug delivery systems, in addition to novel applications for tissue engineering and regenerative medicine [38–41]. Physicochemical traits (size, framework, and molecular makeup) and environmental conditions (pH and heat) may influence degradation processes for compostable PNPs [42]. Thus, polymer stability, use of numerous solvents during preparation, surfactants, and stabilizers to control size, morphology, and surface properties of nanoparticles, proper drug loading via encapsulation, adsorption, and convalescence, and so on are all important aspects of polymeric nanoparticle development. PNPs may be injected systematically by an array of ways, particularly oral, nasal, transdermal, parenteral, pulmonary, and ocular routes, among others [43]. PNPs must satisfy market demand, R&D, manufacturing procedures, clinical studies, and regulatory constraints prior to reaching the market.

1.6 Nanoparticles Based on Metals and Ceramics

The advancement of functional molecular-scale systems is nanotechnology. The Nobel laureate Dr. Richard Feynman initially proposed this idea in 1959, while Professor Norio Taniguchi coined the name "nanotechnology" in 1974 [44]. Nanocomposites are composite materials in which nanoparticles with diameters on the nanometer scale (less than 100 nm) are dispersed throughout matrix that dramatically enhanced mechanical strength, durability, and electrical-thermal conductivity properties even on addition of little amounts (0.5–1% by weight) [45, 46]. The NPs

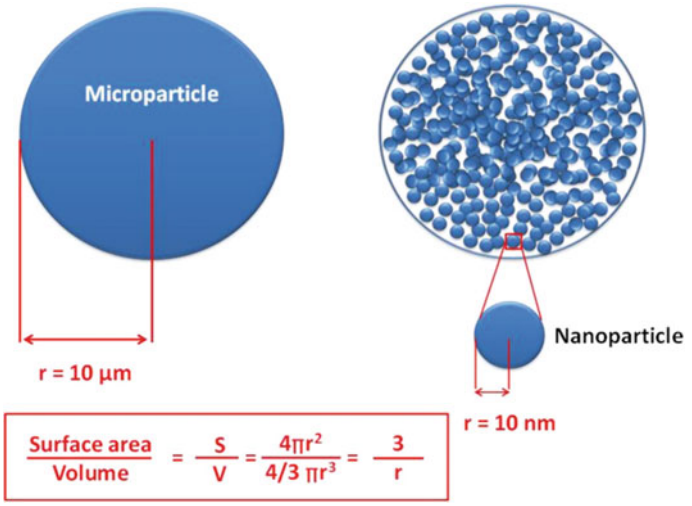

Fig. 1.3 Micro and a nanoparticle surface area to volume ratio. Reproduced with permission from Ref. [47] Copyright 2021, Elsevier

used in wide variety of medical tools and processes, chemical and biological sensing, gas sensing, CO_2 capture, and investigational uses in medicines, testing kits, magnetic resonance imaging (MRI), drug administration, and more.

Nanotechnology relies on nanoparticles (NPs) with nominal diameter of 100 nm or less that exhibit size-dependent physicochemical properties that set them apart from their bulk or submicron/micron-sized counterparts. High reactivity and physicochemical dynamicity are afforded to NPs by their large surface-to-volume proportion (Fig. 1.3). Quantum confinement and increased surface energy are two further NP characteristics that set them apart from bulk in terms of their actions and eventual fates in many systems [47].

On account of their dimensions, form, and chemical composition nanomaterials are grouped generally in multiple categories. Fullerenes and carbon nanotubes (CNTs) are two prevalent types of carbon-based NPs (Fig. 1.4). Fullerenes comprise nanomaterials of globular porous allotropic carbon forms of cages having excellent conductivity, robustness, structure, electron affinity, and flexibility [44].

CNTs are cylindrical structures with just 1–2 nm its diameter. Single-walled (SWNTs), double-walled (DWNTs), along multiwalled (MWNTs) varieties of the rolled sheets, are represented in Fig. 1.5. These materials are typically synthesized by depositing carbon starting materials, notably atomic-scale carbons, vaporized from graphite by laser or electric arc over metal fragments.

Metal NPs are derived entirely from their respective metal predecessors. Nanoparticles made of most alkali metals, comprising silver, gold, palladium, titanium, zinc, and copper, have excellent optical adjustable characteristics and exhibit a wide absorption band in the solar electromagnetic visible region. More specifically, interest

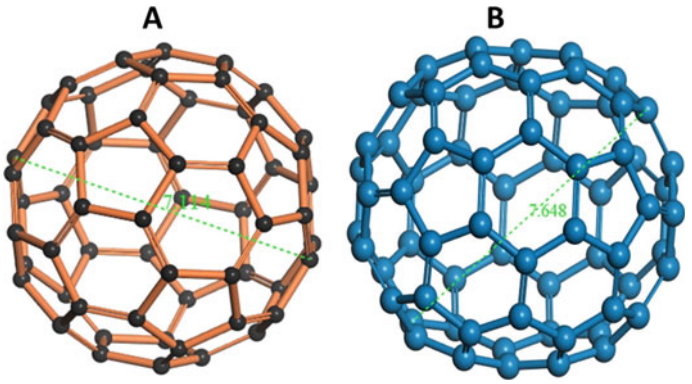

Fig. 1.4 Different forms of fullerenes **a** C_{60} and **b** C_{70}. Reproduced with permission from Ref. [44] Copyright 2019, Elsevier

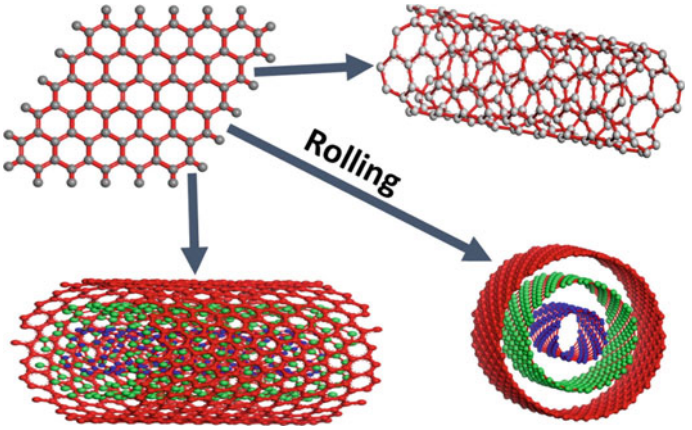

Fig. 1.5 Graphite layer rolling into single and multiwalled CNTs. Reproduced with permission from Ref. [44] Copyright 2019, Elsevier

in nanoparticles of silver and gold has increased as they possess excellent microbicidal effects against pathogenic bacteria, viruses, and other microbes containing nuclei [48, 49]. One of the distinctive characteristics of NPs is their high aspect ratio, resulting in exceptional reactivity and physicochemical dynamics [50]. Other than their size, the main traits of NPs that set them apart from bulk are their quantum confinement and greater surface energy [47]. In addition, their surface is readily functionalized to combine targeted and bioactive molecules by hydrogen bond, covalent and electrostatic interaction along with enhanced therapeutic effectiveness by loading various drugs simultaneously [51].

Modifying a nanoparticle's surface is essential for maintaining its stability and avoiding aggregation that are modified by adhesion, predominantly with thiol groups,

disulfide, amines, nitriles, and carboxyl groups. Chemical viability of organo-sulfur in forming an effective covalent interaction to noble metals is largely responsible for its development and use in surface modification. It has been demonstrated that long-chain polymers like polyethylene glycol have been shown to revamp surface of nanoparticles, reducing vague protein absorption. PEG's intrinsic physicochemical features reduce phagocytes uptake and deposition in non-target tissues, making it an attractive polymer for therapeutic properties. MNPs must undergo significant optimization of their biological compatibility, wettability, adherence, and cytotoxicity before therapeutic delivery processes. [52]. Other nanomaterials consist of doped or undoped metallic oxide particles such as titanium dioxide, silver or zinc oxide, etc. In addition, metal sulfides and metal organic frameworks (MOFs) have recently sparked quite a bit fascination for promising applications around various biological domains (Fig. 1.6). For instance, AgS, CuS, and FeS, along Zn, Cu, and Mn-based MOF widely utilized in various drug delivery and antimicrobial activities [53, 54].

Nanoparticles (NPs) made of ceramics are inorganic, non-metallic, and formed by heating and cooling a precursor material. Amorphous, multifaceted, thick, transparent, and vacuous morphologies are all feasible. New synthetic methods were used for nanoscale ceramics comprising hydroxyapatite (HA), zirconia (ZrO_2), silica (SiO_2), titanium oxide (TiO_2), and alumina (Al_2O_3) in an effort to enhance their physicochemical features while minimizing their cytotoxicity in biological environments. The versatility of nanoparticle administration routes, as well as their high stability and load capacity, hydrophobic and hydrophilic compatibility, and ease of integration, makes them a promising tool in managing drug delivery. Furthermore, it's possible to provide a targeted impact by functionalizing its surfaces with a wide range of organic groups. Numerous domains, covering catalytic/photocatalytic processes, photodegradation of dye, and image processing might

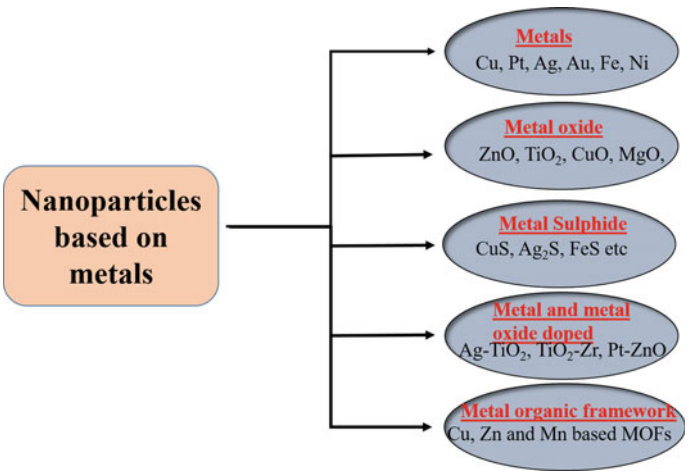

Fig. 1.6 Various types of metal-based nanomaterials

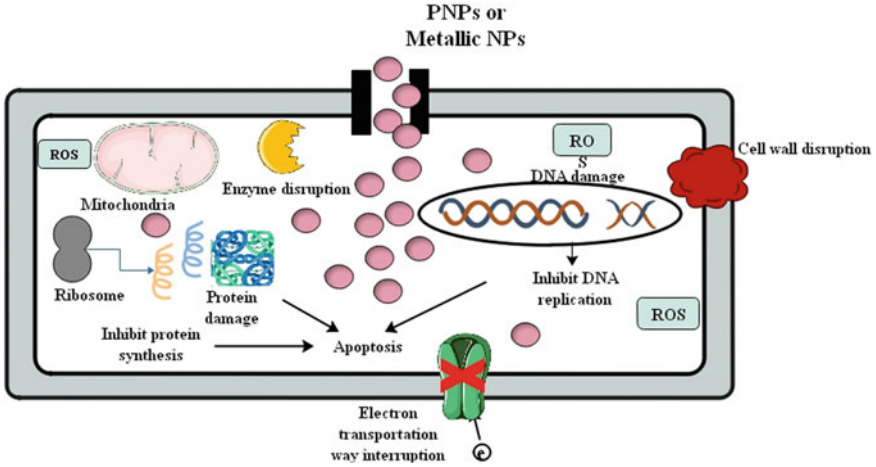

Fig. 1.7 Metal-based nanoparticles general mechanism for antimicrobial potential

benefit from these NPs; hence, scientists are paying special consideration to them. Additionally, their use as antimicrobial agents in diagnostics, drug delivery devices, and medical treatments is on the rise. Some metal oxides like TiO_2 have been utilized to combat transmission of a variety of infectious diseases [46, 55, 56], ZnO, CuO, and copper/TiO_2 dopants with carbon-based allotrope like graphene oxide investigated for bactericidal potential [57, 58]. Similarly, CdS nanoparticles indicated promising antibacterial action with the largest zones of inhibition for *Aspergillus flavus* and *Pseudomonas aeruginosa* [59].

Tiny size and high aspect ratio make polymeric and metal nanoparticles to permeate microbial membranes, where they exert their bactericidal impact. Figure 1.7 depicts general mechanism of immediate nanomaterials release, oxidative stress, and non-oxidative stress that contribute to microbicidal properties. Polymer nanoparticles (PNP) with a significantly lighter weight than conventional polymer composites are a significant alternative to these materials and expanding rapidly as a multidisciplinary research field whose findings have potential to expand polymers' use in industry [60, 61].

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Chapter 2
Fabrication of Polymeric Nanomaterials

Abstract Nanofabrication involves the systematic framework of individual elements or molecules of inorganic or organic substances. Micelles were the first polymeric nanoparticles to be created by polymerization techniques. Multiple techniques developed for synthesizing polymeric nanoparticles, each tailored to specific needs of particular application or set of physicochemical properties of a specific drug. The process of mechanically compressing bulk substances employing template unless original size is greater than nanovalue. Top-down approaches include milling, laser ablation, etching, sputtering, and electroexplosion. The first approach devised for polymeric NPs from preformed polymer was solvent evaporation where a polar organic solvent serves to dissolve polymer and add then active component are dispersed. Emulsification-solvent evaporation is employed for polymeric NPs fabrication with dimensions of approximately 100 nm, also to acquire nanospheres or nanocapsules. Active principles dissolved or dispersed in a polymeric solution to create nanospheres, while drugs dissolved in oil followed by emulsified in an organic polymeric solution to create nanocapsules, which are then dispersed in an external phase. This method operates on the basis of polymer interfacial deposition upon transit of organic solvent passes from lipophilic to aqueous phase. Nanoparticles can be physically labeled with a tag like a dye, magnetic particle, or radioactive marker. Numerous imaging approaches, notably fluorescence microscopy, magnetic resonance imaging (MRI), and positron emission tomography (PET), can detect a physical identification. Methods for chemically labeling nanoparticles involve the attachment to particular functional groups that can react with target molecules or receptors such as to target particular receptors on cancer cells, scientists have coupled polymeric nanoparticles with ligands including folate, transferrin, or epidermal growth factor. Using diverse techniques, such as avidin–biotin, streptavidin–biotin, and covalent bonding, researchers have functionalized polymeric nanoparticles with antibodies that target specific cancer cells, such as breast cancer or melanoma cells.

Keywords Nanofabrication · Physicochemical · Polymeric · MRI · PET · Nanospheres

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2.1 Introduction

PNPs are the ultimate outcome of polymerization events comprising multiple monomer units that may self-assemble into complexes of nanometric dimensions (10–100 nm) by deploying renewable resources and their copolymers, spanning diblock, triblock, multiblock, and radial block copolymers [1, 2]. Micelles were the first polymeric nanoparticles to be created by polymerization techniques [3, 4]. Multiple techniques developed for synthesizing polymeric nanoparticles, each tailored to specific needs of particular application or set of physicochemical properties of a specific drug [5]. Current laboratory synthesis processes for PNPs display batch-to-batch variability in particle size and product quality and are often not repeatable and challenging to implement in industry [6, 7]. Generally, dispersing performed polymers or polymerizing monomers are the two most common approaches [8]. Following that, advanced polymerization methods devised for polymeric-based nanoparticles were stabilized using surfactants [9]. There are several approaches for preparing polymeric nanoparticles, primarily top-down and bottom-up procedures (Fig. 2.1). Depending on their size and structure, it is conceivable to categorize polymer nanoparticle carriers of drugs as either nano/microcapsules or nano/microspheres [9–11]. Nanoparticles comprise fine particles spanning in size from 100 to 2500 nm or ultrafine particles from 1 to 100 nm, whereas emulsion polymerization method produces nanoparticles of 50 to 300 nm dimension. Premade polymers in polymerization process eliminate the risks associated with polymerization, particularly development of undesired oligomers and the formation of hazardous, reactive residues.

Most methods that use premade polymers typically begin by disintegration of polymer within organic solvent that may lead to toxicity and environmental hazards. Overall the final product must be free of solvent residues. Techniques based on polymerization of monomers permit integration of compounds into polymeric NPs with greater efficacy in a single reaction step [8, 13]. In most cases, a nanoemulsion was made by dissolving a drug polymer into an organic solvent that was insoluble in water; the probe-sonication process was one such technique. The natural solvent achieves vaporization under conditions of increased temperature or low pressure by means of rotary evaporator. Subsequently, the resulting nanoparticles are collected for the intent of assessment. Various modifications and enhancements of emulsification strategies seemed documented. For instance, sonication is vital process in forming nanoemulsion that holds vulnerable drugs, as it can increase temperature, rendering the active components inactive. Researchers implemented an on/off cycle for keeping moderate temperature in order to prevent the issues. Figure 2.2 depicts additional examples of general procedures for preparing the drug-polymer nanoparticle [14, 15].

PNPs optimization as well as assembly are complicated and time-consuming [6]. The products are typically derived as aqueous colloidal suspensions, irrespective of preparation approach used. The potential for simultaneous manufacturing of PNPs along with repeatable and tunable physiochemical features is very promising

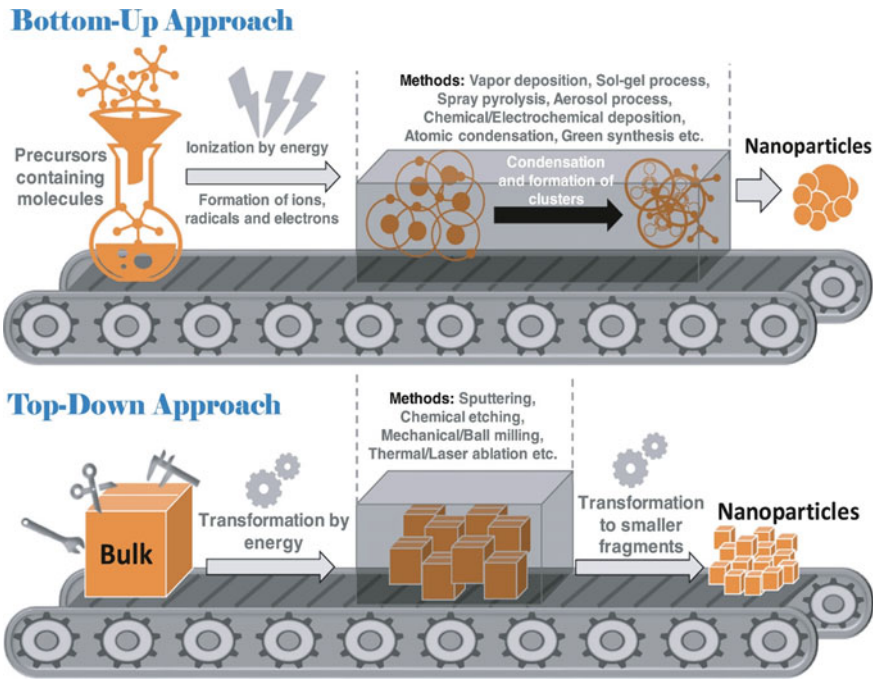

Fig. 2.1 Bottom-up and top-down approach of nanoparticles synthesis: permission to reprint from Ref. [12] Copyright 2021, Elsevier

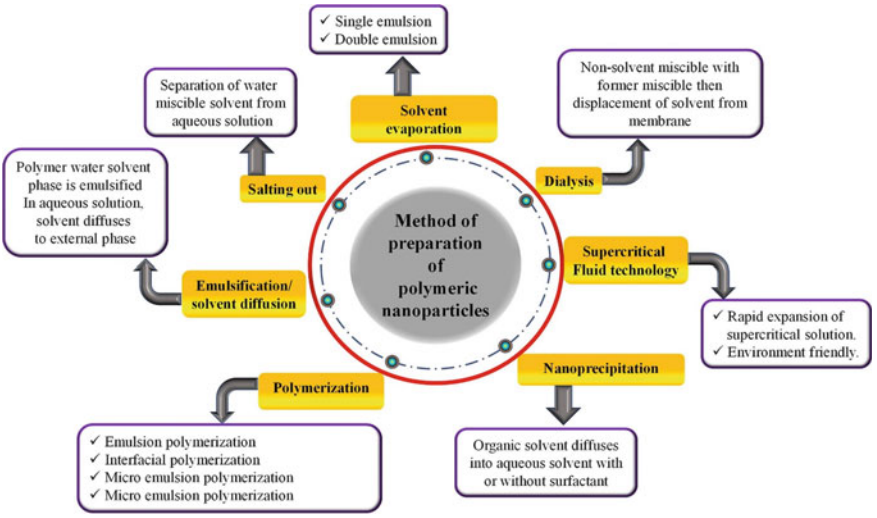

Fig. 2.2 General methods of polymeric nanoparticles preparation. Reproduced with permission from Ref. [16] Copyright 2019, Elsevier

[7, 17]. Inconsistent particle delivery is an ongoing issue caused by barriers associated in manufacturing, describing, and delivering particles with precise physical and chemical properties [15]. Synthesis of complex and dynamic NPs is primary cause of non-reproducibility and irregularity in manufacturing of particles that are unstable, ephemeral, and frequently change in response to environmental or temporal changes [18]. This chapter discusses the diverse methods for producing polymeric nanoparticles (PNPs).

2.2 Top-Down Methods

The process of mechanically compressing bulk substances employing template unless original size is greater than nanovalue [12]. Top-down approaches include milling, laser ablation, etching, sputtering, and electroexplosion [15] (Table 2.1).

2.2.1 Bottom-Up Methods

Nanofabrication involves the systematic framework of individual elements or molecules of inorganic or organic substances, often by autonomous assembly where chemical or biological reducing agents are used for accumulation of atomic particles, molecules or groups of varying dimensions, leading to the synthesis of NPs [12] (Table 2.2).

2.3 Emulsification-Solvent Evaporation

The process of emulsification entails integrating an aqueous solution with insoluble solvent in water and then subjecting to strong shear forces resulting PNPs by evaporating solvent. The first approach devised for polymeric NPs from preformed polymers was solvent evaporation where a polar organic solvent serves to dissolve polymers and add then active component are dispersed (Fig. 2.3). Dichloromethane and chloroform have been extensively utilized, albeit historically more frequently but were replaced with ethyl acetate [20], which is less poisonous and more useful in biomedical applications for its superior toxicological profile. Some techniques continue to employ volatile organic solutions. This method permits the nanospheres fabrication. Due to its adaptability, this approach is often used for drug encasement in polymeric particles as it may accommodate wide variety of drug/polymer pair compositions [15]. It's also prevalent to produce surface active agents (polyvinyl acetate) in aqueous state. In order to develop nanodroplet dispersion, a surfactant is incorporated to disperse organic mixture in aqueous phase via rapid-speed homogenization followed by diffusion in emulsion's continuous phase, the polymer solvent

Table 2.1 Top-down approaches with pros, cons, and general remarks

Top-down methods	Merits	Demerits	General remarks
Optical lithography	Long-standing, established micro/nanofabrication tool especially for chip production, sufficient level of resolution at high throughput	Tradeoff between resist process sensitivity and resolution, involves state-of-the-art expensive clean room-based complex operations	The 193 nm lithography infrastructure already reached a certain level of maturity and sophistication, and the approach could be extended to extreme ultraviolet (EUV) sources to shrink dimension. Also, future developments need to address the growing cost of mask set
E-beam lithography	Popular in research environments, an extremely accurate method and effective nanofabrication tool for < 20 nm nanostructure fabrication with desired shape	Expensive, low throughput, and a slow process (serial writing process), difficult for < 5 nm nanofabrication	E-beam lithography beats the diffraction limit of light, capable of making periodic nanostructure features. In the future, multiple electron beam approaches to lithography would be required to increase the throughput and degree of parallelism
Soft and nanoimprint lithography	Pattern transfer based simple, effective nanofabrication tool for fabricating ultrasmall features (< 10 nm)	Difficult for large-scale production of densely packed nanostructures, also dependent on other lithography techniques to generate the template, and usually not cost effective	Self-assembled nanostructures could be a viable solution to the problem of complex and costly template generation, and for templates of periodic patterns < 10 nm
Block copolymer lithography	A high-throughput, low-cost method, suitable for large-scale densely packed nanostructures, diverse shapes of nanostructures, including spheres, cylinders, lamellae possible to fabricate including parallel assembly	Difficult to make self-assembled nanopatterns with variable periodicity required for many functional applications, usually high defect densities in block copolymer self-assembled patterns	Use of triblock copolymers is promising to generate more exotic nanopattern geometries. Also, functionalization of parts of the block copolymer could be done to achieve hierarchy of nanopatterning in a single step nanofabrication process

(continued)

Table 2.1 (continued)

Top-down methods	Merits	Demerits	General remarks
Scanning probe lithography	High resolution chemical, molecular and mechanical nanopatterning capabilities, accurately controlled nanopatterns in resists for transfer to silicon, ability to manipulate big molecules and individual atoms	Limited for high-throughput applications and manufacturing, an expensive process, particularly in the case of ultrahigh-vacuum-based scanning probe lithography	Scanning probe lithography can be leveraged for advanced bionanofabrication that involves fabrication of highly periodic biomolecular nanostructures

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evaporates by constant magnetic agitation at ambient temperature (for polarized solvents) or by gradual method under low pressure (for dichloromethane and chloroform), leaving behind a suspension of NPs which can be rinsed, collected, and freeze-dried for long-term storage [21, 22]. This technique is non-toxic, produces NPs, and exhibits a swift response time. The substantial energy consumption linked to method may compromise the stability, and necessity for uniformity of drug is a downside.

2.4 Emulsification/Solvent Diffusion

Leroux et al. [24] first introduced this method that entails the development of oil/aqueous emulsion among polymer and drug containing partially water-soluble solvent and surfactant-containing aqueous solution. As a means of establishing primarily thermodynamic stability among two phases at ambient climate, the organic solvent phase utilized was drenched by water. Solvent afterward dissipates from dispersed molecules to outer phase as dispersed with a substantial quantity of water, producing colloidal particles as it permits the fabrication of high-yield, high-reproducibility nanosphere and capsules, as well as particle size control. The subsequent step could be avoided if the solvent has a low enough boiling point or if it can be filtered out. On this process, the droplet size falls quickly on a millisecond time scale and produces NPs having sizes spanning from 80 to 900 nm. It emerged that emulsification process relied mainly on duration, stirrer type, and rotating rate. Conversely, spinning has no effect over ultimate size distribution during the dilution phase. Only sufficient blending is required to achieve homogeneity [25]. This method is highly efficient, scalable, applicable to thermolabile pharmaceuticals, and stresses the encapsulated protein as little as feasible. However, large concentrations of liquid must be eradicated from suspension, and water-soluble medications may

Table 2.2 Bottom-up synthetic techniques with their pros and cons

Bottom-up techniques	Merits	Demerits	General remarks
Atomic layer deposition	Allows digital thickness control to the atomic level precision by depositing one atomic level at a time, pin-hole free nanostructured films over large areas, good reproducibility and adhesion due to the formation of chemical bonds at the first atomic layer	Usually a slow process, also an expensive method due to involvement of vacuum components, difficult to deposit certain metals, multicomponent oxides, certain technologically important semiconductors (Si, Ge, etc.) in a cost-effective way	Although a slow process, it is not detrimental for the fabrication of future generation ultrathin ICs. The stringent requirements for the metal barriers (pure; dense; conductive; conformal; thin) that are employed in modern Cu-based chips can be fulfilled by atomic layer deposition
Sol gel nanofabrication	A low-cost chemical synthesis process-based method, fabrication of a wide variety of nanomaterials including multicomponent materials (glass, ceramic, film, fiber, composite materials)	Not easily scalable,, usually difficult to control synthesis and the subsequent drying steps	A versatile nanofabrication method that can be made scalable with further advances in synthesis step
Molecular self-assembly	Allow self-assembly of deep molecular nanopatterns of width less than 20 nm and with large pattern stretches, generates atomically precise nanosystems	Difficult to design and fabricate nanosystem unlike mechanically directed assembly	Molecular self-assembly of multiple materials may be a useful approach in developing multifunctional nanosystems and devices
Physical and chemical Vapor deposition	Versatile nanofabrication tool for fabrication of nanomaterial including complex multicomponent nanosystem (e.g., nanocomposites), controlled simultaneous deposition of several materials including metal, ceramics, semiconductors, insulators and polymers, high purity nanofilms, a scalable process, possibility to deposit porous nanofilms	Not cost effective because of the expensive vacuum components, high-temperature process and toxic and corrosive gases particularly in the case of chemical vapor deposition	It provides unique opportunity of nanofabrication of highly complex nanostructures made of distinctly different materials with different properties that are not possible to accomplish using most of other nanofabrication techniques. New advances in chemical vapor deposition such as “initiated chemical vapor deposition” (i-CVD) provide unprecedented opportunities of depositing polymers without reduction in molecular weights

(continued)

Table 2.2 (continued)

Bottom-up techniques	Merits	Demerits	General remarks
DNA scaffolding	Allow high precision assembly of nanoscale components into programmable arrangements with much smaller dimensions (less than 10 nm in half-pitch)	Many issues need to explore such as novel units and integration processes compatibility with CMOS fabrication, line-edge roughness, throughput and cost	Very early stage. Ultimate success depends on the willingness of the semiconductor industry in terms of need, infrastructural capital investment, yield and manufacturing cost

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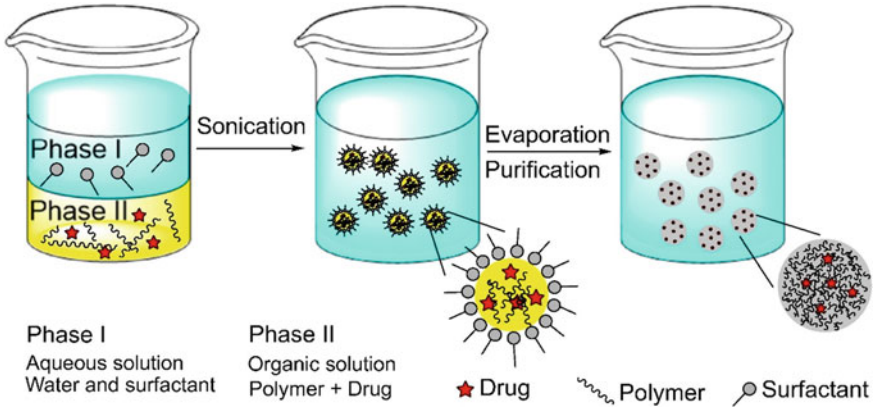

Fig. 2.3 Representation of emulsification-solvent evaporation method. Reproduced with permission from Ref. [23] Copyright 2017, Elsevier

escape into the exterior phase during the emulsifying step which are some of its major concerns [23]. This approach is only pertinent to lipophilic substances and necessitates extensive rinsing.

2.5 Emulsification/reverse Salting-Out

As alternative to diffusion approach, the salting-out method uses phenomenon to remove hydromiscible component from aqueous solution that produces spheres with dimensions amid 170 and 900 nm [26]. The primary distinction is in the component parts: an o/w emulsion comprises a hydrophilic solvent like acetone or ethanol,

whereas the aqueous phase has a gel, salting agent, and colloidal stabilizing agent. In order to produce an o/w emulsion, the aqueous phase must be saturated, thereby restricting solubility of both acetone and water. The sole requirement of this process is presence of a two-phase system along with salting-out agent. Numerous varying compounds, both ions and inert substances such as sucrose, may be used as suitable salting-out agents. Song et al. [27] used NaCl in place of magnesium chloride or acetate as salting-out agent for PLGA nanoparticles formation. Similarly, nanoparticles of PLGA and PLA (poly(lactic acid) nanoparticles with an average dimension of under 200 nm were synthesized by Konan et al. [28] utilizing tetrahydrofuran (THF) as the polymer solvent. For optimal binding of hydrophilic drugs in polymer, twofold emulsion production may be employed. This involves first forming a w/o emulsion and then separating the generated nanoparticles [29]. For concomitant hydrophobic and hydrophilic compounds, biodegradable Janus particles can be used [30]. Zweers and colleagues studied the impact of particle size in an ordinary process and concluded that the best way to regulate particle size is by modifying polymer concentration in external phase [31]. Avoiding chlorinated solvents, which are harmful to both ecological and biological systems, is the principal benefit of the salting-out procedure. The biggest drawbacks are extensive purification processes required owing to use of salts and the fact that it can only be used for encapsulating lipophilic pharmaceuticals. Few recent papers have cited the salting-out approach [23].

2.6 Nanoprecipitation

Solvent displacement, or nanoprecipitation, was pioneered by Fessi et al. [32] and necessitates combination of two indivisible solvents. Organic solvents like acetone and acetonitrile due to their incompatibility with water are readily eradicated via evaporation leaving behind just the polymer in the internal phase [33, 34]. So, emulsification-solvent evaporation is employed for polymeric NPs fabrication with dimensions of approximately 100 nm [35] and also to acquire nanospheres or nanocapsules [36]. Active principles are dissolved or dispersed in a polymeric solution to create nanospheres, while drugs dissolved in oil are followed by emulsified in an organic polymeric solution to create nanocapsules, which are then dispersed in an external phase [26]. This method operates on the basis of polymer interfacial deposition upon transit of organic solvent passes from lipophilic to aqueous phase. Under stirring (in dropwise manner), the polymer is dissolved solvent of intermediate polarity that is water-miscible, and this solution is then added to an aqueous solution. Nanoparticles appear when polymer solution spontaneously diffuses into aqueous phase [37]. This mechanism seems to be driven by Marangoni effect, which increases surface area owing to fast diffusion and forms minute organic solvent beads [23]. The nanodroplets' solvent slowly evaporates, leaving behind nanocapsules or nanospheres of precipitated polymer [38]. Surfactants are often included to maintain the stable colloidal suspension; however, their inclusion is

not essential for nanoparticles synthesis. The resulting nanoparticles are superior to those created using the emulsification-solvent evaporation method due to their more uniform size and narrower size distribution. This technique is advantageous due to its simplicity, speed, and reproducibility. Finding a viable drug/polymer/solvent/non-solvent system that permits efficient nanoparticle formation is the primary obstacle. Troublesome mixing and drug encapsulation are additional obstacles. The automated high-throughput (pipetting robot, inkjet printing) process is an additional innovation in the field of nanoprecipitation [39, 40]. Modifying drug solubility by altering pH or varied solvent content is additional method for enhancing encapsulation efficiency [41].

2.7 Membrane Extrusion

The nanoporous membrane extrusion method leads to NPs with high degree of size control alongside excellent scalability in comparison to the self-emulsification method, which generates massive and polydisperse emulsion particles. Pore measurement, stress, velocity, and fluid interface tension are all variables in membrane extrusion methods. Figure 2.4 depicts four typical methods for extruding nanoporous membranes: (a) vesicles expansion; (b) emulsified membranes; (c) precipitation; and (d) biological membrane extrusion. Ps are produced in a single phase via precipitation extrusion. The inert solution is brought into contact with the disintegrated solute feedback solution at the nanopore outlet, after that, feed solution particles confined in nanopores rapidly precipitate into solid NPs, which are then continuously transported out of nanopore outflow and disseminated in ensuing receiving mixture to consolidate [42]. Compared to synthetic PEGylated NPs, cell membrane-enclosed NPs are better able to proliferate for longer periods of time and bypass the reticuloendothelial system because they make use of the biological activities of the cell membrane and its proteins [43]. This technique yields PNPs with a diameter between 100 and 400 nm.

2.8 Super Critical Fluid Technology (SCF)

When a fluid is squeezed and subjected to heat over its critical temperature and pressure (Tc, Pc), its chemical or physical characteristics change between gas and liquid; such transition occurs in form of a supercritical fluid (SCF). This novel form of matter exhibits gas-like behavior while density or solvating features as liquids. Supercritical carbon dioxide ($scCO_2$) is preferred SCF as it is accessible, cheap, non-combust non-toxic, and ecofriendly [44]. In pharmaceutical research, numerous recent and outstanding evaluations on particle generation, composition, and regulation have been published using SCF. Perrut and Fages et al. reviewed procedures for preparing SCF particles [45, 46], whereas Ginty et al. [47] described advance field of drug

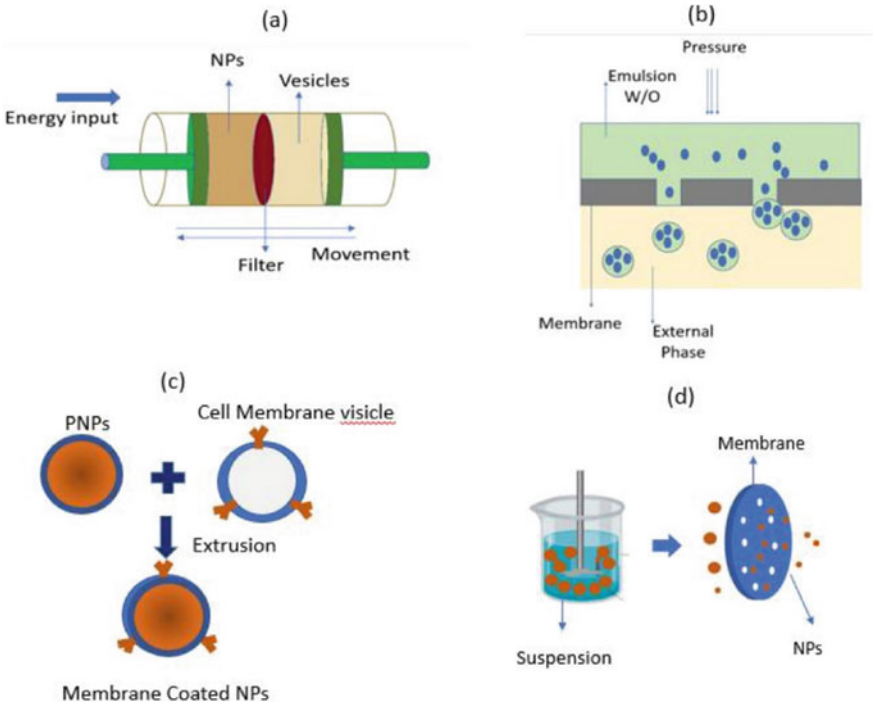

Fig. 2.4 **a** Vesicle extrusion, **b** membrane emulsification, **c**, **d** precipitation and biological membrane extrusion. Reproduced with permission from Ref. [15] Copyright 2022, Elsevier

delivery through implementation of SCF. Mishima et al. [48] provided a summary of producing sustainable particles for pharmaceuticals and gene delivery.

Rapid expansion of supercritical solutions (RESS), gas antisolvent system (GAS), supercritical antisolvent process (SAS) and its variations, and particles generation from gas-saturated solution operations are the most prevalent processing methods utilizing supercritical fluids [15]. The extent to which SCF is used as solvent, solute, or an antisolvent determines the most appropriate technique to apply. Supercritical fluids (SCF) have been explored as promising option considering the use of sustainable technology, ease and reproducibility of scaling up, the excellent regulation of morphological uniformity, and production of high-quality nanomedicines [49].

2.9 Labeling of Polymeric Nanoparticles

Polymeric nanoparticles desirable physicochemical qualities, including small size, large surface area, and tendency to encapsulate, have gained expanding application in medical and pharmaceutical fields. The capability to label or functionalize polymeric nanoparticles with particular molecules or groups allows for targeted delivery to individual cells and tissues. Many different labels, including fluorescent dyes, radiolabels, and magnetic nanoparticles, may be affixed to polymeric nanoparticles. The label specified for nanoparticles will be contingent on their intended use and requisite detection technique.

Nanoparticles can be physically labeled with a tag like a dye, magnetic particle, or radioactive marker. Numerous imaging approaches, notably fluorescence microscopy, magnetic resonance imaging (MRI), and positron emission tomography (PET), can detect a physical identification. Moreover, integrating fluorescent dyes into polymeric nanoparticles during synthesis or conjugating them to nanoparticle's surface following synthesis are approaches used by researchers for labeling. The knack of fluorescent compounds to emit light of diverse wavelengths permits detection by fluorescence microscopy or flow cytometry. Several distinct coupling strategies could potentially be used to introduce fluorescent pigments especially rhodamine, fluorescein, and Cy5.5 into polymeric nanoparticles for labeling optical imaging applications [50]. As labels for magnetic resonance imaging (MRI) of polymeric nanoparticles, magnetic nanoparticles are affixed to surface by various ways, like electrostatic interactions and covalent bonding [51]. Radioactive isotopes, such as ^111In or ^99mTc, may be anchored to polymeric nanoparticles, which can then be detected by imaging modalities such positron emission tomography (PET) utilizing ^64Cu or ^89Zr, or single-photon emission computed tomography (SPECT). It is conceivable to study biodistribution and in *vivo* imaging of polymeric particles by using radiolabels. Among the most commonly used radioisotopes are 125I, 131I, and 99mTc that integrated by chelation methods [52]. Coating polymeric nanoparticles with gold nanoparticles makes them visible in dark-field microscopy and detectable with surface-enhanced Raman scattering (SERS) [53]. Labeling polymeric nanoparticles with bioluminescent proteins like luciferase makes them visible in bioluminescence imaging.

Methods for chemically labeling nanoparticles involve the attachment to particular functional groups that can react with target molecules or receptors such as to target particular receptors on cancer cells; scientists have coupled polymeric nanoparticles with ligands including folate, transferrin, or epidermal growth factor (EGF) [54–56]. Chemical linkers such as biotin-streptavidin, or maleimide-thiol may be used to attach ligands to nanoparticles that can increase uptake and accumulation in certain cells or tissues. Methods for biological labeling encode specific proteins, peptides, or antibodies that recognize and bind to target cells or tissues [57]. Using diverse techniques, such as avidin–biotin, streptavidin–biotin, and covalent bonding, researchers have functionalized polymeric nanoparticles with antibodies that target

specific cancer cells, such as breast cancer or melanoma cells. Nanoparticles modified with antibodies have the potential to preferentially attach to cancer cells, where they may then either kill the cells or deliver therapeutic pharmaceuticals. The labeling strategy used should be tailored to the requisite detection sensitivity and specificity.

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Chapter 3
Properties and Characterization of Polymeric Nanomaterials

Abstract The "characterization" in material science encompasses vast assortment of methods to investigate properties and architecture. The composition and concentration of polymeric NPs, their dimension, layout, interface characteristics, crystalline nature, and dispersed behavior may all vary. Typically, multiple approaches are used to evaluate these properties in an effort to fully characterize the NPs. Numerous methodologies are currently utilized to determine the quality of nanostructured materials fabricated, mainly microscopic (scanning and transmission electron microscopy, atomic force microscopy), spectroscopic (Fourier transform infrared, UV–Vis, fluorescence, or Raman spectroscopy) and X-ray-based methods (X-ray diffraction, X-ray photoelectron spectroscopy, X-ray tomography), NMR, and molecular modeling. The electrical functions of nanosized components are mostly due to quantum mechanical wave behavior of electrons and the absence of scattering centers. The high electrical conductivity of polymers like polyaniline, polypyrrole, and polyacetylene makes them ideal for use in electronics. Mechanical traits of various materials vary greatly and upon comparison to more common materials, metals' mechanical qualities notably strength, flexibility hardness, toughness, pliability, ductility, rigidity, and yield stress are some characteristics that may be measured. The shape and dimensions of PNPs may be investigated by employing microscopy methods like transmission electron microscopy (TEM). Laser scattering (dynamic or static light scattering, laser diffraction), field flow fractionation (FFF), electron microscopy (EM), centrifugation (analytical ultracentrifugation, and centrifugal particle sedimentation), tunable resistive pulse sensing (TRPS), and particle tracking analysis (PTA) are just some of the methods that could be employed to determine average diameter and size distribution of nanoparticles (Caputo et al. in J Control Release 299:31 (2019)) [1]. The most commonly used approach for determining size is dynamic light scattering (DLS).

Keywords Characterization · UV–Vis · NMR · TEM · DLS · TRPS

It is imperative to characterize manufactured nanoparticles in order to corroborate their nanoscale dimension. The "characterization" in material science encompasses vast assortment of methods to investigate properties and architecture. The shape

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Table 3.1 Techniques for physicochemical characteristics of PNPs

Approaches	Physicochemical features
Atomic force microscopy (AFM)	Dimension and its distribution Morphology and structure Fundamentals of aggregates and interfaces
Differential scanning calorimetry (DSC)	Prospective drug-polymer correlations and drug's physicochemical status
Dynamic light scattering (DLS)	Size dispersion in hydrokinetics
Fluorescence spectroscopy	Estimation of critical affiliation ratio Release of drugs in vitro
Infrared spectroscopy (IR)	Bioconjugate framework and configuration, Determination of functional group
Scanning electron microscopy (SEM) Scanning tunneling microscopy (STM)	Dimension and sizing Morphology Agglomeration
Transmission electron microscopy (TEM)	Measurements and proportions Shape diversity
X-ray photoelectron spectroscopy (XPS)	Interface atomic and chemical formulation
Zeta potential	Surface-charge-related robustness
UV–Visible	Optical properties and band gap

and structure of nanomaterials may be determined with use of characterization techniques, and efficiency of such techniques can be assessed. While the majority of technologies are qualitative, some are quantitative. Polymeric nanoparticle characterization lacks both established methodology and FDA regulatory protocols [2]. Nevertheless, it is widely known that the physicochemical features of the particles have a role in the physiological interactions. In order to comprehend the system's behavior in body, it is crucial to develop an adequately accurate image of PNPs. The composition and concentration of polymeric NPs, their dimension, layout, interface characteristics, crystalline nature, and dispersed behavior may all vary. Typically, multiple approaches are used to evaluate these properties in an effort to fully characterize the NPs. Numerous methodologies are currently utilized to determine the quality of nanostructured materials fabricated [3, 4], mainly microscopic (scanning and transmission electron microscopy, atomic force microscopy), spectroscopic (Fourier transform infrared, UV–Vis, fluorescence, or Raman spectroscopy) and X-ray-based methods (X-ray diffraction, X-ray photoelectron spectroscopy, X-ray tomography), NMR, and molecular modeling. This chapter will focus on common and accessible techniques for characterizing PNPs. The primary objective is to provide both a theoretical and practical comprehension of methods used to explain an array of PNP forms. Extensive analytical techniques have been implemented on each method for identifying nanostructured materials as depicted in (Table 3.1).

3.1 Optical Properties

Metal-polymer interactions can be used to combine and, in some cases, synergistically create interesting optical properties of nanomaterials by altering their mechanical behavior; these properties can then be exploited to develop devices with properties such as refractive index change, polarization, optical switching, LEDs, optical lenses, actuators, and solar photovoltaics. Particle size may be adjusted to provide a range of structural, optical, thermal, and electrical characteristics that are well suited to a variety of device uses. Modifying the composite's configuration including the metal proportions and the size and shape of nanoparticles can drastically alter its electrical and optical characteristics [5, 6]. By reducing the size of the particles, the material's overall dimensions are reduced, and the resulting NPs have greater crystal perfections and fewer defects. More recent interest on semiconductor NPs may be attributed to their size-dependent nature as a result of the quantum confinement phenomena [7]. By altering the morphology, chemical composition of surface, or aggregation state, it is possible to influence the material's optical features, which are intrinsically bound to its electronical traits. The linear and nonlinear behavior of these substances may be precisely optimized by modulating crystal dimension and surface chemistry. The manufacturing method gradually evolves into a crucial part of strategy. Polymer lasers, known as solid-state derived lasers, utilize polymers like polymethyl methacrylate (PMMA) and methyl methacrylate (MMA) as the gain medium. Due to excellent surface texture and extreme opacity of those polymers, laser characteristics are determined via laser dye applied to dope matrix of polymers. These organic lasers are highly regarded for their ability to produce small line widths, which are then put to good use in spectroscopy and other analytical procedures. The polymer's refractive index changes with temperature, making it a crucial optical property in laser applications [8, 9].

3.2 Electronic Properties

The electrical functions of nanosized components are mostly due to quantum mechanical wave behavior of electrons and the absence of scattering centers [7]. The high electrical conductivity of polymers like polyaniline, polypyrrole, and polyacetylene makes them ideal for use in electronics. Synthesizing these polymers into nanoparticles is a great way to boost their electrical conductivity as polyethylenedioxythiophene (PEDOT) nanoparticles are employed in numerous forms of electronic devices due to their high electrical conductivity [10]. Polymeric nanoparticles may have a different dielectric constant than bulk polymer because of confinement effect. By controlling their dimensions and geometry, polymeric nanoparticles may be made with broad spectrum of dielectric constants as dielectric constant of polyvinylpyrrolidone (PVP) nanoparticles is 4.8 for those with a diameter of 100 nm and 8.4 for those with a diameter of 20 nm [11].

3.3 Thermal Properties

Differential scanning calorimetry, an aspect of thermal analysis, is a mainstay technique for characterizing polymers. The physicochemical condition and probable associations of drug inside PNPs are often investigated that may detect glass transition, crystallization, and melting, among other phase changes. Many performance metrics may be connected with variations in the melting or glass transitions of a material, which in turn are influenced by changes in material's chemical constituents and atomic structure. In amorphous substances, unstructured areas of partially crystalline components, glass transition temperature (tg) undergo a reversible phase shift from rigid and fragile to softer and malleable form. So, it is crucial technique for gauging the degree of crystallinity in semicrystalline polymers. Plastics heat endurance and impact on reinforcements like flame retarders may be assessed through thermogravimetric analysis. Differential, thermomechanical, dynamic mechanical, and dielectric are various forms of thermal analysis that are often hybrids of aforementioned primary methods. More subtle shifts with temperature that impact intricate modulus or dielectric functionality of subjects may be revealed by dynamic mechanical and dielectric spectrometry, which are merely adaptations of thermal assessment [9]. PNPs may benefit from the mechanical strength provided by polymers in their glassy form, and particle aggregation can be prevented [12].

3.4 Mechanical Properties

The mechanical features of a material persist despite being stressed or loaded in different ways. The mechanical traits of nanocrystalline materials were target of much research due to their very varied features. Measuring the strength of a polymer sheet is often used as an example of characterizing mechanical characteristics in polymers. For stress–strain characteristics of polymer, parameters yield strength and Young's modulus of elasticity are particular relevance [13]. Mechanical traits of various materials vary greatly and upon comparison to more common materials, metals' mechanical qualities notably strength, flexibility hardness, toughness, pliability, ductility, rigidity, and yield stress are some characteristics that may be measured. Metals' brittleness is crucial trait as most inorganic and non-metal substances are fragile with no desired properties of flexibility, robustness, elastic modulus, and toughness. Furthermore, certain organic materials lack brittleness or rigidity since they are malleable substances [7]. There is currently no consensus on mechanical characteristics of spherical polymer nanoparticles as function of size. Polystyrene nanoparticles (200 nm in diameter) with hydrated ionic functional groups were found to have marginally lower compressive moduli than the similar bulk materials [14]. Conversely, research by Paik et al. [15] showed that polypropylene nanoparticles had greater modulus of elasticity than bulk material. Particle elasticity modulus was

considered to alter depending on factors such as its tg, crystal sequence, and structure. However, contrary to commonly held notion that no displacement exists in crystalline nanoparticles, it has been shown that dislocations within particles contribute to shift in mechanical properties of nanomaterials. Ramos et al. [16] experimental results showed that stacking flaws and distortions formed in certain crystallographic orientations led to an increase in toughness and elastic modulus of gold nanoparticles with sixfold symmetry compared to the bulk phase. It's important to note that measuring mechanical properties of each nanomaterial is challenging task, with an array of aspects that might alter final findings. Nanoparticle distribution on ideal firm substrate, particle placement, loading, minimal particle distortion, and other such parameters are all important. Furthermore, several uncertainties, among them those connected with instrument validation and the computation of models, should be taken into account when measuring and computing the mechanical characteristics of nanoparticles using AFM [14].

3.5 Distinctive Properties

Polymeric nanomaterials possess distinct advantages over their bulk counterparts because of their unique properties. Size-dependent effects are increasingly evident at the nanoscale like on macroscale, an alloy of gold solution appears yellow, but when examined at nanolevel, it appears red or violet [7]. At the nanoscale, substances suffer dramatic changes in their electrical properties, in comparison to their bulk form. Also, PNPs' mechanical qualities are much improved in comparison to their bulk counterparts as consequence of increase in crystal symmetry or decrease in crystallographic defects. Nanocomposites are analyzed in either single or multiparticle ensemble of elemental evaluation to determine their chemical composition, which includes not only the atomic elements but also the compounds' natural or manufactured functional groups. Insight into polymerization process, presence of chemical interaction among drug and polymer, and its decomposition can all be gained by determining polymer's molecular weight variation after it has been prepared. Size-exclusion SEC is often used to quantify molecular weight distribution of a polymer [17], whereas static light scattering to measure intensity of light scattered by polymeric NPs thus polymers may acquire a wide variety of new characteristics by having their sizes and morphologies adjusted.

3.6 Ultraviolet–Visible Spectroscopy (UV–Vis)

In order to measure how much light a sample absorbs and scatters, researchers employ ultraviolet–visible spectroscopy (abbreviated as UV/VIS) to calculate extinction (the total amount of light a sample blocks out). PNPs' dimensions, form, concentration,

and aggregation all have an effect on these characteristics. This implies that UV–Visible spectroscopy may be used to identify and characterize polymeric nanoparticles. The quantity of light consumed by specimen may be used as substitute for concentration of analytes using this method. The conjugation and conjugate proportion of biomolecules to nanomedicines have also been assessed by this method. Qasim et al. exploited UV–Visible spectroscopy to investigate the encapsulation of silver nanoparticles in poly-*N*-isopropylacrylamide-based polymeric nanoparticles. Similarly, Iram et al. employed UV–Vis spectroscopy to investigate optical characteristics for MgO and CaO doped cellulose nanocrystals graft acrylic acid and their strong band-to-band absorption [18, 19].

3.7 X-Ray Diffraction (XRD)

In multitude of X-ray modalities, XRD is most significant for deciphering atomic-scale structures of crystalline substances. Besides detecting bulk crystal mass, it is also a great method for evaluating size of individual crystallites in nanostructures. The mean size of crystallite may be calculated using Scherrer formula, i.e., $D = K\lambda/(\beta \cos\theta)$, based on line broadening. Scherrer's constant (denoted by K) is employed to account for particle shape and value been reported to be 0.9, although it varies from particle to particle.

In accordance with Bragg's rule, X-ray diffraction is simply reflection of collimated beam of X-rays impinges onto crystalline planes of material. Wide-angle X-ray scattering approach is used to identify crystalline grains, form, and lattice distortion due to long-range order, although it can only be used to materials that are sufficiently disordered. Even though being an advanced approach applied extensively to identify atomic layout of materials, its applicability is limited because of inability to form crystals and only derive data from single conformation or binding state of sample [20]. The low amplitude of reflected X-rays renders XRD less useful than electron diffractions for components of low atomic number. Structure characterization by X-ray diffraction utilizing femtosecond pulses emanating from hard-X-ray free-electron laser has been stated [21]. This technique has potential in discovering the structures of large molecules that are either not prone to exposure to radiation or do not produce crystals of enough size to be studied using conventional irradiation devices.

3.8 Raman Spectroscopy (RS)

RS is popular method of in situ studies due to its ability to characterize nanomaterials and nanostructures without need for sample preparation and with submicron spatial resolution for transparent materials [22]. RS works on premise of measuring inelastic scattering of incoming light of varying frequencies as a result of its interaction with

electric dipoles in molecule. In this spectra, the lower-frequency photons are referred as Stokes lines, while higher-frequency beams as anti-Stokes lines, since they emit at divergent speeds than incoming photons. It depends on regularity with which polarizabilities shift as result of characteristic vibrations of molecules or atomic clusters. Some intense oscillations allowed in IR result in feeble Raman strips; therefore, the two strategies work well together. This technique complements others as multiple strong vibrations in IR result in weak Raman strips. Such methods allow us to validate recognized compounds in fingerprint area and to identify impurities or unanticipated interactions based on distinctive functional compounds. Recent study of PLGA nanoparticles describes laser trapping/Raman spectroscopy approach on single-particle level that shows effectively integration into the PNPs utilizing just around ten individual PLGA nanoparticles [23].

The fact that water molecules are typically weak Raman scatterers makes it a good option for analyzing biological materials in aqueous solution. Tissue abnormalities may be detected using RS because of the extensive molecular information it provides, which may be utilized to probe conformations and concentrations of tissue components [24]. Fluorescence interference and an extremely minuscule cross section are two more drawbacks of RS, which both need powerful laser stimulation and substantial sample amounts to generate adequate signals [2, 25].

3.9 Fourier Transform Infrared Spectroscopy (FTIR)

Infrared (IR) radiation may be absorbed by molecules in general provided they have time-dependent dipole moment and their oscillation rate is same as received IR radiation. A typical mode is described as stable state of molecular vibrational Hamiltonian and happens after a molecule consumes infrared light and uses that energy to stretch, bend, or twist its covalent bonds. When plotted as a function of incident frequency, the IR spectrum presents an indication of molecule's structure due to oscillations in particles typically involving multiple coupled pairs of atomic particles or covalent connections, and these must be regarded to be amalgamation of ordinary modes [2]. It is common practice to detect the conjugated byproducts, like protein chains anchored to NP substrates, and highlight the dynamic states of linked molecules [26]. Absolute internal reflection and IR are combined in the emerging technique of attenuated total reflection (ATR)-FTIR to study chemical composition of assimilated and deposited species at solid/air or solid/liquid interface without complications of specimen preparation and spectral irreproducibility inherent to traditional IR. When sample at internal reflective interface retains evanescent IR frequencies comparing specimen's vibrational patterns, ATR-FTIR may yield IR absorption spectra that can be used to explore surface or chemical properties [27, 28]. However, since ATR-FTIR spectroscopy has a penetration depth of exactly same degree of magnitude as incoming IR wavelength, it is not highly sensitive surface-analysis approach at nanoscale.

3.10 Fluorescence Spectroscopy

For efficient fluorescence spectroscopy measurements, a fluorescent probe must be incorporated into the medium at very dilute concentrations in order to ensure the presence of the chromophores does not significantly disturb the bulk. The probe is selected on the basis of its sensitivity to variations in its immediate surroundings, as measured by variations in its emission behavior [29]. When stimulated by a certain wavelength, luminescence may be emitted from polymeric nanoparticles that have been tagged with fluorescent dyes or coupled with fluorescent compounds. Information about the nanoparticles' characteristics may be gleaned from the fluorescence's intensity and wavelength. Moreover, size and diameter of nanoparticles may be estimated by measuring their diffusion rate using fluorescence correlation spectroscopy (FCS) [30, 31]. The size, shape, and surface chemistry, durability, and drug release characteristics of polymeric nanoparticles may be determined by studying their fluorescence spectra. For instance, the confined effects on glass transition temperature and physical aging in polystyrene, poly(methyl methacrylate) (PMMA), and poly(2-vinyl pyridine) (P2VP) nanocomposites with silica or alumina nanospheres have been studied using various fluorescence of dyes (pyrene, 1,10-bis(1-pyrene) decane (BPD) or 4-tricyanovinyljulolidene) (TCJ) [32]. The size of nanoparticles along with release kinetics of drugs may be determined by observing their fluorescence and measuring their intensity and breadth using fluorescent dyes bound to their surface.

Particles may be labeled with a fluorescent probe or with fluorescent chemicals that are naturally present in polymer. The excitation light is absorbed by fluorescent molecules, and their subsequent longer-wavelength emission is measured. Several variables including nanoparticles' dimensions and form, kind of fluorescent molecules, and surrounding medium affect spectrum of polymeric nanoparticles. Nanoparticles' emission spectra, for instance, might expand or shift to longer wavelengths as their sizes grow. The presence of charged groups or hydrophobic domains may either suppress or boost the fluorescence emission on the particle's surface [29].

3.11 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) is an approach of determining atomic composition as well structure of surface by analyzing the resulting signals from electron beam's collision with sample [33]. Using secondary and backscattered ions from sample, SEM generates a 3D image of substance under study. After escaping the surface of sample, these errant electrons are often detected using an Everhart–Thornley scintillator-photomultiplier detector. Many natural PNPs cannot be seen under an electron microscope because of their inability to deflect an electron beam. Therefore, a thin metal film (200–300 Å) must be coated onto the sample during preparation in order to form a conductive layer. This technique eliminates surface charging, dampens the effect of temperature changes and strengthens the extraneous

electron output necessary for SEM. A tungsten filament with thin tip of 10 nm may discharge high-energy electrons when placed in electric field, paving the way for low-voltage SEM [34]. This improves both proportion of signal to noise and spatial accuracy approach. Moreover, size, shape, and distribution of PNPs may all be learned with great precision and clarity using SEM. The purity and aggregation of a PNP sample may be inferred from its SEM pictures. The destructive nature of SEM sample preparation is its downside. Furthermore, it is always uncertain whether or not the perceived image is really indicative of the bulk sample in the case of heterogeneous mixtures of nanoparticles.

3.12 Transmission Electron Microscopy (TEM)

The shape and dimensions of PNPs may be investigated by employing microscopy methods like transmission electron microscopy (TEM). In TEM, a 2D image is created when electrons go through the sample [35]. Since the TEM can distinguish between nanocapsules and nanospheres and measure nanocapsule wall thickness [36]. Nanocapsules are spherical in form and manufactured by enclosing an oily core in a thin (about 5 nm) polymeric envelope, whereas nanospheres are spherical and constructed of a solid polymeric structure. TEM is powerful instrument for researching nanocarriers as it can measure the nanocapsules copolymeric layer with nanoscale precision. For instance, Guinebretière et al. [37] produced PCL nanocapsules by an diffusion method, and their TEM measurements indicated a membrane thickness of 1–2 nm. The optimal thickness of specimen for TEM is just 100 nm is one of its limitations. There is a risk of localized overheating and subsequent structural damage in organic samples that absorb kinetic energy. To get around this problem, scientists have developed a method called cryo-TEM, which studies nanoparticle structural arrangement in frozen condition with few adjustments to material [38].

3.13 Atomic Force Microscopy (AFM)

Atomic force microscopy (AFM) commonly referred as scanned probe microscopy (SPM), for characterizing PNPs. Since its inception by Binnig et al., AFM remain invaluable procedure for nanometer-scale surface morphology research and sensitive force measurements. Examples of possible uses besides imaging include the determination of force-driving colloidal dynamics and improved comprehension multiple molecular-level biological phenomena. It provides nanometric-scale, three-dimensional data with a resolution comparable to that of an atomic microscope. This strategy is applied to investigate intricate surface topography of nanomaterials, with sectional examination revealing minute holes and pores [12, 39]. AFM imaging involves the measurement of attraction and repellent forces of an edge probe tip and object's surface [40]. The applied force is detected by anchoring sensor with flexible

cantilever and observing its deflection via laser-photodiode system that hat compares the resulting voltages. A piezoelectric scanner is what really does the scanning, but it also manages where the sample is and how far it moves in relation to the tip. The two most prevalent forms of AFM imaging are contact and striking mode. In former, a horizontal scan is performed at fixed height over the sample, and the cantilever's deflection is recorded. On the other hand, tapping mode has the cantilever gently "tapping" on the surface while scanning at its resonance frequency. Size, shape, and aggregation may all be studied in PNPs using AFM, just as they can with SEM. Since AFM is a non-destructive technique, it may be used on many different substrates without the need for extensive sample preparation. Even so, the concentration, aggregation, and cleanliness of a sample are all impacted by how well it is prepared for examination. PNPs' sizes might be altered, if water evaporated during AFM sample preparation. Nanoparticle coalescence may also be explained by the mica substrate's hydrophilicity, which allows it to retain trace quantities of water even after evaporation, potentially leading to aggregation. Lyophilization on the mica wafer might provide protection against this. The ability to examine biomolecules while immersed in aqueous solutions is only one of the many benefits of AFM. The lateral dimensions of PNPs are often overestimated by AFM because tip is typically bigger over particulates being probed. The height (z-axis) alone should be relied upon for the correct size; hence, care must be used while employing the side measurements. Although AFM is more precise across z-axis, SEM offers superior range of focus. AFM resolution comparable to that of SEM is far more user-friendly, takes up much less room in the lab, and can be purchased for a fraction of the price. Despite their apparent differences, SEM and AFM may have certain similarities. Both methods have lateral resolutions of 5–100 nm and provide images with artifacts. The benefits of one approach could potentially make up for another when utilized in tandem. In order to investigate the surface topology of PLA-Tween 80-10 PNPs, Zhang et al. [12] employed FESEM and AFM (Fig. 3.1) that resulted in uniformity in size and shape of PNPs.

3.14 X-Ray Photoelectron Microscopy (XPS)

The chemical composition of material's surface may be examined by X-ray photoelectron spectroscopy (XPS). Kinetic energy from freed electrons is calculated using Einstein's photoelectric effect and X-ray illumination. The energy of core electrons emitted by an element is one of its defining characteristics and may be used to infer the number of elements and the nature of their chemical bonds. This method also goes by the name of electron spectroscopy for chemical analysis (XPS). The average path of motion of photoelectron is extremely brief, ranging from several to a few nanometers (10 nm), since photoelectron is generated within solid material that may interact with other electrons and be dispersed. As a result, it is often used for qualitative as well as quantitative evaluations of the surface's chemistry [41]. This approach may be utilized for analysis without jeopardizing the safety of the examined materials

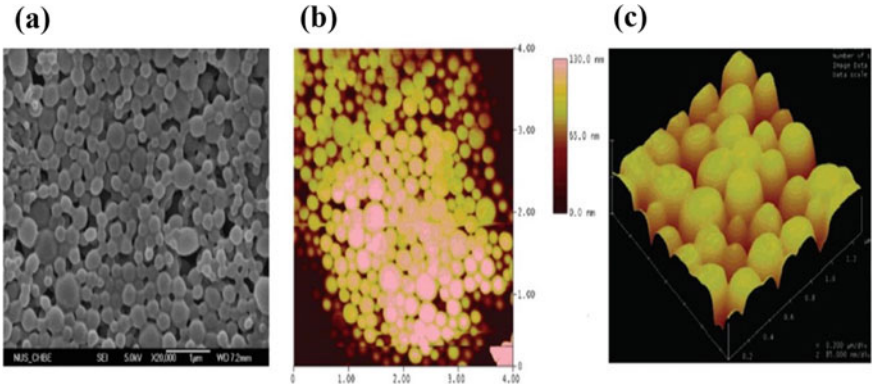

Fig. 3.1 **a** FESEM **b** AFM 2D image and **c** 3D image of PLA-Tween 80-10 PNPs. Reproduced with permission from Ref. [12] Copyright 2017, Elsevier

since it is spectroscopic in nature. XPS examination might be useful for studying solid samples like catalysts, semiconductors, and surface-modified regions as thin films and coatings [42].

3.15 Particle-Sizer

Drug delivery nanoparticles should be of a size (100 nm in diameter) that prevents rapid clearance by the kidneys and blood vessels but is small enough to be removed by mononuclear phagocyte system (MPS) [43]. Laser scattering (dynamic or static light scattering, laser diffraction), field flow fractionation (FFF), electron microscopy (EM), centrifugation (analytical ultracentrifugation, and centrifugal particle sedimentation), tunable resistive pulse sensing (TRPS), and particle tracking analysis (PTA) are just some of the methods that could be employed to determine average diameter and size distribution of nanoparticles [1]. The most commonly used approach for determining size is dynamic light scattering (DLS), but new techniques are constantly being developed [44]. Despite its low resolution, DLS is ideal for a preliminary nanoparticle screening in which sample integrity and stability may be assessed. Complex biological media may be imaged in terms of particle dimension and diameter, although doing so often requires a series of high-resolution measurements. Depending on the biomaterial, agglomeration development or varied average dimensions of particles in reaction may have profound effects on cellular absorption, distribution, toxicity, or destiny. Nanoparticles' pharmacokinetic profiles are influenced by their sizes due to the fact that these factors determine where the particles end up in tissue compartments and how they interact with different biological intermediates and receptors. For instance, renal clearance and non-specific uptake by MPS are both affected by particle size. The opsonization of NPs is influenced by their size

and surface chemistry due to the bending of systems [45]. Particle size impacts how they travel and stick together in the respiratory, digestive, and circulatory systems. Nanoparticles (<100 nm) depart blood vessels by endothelial fenestrations, whereas microparticles are either absorbed by Kupffer cells in hepatocytes or get trapped in capillary beds. Furthermore, nanoparticles smaller than 200 nm may be internalized by clathrin-mediated pathway, whereas nanoparticles bigger than 500 nm may be internalized via the caveolae-mediated system [46, 47].

3.16 Z-Potential

The surface charge and stability of how much aggregation will happen over time of a PNP colloidal solution are often assessed by ζ-potential analysis. Further, it is important variable in detecting ultimate destination of PNP in vivo particularly via interactions with cell membranes. Standard electrophoresis measurements of ζ-potential of suspended fragments are used to calculate the particle surface charge [48]. Electrophoretic mobility is measurement of trajectory of charged particles in response to an applied electrical voltage. Henry's equation establishes a connection between ζ-potential and electrophoretic mobility (μ):

$$\mu = 2\zeta\varepsilon/3\eta_0 * f(kr)$$

Henry's function is denoted by $f(kr)$, ε, dielectric constant, and medium's viscosity by η_0. The stability of a colloidal solution may be quantified by its ζ-potential. Instability, accumulation, coagulation, and flocculation are all conditions indicated by values between -30 and $+30$ mV [20]. Therefore, for large ζ values, electric repulsion makes particle aggregation less probable. The effect of having one or two charged groups on polymers on final size of nanoprecipitated PLGA PNPs was investigated in an intriguing study by Reisch et al. [49]. The authors dropped it from more than 100 to less than 25 nm.

Measurements of ζ-potential showed that numerous charged end groups had been successfully introduced onto the polymer chain. Negative (40 mV) values were observed for PLGA with carboxy and sulfonate end groups, whereas positive (+15 mV) values were for PLGA-NMe3. Surfactants may be used for both electrostatic and steric stabilization of PNPs in aqueous solutions. There are a number of variables that may affect stability and by extension ζ-potential including pH, ionic concentration and strength, alongside types of surface ligands [20]. An increase in potential often results in a rise in PNP uptake. The disintegration of functional structures on surface, adhesion of ionic substances in hydrophilic dissipating media, and solvation effect all have an impact on zeta potential, which in turn indicates surface charge of particles [50]. The electrophoretic mobility in specific solvent measures their zeta potential through Doppler methods, which detect acceleration of particles as voltage indicator. It may be affected by presence of phospholipids, poloxamers, and polymers considered as primary building blocks of PNPs. Although

substantial repulsion effects avert consolidation owing to infrequent contacts among nearby nanomaterials, a reasonably high zeta potential value (|±30 mV|), is required for excellent physicochemical integrity of suspended colloids [51]. Drug association with nanoparticles may be better understood if their zeta potentials are known. Albumin loading into nanospheres made of chitosan and diblock hybrid polymers of ethylene oxide and propylene oxide (PEO-PPO) was reported to be well understood owing to zeta potential by Calvao et al. [52]. He also measured how zeta potential values vary depending on formulation. Thus, NPs' zeta potential may be modified for given use by coating them with surfactant or other materials, such as polyethyleneglicol (PEG) of various molecular weights [53].

3.17 Conclusion

Nanotechnology is an essential field with many potential uses, including environmental remediation, energy generation, and a plethora of other fields. Polymeric nanoparticles, the subject of intense study at moment, are the foundational elements for the development of cutting-edge technology. Polymers and nanostructures may be characterized based on their structure, morphology, and chemistry. For both the controlled production and practical application, an understanding of characterization is essential. Polymeric composition may be predicted with use of data acquired through characterization methods. These methods are useful and nanotechnology's bright future is due to innovative spirit with which it approaches its work; on the other hand, it is constrained by factors including the need for improved methods of materials description. It is vital to integrate several approaches to have a thorough understanding of particles and their characteristics. A better comprehension of selecting assays effective for characterizing materials is provided by brief explanations of each technique, along with their merits and downsides of each.

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Chapter 4
Bovine Mastitis

Abstract Mastitis is a swelling of mammary gland distinguished by tangible, chemical, and typically bacteriological variations in milk and pathological modifications in glandular system in reaction to trauma for the objective of depleting and eliminating the pathogenic agents which triggered the inflammation. Many other kinds of injuries may result in inflammation, involving exposure to chemical irritants, physical trauma, or pathogenic pathogens and their toxins. Milk from cow with bovine mastitis has a somatic cell count (SCC) of more than 2×10^5 cells per ml, and bacteria that might cause mastitis have been identified from milk on many occasions. Besides viral agents, pharmacological, natural, or cognitive stimuli may also trigger aggravation of the mammary glands. Mastitis pathogens are classified as either communicable or environmental, depending on their respective epidemiologies. While the infected mammary is the principal repository for infectious microorganisms, an insalubrious atmosphere is the principal reservoir for environmental mastitis (Hillerton and Berry in J Appl Microbiol 98:1250–1255, 2005). The bacteria *Streptococcus agalactiae, Staphylococcus aureus* subsp. *aureus*, and *Mycoplasma* spp. are all examples of common infectious diseases. In the atmosphere, *streptococci* other than *S. agalactiae*, as *Streptococcus uberis*, *enterococci*, and coagulase-negative *staphylococci* (CNS) are common illnesses. Treatment of mastitis produced by *streptococci* and penicillin-susceptible *staphylococci* with a ß-lactam antibiotic, especially penicillin G, is the first line of defense. Because of the risk of fostering the development of wide-spectrum ß-lactam resistance, first-line treatment options for mastitis should not include wide-spectrum antibiotics like third- or fourth-generation cephalosporins. In acute mastitis caused by *S. aureus* and in acute instances of coliform mastitis, systemic therapy is advised, ideally in conjunction with IMM medication. Effective medication and management of mastitis are greatly aided by early, accurate diagnosis. Management of mastitis outside of the lactation period, removal of persistently infected livestock, good management, and cleanliness are the key control strategies.

Keywords Mastitis · ß-lactam · SCC · Epidemiologies · Cephalosporins · *S. aureus*

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4.1 Introduction

The dairy cattle [1] has striven been considered presumably nature's supreme meal [2], due to its provenance that milk contains virtually every nourishment essential for a neonate (i.e., it's rich in carbohydrate, amino acids, calories, vitamins, minerals, digestive enzymes, and flourishing elements) and is therefore universally consumed [3]. Insufficient supervision, little inherited prospective, malnutrition, poor reproductive efficiency, and mastitis are just some of the major challenges to economic milk yield in dairy cows [4]. Mammary inflammation comes through the Greek words mammae (breast) and mastos (inflammation) [5]. In accordance with National Mastitis Council's (1996) present understanding of bovine mastitis, mastitis is a swelling of mammary gland distinguished by tangible, chemical, and typically bacteriological variations in milk and pathological modifications in glandular system in reaction to trauma for the objective of depleting and eliminating the pathogenic agents which triggered the inflammation. Many other kinds of injuries may result in inflammation, involving exposure to chemical irritants, physical trauma, or pathogenic pathogens and their toxins [6]. Crossbred cows are more prone to mastitis than indigenous livestock and it's probable that the milk the standard characteristic differs among managed and unstructured dairy farms due to vast disparities in handling practices. While it is customary in Ethiopia to utilize a calf to encourage milk production, the National Mastitis Council (NMC) warns that calves may potentially serve as a vector for the spread of mastitis [7].

The majority of global population relies on milk as regular source of nutrition; nevertheless, due to its substantial bacterial load and suitability as a substrate for bacterial progression, raw milk constitutes a public health danger once ingested. Since, 1917 [8], researchers have been keeping a close eye on cattle mastitis, a productivity disease with several etiologies. Disease of the mammary glands, known as mastitis [9] in its many forms (including subclinical to clinical mastitis), affects dairy cattle across the globe. It has several etiological maladies, although bacteria are often thought of being the root of the problem when milk output drops. Mastitis is predicted to cause a yearly economic loss of US$ 200 per cattle, according to research by Costello [10]. According to studies, [11] yearly financial losses in India owing to asymptomatic and clinical mastitis are US$ 98,228 million, or 7165.51 crore Indian currency.

Mastitis has been documented in a wide variety of animal breeds; however, it frequently occurs in humans and farmed milking animals. Milk from cow with bovine mastitis has a somatic cell count (SCC) of more than 2×10^5 cells per ml, and bacteria that might cause mastitis have been identified from milk on many occasions [12, 13]. Besides viral agents, pharmacological, natural, or cognitive stimuli may also trigger aggravation of the mammary glands. As previously discussed [14, 15], mastitis is complex illness that may appear clinically or subclinically and is well-acknowledged as a significant source of economic reductions in the dairy industry.

Non-infectious origins of glandular inflammation include chemical, natural, and agonizing events. When present, infectious mastitis reduces milk supply and causes

alterations in milk chemistry, making it a significant illness in the species deemed susceptible to it. For instance, mastitis in humans is linked to premature weaning because of corresponding indications which can in turn induce stunted infant growth [16]. Conversely, mastitis in milking cows is linked to decreased milk production, altered milk formulation, and decreased milk standard [1, 17] additionally, acute clinical mastitis in pigs is related to decreased piglet development and mortality. [18]. Infectious mastitis, often known as mastitis, is a prevalent bacterial illness of breast that is typically diagnosed in nursing mothers. Abnormal milk discharges regardless of topical or systemic indications of inflammation are also diagnostic of clinical mastitis. The prevalence of subclinical mastitis in milking cows may be higher than among different species. Indications of inflammation are assessed as part of milk value and production enhancement efforts, and therefore this could be particularly relevant in commercialized dairy production facilities. Although there are fewer instances of subclinical mastitis in various animals, around 45% of females in certain human groups may have subclinical intramammary infection (IMI) [16].

The financial expense due to these illnesses is connected to both primary and secondary costs. Treatment expenses, wasted milk, herdsman hours, livestock mortality, and expenditures attributed to recurrent episodes of mastitis are all examples of direct losses. Indirect effects encompass things like lower milk output and competence, more culling, lower rates, premature drying out, animal care concerns, and health issues [19]. Milk production drops by 30% each quadrant in mastitis-affected dairy cattle, which may amount to a loss of 15% per milking cattle per lactation [5]. This makes mastitis particularly significant issue impacting the global dairy sector. To this day, mastitis remains the most costly illness affecting dairy cattle, responsible for 38% of total direct expenditures associated with the most frequent production disorders [20]. Losses due to medication, culling, mortality, and reduced milk output from clinical mastitis are extremely arduous to evaluate. Recent studies have put the approximate cost of treating clinical mastitis at about £175 [21]. Clinical mastitis among the UK's dairy herds affects the industry almost £168 million per year, anticipating a mean incidence of 40 cases per 100 cows per year. A recent research found that mastitis caused the deaths of 0.6% of milking cows annually [22]. Losses caused by mastitis are expected to cost the global economy over $35 billion annually. Milk production deficits attributable to subclinical mastitis and greater cow replacement expenses due to elevated somatic cell counts (SCCs) have been projected to add another $960 million to the yearly expenses of mastitis in the USA [23]. In the USA and California, average value of treating clinical mastitis is $107 and $200, accordingly [24]. The mean yearly expense of subclinical mastitis in Scottish dairy farms was 100 GBP/cow [25], due to substantial bulk tank SCC, whereas in UK and Netherlands, the mean yearly earnings loss was projected to be between 42 and 84% GBP/cow [26] and about 59% EUR/cow [27].

The least prevalent reason of mortality in mature milking cows is mastitis [20]. Somatic cell count (SCC) beyond 200,000 cells/mL is inversely proportional to yield, with a 2.5% drop in production for every 100,000 cells/mL rise in SCC, as shown by [15]. The advantages of keeping SCCs around 90,000 cells/mL have been established by other researchers [28]. Furthermore, it has been established that

both overt and covert cases of mastitis possess a negative impact on future fertility [29]. The public health significance of mastitis shouldn't be overlooked just because of its monetary repercussions. The widespread adoption of antibiotics for mastitis cure and management may have consequences for human wellness by increasing the likelihood that antibiotic-resistant isolates of bacteria might emerge and spread within food chain [30]. Whereas, zoonotic pathogen transmission via milk is very uncommon in modern period due to widespread pasteurization, it does exist, particularly in growing unpasteurized dairy food sector and in event of a pasteurization abandonment.

Infectious pathogens during milking are often thought to be the primary cause of mastitis. Pathogenic microorganisms are one form of infectious entity that is often present in the atmosphere of dairy cattle and poses a risk toward milk production of dairy cattle. Furthermore, milk output might drop by 10–12% per cow every lactation for every infected quarter [5]. The economic consequences of bovine mastitis stem from the disease's effect on milk quality and production [19, 31], which seems to differ with each suspected pathogen. Both primary and secondary costs have a significant influence on the economy when a disease spreads. Milk spoilage owing to bacterial contamination, the usage of antibiotics in therapy, or visual adulteration, as well as treatment costs, are direct expenses. Farmers often fail to account for indirect costs since they are not obvious [19, 32–34].

Bovine mastitis is an ubiquitous illness that cannot be removed fully, unlike infections like foot-and-mouth that may be obliterated from a system by culling and immunization programs. Definitive annihilation is quite improbable due to the diversity of microorganisms capable of causing this illness and widespread nature of such organisms. Therefore, a comprehensive control plan [35] can only be effective if the global distribution of the illness and its causative agents are well-understood.

4.2 Bovine Udder Anatomy

The mammary gland is female-specific gland that developed to produce and secrete milk with the objective of nourishing young. However, natural selection has led toward nursing dairy cow producing milk amounts that much surpass the nutritional needs of the newborn. In comparison with the typical commercial milking cow, which produces about 4 times as much milk, the bovine calf intakes around 11 kg milk at 30 days of age if given gratuitously [36]. Milk contains a wide variety of essential elements, including protein, carbs, lipids, minerals, and vitamins, and is produced in large quantities by maternal gland [37]. The mammary gland has a very specific anatomy because it produces milk in a very specific way. The vascular endothelium plays a crucial role in mammary gland development, growth, and the generation of milk. Endothelial cells in mammary capillaries create a single, tiny layer that acts as a semipermeable impediment, allowing the body to take in oxygen and exhale carbon dioxide while also transferring solutes and biomolecules necessary for cell vitality and metabolism [38, 39]. Endothelial cells also promote abundant milk generation

and release by speeding up the absorption of blood-derived ingredients as amino acids and glucose [40, 41]. Endothelial cells sustain the vessels and underneath milk-producing tissue by directly orchestrating arterial tone, blood mobility, and arterial permeability [39].

During mammogenesis, the earliest phase of mammary gland growth, an intricate epithelial and endothelial structure is established. The capacity of mammary gland to release milk normally has been shown to be impaired when the normal course of mice mammogenesis is interrupted [42]. Mammogenesis coincides with the initial phase of breastfeeding, which itself starts during gestation and continues after birth of subsequent neonate. The formation of cattle mammary endothelium may be deduced from investigations of mammogenesis in rodents and rats [42–45]. Additionally, myoepithelial parts and connective tissue, the breast endothelium produces a thinner layer of simple squamous endothelial cells that contribute to mammary capillaries. Mammary gland angiogenesis is distinctive due to the presence of basketlike capillary networks around clusters of alveoli resembling bronchi [43]. Underneath each alveolus are networks of capillaries that increase the surface region available for gaseous exchange. The endothelium surface is mainly continued with occasional patches of fenestration to promote substitution and maintain a particular barrier. In contrast to the discontinuous endothelium, which enables the unrestricted flow of big solutes and proteins, the perpetual endothelium only permits the interchange of water and incredibly tiny molecules [46–48]. Endothelial cells have fenestrations, or tiny openings, that allow proteins and other minute molecules to flow through. The cattle mammary gland seems to retain a structure analogous to mouse and human mammary endothelium [49, 50]. This endothelium forms a semipermeable barrier throughout growth and is mostly consistent with some regions of fenestration. In initial pregnancy, endothelial cells in mice models develop microvilli and peripheral folds, increasing their surface region and facilitating the passage of more fluid [49]. The development of microvilli and peripheral folds, small fenestrations, and basket-like capillary networks around alveoli provide convincing indication that breast structure is crucial to proper mammary function (Fig. 4.1; Table 4.1).

The mammary gland acquires the ability to produce milk via a process known as lactogenesis. It progresses in two phases. The gland develops the ability to release milk, tiny blood, and urine about the time of mid-pregnancy [52]. The first step of lactogenesis [53] describes this development. The second stage of lactogenesis, marked by the production of profuse milk secretions, begins around the fourth postnatal day. The "coming in" of milk refers toward significant volume rise that occurs about 40 h postpartum. If milk is regularly drained from the mammary gland, the milk production may be maintained for a longer period of time. Maintaining milk production requires prolactin, while let-down, the process by which babies remove milk from the gland, is mediated by oxytocin. The referred to as "feedback inhibitor of lactation" [54] is a local substance released into milk that may regulate milk production to meet the needs of the newborn.

Regeneration of cattle mammary tissues during involution is necessary for maximum milk yield in subsequent lactation [55]. Consistently milking dairy cattle reduced production by around 75% relative to the same cow provided a 60-day

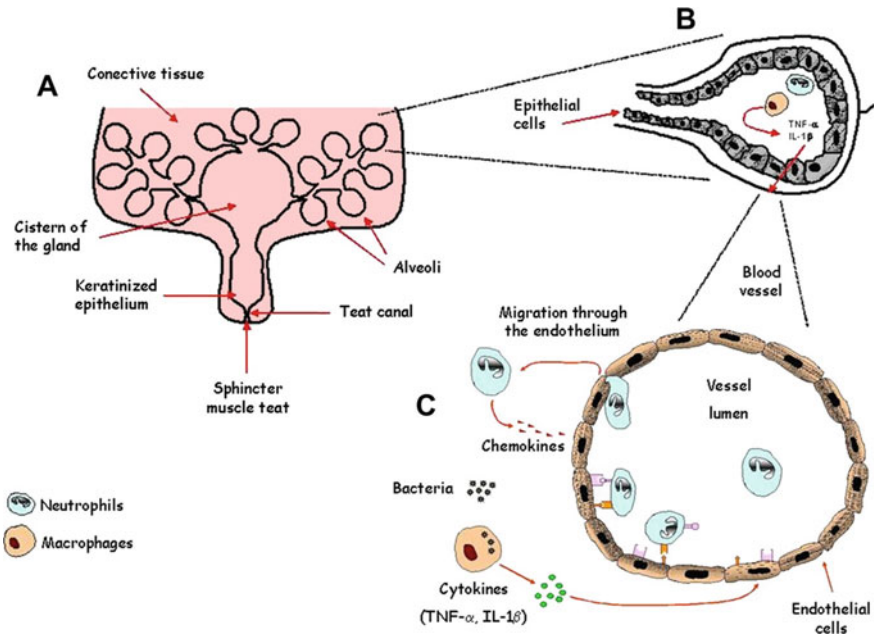

Fig. 4.1 **a** Schematic diagram of the bovine mammary gland showing the most important anatomic factors that act as defense barriers. The teat sphincter muscle represents the first line of defense, whereas the keratinized epithelium of the teat cistern is considered the second line. **b** Cellular and soluble factors that participate in the innate immune response of the mammary gland. Macrophages located in the alveoli phagocytize bacteria that enter the mammary gland cistern. Activated macrophages release cytokines such as TNF-α and IL-1ß. **c** Endothelial cells from blood vessels adjacent to alveoli express adhesion molecules in response to pro-inflammatory cytokines; this, in turn, facilitates neutrophil recruitment from the bloodstream to the site of infection in order to eliminate the invading bacteria

dry interval before calving [56]. Loss of cellular complexity, reduced cell quantity, and capacity to produce milk are all symptoms of involution that have been seen in several species [55, 57, 58]. It has been shown that epithelial cells undergo rapid apoptosis after milking has stopped in mammary tissue of mice, goats, and dairy cows [59]. In contrast, numerous bovine investigations show minor epithelial cell lost during development despite a significant reduction in alveolar lumen space [55]. Nonetheless, earlier studies in nursing goats show that milk buildup in the mammary gland may trigger activation of epithelial cell death [60]. Although it is unclear if bovine capillaries regress during involution, variations in endothelium following mouse and sheep development may be utilized to extrapolate modifications in cattle. The capillary integrity of the involuting murine mammary gland drops to levels seen in the first trimester [61]. It has been hypothesized from studies conducted on sheep that endothelial apoptosis contributes to a reduction in mammary capillary density. Endothelial apoptosis and the existence of apoptotic structures inside the

Table 4.1 Cytokines associated with the immune response of bovine mammary gland infected with *Escherichia coli* or *Staphylococcus aureus*

Cytokine	Source	Function	Type of mastitis and pathogen bacterium
IL-1ß	Macrophages and epithelial cells	Neutrophil recruitment to the mammary gland	Clinical by *E. coli*
IL-2	$CD4^+$ lymphocytes	Induce growing and differentiation of B lymphocytes activates NK cells	Subclinical by *S. aureus*
		Activates $CD8^+$ lymphocytes	ND
IL-6	Macrophages	Regulates acute phase protein synthesis	Clinical by *E. coli*
		Favors the influx of monocytes to the mammary gland	
IL-8	Monocytes, T lymphocytes, macrophages, epithelial and endothelial cells	Chemokine important in neutrophil recruitment to the mammary gland	Clinical by *E. coli*
			Subclinical by *S. aureus*
IL-12	Dendritic cells and T lymphocytes	Regulates differentiation of T lymphocytes	ND
IFN-γ	$CD4^+$ and $CD8^+$ lymphocytes, and NK cells	Activates T lymphocytes	ND
		Induces production of IL-12 Mediates activation of neutrophils	
TNF-α	Macrophages, neutrophils, and epithelial cells	Induces expression of adhesion molecules in endothelial cells	Clinical by *E. coli*

Interleukin (IL; IL-1ß, IL-2, IL-6, IL-8, IL-12); Interferon gamma (IFN-γ); Tumor necrosis factor alpha (TNF-α). ND, not determined

cytoplasm of otherwise viable endothelial cells were both reported during progression of the ovine breast gland [62]. Remarkably, in mouse studies of involution, epithelial cell death occurred first, followed by endothelial cell death. In the mouse mammary gland, capillary shrinkage was seen in regardless of apoptotic endothelial cells, suggesting that apoptosis does not constitute the main cause of reduced vascular networks [57]. When milk production quits, or in most however not all species, when prolactin is removed, involution ensues in the lactation gland. Milk production stops, the mammary epithelium undergoes apoptosis [63], and gland undergoes particular modifications to return to its pre-pregnancy form; these processes follow a predictable pattern, similar to lactogenesis.

4.3 Bacteria Classification and Mastitis Epidemiology

Mastitis pathogens are classified as either communicable or environmental, depending on their respective epidemiologies. While the infected mammary is the principal repository for infectious microorganisms, an insalubrious atmosphere is the principal reservoir for environmental mastitis [1]. The bacteria *Streptococcus agalactiae, Staphylococcus aureus* subsp. *Aureus*, and *Mycoplasma* spp. are all examples of common infectious diseases. In the atmosphere, *streptococci* other than *S. agalactiae* as *Streptococcus uberis*, *enterococci*, and coagulase-negative *staphylococci* (CNS) are common illnesses [64]. The majority of illnesses originate by *Staphylococci, Streptococci, and Enterobacteriaceae* [5]; however, over 140 distinct microbes have been identified from bovine intramammary diseases. *Staphylococcus aureus* (*S. aureus*) is the main bacterium in bovine mastitis; however, *Streptococcus* species including *S. agalactiae* and *S. dysgalactiae* are also common causes. Relying on the infecting isolates, bovine mastitis may manifest as acute suppurative, gangrenous, or chronic mastitis. Clinical, or overt, and subclinical, or concealed, types of Mastitis are both possible [5]. Subclinical mastitis is 15–40 times more typical than clinical and results in substantial economic losses for the vast majority of dairy farms [65]. The specific pathogens responsible for disease's manifestation are well-documented. Many other types of bacteria may cause clinical mastitis, although *S. uberis, Escherichia coli, Klebsiella* spp., *Pseudomonas aeruginosa,* and pyogenic microbes are the most common suspects [64]. Conversely, *S. agalactiae, CNS,* and *Enterococcus* spp. are linked to asymptomatic mastitis [66]. However, *S. aureus* has been identified as a causal agent of both overt and covert forms of mastitis [65]. Bovine mastitis is more common on farms with greater number of cows, according to a recent study [67]. Prevalence is greater in initial lactation compared to mid-lactation and in animals with wounds and tick infestations around udder and teats compared to cows without these characteristics [68]. The frequency of subclinical Mastitis varies from 21.1 to 86.2%, according to studies done in various countries throughout the globe [69, 70] (Fig. 4.2).

4.4 Etiology of Mastitis

Subclinical mastitis is most often caused by coagulase-negative and positive *staphylococci,* environmental *streptococci,* and coliforms in nursing cows [72]. Prenatally and postnatally, these bacteria are responsible for IMI in heifers. The occurrence of IMI in cow mammary glands before and after giving birth varies greatly across investigations. Prevalence of IMI following delivery varied widely, from 13.3% to more than 57.0%. Despite being the leading source of IMI in milking cattle, coagulase-negative *staphylococci* (CNS) have been characterized as opportunistic mastitis pathogenic organisms, resident colonizers on teat surface, and seldom-producing clinical mastitis [73]. Pre- and postoperative, CNS constituted most common etiology

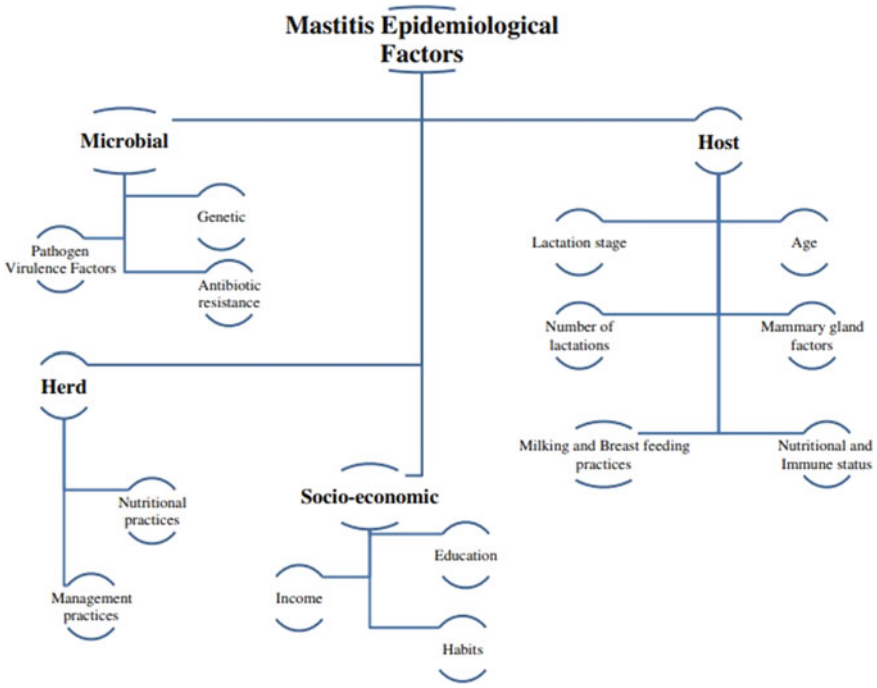

Fig. 4.2 Epidemiological factors influencing mastitis etiology and pathophysiology. Reproduced with permission from Ref. [71]. Copyright 2011, Springer Science Business Media, LLC

of IMI in heifers. The median prevalence of subclinical CNS IMI before and after delivery, respectively, was 31.1% and 27.9%. *S. aureus* is the most common causative organism of mastitis in CPS. CPS mastitis and *S. aureus* mastitis are commonly used indiscriminately due to high degree of similarity between the two conditions. Numerous CPS exist, however, and they're not all related to *S. aureus*. According to Roberson et al. [74], *S. aureus* accounted for 98% of CPS inducing IMI in multiparous cattle in farms with a greater frequency of CPS, whereas *Staphylococcus hyicus* accounted for just 2%. *S. aureus* accounted for 61% in multiparous cattle in less prevalence groups, *S. hyicus* for 38%, and *S. intermedius* for 1%. Herds having a higher than 6% prevalence of CPS IMI were considered to have a greater prevalence. Subclinical *S. aureus* IMI was less common in heifers after giving birth in less prevalence populations (61% vs. 69%) [74]. The fact that multiparous animals with a higher prevalence of *S. aureus* had a higher ratio of *S. aureus* to *S. hyicus* than primiparous populations with a high prevalence of *S. aureus* demonstrates the infectious character of *S. aureus*. Milking period in multiparous cows is likely the primary time this chemical is spread. Particularly for heifers, CPS linked with mastitis tends to be *S. hyicus* in low prevalent herds than in high, when milking time cleanliness and other control techniques may have been used. Prepartum CPS IMI in heifers was anywhere from 0.4 to 14.9%, whereas parturition IMI was anywhere from 0.6 to

8.0%. CPS IMI seems to be more common in heifers prior calving than thereafter, since the observed IMI prevalence span is narrower after calving than prior. In addition, there were more investigations revealing a CPS IMI below 1.0% before delivery than after. The average prevalence of CPS IMI before pregnancy was 3.2%, whereas it was 2.8% after delivery. Compared to CPS, the average prevalence of IMI due to environmental infections was 5.7% and 5.6% before and after delivery, respectively. Significantly infectious mastitis pathogens include *S. aureus, S agalactiae*, and *Mycoplasma.* These infectious mastitis germs may quickly propagate to many cows, leading to a significant increase in bulk tank SCC. Strains of *Streptococcus, Staphylococcus*, and environmental "*streps*" (*E. streps*) are common over teat skin and may cause mild cases of mastitis. Bulk tank milk often contains them, and increased numbers may indicate a sanitary issue with the teats or an infected environment. *S. dysgalactiae* is the least frequent streptococcal species in BTC, but many *E. streps* are *S. uberis*. Over 10% of *S. uberis* mastitis patients in one study with an effort to quantify microbial shedding rates exhibited counts among 10^6 and 10^8 cfu/mL [75]. Dairy environments often arbour *E. coli, Klebsiella, and Serratia* in waste, bedding, and even water sources. They are a potential source of high bacterial numbers and may induce severe mastitis. High levels of these microbes in milk are more frequently the result of contamination from filthy teats or manure. It is not safe to infer that the existence of such microbes in BTC indicates mastitis. While *prototheca* are generally harmless, several species have been linked to mastitis in livestock. They are often linked to the presence of polluted surface water in a dairy setting. Despite its reputation as an environmental mastitis microbe, *Prototheca* may transfer between cow to cow resembling contagious mastitis. *Prototheca* mastitis is often misdiagnosed and may result in either constant, minimal, or periodic shedding of pathogen. The detection of this microorganism in BTC raises the possibility of a widespread herd illness. *Pseudomonas aeruginosa* (*P. aeruginosa*) is unusual, yet may cause serious damage and spread easily. It's quite likely that contaminated sources contributed to its appearance in bulk milk. Water contamination is a common cause of infection for cows. Biofilms of *P. aeruginosa* may form in milking facility and other dairy systems. Pseudomonas spp. are common in dairy ecosystem, although they do not cause any diseases. The abscess-causing microbe *Arcanobacterium pyogenes* is most prevalent in udder, skin, and uterus of cattle. Extremely seldom does mastitis result from yeast. They're often linked to udder care tools that have been tainted with bacteria. Different species of Bacillus are generally harmless to humans. These microbes can survive pasteurization in the laboratory because they are heat-resistant spores. They may develop in dirt residues on stainless steel milking machinery, but they usually get into the milk via skin of contaminated teat. BTC is particularly useful for detecting *S. aureus, S. agalactiae*, and Mycoplasma, the three most common infectious mastitis organisms. Low-level recognition of these infections ought to be a goal of culture techniques used. Large amounts of germs from teat surface and dirt further dilute the bacterial count from contaminated quarters, making diagnosis difficult. To improve sensitivity for identifying such microbes, it is advised that extra sample volume be utilized alongside with selective medium. It has been suggested that using *Staphylococcus*-selective agar, [76] modified Edwards medium, [77], and broth-enhanced

Mycoplasma cultures [78] may increase the efficacy of tests for the presence of such bacteria. Half a plate of selective medium is inoculated with 100–200 L to indicate herd illness at an earlier stage. Using *Prototheca* isolation medium dramatically improves its detectability in BTC [79]. Farmers may still benefit from the testing service even if the BTC only informs the possibility of infectious mastitis pathogens in bulk tank milk according to culture findings of any of the aforementioned selective agars.

4.5 Predisposing Factors and Disease Characteristics

The prevalence of mastitis is affected by several variables, including the quantity of lactating cows, the production phases of cows, herd supervision, environment's humidity and temperature, variations in species, and the milking features. Risk variables in husbandry and surroundings produce mastitis by lowering the cow's regional and systemic shields and defenses and/or boosting the udder's susceptibility to microbes [80].

4.5.1 Environmental Factors

The circumstances as congestion, improper ventilation, insufficient manure expulsion from the exterior of stalls, feeding domains, and physical activity sections, inadequately maintained liberated stalls, possession of farm reservoirs or filthy exercise lots, unhygienic calves pens, and general absence of farm standards and hygiene may increase accessibility [81]. Teat ends are often exposed to environmental infections via contact with settling contents. Variations in pollutants (and hence the accessibility of food), humidity, and temperature all affect the quantity of microbes in settling [82]. Sand or pulverized limestone, two examples of low-moisture inorganic substances, are preferred over minced organic substances. In general, fewer germs may be found in bedding that has been allowed to air dry. Pathogen development is stimulated by higher temperatures and hindered by lower ones [81] (Fig. 4.3).

4.5.2 Host Factors (Cow Factors)

The incidence of bovine mastitis was observed to vary significantly with longevity, parity, and milking phase. The infection rate among adult cows was 93.2%, whereas that among young adults was 65%. Infection rates were highest during initial lactation period (87.2%), followed by the middle lactation phase (65.9%), and finally late lactation period (73.1%). Similarly, cows possessing a calf load of at least five appeared

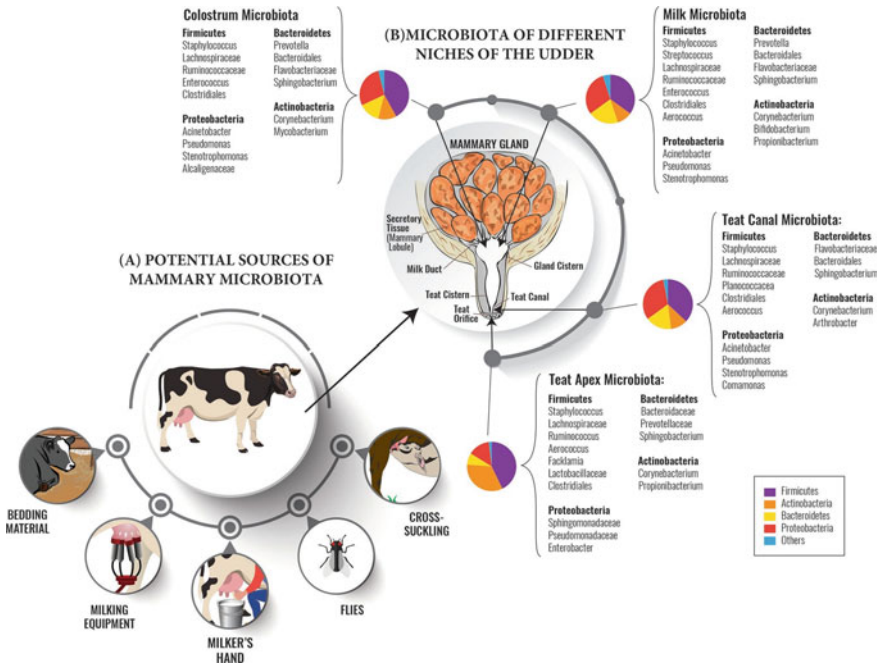

Fig. 4.3 Potential sources and composition of udder microbiota. **a** Environmental sources of microbes contributing to microbiota of various niches of the udder. **b** Microbiota composition of various niches of the udder. Proportions of main bacterial phyla were estimated based on studies that used 16S rRNA gene sequencing to explore udder microbiota. Color version available online. Reproduced with permission from Ref. [83]. 2018 American Dairy Science Association

more vulnerable than those with an average calf load [80]. Cows possessing a pendulous udder are more likely to get mastitis than without one. A microorganism may readily attach with the teat and gain entrance to glandular tissue because of the pendulous udder, making it vulnerable to harm. Cows with teat infections have a higher disease incidence compared to healthy teats. SCM is more common in high-producing cows compared to low- to medium-yielding ones [84]. When contrasted to the lactation interval, the dry duration has a higher incidence of new intramammary illnesses brought on by atmospheric *streptococci* and coli types. The mammary tissue goes through a series of alterations that affect the cow's capacity to resist microbial infection throughout dry phase. Intramammary infections are most common in two weeks following drying off and two weeks leading up to calving. Clinical manifestations of infections obtained during dry periods are common. Sixty-five percent of coliform clinical episodes in initial two months of lactation are due to intramammary complications that began during dry season, according to research. The primary two months following calving, 56% of clinical cases are caused by *streptococcal* infections in dry season [82]. Therefore, the dry season and early lactation should be the primary focus

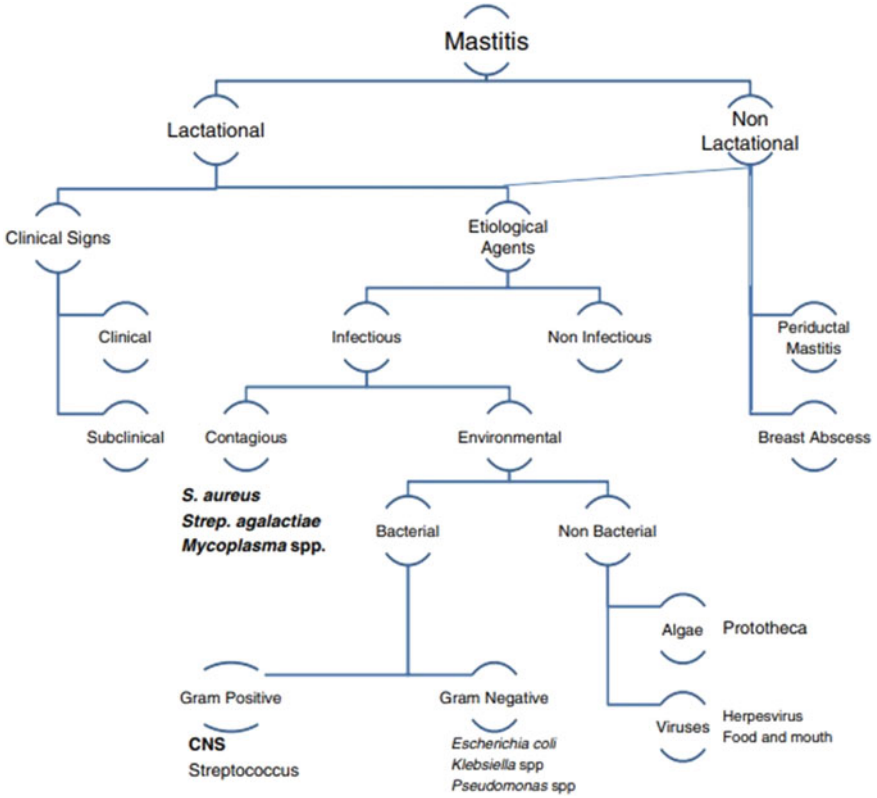

Fig. 4.4 Mastitis classification across species depending on lactation stage, clinical signs, and etiological agent. CNS, coagulase-negative *Staphylococcus.* Reproduced with permission from Ref. [71]. Copyright 2011, Springer Science Business Media, LLC

of livestock management techniques aimed at preventing environmental mastitis [81] (Fig. 4.4).

4.5.3 Agent/Pathogen Factors

Virulence metrics are required for bacterial colonization, multiplication, and persistence in udder. Toxins, bindings, invading forces capsule formation, and resistance to serum complement are all among them [85]. There are three main functions of virulence parameters, including those that shield bacteria from the host's defense system and drugs, those resulting in tissue deterioration, and those which promote bacterial adherence to host cells [86].

4.5.4 Genetic Aspects of Mastitis

There is a hereditary component to milking cow's vulnerability to or resistance against mastitis. The mammary gland's integrity is negatively impacted by selection for higher milk production [87]. Under this way, the illness mostly affects high-production herds under intensive husbandry.

4.5.5 Type of Purpose and Breed of Cows

Worldwide, mastitis is an extremely troublesome issue for dairy farmers. It may show up unexpectedly in any milking herd, particularly among the well-run ones, making every herd vulnerable. Beef cows' udder glands produce the milk their calves rely on for sustenance. Calves normally empty the udder of milking cows many times per day, often as many as a dozen times per day, with no evidence of mechanical injury to their teats or serious illnesses. This is why beef cow herds don't have as stringent mastitis monitoring protocols.

4.5.6 Udder Structure

Infections are more inclined to spread in udders if the lobes have not formed evenly. Long teats, being more vulnerable to injury, are linked with a higher incidence of inflammation. Teats and teat ducts are narrower and shorter in cows having mastitis, whereas the base and circumference of the teats are greater. There's a correlation between the illness and the size of teat canal. Because the muscles responsible for sealing the udder become more rigid as its cross-sectional region becomes bigger, the udder might stay open for a longer period of time post-milking, leaving the animal more vulnerable for illness (Fig. 4.5).

4.5.7 Age of Cows and Stage of Lactation

Cows' age and milking status both constitute significant variables in mastitis development. Milking more often over a longer period of time causes the teat canal to become larger, or the passage may stay inevitably partially open in older animals. Impairment to breast epithelium from prior inflammatory conditions has been related to age, with older cows showing greater susceptibility despite effective therapy. A higher incidence of clinical mastitis postpartum is shown in heifers with contaminated udders before to parturition, by as much as a factor of four compared to heifers from healthy cows. This is likely because oxidative stress rises and antioxidant response

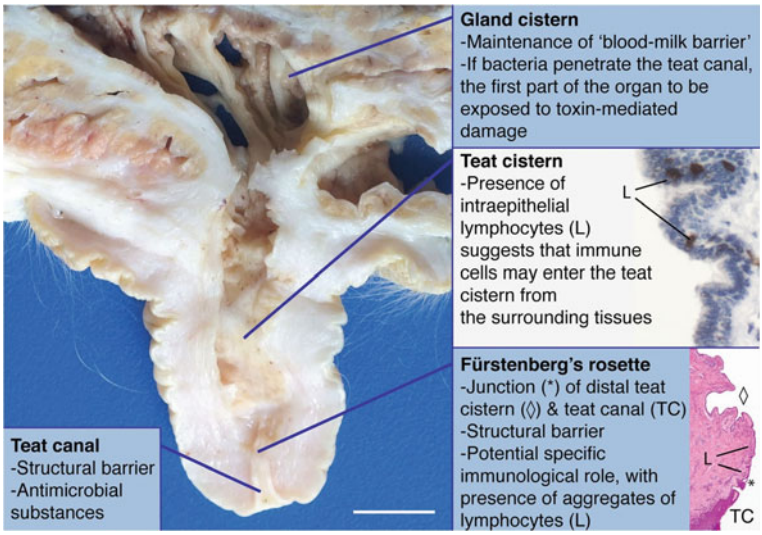

Fig. 4.5 Structural defenses of the bovine mammary gland. Sagittal section through the distal gland cistern, teat cistern, and teat canal of a periparturient Holstein Friesian dairy cow; formalin fixed tissue; scale bar: 10 mm. Teat cistern immunohistochemical inset: Sagittal section through the teat cistern. Scattered intraepithelial lymphocytes expressing CD3 are present multifocally. Immunohistochemical staining for CD3 with hematoxylin counterstain. Fürstenberg's rosette histological inset: Sagittal section through the teat canal (TC)—distal teat cistern (diamond) junction, Fürstenberg's rosette (*) of a Holstein Friesian dairy cow 45 dI. Groupings of small to moderate numbers of lymphocytes (L) are present multifocally. Hematoxylin and eosin stain; dI: days involution, with concurrent pregnancy until abortion at approximately 31dI. Reproduced with permission from Ref. [88]. Copyright © 2018, The Author(s)

systems are less effective just as breastfeeding begins. Milking equipment and cleanliness: Keeping a clean milking area is crucial for preventing the spread of contagious mastitis throughout the herd.

4.5.8 Dry Period

In addition to being a time when mastitis is more likely to develop, the dry phase is regarded to be a crucial time in determining the udder's overall health [89]. Transformation within the udder's glandular structure is the primary physiological and endocrine change that occurs during this time. For a 305-day nursing cycle, experts recommend a dry phase of 40–60 days. Somatic cell count in milk rises when the dry time is eliminated or shortened because the mammary is less able to withstand inflammatory conditions.

4.5.9 Body Condition Score of Animals

Chronic inefficiencies in calories, amino acids, nutrients, or supplements have constantly been linked to elevated disease vulnerability as an indicator of suppressed immune response; high-yielding milk producers typically demonstrate a negative energy equilibrium after birth, which can impact the body's defenses and metabolic rate of individual.

4.5.10 Milk Yield

Herds exhibiting a small bulk milk somatic cell number were more likely to develop acute mastitis during the initial lactation if milk output from the preceding lactation was substantial (305 days) [90]. Milk containing high protein level on day before drying out has been linked to CM developing early in lactation. This might indicate that the udder is receiving more nutrients than usual, which would slow the degeneration of udder tissue.

4.5.11 Hygiene Scoring

The living conditions of dairy cows are crucial to their well-being. The key to ensuring that milking cows have long and productive existence is providing them with a secure and pleasant housing. Several evaluation methodologies quantify dung contamination in various cow body parts to determine the shelter's cleanliness [91]. Low standards of cleanliness may lead to an increase in mastitis and progression of lameness in milking cows. Researchers found a correlation between a hygienic housing, healthy cows, and a small quantity of somatic cells in milk they produced [92]. Environmental mastitis could be affected by decisions made at group level, such as the kind of settling used, the extent with which slurry is removed from alleyways, and the style of housing used.

4.6 Pathogenesis of Disease

An inflamed mammary tissue looks like a normal response to an infection, given that all breast infections enter gland by teat canal. Nevertheless, mastitis progression is considerably nuanced than this, and usually best understood as a three-stage process consisting of invasion, infection, and inflammation. The occurrence of mastitis may

be reduced via careful management, especially through the implementation of excellent hygiene practices, most effectively during the invasion stage of disease's life cycle.

During invasion phase, bacteria go down the teat canal and into the milk. The infected state is characterized by fast pathogen proliferation and tissue invasion inside the breasts. Once a bacteria number has been specified in teat canal, it might multiply and spread down the teat duct and into adjacent mammary tissue, infecting tissue either often or seldom, relying on tissue's sensitivity. Endotoxins are produced when some organisms multiply; for example, coliform mastitis results in significant systemic consequences with very little inflammatory responses.

Clinical mastitis, caused by inflammation in response to infection, manifests at varied rates of udder abnormality and systemic impact, from light to per acute; coarse and asymptomatic milk defects manifest at distinct stages of inflammation. Udder defects include extreme enlargement, elevated body temperature, and sometimes necrosis in the acute and per acute phases, and abscess development and glandular degeneration in chronic phases. Systemic manifestations are caused by inflammatory mediators. A reduction in milk supply, the abundance of inflammatory products, and noticeable variations in milk chemistry are all examples of severe milk aberrations [67] (Fig. 4.6).

An elevated somatic cell number is gold standard for gauging milk integrity and mammary wellness, and it also happens to be the most severe subclinical aberration of milk. Neutrophils (11%), macrophages (66–88%), lymphocytes (10–27%), and epithelial cells (0.7%) of milk somatic cells are all found in normal gland. Inflammatory mastitis is characterized by the presence of neutrophils, which make up over 90% of entire glandular leukocytes. When neutrophils arrive at a location of infection, they engulf and destroy microbes. To eradicate bacteria, neutrophils use an oxygen-dependent destruction process which results in respiratory burst that generates hydroxyl and oxygen radicals. The somatic cell count (sce) in milk from a healthy breastfeeding mammalian gland is below 100,000 cells/mL. Since the quantity of neutrophils increases following intramammary illness while, the amount of glandular discharge decreases, the glandular sec may rise to over 1,000,000 cells/mL of milk. A rapid migratory reaction from neutrophils and their ability to kill bacteria at point of illness are crucially associated with the extent and longevity of mastitis [67].

4.7 Clinical Signs and Symptoms

Various microorganisms produce chronic, subclinical, subacute, acute, and peracute versions of the illness, making clinical distinction between the many etiologies of mastitis challenging. Even in a highly specialized hospital setting and following modifications to account for local variables, the best clinical efficiency that can be achieved is still around 70% [94], which is not precise enough to be clinically helpful.

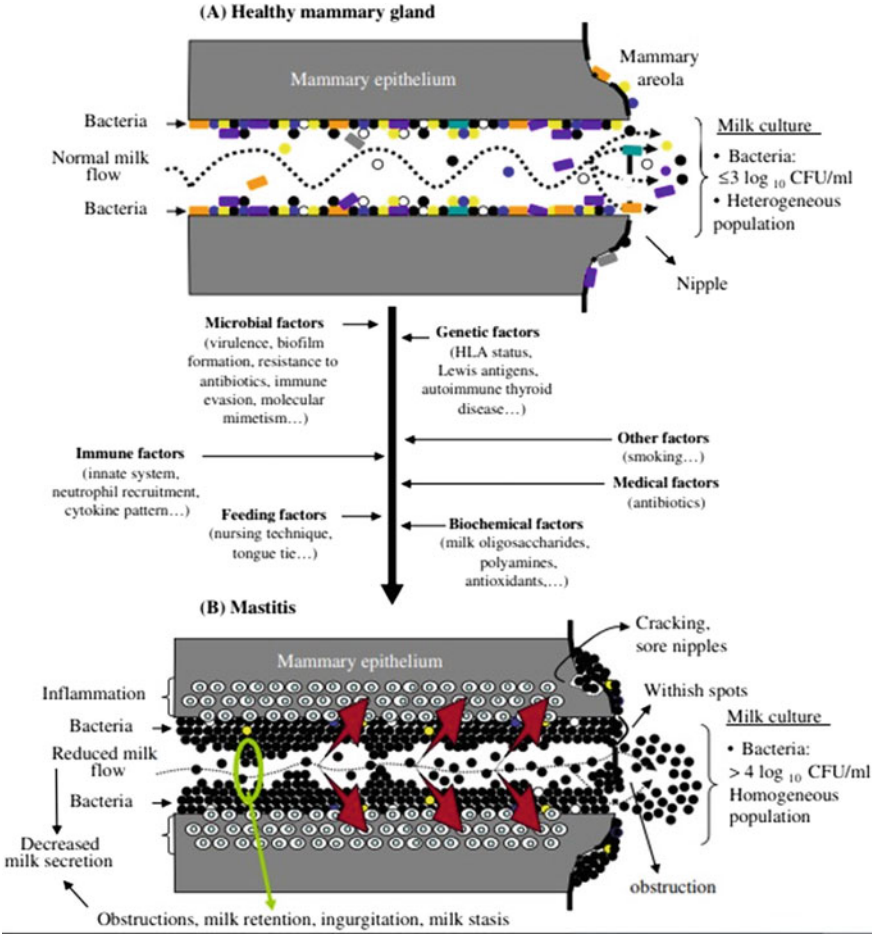

Fig. 4.6 Schematic representation of the etiopathogeny of human mastitis as suggested by the "disbiosis" model [93]; human mammary epithelium in physiological conditions (**a**) and during mastitis (**b**). *Red arrows* indicate the typical burning and/or needle-like pain Reproduced with permission from Ref. [71]. Copyright 2011, Springer Science Business Media, LLC

As such, prior to implementing pathogen-specific therapy, a bacteriological culture of milk specimen from afflicted gland is necessary.

Clinical mastitis episodes are also categorized according to their severity and duration. The findings of a physical assessment alone may diagnose clinical mastitis, and a helpful characterization of the condition is an unfavorable response to the query "would you drink this?" Simply put, "undrinkable" is a straightforward and universal notion for describing clinical mastitis, as cow's milk exhibiting this condition is unfit for human consumption. Clinical mastitis recurrence must be a minimum of 14 days apart from the last episode. Mastitis manifests itself clinically as altered mammary gland production, mammary gland stature, uniformity, and warmth, and

usually a systemic response. According to the aforementioned classification scheme [67], clinical mastitis may be subdivided down into three distinct subtypes: aberrant milk, atypical gland, and aberrant cow (systemic illness).

Evidently anomalous milk is not fit for human consumption. A malignant gland will be noticeably bigger and stiffer than surrounding tissue. Symptoms of an atypical cow include fever, depression, loss of appetite, and reduced milk output. Superior clinical usefulness, universal ease of use, and a solid pathophysiological foundation for therapy are all hallmarks of this three-part classification method. Specifically, it's possible to create ideal treatment regimens for each of the three stages of clinical mastitis. While several classification schemes have been proposed, none compare to the secretion-gland-cow system for their ease of use and applicability.

Clinical mastitis episodes may also be characterized based on how severe they were and how long they sustained.

Depending on the extent, it is classified as either

(a) Peracute, identified by intense inflammation, bruising, heat, and discomfort in quarter
(b) Acute, without an apparent systematic response
(c) Subacute, distinguished by minimal inflammation with enduring anomaly of the milk.

Timeframe is defined as:

Temporary (as in *E. coli* and *Klebsiella* spp.)
Recurrent (as in *S. aureus* and *S. dysgalactiae*).
Permanent (as in *S. agalactiae* and *M. bovis*).

A striped cup with a shining black plate is ideal for examining the milk since it allows for the easy identification of discoloration, blood clots, granules, and foul-smelling material. It is common practice for the herdsman to milk it initially streams upon surface; in particular parlors, black tiles are put on the floor so that the milk may be easily examined for signs of mastitis. As long as the surface is regularly cleaned, it seems safe to continue the routine. Blood stains or wateriness are also possible causes of pigmentation in nursing quarter, with the latter often suggesting chronic mastitis. Slight wateriness in the initial few creeks is not cause for concern, but if it lasts for two or three it is noteworthy. Bovine mastitis endures many unanswered questions, one of which involves how to medicate a cow who has aberrant discharge in its initial two or three milking but then produces milk that looks normal after that. Even if modest and only visible in initial few streams, particles or flakes remain substantial, suggesting a considerable degree of inflammation, which are almost always characterized by discoloration. The presence of blood clots or tiny lumps of wax in milk within initial few days following calving, particularly in heifers, is not indicative of mastitis. Cattle with flakes after milking could have mammary tuberculosis [67].

4.8 Treatment

4.8.1 Effective Clinical Mastitis Medication

Ideally, mastitis therapy would be directed toward the offending bacteria, but in the event of an emergency, treatment would be commenced determined by herd statistics and individual expertise. The correct antibiotic might be chosen with the help of a speedy or on-farm bacteriological diagnostic. Veterinarians possessing experience on farm have to determine treatment regimens and medicine choices [95, 96]. The prudent use of antibiotics might be encouraged by the implementation of documented procedures for mastitis therapy on farm [97, 98]. Herds experiencing infectious mastitis may take use of particular somatic cell number statistics, the California Mastitis Assessment, and bacteriological specimens to track the medication response of cattle. Narrow-spectrum antibacterial agents are often more effective than broad-spectrum ones. Recommendations for the appropriate use of antimicrobials in the cure of mastitis have been created [98]. Treatment of mastitis produced by *streptococci* and penicillin-susceptible *staphylococci* with a ß-lactam antibiotic, especially penicillin G, is the first line of defense. Because of the risk of fostering the development of wide-spectrum ß-lactam resistance, first-line treatment options for mastitis should not include wide-spectrum antibiotics like third- or fourth-generation cephalosporins. In acute mastitis caused by *S. aureus* and in acute instances of coliform mastitis, systemic therapy is advised, ideally in conjunction with IMM medication [99]. Cure rates for mastitis are low in part because the typical treatment is too brief. In mastitis caused by *S. aureus* and *S. uberis*, treatment time should often be increased to increase the likelihood of a successful outcome [100]. The suggested treatment period for clinical mastitis is minimum of three days, which is greater than labeled therapies in various countries. Treatments for mastitis should be based on verification, meaning that both the effectiveness of individual products and the duration of necessary therapy [101].

Bacterial recovery rates recorded in New Zealand ranged from 40 to 80% [102]. When intramammary treatments of boracic acid, acriflavine, iodine, and sulfonamides were used before penicillins were widely available. “Blitz” treatment and targeted DCT were examined, and dose–response studies with intramammary penicillin were conducted across clinically and subclinically infected areas [103]. Treatment of clinical mastitis during initial lactation with either intramammary administration or parenteral insertion of penicillin-derived drug results in equal rates of infection clearance across the two routes of administration [104].

Infectious agents may be treated with a variety of antibiotics, notably penicillin and streptomycin. The sole effective treatment is administration into the udder; nevertheless, this has a low success rate. This happens as the drug is not able to go as far because of widespread tissue loss in immediate area. Erythromycin, since it spreads

more quickly, has been cited as a more effective alternative. Even metronidazole-based anti-anaerobe treatments have been reported to work. If you have a breast-feeding animal, chlortetracycline may be helpful, but you shouldn't give it to a dry gland since it will kill the gland's secretory activity.

4.8.2 Subclinical Mastitis Treatment

Antibiotic management of subclinical mastitis is usually not cost-effective during nursing due to substantial treatment expenses and low effectiveness. Antimicrobial therapy resulted in 75% bacteriological cure rate in large study of subclinical mastitis subjects [105], whereas no therapy resulted in 68% recovery rate. For mastitis caused by *streptococci* exclusively, the minimal assist applied; for mastitis caused by *S. aureus*, antibacterial agents were equivalent to no therapy at all. There will be no change in herd's mastitis rate by treating subclinical cases unless other preventative measures are also implemented. In most cases, treating cows for high somatic cell counts has been proven to have little impact on milk output [7, 106]. Subclinical mastitis is treated in herds when issues are triggered by highly infectious bacteria like *S. aureus* or *S. agalactiae* [95]. Mastitis has been treated with antibiotics for over fifty years, but there is no agreement on which antibiotics are the most effective, safest, and cost-effective. Evidence-based medication, which has recently been implemented to veterinary medicine [101], should be used for mastitis treatment as well. Dairy cows provide milk for human consumption, and therefore it's important to think about how this can affect the public's health. Because antimicrobial therapies for dairy cows result in milk remnants avoiding residues is crucial part of mastitis therapy [95]. Specifically, when antibiotic is given systemically, choosing a material with a reduced minimum inhibitory concentration level for target organism is preferred. Since phagocytosis is disrupted in mammary gland, the antibiotic in question should kill bacteria rather than just stop them from multiplying [107]. Traditionally, in vitro testing for antimicrobial sensitivity was seen as a necessary first step before beginning therapy. However, relieving bovine mastitis successfully in vivo depends on more than just action in vitro. However, this may vary by area, and antibiotic resistance among mastitis organisms has not yet arisen as clinically important concern. The pervasive penicillin G-resistance of *staphylococci*, especially *S. aureus*, is the main issue [108, 109]. Treatment success rates for mastitis produced by penicillin-resistant *S. aureus* variants sound to be lower than those for mastitis generated by penicillin-susceptible variants [110]. It is unclear whether this is related to pharmacological issues with the medications themselves or to virulence factors likely associated with the beta-lactamase gene in the resistant strains [111]. Penicillin G-resistant *staphylococci* should be tested for in vitro ß-lactamase activity before therapy [108]. Coagulase-negative *staphylococci* are more resistant than *S. aureus* and may rapidly acquire many drug resistance phenotypes [96]. Despite the continued susceptibility of *streptococci* producing mastitis to penicillin G, evidence of increasing tolerance to macrolides and lincosamides has been observed [112].

4.9 Prevention and Control

The endemic nature of bovine mastitis makes it impossible to totally eliminate the illness. It is quite improbable that this illness will ever be eradicated because of the diversity and prevalence of the microbes that trigger it [113]. Thus, a comprehensive control plan [114] can only be effective if the epidemiology of the illness and its causative agents are well understood. Efficient mastitis management has resulted from the implementation of comprehensive herd health control strategies [115]. Effective medication and management of mastitis are greatly aided by early, accurate diagnosis. Management of mastitis outside of the lactation period, removal of persistently infected livestock, good management and cleanliness are the key control strategies [113]. Reducing the likelihood that teats may come into contact with milk from sick cows is crucial to effective management of infectious mastitis bacteria. Reduce the quantity of bacteria that come into contact with teat, boost the cow's immune system, and immerse the teat in germicidal solution before milking to prevent environmental mastitis. The ideal conditions for animals are ones that are spotless and dry. Therapy of clinical and subclinical illnesses in milking cows often involves the use of antimicrobial agents [116]. Post-milking, the teat canal might stay open for another couple of hours before sealing back up. To keep the cows upright post-milking, new food and drink must be provided, and if the cows must lay down, the stalls must be sanitized and well-bedded [113]. Most dairy producers don't bother weaning their calves and instead have their calves suck directly from the dam's udder, which may lead to udder injury and infection. Infectious agents may enter the teat when a calf is nursing. In dairy animals, it is crucial that calves not be allowed to suckle their mothers. To reduce contact with infections in mammary gland, it is important to have sufficient airflow and cleanliness in farm facility [117]. To prevent the transmission of illness, the milker's hands must be thoroughly cleansed, dried, and disinfected after each milking. All milking equipment must be spotless and dry before use. It's important to have dry bedding. Urine and feces should be cleaned up right away since they are a perpetual breeding ground for disease on the farm [113]. The National Mastitis Council of the USA and Canada has recently upgraded its original five-point plan to 10 points, including seventy-three bullet points. The ten items are as follows:

(a) setting udder health objectives
(b) retaining the milking space dry, sanitary, and comfortable
(c) practicing safe milking practices
(d) caring for and using milking machinery properly
(e) keeping accurate records
(f) handling clinical mastitis throughout lactation
(g) handling dry cows well
(h) maintaining biosecurity for transmissible pathogens and euthanizing permanently infected cows and

(i) Management measures as dry cow therapy, milking method, post-milking teat immersion, and antibiotic therapy for clinical mastitis have been shown to significantly reduce mastitis incidences and bulk tank milk SCC [85].

Chronic subclinical illnesses have been widely accepted as key hurdle in management of mastitis in milking farms [1], despite the fact that subclinical mastitis is most common type affecting cows. Milking affected animals last and keeping them from sitting down post-milking are other common techniques for reducing the spread of infectious and environmental mastitis. If you feed them just after you milk them, you can guarantee that they will stand for the whole 30 min [118]. Dairy cows may be more susceptible to pathogens [119] because most Ethiopian families utilize manual milking and do not sanitize their hands, udders, or teats prior milking.

4.9.1 Contagious Mastitis Control

Infections with *S. aureus* continue to be the primary cause of mastitis in milking cows. The success rate of antibiotic treatment while lactation is quite poor. Many animals that get infected must be culled because they develop chronic diseases. Effective mastitis management measures, as teat immersing and dry animal care, may eliminate *S. agalactiae* from dairy farms. The udder, rumen, waste, and barnyard are all possible habitats for *Streptococcus dysgalactiae.* They are relatively sensitive to antibiotics and may be managed with improved cleanliness [120]. Post-milking teat immersion, complete dry cow treatment, elimination, medication of clinical instances and adequate inspection of milking machinery are the most common methods for preventing the spread of infectious mastitis in milking farms [67] (Fig. 4.7).

4.9.2 Environmental Mastitis Control

Decreasing the teat end's susceptibility to atmospheric infections and increasing cow's immunity against intramammary infection are both effective strategies for minimizing environmental mastitis. Hazardous microorganisms may thrive in natural bedding resources, feces, and moist or damp environments in sheds and meadows. Maintaining proper cleanliness during milking may have an effect on how much of the teat end is exposed. Overall, the risk of vulnerability is reduced when all places are neat, cold, and dry. The calving region must be free of any potentially harmful substances, such as dung, dirt, or pools of stale water. Optimal resistance is achieved by reducing stress, protecting the teats, and eating diet high in antioxidant nutrients like vitamin E and selenium. Following milking, it's best to use a germicidal immersing on the teats to prevent infection. The use of germicidal or a shield dips to prevent environmental mastitis within dry period has been met with limited success,

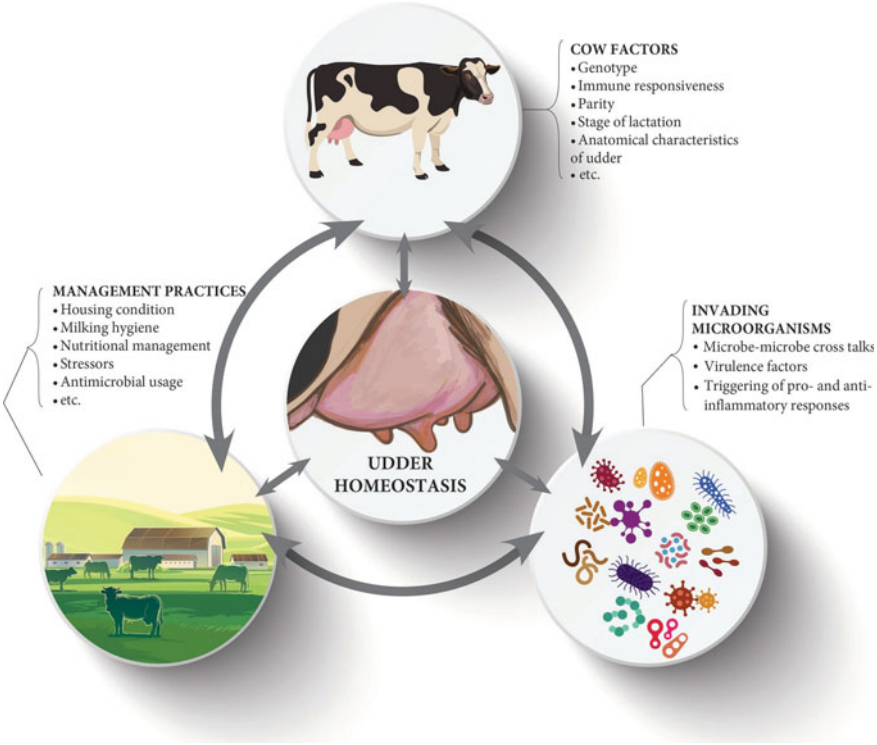

Fig. 4.7 Determinants of udder homeostasis. Defense mechanisms of the udder against microbial colonization are modulated by a complex interaction network among **a** cow genotype, physiological status, and udder characteristics; **b** invading microorganisms, their cross talks, and virulence factors; and **c** management practices that can influence metabolic and immune homeostasis of the cow. Color version available online. Reproduced with permission from Ref. [83]. 2018 American Dairy Science Association

and antibiotic treatment during milking or dry period is of limited benefit in this regard [121]. However, drying off all animal quarters can aid in the prevention of environmental streptococci upon early dry period.

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Chapter 5
Antibiotic Drug Resistance

Abstract Antibiotics inhibit the growth or reproduction of microbes, enabling immune system and other defense mechanisms to finish their task. Common mechanisms for operation include acting as a membrane disrupting drug, impeding protein production, DNA formation, RNA synthesizing, and bacterial cell production overall. Antibiotic usage has resulted in multidrug-resistant (MDR) strains of several microbial species linked to human illness outbreaks. For instance, multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) has emerged as a prominent disease in both establishing and established countries. Nosocomial sickness (infections contracted while in hospital) can also be quite dangerous. These encompass infections caused by bacteria like *Acinetobacter baumannii*, *Burkholderia cepacia*, *Campylobacter jejuni*, *Citrobacter freundii*, *Clostridium difficile*, *Enterobacter* spp., *Enterococcus faecium*, *Enterococcus faecalis*, *Escherichia* "superbugs" are microorganisms that cause more severe illnesses and deaths than normal because they have acquired mutations that make them resistant to antibiotic classes most commonly used to treat them. Certain ß-lactam antibacterials are derived from fungi and kill bacteria by blocking the enzyme transpeptidase. Inhibition of topoisomerase II (DNA gyrase) and topoisomerase IV, quinolones prevent bacteria from producing new DNA. Sulfonamides are structurally similar to the bacterial DHF precursor p-aminobenzoic acid (PABA). By acting as fierce inhibitors of PABA consumption, sulfonamides reduce DHF production. Antibiotic usage has several positive effects, including increased milk production from healthier cows, decreased rates of illness, morbidity, and death, reduced pathogen loads, and more milk of a higher quality and quantity. However, there are concerns that antibiotics used in agriculture may contribute to the rise of bacteria resistant to antimicrobial drugs, which might have an effect on the treatment of illnesses that affect humans. Antibiotic residues are also more common in milk produced by cows who have been treated for mastitis. Bacteria employ four main methods for combating antibiotics: (1) reducing the amount of medicine taken in; (2) altering the drug's target; (3) rendering the drug ineffective; and (4) engaging in active drug efflux.

Keywords Transpeptidase · MDR · Antibiotics · DNA · PABA · DHF

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5.1 Introduction

Many effective active compounds derived from plants have a long history of usage as medicines and have been used for centuries. The development of penicillins at beginning of twentieth century marked the beginning of search for drugs derived from microorganisms. The vast majority of today's pharmaceuticals have their initial structural inspiration in naturally occurring compounds produced by bacteria. Numerous medical conditions may be diagnosed, symptoms mitigated, diseases treated, prevented, and even pain alleviated with the administration of drugs developed from bacterial secondary by-products. Tens of millions of soil microbes were likely screened throughout golden age of bacterial organic product discovery (1940–1970) [1], a massive undertaking that yielded a substantial number of known bacterial metabolites [2, 3]. Antimicrobial drugs like erythromycin, streptomycin, tetracycline, and vancomycin, as well as chemotherapy treatments like doxorubicin, fall within this category. Ninety percent of the medicines used in hospitals today are discovered in microbes [3, 4] (Fig. 5.1).

Over 23,000 natural compounds having antibacterial action are known to be generated by microbes, but only 25,000 have been identified from larger species [4]. Only a small fraction of these compounds—perhaps hundreds—are actually employed in actual patient care [6]. Antibiotic production rates among eubacteria are only vaguely calculated, though *actinobacteria* seems to be the best producers (Fig. 5.2).

The most successful drug discovery programs have used one of three strategies:

(1) Traditionally, complete cell-assays were employed for screening materials, crude extracts, or refined compounds for biological effectiveness without specifying the pharmacological target. Following a chemical with biological activity has been discovered, researchers go further into analyzing its target and mechanism of execution. Bioactive-guided assessment, classical medicine, advanced pharmacology, and

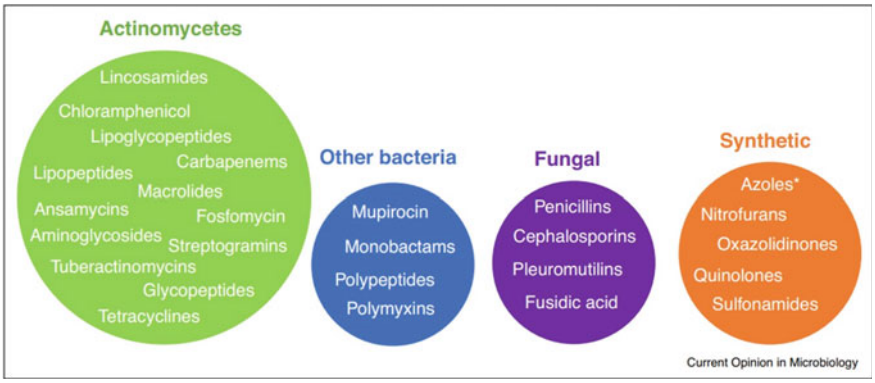

Fig. 5.1 Most clinically relevant classes of antibiotic are derived from natural products. Reproduced with permission from Ref. [5]. Copyright 2020 The Authors. Published by Elsevier Ltd.

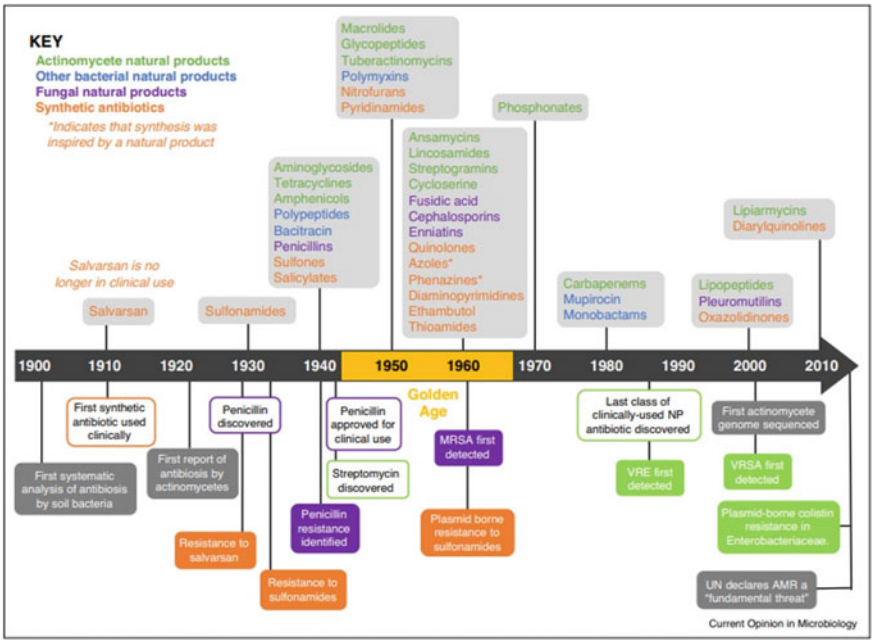

Fig. 5.2 Timeline showing the decade new classes of antibiotic reached the clinic. The antibiotics are colored per their source: green = actinomycetes, blue = other bacteria, purple = fungi and orange = synthetic. At the bottom of the timeline are key dates relating to antibiotic discovery and antimicrobial resistance, including the first reports of drug-resistant strains methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *enterococci* (VRE), vancomycin-resistant *S. aureus* (VRSA) and plasmid-borne colistin resistance in *Enterobacteriaceae*. Reproduced with permission from Ref. [5]. Copyright 2020 The Authors. Published by Elsevier Ltd.

morphological medicine exploration are all terms used to describe this approach [7, 8].

(2) Chemical scrutinizing is an alternative method for discovering new therapeutic compounds; it seeks to find unique, chemically varied molecules regardless to their biological significance. Synthetic databases or natural compounds (such as microbial metabolites) may both be employed in this method. High-performance liquid chromatography, mass spectrometry (MS), and nuclear magnetic resonance spectroscopy are only a few of the analytical modalities used for this purpose. Therefore, the explanation of structure is key step in preventing the reemergence of previously identified drug. These days, dereplication is made much easier with the availability of extensive databases including mass spectra for recognized molecules. Whether a novel compound is discovered in this way, it will be put through tests to see whether it has any biological (antibiotic) action.

(3) Target-oriented assessments in contrary to chemical evaluation, seeks to find substances that act upon a previously established molecular receptor. Thus, the objective is cell or structural framework in the disease process that investigational medicine

is designed to alter. It's important to keep into consideration target's various characteristics. An excellent bactericidal objective, for instance, might be found in many different types of bacteria but have no human counterpart. The position of receiver within the bacterium, as well as the incidence of resistance to newly developed compounds, are additional significant characteristics. Since high-level, target-based susceptibility to such chemicals does not emerge by single-step genetic variations, it is necessary to pick several objectives or goals encapsulated by many genes.

Antibiotics inhibit the growth or reproduction of microbes, enabling immune system and other defense mechanisms to finish their task. Common mechanisms for operation include acting as a membrane disrupting drug, impeding protein production, DNA formation, RNA synthesizing, and bacterial cell production overall [9]. Suppression of protein production may also occur if drugs attach to the bacterium within cell wall and are then transported into the translational sites through energy-intensive transit processes [10]. Antimicrobials are undeniably a gift to human culture that have preserved millions of lives by fighting off diseases and other microorganisms [11]. Antibiotics come in a wide variety, and many of them have been put to beneficial utilization throughout the years. In the middle of the twentieth decade, antimicrobials were hailed as a miracle medicine. There was hope at the moment that spread of illness might soon be stopped entirely. Alexander Fleming and Paul Ehrlich's names are often used together as though they were corresponding to the start of contemporary "antibiotic era" [12]. Antibiotics were formerly thought to be a panacea since they could kill disease-causing microorganisms without harming the host. Fleming was first to warn about dangers of overusing or short-term penicillin therapy [12]. This is why the decades between the 1950s and the 1970s were heralded as the "golden age" of antimicrobial exploration [13]. In the 60 years after their invention, manufacturers have mass-produced millions of metric tons of antibacterial from emerging groups. Antibiotics are in more desire than ever before, and this has opened the door to unapproved and cost-effective use. However, antibiotic resistance has emerged in large part owing to careless and excessive use of these drugs [14]. In the past, resistant bacteria appeared in accordance with number of novel antibacterial drugs that were developed. Developing and re-emerging susceptibility of bacteria worldwide has shifted the emphasis of disease control efforts toward the adaptation of currently available antibiotics [13].

Inappropriate utilization of medications has hastened the development of bacteria resistant to these substances and of genes that allow them to do so, lowering the effectiveness of these drugs as treatments for human and animal diseases [15]. Global antibiotic durability is a public welfare emergency that needs immediate attention, according to the World Health Organization [16]. The development of antibiotic resilience has been linked to the loss of an antibiotic's bactericidal effects. Antibiotic- "resistant" bacteria tend to grow even when exposed to therapeutic quantities of drugs [17]. When microbes can reproduce despite the usage of antibiotics, we say that they are antibiotic-resistant. Normal concentrations of antibacterial drugs are efficient for them, but as the microorganisms grow less responsive or resistant, a larger dose of same medicine is required for treatment. Antibacterial resistance emerged soon following the development of novel antibacterial chemicals [18]. It is

possible for bacteria to develop resistance to antibiotics by the mechanism of natural selection [19]. One research, for instance, demonstrated that antibiotics were widely used to cure non-cholera diarrhea sickness in Thailand, such as sulfamethoxazole and trimethoprim (TMP-SMZ), ampicillin, and tetracycline, have no such function now [20]. In addition, a research done in Bangladesh demonstrated the efficacy of same medications in curing them [21]. Indeed, records of susceptibility existed prior to antibiotics were used to combat the illness [22]. The rise of antibiotic-resistant bacteria is a direct result of their careless overuse. Therapeutic usage of sulfonamides has been prompted by the appearance of resistant strains since the drugs' release in 1937. Nonetheless, reports of sulfonamide resilience date back to 1930s, revealing a process of resilience that persists at work today, over eighty years later [14]. Aminoglycoside-resistant variants of *S. aureus* emerged around six years after the discovery of these drugs [23]. Methicillin, a prototype of semisynthetic penicillinase-resistant penicillin, developed in 1961 to combat penicillinase-producing Staphylococcus aureus isolates, although reports of methicillin resistance quickly emerged [24].

Among most frequently administered antibiotics, β-lactams cure a wide variety of microbial illnesses by blocking proliferation of microbial cell envelope. Penicillin and related β-lactam antibacterial are possibly most significant medications ever because of the great influence they have had on health all over the world by curing bacterial illnesses [25]. The contention to β-lactam antimicrobial agents is a serious problem [26], as these drugs are effective against a wide variety of bacteria and have a low risk of harmful effects in humans. However, bacteria and additional infection-causing microorganisms evolved remarkably sophisticated mechanisms for developing contention to antibiotics and additional antibacterial medications. Increasing antibiotic usage and abuse across a wide range of medical conditions is largely to blame [27]. 70% of nosocomial infections are caused by bacteria which were impervious to at least one treatment; certain germs are discovered to be impervious to practically all designated medicines and are therefore addressed only by select medicines which are potentially hazardous. *S. aureus* and *pneumococcus* are two types of bacteria that cause serious illnesses in population and have become more resistant to antibiotics, according to studies [28]. There is a notable level of microbial resistance to antimicrobial agents, as shown by studies [29], including various types of bacteria as *Acinetobacter*, *Proteus*, *Escherichia coli*, *Klebsiella*, and *Pseudomonas*.

Additionally, fluoroquinolones were first developed to combat the spread of gram-negative microbial ailments in 1980s, but it was subsequently discovered, due to development of fluoroquinolones contention, that these medicines were also utilized to address gram-positive disorders [30]. Resilience to quinolones was developed by a gradual accumulation of chromosomal changes, most noticeably within methicillin-resistant isolates. Clinically significant isolates of Vancomycin-resistant Staphylococcus aureus (VRSA) were first discovered in 2002, 44 years before Vancomycin was first introduced in market [31].

Antibiotic usage has resulted in multidrug-resistant (MDR) strains of several microbial species linked to human illness outbreaks. For instance, multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) has emerged as a prominent disease in both

establishing and established countries. Nosocomial sickness (infections contracted while in hospital) can also be quite dangerous. These encompass infections caused by bacteria like *Acinetobacter baumannii*, *Burkholderia cepacia*, *Campylobacter jejuni*, *Citrobacter freundii*, *Clostridium difficile*, *Enterobacter* spp., *Enterococcus faecium*, *Enterococcus faecalis*, *Escherichia* "superbugs" are microorganisms that cause more severe illnesses and deaths than normal because they have acquired mutations that make them resistant to antibiotic classes most commonly used to treat them. As a result, the number of alternatives to therapy for such microorganisms decreases, and the length of time spent in hospital increases in both expense and duration. There have been situations when superresistant bacteria have also become more virulent and contagious. Antibiotic resilience is an indicator of pathogenicity in the real world [13].

Tuberculosis is prototypical human disease; it has coevolved with humans and now affects almost third of global population. Despite the fact that streptomycin and isoniazid were discovered and quickly used as effective therapies, resilience quickly emerged. The most common gram-negative pathogens include *Escherichia coli*, *Salmonella enterica*, and *Klebsiella pneumoniae*; they are responsible for a wide range of ailments in individuals plus animals, and there exists a significant association among antibacterial use in treating such illnesses and the emergence of antibiotic retaliation across the last fifty years. This is most pronounced with ß-lactam family of antibacterial and ß-lactamases that inactivate them. To date, a multitude of ß-lactamases associated with resistance have been categorized into several classes and subclasses, equating to thousands of diverse varieties. They include discovery of new groups of genes and subsequent explosions of mutants that resulted from those discoveries [32–36]. The development and spread of opposition to ß-lactam antibacterials across intestinal microbes in both public and hospital illnesses have been largely attributed to HGT. *Pseudomonas aeruginosa* has progressed form burn wound illness to significant nosocomial hazard in the context of hospital-acquired illnesses. Additionally, the discovery of novel antibiotic compounds coincided with evolution of pathways that make antibiotics ineffective, jeopardizing potentially promising therapies (as ß-lactams and aminoglycosides). People suffering cystic fibrosis [37] have significant risk from *P. aeruginosa* since this infection is very insistent and may evade human immune responses. The prolonged use of antibiotics in cure of cystic fibrosis is linked to emergence of resilience. *Acinetobacter baumannii*, another gram-negative bacterium, is becoming recognized as a major nosocomial threat. Similar to *pseudomonads*, it contains a set of r genes and virulence factors that contribute to increased fatality and illness [38]. Numerous varieties of *Acinetobacter* are inherently proficient for DNA absorption and exhibit significant instances of organic transformation, which is assumed to be where organisms' infectious qualities originate. Subsequent genome sequencing investigations have shown that certain *A. baumannii* variants contain a minimum of 28 genomic features expressing antibiotic resilience determinants, along with over fifty percent of these incorporates also carry virulence activities in a variety of type IV secretion mechanisms [39, 40]. *Staphylococcus aureus*, a gram-positive bacterium, is most infamous superbug at the moment. The degree to which the virus's negative image is attributable to its considerable media

attention raises questions about whether it is the most dangerous superbug or not. Approximately 30% of the population carries *S. aureus* as respiratory commensal, and exposure of *S. aureus* has long been related to prevalent skin illnesses like blisters.

5.2 Antibiotic Types

5.2.1 Narrow-Spectrum Antibiotics

A narrow range describes chemotherapeutic drugs that are effective against just one or a small number of bacteria. For instance, the antibacterial drug isoniazid exclusively kills mycobacterium [41].

5.2.2 Extended-Spectrum Antibiotics

Antibiotics with broad-spectrum activity are those which are enforceable not just toward gram-positive microbes but also for a large subset of gram-negative microorganisms. Since it is effective against both gram-positive and certain gram-negative microorganisms, ampicillin is often cited as an instance of an antibiotic possessing a broad spectrum [41].

5.2.3 Broad-Spectrum Antibiotics

Antibiotics with a broad range of activity are those that are effective against many different types of bacteria. Superinfections of microbes like *Candida albicans*, whose development is generally held in control by the existence of additional microbes, might result from the use of broad-spectrum antibacterial drugs [41].

5.2.4 Combinations of Antimicrobial Drugs

It is preferable from a therapeutic standpoint to use single medication that has highest degree of specificity for infectious agent. The risk of an outbreak is decreased, drug resistance is impaired, and toxicity is kept to a minimum by adopting this approach. However, there are contexts when many medications are used together. Drug combinations are helpful in many medical contexts; one such scenario is TB therapy [41].

5.2.5 *Prophylactic Antibiotics*

In particular medical settings, antibiotic prophylaxis is more appropriate than therapy. Antimicrobial drugs should be used with caution to prevent the development of resistant microbes and superinfection; thus, preventive usage is limited to instances when the advantages will exceed risk. Prophylaxis should be taken for as long as there is a chance of disease [41].

5.3 Antibiotic Classification

Approximately 2,140,000 extracellular mediators have been found across all species and categorized according to their roles, framework, and biosynthetic pathway since the conclusion of 2019. It is well-established that microbes create >50,000 chemicals, among which around 22,000–23,000 contain bioactive characteristics and approximately 17,000 are antibacterials [42]. Steroids (hydrocortisone) and terpenoids (menthol, thymol) are two examples of extracellular compounds; others include fatty acid-derived compounds (monocaprin) and polyketides (erythromycin A), alkaloids (metronidazole, quinolones), non-ribosomal polypeptides (polymyxin, teixobactin, vancomycin), and enzymatic substrates [43].

5.3.1 *Classification According to Origin*

Antibiotics may first be sorted into groups determined on where they came from, or more precisely, what kinds of microbes made them. This approach of categorization has been examined in [44].

The ability to classify virtually all antibiotics into a few broad categories is a benefit of this approach, but it precludes any additional logical classification beyond classifying them into their respective phylogenetic families.

The aforementioned are some reasons for why it is insufficient to just classify entities based on their origin:

a. The capacity to produce antibiotics is not, as is often believed, an inherent, persistent characteristic of any specific microbe.
b. Many different compounds with varying chemical structures and biological activities are produced by different species within the same genus. It is currently difficult to classify over 2,000 Streptomyces-derived pharmaceuticals in this manner. There is not any definitive classification established for the Streptomyces genus alone. Antimicrobial characteristics generated by Streptomyces isolates from identical series showed no association with one another. Likewise, the Streptomycetales' evolutionary connection is not well understood. As a result, Streptomyces antimicrobial drugs should be categorized only according to their chemical

makeup, as recommended in [45]. Antibiotics from families *Aspergillaceae* and *Bacillaceae* are in an identical predicament.

c. Antibiotics with almost equivalent structures may be generated by species from widely diverse phylogenetic backgrounds. Antimicrobial with similar structural features have been produced by many different microbial species. Cephalosporin C and penicillins are solely developed by fungi; meanwhile, in the last few years, five cephalosporin C skeleton-containing antibacterials were determined from *Streptomyces* isolates in culture. Iodinin is generated by *Pseudomonales*, *Eubacteriales*, and *Actinomycetales*; its methoxy derivative, myxin, is developed by *Streptomyces creatures* [46, 47].
d. Antibiotics can be produced by a species in a wide variety. Bacillus subtilis produces 66 distinct antimicrobial compounds, whereas *Bacillus brevis* produces 23. These antibiotics are mostly polypeptide in nature. *Pseudomonas aeruginosa* isolates create roughly 40 different kinds of antibiotics, mostly of N-heterocyclic structure; species corresponding to the *Streptomyces hygroscopicus* or *Streptomyces griseus* series constitute a collection of diverse antibiotic compounds. As an example, approximately 19 distinct species of Streptomyces generate oxytetracycline, despite the fact that they all belong to the particular same genera.

5.3.2 Classification According to Biosynthesis

The biosynthetic pathway categorization [48] is grounded in extensive theory and biochemistry [49, 50]. Subsequently, the potential for systematization is constrained by small number of drugs studied (the formation of only roughly 200 antibacterial is revealed). Further, complications arise due to the fact that many diverse molecules (macrolides, polyenes, tetracyclines, terpenes, etc.) are metabolized by comparable, generic biosynthetic routes. When various metabolic processes are used to construct distinct parts of molecule, an integrated biogenesis pathway is outcome. There is tight relationship between chemical a framework and the biosynthetic categorization. Biosynthetic processes that produce comparable or remarkably comparable chemical patterns are common. Unlike chemical categorization, which is based on the last outcome of precise chemical makeup, the categorization in accordance with biosynthesis is dependent on usually unpredictable course of metabolism. The connection between biosynthetic categorization and provenance is clear in specific examples; related microbial communities often produce analogues of one another's chemical structures through analogous metabolic processes.

5.3.3 Spectrum of Activity

Classifying antibiotics relying on their range of action is a tried and true method with deep practical significance. Practically, major significant antibiotic has a well-characterized range of action. Antibiotics are typically tested for their ability to kill a wide variety of microbes, viruses, molds, yeasts, protozoans, and other creatures, as *rickettsiae*, *Mycoplasma*, and *spirochetes*, as part of the procedure of determining their antimicrobial range. Antitumor, antiviral, insecticidal, etc., action is often present in combination with antibacterial properties in certain microbial compounds. Particular antibacterial medications (like sarcidin, validamycin, and saramycetin) are selectively active toward a few specific species, while others (like penicillin and tetracycline) are efficient toward a wide range of microbes, molds, and yeast cells. Paromomycin, for example, is usually grouped with antiprotozoal medicines, despite the fact its whole variety of exertion is virtually comparable with additional aminoglycosides (referred to as antimicrobial antibiotics); e.g., with neomycin, kanamycin, excluding that it demonstrates significantly greater antiprotozoal action compared to both of them. This creates issue with the obvious categorization of therapeutic antibacterial.

5.3.4 Classification According to Mechanism of Action

Theoretical researchers implemented significant importance on categorizing things according to their mechanisms of activation. Antibiotics with a well-established means for effectiveness are abundant, as shown by numerous attempts to classify them [50, 51]. The study of molecular science has subsequently attracted a lot of attention. Though much remains unclear despite the wealth of data collected, method for effectiveness of around 200 antibiotics has been explained in some detail. Commercially available antibiotics are the sole medications for which the mode of action can be considered fully understood. There is still much confusion on several issues.

a. It is frequently challenging to distinguish between main and additional procedures in tests aimed at elucidating a mode of action.
b. It is challenging to create functional cell replicas in cell-free environment. The processes at work in an organism are different from those in test tube. Most experiments are conducted in cell-free environment that has certain differences from the real thing.
c. More than one mode of effectiveness is present in certain antibiotics; gramicidin S, for example, interferes with membrane activity and aerobic phosphorylation.
d. Compounds with radically different chemical structures often have same mechanism of action, adding to the complexity of situation. In particular, both aminoglycosides and macrolides block the formation of proteins by attacking 30 S ribosomes.

5.3.5 Classification According to Physicochemical Properties

Only structural or chemical characteristics should be considered when categorizing antibacterial drugs. Water dissolution, organic chemical dissociation, acidity, basicity, amphoteric nature, signal qualities, and the presence of *N* or additional components are all ways in which antibacterial drugs may be distinguished from one another. Some work was also done to try to organize antibacterials into functional divisions and other categories based on their molecular structure and basic analyses. The assessment and speedy discovery of novel antibiotics is greatly aided by ranking antibiotics utilizing physicochemical parameters like UV, IR, NMR spectra, paper-chromatographic or thin-layer chromatographic measurements. Although these categories are helpful in practice, they do not advance theorizing in any meaningful way. Furthermore, it is important to acknowledge that many methods discussed may not yet be suitable for implementation within a comprehensive framework owing to the lack of pertinent scientific evidence.

5.4 Mechanism of Action of Antibiotics

5.4.1 Inhibitors of Cell Wall Synthesis

The majority of organisms possess cell wall, a stiff barrier around cell which shields from harmful environmental factors and hinders plasma layer from bursting due to excessive interior osmotic pressure. Murein (peptidoglycan) matrix is responsible for a great deal of skeletal strength of cell walls. This is an intricate large molecules assembled from simpler constituent parts. *N*-acetylglucosamine and *N*-acetyl-muramic acid, both of which include amino sugar molecules and carry peptide ties, are the building blocks. The components are generated inside the bacterium, moved across cell exterior membrane, and integrated outside cell. The enzymatic transpeptidase forms bonds between amino sugar molecules in two neighboring peptide tracts. Since animal and human cells do not contain cell wall, antimicrobial medicines that interfere with the production of cell barrier are effective against bacteria. They inhibit the growth and multiplication of microorganisms, rendering them harmless. Penicillins, cephalosporins, bacitracin, and vancomycin are all examples of ß-lactam antibacterials [52] (Fig. 5.3).

Penicillins (A)

Penicillin G (benzyl-penicillin) is an ancestor of such class of antibiotics. This is cultivated by mold fungus, namely Penicillium notatum. Most penicillins endure an identical arrangement made up of thiazolidine and 4-membered ß-lactam circle, and penicillin G represents no exception. Six-APA is not antimicrobial by alone. The

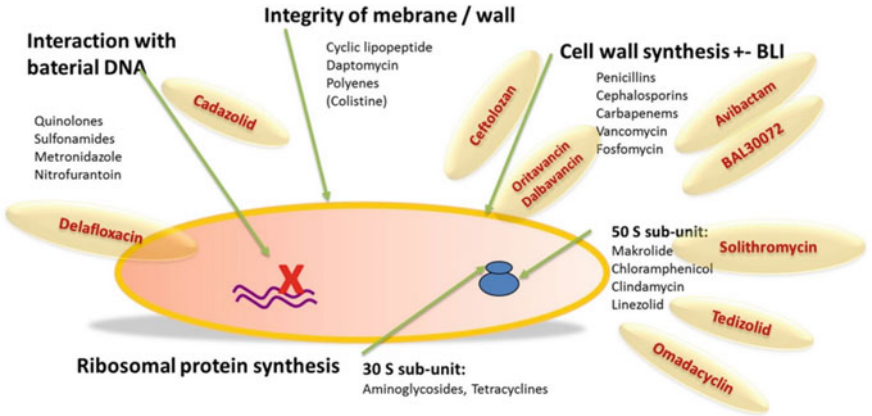

Fig. 5.3 Schematic antibiotic action: new compounds in an overview. Reproduced with permission from Ref. [53]. Copyright © 2015, The Author(s)

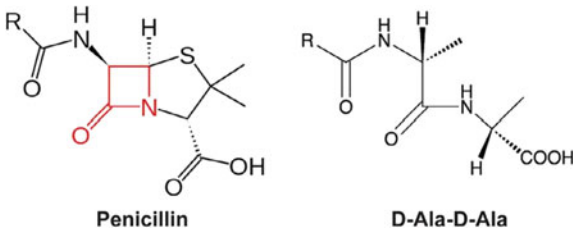

Fig. 5.4 Resemblance of beta-lactam antibiotics to D-Ala-D-Ala [D-alanyl-D-alanine]. Reproduced with permission from Ref. [55]. Copyright 2020 Elsevier Inc.

transpeptidase activity of bacteria is disrupted by penicillins, which causes cell wall formation to stop. Penicillins are actually bactericidal because microbes expand and explode owing to cell wall flaws during the development and proliferation phase. Penicillins are typically safe; the usual single dosage of penicillin G is between 0.6 g intramuscularly ($= 10^6$ international units, 1 Mega I.U.) and 60 g when administered intravenously. The majority of serious side effects stem from hypersensitivity [52, 54] (Fig. 5.4).

Cephalosporins

Certain ß-lactam antibacterials are derived from fungi and kill bacteria by blocking the enzyme transpeptidase. Cephalexin (gray rectangle) is an example of one of these antibiotics that shares their fundamental architecture with others: 7-aminocephalosporanic acid. Although cephalosporins are steadily acid-stable, plenty of these are also poorly assimilated. Most of these drugs, notably the highly active ones, are exclusively utilized in healthcare facilities due to the fact that they must be administered parenterally. Some, like cephalexin, may even be taken orally.

Cephalosporins are impervious to penicillinase; however, there are bacteria that can produce cephalosporinase. Although, certain analogues are impervious toward the ß-lactamase as well. The cephalosporins are effective against a wide variety of microorganisms. Microbes resilient to a wide range of antimicrobials are not immune to contemporary versions (such cefotaxime, cefmenoxin, cefoperazone, ceftriaxone, ceftazidime, and moxalactam). The majority of people tolerate cephalosporins quite well. All of them may trigger an allergic response, and some of them can also induce kidney damage, alcohol intolerance, and excessive bleeding (vitamin K antagonism) [52, 54].

Other Inhibitors of Cell Wall Synthesis

Bacitracin and vancomycin are effective exclusively toward gram-positive microbes because they block the transfer of peptidoglycans inside cytoplasmic layer. Bacitracin is a polypeptide compound known for its significant nephrotoxicity, and as a result, it is only used topically. Glycopeptide vancomycin represents the medicine recommended for (oral) management of bacterial intestinal swellings that might develop as a side effect of antimicrobial rehabilitation (pseudomembranous enterocolitis resulting from *Clostridium difficile*). There is no absorption [54].

5.4.2 Protein Synthesis Inhibitors

The process of transcription converts the information in bacteria's genome into messenger RNA (mRNA). The mechanism through which proteins are synthesized from messenger RNA (mRNA) is called translation, and it takes place inside ribosome of the cell. Protein synthesis is aided by ribosomes and other cytoplasmic entities. The ribonucleo-protein subunits 30S and 50S combine to form the microbial 70S ribosome [56]. The antibiotics inhibit protein synthesis of microorganisms by interfering with either the 30S or 50S subunit of ribosome [57, 58].

Tetracyclines

Tetracyclines are protein-synthesis-inhibiting antibacterial drugs with a wide range of activity. They have efficacy toward a wide range of bacteria, both gram-positive and gram-negative, as well as certain protozoa as amebas. Most tetracyclines possess comparable antimicrobial properties; however, certain tetracycline-resistant organisms may still be vulnerable to doxycycline and minocycline because these medicines are carried at a slower rate by mechanism liable for resistance. A few changes in clinical effectiveness may be attributed to variations in medication absorption, dispersion, and elimination. Tetracyclines are taken up by bacteria in two ways: passive

absorption and dynamic transport, which requires energy. The medication accumulates within the cells of those who are vulnerable to it. Tetracyclines inhibit translation by binding irrevocably to 30S component of microbial ribosome, preventing aminoacyl-tRNA from adhering to recipient region on the mRNA-ribosome complex upon entering the cell. As a result, the peptide is unable to incorporate any more amino acids [54].

Aminoglycosides

Proteins with incorrect sequences are synthesized due to aminoglycoside-induced binding of "wrong" t-RNA-AA complexes. The aminoglycosides kill bacteria. Their range of action consists mostly of gram-negative bacteria. The most common antibiotics for treating TB includes streptomycin and kanamycin [52]. Initially isolated from different streptomyces varieties, aminoglycosides are a class of antibacterial which kill bacteria that exhibit similar chemical, bactericidal, pharmacologic, and toxicological properties. Streptomycin, neomycin, kanamycin, amikacin, gentamicin, tobramycin, sisomicin, netilmicin, and others fall within this category [41].

Chloramphenicol

Chloramphenicol hinders peptide synthetase. It encompasses bacteriostatic action contrary to a broad range of bacteria. The chemically simple molecule is presently developed artificially [52].

Erythromycin

Erythromycin inhibits ribosome progression. Its main effect is bacteriostatic, indicating it kills off gram-positive bacteria. The acid-labile base € is often provided in the form of a salt (*E. stearate*) or an ester (e.g., *E. succinate*) for oral administration. Erythromycin has a high safety profile. In cases of penicillin intolerance or resistance, this antibiotic is a good alternative. Derivatives like azithromycin, clarithromycin, and roxithromycin have improved acid resilience and bioavailability. The chemicals, described as josamycin and spiramycin, are examples of macrolide antibiotics. Motiline (! Interprandial bowel motility) is a hormone secreted by gut, and erythromycin acts similarly [41, 52, 54].

Clindamycin

The antimicrobial effects of clindamycin are comparable to those of erythromycin. Bacteriostatic effects are shown most strongly against gram-positive aerobic and

anaerobic infections. In contrast to lincomycin, which is naturally produced by Streptomyces bacteria, clindamycin is a semisynthetic chloro counterpart. Clindamycin is chosen over lincomycin because it absorbs more effectively when ingested orally and because it is more effective against germs. Both have good depth of penetration in bone [52].

5.4.3 DNA Replication Inhibitors

Quinolones

Synthetic fluorinated analogues of nalidixic acid, the major quinolones possess paramount importance. Antimicrobial activity has been shown against both gram-positive and gram-negative microorganisms. As a result of their inhibition of topoisomerase II (DNA gyrase) and topoisomerase IV, quinolones prevent bacteria from producing new DNA. Transcription and duplication cannot proceed normally when DNA gyrase is inhibited because positive supercoils cannot unwind. Blocking topoisomerase IV prevents the correct distribution of duplicated chromosomes to sister cells. Nalidixic acid, oxolinic acid, and cinoxacin were the first quinolones; however, they were not able to produce broad antibacterial extents. Nevertheless, nalidixic acid and cinoxacin continue to be viable alternatives for the treatment of lower urinary tract infections. Antimicrobial action is dramatically enhanced in fluorinated analogues (ciprofloxacin, levofloxacin, and others) relative to nalidixic acid, and microbicidal concentrations are reached in bloodstreams and tissues. Since their inception, fluoroquinolones have been studied and used for their potentiation of gram-negative aerobic bacteria while having a much lower effect on gram-positive bacteria. There are a number of modern antibiotics with enhanced efficacy toward gram-positive cocci. This differential effect on gram-negative and positive bacteria is helpful for categorizing pathogens. With MICs four- to eight-times larger compared to ciprofloxacin, the original medication, norfloxacin is less active among fluoroquinolones toward gram-negative and gram-positive pathogens. A second category of comparable medicines, including ciprofloxacin, enoxacin, lomefloxacin, levofloxacin, ofloxacin, and pefloxacin, has intermediate to high efficacy over gram-positive bacteria but exceptional gram-negative action [54] (Table 5.1).

5.4.4 Folic Acid Metabolism Inhibitors

Sulfonamides

Sulfonamides are structurally similar to the bacterial DHF precursor p-aminobenzoic acid (PABA). By acting as fierce inhibitors of PABA consumption, sulfonamides reduce DHF production. Numerous microbes are DHF deficient because they cannot

Table 5.1 Mode of action of different classes of antibiotics [59]

Mode of action	Targets	Drug class	Specific drugs example
Cell wall synthesis inhibition	Penicillin-binding protein	β-lactams	Penicillin G, amoxicillin, and cephalosporin C
Inhibition of protein synthesis	Peptidoglycan subunits	Glycopeptides	Vancomycin
	30 s subunit	Aminoglycosides and tetracyclines	Streptomycin, gentamicin, neomycin, tetracycline, and doxycycline
	50 s subunit	Macrolides, chloramphenicol, and oxazolidinones	Erythromycin, azithromycin, chloramphenicol, and linezolid
Inhibition of nucleic acid synthesis	RNA	Rifamycin	Rifampin
	DNA	Fluoroquinolones	Ciprofloxacin and ofloxacin
Anti-metabolites	Folic acid synthesis enzymes	Sulfonamides and trimethoprim	Sulfamethoxazole, dapsone, and trimethoprim
Disrupt membranes	Lipopolysaccharides	Polymyxins	Polymyxin B and colistin

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acquire DHF from an external source (exogenous folate). Therefore, sulfonamides have bacteriostatic action toward a wide variety of microorganisms. Synthetic sulfonamides are made in a chemical laboratory. To varying degrees, sulfonamides are effective against gram-positive and gram-negative bacteria, as well as nocardia, Chlamydia trachomatis, and even certain protozoa. The growth of *E. coli*, Klebsiella, Salmonella, Shigella, and Enterobacter, among other intestinal bacteria, is reduced. Sulfonamides, however, do not impede rickettsiae but rather enhance their proliferation [54]. The pharmacokinetic parameters of every specific sulfonamide are determined by its residue R. When used orally, sulfonamides are absorbed well. They are excreted by the kidneys after being digested. Variation in excretion rates might lead to a large range in effective period. When it comes to treating bacterial bowel infections, some family members excel because they are poorly absorbed by the stomach. Contraindicated in final weeks of pregnancy and in infants due to the risk of kernicterus, allergic responses may cause serious skin damage and can displace other medications bound to plasma proteins. Sulfonamides are seldom employed currently [52] because resistant bacteria often arise (Fig. 5.5).

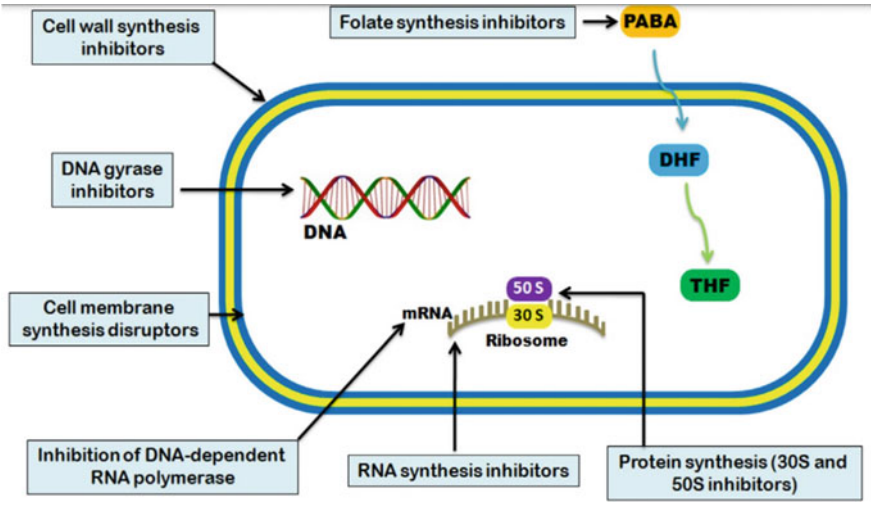

Fig. 5.5 Mode of action of antibiotics. Reproduced with permission from Ref. [60]. Copyright 2021 The Authors. Published by Elsevier Ltd on behalf of King Saud Bin Abdulaziz University for Health Sciences

Trimethoprim

Trimethoprim blocks microbial DHF reductase, while the human enzyme is much less susceptible (very rarely leading to bone marrow depletion) compared to the microbial one. Trimethoprim, a 2,4-diaminopyrimidine, exhibits bacteriostatic action toward many different types of bacteria. Most often, it is found in cotrimoxazole combinations. Approximately 50,000 times more effectively than the identical enzyme in human cells, the bacterial dihydrofolic acid reductase is inhibited by the trimethoxybenzylpyrimidine trimethoprim. Another benzylpyrimidine, pyrimethamine, inhibits the action of protozoal dihydrofolic acid reductase more effectively than mammalian cell dihydrofolic acid reductase. When sulfonamides are combined with trimethoprim or pyrimethamine, successive blockage in this metabolic process occurs, resulting in a significant synergistic increase in the action of both medicines [52, 54] (Table 5.2).

5.5 Drug Resistance and Bovine Mastitis

Antibiotics are routinely given to livestock to prevent illness and boost productivity. Mastitis, which may be caused by both gram-positive and gram-negative bacteria, is treated and prevented on dairy farms using antibiotics such penicillin, cephalosporin, streptomycin, tetracycline, and many more. During the dry season, antibiotics are often given to whole herds as a preventative measure against mastitis. Antibiotic usage has several positive effects, including increased milk production from healthier

Table 5.2 Glossary of antibiotic resistance elements

Gene	Associated enzyme activity	Targeted antibiotics	Notes
bla	Hydrolase	b-Lactams	Widely distributed with many varieties, e.g. TEM, SHV
ctx-M	Hydrolase	b-Lactams	Linked to resistance to cephalosporins containing an oxyimino group such as cephotaxime
cat	Acetyltransferase	Chloramphenicol	
sul	Dihydropterate synthase	Sulfonamides	Drug resistance version of susceptible cellular enzyme
tetO, *tetW*	Immunity protein	Tetracyclines	Bind to the bacterial ribosome and decrease affinity for tetracyclines
qnrA	Immunity protein	Fluoroquinolones	Protects DNA gyrase from fluroquinolone antibiotics
vanR, v*ans*, *vanH*, *vanA*, *vanH*	Cell wall biosynthesis proteins	Glycopeptides	Synthesis of an altered peptidoglycan with reduced affinity for glycopeptide antibiotics

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cows, decreased rates of illness, morbidity, and death, reduced pathogen loads, and more milk of a higher quality and quantity. However, there are concerns that antibiotics used in agriculture may contribute to the rise of bacteria resistant to antimicrobial drugs, which might have an effect on the treatment of illnesses that affect humans. Antibiotic residues are also more common in milk produced by cows who have been treated for mastitis [61]. Cloxacillin and other penicillinase-resistant penicillins have been developed in an attempt to combat staphylococcal resistance. These resistant penicillins are, on a molar basis, much less potent than penicillin G. The majority of *staphylococc*i and *streptococci* may still be killed by penicillin G. Antibiotics besides penicillin have also been used to combat penicillin-resistant staphylococci;

examples include the macrolides erythromycin, tylosin, and spiramycin. Antibiotic resistance is a potential threat as well. There have been advances in treating mastitis, and penicillinase-resistant penicillins such cloxacillin are often utilized in mastitis treatment, particularly in dry cow formulations [62]. Since gram-negative germs are killed off by drying up the udder, using broad-spectrum antibiotics for this purpose is illogical. Combining a ß-lactam antibiotic with a particular inhibitor of bacterial ß-lactamase is another treatment method for ß-lactamase-producing bacteria. Inhibitors such as clavulanic acid are used. The inhibitor prevents the destruction of the co-administered antibiotic by inhibiting microbial lactamase in an irreversible manner but has extremely low levels of antibiotic action on its own. Inhibitors are most effective when used in conjunction with broad-spectrum penicillins such amoxicillin [62].

5.6 Types of Resistance

Impaired medication absorption, modified drug sights, drug suppression, and effective drug efflux are four main mechanisms by which microorganisms develop resistance to antimicrobials. Strategies of inherent resilience involve hampering absorption, while those of acquired immunity may entail modifying drug targets, suppressing them, or releasing them. Due to basic anatomical variations, the strategies used by gram-negative bacteria and gram-positive bacteria are different. All four major methods are used by gram-negative bacteria, while gram-positive bacteria seldom utilize uptake inhibition and lack the ability to produce specific kinds of drug efflux mechanisms [63, 64].

5.6.1 Intrinsic Resistance

Antibiotic resistance may be conferred to certain bacterial genera (or species) by virtue of particular structural/functional properties. These bacterial communities often lack the target location for the antibiotic, rendering it useless against them.

Mycoplasma spp., for instance, are resistant to beta-lactam antibiotics and glycopeptides because they lack a cell wall. In addition, the outer barrier surrounding bacterial cells prevents the antibiotic from penetrating the cells. The capacity of certain bacterial species to develop enzymes that are able to inactivate the antibiotics (for example, AmpC –lactamase in E. coli) may also contribute to antibiotic resistance [65].

5.6.2 Acquired Resistance

By exchanging genetic information with antibiotic-resistant bacteria, naturally resistant microorganisms may acquire resistance to such drugs. Acquired resistance often occurs via one of three main pathways [65].

5.6.3 Enzymatic Deviation or Antibiotic Inactivation

In both gram-positive and gram-negative bacteria, enzyme modifications and antibiotic inactivation are seen. One way that bacteria are able to counteract antibiotics is by a process called enzymatic modification, in which acetyl, adenyl, or phosphate groups are added to a particular spot on the antibiotic, rendering it chemically inactive and preventing it from binding to the target site. Phosphorylation, for instance, is seen in macrolides, while aminoglycosides undergo either acetylation, adenylation, or phosphorylation [66]. Enzymatic inactivation involves the hydrolytic cleavage activity of antibiotics (like ß-lactamases against penicillins and cephalosporins) [35, 67], which occurs as a result of direct binding of the bacterial enzymes to the antibiotics.

5.6.4 Decreased Intracellular Bactericidal Deposits

Bacteria may limit the aggregation of antibiotics within their cells in two ways: by decreasing the rate at which drugs enter into cells, or by increasing rate at which they leave cells, known as efflux. Antibiotics as tetracycline and β-lactam penetrate *E. coli* via the outermost membrane porin OmpF, whereas carbapenems enter *Pseudomonas aeruginosa* through the exterior membrane porin OmpD [65]. Porin genes have been down-regulated, structurally modified, or functionally deleted in gram-negative bacteria, resulting in a decreased inflow (also known as the permeability barrier against antibiotics). Antibiotics are actively secreted from the cells by multidrug transporters like the AcrAB-TolC transporter in E. coli [65, 68], which is typically utilized by virtually all microbial cells to bring hazardous compounds from cellular metabolism out of the cells. The major facilitator superfamily (MFS), ATP-binding cassette (ABC), and multidrug and toxic compound extrusion (MATE) families are all examples of transporter families that play a role in bacterial resistance [68, 69].

5.6.5 Antibiotic Target Region Modifications

Target locations have undergone alterations like as both gram-positive and gram-negative bacteria may acquire resistance to fluoroquinolones due to mutations in

the quinolone-resistance-determining region (QRDR) of the DNA gyrase (topoisomerase II and topoisomerase IV) [70, 71]. Erm methylases, which modify the chemical structure of RNA, have been shown to be successful in generating resistance to macrolides, lincosamides, and streptogramin B antibiotics in both gram-positive and gram-negative bacteria [72]. Methylation of the cfr gene is responsible for resistance in several bacteria [73, 74]. This includes the gram-positive and gram-negative bacteria *Staphylococcus, Enterococcus, Bacillus, Escherichia coli*, and *Proteus vulgaris.* Sulphonamide and trimethoprim resistance often involve the substitution of a drug-resistant target for the original, sensitive target. The genes sul1, sul2, and sul3 in gram-negative bacteria code for dihydropteroate synthases, which provide resistance to sulphonamides [65, 75]. Additionally, *Staphylococcus* spp. have the genes mecA and mecC, which encode for an alternative penicillin-binding protein with much less affinity for all ß-lactam antibiotics [75, 76].

5.7 Methods of Drug Resistance

Bacteria employ four main methods for combating antibiotics: (1) reducing the amount of medicine taken in; (2) altering the drug's target; (3) rendering the drug ineffective; and (4) engaging in active drug efflux. Limiting uptake, inactivation, and efflux are all mechanisms that can be used to achieve intrinsic resistance, whereas drug target modification, inactivation, and efflux are all processes that may be used to achieve acquired resistance. Gram-negative and gram-positive bacteria use distinct routes owing to the framework and additional differences. Gram-positive bacteria, typically deficient in LPS outer layer and drug efflux pathways [58, 59], embrace only two of the four major pathways [63, 64].

5.7.1 Decreasing Drug Uptake

The capacity of bacteria to restrict their own absorption of antimicrobial drugs varies naturally, as was previously noted. In gram-negative bacteria, the LPS layer's composition and activity prevent some substances from penetrating. This means that these bacteria have developed innate resistance to many kinds of potent antibacterial agents [77]. Mycobacteria contain a lipid-rich outer layer that facilitates of hydrophobic drugs as rifampicin and fluoroquinolones [78, 79]. B-lactams and glycopeptides are ineffective against Mycoplasma and other cell wall-deficient pathogens [80], as well as all other medicines that target the cell wall. Since gram-positive bacteria lack a cell wall, there is less of an effort to limit their access to antibiotics. *Enterococci* exhibit inherent resistance to aminoglycosides due to the difficulties polar molecules have in entering the cell wall. Recently, vancomycin-resistance emerged in *S. aureus*, another gram-positive bacterium. There are two ways that *S. aureus* defends itself against vancomycin, and one of them is an intermediate resistance provided by a

thicker cell wall that makes it hard for the antibiotic to penetrate the cell. VISA strains [78, 81] are the name given to these specific varieties. Porin pathways are often used by microbes possessing substantial exterior coverings to allow substances to enter inside the cell. The porin pathways in gram-negative bacteria may allow hydrophilic substances to enter the cell [82]. Reducing the total amount of porins or introducing mutations that alter the channel's selectivity are the two primary mechanisms by which porin alterations restrict drug absorption [83]. Reduction in the quantity of porins (and sometimes the cessation of synthesis of some porins) is a recognized mechanism by which members of the *Enterobacteriaceae* family acquire resistance. As a group, these bacteria exhibit carbapenem resistance by decreasing their porin number [84, 85]. Resistance to imipenem and certain cephalosporins has been shown in Escherichia coli due to mutations that alter the porin channel, and to β-lactams and tetracycline in Neisseria gonorrhoeae [84, 86].

5.7.2 Modification of Drug Targets

Antibiotics may operate on a broad number of receptors inside a germs cell, and germs can also modify a wide range of targets within cell to become resistant to such drugs. Resilience toward β-lactam drugs deployed predominantly against gram-positive bacteria might arise through alterations in composition or number of PBPs (penicillin-binding proteins). As transpeptidases, the PBPs regulate the production of peptidoglycan in cell exterior. Alteration in PBP abounding (the rise in PBPs with lower drug-binding capability, or a decline in PBPs with equivalent drug interaction) impacts the amount of drug accessible for adhering to substrate. If the arrangement of drug-binding protein is changed (for instance, PBP2a in S. aureus by adoption of mecA gene), the protein may lose some or all of its binding ability to drug [87]. The lipopeptides (like daptomycin) act by depolarizing the cell membrane, whereas the glycopeptides (like vancomycin) block cell wall formation. These antibiotics are ineffective against gram-negative bacteria (those with a thick LPS coating) [88]. *Enterococci* (VRE; vancomycin-resistant *enterococci*) and *S. aureus* (MRSA) are two bacteria that have developed resistance to the antibiotic vancomycin. To become resistant to vancomycin, bacteria must acquire van genes, which cause a change in peptidoglycan precursor composition and weaken the antibiotic's adherence to molecule [88].

5.7.3 Drug Inactivation

Both bacterial degradation and the addition of a chemical moiety to a medicine may reduce its efficacy. The beta-lactamases are an extremely diverse group of enzymes that hydrolyze many drugs. The tetX gene allows for the hydrolysis of tetracycline,

another medication that may be rendered ineffective in this way [89, 90]. Typically, acetyl, phosphoryl, and adenyl groups are transferred to the medication in order to render it inactive. Numerous transferases have been isolated and characterized. Acetylation is substantially popular approach since it has been found to be efficient toward a wide variety of medications. Several studies have shown that phosphorylation and adenylation are often used against the aminoglycosides [90–92].

5.7.4 β-lactamases

The beta-lactam class of antibiotics is the most popular choice for treating microbial infections. This class of drugs is characterized by a common structural feature: a β-lactam ring with four positions. Three broad processes contribute to the development of resistance to β-lactam antibiotics. The three main mechanisms of resistance are (1) extrusion of β-lactam medicines by efflux channels; (2) breakdown of drug by β-lactamase enzymes; and (3) the prevention of the interaction between the target PBP and the drug, usually by modifying the ability of the drug to bind to the PBP (this is mediated by alterations to existing PBPs or acquisition of other PBPs) [93, 94]. Penicillinases and cephalosporinases, now known as β-lactamases, are enzymes that hydrolyze a particular spot in the –lactam ring structure, rendering the medicine ineffective. Drugs with an open ring structure are ineffective in binding to PBP proteins. The ß-lactamases that are now known to exist are quite common and can deactivate all beta-lactam medications. Most gram-negative bacteria develop resistance to –lactam antibiotics, such as penicillin and cephalosporins, via the development of ß-lactamases [35, 89]. These enzymes might be acquired by the bacterium through plasmid or present on the chromosome. Gram-negative bacteria belonging to the family *Enterobacteriaceae* often have –lactamase genes on their chromosomes. *Aeromonas spp*., Acinetobacter spp., and Pseudomonas spp. are a few more gram-negative bacteria that have them. Some gram-positive bacteria, including *S. aureus, Enterococcus faecalis,* and *Enterococcus faecium*, may also carry β-lactamase genes on plasmids [63, 95]. However, this is not the case for the vast majority of gram-positive bacteria.

5.7.5 Drug Efflux

Bacterial chromosomes contain genes responsible for efflux channels. Whereas, few are constantly made, others are only made or released at higher levels in reaction to external stimuli or in the context of a specific substrate (high-level resistance is often due to a mutation that changes the transport channel). Many efflux channels will transport a wide range of molecules (multidrug [MDR] efflux channels), with their primary role being to cleanse the bacterial cell of hazardous substances. Carbon availability affects the resistance capabilities of several of such pumps [77, 96]. The

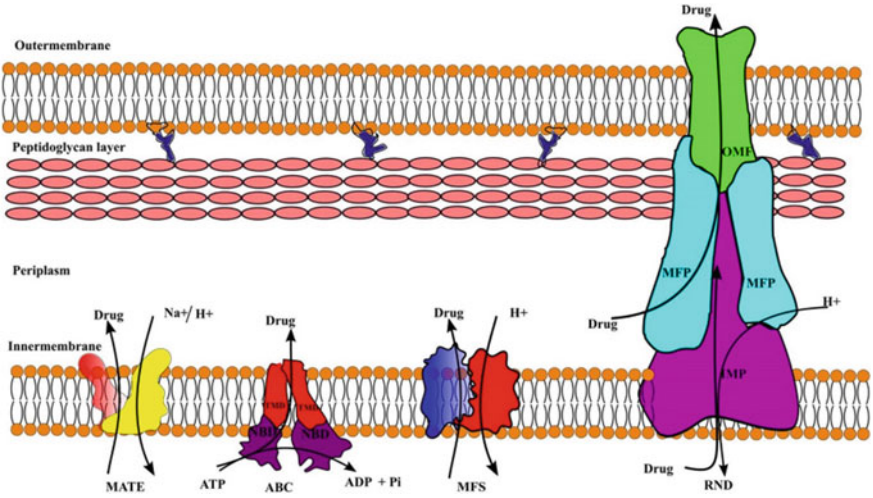

Fig. 5.6 Plasma Membrane View of Multidrug Efflux pumps in H. pylori. Reproduced with permission from Ref. [99]. Copyright 2021 Elsevier Ltd. All rights reserved. Selection and peer-review under responsibility of the scientific committee of the International Conference on Advances in Materials Research 2019

majority of bacteria have a wide variety of efflux channels types. Bacterial efflux channels can be broken down into five distinct families according to their structural similarities and energy requirements. These categories are the ATP-binding cassette (ABC) category, multidrug and toxic compound extrusion (MATE) category, the small multidrug resistance (SMR), the major facilitator superfamily (MFS), and resistance-nodulation-cell division (RND) category. Efflux channel families tend to be made up of single-component channel that shuttle substrates across the cytoplasmic membrane. Almost exclusively seen in gram-negative bacteria, multicomponent pumps belonging to the RND family efflux substrate over the whole cell envelope in concert with a periplasmic membrane fusion protein (MFP) and an outer membrane protein (OMP-porin) [77, 97, 98] (Fig. 5.6).

The efflux category of gram-negative bacteria includes many more members that may behave as multicomponent channels. MacB, an ABC group member, is a component of a tripartite pump (MacAB-TolC) that clears cell of macrolide antibacterials. EmrB, a component of the MFS, functions as a tripartite pump (EmrAB-TolC) in E. coli to facilitate the removal of nalidixic acid [100, 101].

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Chapter 6
Polymeric Nanoparticles for Bovine Mastitogens

Abstract Antibiotic-resistant "superbacteria" and "superfungi" have sparked worldwide concern and fear. Drug resistance is an increasingly urgent issue. The rate of recovery and lifespan rate of sick people may be greatly increased with the use of prompt detection and targeted destruction of germs. Therefore, it is very important to preserve human wellness by creating innovative, quick infection screening technologies, and extremely efficient antibacterial compounds. Due to their wide conjugated plane along primary chain, conjugate polymers (CPs) are able to absorb more light and boost signals more effectively than tiny molecule fluorescent materials. Moreover, CPs possess a greater capability to activate to ambient oxygen and produce ROS, making them particularly well-suited for applications such as antimicrobial photosensitive products and fluorescent detecting products. This is due to their adaptable structure, simple post-modification, significant light absorption limit, and superior light stability. Chitosan, a kind of natural polymer, is the most used nanocarrier for the delivery of antibiotics and other antibacterial medications. Possible antimicrobial strategies of Ch-coated silver and gold nanoparticles include breakdown of cell exterior wall and membrane, escape of intracellular electrolytes and low-molecular-weight proteins, the interaction of critical bacterial nutrients with Ch, blocking mRNA and amino acids formation via linking to microbial DNA, alteration of makeup and operation of protein molecules, and generation of reactive oxygen species (ROS). The rise of multidrug-resistant pathogenic microorganisms poses a significant risk to global public health. Therefore, there is a critical need for the rapid generation of new, effective, and safe antimicrobial mediators. Polymeric nanoparticles (NPs) represent the future of nanomedicine, which will enhance and ease the use of traditional medicines to aid people on a local and global scale.

Keywords Resistance · Polymers · ROS · Nanoparticles · Polymeric · mRNA

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6.1 Introduction

An ever-increasing number of microbes have developed from bacterial reservoirs, such as infections in animals or carriers, and subsequently spread to individuals, resulting in evolving pathogenic illnesses, during the last several decades. Organisms that threaten global human health are spreading at an alarming rate due to integration and climate disruption [1, 2]. Infectious illness death rates have been climbing steadily in recent years. Major impacts on public well-being and sanitation are seen when pathogenic microbes come into contact with people via the air, meals, water, or medical equipment [3, 4]. When it comes to the concealed hazards that compromise the well-being of humans, infectious bacteria and fungus are at the top of the list. Diseases triggered by infectious microorganisms have been successfully treated due to the development of several antibiotics throughout the past few years. However, there are currently very few efficient medications accessible for clinical care of very ill persons with drug-resistant bacterial diseases [5–8] owing to misuse of medications and the rise of the development of drug-resistant bacteria and fungi. Researchers have been trying to discover new antibiotics, but their progress has been far slower than that of antibiotic-resistant pathogens. Antibiotic-resistant "superbugs" and "superfungi" have emerged in certain regions [9, 10]. Antibiotic-resistant "superbacteria" and "superfungi" have sparked worldwide concern and fear. Drug resistance is an increasingly urgent issue. The rate of recovery and lifespan rate of sick people may be greatly increased with the use of prompt detection and targeted destruction of germs. Therefore, it is very important to preserve human wellness by creating innovative, quick infection screening technologies and extremely efficient antibacterial compounds. Due to their wide conjugated plane along primary chain, conjugate polymers (CPs) are able to absorb more light and boost signals more effectively than tiny molecule fluorescent materials [11]. Moreover, CPs possess a greater capability to activate to ambient oxygen and produce ROS, making them particularly well-suited for applications such as antimicrobial photosensitive products and fluorescent detecting products. This is due to their adaptable structure, simple post-modification, significant light absorption limit, and superior light stability [12].

Bioactive chemicals are notoriously prone to losing their pharmacological efficacy because of their insolubility and delivery method. The pharmacokinetics, bioavailability, biodistribution, metabolic stability, elimination safety, and relieving range of a drug must all be optimal [13], and thus it's important to get those aspects right throughout the drug development process. Antimicrobial medication half-life and bioavailability should be improved, while the dosage given should be decreased, in the setting of infectious disorders [14, 15]. Traditional antimicrobials are disseminated systemically through the circulation after injection, but a large proportion of the medication is rapidly cleared and inactivated. However, nanosystems loaded with drugs may remain in the bloodstream for much longer and then go directly to the desired organ or tissue. By administering the right amount of medicine, undesirable plasma variations and their consequences may be avoided. In addition, the nanoscale nature of these devices improves drug penetration across tissue barriers and protects

it until cellular absorption and tailored release [13, 14, 16, 17]. The basic methods implicated in regulated dispersion of antibiotics include diffusion-based evacuation, elution-based launch, and chemically or stimulus-controlled dissipate. Controlled release of drugs from nanosystems responds to many different kinds of inputs. Polymeric nanoparticles for antimicrobial applications bioactive chemicals are notoriously prone to losing their pharmacological efficacy because of their insolubility and delivery method. To be effective, medications must also exhibit optimal pharmacokinetics, bioavailability, pharmacokinetics, metabolic function, elimination, toxicities, and curative impact persistence [13], and thus it's important to get those aspects right throughout the drug development process. Antimicrobial medication half-life and bioavailability should be improved, while the dosage given should be decreased, in the setting of infectious disorders [14, 15]. Traditional antimicrobials are disseminated systemically through the circulation after injection, but a large proportion of the medication is rapidly cleared and inactivated. However, nanosystems loaded with drugs may remain in the bloodstream for much longer and then go directly to the desired organ or tissue. By administering the right amount of medicine, undesirable plasma variations and their consequences may be avoided. In addition, the nanoscale nature of these devices improves drug penetration across tissue barriers while simultaneously protecting it until cellular absorption and tailored release [13, 17]. The basic methods implicated in enforced distribution of antimicrobial agents include diffusion-based, elution-based, and chemically or stimulus-controlled liberation [16]. To that end, nanostructured systems seem like a promising strategy for fighting antimicrobial resistance and creating effective new therapies. For the reasons stated above, polymeric nanosystems are the best option for controlled and accurate drug release at the intended areas because of their biological suitability, medication retrieval, biological degradation, and durability all improve [18–20]. Collagen, chitosan, gelatin, and albumin are all natural sources from which polymeric nanosystems may be derived, as can polyethylene glycol, polylactic acid, poly(lactic-co-glycolic acid), and polycaprolactone (PCL) [18, 21]. Nanoparticles, micelles, vesicles, dendrimers, and hybrid inorganic-polymer nanosystems are just a few of the shapes, and they may take throughout development [22].

6.2 Polymeric Nanoparticles as Antimicrobials

Biosynthesis is essential for microbial development since it is the mechanism by which cell converts chemical substances that absorbs in through pores into biomass. A bigger cell size, DNA replication, and subsequent cell division into two sister cells are all results of a higher biomass [23]. Microbial proliferation may be broken down into two separate phases: the planktonic rise period, associated with free-swimming single-celled microbes that are not adhered to external areas and the biofilm proliferation stage, characterized by the development of a sessile community that is adhered to surfaces and exhibits increased resistance to external factors [23, 24]. Both microbial colonization and biofilm formation are concerning, but biofilm development is more

worrisome because it allows germs to persist in ulphateble conditions and spread to new sites, increasing the likelihood of chronic, life-threatening infections [24, 25]. For this reason, the following discussions focus on research aiming to use polymeric nanoparticles to inhibit the development of both planktonic bacteria and biofilms. Articles were selected based on their use of the terms "polymeric nanoparticles" and "antibacterial" or "biofilm" in Scopus-indexed studies published after 2018. This led to the identification of 24 relevant research, which were then sorted into categories based on whether or not the polymer utilized was natural or synthetic. Chitosan, a kind of natural polymer, is the most used nanocarrier for the delivery of antibiotics and other antibacterial medications. Gentamycin-coated phosphatidylcholine-chitosan hybrid nanoparticles were created, for instance, by Qiu et al. The lipid component was included due to the possibility of lipid medication carriers fusing with the bacterial phospholipid membrane. The findings showed that the synthesized system could stop the spread of both gram-positive and gram-negative bacteria and prevent biofilm formation [26]. The antimicrobial activities of gentamycin possessing chitosan nanoparticles were also studied by Alruwaili et al. [27], who then dispersed these nanoparticles in pH-sensitive Carbopol polymer solutions to generate sol–gel systems for ocular administration. Through ionic gelation and polyelectrolyte complexation, Ciro et al. [28] created chitosan-polyanion nanostructures filled by ampicillin. The anionic polyelectrolytes corresponded to potassium and sodium salts of poly(maleic acid-alt-ethylene) and poly(maleic acid-alt-octadecene). Synthesizing supramolecular polyelectrolyte complexes for antibacterial use, Evangelista et al. used the repulsion involving the $–SO_3$ units of carrageenan and positive $–NH^{3+}$ units of cyclodextrin-grafted chitosan. Since cyclodextrin may form host–guest inclusion complexes with silver sulfadiazine molecules, allowing the release of silver ions against bacterial cells [29], it was employed in this study. In a separate investigation, Walvekar et al. [30] looked into the efficacy of hyaluronic acid-oleylamine conjugates as drug nanocarriers against methicillin-resistant Staphylococcus aureus. In order encompass antimicrobial called vancomycin, polymersomes were utilized. These nanocapsules are made up of hydrophilic resins coupled by extended fatty acids and may construct themselves into round drug carriers. Oliveira et al. also developed a two-layer biomembrane for wound care; the primary surface was made of chitosan, hydroxypropyl methylcellulose, and lidocaine chloride; the subsequent covering was made of polymyxin B sulfate antibiotic-containing sodium alginate nanoparticles [31] also developed a two-layer biomembrane for wound care; the primary surface was made of chitosan, hydroxypropyl methylcellulose, and lidocaine chloride; the subsequent covering was made of polymyxin B sulfate antibiotic-containing sodium alginate nanoparticles [31].

For the administration of ampicillin, additional kind of polymeric nanoparticle is possible; these are complex polyelectrolyte nanoparticles generated from the polymeric salts obtained from Eudragit-E100TM and sodium salt of poly(maleic acid-alt-octadecene) [32]. These nanoparticles include an integral bactericidal function core made up of quaternary ammonium salt copolymers and small molecule iodine. Chitosan remains a desirable option for the development of antimicrobial remedies

due to its positively charged surface, which offers intrinsic microbicidal characteristics, and possibility to produce nanoparticles with dimensions below 200 nm. Because chitosan is a biodegradable, naturally occurring polymer, it may be safely injected into the body and used to manage the release of drugs without producing any harmful by-products [33].

6.3 In-Vitro Experimentation of Polymeric Nanoparticles

Antibiotic resistance among bacteria is a major health concern worldwide. In many cases, multidrug-resistant bacteria cause diseases that cannot be treated, resulting to the deaths of countless individuals throughout the globe [34]. According to World Health Organization, drugs-resistant diseases kill around 700,000 individuals annually; of these deaths, 230,000 are attributable to bacterial multidrug-resistant TB. Antimicrobial resistance has the potential to put an additional 24 million people into scarcity by 2030, and by 2050, drug-resistant illnesses might be responsible for 10 million annual fatalities. As a result, there is a pressing need for the discovery of new, secure, and efficient antibacterial medicines to manage multidrug-resistant bacteria and treat infectious illnesses. Potential and effective antibacterial agents that combat these issues might be Ch-coated polymeric silver and gold nanoparticles. There are several gram-positive and gram-negative harmful bacteria that are no match for the biopolymer Ch's potent antibacterial properties [35, 36]. Avadi et al. [37] reported that Ch has potent antibacterial action against virulent strains of *E. coli*. Researchers Costa et al. [38] found that bioactive Ch efficiently suppresses the development of six oral invasive microbial isolates with low microbial inhibitory concentrations (MICs). These strains included *Prevotella buccae* (CCUG 15,401), *Tannerella forsythia* (CCUG 51,269), *Aggregatibacter actinomycetemcomitans* (CCUG 13,227), *Streptococcus mutans* (CCUG 45). Using a total of 31 typical foodborne pathogens, Jiang et al. [39] studied antibacterial action of two water-soluble chitosans and found that chitosans utilized successfully suppressed most of the infections. The antibacterial activity of Ch has also been shown against a variety of pathogenic gram-positive and gram-negative bacteria [36, 40, 41]. There exist a variety of investigations showing that silver and gold nanoparticles are efficient [42, 43] in halting the expansion of multidrug-resistant bacteria. Huq [44] found that biosynthesized AgNPs using *Lysinibacillus xylanilyticus* MAHUQ-40 were effective against drug-resistant human infections as *Vibrio parahaemolyticus* and *Salmonella typhimurium*. Huq and Akter [45] demonstrated conceivable antibacterial properties of AgNPs synthesized by microbes toward multidrug-resistant diseases *K. engalia* and *S. enteritidis*. The microdilution technique was used to determine the minimum inhibitory and minimum bactericidal concentrations (MICs and MBCs), respectively [45]. Antibacterial activity against *B. subtilis*, *E. coli*, and *K. engalia* was studied by Hasnain et al. [46], who reported on the production of AuNPs induced by panchagavya extract. They discovered that biosynthesized AuNPs mediated by panchagavya extract were highly antibacterial against all three of these dangerous pathogens [47]. Even in trace amounts, silver

and gold as metals are poisonous [48]. Ch, a bioactive polymer, is notable for being nontoxic, biodegradable, biocompatible, low immunogenic, and hemostatic [49–51]. By conjugating bioactive Ch with either bioactive silver nanoparticles or bioactive gold nanoparticles, the efficacy and endurance of biologically active Ch will be substantially increased, despite the adverse effects of silver and gold nanoparticles will be considerably decreased. It has been reported by Potara et al. [52] that the addition of Ch strengthens the AgNPs and prevents them from aggregating. Furthermore, Ch raises AgNPs' surface charge, which enhances their binding to the negative charges on microbial cell surfaces [52]. Researchers Saha et al. [53] found that adding Ch to biosynthesized AuNPs improved their durability and performance. Biosynthesized Ch-AgNPs have been shown to be more effective against pathogenic bacteria than biosynthesized AgNPs, according to research by Shinde et al. [54]. Ch AgNPs were likewise proven to be nontoxic to normal cells. Both AgNPs and Ch-AgNPs were synthesized using Prunus cerasus leaf extract, and their antibacterial activity was tested against strains of bacteria that are resistant to many antibiotics. This investigation showed that compared to AgNPs alone, Ch-AgNPs were more efficient at inhibiting the development of multidrug-resistant pathogenic bacterial strains [54]. Ch silver nanocomposites mediated by plant extracts have been shown to be antibacterial against a variety of pathogenic bacteria, including *Klebsiella planticola* (MTCC 2277), *Bacillus subtilis* (MTCC 3053), *Enterococcus faecalis* (ATCC 8043), *Escherichia coli* (ATCC 8739), and *Pseudomonas aeruginosa* (ATCC 9083). The biosynthesized Ch-AgNPs exhibit substantial antibacterial activity because of the presence of small-sized silver nanoparticles on the surface of Ch [55]. This is evidenced by their efficacy against all of the pathogens tested. In order to inhibit the pathogenic *B. cereus*, *S. aureus*, and *E. coli*, Saruchi et al. [56] employed *Saccharum officinarum* plant extract to green synthesis Ch-AgNPs. They concluded that the biosynthesized Ch-silver nanocomposite might be employed as a medication to possibly control different pathogenic bacteria [56], based on their findings that the bionanocomposites are potentially extremely effective against all tested pathogenic strains of bacteria. Fuster et al. [57] investigated antimicrobial effectiveness of Ch-AuNPs toward four microbial strains: gram-negative *E. coli* ATCC 25,922 and medical strain of *E. coli* 11,046 (CI-EC), and gram-positive *S. aureus* ATCC 29,213 and methicillin-resistant *S. aureus* ATCC 43,300. When evaluated for a panel of harmful microbial strains, Ch-AuNPs demonstrated potent antimicrobial capacity, suggesting that these tiny structures may be effective in the battle toward microbial illnesses [57]. A number of AgNPs were produced by Rezazadeh et al. [58] and evaluated for antimicrobial properties toward four virulent microbial isolates (*E. coli*, *Proteus*, *Salmonella*, and *B. cereus*) employing disk diffusion method. Comparison of the antibacterial effects of a variety of AgNPs, including Ch-AgNPs mediated by algae extract (biological AgNPs), AgNPs mediated by algae extract (algae mediated AgNPs), only-Ch-mediated AgNPs, chemically synthesized AgNPs (chemical AgNPs), and $AgNO_3$ solution. In comparison with other AgNPs, algae-Ch extract, and $AgNO_3$ precursor, the findings demonstrated that the Ch-AgNPs (biological AgNPs) mediated by algae extract display better efficiency against all four chosen bacterial strains [58]. The Ch-AgNPs mediated by algal extract were

the most effective against all four pathogenic microbial isolates examined (*E. coli*, *Proteus*, *Salmonella*, and *B. cereus*), with zones of inhibition of 21, 20, 18, and 17 mm, respectively. The biomolecules that coat the surface of biological AgNPs are found in the extract of marine algae. It stands to reason that the antimicrobial efficacy of these bioactive AgNPs will improve after being coated with biopolymer Ch due to an increase in their biological applicability and biocompatibility [59]. Pagonis et al. investigated the photodynamic impacts of methylene blue (MB)-loaded poly(lactic-co-glycolic acid) (PLGA) NPs toward *E. faecalis* in vitro. In addition, the photodynamic impacts of such NPs on *E. faecalis* biofilms in extracted teeth were studied using a laboratory disease model. Through the use of TEM, we analyzed the absorption and distribution of NPs in dissociated *E. faecalis* after maturation by PLGA linked with colloidal gold granules for 2.5, 5, and 10 min. After 10 min of pretreatment with MB-loaded NPs, *E. faecalis* species in the planktonic phase and infected root canals were made photosensitive to red light at 665 nm. All three time points demonstrated a greater concentration of NPs on the surfaces of bacterial cell walls. Scientists discovered that when light was combined with MB-loaded NPs, microbial numbers dropped in both the root channels and planktonic period. A new investigation suggests that the utilization of PLGA NPs coupled with photoactive drugs might improve antibacterial dental therapy. Nevertheless, they also mentioned that serum proteins in growth media can affect the photodynamic outcomes of MB-loaded PLGA NPs [60]. An further study from 2014 looked at the efficacy of a novel photosensitizer (rose bengal functionalized chitosan NPs) in killing microorganisms in setting of root canal substances proven to impair the disinfectant's efficacy. Shrestha et al. produced CSRBnp and studied its characteristics (including dimensions, charge, and oxygen singlet emissions). The antimicrobial capacity of CSRBnp was tested on planktonic *E. faecalis* in the presence of several inhibitors, as dentin, dentin-matrix, pulp tissue, microbial lipopolysaccharides, and bovine serum albumin (BSA). Methylene blue, a cationic photosensitizer, and rose bengal [RB], an anionic photosensitizer, were used with inhibitors. Singlet oxygen release was shown to be slower for CSRBnp compared to methylene blue and RB. The authors of this study discovered that the pulp and BSA considerably reduced the bactericidal activity of all three photosensitizers used in photodynamic treatment. When tested 24 h after photodynamic treatment, CSRBnp showed a residual effect and completely eradicated the germs. According to Shrestha et al., in addition to having a higher adhesion for microbe cell interfaces and releasing singlet oxygen upon RB photoactivation, these properties allow CSRBnp to exert antimicrobial effects despite the context of tissue inhibitors. The researchers came to the conclusion that CSRBnp are an effective new antibacterial agent [61] for disinfecting root canals. In a separate research, Shrestha and colleagues looked into the feasibility of using bursting cavitation bubbles to transport antibacterial NPs into dentinal tubules, where they could enhance root canal disinfection. There were two phases to the experimentation: transporting the small porcelain particles along a simulated tubular medium was first investigated using exploding cavitation waves. To further explore this, we used root-dentin portions to evaluate the efficacy of 27 kHz HIFU for 2 min in transporting antimicrobial NPs inside dental tubules. Subsequently,

slices were analyzed using field emission scanning electron microscopy and energy-dispersive X-ray spectroscopy. In category 1's experiment, porcelain pellets were effectively fed down across the entire dimensions of tubular conduit by breaking cavitation bubbles with HIFU. In phase 2 trials, they showed that antimicrobial NPs were able to penetrate the dentinal tubules to thicknesses of up to 1 km owing to the bursting cavitation bubbles therapy with HIFU. This study proposed that crumbling bubbles of cavitation created by HIFU may be used to deliver antimicrobial NPs into the dentinal canals, hence improving decontamination in dentistry [62]. Triclosan is an antibacterial medication found to kill many types of bacteria responsible for tooth deposits. For the purpose of treating gum disease, triclosan-loaded nanoparticles (NPs) were produced from poly(d,l-lactide-co-glycolide), poly(d,l-lactide), and cellulose acetate phthalate (CAP) using the emulsification-diffusion method. The captivity effectiveness of solid NPs created by Pinon-Segundo et al. was more than 63.8%. PLGA-NPs had a larger mean diameter when TCS was added, and the temperature at which glass transitions was decreased as a result. In in vitro release studies, NPs quickly released TCS owing to their large contact region. TCS-NPs may help decrease gingival inflammation, according to preliminary in vivo study findings; nevertheless, additional study is required to determine the efficacy of such carriers as drug delivery vehicles for periodontal disease treatment [63].

6.4 Mechanism of Action of Polymeric Nanoparticles

The methods by which chitosan and its analogs exert antibacterial properties are not yet fully understood. Several variables acting in a sequential and autonomous mode are known to affect chitosan's antibacterial impact. In what follows, we'll talk about how exactly chitosan kills germs.

The antimicrobial capacity of Ch-doped polymeric silver or gold nanostructures is highly variable depending on many factors such as the variety of Ch employed, its molecular weight, form or dimension of silver or gold nanoparticles, molecular proportion of Ch to silver or gold nanoparticles, and production circumstances (pH, temperature, etc.) [57, 64]. As a result of interacting with negatively polarized outer membranes of gram-negative and gram-positive bacteria [57, 64], Ch and silver or gold particles have antimicrobial properties. Ch-coated polymeric silver or gold nanoparticles limit bacterial growth; nevertheless, the mechanism by which this occurs is not well-known. In order to explain how Ch may kill both gram-negative and gram-positive microbes, many hypotheses have been proposed. Hydrolysis of microbes cell exterior wall peptidoglycans and diffusion of intracellular electrolytes like potassium ions, peptides, and low-molecular-weight proteins are among the most likely outcomes of collision between positively charged Ch molecules (NH^{3+} groups) and negatively charged germs cellular layers [57, 65]. As stated by Sebti et al. [66], once Ch penetrates the nucleus of microbes via the cell wall, it binds to bacterial DNA and inhibits mRNA and proteins synthesis. One additional method is to use Ch

to chelate vital minerals for microorganisms [67]. Ch's strong metal-binding capacities, as discovered by Wang et al. [68], impact the association of various crucial metallic elements with Ch inside microbial cell and consequently limit microbial growth. Ch, a strongly charged bioactive polymer, improves microbial interaction, and silver or gold nanoparticles, both positively charged, breach the microbial cell barrier with increased effectiveness when employed simultaneously. Increasing the capacity for biocompatibility and antimicrobial impact of silver or gold nanoparticles by combining them with biopolymer Ch has been shown [65, 69]. Silver ions are negatively charged and may disrupt the makeup and operations of DNA and proteins by interacting with positively charged ions [64]. The antimicrobial properties of Ch-AuNPs have been postulated to be caused by electrostatic interactions between the particles and the membranes of bacteria [57]. When these molecules interact, they change the structure of microbial membrane, rendering it dysfunctional. There is yet no clear understanding regarding how these tiny particles achieve their antimicrobial activities. Possible antimicrobial strategies of Ch-coated silver and gold nanoparticles include breakdown of cell exterior wall and membrane, escape of intracellular electrolytes and low-molecular-weight proteins, the interaction of critical bacterial nutrients with Ch, blocking mRNA and amino acids formation via linking to microbial DNA, alteration of makeup and operation of protein molecules, and generation of reactive oxygen species (ROS). Using the strategies outlined above, [70] silver and gold nanoparticles coated with Ch may inhibit the growth of dangerous bacteria and finally kill them.

Too far, the mechanism by which chitosan and its derivatives inhibit bacterial growth remains unknown. Numerous factors, acting in a chronological and independent fashion, are known to regulate chitosan's antimicrobial properties.

6.4.1 Part of Chitosan

Chitosan's antibacterial action relies on its polycationic composition. The antimicrobial operation of chitosan and its metabolites is caused by electrostatic attraction among polycationic framework and solely anionic features of microorganisms' exterior (like gram-negative lipopolysaccharide and cell exterior amino acids) (Fig. 6.1), as the pKa of chitosan and its variants is smaller compared to pH of surroundings. Since the pKa of chitosan may be altered by the grafted groups of particular derivatives, leading to protonation at higher pH value [71], the polycationic structure arises needlessly under acidic environments. Chitosan's antibacterial function improves when its positive charge density is bolstered, as shown with quaternized chitosan [72–74] and chitosan metal complex [75–77]. However, chitosan's antimicrobial properties are compromised or eliminated if its polycationic characteristic is removed or inverted. Electrostatic interaction is also affected by number of amino acids that protonate at position C^{-2} on chitosan foundations. A high concentration of amino acids may improve antibacterial efficacy. So, naturally occurring chitosan with more substantial amount of disulfide (DD) has a more potent suppressive effect

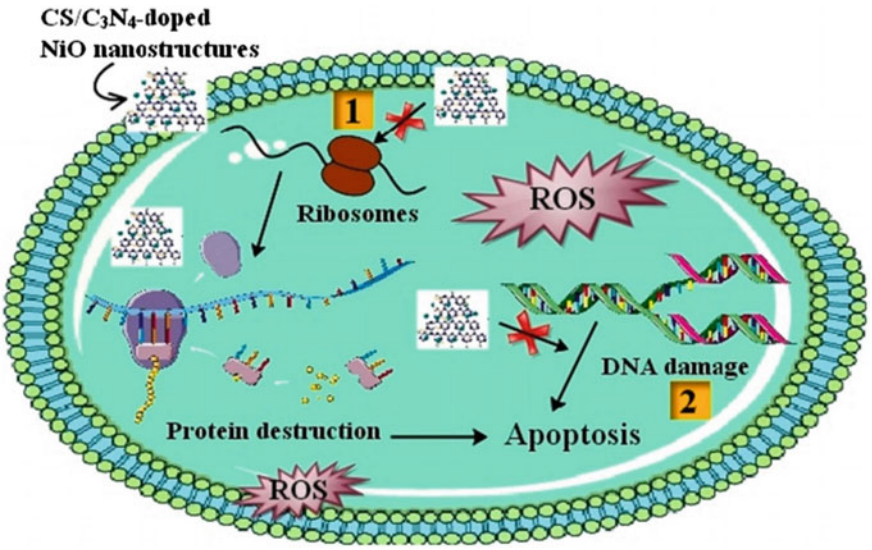

Fig. 6.1 Antibacterial activity of synthesized nanocatalyst. Reproduced with permission from Ref. [82]. Copyright 2022 Elsevier B.V.

than chitosan with a reduced DD. The carboxyl-negative charges within a microbial cell wall interface extensively with the positively-charged asparagine *N*-conjugated chitosan oligosaccharide [78]. Guanidinylated chitosan was produced by replacing amino with formamidine to increase the number of amino groups, and it was shown to be more effective against bacteria than chitosan itself [79, 80]. *N*-carboxyethyl chitosan, on the other hand, lacked any antibacterial action at doses up to 20 mg/mL [81], perhaps because of graft on the amino groups.

6.4.2 Part of Microorganism

The outer membrane (OM) of gram-negative bacteria is hydrophilic because it includes lipopolysaccharide (LPS). The lipid constituents and innermost portion of LPS molecules include anionic compounds (phosphate, carboxyl) that assist in the durability of LPS coating via electrostatic bonds with divalent cations [83]. When chelating substances like ethylenediaminetetraacetic acid remove these cations, the OM becomes unstable and LPS molecules are released. Gram-negative bacteria are highly resistant to hydrophobic medicines and harmful medications because the OM acts as a penetrating barrier against macromolecules and hydrophobic substances. Therefore, for a substance to have bactericidal action against gram-negative bacteria, it must be able to overcome the OM [84]. Gram-positive bacteria have teichoic acid (TA) and peptidoglycan (PG) in their cell walls. Gram-positive bacteria rely on the

polyanionic polymer TA, which crosses the cell wall to make contact with the phospholipid bilayer (PG). They may be anchored into the outer leaflet of the cytoplasmic membrane through a glycolipid (lipoteichoic acids, LTA) [85] or they can be covalently attached to *N*-acetylmuramic acid of the peptidoglycan layer (wall teichoic acids). The structural integrity of the cell wall is due to TA since it contains poly (glycerol phosphate) anion groups. Furthermore, it is essential for the activity of several enzymes that reside in membranes. LPS, an analogue of TA, has a comparable effect on the cell wall of gram-negative bacteria. The antibacterial properties of chitosan are highly correlated with features of the cell membrane. The surface of a bacterium is not like the smooth surface of a sphere because it is structurally complex and chemically diverse. Even bacteria lacking surface appendages like pili, fimbriae, or flagella nevertheless contain polymers that may protrude from the surface, such as lipopolysaccharide (LPS), mycolic acid, lipoteichoic acid (LTA), capsular polysaccharides (CPS), or proteins [86]. Strong attachment may be caused by short-range interactions of a different kind, such as hydrogen bonding [87], and these polymers are heavily involved in interactions with surfaces (called polymer interactions). Even if there is no overall attractive force between the cells, polymers may nonetheless cover great distances to bind them together [88]. Cell surface polyanions contribute to the electrostatic interactions between chitosan and its derivatives. More chitosan was absorbed by gram-negative bacteria because their cell surfaces had a larger negative charge [89]. This meant that the inhibitory impact of the chitosan was stronger against the gram-negative bacteria. The hydrophobicity of a cell surface is also important for bacterial interactions with surfaces, such as adhesion and floc formation [90]. Both gram-negative and gram-positive bacterial cell walls are vulnerable to antibacterial agents because of interactions at the cell surface, which damage the cell wall or outer membrane (OM) initially. Chitosan's ability to interfere with membrane functions in gram-positive bacteria may depend on its ability to cross the lipid bilayer of cell wall by LTA [85]. The LPS and proteins that make up gram-negative bacteria's external membrane (OM) rely on electrostatic connections with divalent cations to stay intact. As the pH is lower than the pKa of chitosan and its derivatives, polycations may compete with divalent metals for binding with polyanions. When the pH rises over the pKa value, however, chelation takes over. Substituting Mg^{2+} and Ca^{2+} ions for those that exist in cell wall may damage it's framework or reduce the capacity of degradative enzymes. Multiple techniques have provided evidence for the breakdown of cell wall structure. *N*-phenyl-1-naphthylamine (NPN) is a hydrophobic probe that is often not allowed in OM. NPN may divide into disturbed OM, as seen by a rise in fluorescence [59, 91–93], indicating that the OM has been damaged and is no longer functioning. Nano-indentation investigations utilizing atomic force microscopy to examine the antimicrobial properties presented impact of chitosan for both *E. coli* and *S. aureus*. Cells subjected to chitosan polymers demonstrated broader deflecting arcs than unaltered bacteria, demonstrating that depression or encapsulation of cells happened because of less rigid cells following therapies. These findings are consistent with a weakening of the cell wall as a consequence of cell wall damage or cell lysis [94]. When a cell membrane is no longer shielded by a

cell wall, it is vulnerable to environmental influences. As a result, membrane permeability may be substantially altered, which in turn can affect the activities of the cell membrane [84]. Cell membranes are essentially negatively charged bilayers of phospholipid; however, chitosan would slightly change their accessibility. As a result of the enforceable, the surface charge of microbe is soon nullified and possibly inverted [95]. Denatured membrane proteins and phospholipid bilayer penetration may be initiated by subsequent interactions. Because of the increased membrane permeability, the cell membrane becomes unstable, intracellular chemicals seep out, and the cell dies. Antibacterial actions are thought to involve membrane proteins. Indirectly reflecting the shift in membrane composition, one CM-based investigation discovered conformation modifications of membrane proteins. OCMs altered the structure of membrane proteins, exposing phenylalanine (Phe) residues found within, as shown by an increase in the fluorescence of the bacterial solution after treatment [84]. It has been shown that whey protein inhibits chitosan's antibacterial action [96]. This finding is consistent with the fact that chitosans (regardless of Mw) will be used only in foods with low protein content [97]. Intracellular components leaking out is a sure sign that the bacterial cytoplasmic membrane has been compromised. Increased absorbance at 260 nm [95], electrical conductivity of cell suspension [84], and cytoplasmic–galactosidase release [59, 91–93] are all indicators of leakage. Microscopic pictures also showed membrane breakdown in the cells [84, 94]. Leakage from cationic biocides occurs in a specific order: first, LMw compounds like potassium ions and phosphate seep out, followed by nucleotides like DNA, RNA, and other components [98, 99]. Phospholipids and proteins are likely the chitosan interaction targets on the cell membrane [84, 95]. There are three main ways in which cationic polymers interact with negatively charged lipids [100]. Polylysine is an example of the first kind of surface binding, which seems to occur owing to the electrostatic repulsion between like-charged molecules. This contact is negligible in terms of its effect on the lipid's phase transition temperature and may be eliminated by increasing the ionic strength. The polymer expands, the phase transition temperature drops, and the membrane's permeability is changed as a result of the second kind of interaction, which also includes charge interactions and the polymer penetrating the bilayer. Polymers may fully disrupt the membrane in the third mode of contact, preventing the lipids from going through a phase transition. Micelles or other aggregates made up of both polymer and lipid may develop under these conditions. The data presented so far suggests that chitosan's action is connected to phase separation, like that of other cationic biocides [95]. The biocidal effect of chitosan and its derivatives may be explained by the roles played by the polymers in each of the component processes. A cationic biocide's fatal effect, for instance, may be broken down into the following simple events: Adsorption onto the surface of the bacterial cell, diffusion through the cell wall, attachment to the cytoplasmic membrane, rupture of the cytoplasmic membrane, cytoplasmic component leakage, and finally cell death [101]. While many researchers have looked at the method of action from a morphological and physicochemical perspective, very few have taken a molecular perspective. From a metabolic and energy-transit perspective, molecular techniques and considerations are anticipated to provide a sneak view into the mechanism of antibacterial activity.

Recent research [85] examined the transcriptional response pattern to chitosan and found that the level of expression for 166 open reading frames changed significantly (at a false discovery rate of 0.64%). The genetic expression profiles clearly showed that the bacterial growth rate was decreased after chitosan treatment. This information on transcriptional responses is circumstantial proof that chitosan therapy disrupts cellular energy metabolism. Chitosan is thought to cause secondary cellular effects after binding to cell wall polymers. Microbial surface integrity is destabilized and disrupted (by unclear means), allowing cellular constituents to seep out despite inducing the creation of discrete pores in outer layer. Oxygen reduction is hampered, and the cells are forced to switch to anaerobic energy production, both of which are consequences of the disruption of appropriate functional structure of electron transport chain. The cellular machinery as a whole might become dysfunctional as a result [100].

6.5 Polymeric Nanoparticles: A Future Prospective

The misuse and abuse of antimicrobial medications have resulted in microbial resistance, which has become a crucial and severe health concern that has resulted in many fatalities throughout the globe. New, potentially more effective antibacterial agents have been developed with the use of nanotechnology. Polymeric nanoparticles in particular have been the subject of much study because of their dual role as a drug nanocarrier and an antibacterial agent, which they may do by either passive or active targeting of certain microbial strains. Many different types of microorganisms are being studied for their potential use in antimicrobial medicines. Due to the absence of effective antiviral medicines, the present COVID-19 pandemic crisis has highlighted the need for further development of polymeric nanoparticles for use in such applications. The coronavirus might be effectively destroyed by polymeric nanoparticles loaded with an antiviral drug, which would then be released in sufficient numbers to eradicate the infection. The benefits of polymer-based nanoparticles improved biological compatibility, biodegradability, and elimination from human bodies, for example must be balanced against the limits of the presently available systems. In particular, the production process is made more difficult by the dimension and size distribution of polymeric nanoparticles. One such option that might result in the creation of more uniform and diminutive nanoparticles is the use of microfluidic techniques. Further, by enabling drug encapsulation and functionalization in a single process, typical synthetic reaction stages might be eliminated, and nanoparticle stability could be optimized by control of surface charge (Fig. 6.2).

The rise of multidrug-resistant pathogenic microorganisms poses a significant risk to global public health. Therefore, there is a critical need for the rapid generation of new, effective, and safe antimicrobial mediators (Fig. 6.3).

Biodegradable, biocompatible, highly active, stable, and little poisonous, Ch-coated polymeric silver and gold nanoparticles are a new class of bioactive hybrid materials in medicine. Ch is an effective bactericidal biopolymer and biocompatible

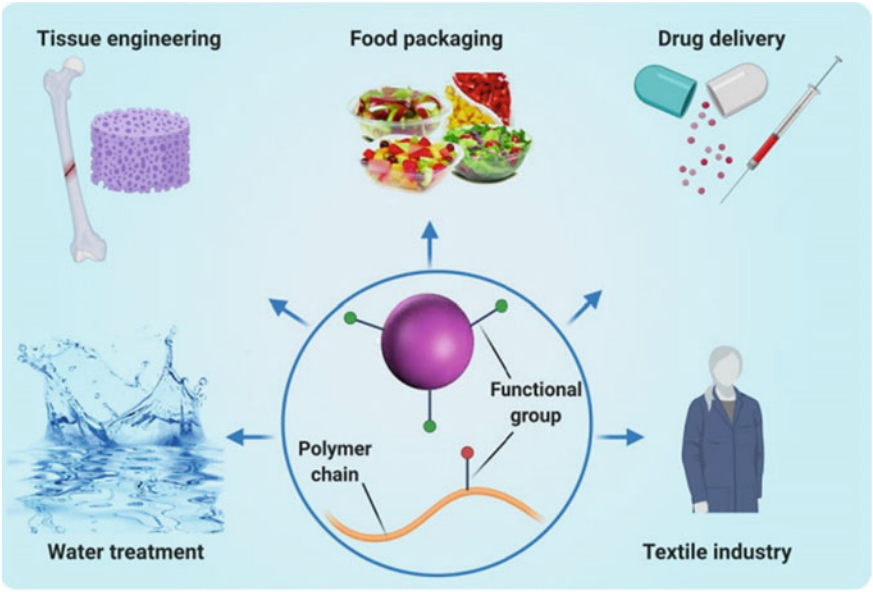

Fig. 6.2 Fields related to polymer functionalization. Reproduced with permission from Ref. [102]. Copyright © 2020, The Author(s)

polymer that may be used to treat a broad range of microbial infections. Biosynthesized silver and gold nanoparticles (AgNPs and AuNPs, respectively) are very effective bactericidal agents across a diverse array of infectious microorganisms, even those impervious to many antimicrobial classes. The harmony, contaminants, and efficacy of Ch-coated nanomaterials are all enhanced by coupling with silver or gold nanoparticle (AgNPs or AuNPs). Recent studies validated the superior stability and efficacy of Ch-coated silver and gold nanoparticles toward pathogenic microorganisms. Biosynthesized polymeric silver or gold nanoparticles with a Ch-coating have been stressed for their antimicrobial qualities. Ch-doped polymeric silver or gold that has been biosynthesized has certain drawbacks.

First, the Ch type and molecular weight, Ch concentration, Ag/Au salt concentration, and plant/microbe extract concentration. Both the synthesis and the antibacterial activity are affected by these variables. Second, plants and microbes with therapeutic or pharmacological properties, as well as probiotics and other helpful microorganisms, should be used in the production of Ch-coated silver or gold nanoparticles. Third, the best possible conditions for synthesis should be maintained, including temperature, pH, time, etc. However, it is critical to investigate potential cytotoxic influence on human cells during biosynthesis, since some studies have shown Ch-coated polymeric silver or gold nanoparticles to be harmless and secure for usage. Subsequently, Ch-coated polymeric silver and gold nanoparticles may prove to be a valuable asset in the field of nanomedicine for the treatment of MDR-pathogens [70].

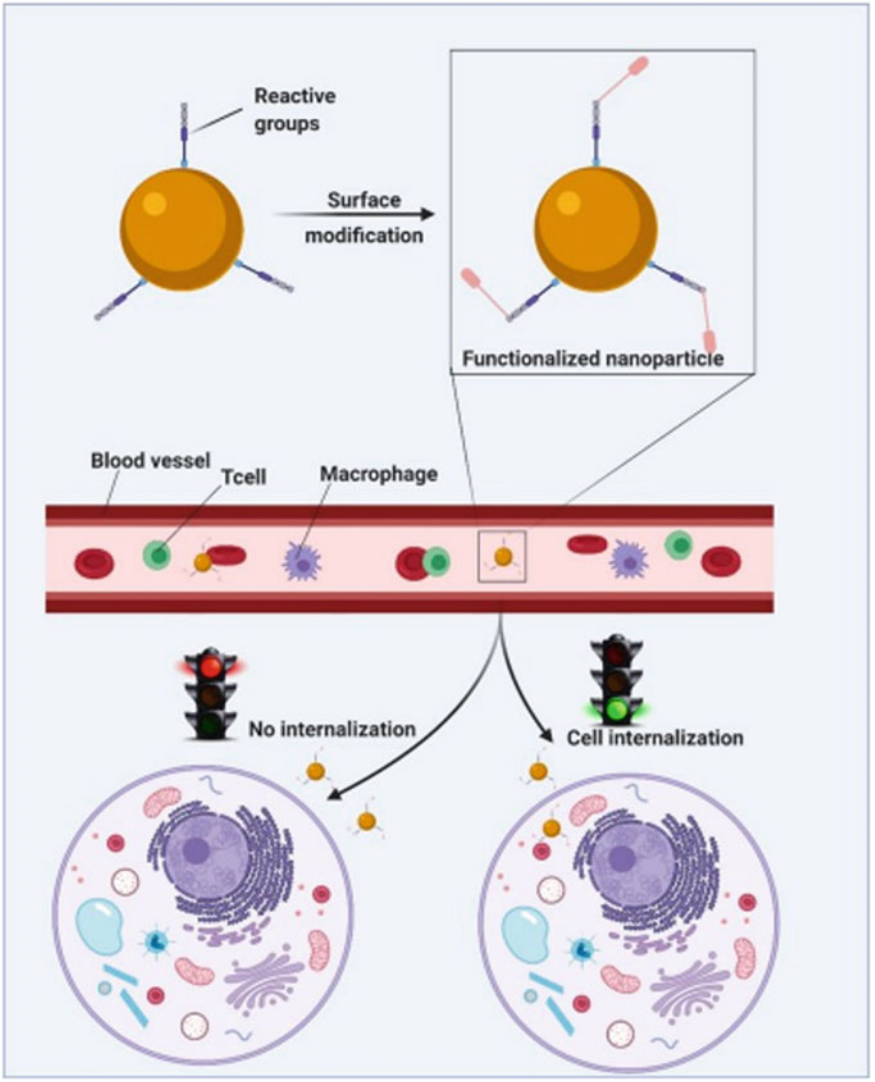

Fig. 6.3 Effect of surface functionalization on cell internalization. Surface-modified nanomaterials could allow or avoid cell internalization. Reproduced with permission from Ref. [102]. Copyright © 2020, The Author(s)

Some of the problems that will need to be fixed in order for polymeric nanothernostics to progress are as follows: (a) divergences in adding and monitoring because of the conjunction and attributes of chemotherapy medications and monitoring chemicals; (b) steric blockages owing to adherence of drug molecules with intended ligands, that can disrupt with attaching need of nanoformulation, triggering reduce selectivity; and (c) inadequate solubility owing to hydrophobic substances molecules packed on

silica surface [103]. However, these concerns will be included into the theranostic study in order to overcome the technical obstacles that stand in the way of actual clinical applications. There will also be a transition from "anatomical imaging" to "molecular imaging" in the imaging approaches that make use of nanoformulations. All cancers caused by individual gene mutations will be curable when these cutting-edge medicines have been developed [104].

The biopharmaceutical sector may have found a realistic and promising technique in nanoparticulate drug delivery devices. When compared to more standard medication delivery methods, they come out on top. They improve the solubility, permeability, and bioavailability of many powerful medications that would be difficult to administer orally without them. Increased patient compliance and fewer missed doses are two additional benefits of nanoparticle medication delivery systems. Numerous biological agents with low water solubility, permeability, and bioavailability may benefit from nanoparticulate drug delivery technologies in the near future. By maintaining stability and retaining the structure of these medications, nanoparticles help mitigate some of their specific challenges. Also, nanoparticles allow for targeted distribution and regulated release, making for a highly clever therapy [105].

Polymeric nanoparticles (NPs) represent the future of nanomedicine, which will enhance and ease the use of traditional medicines to aid people on a local and global scale. We should expect significant advancements in disease detection, therapy, and prevention as a result of ongoing preclinical and clinical research on polymeric NPs [106].

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Index

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