Suresh Kumar Gahlawat
Joginder Singh Duhan · Raj Kumar Salar
Priyanka Siwach · Suresh Kumar · Pawan Kaur
Editors

Advances in Animal Biotechnology and its Applications

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Preface

The book, entitled *Advances in Animal Biotechnology and its Applications*, is a compilation of 22 chapters written by highly experienced authors from leading universities of India related to genomics, bionanotechnology, drug designing, diagnostics, therapeutics, food and environmental biotechnology, etc. These chapters have been divided into five sections, and the text is more than a compendium of information. These chapters will cover the fundamentals of technical advances in genome editing, gene silencing, in silico drug designing, nanoparticles, tissue engineering, cryopreservation, molecular approaches in diagnostics and therapeutics, etc. The contents are highly informative and useful and will provide an overview of prospects and applications while emphasizing modern, cutting-edge, and emerging areas of animal biotechnology. A list of references at the end of each chapter is provided for the readers to learn more about a particular topic. Typically, these references include basic research, research papers, review articles, and articles from the popular literature. This book will be useful for students, teachers, and researchers in the various disciplines of life sciences, agricultural sciences, medicine, and biotechnology in universities, research institutions, and biotech companies.

The editors are grateful to the contributing authors for providing necessary expertise and commitment. Without their contribution, this book would not have been possible. Our sincere thanks go to the University Grants Commission, New Delhi, for sponsoring the National Conference on Biotechnology: Emerging Trends, which was organized by the Department of Biotechnology, Chaudhary Devi Lal University, Sirsa, India, during February 2016 and attended by most contributing authors. Last but not least, we also thank the university administration and our family members for their constant support and encouragement while we were working on this project.

Sirsa, Haryana, India

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About the Editors

Dr. Suresh Kumar Gahlawat is Professor and Ex- Dean, Faculty of Life Science at Chaudhary Devi Lal University, Sirsa, India. Renowned for his pioneering work in the fields of Marine Biotechnology, Bacteriology, and Virology, he has published more than 60 manuscripts in various national and international peer-reviewed journals. He also serves as a referee for a number of international journals, including the *International Journal of Aquaculture*, Netherlands, and the *Journal of Applied Microbiology*. In addition, he has chaired numerous national and international conferences, symposia, workshops, and technical meetings and has delivered a number of keynotes and invited lectures. He is an active member of various international scientific organizations and societies including the Association of Microbiologists of India. To date, he has authored more than 10 books and manuals in the field of Biotechnology and Fisheries. He is the recipient of several research, teaching, and service awards, including DBT Overseas Associateship and Boyscasts fellowship.

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Part I

Genomics

1 Prediction Expression Analysis and Target Validation of Bacterial Small RNAs

Puneet Kaur and Praveen P. Balgir

1.1 Introduction

Small regulatory noncoding RNAs (sRNAs) other than transfer (tRNA) and ribosomal (rRNA) ranging from approximately 50 to 500 nts in length are encoded by intergenic regions (IGRs) and are transcribed from their own promoters. Their transcription usually terminates at a strong Rho-independent terminator. They regulate gene expression by base pairing with other RNAs with or without protein partners like Hfq, Csr, etc. The base pairing could be partial or imperfect in case of *trans*-acting sRNAs, while *cis*-acting sRNAs have regions of perfect complementarity with the target RNAs. In earlier studies, sRNAs were thought to be *trans*-acting and noncoding in nature, which usually required protein partners like RNA chaperone protein, Hfq, to modulate translation or stability of target mRNAs, but later many *cis*-acting, coding, and Hfq-independent sRNAs came to light (Liu and Camilli 2010). It is well established that these sRNAs are induced and differentially expressed under stress or specific environmental conditions indicating their involvement in adaptation to changing conditions (Gottesman et al. 2006). They are involved in many cellular processes like iron homeostasis, carbon metabolism, quorum sensing, sporulation, virulence, stress response, etc. (Michaux et al. 2014b). These sRNAs have attracted scientists from all over the world since their discovery in *Escherichia coli* in the year 2001 (Argaman et al. 2001; Rivas et al. 2001; Wassarman et al. 2001). Fifteen years later, the field has expanded immensely, and today, it boasts of databases like sRNAMap (Huang et al. 2009), sRNATarBase (Cao et al. 2010), BSRD (Li et al. 2013), and sRNAdb (Pischimarov et al. 2012) that are available along with a long list of organisms explored for sRNAs. The present chapter is focused on the different in silico tools for prediction of putative sRNAs along with techniques and strategies used to isolate and validate them. Further, tools and techniques for prediction and validation of their targets are discussed (Fig. 1.1).

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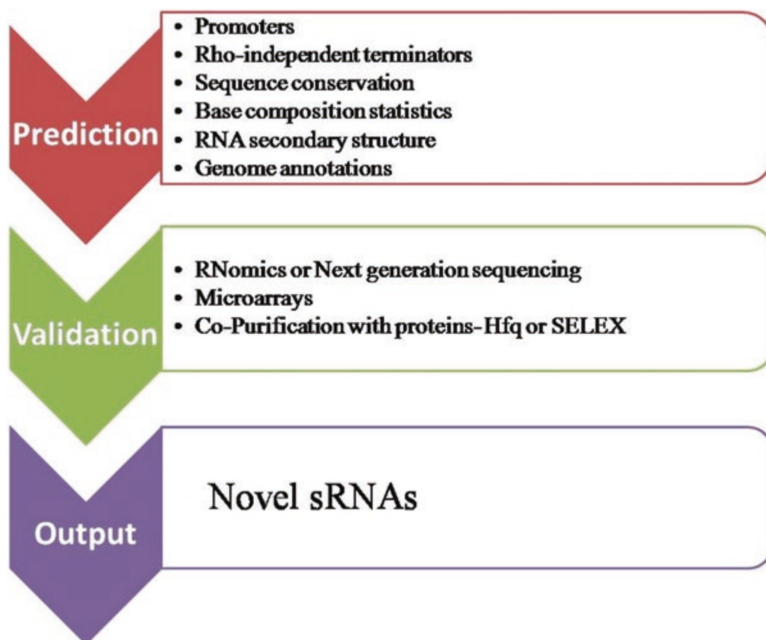

Fig. 1.1 Overview of prediction and validation strategies used for sRNA identification

1.2 sRNA Prediction and Validation

1.2.1 In Silico Prediction of Small RNA-Encoding Genes

The accidental discovery of ~12 sRNAs in *E. coli* (Gottesman 2004) paved the way for systematic bio-computational searches for sRNAs by several groups in 2001. Argaman et al. (2001) predicted sRNAs in *E. coli* based on the criteria: presence of sigma 70 promoter and Rho-independent terminator in IGRs, sequence conservation among closely related species (*Salmonella*, *Yersinia*, and *Klebsiella*), and the genomic location of putative sRNA genes to discriminate and exclude conserved mRNA leaders or trailers from sRNA list. These criteria lead to the prediction of 24 sRNA candidates, out of which 23 were experimentally tested with northern blotting and 5′ and 3′ RACE and 14 were confirmed. Wassarman et al. (2001) used a similar criterion in combination with co-immunoprecipitation and microarray experiment and listed 59 candidate sRNAs. Northern analysis confirmed 23 sRNAs of which 17 were considered as novel sRNAs and 6 were predicted to be small protein-coding genes based on the presence of putative Shine-Dalgarno sequences and reading frame conservation.

The increasing availability of complete genome sequences has led the way for in silico searches of sRNA in different bacteria. Genome-wide searches for putative sRNA encoding genes included many features such as conserved intergenic sequences, promoters, terminators and GC-rich regions. Step by step searches limit

the rate at which these searches can be conducted. To address this problem, novel algorithms were developed to conduct genome-wide predictive searches for sRNAs, but the ratio of predicted to confirmed sRNA is low, and a large number of sRNAs are waiting to be verified experimentally. Some algorithms and softwares for sRNA predictions are described below.

QRNA developed by Rivas et al. (2001) is powerful computational approach to detect sRNAs based on secondary structure conservation. This was the first widely used computational software for sRNA predictions. It uses three probabilistic models, i.e., for RNA-structure-constrained, coding-constrained, and position-independent evolution, to examine the mutation pattern in a pairwise sequence alignment which is further classified as coding or noncoding RNA according to the Bayesian posterior probability. It can be downloaded from http://www.genetics.wustl.edu/eddy/software web address. Rivas et al. (2001) developed and implemented QRNA to screen the complete genome of *E. coli* K12 MG1655 for sRNA candidates and predicted 275 candidates. Of these, 49 were assayed experimentally and 11 were found to express small transcripts of unknown function. Here, the Northern analysis was limited to only one growth condition, i.e., exponential phase, as compared to broader set of growth conditions in earlier two studies which could have increased their number of confirmed candidates. QRNA algorithm was applied for predicting sRNAs in *Bacillus subtilis* (Silvaggi et al. 2006), *Burkholderia cenocepacia* J2315 (Coenye et al. 2007), *Streptococcus pyogenes* (Tesorero et al. 2013), *Deinococcus radiodurans* (Tsai et al. 2015), etc.

Pichon and Felden (2003) developed a computational tool – Intergenic Sequence Inspector (ISI) – that helped in identifying bacterial sRNAs. It automatically extracted intergenic sequences from genome sequence files of NCBI and used BLAST to compare these sequences with those of closely related species and finally displays sequence and structural signatures of RNA genes including putative promoters, terminators, RNA secondary structure predictions, and percent GC content. It is available for download at web address – http://www.biochpharma.univ-rennes1.fr/. It was used for sRNA prediction in *Staphylococcus aureus* (Pichon and Felden 2005) and *Sinorhizobium meliloti* (Del Val et al. 2007).

Washietl et al. (2005) developed RNAz program which combined comparative sequence analysis and structure prediction for predicting new sRNAs. Their approach consists of combining primary sequence homologies and thermodynamic stability of secondary structure to score and detect putative sRNA sequences. The program can be downloaded from web address – www.tbi.univie.ac.at/~wash/RNAz. *Sinorhizobium meliloti* (Del Val et al. 2007), *Synechocystis* sp. PCC6803 (Voss et al. 2009), *Shigella flexneri strain 301* (Peng et al. 2011), and *Saccharopolyspora erythraea* (Liu et al. 2013) are few bacteria for which RNAz has been applied for sRNA predictions.

sRNAPredict (Livny et al. 2005) is a complete software for sRNA prediction. This program identifies candidate loci by extracting the sequences based on regions of sequence conservation and intrinsic transcription terminators from output files of programs such as BLAST, RNAMotif, and TransTerm. sRNAPredict is available for download at http://www.tufts.edu/sackler/waldorlab/sRNAPredict/. An improved

version of this tool named sRNAPredict2 was used to predict several new sRNAs in 10 different pathogenic bacterial species and verified 17 sRNAs in *Pseudomonas aeruginosa* (Livny et al. 2006). This tool has been applied for sRNA prediction in other bacteria such as *Clostridium acetobutylicum* (Chen et al. 2011), *Streptococcus pneumoniae* (Tsui et al. 2010), *Streptococcus mutans* (Xia et al. 2012), *Streptococcus pyogenes* (Tesorero et al. 2013), etc.

SIPHT (sRNA identification protocol using high-throughput technologies) is also a very powerful tool for sRNA prediction (Livny et al. 2008). It utilizes automatic workflow to enable a single user to identify sRNAs. This is the web interface to sRNApredict3 software that identifies candidate intergenic loci based on the intergenic conservation and Rho-independent terminators and then annotates each locus for many features that provides information regarding the strength of its prediction and potential biological functions. SIPHT is available at http://bio.cs.wisc.edu/sRNA. SIPHT has been successfully applied to predict sRNA in a number of bacteria, viz., *Burkholderia pseudomallei* (Khoo et al. 2012), *Brucella abortus* (Dong et al. 2014), *Zymomonas mobilis* (Cho et al. 2014), *Pectobacterium atrosepticum* (Kwenda et al. 2016), etc.

Nucleic acid phylogenetic profiling (NAPP) is also a web interface which performs high-throughput noncoding sequence identification and functional characterization (Marchais et al. 2009). It clusters noncoding sequences based on their conservation profiles in a large number of genomes and identifies noncoding RNAs by their inclusion in clusters enriched for known noncoding RNAs. NAPP was used for sRNA identification in *Staphylococcus aureus* and 48 sequences meeting a set criterion were chosen, out of which 24 were randomly selected for experimental validation using Northern blotting to confirm 7 novel sRNAs. It was used for sRNA prediction in *Brucella abortus* (Dong et al. 2014), *Burkholderia thailandensis* (Stubben et al. 2014), etc.

Raasch et al. (2010) integrated existing methods and tools for small RNA detection as Moses software. BLAST, word frequency analysis (for GC-content analysis), RNAz (using ClustalW for alignments), RNAfold, Dynalign are the modules that Moses provide. Twenty sRNAs in *Streptococcus pyogenes* NZ131 were predicted using this tool, of these, four were experimentally verified using RT-PCR (Raasch et al. 2010). It is available at http://www.sbi.uni-rostock.de/moses.

Another computational tool, sRNA scanner, was developed by Sridhar et al. (2010) based on position weight matrix (PWM). It uses pre-computed PWM derived from *E. coli* K-12 sRNA promoter and rho-independent terminator signals to identify intergenic small RNAs through sliding window based genome scans. sRNA scanner is available at http://bicmku.in:8081/sRNAscanner or http://cluster.physics.iisc.ernet.in/sRNAscanner/. The tool has been used to predict sRNAs in bacteria like *Bacillus cereus* (Babu et al. 2011), *Burkholderia pseudomallei* (Khoo et al. 2012), *Streptococcus pyogenes* (Patenge et al. 2012), *Streptococcus mutans* (Zeng et al. 2013), etc.

NOCORNAc (noncoding RNA characterization) developed by Herbig and Nieselt (2011) also facilitates the automated prediction and characterization of functional ncRNAs. It integrates SIDD model (stress-induced duplex

destabilization) for transcription start site prediction and TransTermHP for Rho-independent terminator prediction. It can also perform RNA-RNA interaction predictions using IntaRNA. The searches are not restricted to intergenic regions for prediction of ncRNA transcripts. The software is available at http://www.zbit.uni-tuebingen.de/pas/nocornac.htm. NOCORNAc has been successfully applied for sRNA predictions in *Streptomyces coelicolor* (Herbig and Nieselt 2011), *Staphylococcus equorum* (Schuster et al. 2013), and *Saccharopolyspora erythraea* (Liu et al. 2013).

1.2.2 Microarray-Based Detection and Validation of sRNAs

Microarrays are a powerful method for identifying new sRNA candidates and studying their expression patterns. Arrays for sRNA detection are required to include probes specific to IGRs as well as open reading frames from both sense and antisense strands for efficient identification. The first use of microarray for sRNA detection came in 2001 when Wasserman and co-workers used high-density oligonucleotide probe array in combination with comparative genomics and identified growth phase dependent sRNAs in *E. coli*. In another approach, Zhang et al. (2003) used RNA isolated by co-immunoprecipitation with global sRNA regulator Hfq to detect sRNAs directly on microarrays. The sensitivity of this method was demonstrated by detection of oxidative stress-induced sRNA-OxyS present in very low concentrations under growth conditions used in the study. In two more cases, size fractionation was used for sRNA enrichment before library construction. The newly discovered sRNAs were missed in previous bio-computational searches as they were not conserved through different *E. coli* strains and were not encoded from IGRs (Vogel et al. 2003; Kawano et al. 2005). Some research groups used low-density arrays with probes designed for defined set of sRNA, mRNA, tRNA, and rRNA regions. While others used high-density arrays which cover both sense and antisense strands of whole genome including IGRs, low-density arrays were used by Pichon and Felden (2005) to monitor sRNA expression patterns of four different pathogenic strains of *Staphylococcus aureus*, viz., MRSA252, COL, N315, and 502A. Other examples of the use of low-density arrays include *Bacillus subtilis* (Silvaggi et al. 2006), *Streptomyces coelicolor* (Panek et al. 2008), and *Enterococcus faecalis* (Shioya et al. 2011) where arrays were designed for IGRS only in case of *Bacillus subtilis* and *Enterococcus faecalis*, and for *Streptomyces coelicolor* only bio-informatically predicted sRNA genes were spotted on arrays. Low-density arrays are a cost-effective alternate to high-density arrays, but high-density arrays are preferred by scientists for accurate analysis of sRNAs as it can help discriminate sRNAs encoded by intergenic regions from processed untranslated regions. Examples of whole genome tiling arrays are shown in Table 1.1.

Some researchers used two-color microarray to distinguish small RNA fraction from that of long RNA fraction by labeling the former with Cy3 and latter with Cy5 fluorescent markers. This two color microarrays were used for validating sRNAs in *Sinorhizobium meliloti* (Valverde et al. 2008; Schluter et al. 2010). Although,

Table 1.1 List of microorganisms explored for sRNAs using whole genome microarrays

S. no.	Species	Predicted	Tested	Verified	Identification approach	References
1.	*Burkholderia cenocepacia* J2315	213	213	4	Comparative genomics approach, microarray, qPCR	Coenye et al. (2007)
2.	*Caulobacter crescentus*	300	27	27	Microarray, Northern analysis, 5′ RACE	Landt et al. (2008)
3.	*Streptococcus pyogenes* M1T1	75	32	7	Microarray, qRT-PCR, western blot	Perez et al. (2009)
4.	*Rhizobium etli*	447	89	4	Microarray, qPCR, Northern analysis, 5′ RACE	Vercruysse et al. (2011)
5.	*Streptococcus pneumoniae* TIGR4	50	14	13	Microarray, qPCR	Kumar et al. (2010)
6.	*Shigella flexneri* strain 301	256	18	9	RNAz, microarray, RT-PCR, Northern analysis	Peng et al. (2011)
7.	*Porphyromonas gingivalis*	37	2	1	Microarray, RNA sequencing, Northern analysis	Phillips et al. (2014)

microarray is widely used for sRNA detection but there are certain limitations to this method. Short (less than 50 nt) and highly structured sRNAs might be harder to detect. To overcome this, sRNA enrichment is always recommended to enhance the amount of sRNAs with low abundances and reduce larger RNAs with similar sequences to sRNAs, which finally improves the signal-to-noise ratio over background. Also, validation of the microarray data by techniques such as Northern blotting, RACE-PCR, and real-time PCR is always recommended.

1.3 RNomics and Next-Generation Sequencing

RNomics approach of sequencing of cDNA clones prepared from size fractionation of total cellular RNA helped to discover new sRNAs in bacteria. In first such studies in *E. coli*, total RNA from three different growth phases was size fractionated (50–500 nt) by gel extraction, reverse transcribed, cloned to construct libraries, and were analyzed by conventional Sanger sequencing (Vogel et al. 2003). In another study of *E. coli*, total RNA of size ranging from 30 to 65 nts was cloned for library construction (Kawano et al. 2005). Both the studies uncovered new sRNAs from IGRs and from 5′- to 3′-UTR of ORFs including riboswitches as well. One major limitation of this approach was the need to eliminate highly abundant tRNAs and rRNAs. Also, highly structured sRNAs are not easily reverse-transcribed, and less structured ones are over presented in libraries. Therefore, such libraries don’t reflect the

Table 1.2 List of microorganisms validated for sRNA using sequencing approach

Sr. No	Species	Predicted	Tested	Verified	Identification approach	Reference
1.	*Bacillus anthracis*	2	2	2	RNA-sequencing, qPCR	McKenzie et al. (2014)
2.	*Deinococcus radiodurans*	199	54	41	Deep RNA sequencing, Northern analysis, RT-PCR	Tsai et al. (2015)
3.	*Neisseria gonorrhoeae*	232	10	7	Own bioinformatic approach, RNA sequencing, Northern analysis	McClure et al. (2014)
4.	*Zymomonas mobilis*	106	106	15	Deep RNA sequencing, SIPHT, Northern analysis, deep 5′ and 3′ RACE	Cho et al. (2014)
5.	*Rickettsia prowazekii*	26	9	6	SIPHT,RNA-sequencing, RT-PCR	Schroeder et al. (2015)
6.	*Pectobacterium atrosepticum*	137	8	8	SIPHT, RNA sequencing, RT-PCR, qPCR	Kwenda et al. (2016)
7.	*Ruegeria pomeroyi*	99	16	14	RNA-sequencing, qPCR, Northern analysis	Rivers et al. (2016)

true abundance of all sRNAs. Other novel strategies are used to replace the time-consuming cDNA cloning step and the costly Sanger sequencing with high-throughput parallel sequencing of hundreds and thousands of cDNA fragments.

Pyrosequencing was first used in *Salmonella* to uncover Hfq-associated sRNAs (Sittka et al. 2008). Later in 2009, 454 pyrosequencing was used to discover sRNAs in *Rhodobacter sphaeroides* (Berghoff et al. 2009) and *Vibrio cholerae* (Liu et al. 2009). In *Rhodobacter sphaeroides*, 18 sRNAs from IGRs and 2 antisense RNAs were discovered. Four of the identified sRNAs were found to be important in gene regulation in the oxidative and photooxidative stress response. Liu et al. (2009) introduced a new protocol for sRNA enrichment by depletion of the small-sized 5S rRNA and tRNAs by oligonucleotide-guided RNase H treatment in the size-fractionated RNA followed by direct cloning and parallel sequencing in *Vibrio cholerae*. They identified 20 previously known sRNAs, 500 new and putative intergenic sRNAs and 127 putative antisense sRNAs. This strategy was later followed by Acebo and co-workers (2012) to identify 68 novel sRNAs in human pathogen *Streptococcus pneumoniae.* Sequencing studies of pathogenic strains of *Listeria monocytogenes* (Mraheil et al. 2011), *Legionella pneumophila* (Weissenmayer et al. 2011), *Yersinia pseudotuberculosis* (Koo et al. 2011), *Xanthomonas campestris* (Schmidtke et al. 2011), *Yersinia pestis* (Yan et al. 2013), and *Erwinia amylovora* (Zeng and Sundin 2014) revealed sRNAs involved in virulence. Other examples of sRNA prediction using sequencing approach are listed in Table 1.2.

Another technique differential RNA sequencing (dRNA-Seq) differentiates primary transcripts from processed RNAs. This is achieved by depleting processed RNA by subjecting the RNA sample to terminator exonuclease. The dRNA-Seq approach verified the expression of seven sRNAs in *Bradyrhizobium japonicum* (Madhugiri et al. 2012). In parallel, RNA-Seq and differential 5′-end RNA-Seq approach discovered a large number of previously unknown sRNAs and also unraveled their transcription start sites at the genome-wide level in *Clostridium difficile* (Soutourina et al. 2013). Genome-wide survey in *Corynebacterium glutamicum* by dRNA-Seq identified 543 *cis* antisense sRNAs and 262 *trans*-encoded sRNAs (Mentz et al. 2013). Recently, van der Meulen et al. (2016) uncovered 375 novel RNAs including sRNAs, asRNAs, long 5′-UTRs, putative regulatory 3′-UTRs, novel (small) ORFs, internal promoters, transcription start sites, and operon structures with dRNA-Seq technology in *Lactococcus lactis*.

1.4 Co-purification with Proteins: Hfq

Many of the sRNAs known to date are found as ribonucleoprotein complexes as they require protein partners for accurate functioning and intracellular stability and to modify the activity of target mRNAs or proteins. Such sRNAs can be co-immunoprecipitated with protein partners like Hfq using partner protein-specific antibodies. First such study was conducted by Zhang et al. (2003), where sRNAs were co-immunoprecipitated with anti-Hfq antibodies followed by microarray analysis. Many previously known and some new sRNAs expressed at lower levels were also discovered. Later studies in *Listeria monocytogenes* (Christiansen et al. 2006), *Pseudomonas aeruginosa* (Sonnleitner et al. 2008), and *Haloferax volcanii* (Fischer et al. 2011) discovered sRNAs by immunoprecipitation with Hfq followed by direct enzymatic RNA sequencing, conventional RNomics, and Microarrays, respectively. This technique is much suitable for intrinsically stable sRNAs as sRNAs with a shorter half-life could not be detected due to long incubation steps involved. A modification of this method was introduced to overcome the limited availability of species-specific Hfq antibodies where an epitope was tagged to the chromosomal hfq gene of *Salmonella*, and Hfq-associated RNA was analyzed after co-immunoprecipitation with a commercial anti-epitope antibody by RNA-Seq (Sittka et al. 2008). In another modification of the method, Hfq proteins from two eubacteria (*Neisseria meningitidis*, *Aquifex aeolicus*) and an archaeon (*Methanocaldococcus jannaschii*) were expressed in Hfq mutant *Salmonella* (Sittka et al. 2009).These foreign Hfq proteins were able to detect some of the previously unknown and less abundant sRNAs. This technique could also be used in bacteria which normally do not express Hfq. However in *Salmonella*, the epitope did not interfere with the virulence or hfq functions, but prior testing is always recommended for other species.

The genomic systematic evolution of ligands by exponential enrichment (SELEX) approach covers all the sRNAs encoded by an entire genome irrespective of their expression. In an attempt to identify new Hfq-binding sRNAs in *E. coli*, a library of random sequences of 50–500 nts long of entire genome were transcribed

in vitro, and then the resulting RNA pool is subjected to rounds of Hfq binding, partitioning, and re-amplification. Specific Hfq interaction of enriched RNA was then determined in vivo using a yeast three-hybrid system (Lorenz et al. 2006). This approach detected many less abundant antisense RNAs, but it hardly covered some of the previously known Hfq-associated sRNAs.

1.5 Target Prediction and Validation

sRNA physically interacts with its mRNA or protein targets to alter their translation, stability, or function; in other words, the target is translationally activated or inhibited as a consequence of binding to sRNAs. Binding of sRNA with its target is the main aspect exploited for bioinformatic prediction of mRNA targets. However experimental approaches based on monitoring decreased or increased abundance level of target mRNAs by transcription analysis using microarrays or sequencing technologies or proteome analysis are also adopted by researchers.

1.5.1 In Silico Target Prediction

One simple in silico way to detect target mRNA based on sRNA-mRNA interaction is BlastN or Fasta3 searches (Chen et al. 2004; Pichon and Felden 2005). Many dedicated algorithms available online for efficient prediction of target mRNAs are also available.

TargetRNA developed by Tjaden and co-workers (2008) consists of an individual base pair model and a stacked base pair model for calculating optimal hybridization scores and corresponding P-value between a sRNA and all the mRNAs of a given genome. More precisely, for a given sRNA, all potential sRNA-target mRNA interactions will be considered, and the top 10 or 50 interactions with smallest P values will be used to interpret putative candidate mRNA targets with graphical representation of predicted interaction. The program is freely available at http://snowwhite.wellesley.edu/targetRNA/. *Agrobacterium tumefaciens* (Wilms et al. 2011), *Mycobacterium tuberculosis* (Pellin et al. 2012), *Salmonella enterica* (Hebrard et al. 2012), and *Listeria monocytogenes* (Wurtzel et al. 2012) are some of the microorganisms for which TargetRNA tool was used for mRNA target prediction.

sRNATarget by Cao et al. (2009) constructed two prediction models, sRNATargetNB and sRNATargetSVM, using Naive Bayes method and support vector machine (SVM), respectively, only for sRNAs negatively regulating their targets. According to authors, the sensitivity, accuracy, and specificity was 40.90%, 93.03%, and 93.71% for sRNATargetNB and 72.73%, 80.55%, and 80.65% for sRNATargetSVM, respectively. sRNATargetNB was much faster than sRNATargetSVM and was used to construct the web server-sRNATarget. It is publicly available at http://ccb.bmi.ac.cn/srnatarget/. *Xanthomonas campestris* (Jiang et al. 2010), *Agrobacterium tumefaciens* (Wilms et al. 2011), *Mycobacterium tuberculosis*

(Pellin et al. 2012),and *Enterococcus faecalis* (Shioya et al. 2011) are few bacteria in which sRNATarget has been used for target predictions.

IntaRNA (interacting RNAs) by Busch et al. (2008) incorporated accessibility of binding sites of two RNA molecules and a user-definable seed and introduced IntaRNA. It finds the optimal structure with the binding sites of two RNA molecules and minimum folding energy. It is freely accessible at http://rna.informatik.uni-freiburg.de. It has been used for predictions in *Enterococcus faecalis* (Shioya et al. 2011), *Yersinia pseudotuberculosis* (Koo et al. 2011), *Synechococcus* sp. WH7803 (Gierga et al. 2012), *Streptococcus pyogenes* (Tesorero et al. 2013), etc.

sTarpicker is based on a two-step model for hybridization between sRNA and its mRNA target (Ying et al. 2011). In this method, all the possible sRNA-target duplexes are screened and stable ones are selected. In next step, hybridization between the duplexes is extended to identify the entire binding site. Finally, an ensemble classifier is constructed to distinguish true interactions from pseudo interactions using machine learning methods. Thermodynamic stabilities and site accessibility of the sRNAs and targets are also considered for hybridization energy calculation of seed regions and binding regions. sTarPicker is available online at http://ccb.bmi.ac.cn/starpicker/. The program has been used for predicting interactions in *Streptococcus mutans* (Xia et al. 2012), *Brucella abortus* (Dong et al. 2014), *Agrobacterium fabrum* (Dequivre et al. 2015), *Shigella flexneri* (Wang et al. 2016), etc.

RNAPredator developed by Eggenhofer et al. (2011) predicts sRNA targets using a dynamic programming approach RNAplex, whose predictive performance is similar to more complex methods but is three times faster than IntaRNA. It considers accessibility of target for better specificity of prediction. It is available at http://rna.tbi.univie.ac.at/RNApredator. *Streptococcus mutans* (Xia et al. 2012), *Pseudomonas syringae* (Park et al. 2014), *Bacillus anthracis* (McKenzie et al. 2014), and *Agrobacterium fabrum* (Dequivre et al. 2015) are few microorganisms for which RNApredator has been applied for mRNA target prediction.

CopraRNA (Comparative prediction algorithm for small RNA targets) incorporates and extends the functionality of IntaRNA to predict targets, interaction domains of bacterial small RNA molecules (Wright et al. 2013). It uses conservation of target regulation for prediction. Functional enrichment of predicted targets and visualization of interacting regions are some of the post-processing steps available. CopraRNA results were able to identify targets and characterize physiological functions of the sRNAs as efficiently as experimental methods like microarray. It is freely accessible at http://rna.informatik.uni-freiburg.de. CopraRNA has been used for target predictions in *Burkholderia thailandensis* (Stubben et al. 2014), *Enterococcus faecalis* (Michaux et al. 2014a), *Bacillus subtilis* (Durand et al. 2015), *Rickettsia prowazekii* (Schroeder et al. 2015), etc.

TargetRNA2 considers many factors while searching for targets of RNA regulation, like conservation of the sRNA in other bacteria, secondary structure of sRNA and each candidate mRNA target, and hybridization energy of interaction (Kery et al. 2014). It also has ability to integrate RNA-Seq data, if available. There is more than half reduction in false-positive predictions if RNA-Seq data is incorporated in

TargetRNA2. The TargetRNA2 web server is available at http://cs.wellesley.edu/~btjaden/TargetRNA2. It has been used for predictions in *Zymomonas mobilis* (Cho et al. 2014), *Bacillus subtilis* (Durand et al. 2015), *Rickettsia prowazekii* (Schroeder et al. 2015), *Ruegeria pomeroyi* (Rivers et al. 2016), etc.

1.5.2 Experimental Validation of Targets

All the above mentioned computational studies provide a hint of possible targets of a sRNA; however, experimental studies are essentially required for final validation. Impact of sRNA on its target (mRNA or protein) can be monitored by proteome or transcriptome analysis. For instance, inhibition of a mRNA target as a consequence of binding with sRNA can be confirmed by comparing the abundance level of mRNA in a strain overexpressing the respective sRNA and wild-type strain using microarrays (Masse et al. 2005). General approach for sRNA detection by co-immunoprecipitation of cellular RNA with the Hfq followed by microarray analysis detected some mRNA fragments as putative targets of such sRNAs of *E. coli* (Zhang et al. 2003). So, sRNAs can be used as bait to capture the mRNA regulated by it. In one such study, in vitro transcribed sRNA was immobilized with His-tagged Hfq and was used to capture targets from cellular mRNA extracts of *E. coli* by affinity purification (Antal et al. 2005). In another similar approach, in vitro transcribed sRNA was biotinylated and bound to streptavidin beads and was used to capture mRNAs from the cellular extracts of *E. coli*, which were later detected on whole genome microarrays (Douchin et al. 2006). For mRNA target identification using proteome analysis, the protein extracts of a wild-type strain are compared with that of a mutant strain either lacking or overexpressing a sRNA. Michaux and co-workers (2014) performed a 2D PAGE (polyacrylamide gel electrophoresis) followed by mass spectrometry (MS) to compare the cytoplasmic proteomes of sRNA mutant strains with wild-type strains to uncover the regulated targets of that particular sRNAs of *E. faecalis*. Tesorero and co-workers (2013) employed differential RNA sequencing analysis to screen targets for a sRNA of *Streptococcus pyogenes* by comparing transcriptome analysis of sRNA deletion mutant with that of the wild-type strain.

1.6 Conclusion and Future Perspectives

The methods reviewed above can search and identify sRNAs in any bacterial genome. As the number of known sRNAs has greatly increased in recent years, functional characterization of most of them is lagging behind. Computational tools based on comparative genomics have facilitated uncovering of sRNAs in diverse species to a great extent. One technical limitation is that the result output includes a large number of false-positives predictions, also none of the existing tools is able to find all the experimentally validated sRNAs; however, they provide a good beginning point for sRNA analysis. Experimental approaches also have many caveats,

like conventional RNomics might miss the highly structured sRNAs as they might not get reverse transcribed to cDNA due to structural constraints. For microarrays, sRNA enrichment is essentially required to improve signal-to-noise ratio. Also microarrays are limited to prediction of sRNAs in completely sequenced genomes; in contrast, next-generation sequencing is a more promising alternative. Another major limitation for all the methods except genomic SELEX is related to conditions for expression of sRNA. All developmental stages and different possible growth conditions should be explored as sRNAs are expressed under unique physiological conditions and may be missed by screening in standard growth conditions. Whereas different strategies for sRNA searches are well established now, target identification and validation is a much less explored aspect. The focus should be shifted from identification to functional characterization so as to successfully construct regulatory networks (or pathways) for sRNAs and targets.

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References

Acebo P, Martin-Galiano AJ, Navarro S, Zaballos A, Amblar M (2012) Identification of 88 regulatory small RNAs in the TIGR4 strain of the human pathogen *Streptococcus pneumoniae*. RNA 18:530–546

Antal M, Bordeau V, Douchin V, Felden B (2005) A small bacterial RNA regulates a putative ABC transporter. J BiolChem 280:7901–7908

Argaman L, Hershberg R, Vogel J, Bejerano G et al (2001) Novel small RNA encoding genes in the intergenic regions of *Escherichia coli*. Curr Biol 11:941–950

Babu MM, Sridhar J, Gunasekaran P (2011) Global transcriptome analysis of *Bacillus cereus* ATCC 14579 in response to silver nitrate stress. J Nanobiotechnol 9(1):1–2

Berghoff BA, Glaeser J, Sharma CM, Vogel J, Klug G (2009) Photooxidative stress-induced and abundant small RNAs in *Rhodobacter sphaeroides*. Mol Microbiol 74(6):1497–1512

Busch A, Richter AS, Backofen R (2008) IntaRNA: efficient prediction of bacterial sRNA targets incorporating target site accessibility and seed regions. Bioinformatics 24:2849–2856

Cao Y, Zhao Y, Cha L, Ying X et al (2009) sRNATarget: a web server for prediction of bacterial sRNA targets. Bioinformation 3:364–366

Cao Y, Wu J, Liu Q, Zhao Y et al (2010) sRNATarBase: a comprehensive database of bacterial sRNA targets verified by experiments. RNA 16(11):2051–2057

Chen S, Zhang A, Blyn LB, Storz G (2004) MicC, a second small-RNA regulator of Omp protein expression in *Escherichia coli*. J Bacteriol 186:6689–6697

Chen Y, Indurthi DC, Jones SW, Papoutsakis ET (2011) Small RNAs in the genus clostridium. MBio 2(1):e00340–e00310

Cho SH, Lei R, Henninger TD, Contreras LM (2014) Discovery of ethanol-responsive small RNAs in *Zymomonas mobilis*. Appl Environ Microbiol 80(14):4189–4198

Christiansen JK, Nielsen JS, Ebersbach T, Valentin-Hansen P et al (2006) Identification of small Hfq-binding RNAs in *Listeria monocytogenes*. RNA 12:1383–1396

Coenye T, Drevinek P, Mahenthiralingam E, Shah SA et al (2007) Identification of putative non-coding RNA genes in the *Burkholderia cenocepacia* J2315 genome. FEMS Microbiol Letter 276(1):83–92

del Val C, Rivas E, Torres-Quesada O, Toro N, Jimenez-Zurdo JI (2007) Identification of differentially expressed small non-coding RNAs in the legume endosymbiont *Sinorhizobium meliloti* by comparative genomics. Mol Microbiol 66:1080–1091

Dequivre M, Diel B, Villard C, Sismeiro O et al (2015) Small RNA deep-sequencing analyses reveal a new regulator of virulence in *Agrobacterium fabrum* C58. Mol Plant-Microbe Interact 28(5):580–589

Dong H, Peng X, Wang N, Wu Q (2014) Identification of novel sRNAs in *Brucella abortus* 2308. FEMS Microbiol Lett 354:119–125

Douchin V, Bohn C, Bouloc P (2006) Down-regulation of porins by a small RNA bypasses the essentiality of the regulated intramembrane proteolysis protease RseP in *Escherichia coli*. J BiolChem 281:12253–12259

Durand S, Braun F, Lioliou E, Romilly C et al (2015) A nitric oxide regulated small RNA controls expression of genes involved in redox homeostasis in *Bacillus subtilis*. PLoS Genet 11(2):e1004957

Eggenhofer F, Tafer H, Stadler PF, Hofacker IL (2011) RNApredator: fast accessibility-based prediction of sRNA targets. Nucleic Acids Res 39:W149–W154

Fischer S, Benz J, Späth B, Jellen-Ritter A et al (2011) Regulatory RNAs in *Haloferax volcanii*. Biochem Soc Trans 39(1):159–162

Gierga G, Voss B, Hess WR (2012) Non-coding RNAs in marine *Synechococcus* and their regulation under environmentally relevant stress conditions. ISME J 6(8):1544–1557

Gottesman S (2004) The small RNA regulators of *Escherichia coli*: roles and mechanisms. Annu Rev Microbiol 58:303–328

Gottesman S, McCullen CA, Guillier M, Vanderpool CK et al (2006) Small RNA regulators and the bacterial response to stress. Cold Spring Harbor Symp Quant Biol 71:1–11

Hébrard M, Kröger C, Srikumar S, Colgan A et al (2012) sRNAs and the virulence of *Salmonella enterica* serovar *typhimurium*. RNA Biol 9(4):437–445

Herbig A, Nieselt K (2011) nocoRNAc: characterization of non-coding RNAs in prokaryotes. BMC Bioinforma 12(1):1

Huang HY, Chang HY, Chou CH, Tseng CP et al (2009) sRNAMap: genomic maps for small non-coding RNAs, their regulators and their targets in microbial genomes. Nucleic Acids Res 37(suppl 1):D150–D154

Jiang R-P, Tang D-J, Chen X-L, He Y-Q, Feng J-X, Jiang B-L, Lu G-T, Lin M, Tang J-L (2010) Identification of four novel small non-coding RNAs from Xanthomonas campestris pathovar campestris. BMC Genomics 11(1):316

Kawano M, Reynolds AA, Miranda-Rios J, Storz G (2005) Detection of 5′- and 3′-UTR-derived small RNAs and cis-encoded antisense RNAs in *Escherichia coli*. Nucleic Acids Res 33:1040–1050

Kery MB, Feldman M, Livny J, Tjaden B (2014) TargetRNA2: identifying targets of small regulatory RNAs in bacteria. Nucleic Acids Res 42(W1):W124–W129

Khoo JS, Chai SF, Mohamed R et al (2012) Computational discovery and RT-PCR validation of novel *Burkholderia* conserved and *Burkholderia pseudomallei* unique sRNAs. BMC Genomics 13(Suppl 7):S13

Koo JT, Alleyne TM, Schiano CA et al (2011) Global discovery of small RNAs in *Yersinia pseudotuberculosis* identifies *Yersinia*-specific small, noncoding RNAs required for virulence. Proc Natl Acad Sci U S A 108(37):E709–E717

Kumar R, Shah P, Swiatlo E, Burgess SC et al (2010) Identification of novel non-coding small RNAs from *Streptococcus pneumoniae* TIGR4 using high-resolution genome tiling arrays. BMC Genomics 11:350

Kwenda S, Gorshkov V, Ramesh AM, Naidoo S et al (2016) Discovery and profiling of small RNAs responsive to stress conditions in the plant pathogen *Pectobacterium atrosepticum*. BMC Genomics 17(1):1

Landt SG, Abeliuk E, McGrath PT, Lesley JA et al (2008) Small non-coding RNAs in *Caulobacter crescentus*. Mol Microbiol 68(3):600–614

Li L, Huang D, Cheung MK, Nong W et al (2013) BSRD: a repository for bacterial small regulatory RNA. Nucleic Acids Res 41(D1):D233–D238

Liu JM, Camilli A (2010) A broadening world of bacterial small RNAs. Curr Opin Microbiol 13:18–23

Liu JM, Livny J, Lawrence MS, Kimball MD et al (2009) Experimental discovery of sRNAs in *Vibrio cholerae* by direct cloning, 5S/tRNA depletion and parallel sequencing. Nucleic Acids Res 37(6):e46

Liu WB, Shi Y, Yao LL, Zhou Y, Ye BC (2013) Prediction and characterization of small noncoding RNAs related to secondary metabolites in *Saccharopolyspora erythraea*. PLoS One 8(11):e80676

Livny J, Fogel MA, Davis BM, Waldor MK (2005) sRNAPredict: an integrative computational approach to identify sRNAs in bacterial genomes. Nucleic Acids Res 13:4096–4105

Livny J, Brencic A, Lory S, Waldor MK (2006) Identification of 17 *Pseudomonas aeruginosa* sRNAs and prediction of sRNA-encoding genes in 10 diverse pathogens using the bioinformatic tool sRNAPredict2. Nucleic Acids Res 34(12):3484–3493

Livny J, Teonadi H, Livny M, Waldor MK (2008) High-throughput, kingdom-wide prediction and annotation of bacterial non-coding RNAs. PLoS One 3(9):e3197

Lorenz C, von Pelchrzim F, Schroeder R (2006) Genomic systematic evolution of ligands by exponential enrichment (genomic SELEX) for the identification of protein-binding RNAs independent of their expression levels. Nat Protoc 1:2204–2212

Madhugiri R, Pessi G, Voss B, Hahn J et al (2012) Small RNAs of the *Bradyrhizobium/Rhodopseudomonas* lineage and their analysis. RNA Biol 9(1):47–58

Marchais A, Naville M, Bohn C, Bouloc P, Gautheret D (2009) Single-pass classification of all noncoding sequences in a bacterial genome using phylogenetic profiles. Genome Res 19(6):1084–1092

Masse E, Vanderpool CK, Gottesman S (2005) Effect of RyhB small RNA on global iron use in *Escherichia coli*. J Bacteriol 187(20):6962–6971

McClure R, Tjaden B, Genco C (2014) Identification of sRNAs expressed by the human pathogen *Neisseria gonorrhoeae* under disparate growth conditions. Front Microbiol 5:456

McKenzie AT, Pomerantsev AP, Sastalla I, Martens C et al (2014) Transcriptome analysis identifies *Bacillus anthracis* genes that respond to CO 2 through an AtxA-dependent mechanism. BMC Genomics 15(1):1

Mentz A, Neshat A, Pfeifer-Sancar K, Pühler A et al (2013) Comprehensive discovery and characterization of small RNAs in *Corynebacterium glutamicum* ATCC 13032. BMC Genomics 14(1):714

Michaux C, Hartke A, Martini C, Reiss S et al (2014a) Involvement of *Enterococcus faecalis* small RNAs in stress response and virulence. Infect Immun 82(9):3599–3611

Michaux C, Verneuil N, Hartke A, Giard JC (2014b) Physiological roles of small RNA molecules. Microbiology 160:1007–1019

Mraheil MA, Billion A, Mohamed W, Mukherjee K et al (2011) The intracellular sRNA transcriptome of *Listeria monocytogenes* during growth in macrophages. Nucleic Acids Res 39:4235–4248

Panek J, Bobek J, Mikulik K, Basler M, Vohradsky J (2008) Biocomputational prediction of small non-coding RNAs in *Streptomyces*. BMC Genomics 9:217

Park SH, Bao Z, Butcher BG et al (2014) Analysis of the small RNA spf in the plant pathogen *Pseudomonas syringae* pv. tomato strain DC3000. Microbiology 160(5):941–953

Patenge N, Billion A, Raasch P, Normann J et al (2012) Identification of novel growth phase-and media-dependent small non-coding RNAs in *Streptococcus pyogenes* M49 using intergenic tiling arrays. BMC Genomics 13(1):1

Pellin D, Miotto P, Ambrosi A, Cirillo DM, Di Serio C (2012) A genome-wide identification analysis of small regulatory RNAs in *Mycobacterium tuberculosis* by RNA-Seq and conservation analysis. PloS ONE 7(3):e32723

Peng J, Yang J, Jin Q (2011) An integrated approach for finding overlooked genes in *Shigella*. PLoS One 6(4):e18509

Perez N, Trevino J, Liu Z, Ho SC et al (2009) A genome-wide analysis of small regulatory RNAs in the human pathogen group a *Streptococcus*. PLoS One 4:e7668

Phillips P, Progulske-Fox A, Grieshaber S, Grieshaber N (2014) Expression of *Porphyromonas gingivalis* small RNA in response to hemin availability identified using microarray and RNA-seq analysis. FEMS Microbiol Lett 351(2):202–208

Pichon C, Felden B (2003) Intergenic sequence inspector: searching and identifying bacterial RNAs. Bioinformatics 19:1707–1709

Pichon C, Felden B (2005) Small RNA genes expressed from *Staphylococcus aureus* genomic and pathogenicity islands with specific expression among pathogenic strains. Proc Natl Acad Sci U S A 102(40):14249–14254

Pischimarov J, Kuenne C, Billion A, Hemberger J et al (2012) sRNAdb: a small non-coding RNA database for gram-positive bacteria. BMC Genomics 13(1):384

Raasch P, Schmitz U, Patenge N, Vera J et al (2010) Non-coding RNA detection methods combined to improve usability, reproducibility and precision. BMC Bioinformatics 11:491

Rivas E, Klein RJ, Jones TA, Eddy SR (2001) Computational identification of noncoding RNAs in *Escherichia coli* by comparative genomics. Curr Biol 11:1369–1373

Rivers AR, Burns AS, Chan LK, Moran MA (2016) Experimental identification of small non-coding RNAs in the model marine bacterium *Ruegeria pomeroyi* DSS-3. Front Microbiol 7:380

Schlüter JP, Reinkensmeier J, Daschkey S et al (2010) A genome-wide survey of sRNAs in the symbiotic nitrogen-fixing alpha-proteobacterium *Sinorhizobium meliloti*. BMC Genomics 11(1):1

Schmidtke C, Findeiß S, Sharma CM, Kuhfuß J et al (2011) Genome-wide transcriptome analysis of the plant pathogen *Xanthomonas* identifies sRNAs with putative virulence functions. Nucleic Acids Res 40(5):gkr904

Schroeder CL, Narra HP, Rojas M, Sahni A et al (2015) Bacterial small RNAs in the genus *Rickettsia*. BMC Genomics 16(1):1

Schuster CF, Park JH, Prax M, Herbig A et al (2013) Characterization of a mazEF toxin-antitoxin homologue from *Staphylococcus equorum*. J Bacteriol 195(1):115–125

Shioya K, Michaux C, Kuenne C, Hain T et al (2011) Genome-wide identification of small RNAs in the opportunistic pathogen *Enterococcus faecalis* V583. PLoSONE 6(9):e23948

Silvaggi JM, Perkins JB, Losick R (2006) Genes for small noncoding RNAs under sporulation control in *Bacillus subtilis*. J Bacteriol 188:532–541

Sittka A, Lucchini S, Papenfort K, Sharma CM et al (2008) Deep sequencing analysis of small noncoding RNA and mRNA targets of the global posttranscriptional regulator, Hfq. PLoS Genet 4(8):e1000163

Sittka A, Sharma CM, Rolle K, Vogel J (2009) Deep sequencing of *Salmonella* RNA associated with heterologous Hfq proteins in vivo reveals small RNAs as a major target class and identifies RNA processing phenotypes. RNA Biol 6(3):266–275

Sonnleitner E, Sorger-Domenigg T, Madej MJ, Findeiss S et al (2008) Detection of small RNAs in *Pseudomonas aeruginosa* by RNomics and structure-based bioinformatic tools. Microbiology 154:3175–3187

Soutourina OA, Monot M, Boudry P, Saujet L et al (2013) Genome-wide identification of regulatory RNAs in the human pathogen *Clostridium difficile*. PLoS Genet 9(5):e1003493

Sridhar J, Ramkumar S, Narmada Sabarinathan R, Hong-Yu O (2010) sRNAscanner: a computational tool for intergenic small RNA detection in bacterial genomes. PLoS ONE5 8:e11970

Stubben CJ, Micheva-Viteva SN, Shou Y, Buddenborg SK et al (2014) Differential expression of small RNAs from *Burkholderia thailandensis* in response to varying environmental and stress conditions. BMC Genomics 15(1):1

Tesorero RA, Yu N, Wright JO, Svencionis JP et al (2013) Novel regulatory small RNAs in *Streptococcus pyogenes*. PLoS One 8(6):e64021

Tjaden B (2008) TargetRNA: a tool for predicting targets of small RNA action in bacteria. Nucleic Acids Res 36:W109–W113

Tsai CH, Liao R, Chou B, Contreras LM (2015) Transcriptional analysis of *Deinococcus radiodurans* reveals novel small RNAs that are differentially expressed under ionizing radiation. Appl Environ Microbiol 81(5):1754–1764
Tsui HC, Mukherjee D, Ray VA, Sham LT et al (2010) Identification and characterization of noncoding small RNAs in *Streptococcus pneumoniae* serotype 2 strain D39. J Bacteriol 192:264–279
Valverde C, Livny J, Schluter JP, Reinkensmeier J et al (2008) Prediction of *Sinorhizobium meliloti* sRNA genes and experimental detection in strain 2011. BMC Genomics 9:416
van der Meulen SB, de Jong A, Kok J (2016) Transcriptome landscape of *Lactococcus lactis* reveals many novel RNAs including a small regulatory RNA involved in carbon uptake and metabolism. RNA Biol 13(3):353–366
Vercruysse M, Fauvart M, Cloots L, Engelen K et al (2011) Genome-wide detection of predicted non-coding RNAs in *Rhizobium etli* expressed during free-living and host-associated growth using a high-resolution tiling array. BMC Genomics 11(1):53
Vogel J, Bartels V, Tang TH, Churakov G et al (2003) RNomics in *Escherichia coli* detects new sRNA species and indicates parallel transcriptional output in bacteria. Nucleic Acids Res 31:6435–6443
Voss B, Georg J, Schön V, Ude S, Hess WR (2009) Biocomputational prediction of non-coding RNAs in model cyanobacteria. BMC Genomics 10:123
Wang L, Yang G, Qi L, Li X et al (2016) A novel small RNA regulates tolerance and virulence in *Shigella flexneri* by responding to acidic environmental changes. Front Cell Infect Microbiol 6:24
Washietl S, Hofacker IL, Stadler PF (2005) Fast and reliable prediction of noncoding RNAs. Proc Natl Acad Sci U S A 102:2454–2459
Wassarman KM, Repoila F, Rosenow C, Storz G, Gottesman S (2001) Identification of novel small RNAs using comparative genomics and microarrays. Genes Dev 15:1637–1651
Weissenmayer BA, Prendergast JGD, Lohan AJ, Loftus BJ (2011) Sequencing illustrates the transcriptional response of *Legionella pneumophila* during infection and identifies seventy novel small non-coding RNAs. PLoS One 6(3):e17570
Wilms I, Voss B, Hess WR, Leichert LI, Narberhaus F (2011) Small RNA mediated control of the *Agrobacterium tumefaciens* GABA binding protein. Mol Microbiol 80(2):492–506
Wright PR, Richter AS, Papenfort K, Mann M et al (2013) Comparative genomics boosts target prediction for bacterial small RNAs. Proc Natl Acad Sci U S A 110(37):E3487–E3496
Wurtzel O, Sesto N, Mellin JR, Karunker I et al (2012) Comparative transcriptomics of pathogenic and non-pathogenic *Listeria* species. Mol Syst Biol 8(1):583
Xia L, Xia W, Li S, Li W et al (2012) Identification and expression of small non-coding RNA, L10-Leader, in different growth phases of *Streptococcus mutans*. Nucleic Acid Ther 22:177–186
Yan Y, Su S, Meng X, Ji X et al (2013) Determination of sRNA expressions by RNA-seq in *Yersinia pestis* grown in vitro and during infection. PLoS One 8(9):e74495
Ying X, Cao Y, Wu J, Liu Q et al (2011) STarPicker: a method for efficient prediction of bacterial sRNA targets based on a two-step model for hybridization. PLoS One 6:e22705
Zeng Q, Sundin GW (2014) Genome-wide identification of Hfq-regulated small RNAs in the fire blight pathogen *Erwinia amylovora* discovered small RNAs with virulence regulatory function. BMC Genomics 15(1):1
Zeng L, Choi SC, Danko CG, Siepel A et al (2013) Gene regulation by CcpA and catabolite repression explored by RNA-Seq in *Streptococcus mutans*. PLoS One 8(3):e60465
Zhang A, Wassarman KM, Rosenow C, Tjaden BC et al (2003) Global analysis of small RNA and mRNA targets of Hfq. Mol Microbiol 50:1111–1124

Genome Editing

2

Praveen P. Balgir, Suman Rani, and Vishal

2.1 Introduction

Our understanding of basic genetics has improved immensely with time. Detailed analyses of mode of action of the DNA repair machinery of prokaryotes and eukaryotes have brought out the possibility of designing systems to carry out modifications of genome in live cells. The advent of whole genome sequencing has revealed quite a few secrets of the microbial world too. The discoveries of protective mechanisms employed by microbes against the phage invaders have led to the development of technologies that can edit the genome with precision. Reengineered nucleases now allow deletion, insertion, and modification at one or more chosen genes, in a hitherto unattainable precision in live whole cells. The field is expanding at a very rapid pace, and this review attempts at taking a bird's eye view of these developments.

This technology is now known as "genome editing" and is based on the combined use of engineered nucleases made up of target sequence-specific DNA-binding domains and a nonspecific DNA cleavage domain (Carroll 2011). These multi-domain nucleases enable efficient, targeted, precise DNA alterations by cutting targeted DNA double strands (DSBs) and inducing cellular DNA repair machinery, to make necessary changes. Thus, error-prone nonhomologous end joining (NHEJ) and homology-directed repair (HDR) are then carried out leading to desired change, viz., deletion, insertion, mutation, etc., at the site (Wyman and Kanaar 2006). Genome editing technologies have been broadly based on specially designed nucleases such as:

1. Meganucleases reviewed by Stoddard (2011)
2. Zinc-finger nucleases (ZFN) as detailed in review of Urnov et al. (2010)

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3. Transcription activator-like effector nucleases, the TALENs described in review by Bogdanove and Voytas (2011) and Scharenberg et al. (2013)
4. Clustered regularly interspaced short palindromic repeat (CRISPR)-associated nuclease Cas9 described in the review by Doudna and Charpentier (2014) and Hsu et al. (2014)

2.2 The Meganucleases

Meganucleases are endodeoxyribonucleases having large recognition sites, of double-stranded DNA sequences ranging from 12 to 40 base pair long. Such sites are rare in most genomes, occurring only once per genome (Stoddard 2011). Meganucleases can replace, eliminate, or modify sequences in a highly targeted way. For making desired tools, the recognition sequences can be modified through protein engineering. Based on sequence and structure motifs, meganucleases have been divided into five families: LAGLIDADG, GIY-YIG, HNH, His-Cys box, and PD-(D/E) XK. The most well-characterized family is that of the LAGLIDADG proteins, which occur in all kingdoms of life. Its gene is generally encoded within introns or inteins and sometimes reported as free-standing member too. Naturally occurring members of this group have been attributed to perform either the function of RNA maturases carrying out splicing of their own intron or as highly specific endonucleases capable of recognizing and cleaving the exon-exon junction sequence around their own intron; thus, they are also named as “homing endonuclease.” Since these enzymes have long recognition sequences, their off-target effects are minimized. Meganucleases such as I-Sce I (from baker’s yeast), having an 18 bp recognition sequence, have been widely used in research. Recently, meganucleases for genome engineering have been modified by incorporating the DNA-binding domain from TALEs. These hybrid “megaTALs” combine the ease of high DNA-binding specificity and engineering potential of the TALE with the high cleavage efficiency of meganucleases.

2.3 Zinc-Finger Nucleases (ZFN)

The Cys2-His2 zinc-finger domain is the most studied DNA-binding motifs found in eukaryotic transcription factors and other DNA-binding proteins. A single zinc-finger is made up of about 30 amino acids in a conserved ββα configuration (Beerli and Barbas 2002). A number of amino acids on the surface of the α-helix typically contact 3 bp in the major groove of DNA, with diverse degree of selectivity. Large combinatorial libraries or rational designing has been used for “modular assembly” approach. In these designs, more than three zinc-finger domains, each targeting a triplet of DNA sequences, are linked together in tandem such that they recognize specific DNA sequences. The basis of designing of zinc-finger proteins was the development of nonnatural arrays consisting of more than three zinc-finger domains. The discovery of the structure of a highly conserved linker sequence allowed construction of synthetic zinc-finger proteins that bind 9–18 bps of DNA sequences

instead of the triplet (Liu et al. 1997). Since 18 bp of DNA sequence confers specificity even within large genomes, up to 68 billion bp of DNA, this method allowed for targeting of specific regions within the human genome also (Beerli et al. 2000). Further refinement of the technology involved the development of zinc-finger nickases (ZFNickases) (Kim et al. 2012; Wang et al. 2012; Ramirez et al. 2012) taking advantage of the discovery that nicking of DNA induced HDR only and not the error-prone NHEJ repair pathway. Hence, the approach leads to fewer off-target effects. However, the use of ZFNickases for HDR remains low as compared to that with traditional ZFNs.

2.4 Transcription Activator-Like Effector Nucleases, the TALENs

Transcription activator-like effectors (TALEs) are naturally occurring proteins discovered in the plant pathogenic bacteria of genus *Xanthomonas*. TALEs are constituted of 33–35 amino-acid repeats that specifically recognize a single base pair and constitute its specific DNA-binding domain. This DNA-binding specificity of TALE is determined by two hypervariable amino acids known as the repeat-variable di-residues (RVDs). As in case of zinc fingers, modules of TALE repeats are linked together as recognition domain for contiguous DNA sequences. TALE–DNA-binding repeats have greater design flexibility as compared to the triplet-based zinc-finger proteins as they can recognize single base pairs.

These TALEs DNA-binding modules are fused to the cleavage domain of the FokI endonuclease to produce a variety of TALENs for binding to target DNA and create DSBs. TALENs can be used in genome editing only if FokI cleavage domain dimerize which will then cleave both strands of the target DNA leading to its deletion. Hence, two TALENs are combined to target the opposite DNA strands in a tail-to-tail orientation, maintaining proper spacing between the two binding sites (Li et al. 2011; Mahfouz et al. 2011). However, the main problem affecting efficiency of TALENs is the requirement of FokI domain to self-dimerize, thus requiring the co-expression of two separate TALEN genes for forming a functional heterodimer around the target sequence. The spacer length between homodimer and heterodimers influences the efficiency of binding and is dependent on the linker length, and the TALENs are found to be functional with spacer ranging from 12 to 30 bp. Mostly, longer linkers are designed with longer spacers and vice versa. Application of TALENS has yielded desired results in yeast, plants, Drosophila, and mammalian cells (Bedell et al. 2012; Carlson et al. 2012; Cermak et al. 2011; Li et al. 2012; Liu et al. 2012). Recently, a study has reported the construction of a library of TALENs targeting 18,740 human protein-coding genes (Kim et al. 2013). The cloning of repeat TALE arrays presents an increased risk of off-target effects, due to extensive identical repeat sequences.

The designing of varied ZFNs and TALENs is based on their DNA-binding domains which can recognize virtually any sequence. These DNA-binding modules can then be combined with various effector domains to affect genomic structural and functional changes. Thus, these can be combined with DNA modulating

nucleases, transcriptional activators, repressors, or recombinases, transposases, DNA and histone methyltransferases, and histone acetyltransferases, each resulting in specific functional alteration of specific gene/genes. Hence, to carry out gene alteration, the designing of DNA-binding specificity of zinc finger and TALE protein is of utmost importance.

2.5 The CRISPR-Cas (CRISPR-Associated Proteins) System

The CRISPR-Cas (CRISPR-associated proteins) system is found in bacterial and archaeal genomes as a defense mechanism against invading phages and plasmids. CRISPR-Cas system was first observed in 1987 (Ishino et al. 1987) in *Escherichia coli*. The hypothesis that CRISPRs might form an immune system against phages and plasmids was confirmed in 2005, when three groups independently reported that spacer sequences possessed homology with foreign genetic material. The first experimental conformation of CRISPR-Cas-mediated adaptive immunity emerged in 2007 with the isolation of phage-resistant *Streptococcus thermophilus* cells possessing new CRISPR spacers after a phage challenge (Denomy and Davidson 2014).

The natural CRISPR-Cas is a bacterial defense system carried out in multiple steps by which specific small fragments of foreign genetic material are first recognized as non-self and then inserted into the host genome between short DNA repeats. Later, these fragments/spacers, in association with host Cas proteins, are used as an adaptive immune system by which newly entered foreign genetic materials are recognized and degraded or silenced (Bhaya et al. 2011). This system can be used to model experimentation to study coevolution of immunity and resistance simultaneously, arising naturally between the host and the invading virus within a short time scale in the lab. Even evidence of past genetic invasions can be deduced in some cases. The ability to acquire foreign DNA and use it to fight against invading pathogens is an acquired and heritable immune system, reminiscent of a Lamarckian mode of evolution (Koonin and Wolf 2009).

1. Phage or plasmid entry recognition and insertion of foreign DNA fragment
2. Recognition and cleavage of foreign DNA

Three major types of CRISPR/Cas systems (Makarova et al. 2011; Wiedenheft et al. 2012) are found in bacteria with differing repeat sequences, Cas proteins, and their mode of action. These systems are grouped as types I–III and subtypes types I–E. The three types are readily distinguishable by virtue of the presence of their unique signature genes in each, viz., Cas3 in type I systems, Cas9 in type II, and Cas10 in type III systems, respectively. In spite of the diversity of the *cas* genes in the organisms containing CRISPRs, the loci have common structural features. All CRISPR sites consist of multiple repeated sequences ranging from 21 to 48 bp, separated by 26–72 bp variable spacer sequences with *cas* genes located adjacent to the CRISPR locus (Denomy and Davidson 2014) (Fig. 2.1).

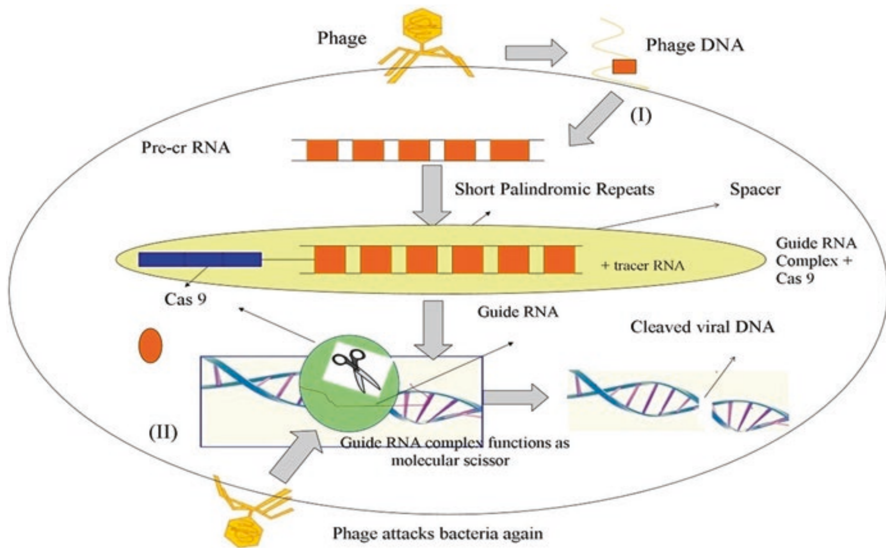

Fig. 2.1 CRISPR-Cas9-mediated bacterial immune defense

2.5.1 Type I CRISPR-Associated Systems

Type I CRISPR-Cas system has wide distribution among bacteria and archaea and has six different subtypes (A–F). All these subtypes encode *cas*3 gene which consists of two domains, namely, N-terminal HD phosphorylase/nuclease domain and C-terminal DExH helicase domain (Makarova et al. 2006). In subtypes A, B, and D, different genes encode these two helicase and nuclease domains. These genes work together by unwinding ds DNA (helicase domain) and cleaving ssDNA (HD domain) of targets, depending upon ATP and Mg^{2+} ions (Richter et al. 2013). Cas 3 alone can't protect the cell from infection independently. Several other subtype-specific cas proteins combine into crRNA-guided surveillance complexes known as cascade (CRISPR-associated complex for antiviral defense) in *E.coli* K12 (Sorek et al. 2013). This complex (type I–E cascade) binds target sequences complimentary to the crRNA spacer. Later on, cascade recruits Cas 3 to cleave the target DNA molecule. Richter et al. (2013) discovered the importance of "seed sequence," positioned at 5′ end represented by 6–12th nucleotides of the crRNA spacer sequence, to be the most crucial for target binding. Even a single point mutation of seed sequence nucleotide can result in lower binding efficiency. Apart from these changes in the crRNA spacer sequences subsequent to the seed sequence are inconsequential, and the mutated crRNA can still bind. In order to protect host genomic encoded CRISPR cluster from degradation by cascade, cas3 complex, the 5′ tag of crRNA, and the PAM sequence that is located upstream of viral protospacer should not be complimentary. To differentiate between self- and non-self-target, cascade complex recognizes and binds to specific PAM sequence that results in helical destabilization

and strand infringement of the matching seed sequence. Type I PAM sequence is 2–3 nucleotides long and is different in different subtypes and organisms (Richter et al. 2013). Cascade is a ribonucleoprotein complex made up of 11 subunits, of 405KD, in which five subunits are functionally essential Cas proteins. One of the subunits is a CRISPR-specific endoribonuclease Cas6e (also known as CasE or Cse3) that is responsible for creating 61 nucleotide mature short crRNA from the long precursor CRISPR RNA molecules. Cas6e and the crRNA are also required for assembly of the other Cas proteins such as Cse1, Cse2, Cas7, and Cas5. Using cryo-electron microscopy (cryo-EM), researchers found the structure of cascade complex. This structure explains how Cas protein provides protection to crRNA from degradation, when crRNA is available for base pairing with invading nucleic acid. A similar complex is present in *S. solfataricus* (type I-A). In *E. coli* (type I-E) and *S. solfataricus* (type I-A) complexes, Cas7 is joined into a right-handed helix along the ribose-phosphate backbone of crRNA, forming a ribonucleoprotein that is structurally similar to the RecA nucleoprotein filament. The crRNA leads the cascade to its dsDNA target sequence where it base pairs with its complementary DNA strand, expelling the noncomplementary strand forming a R loop. Base pairing in the target-bound complex extends along the length of the crRNA, resulting in a chain of short helical segments; as formed during interaction mediated by the RecA nucleoprotein filament. Cas7 pre-positions crRNA in a stretched conformation that is optimal for strand invasion and exchange. In several type I systems, large cascade complexes have been found and low-quality structures are available for the complexes from *S. solfataricus* (type IA), *Bacillus halodurans* (type I-C), and *Pseudomonas aeruginosa* (type I-F). Although these structures predict that type I complexes may share a similar Cas7-based helical form, each complex is morphologically distinct, and differences in their nucleic acid-binding properties have been reported (Sorek et al. 2013).

2.5.2 Type II CRISPR: Associated System

Type II systems have been found only in bacterial genomes (Makarova et al. 2011). These systems are characterized by the presence of minimal set of *cas* genes: *cas9*, *cas1*, *cas2*, and csn2 (type IIA) or *cas4* (type II-B). In these systems, large multifunctional protein, encoded by the characteristic protein of the system, namely, cas9, participates in both crRNA synthesis and in the degradation of foreign DNA. This system is different from type I CRISPR-associated system because here synthesis of crRNA requires a trans-activating crRNA (tracrRNA). TracrRNA is transcribed from the CRISPR loci and contains a 25 nt long sequence which is complementary to the crRNA repeat sequence. Pairing between the tracrRNA and the crRNA repeats is favored by cas9, results in dsRNA that is recognized and degraded by the host RNase III enzyme (Sorek et al. 2013). This degradation leads to generation of mature crRNA of 20 nucleotide spacer 5′ tags and 19–22 nucleotides

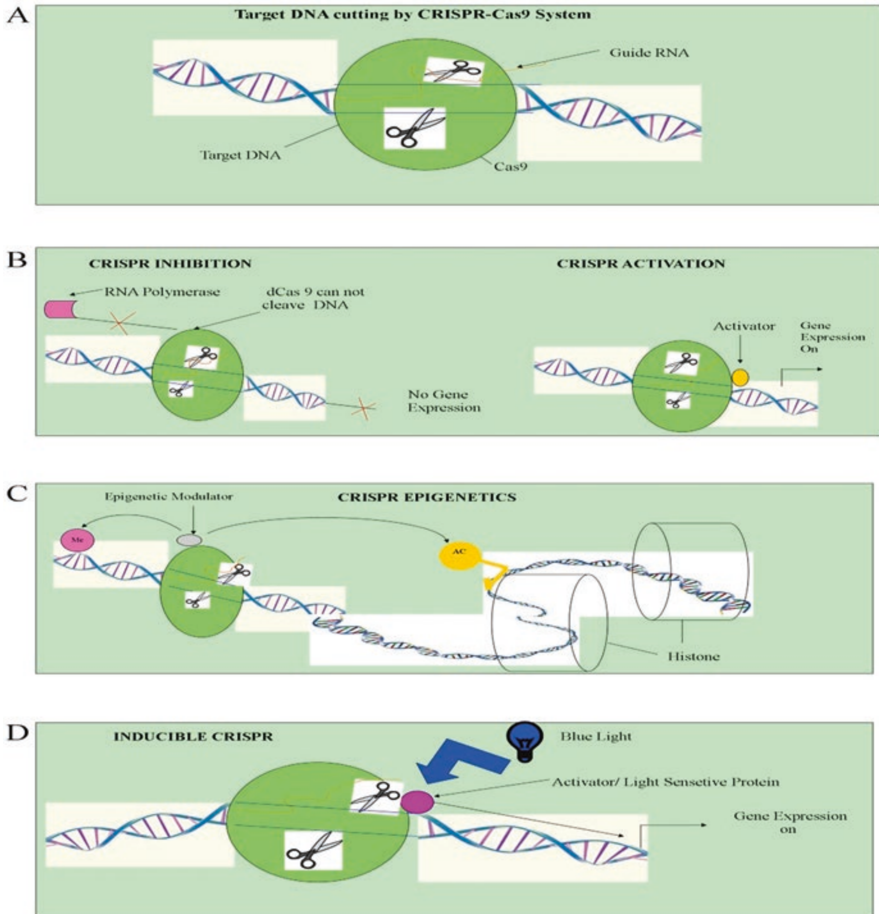

Fig. 2.2 (**a**) Target DNA cutting by CRISPR-Cas9 system. (**b**) CRISPR inhibition and CRISPR. (**c**) CRISPR epigenetics. (**d**) Inducible CRISPR

repeats 3′ tags. In the interference mechanism cleavage of target dsDNA require cas9, crRNA, and tracrRNA. For the cleavage of DNA strand that is complementary to the crRNA, cas9 have Mcr/HNH nuclease domain, and for noncomplementary strand, it has RuvC-like (RNase H fold) domain. For the degradation of foreign/viral DNA, type II system requires a conserved 5 nucleotide long PAM sequence (NGGNG) to be present immediately downstream of protospacer (the phage repeat spacers present at CRISPR). Complementary strand for cleavage is recognized at 3 nucleotides upstream of the PAM sequence, and the noncomplementary strand of DNA is at some other sites within three to eight base pairs upstream of PAM producing blunt-ended product (Richter et al. 2013) (Fig. 2.2).

2.5.3 Type III CRISPR-Associated System

Type III system has two subtypes type III-A and type III-B (Makarova et al. 2013). These systems are mainly present in archaea. Type III B system is never present alone and but is always in combination with other CRISPR subtypes. These both systems encode *cas10* and *cas 6* genes of which cas6 is a CRISPR-specific endoribonuclease and cas 10 is involved in interference mechanism (Sorek et al. 2013). Cas10 encodes a HD nuclease domain that helps in cleavage of target ssDNA. The type III-A system of *Staphylococcus epidermidis* targets DNA. This system has five Cmr proteins. The system does not require specific PAM sequence for recognition. Type III-B system of *Pyrococcus furiosus* targets RNA molecules. This system has six proteins (Cmr1–Cmr6) in ribonucleoprotein interference complex. A similar Cmr complex with seven proteins (Cmr1–Cmr7) was identified for *Sulfolobus solfataricus* which carried out showed endonucleolytic cleavage of invading RNA at UA dinucleotides. This fundamental difference highlights the functional diversity present even within the same CRISPR/Cas types (Richter et al. 2013).

CRISPR-Cas9 has taken center stage since no other enzyme system has comparable specificity and processivity. It can be guided directly to sequence of choice using small guide RNA (gRNA). The Cas enzyme cleaves and then the cell's repair machinery takes over but repairs with mistakes. However, if a template DNA is provided, the repair process can introduce the desired change in the gene, i.e., the gene can be edited based on the template DNA provided. Apart from editing, this novel tool can provide a whole repertoire of tools to manipulate one or more DNA sequences simultaneously in cells and even whole organism.

2.6 Delivery of Editing Machinery

As discussed in this chapter, site-specific nucleases have been proven to be capable of bringing about desired changes to specific genomic sequences; a major limitation in application of the technology is the methodology to deliver this gene-altering machinery to specific cells and tissues. Many of the recombinant DNA transformation methods used for delivery of nucleic acid therapeutics in gene therapy have been tried for gene editing also. These are usually cell type specific and include delivery by plasmid DNA transformation, viral vectors transfection, or in vitro transcribed mRNA liposomal delivery. However, presently reported drawbacks of viral and nonviral gene delivery systems are also the road blocks in applying site-specific nucleases for in vivo genome editing. Many cultured cells cannot withstand these methods and are rendered unviable during transfection of plasmid DNA or mRNA by electroporation or cationic liposomes and hence can be used for limited cell types only. Viral vectors also have their own problems as they can carry limited DNA, are potentially immunogenic, and involve additional biosafety issues. Despite all these issues, clinical trials are ongoing (Perez et al. 2008). Future endeavor would need to be focused on improving delivery methods.

2.7 Applications

2.7.1 Xenotransplantation

Since the 1960s, xenotransplantation has been an attractive proposition for tens of thousands of patients around the world, with organ failures, who die while waiting for a suitable human donor. However, a steady supply of farm-grown organs using genome editing can be expected to allow doctors to place recipients on immune-suppressant drugs days ahead of surgery, improving their survival rates. To date, the immune system has turned out to be more complex and by far the biggest hurdle in any such endeavor. The baboons that received pig organs never survived longer than a few weeks although the researchers have developed α-gal knockdown pigs along with insertion of five human genes in the transgenic donor pigs (Iwase et al. 2015). Genome editing has brought the whole field into limelight once again.

2.7.2 Classical and Contemporary Approaches for Establishing Gene Function

Targeted gene inactivation via homologous recombination has been a powerful method capable of evaluating gene function (Carroll 2011). Mutated Cas9 produced in the lab of Qi et al. (2013) binds DNA specified by the gRNA but does not cleave it instead it turns the gene expression off in this process, by inhibiting transcription from the bound DNA. Similarly, mutated Cas9 can bind a gene activation protein domain, giving it a new activation function. Some further modifications of the same provided the tool that could turn off and on the gene expression at will (Gilbert et al. 2014). The use of longer gRNAs has also given rise to new tools that can activate or suppress more than one gene in the same experiment (Zalatan et al. 2015). Such hacked CRISPRs have been used to rapidly generate hundreds of varieties of cell lines with each targeting a specific gene. These cell lines have been used to study influence of gene expression switching on and off to understand molecular mechanism underlying complex diseases like Alzheimer's disease. RNA interference (RNAi) provides for gene silencing and modulation of gene expression, but its off-target effects by comparison make CRISPR a more specific tool.

Further, scientists have used CRISPRS with longer gRNAs to tweak gene expression simultaneously in more than one gene, allowing the CRISPR technology to be the best available option for carrying out metabolic engineering.

2.7.3 Epigenetics and CRISPRs

Mutated Cas9 was used to add an acetyl group to histones at specific sequences in the genome opening a full new field of study of the influences of the epigenome in disease state or delineate environmental influences, interacting with genome

via epigenome. These new tools will allow inferences to be made with certainty as to the influence of environmental factors in modulating the epigenome and influencing gene expression.

2.7.4 CRISPR and the Noncoding Genome

Another new area of application of CRISPR-Cas9 is throwing light on the dark matter of the genome. This dark matter constitutes nearly 98% of the noncoding genome. However till now, scientists are only partly aware of its gene regulation, chromatin organization, etc. The functional annotation of such regions of the genome has been made possible by CRISPR targeting them precisely.

2.7.5 CRISPR: Cas9 in Cell and Tissue Differentiation

An interesting development wherein a Cas9 tool modified to respond to light or chemical switch to be activated make the tool to be controlled more finely. Most of these applications have been tried on progenitor cell types, including embryonic stem (ES) cells (Lombardo et al. 2007) and induced pluripotent stem (iPS) cells (Hockemeyer et al. 2009, 2011). With an eye on future therapeutic applications involving moddeling of broad range of genetic disorders further development of such tools look promising (Ding et al. 2013) (Fig. 2.3).

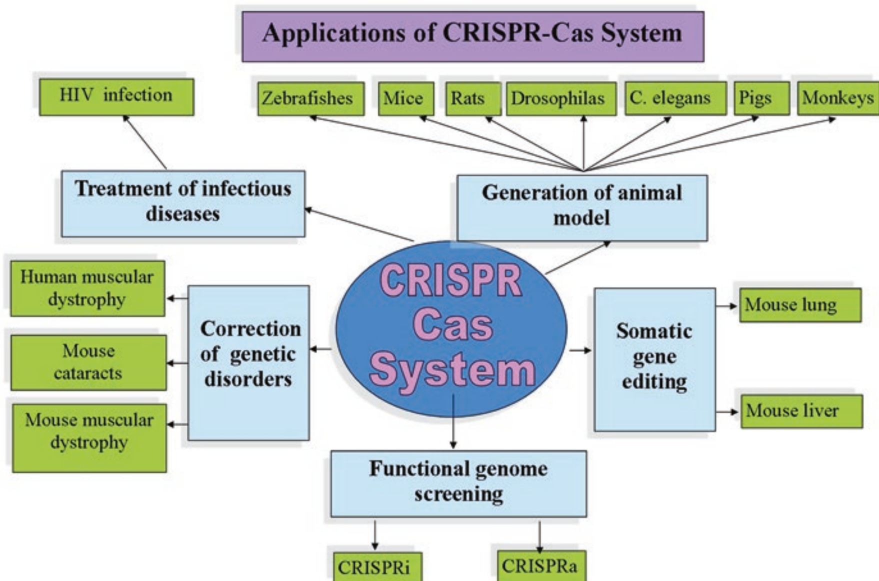

Fig. 2.3 Applications of CRISPR-Cas system

2.8 Challenges to Be Solved

In mammalian cells, for CRISPR-Cas9 to function efficiently, presence of PAM sequences in the DNA at the chosen site for editing is required, and this information needs to be incorporated while designing gRNA which is the challenge for designing of the optimal RNA scaffold. The challenge of optimal methods for delivering the editing machinery into cells and organisms is still not resolved.

2.9 Conclusion

Since the mechanism of recombination and transposition described above uses a prokaryotic system for genome editing in eukaryotic cells, it does not interfere in cellular DNA repair pathways; hence, these approaches are functional in nearly all cell types and cell cycle stages. There is possibility of improving efficiency of these processes by directed evolution. The technology has opened up hitherto unachievable possibilities in basic biological processes, medicine, agriculture, and biotechnology within reach of scientists. Therapeutic genome editing of diseased cells and tissues is expected to result in the deletion or editing of deleterious mutations or replacement with protective mutations, with certainty, giving hope to scientists to treat hitherto untreatable diseases.

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References

Bedell VM, Wang Y, Campbell JM, Poshusta TL et al (2012) In vivo genome editing using a high-efficiency TALEN system. Nature 491:114–118
Beerli RR, Barbas CF (2002) Engineering polydactyl zinc finger transcription factors. Nat Biotechnol 20:135–141
Beerli RR, Dreier B, Barbas CF (2000) Positive and negative regulation of endogenous genes by designed transcription factors. Proc Natl Acad Sci USA 97(4):1495–1500
Bhaya D, Davison M, Barrangou R (2011) CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. Annu Rev Genet 45:273–297
Bogdanove AJ, Voytas DF (2011) TAL effectors: customizable proteins for DNA targeting. Science 333(6051):1843–1846
Carlson DF, Tan W, Lillico SG, Stverakova D et al (2012) Efficient TALEN-mediated gene knockout in livestock. Proc Natl Acad Sci USA 109:17382–17387
Carroll D (2011) Genome engineering with zinc-finger nucleases. Genetics 188:773–7823
Cermak T, Doyle EL, Christian M et al (2011) Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic Acids Res 39(12):e82
Denomy JB, Davidson AR (2014) To acquire or resist: the complex biological effects of CRISPR–Cas systems. Trends Microbiol 22(4):218–225
Ding Q, Lee YK, Schaefer EA, Peters DT et al (2013) A TALEN genome-editing system for generating human stem cell-based disease models. Stem Cells 12(2):225–238

Doudna JA, Charpentier E (2014) The new frontier of genome engineering with CRISPR-Cas9. Science 346(6213):1258096

Gilbert LA, Horlbeck MA, Adamson B et al (2014) Genome-scale CRISPR-mediated control of gene repression and activation. Cell 159(3):647–661

Hockemeyer D, Soldner F, Beard C et al (2009) Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. Nat Biotechnol 27(9):851–857

Hockemeyer D, Wang H, Kiani S, Lai CS et al (2011) Genetic engineering of human pluripotent cells using TALE nucleases. Nat Biotechnol 29(8):731–734

Hsu PD, Lander ES, Zhang F (2014) Development and applications of CRISPR-Cas9 for genome engineering. Cell 157(6):1262–1278

Ishino Y, Shinagawa H, Makino K et al (1987) Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product. J Bacteriol 169:5429–5433

Iwase H, Liu H, Wijkstrom M et al (2015) Pig kidney graft survival in a baboon for 136 days: longest life-supporting organ graft survival to date. Xenotransplantation 22(4):302–309

Kim E, Kim S, Kim DH, Choi BS et al (2012) Precision genome engineering with programmable DNA-nicking enzymes. Genome Res 22(7):1327–1333

Kim Y, Kweon J, Kim A, Chon JK et al (2013) A library of TAL effector nucleases spanning the human genome. Nat Biotechnol 31(3):251–258

Koonin EV, Wolf IY (2009) Is evolution Darwinian or/and Lamarckian? Biol Direct 4:42

Li T, Huang S, Jiang WZ et al (2011) TAL nucleases (TALNs): hybrid proteins composed of TAL effectors and FokI DNA-cleavage domain. Nucleic Acids Res 39:359–372

Li T, Liu B, Spalding MH et al (2012) High-efficiency TALEN-based gene editing produces disease-resistant rice. Nat Biotechnol 30:390–392

Liu Q, Segal DJ, Ghiara JB, Barbas CF (1997) Design of polydactyl zinc-finger proteins for unique addressing within complex genomes. Proc Natl Acad Sci USA 94(11):5525–5530

Liu J, Li C, Yu Z, Huang P et al (2012) Efficient and specific modifications of the Drosophila genome by means of an easy TALEN strategy. J Genet Genomics 39:209–215

Lombardo A, Genovese P, Beausejour CM et al (2007) Gene editing in human stem cells using zinc-finger nucleases and integrase-defective lentiviral vector delivery. Nat Biotechnol 25(11):1298–1306

Mahfouz MM, Li L, Shamimuzzaman M, Wibowo A et al (2011) De novo-engineered transcription activator-like effector (TALE) hybrid nuclease with novel DNA binding specificity creates double-strand breaks. Proc Natl Acad Sci USA 108:2623–2628

Makarova KS, Grishin NV, Shabalina SA et al (2006) A putative RNA interference-based immune system in prokaryotes: computational analysis of the predicted enzymatic machinery, functional analogies with eukaryotic RNAi, and hypothetical mechanisms of action. Biol Direct 1:7

Makarova KS, Haft DH, Barrangou R et al (2011) Evolution and classification of the CRISPR-Cas systems. Nat Rev Microbiol 9(6):467–477

Makarova KS, Wolf YI, Koonin EV (2013) The basic building blocks and evolution of CRISPR-cas systems. Biochem Soc T 41:1392–1400

Perez EE, Wang J, Miller JC et al (2008) Establishment of HIV-1 resistance in $CD4^+$ T cells by genome editing using zinc-finger nucleases. Nat Biotechnol 26(7):808–816

Qi LS, Larson MH, Gilbert LA et al (2013) Repurposing CRISPR as an RNA-guided platform for sequence specific control of gene expression. Cell 152(5):1173–1183

Ramirez CL, Certo MT, Mussolino C, Goodwin MJ et al (2012) Engineered zinc finger nickases induce homology-directed repair with reduced mutagenic effects. Nucleic Acids Res 40(12):5560–5568

Richter H, Randau L, Plagens A (2013) Exploiting CRISPR/Cas: interference mechanisms and applications. Int J Mol Sci 14:14518–14531

Scharenberg AM, Duchateau P, Smith J (2013) Genome engineering with TAL-effector nucleases and alternative modular nuclease technologies. Curr Gene Ther 13(4):291–303

Sorek R, Lawrence CM, Wiedenheft B (2013) CRISPR-mediated adaptive immune systems in bacteria and archaea. Annu Rev Biochem 82:237–266

Stoddard BL (2011) Homing endonucleases: from microbial genetic invaders to reagents for targeted DNA modification. Structure 19(1):7–15

Urnov FD, Rebar EJ, Holmes MC et al (2010) Genome editing with engineered zinc finger nucleases. Nat Rev Genet 11(9):636–646

Wang J, Friedman G, Doyon Y et al (2012) Targeted gene addition to a predetermined site in the human genome using a ZFN-based nicking enzyme. Genome Res 22(7):1316–1326

Wiedenheft B, Samuel H, Sternberg, Doudna JA (2012) RNA-guided genetic silencing systems in bacteria and archaea. Nature 482:331–338

Wyman C, Kanaar R (2006) DNA double-strand break repair: all's well that ends well. Annu Rev Genet 40:363–383

Zalatan JG, Lee ME, Almeida R, Gilbert LA, Whitehead EH, La Russa M et al (2015) Engineering complex synthetic transcriptional programs with CRISPR RNA scaffolds. Cell 160:339–350

3 Silencing Human VCAM 1 Gene

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3.1 Introduction

Vascular cell adhesion molecule 1 (VCAM-1), also called CD106 (cluster of differentiation 106), is a human protein that regulates adhesion and migration of lymphocyte, monocytes, basophils and eosinophils to vascular endothelium. During this process, binding of leukocytes to endothelial cells occurs via low-affinity adhesion. Due to blood flow force and the low-affinity adhesion, rolling of leukocytes on endothelial cells occurs (Springer 1994). The bound leukocytes finally migrate in-between endothelial cells (ECs). One such endothelial cell adhesion molecule mediating leukocyte migration is VCAM-1 (Springer 1995).

3.2 Discovery of VCAM1

In 1989, two groups working independently discovered VCAM-1. Firstly, it was named inducible cell adhesion molecule 110 (INCAM-110) due to inducibility of interleukin-1 (IL-1) or tumour necrosis factor-α (TNF-α) on human umbilical vein endothelial cells (HUVEC), but later on, it was named VCAM-1 because of its ability to mediate a firm adhesion of melanoma cells (Osborn et al. 1989; Rice and Bevilacqua 1989). The first ligand for VCAM-1 mediating adhesion of B cells to the lymphoid germinal centres was identified in 1990 as VLA-4 (very late activation antigen-4)/integrin α4β1 (Freedman et al. 1990). Later on, various VCAM-1 binding integrins like α4β7, αMβ2, α9β1 and αDβ2 were identified, but VLA-4 emerged to be the best investigated one (Barthel et al. 2006; Chiu et al. 1995; Costa et al. 2012; Kilger et al. 1995; Kon et al. 2011; Walsh et al. 1996).

The importance of VCAM-1 in mediating the immune response was further elucidated with research articles documenting that VCAM-1 binds eosinophils and

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basophils, and monocytes (Bochner et al. 1991; Carlos et al. 1991). VCAM-1 was also implicated in cancer progression as it was shown that tumour cells could bind to the endothelium through binding of their surface, α4β1 (Taichman et al. 1991). It was later discovered that VCAM-1 could also bind to α4β7 expressed on T lymphocytes (Ruegg et al. 1992).

3.3 Gene VCAM-1

The human genome contains a single copy of VCAM-1 having nine exons across about 25 kb of DNA. Out of the 9, exons 2–8 contain C2 or H-type immunoglobulin domains. Due to alternate mRNA splicing, two different VCAM transcripts that encode different isoforms in humans are generated (Hession et al. 1991). Upstream from the transcriptional start site is located a consensus TATAA box. The promoter contains two NF-κB consensus binding sites located at −57 and −78 upstream of the transcription start site, GATA family transcription factor sites at −239 and −253 and an activator protein 1 (AP1) binding site at −490. The VCAM 1 gene is located at 1p31–32 region of chromosome 1 (Cybulsky et al. 1991). A study performed by Neish et al. (1992) shows the Ets binding site at −1026, −976 and −216, as well as octamer-like sequences at −1547, −1175 and −724.

3.3.1 VCAM 1 Gene Expression Pattern

VCAM-1 gene expression mainly restricted to endothelial cells in large and small blood vessels after cytokine stimulation. However, VCAM-1 has also been shown to be constitutively expressed on vascular smooth muscle cells (VSMCs) as well as cells outside of the vascular endothelium such as epithelial cells, monocyte-derived cells, mesangial cells and dendritic cells (DC) of lymphoid tissue and in the skin (Khachigian et al. 1995; Rice et al. 1991; Ruegg et al. 1992; Simionescu and Simionescu 1993).

3.3.2 Regulation of VCAM-1 Expression

MicroRNA miR126 identified by Harris and co-workers in 2008 is expressed in 3′ UTR of VCAM-1 in endothelial cells, and they have found that transfecting miR126 in endothelial cells causes increase in VCAM1 expression stimulated by TNF-α. Also, overexpression of miR-126 reduced VCAM 1 gene expression, and thereby declining endogenous miR126 levels amplified the leukocyte adherence to endothelial cells and prompt the conclusion that miR126 controls VCAM1 expression (Harris et al. 2008).

3.3.3 Gene Silencing

"RNA interference" (RNAi) has become an important tool in cell and molecular biology for gene knockdown studies. The phenomenon of RNA interference has been widely applied in the field of functional genomics. The term RNAi was coined after the groundbreaking discovery that introduction of double-stranded RNA (dsRNA) into the nematode model *Caenorhabditis elegans* leads to silencing of specific genes which are highly homologous in sequence to the delivered dsRNA (Fire et al. 1998). In mammalian cells, RNAi was first employed as a tool to induce the silencing of the targeted gene (Elbashir et al. 2001). Small interference RNAs (siRNAs) were initially seen amid transgene virus-induced inhibition in plants (Mello and Conte 2004), reliable with a characteristic part in genome defence. In 2002 and 2003, centromeres, transposons and other repetitive sequences were revealed as another wellspring of siRNAs (Lippman and Martienssen 2004). The functional studies in plants prompted the revelation of trans-acting siRNAs (ta-siRNAs) that are cut from particular genomic transcripts and direct discrete arrangements of target genes (Allen et al. 2005; Vazquez et al. 2004). Thereafter in 2008, different sources of endogenous siRNAs (endo-siRNAs) have been recognized (Golden et al. 2008). Synthetic siRNAs impact the normally occurring RNAi process in such a way that is steady and unsurprisingly concerning the degree of action (Bumcrot et al. 2006).

3.4 VCAM1 Function

One of the first studies in 1996 correlated the epicardial dissolution, leading to myocardial thinning to lack of VCAM1 and α4 integrin (Olson and Srivastava 1996). Control and localization of marginal zone B cells were studied in 2002, and it has been found that ligands VCAM-1 and ICAM-1 bind to B cells. Inhibition of α4β1 results in selective and rapid release of B cells from marginal zone. In addition, lipopolysaccharides initiate re-localization of marginal zone B cells which involves downregulation of integrin-mediated adhesion (Lu and Cyster 2002). During vascularization, α4β1integrin and VCAM 1 help in cell-cell adhesion process (Garmy-Susini et al. 2005). Fungus-derived cyclic heptadepsipeptide (cyclopeptolide) known as CAM741 molecule discovered in 2005 inhibits the biosynthesis of VCAM 1 by blocking the process of co-translational translocation (Besemer et al. 2005).

3.4.1 VCAM-1 in Atherosclerosis

The evidence for involvement of VCAM-1 in atherosclerosis came from the development of VCAM-1 knockout mice. The fourth domain of VCAM-1 was disrupted to produce VCAM-1 domain 4-deficient (D4D) allele. In this manner, VCAM-1$^{D4D/D4D}$ and VCAM-1$^{(+/+)}$ mice were produced from heterozygous cross between these and were nourished on high-cholesterol diet. ICAM-1 deficiency either alone or in

combination with VCAM-1 did not alter lesion formation. It was observed by Cybulsky et al. (1991) that both VCAM-1 and ICAM-1 genes are upregulated during atherosclerosis. However, the role of VCAM-1 proved to be more important for the recruitment of monocytes at the site in vivo, for the initiation of atherosclerotic lesion development.

Consistent with these findings, Lessner et al. (2002) also showed that the adhesion molecules (both VCAM-1 and ICAM-1) play a significant role in monocyte recruitment mainly during lesion initiation. Besides the formation of atherosclerotic lesions, VCAM-1 also plays a role in neointima formation after arterial injury (Manka et al. 1999). Significant medial thickening was observed in Apo-E-deficient mice fed with a western-type diet for 4 weeks. The studies showed that arterial injury increases the expression of VCAM-1.

It has been revealed that during atherosclerosis, allograft rejection, infection and asthmatic responses, VCAM-1 activates signals inside the endothelial cells that resulted in the opening of an "endothelial cell entryway" through which leukocytes can migrate. These signals are mediated by reactive oxygen species and are regulated by cytokines (Cook-Mills 2002). It has been observed that human vascular endothelial cells can be efficiently transfected with siRNAs with low toxicity effect. siRNAs antagonistic to ICAM-1, VCAM-1 and E-selectin can inhibit the gene expression on ECs. Transfecting ICAM-1, VCAM-1 and E-selectin with siRNAs antagonistic to their respective genes can decrease the gene expression at mRNA and protein level on the ECs (Walker et al. 2007).

VCAM-1, additionally, being communicated by endothelial cells for enlistment of leukocytes amid irritation, it is likewise copiously communicated by smooth muscle cells in atherosclerotic injuries and in harmed courses. Along these lines, the part of VCAM-1 in smooth muscle cell movement was analysed by confining smooth muscle cells from the aorta of C57BL/6 mice and transfecting them with siRNAs focusing on VCAM-1. This brought about a huge decrease in the quantity of moved cells.

VCAM-1 is equally expressed by smooth muscle cells (SMCs) in atherosclerotic lesions and in injured arteries as is expressed by ECs for recruitment of leukocytes during inflammation. So, its role in smooth muscle cell migration was analysed by isolating SMCs from the aorta of C57BL/6 mice and transfecting them with siRNAs antagonistic to VCAM-1. Therefore, the number of relocated SMCs was critically decreased. Thus, these studies indicated that VCAM-1 is necessary for the migration of smooth muscle cells (Petersen et al. 2008).

In a study of 252 patients, increased serum levels of soluble VCAM-1 predicted periodic ischemic stroke (Campbell et al. 2006). The patients with high levels of VCAM-1 and proBNP (pro-brain natriuretic peptide) levels are 3.6 times more prone to periodic ischemic stroke than the ones having lower levels of both biological markers.

Due to its involvement in cellular differentiation, the effect of female cigarette smoking on VCAM-1 was studied. It was observed that smoking stimulated overexpression of VCAM-1 in endothelial cell surface which may cause atherosclerosis due to the accumulation of adhesion protein. On the other hand, the expression of

VCAM-1 was inhibited by smoking in B cells which interrupted the normal immune signal transduction and response (Pan et al. 2010). Thus, silencing of VCAM-1 gene can have implications on the progression of atherosclerosis.

Sun et al. (2016) studied the effect of salusin-β, a stimulator involved in the progression of atherosclerosis and found an enhancement in the expressions and activity of acyl coenzyme A:cholesterol acyltransferase-1 (ACAT-1) and VCAM-1 in vascular smooth muscle cells (VSMCs). Monocyte adhesion to VSMCs due to the salusin-β was inhibited by silencing VCAM-1. Inhibition of NFκB was observed to prevent salusin-β-induced ACAT-1 and VCAM-1 upregulation, formation of foam cells and adhesion of monocytes to VSMCs. They concluded that monocyte adhesion and formation of foam cell are induced by salusin-β in VSMCs through miR155/NOX2/NFκB-mediated ACAT-1 and VCAM-1 expressions.

3.4.2 Other Roles of VCAM-1

3.4.2.1 Cell Differentiation

VCAM-1 has also been shown to play an important role in the development of B-cell differentiation (Pan et al. 2010). It adheres the pro-B cells to stromal cells, which in turn results in the conversion of pro-B cells into pre-B cells (Funk et al. 1994).

3.4.2.2 Cardiac Therapy

Qu et al. (2009) demonstrated that VCAM1-induced perivascular changes are also involved in pathogenesis of neointimal hyperplasia in rats following de-endothelialization of carotid surgically, and thus, VCAM-1 siRNA may be used by local delivery during carotid endarterectomy operation to reduce restenosis.

Radecke et al. (2015) investigated the expression of VCAM-1 on coronary artery endothelial cells (CAECs) and compared this with receptor expression on cultured human aortic endothelial cells (HAECs) stimulated with TNFα. VCAM-1 is upregulated on EC microparticles (EMPs) and CAECs. MI patients have high VCAM-1 expression on CAECs (73%) and on EMPs (79%). Increased VCAM-1 expression is a threat for the plaque instability prompting MI. Increased VCAM-1 expression on EC and formation of EMP at the site of coronary plaque is positively correlated with the extent of vascular inflammation in patients with myocardial infarction.

TNF-α triggers the signal transductions of Nox/ROS, MAPKs and AP-1 for upregulation of VCAM-1 in human cardiac fibroblasts (HCFs). This leads to the increased adhesion and association of monocytes to HCFs. VCAM-1 expression in HCFs stimulated by TNF-α increases the inflammatory response and thus supporting the theory that TNF-α assumes a key role in the enhancement and establishment of heart diseases.

3.4.2.3 Graft Rejection

Singh et al. (2005) showed that the function of cell surface adhesion can be down-regulated by VCAM-1 shedding by proteolytic cleavage. Alam et al. (2006) carried

out study on graft rejection in mouse. They silenced VCAM-1 gene by vector-based siRNA approach. The study showed that the graft rejection is based on leukocyte recruitment on the endothelium via adhesion molecules. Thus, survival of graft can be prolonged by inhibiting these interactions.

3.4.2.4 Cancer Metastasis

Besides being involved in atherosclerosis, other biological functions have been assigned to VCAM-1. Studies have shown that VCAM-1, together with Caveolin-1, plays a critical role in the metastasis of gastric tumours. Colocalizing the VCAM-1 with Caveolin-1 and designing the siRNA against Caveolin-1 resulted in a significant decrease in the expression of Caveolin-1 and subsequently decreased adherence of VCAM-1 to gastric tumour cells (Shin et al. 2006).

Moreover, VCAM-1 is also associated with the metastasis of ovarian cancer. This was clear from the observation that VCAM-1 expression was found on the mesothelium of the women suffering from ovarian cancer. Highly tumorigenic cells (SKOV-3 cells and ES-2) were used that efficiently transmigrate through the layer of mesothelial cells. Using antibodies against VCAM-1 and designing siRNAs against VCAM-1, significant inhibition of movement of SKOV-3 cells was observed. Thus, it became evident that VCAM-1 regulated the ovarian cancer metastasis (Slack-Davis et al. 2009).

3.4.2.5 Cancer Therapy

A study by Klemke et al. (2007) brought to light the mechanism of transendothelial migration across endothelial cell layer by highly metastatic melanoma cell line. Such cells were observed to express α4β1 integrin which is the receptor for VCAM1. Thus, high expression of α4β1 integrin on melanoma cells leads to enhanced extravasation, aided by their high affinity to the activated endothelial cells expressing VCAM-1. The results were supported by the experiment blocking the α4β1 integrin on tumour cells using antibodies and/or knocking down VCAM-1 on endothelial cells using siRNA. The study also pointed to siRNA-based therapeutics for interfering with tumour metastasis.

Huang et al. (2013) studied VCAM-1 expression in ovarian cancer cell lines. With respect to surgery and chemotherapeutic drugs, the high expression of VCAM-1 found to be correlated in older patients. Overexpression of VCAM-1 in ovarian cancer cells in contrast to their respective control cells resulted in increased cell migration and enhanced growth of xenograft tumours in mice.

In the study by Wang et al. (2014), knockdown of endogenous VCAM-1 expression in metastatic breast cancer cell line MDAMB231 reduced cell proliferation and inhibited TGFβ1 or IL-6 mediated cell migration as well. The low level of VCAM1 increased chemosensitivity of the cell line. Thus, in SCID xenograft mouse model, silencing the VCAM-1 levels reduced the formation of tumour (Wang et al. 2014).

3.4.2.6 Novel Anti-inflammatory Molecule

Kowalski et al. (2013) studied the potential of delivering anti-inflammatory siRNA molecules. They used successfully a new generation of liposomes based on cationic amphiphile SAINT-C18 (1-methyl-4-(cis-9-dioleyl)methyl-pyridinium-chloride) molecule, SAINT-O-Some. The liposomes were used for selective functional delivery of siRNA antagonistic to VCAM 1 and E-selectin, into inflamed primary endothelial cells. Nanoparticles formed of SAINT-O-Somes carrying siRNAs of size 106 nm and antagonistic to the endothelial gene degraded the targeted mRNA and the protein without having any cytotoxicity.

3.4.2.7 Stem Cell Activation

Aomatsu et al. (2014) observed that PDGFRβ mediated the upregulation of VCAM-1 expression in human bone marrow-derived mesenchymal stem cells. The overexpression of N-cadherin, a structural molecule in adherence junctions in MSCs, enhanced VCAM-1 expression and activated intracellular signalling factor, Src by its phosphorylation. Another study identified a novel role for signalling through the macrophage colony-stimulating factor receptor (M-CSFR) in the formation of splenic myelopoiesis. Interfering with this, receptor inhibited macrophage functions in the splenic haematopoietic region. These cells expressed VCAM-1 to retain haematopoietic stem and progenitor cell (HSPCs) in the red pulp in the spleen. Interference of this pathway by silencing either M-CSFR or VCAM-1 empowered HSPCs to get away into the blood and thereby reducing circulating monocytes (Dutta et al. 2015).

3.4.2.8 Other Diseases

Garcia-Bernal et al. (2006) observed that the spleen and lymph node T cells have increased α4 expression under sheer stress resulting in higher levels of adhesion molecule VCAM-1. Increased α4 expression has been correlated with T-cell memory phenotype and enhancement in adhesion to α4β1 ligands.

Walker et al. (2011) standardized effectiveness of delivering by transfection to cells with a protective medium containing a combination of specific siRNA sequences against VCAM-1, ICAM and E-selectin in human pulmonary microvascular cells.

Lee et al. (2012) studied expression of lipopolysaccharide-induced VCAM-1, mediated through TLR4/MyD88/c-Src/NADPH oxidase/ROS/p38 MAPK-dependent p300 and ATF2 pathway, associated with induction of monocyte adhesion to kidney cells. It was proposed that blocking these pathways may result in reduced monocyte adhesion via VCAM-1 and modulation of the inflammatory responses in renal diseases.

The VCAM1 protein belonging to the Ig superfamily expressed by the cytokine-activated endothelial cells is a molecule with many functions and is thus involved in a number of disease aetiologies, making it a target for drug development for diseases as diverse as atherosclerosis and cancer.

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References

Alam AK, Florey O, Weber M, Pillai RG, Chan C, Tan PH, Lechler RI, McClure MO, Haskard DO, George AJ (2006) Knockdown of mouse VCAM-1 by vector-based siRNA. Transpl Immunol 16(3–4):185–193

Allen E, Xie Z, Gustafson AM, Carrington JC (2005) microRNA-directed phasing during trans-acting siRNA biogenesis in plants. Cell 121(2):207–221

Aomatsu E, Chosa N, Nishihira S, Sugiyama Y, Miura H, Ishisaki A (2014) Cell-cell adhesion through N-cadherin enhances VCAM-1 expression via PDGFR beta in a ligand-independent manner in mesenchymal stem cells. Int J Mol Med 33(3):565–572

Barthel SR, Annis DS, Mosher DF, Johansson MW (2006) Differential engagement of modules 1 and 4 of vascular cell adhesion molecule-1 (CD106) by integrins alpha4beta1 (CD49d/29) and alphaMbeta2 (CD11b/18) of eosinophils. J Biol Chem 281(43):32175–32187

Besemer J, Harant H, Wang S, Oberhauser B, Marquardt K, Foster CA, Schreiner EP, de Vries JE, Dascher-Nadel C, Lindley IJ (2005) Selective inhibition of cotranslational translocation of vascular cell adhesion molecule 1. Nature 436(7048):290–293

Bochner BS, Luscinskas FW, Gimbrone MA Jr, Newman W, Sterbinsky SA, Derse-Anthony CP, Klunk D, Schleimer RP (1991) Adhesion of human basophils, eosinophils, and neutrophils to interleukin 1-activated human vascular endothelial cells: contributions of endothelial cell adhesion molecules. J Exp Med 173(6):1553–1557

Bumcrot D, Manoharan M, Koteliansky V, Sah DW (2006) RNAi therapeutics: a potential new class of pharmaceutical drugs. Nat Chem Biol 2(12):711–719

Campbell DJ, Woodward M, Chalmers JP, Colman SA, Jenkins AJ, Kemp BE, Neal BC, Patel A, MacMahon SW (2006) Soluble vascular cell adhesion molecule 1 and N-terminal pro-B-type natriuretic peptide in predicting ischemic stroke in patients with cerebrovascular disease. Arch Neurol 63(1):60–65

Carlos T, Kovach N, Schwartz B, Rosa M, Newman B, Wayner E, Benjamin C, Osborn L, Lobb R, Harlan J (1991) Human monocytes bind to two cytokine-induced adhesive ligands on cultured human endothelial cells: endothelial-leukocyte adhesion molecule-1 and vascular cell adhesion molecule-1. Blood 77(10):2266–2271

Chiu HH, Crowe DT, Renz ME, Presta LG, Jones S, Weissman IL, Fong S (1995) Similar but nonidentical amino acid residues on vascular cell adhesion molecule-1 are involved in the interaction with alpha 4 beta 1 and alpha 4 beta 7 under different activity states. J Immunol 155(11):5257–5267

Cook-Mills JM (2002) VCAM-1 signals during lymphocyte migration: role of reactive oxygen species. Mol Immunol 39(9):499–508

Costa MF, Bornstein VU, Candea AL, Henriques-Pons A, Henriques MG, Penido C (2012) CCL25 induces alpha(4)beta(7) integrin-dependent migration of IL-17(+) gamma delta T lymphocytes during an allergic reaction. Eur J Immunol 42(5):1250–1260

Cybulsky MI, Fries JW, Williams AJ, Sultan P, Eddy R, Byers M, Shows T, Gimbrone MA Jr, Collins T (1991) Gene structure, chromosomal location, and basis for alternative mRNA splicing of the human VCAM1 gene. Proc Natl Acad Sci U S A 88(17):7859–7863

Dutta P, Hoyer FF, Grigoryeva LS, Sager HB, Leuschner F, Courties G, Borodovsky A, Novobrantseva T, Ruda VM, Fitzgerald K, Iwamoto Y, Wojtkiewicz G, Sun Y, Da Silva N, Libby P, Anderson DG, Swirski FK, Weissleder R, Nahrendorf M (2015) Macrophages retain hematopoietic stem cells in the spleen via VCAM-1. J Exp Med 212(4):497–512

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411(6836):494–498

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391(6669):806–811

Freedman AS, Munro JM, Rice GE, Bevilacqua MP, Morimoto C, McIntyre BW, Rhynhart K, Pober JS, Nadler LM (1990) Adhesion of human B cells to germinal centers in vitro involves VLA-4 and INCAM-110. Science 249(4972):1030–1033

Funk PE, Kincade PW, Witte PL (1994) Native associations of early hematopoietic stem cells and stromal cells isolated in bone marrow cell aggregates. Blood 83(2):361–369

Garcia-Bernal D, Sotillo-Mallo E, Nombela-Arrieta C, Samaniego R, Fukui Y, Stein JV, Teixido J (2006) DOCK2 is required for chemokine-promoted human T lymphocyte adhesion under shear stress mediated by the integrin alpha4beta1. J Immunol 177(8):5215–5225

Garmy-Susini B, Jin H, Zhu Y, Sung RJ, Hwang R, Varner J (2005) Integrin alpha4beta1-VCAM-1-mediated adhesion between endothelial and mural cells is required for blood vessel maturation. J Clin Invest 115(6):1542–1551

Golden DE, Gerbasi VR, Sontheimer EJ (2008) An inside job for siRNAs. Mol Cell 31(3):309–312

Harris TA, Yamakuchi M, Ferlito M, Mendell JT, Lowenstein CJ (2008) MicroRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1. Proc Natl Acad Sci U S A 105(5):1516–1521

Hession C, Tizard R, Vassallo C, Schiffer SB, Goff D, Moy P, Chi-Rosso G, Luhowskyj S, Lobb R, Osborn L (1991) Cloning of an alternate form of vascular cell adhesion molecule-1 (VCAM1). J Biol Chem 266(11):6682–6685

Huang J, Zhang J, Li H, Lu Z, Shan W, Mercado-Uribe I, Liu J (2013) VCAM1 expression correlated with tumorigenesis and poor prognosis in high grade serous ovarian cancer. Am J Transl Res 5(3):336–346

Khachigian LM, Collins T, Fries JW (1995) Nuclear factor-kappa B mediates induction of vascular cell adhesion molecule-1 in glomerular mesangial cells. Biochem Biophys Res Commun 206(2):462–467

Kilger G, Needham LA, Nielsen PJ, Clements J, Vestweber D, Holzmann B (1995) Differential regulation of alpha 4 integrin-dependent binding to domains 1 and 4 of vascular cell adhesion molecule-1. J Biol Chem 270(11):5979–5984

Klemke M, Weschenfelder T, Konstandin MH, Samstag Y (2007) High affinity interaction of integrin alpha4beta1 (VLA-4) and vascular cell adhesion molecule 1 (VCAM-1) enhances migration of human melanoma cells across activated endothelial cell layers. J Cell Physiol 212(2):368–374

Kon S, Atakilit A, Sheppard D (2011) Short form of alpha9 promotes alpha9beta1 integrin-dependent cell adhesion by modulating the function of the full-length alpha9 subunit. Exp Cell Res 317(12):1774–1784

Kowalski PS, Lintermans LL, Morselt HW, Leus NG, Ruiters MH, Molema G, Kamps JA (2013) Anti-VCAM-1 and anti-E-selectin SAINT-O-Somes for selective delivery of siRNA into inflammation-activated primary endothelial cells. Mol Pharm 10(8):3033–3044

Lee IT, Shih RH, Lin CC, Chen JT, Yang CM (2012) Role of TLR4/NADPH oxidase/ROS-activated p38 MAPK in VCAM-1 expression induced by lipopolysaccharide in human renal mesangial cells. Cell Commun Signal 10(1):33

Lessner SM, Prado HL, Waller EK, Galis ZS (2002) Atherosclerotic lesions grow through recruitment and proliferation of circulating monocytes in a murine model. Am J Pathol 160(6):2145–2155

Lippman Z, Martienssen R (2004) The role of RNA interference in heterochromatic silencing. Nature 431(7006):364–370

Lu TT, Cyster JG (2002) Integrin-mediated long-term B cell retention in the splenic marginal zone. Science 297(5580):409–412

Manka DR, Wiegman P, Din S, Sanders JM, Green SA, Gimple LW, Ragosta M, Powers ER, Ley K, Sarembock IJ (1999) Arterial injury increases expression of inflammatory adhesion molecules in the carotid arteries of apolipoprotein-E-deficient mice. J Vasc Res 36(5):372–378
Mello CC, Conte D Jr (2004) Revealing the world of RNA interference. Nature 431(7006):338–342
Neish AS, Williams AJ, Palmer HJ, Whitley MZ, Collins T (1992) Functional analysis of the human vascular cell adhesion molecule 1 promoter. J Exp Med 176(6):1583–1593
Olson EN, Srivastava D (1996) Molecular pathways controlling heart development. Science 272(5262):671–676
Osborn L, Hession C, Tizard R, Vassallo C, Luhowskyj S, Chi-Rosso G, Lobb R (1989) Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell 59(6):1203–1211
Pan F, Yang T-L, Chen X-D, Chen Y, Gao G, Liu Y-Z, Pei Y-F, Sha B-Y, Jiang Y, Xu C, Recker RR, Deng H-W (2010) Impact of female cigarette smoking on circulating B cells in vivo: the suppressed ICOSLG, TCF3, and VCAM1 gene functional network may inhibit normal cell function. Immunogenetics 62(4):237–251
Petersen EJ, Miyoshi T, Yuan Z, Hirohata S, Li JZ, Shi W, Angle JF (2008) siRNA silencing reveals role of vascular cell adhesion molecule-1 in vascular smooth muscle cell migration. Atherosclerosis 198(2):301–306
Qu Y, Shi X, Zhang H, Sun W, Han S, Yu C, Li J (2009) VCAM-1 siRNA reduces neointimal formation after surgical mechanical injury of the rat carotid artery. J Vasc Surg 50(6):1452–1458
Radecke CE, Warrick AE, Singh GD, Rogers JH, Simon SI, Armstrong EJ (2015) Coronary artery endothelial cells and microparticles increase expression of VCAM-1 in myocardial infarction. Thromb Haemost 113(3):605–616
Rice GE, Bevilacqua MP (1989) An inducible endothelial cell surface glycoprotein mediates melanoma adhesion. Science 246(4935):1303–1306
Rice GE, Munro JM, Corless C, Bevilacqua MP (1991) Vascular and nonvascular expression of INCAM-110. A target for mononuclear leukocyte adhesion in normal and inflamed human tissues. Am J Pathol 138(2):385–393
Ruegg C, Postigo AA, Sikorski EE, Butcher EC, Pytela R, Erle DJ (1992) Role of integrin alpha 4 beta 7/alpha 4 beta P in lymphocyte adherence to fibronectin and VCAM-1 and in homotypic cell clustering. J Cell Biol 117(1):179–189
Shin J, Kim J, Ryu B, Chi SG, Park H (2006) Caveolin-1 is associated with VCAM-1 dependent adhesion of gastric cancer cells to endothelial cells. Cell Physiol Biochem 17(5–6):211–220
Simionescu M, Simionescu N (1993) Proatherosclerotic events: pathobiochemical changes occurring in the arterial wall before monocyte migration. FASEB J 7(14):1359–1366
Singh RJ, Mason JC, Lidington EA, Edwards DR, Nuttall RK, Khokha R, Knauper V, Murphy G, Gavrilovic J (2005) Cytokine stimulated vascular cell adhesion molecule-1 (VCAM-1) ectodomain release is regulated by TIMP-3. Cardiovasc Res 67(1):39–49
Slack-Davis JK, Atkins KA, Harrer C, Hershey ED, Conaway M (2009) Vascular cell adhesion molecule-1 is a regulator of ovarian cancer peritoneal metastasis. Cancer Res 69(4):1469–1476
Springer TA (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 76(2):301–314
Springer TA (1995) Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. Annu Rev Physiol 57:827–872
Sun H-J, Zhao M-X, Liu T-Y, Ren X-S, Chen Q, Li Y-H, Kang Y-M, Zhu G-Q (2016) Salusin-β induces foam cell formation and monocyte adhesion in human vascular smooth muscle cells via miR155/NOX2/NFκB pathway. Sci Rep 6:23596
Taichman DB, Cybulsky MI, Djaffar I, Longenecker BM, Teixido J, Rice GE, Aruffo A, Bevilacqua MP (1991) Tumor cell surface alpha 4 beta 1 integrin mediates adhesion to vascular endothelium: demonstration of an interaction with the N-terminal domains of INCAM-110/VCAM-1. Cell Regul 2(5):347–355
Vazquez F, Vaucheret H, Rajagopalan R, Lepers C, Gasciolli V, Mallory AC, Hilbert JL, Bartel DP, Crete P (2004) Endogenous trans-acting siRNAs regulate the accumulation of Arabidopsis mRNAs. Mol Cell 16(1):69–79

Walker T, Wendel HP, Tetzloff L, Raabe C, Heidenreich O, Simon P, Scheule AM, Ziemer G (2007) Inhibition of adhesion molecule expression on human venous endothelial cells by non-viral siRNA transfection. J Cell Mol Med 11(1):139–147

Walker T, Siegel J, Nolte A, Hartmann S, Kornberger A, Steger V, Wendel HP (2011) Small interfering RNA efficiently suppresses adhesion molecule expression on pulmonary microvascular endothelium. J Nucleic Acids 2011:694789

Walsh GM, Symon FA, Lazarovils AL, Wardlaw AJ (1996) Integrin alpha 4 beta 7 mediates human eosinophil interaction with MAdCAM-1, VCAM-1 and fibronectin. Immunology 89(1):112–119

Wang PC, Weng CC, Hou YS, Jian SF, Fang KT, Hou MF, Cheng KH (2014) Activation of VCAM-1 and its associated molecule CD44 leads to increased malignant potential of breast cancer cells. Int J Mol Sci 15(3):3560–3579

In Silico Analysis of Human AGTR1 Gene and Precision Medicine Among Hypertensive Population

4

Praveen P. Balgir and Jaspreet Kaur

4.1 Introduction

4.1.1 Renin-Angiotensin-Aldosterone System (RAAS) Pathway

RAAS is critical to the control of blood pressure and is the target of several types of antihypertensive drugs. RAAS pathway (Fig. 4.1) depicts the pharmacodynamics (PD) of RAAS-regulating drugs including candidate genes for the pharmacogenetics (PGx) of ACE inhibitors, angiotensin receptor blockers (ARBs), and rennin inhibitor aliskiren and aldosterone receptor antagonists.

RAAS involves the conversion of angiotensinogen to angiotensin I (Ang I) by renin and its subsequent conversion to angiotensin II (Ang II) by angiotensin-converting enzyme (ACE). Ang II activates the angiotensin II receptor type 1 (AT1) to induce aldosterone synthesis, increasing water and salt resorption and potassium excretion in the kidney with resultant increase in blood pressure (Weir 2007).

ACE inhibitors target the ACE protein by binding to the Zn^{2+} contained in the ACE enzyme and block the conversion of angiotensin I into angiotensin II. This results in downstream reduction of Ang II, decreasing aldosterone secretion and reducing blood pressure. The antihypertensive activity of the ACE inhibitors is due to various effects. Firstly, inhibition of angiotensin II at both systemic and tissue levels leads to reduction in plasma aldosterone together with vasodilatation, resulting in increased natriuresis and diuresis. Secondly, Since ACE is identical to kininase II (responsible for the breakdown of bradykinin), the inhibition of this enzyme leads to an increase in the circulating levels of bradykinin, which in turn causes peripheral vasodilatation.

It also stimulates the secretion of prostaglandins (PGE2, PGI2), which induce vasodilation. The ACE inhibitors have an inhibitory effect on the release of

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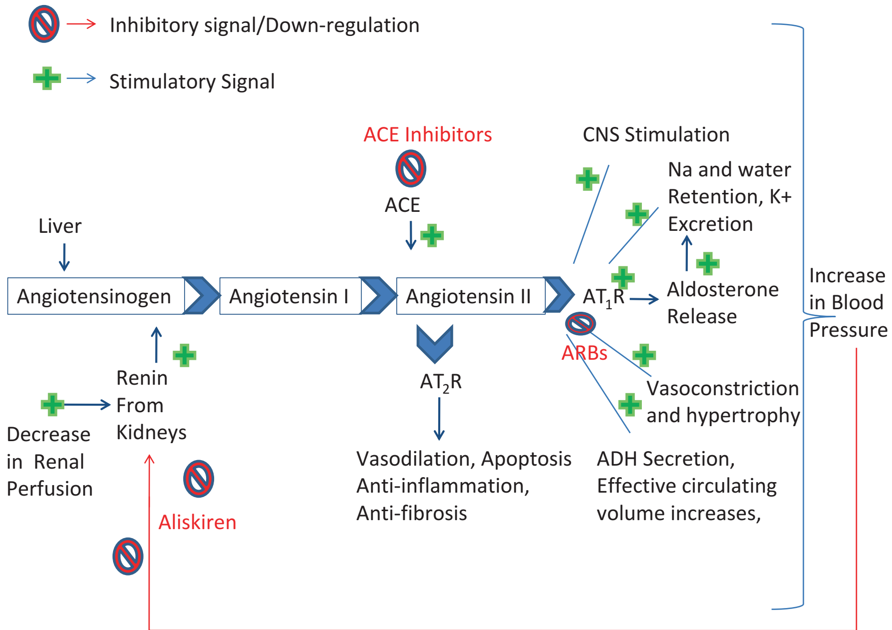

Fig. 4.1 Showing the RAAS pathway. (Adapted from KEGG pathways) (*AT_1R* Angiotensin type 1 receptor, *AT_2R* Angiotensin type 2 receptor, *ARBs* Angiotensin receptor blockers, Renin Inhibitors, *CNS* Central Nervous System). ⃠ = Inhibitory Signal, ✚ = Stimulatory Signal

antidiuretic hormone (ADH) and reduce both central and peripheral sympathetic nervous system activity.

The ARBs represent a relatively newer class of antihypertensive agents. Selective inhibition of the AT_1 receptor has a series of clinical advantages. ARBs do not bind to AGTR2; they are selective for AGTR1. Activation of AGTR1 and downstream signaling including release of calcium lead to transcriptional upregulation of CYP11B2 (aldosterone synthase) and consequent synthesis of aldosterone. The inhibition of aldosterone by ACE inhibitors is incomplete since alternative pathways can still generate Ang II via chymase (CMA1) or cathepsin G (CTSG) degradation of Ang I. This is known as aldosterone escape (Weir 2007). There is also inhibition of the effects of angiotensin II, developed through non-ACE-dependent pathways. Furthermore, free angiotensin II, which finds the AT_1 receptors occupied, can exert its effects on the AT_2 receptors, which mediate vasodilatation and improvement of vascular and cardiac function; ARBs preserve the activity of the AT4-angiotensin IV system and the synthesis of angiotensin 1–7, both mechanisms are involved in vasodilatation. Since, in this case ACE is not inhibited; there isn't an increase in bradykinin concentration, which results in decrease of dry cough, a typical side effect of ACE inhibitors, and consequently a possibly better adherence to therapy by patients. Finally, ARBs also have some other beneficial effects at the systemic level, independently of their binding to the AT_1 receptor; they have been

shown to be able to activate the peroxisome proliferator-activated receptor-γ (PPAR-gamma) and can also induce the release of adiponectin from adipocytes. These effects cause an increase in the sensitivity to insulin, reducing the levels of circulating lipids, and promote anti-inflammatory activity (Nickenig et al. 2006).

4.1.2 Angiotensin II Receptor Type 1 (AGTR1) Gene

The AGTR1 belongs to the family of the angiotensin group of GPCR (G protein-coupled receptors) and shows the seven hydrophobic transmembrane domains forming R-helices in the lipid bilayer of the cell membrane. AGTR1 is composed of five exons, with the first four encoding the 5′ untranslated region (5′UTR) and the fifth being the coding region. The size of the exons ranges from 59 to 2014 bp (Guo et al. 1994). AGTR1 gene is localized to chr 3q24–q24 (*Genomic coordinates* (*GRCh38*): 3:148,697,870–148,743,002(NCBI)). The *AGTR1* product is found mainly in vascular smooth muscle cells and heart, adrenal gland, and kidney. The short isoform is composed of 359 amino acids and has a molecular mass of 41.1 kDa. The longer isoform is composed of 391 amino acids and is an additional 3.8 kDa larger. The protein sequence of the gene product is well conserved (Furuta et al. 1992). Large numbers of SNPs were predicted for AGTR1 gene. There are total 207 coding variants of which 132 are nsSNPs, and the rest of SNPs are synonymous.

4.1.3 Pharmacogenetic Study of AGTR1 Gene

Variation in response to drugs may result in poor control of blood pressure and lead to metabolic disorders like myocardial infraction, diabetes, kidney problems, thyroid, and high lipid profile. PGx offers the potential to achieve better blood pressure control in hypertensive patients, prevent organ damage in cardiovascular and renal diseases, and reduce side effects.

Individual variability in drug efficacy and drug safety is a main challenge in current clinical practice and drug development. The studies of pharmacogenetics focus on relations between genotypes and drug response to account for phenotypic variations of clinical importance in therapy. The concurrence of pharmacogenetics and human genomics in recent years has dramatically increased the discovery of new genetic variations that reveal the mechanisms underlying variability in drug response. The knowledge on genome-disease and genome-drug interactions thereby increases the hope of precision medicine (Ma and Lu 2011).

Thus, attention has been focused on identifying non-synonymous single nucleotide polymorphisms (nsSNPs) with potential impact on structure and function of the encoded drug target protein (Johnson et al. 2005). The SNP database contains predictions for all SNPs in a gene sequence. However in order to identify SNPs with functional impact on the protein, *in silico* analysis can be carried out on the predicted SNPs. Present study was designed to predict potentially deleterious and damaging mutations in proteins caused due to nsSNPs, which can modulate drug

Table 4.1 Pharmacogenetic studies of AGTR1 gene among hypertensive patients

S. no.	Study	Results
1.	Genetic contribution to the acute effects of angiotensin II type 1 receptor blockade (Spiering et al. 2005)	Systemic and renal hemodynamic responses to acute AT1R blockade are genetically determined
2.	Effects of AGTR1 A1166C gene polymorphism in patients with heart failure treated with candesartan (de Denus et al. 2008)	AGTR1 A1166C polymorphism could influence the response to candesartan in patients with heart failure who are receiving ACE inhibitors
3.	RAS polymorphisms relation to antihypertensive drugs (Suonsyrjä et al. 2009)	Polymorphisms of AGT, ACE, and AGTR1 do not predict BP responses to amlodipine, bisoprolol, HCT, and losartan in white hypertensive men
4.	Polymorphisms in RAS and salt-sensitivity genes influenced risk of diabetes associated with thiazides (Bozkurt et al. 2009)	Risk of diabetes due to thiazide use was not increased among AGTR1 1166 CC. ACE 4656 GG genotype enhanced the risk of diabetes due to thiazides
5.	Interactions of AGT, AGTR1, and ACE2 gene polymorphisms affect antihypertensive drugs (Chen et al. 2011)	Gender-specific gene-gene interactions of the AGT, AGTR1, and ACE2 genes were associated with individual variation of response to benazepril
6.	Effect of irbesartan concentration and AGT1R gene polymorphisms on blood pressure (BP) (Jiang et al. 2011)	Patients carrying allele C of rs5186 showed positive association between irbesartan concentration and BP response, but this was not seen in patients with AA genotype
7.	Relationship between renin-angiotensin system genotypes and ACE inhibitors in Chinese patients with coronary artery disease (CAD) (Lee et al. 2013)	ACE inhibitors were associated with a significant decrease in major cardiovascular events in Chinese patients diagnosed with CAD
8.	To investigate the human pharmacogenetic variation related to antihypertensive drugs (Polimanti et al. 2014)	Data suggested that rare variants mainly determine the functionality of genes related to antihypertensive drugs
9.	To study CAD patients among whom treatment reveals benefits of ACE-inhibitor therapy variation between individuals (Oemrawsingh et al. 2016)	Data suggest that long-term perindopril prescription in patients with a PGX score (pharmacogenetic risk score) of 0–2 is cost-effective

response by interfering with drug binding or metabolism, using different bioinformatic tools. Knowledge of such genetic variability in drug action, especially those for diseases with complex etiology, will help in conquering the obstacles and holds promise for achieving the ultimate goal of effective and safe medication to targeted patients with appropriate genotypes (Table 4.1).

4.1.4 *In Silico* Analysis of nsSNPs Using Different Bioinformatics Tools

A stepwise computational approach was employed to study the amino acid sites that are potent drug targets and may be involved in protein stability. In order to identify functional nsSNPs of human AGTR1 gene, data were retrieved from Entrez on the National Center for Biotechnology Information (NCBI) website. The UniProt accession number (P30556) was obtained from the Swiss-Prot database (http://expasy.org), and FASTA format of proteins were downloaded from UniProt (http://www.uniprot.org/).

4.1.4.1 Tools for *In Silico* Analysis of Protein Structure and Function

In silico analysis of AGTR1 variants was performed to get information about their effects on gene structure and function. Deleterious effects of AGTR1 variants were determined using F-SNP database. To predict the effect nsSNPs present on functionally important sites, several computational algorithms like Sorting Intolerant from Tolerant (SIFT) and Polymorphism Phenotyping (PolyPhen-2) were used.

SIFT is a program for predicting whether a SNP has an effect on protein structure. SIFT assumes that sequences of proteins have been conserved throughout evolution, and therefore any nsSNP at these sites may potentially affect the protein function. Thus, SIFT uses sequence homology to predict effects of substitutions at each position of the protein (Ng and Henikoff 2003). PolyPhen-2 (Polymorphism Phenotyping v2) is an algorithm which analyze all possible effects of an amino acid substitution on the stability and function of human proteins using physical, structural, and comparative evolutionary (Johnson et al. 2005; Zhu et al. 2008).

MutPred works by a random forest algorithm based on many features of protein structure and function like the probabilities of gain or loss of properties including loss of solvent accessibility, loss of catalytic residue, loss of stability, and gain of methylation site. The MutPred score is the prediction of the probability that a single amino acid change is deleterious/disease-associated (http://mutpred.mutdb.org/) (Table 4.2).

Firstly, amino acid positions involved glycosylation, disulfide bonding, and lipidation drug binding were retrieved from UniProt. In order to identify functional nsSNPs of human AGTR1 gene on these sites, SNP database was accessed. nsSNPs predicted on these sites were then analyzed using different *in silico* tools (Table 4.3).

In AGTR1, out of 27 nsSNPs 13 are predicted to be deleterious by both mentioned tools compiled in Table 4.3.The selected most probably damaging nsSNPs could be prioritized in further studies of the functional properties of the mutated receptor and have no population data available.

ARBs bind to the AT_1R in the region that partially overlaps with Ang II binding (K102, H166, R167, K199, and D263) and some other residues (V108, N111, A163, and S252) lying in the second extracellular loop (ECL2) and in the helix (Noda et al. 1995; Yamano et al. 1995; Le et al. 2003; Banday et al. 2005). Thus, SNPs present in these sites and nearby have the potential to alter drug binding and its functionality.

Table 4.2 Amino acid position in domains of AGTR1 protein involved in drug binding and protein stability (http://www.uniprot.org/)

Amino acid position	Description	Domain	nsSNP predicted (SNP ID)
4–4	Glycosylation	Topological domain extracellular	No SNP predicted
101–180	Disulfide bond	103–124 Transmembrane (helical)	rs753149496, rs748117430, rs545751404, rs747975618, rs774646145, and rs775810028
		125-–142 Topological domain (cytoplasmic)	rs570903965, rs764518060, rs751231086, rs201745152
		143–162 Transmembrane (helical)	rs373362261, rs753570924, rs201151143, rs140542820, rs550259107, rs144141909, rs368534001, rs762020328, rs770227887
		163–192 Topological domain (extracellular)	rs773829709, rs369846514, rs766907479, rs56257794, rs760594254, rs763952495, and rs756951904
176–176	Glycosylation	Topological domain (extracellular)	No nsSNP predicted
188–188	Glycosylation	Topological domain (extracellular)	No nsSNP predicted
355–355	Lipidation	Topological domain (cytoplasmic)	No nsSNP predicted
163,192,198, 220, 226	Drug-binding sites	Topological domain (extracellular)	rs12721226
		Transmembrane (helical)	
		Topological domain (cytoplasmic)	

The alanine at position 163 interacts with the antihypertensive antagonist, losartan. A163 is present in transmembrane domain four (TMD4) in the binding pocket. MutPred inferred it as deleterious mutation ($p = 0.234$) with potential of disrupting molecular interaction (p score <0.05) along with gain of MoRF binding ($p = 0.5978$), gain of glycosylation ($p = 0.0468$), loss of stability ($p = 0.3273$), and their loss of sheet ($p = 0.3653$) and helix ($p = 0.3949$) also.

Arsenault et al. (2010) evaluated the pharmacokinetics of ARB class of drugs in an in vitro cell-based assay, by testing its affinity by dose displacement of Ang II analogs in COS-7 cells expressing either wild-type human AT_1 (hAT1) A163 or T163h AT_1.The expressions of the receptors were evaluated by saturation binding, and the efficacies were assessed by measuring the [^{3}H]-inositol phosphate production. The results showed that the T163h AT_1 receptor is comparable in affinity, expression, and efficacy with that of native A163 h AT_1 toward peptide ligands. The affinities were also tested with non-peptide antagonists losartan, L-158809,

Table 4.3 Prediction scores from SIFT and PolyPhen-2 of the nsSNPs selected for AGTR1 gene

S. no	SNP ID	Amino acid position	SIFT score	PolyPhen-2 score
1.	rs753149496	L105I	0 Deleterious	0.999 Probably damaging
2.	rs748117430	D109A	0 Deleterious	0.999 Probably damaging
3.	rs545751404	C111R	0.02 Deleterious	0.761 Possibly damaging
4.	rs747975618	F112C	0.25 Tolerated	1 Probably damaging
5.	rs774646145	A120T	0 Deleterious	1 Probably damaging
6.	rs775810028	Y122C	0 Deleterious	0.956 Probably damaging
7.	rs570903965	M125V	0.24 Tolerated	0.764 Possibly damaging
8.	rs764518060	R128H	0.76 Tolerated	0.003 Benign
9.	rs751231086	C136R	0 Deleterious	1 Probably damaging
10.	rs201745152	I138F	0.02 Deleterious	0.076 Benign
11.	rs373362261	V143I	0.46 Tolerated	0.025 Benign
12.	rs753570924	S144R	0.05 Tolerated	0.894 Possibly damaging
13.	rs201151143	L147R	0 Deleterious	0.996 Probably damaging
14.	rs140542820	A149T	0.36 Tolerated	0.114 Benign
15.	rs550259107	S150T	0.08 Tolerated	0.654 Possibly damaging
16.	rs144141909	T155R	0 Deleterious	0.993 Probably damaging
17.	rs368534001	I159V	0.82 Tolerated	0.336 Benign
18.	rs762020328	R161Q	0 Deleterious	1 Probably damaging
19.	rs770227887	Y162C	0.02 Deleterious	1 Probably damaging
20.	rs12721226	A163T	0 Tolerated	0.32 Benign
21.	rs773829709	H167N	0 Deleterious	0.966 Probably damaging
22.	rs369846514	M169V	0.7 Tolerated	0.026 Benign
23.	rs766907479	S171Y	0 Deleterious	0.988 Probably damaging
24.	rs56257794	R172H	0.03 Deleterious	0.999 Probably damaging
25.	rs760594254	R174Q	0.24 Tolerated	0.521 Possibly damaging
26.	rs763952495	R175C	0 Deleterious	1 Probably damaging
27.	rs756951904	L178I	0.43 Tolerated	0.046 Benign

valsartan, telmisartan, irbesartan, candesartan, and EXP3174 losartan and EXP3174, which displayed a sevenfold loss in affinity toward T163h AT_1. The ability of losartan to inhibit Ang II-induced inositol triphosphate production also confirmed a loss in efficacy. Molecular modeling showed a higher steric and hydrophilic hindrance of the T163h AT_1-losartan complex, empirically validating the results of the *in silico* analysis mentioned in Tables 4.2 and 4.3.

Punjabi population was screened for distribution of rs12721226 of AGTR1 gene, from different districts of Punjab (Ropar, Patiala, Nawasehar, Fazillka, Muktsar, Barnala) among 300 hypertensive and 100 normotensive individuals. Study has been approved by Institutional clinical ethical committee (ICEC/4/2011). PCR-RFLP method was used for analysis, and detection of polymorphism was carried out on 10% PAGE. The genotype frequencies observed among 300 hypertensive subjects are 286 GG, 4 GA, and 0 AA and 100 GG, 0 GA, and 0 AA among 100 normal subjects**.** The frequencies of the G and A alleles were 0.96 and 0.04 in hypertensive subjects and 1.0 and 0 in normotensive, respectively. Four patients

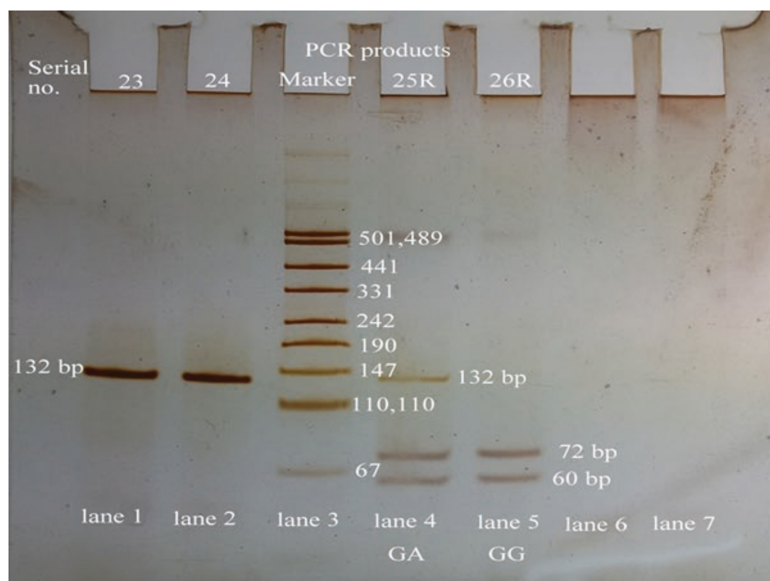

Fig. 4.2 Genotyping after digestion of 132 bp product (lane 1and 2) of rs12721226 of AGTR1 gene with Alu I (restriction enzyme) in lane 4 heterozygote and in homozygote in lane 5 and molecular marker in lane 3

who were found to be heterozygotes were on ARBs, and two of them were also found to be suffering from other metabolic disorder, viz., diabetes (Fig. 4.2).

This SNP is also validated in 1000 genomes database. The population study obtained from NCBI site for this particular SNP shows that in Asian, sub-Saharan Asian, and Europeans, frequency of G allele is 1, and frequency of A allele is 0, that is, they are monomorphic at this locus, but in African Americans, frequency of G allele is 0.978, and frequency of A allele is 0.022, respectively.

Thus, these nsSNPs substantially influence ARB binding and result in modulation of their clinical responses in populations who are polymorphic at this locus. This will help in an appropriate interpretation of pharmacogenetic implication for antihypertensive drugs especially ACE inhibitors and ARBs at population level and for individual patients and drug developers bringing them closer to precision medicine.

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References

Arsenault J, Lehoux J, Lanthier L, Cabana J et al (2010) A single-nucleotide polymorphism of alanine to threonine at position 163 of the human angiotensin II type 1 receptor impairs Losartan affinity. Pharmacogenet Genomics 20(6):377–388

Banday AA, Siddiqui AH, Menezes MM, Hussain T (2005) Insulin treatment enhances AT1 receptor function in OK cells. Am J Physiol Ren Physiol 288:F1213–F1219

Bozkurt O, de Boer A, Grobbee DE, de Leeuw PW et al (2009) Variation in Renin-Angiotensin system and salt-sensitivity genes and the risk of diabetes mellitus associated with the use of thiazide diuretics. Am J Hypertens 22(5):545–551

Chen Q, Yu CQ, Tang X, Chen DF et al (2011) Interactions of renin-angiotensin system gene polymorphisms and antihypertensive effect of benazepril in Chinese population. Pharmacogenomics 12(5):735–743

de Denus S, Zakrzewski-Jakubiak M, Dubé MP, Bélanger F et al (2008) Effects of AGTR1 A1166C gene polymorphism in patients with heart failure treated with candesartan. Ann Pharmacother 42(7):925–932

Furuta H, Guo D, Inagami T (1992) Molecular cloning and sequencing of the gene encoding human angiotensin II type 1 receptor. Biochem Biophys Res Commun 183:8–13

Guo DF, Furuta H, Mizukoshi M, Inagami T (1994) The genomic organization of human angiotensin II type 1 receptor. Biochem Biophys Res Commun 200:313–319

Jiang S, Hsu YH, Venners SA, Zhang Y et al (2011) Interactive effect of angiotensin II type 1 receptor (AGT1R) polymorphisms and plasma irbesartan concentration on antihypertensive therapeutic responses to irbesartan. J Hypertens 29(5):890–895

Johnson MM, Houck J, Chen C (2005) Screening for deleterious non-synonymous single-nucleotide polymorphisms in genes involved in steroid hormone metabolism and response. Cancer Epidemiol Biomark Prev 14(5):1326–1329

Le MT, Vanderheyden PM, Szaszak M, Hunyady L et al (2003) Peptide and nonpeptide antagonist interaction with constitutively active human AT1 receptors. Biochem Pharmacol 65:1329–1338

Lee JK, Wu CK, Tsai CT, Lin LY et al (2013) Genetic variation-optimized treatment benefit of angiotensin-converting enzyme inhibitors in patients with stable coronary artery disease: a 12-year follow-up study. Pharmacogenet Genomics 23(4):181–189

Ma Q and Lu AY (2011) Pharmacogenetics, pharmacogenomics, and individualized medicine. Pharmacol Rev 63:437–459

Ng PC, Henikoff S (2003) SIFT: predicting amino acid changes that affect protein function. Nucleic Acids Res 31(13):3812–3814

Nickenig G, Ostergren J, Struijker-Boudier H (2006) Clinical evidence for the cardiovascular benefits of angiotensin receptor blockers. J Renin-Angiotensin-Aldosterone Syst 7(Suppl 1):S1–S7

Noda K, Saad Y, Kinoshita A, Boyle TP et al (1995) Tetrazole and carboxylate groups of angiotensin receptor antagonists bind to the same subsite by different mechanisms. J Biol Chem 270:2284–2289

Oemrawsingh RM, Akkerhuis KM, Van Vark LC et al (2016) Individualized Angiotensin-Converting Enzyme (ACE)-inhibitor therapy in stable coronary artery disease based on clinical and pharmacogenetic determinants: the Perindopril Genetic (PERGENE) risk model. Pharmacogenet Genomics 23(4):181–189

Polimanti R, Iorio A, Piacentini S, Manfellotto D, Fuciarelli M (2014) Human pharmacogenomic variation of antihypertensive drugs: from population genetics to personalized medicine. Pharmacogenomics 15(2):157–167

Spiering W, Kroon AA, Fuss-Lejeune MJ, de Leeuw PW (2005) Genetic contribution to the acute effects of angiotensin II type 1 receptor blockade. J Hypertens 23(4):753–758

Suonsyrjä T, Hannila-Handelberg T, Fodstad H et al (2009) Renin-angiotensin system and alpha-adducin gene polymorphisms and their relation to responses to antihypertensive drugs: results from the GENRES study. Am J Hypertens 22(2):169–175

Weir MR (2007) Effects of renin-angiotensin system inhibition on end-organ protection: can we do better? Clin Ther 29(9):1803–1824
Yamano Y, Ohyama K, Kikyo M, Sano T et al (1995) Mutagenesis and the molecular modeling of the rat angiotensin II receptor (AT1). J Biol Chem 270:14024–14030
Zhu Y, Hoffman A, Wu X, Zhang H et al (2008) Correlating observed odds ratios from lung cancer case-control studies to SNP functional scores predicted by bioinformatic tools. Mutat Res 639(1–2):80–88

Weblinks

genetics.bwh.harvard.edu/pph2
http://expasy.org
http://mutpred.mutdb.org
http://mutpred.mutdb.org
http://sift.jcvi.org
http://www.ncbi.nlm.nih.gov/snp
http://www.uniprot.org

Part II

Bionanotechnology and Drug Designing

In-Silico Drug Designing: Transition to Modern-Day Drug Discovery

5

Kiran Nehra, Preti Yadav, and Joginder Singh Duhan

5.1 Introduction

Today the rapid advances made in the area of molecular biology, bioinformatics, genomics and proteomics has enabled a deeper understanding of various diseases in the human body. Human body is an apt example of a highly systemic and complex machine equipped with all essential components including various chemicals, enzymes, signalling molecules and other necessary biological components and processes required for its proper functioning. But there are times when these components and processes fail to function properly resulting in undesirable effects. Hence to reinforce the normal, original state of the body, body is administered with external medication, popularly known as 'drugs' (Martis and Somani 2012). Drug is a chemical entity which when interacts with the molecular target brings a change in the form of amelioration of the disease. Millions of lives are lost due to lack of drugs pertaining to life-threatening diseases which are coming up at an ever increasing pace. Earlier drug designing process was time-consuming and a costly endeavour with high percentage of failures. But today, with the rapid developments taking place in the field of bioinformatics and genomics and proteomics, structures of various protein molecules which can act as drug targets have become available online; and it has become a lot easier to design an effective, accurate and reliable drug in silico (Singh 2014). The structural information of target proteins when combined with various computational tools helps in accurate identification of active binding sites on the target, and this information is in turn used in identification of potential drug candidates which can effectively fit and interact with these sites (Bharath et al. 2011). To meet the challenges faced by scientists in developing ideal drugs, in silico

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drug designing has, therefore, become a highly efficient multidisciplinary technology, which not only provides valuable drugs at low capital and time factors but also makes pharmaceutical companies a highly profitable business (Kumar 2013).

The present chapter deals with the drug designing technology including the complete journey of a chemical compound commencing with its discovery till it is crowned as a potential drug. The entire chapter is divided into different sections beginning with traditional drug discovery and the limitations associated with it which demands for a need for an alternative technology; followed by various stages of drug designing which include target identification and validation, lead identification and validation and in silico docking studies; and finally a note on the various clinical trials through which a drug needs to undergo before its release in the market.

5.2 Traditional Drug Discovery Process: Limitations

The traditional drug discovery is intense, exhaustive and an extremely costly task, as it banks on hit and trial testing of chemical compounds (Laeeq et al. 2014). It generally takes 14–16 years of intensive experimentation for a drug candidate to complete its journey from its initial discovery till its landing in the market (Fig. 5.1) (Rahman et al. 2012). On an average, the clinical trials consume 6–8 years to confirm the utility of the discovered molecule as a drug. Apart from the time factor,

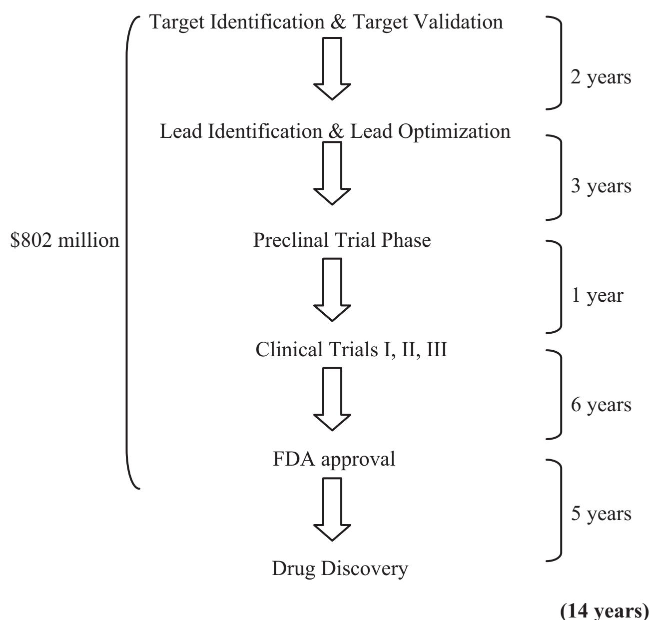

Fig. 5.1 Traditional drug discovery pathway

another major limitation is the cost factor. The average cost to transform a molecule to a successful drug is estimated to be $802 million (Gupta et al. 2011) which mainly involves the cost of failure of thousands of compounds screened in the initial stages of drug discovery. The pharmaceutical companies invest an enormous amount of money for the discovery of a single drug; if the drug fails at any stage during the process, the company has to bear immense losses. As the amount of these losses is highly intimidating, there is an urgent need for the use of highly sophisticated technologies which can be a relief to the pharmaceutical industries and can bring hope to millions of patients.

5.3 In Silico Drug Designing: An Alternative to Traditional Drug Discovery

In silico drug designing is an emerging technology in the field of medicine which is on its way to replace the conventional drug discovery completely, making it a less risky business with the involvement of computers and various softwares to eliminate the failures and to predict the possible outcomes. It is a process involving formulation of drugs for the biological targets in silico in the laboratories using softwares (Table 5.1). With the availability of more and more protein structures deposited online using techniques such as NMR and X-ray crystallography, and the emergence of bioinformatics tools such as CADD, molecular docking and other computer-aided tools (Mandal et al. 2009), the search for active binding sites also known as 'pockets' in the target molecules, and the potential drug molecules which can bind to these sites/pockets, has become comparatively easy. This can be a milestone towards treating life-threatening diseases such as arthritis, malaria, cancer, tuberculosis, neurodegeneration impairment and AIDS (Gupta et al. 2011). Lead identification and its exploitation by changing its structural features which is popularly known as 'drug tailoring' serves as the basis of drug designing.

Table 5.1 Drug designing softwares used in different stages of in silico drug designing

Feature/purpose	Software tools
Molecular modelling	CHARMM, GROMACS, Amber
Target prediction	PredictFX, ChemProt, SEA
Binding site prediction	FINDSITE, fpocket, MED-SuMo
Screening	Pharmer, Catalyst, PharmaGist
Ligand design	GANDI, LUDI, SPROUT
Docking	AutoDock, DOCK, GOLD
Binding free energy estimation	HYDE, X-score, NNScore
QSAR	cQSAR, cLogP, MOLEdb
ADME toxicity	QikProp, VolSurf, GastroPlus

5.4 Drug Designing Approaches

Drug designing starts once a target molecule is confirmed. NMR spectroscopy coupled with various molecular modelling techniques is used to design drugs based upon the detailed information about target and ligand (Dutta et al. 2010). Depending upon the information available about the two molecules, two approaches can be followed for designing a drug. If information is available on known ligands of the target protein molecule, then ligand-based drug designing method is considered, but if information on 3D structure of the molecule is available, then structure-based drug designing proceeds (Lee et al. 2011).

5.4.1 Ligand-Based Drug Designing

In the absence of structural information of the target molecule, ligand-based drug designing banks on the knowledge of known inhibitors which exhibit a binding with the target receptor. Various compounds with structures similar to known inhibitors can be easily identified from chemical databases followed by their linking to the target molecule to produce a high-affinity drug lead (Hajduk 2006). A pharmacophore model is then developed using molecules which bind to the target, which in turn is used to study the minimum necessary characteristics which must be possessed by compounds to bind to the target (Fig. 5.2). Therefore, this method is also known as 'indirect drug designing' as new molecules are designed based on the model developed which in turn is based on the knowledge of what binds to the target. The theory of pharmacophore was given as early as in 1909 by Ehrlich, who defined the pharmacophore as 'a molecular framework that carries (phoros) the essential features responsible for a drug' (pharmacon) biological activity' (Yang 2010). Some other successfully developed ligand-based methods have been based on similarity and substructure searching, 3D shape matching or pharmacophore matching.

5.4.2 Structure-Based Drug Designing

Structure-based drug designing makes use of the 3D structure of the target receptor to hunt potential drug candidates who can interact and change the target function making it more specific and accurate in action. This information is an essential component in novel drug designing and in prediction of interactions between the drugs and the target (Dutta et al. 2010). Therefore, this method is also known as direct drug designing. It involves docking of candidate drugs and calculating electrostatic fit by studying binding affinity and selectivity to the target. Based on the fit and binding affinity, scores are generated, and the compounds are ranked according to the scores. The compound with the highest score ranks first and is considered to be the best fit ligand and a competent drug. Two main categories of structure-based drug designing are:

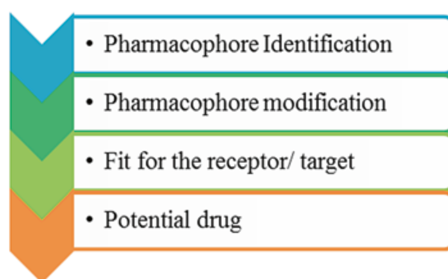

Fig. 5.2 Ligand-based drug designing

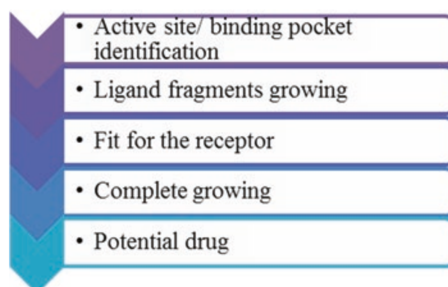

Fig. 5.3 Structure-based drug designing

(a) Finding ligands for the biological target through extensive database searching to find those chemical compounds which produce the best fit by binding with the pocket of the receptor effectively.
(b) Building ligands in stepwise manner by assembling small pieces (atoms or molecular fragments) within the constraints of the binding pocket. The method leads to discovery of novel structures which are not present in any chemical database (Fig. 5.3).

An important aspect of both drug designing approaches is high stringency. The drug designing process in both the approaches is made very selective at initial stages of the drug discovery so as to pass only those drug candidates which have the highest probability of becoming a drug and eliminating all the others (Gmuender 2002). Increasing the number of potential drug candidates does not increase the number of successful drug molecules; rather, this becomes a major cause for a dramatic cost increase in the drug designing process, as even today there are numerous drug molecules awaiting a preclinical trial. Therefore, increasing the number of potential drug molecules down the pipeline does not always guarantee a drug. On the contrary, emphasis should always be laid towards discovering a drug molecule with maximum potency and minimal side effects (Roy et al. 2001). The in silico drug designing process comprises of five main stages; these have been detailed in the subsequent sections.

5.5 In Silico Drug Designing: Developmental Stages

Drug designing process starts with the identification of a biological target which is responsible for causing the disease or against which a drug is to be identified. This identified target is then targeted for the correct lead molecule identification which can firmly bind to it and act as drug to cure the disease. This potential drug molecule is then carried forward to preclinical trials which are conducted on animals, and if the drug shows promising results, it is carried to the next stage which is the conduct of clinical trials on humans. The following sections give a stepwise detailed account of the drug designing procedure which results in the finding of a potential drug candidate.

5.5.1 Target Identification

Target identification marks the first step in the drug designing process. A target is a biological entity present inside the body, which when combines with the potential drug candidate produces therapeutic effect in the form of either alleviation or prevention of the disease. Receptor is the alternative name given to the target molecule. A disease is caused either due to abnormal production of reaction intermediates or malfunctioning of biochemical systems. The most important biological targets are enzymes, hormones and other protein regulators which play an important role in the regulation of many biochemical pathways (Martis and Somani 2012). An ideal target molecule is one that is closely linked to human diseases and binds to a small molecule in order to carry out a function (Gupta et al. 2011).

Identification of a valid target among numerous candidate molecules is a serious challenge (Kumar 2013). However, this can be easened by literature surveying, by analysing various biochemical pathways and by conducting genomic and proteomic studies. Using proteomic approach, protein expression profiles of various compounds are intensively studied and compared. However, this method is not reliable and has a lower percentage of success in target discovery process, as it is time and man power intensive (Bharath et al. 2011). Therefore, to eliminate the risks involved, complementary methods such as a series of computational (in silico) tools (Table 5.1) have been developed for valid and accurate target identification.

5.5.2 Target Validation

Once the target identification is done, it becomes absolutely essential to check the correctness of the target identified in terms of its desired therapeutic effect. Target validation is an extensive and rigorous evaluation to confirm that modulation of target identified will have the desired clinical outcome. It also involves determining the physiological effect produced due to the modulation of the target's function. Target validation also checks the presence of any secondary target which might bind to the drug leading to an undesirable and adverse reaction. The characteristic

property of an ideal drug molecule is its specificity towards the biological receptor, but this is not always the case. There are chances that a drug instead of binding to a single biological target binds to other biological targets leading to unwanted pharmacological actions. Therefore the binding of a drug to other targets needs to be strictly minimized.

5.5.3 Lead Identification

Leads are specific compounds which when bind with the identified biological target produce the desired therapeutic action. Leads possess the basic structural requirements for interacting with the target and for exhibiting the desired action. The process of lead identification involves high-throughput screening of a large chemical compound library containing synthesized or natural compounds. Earlier, lead identification was done by screening huge chemical compound libraries through computer-aided drug designing (CADD) followed by wet lab experimentation to identify potential drug candidates. But, recent advances in drug discovery involving molecular docking have modified the existing methodology. Docking involves computational determination of the binding affinity between a ligand and the target protein molecule. It helps in finding the best potential ligand or protein molecule which is near to becoming a drug. However, before docking, virtual screening has to be performed. Virtual screening is a computer-based method of screening huge libraries of compounds using computer-based softwares. Today virtual screening is the first choice for screening a large number of chemical compounds within a reasonable time frame. Large compound libraries can be easily accessed from both public and private domains to aid virtual screening. The lead identification directed towards a biological target may arise from two sources:

(a) Virtual screening of chemical libraries of diverse compounds against a molecular target
(b) Modification of the natural receptor ligand or enzyme substrate either structurally or chemically

Upon identification of a lead compound, efforts are made towards further refinement by developing a structure-activity relationship (SAR) for properties of the molecule such as activity, efficacy, selectivity, bioavailability and pharmacokinetics (Fig. 5.4).

5.5.4 Lead Optimization

Once the lead compound is identified, it is altered, refined and optimized through a series of complex processes so as to get a more refined compound with improved drug-like characteristics. The goal of lead optimization is to optimize a drug molecule with improved potency and reduced side effects, bioavailability, binding

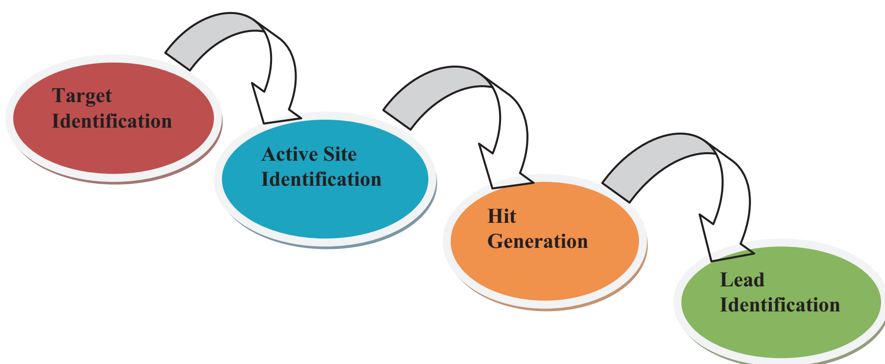

Fig. 5.4 Lead identification pathway

affinity for the target, cell permeability and metabolism. The process of lead optimization is an iterative method. When structural information about the target is lacking, lead optimization is carried out using different approaches such as (Khan and Firoz 2015):

1. 3D/2D quantitative structure-activity relationship (QSAR)
2. Structure-based drug designing (SBDD)
3. Computer-aided drug designing (CADD)

The QSAR approach is based on physicochemical properties of the drug molecule which have the capacity to influence drug action. The pharmacophoric features which play an important role in QSAR studies include hydrophobic and aromatic nature of the molecule, hydrogen bond acceptors, hydrogen bond donors and positive and negative ionizable groups (Dutta et al. 2010). All these approaches generate enormous amount of data, which is carefully studied and then used in the optimization of the lead candidate possessing the best probable structure, with most desirable action. SBDD is used when the 3D structure of the target is available and then the potential drug candidate is docked with it. The one with the best hit is selected and carried forward to different stages. If the 3D structure of the target is not available, then homology modelling is used to create one using structure of a highly homologous protein whose structure is already known. Computer-aided drug designing involves the usage of computers in identification of target molecule and designing of ligand which suits it best. It also predicts the receptor-ligand interaction and predicts the best model with highest possible biological activity (Martis and Somani 2012).

5.5.5 In Silico Pharmacokinetic Studies: ADMET (Absorption, Distribution, Metabolism, Excretion, Toxicity) Prediction

Pharmacokinetics (PK) and pharmacodynamics (PD) play a crucial role in the success of a molecule to become a drug. These studies should be initiated in the preliminary stages of drug development as poor pharmacokinetic and toxicity studies can lead to later stage drug failure which is very costly to bear by any company. The different stages in ADME start with the penetration of drug through various physiological barriers such as blood-brain barrier, gastrointestinal barrier, transportation to its site of action, degradation by different enzymes and lastly excretion out of the body (Rahman et al. 2012). In silico tools (Table 5.1) along with combinatorial chemistry and high-throughput screening (HTS) can be used to predict the most relevant pharmacokinetic, distribution, toxicity and metabolic characteristics of a large number of compounds, thereby quickening the drug discovery process (Kore et al. 2012).

5.6 Clinical Studies

Once a potential drug candidate is selected, it is further tested down the pipeline for checking its efficacy and safety by first conducting preclinical trials on animals, and if the drug clears this stage, then it is further tested on humans.

5.6.1 Preclinical Studies

The primary goal of preclinical studies is to ensure the safety and efficacy of newly discovered drug candidates, in order to begin clinical studies on humans. A newly discovered drug can be tested on human body only after it has been supported by experimental proof of pharmacology and toxicology from animal studies. The preclinical stage involves extensive studies on animal models to get information on various aspects such as safe minimal starting dose with minimum toxicity and side effects, sensitivity to different organs of the body and the kinetics of drug distribution and metabolism of the drug inside the body (Martis and Somani 2012). Preclinical studies are carried out either in vitro on cell lines or in vivo on live animals. However, a small amount of drug is insufficient in this preclinical stage; therefore, upscaling of the drug should be done to meet the large drug demand during clinical trials.

5.6.2 Phase I, II, III and IV Clinical Trials

The preclinical studies are followed by clinical trials. But, before proceeding further into clinical trials, an investigational new drug (IND) application needs to be filed by the innovator with FDA. The application includes the outcome of preclinical

work containing details on how the investigational medicine works in the body and any potential side effects associated with it (Martis and Somani 2012). Apart from the drug manufacturing details, the application also contains detailed and complete plan of conducting the clinical trials. After this only, the actual testing of the drug on human volunteers is conducted to study the effect of the drug on the human body with respect to its toxicological effects and other safety parameters, as the results obtained through animal model cannot be exactly implemented on humans. This stage comprises studies of four phases, phase I, phase II, phase III and phase IV, after the drug has been released into the market.

5.6.3 Phase I Clinical Trial

This phase of clinical trials is conducted on a small number (50–100) of healthy volunteers who are generally compensated financially for their participation in these drug trials. The main goal of this phase of clinical trials is to evaluate the safety and efficacy of the drug when administered in humans. Here, the PK of the drug which involves studies on absorption, metabolization and elimination from the body are intensively studied based on the samples in the form of urine, blood and stool collected from the tested patients. Along with PK, PD studies are also carried out with main emphasis on the side effects caused due to the drug intake. The patient is carefully monitored round the clock for changes in behavioural pattern and vital signs to determine the safe dosing range and to check whether the drug should be promoted on to the next stage of drug development. The main aim of phase I clinical studies is to establish the safe dosage range (Chhabra et al. 2012). This trial usually takes several months to a year and around US$10 million to enter into the next phase.

5.6.4 Phase II Clinical Trial

Phase II clinical trials are conducted in 100–500 volunteers with the target disease. The drug is given to the diseased human body in variable dose strength and schedule to check the optimum dosage, safety, efficacy, tolerability and PK and PD studies of the administered drug. Those drugs which fail to show the desired effect on patients are quickly eliminated, and the ones showing promising signs are taken ahead for much larger phase III trials. It generally takes 1–2 years and US$20 million for testing to promote the drug to the next clinical trial phase.

5.6.5 Phase III Clinical Trial

Phase III clinical trial is an extended version of phase II trials, enrolling 1000–5000 individuals with the target disease at numerous clinical trial sites around the world. This trial covers a large population and multiple site studies including hospitals and health centres around the world to collect and process data about the effect of the

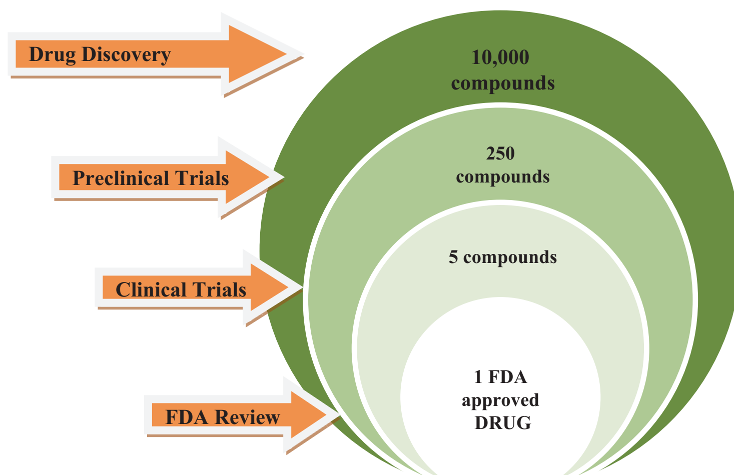

Fig. 5.5 Screening of chemical compounds in various stages of drug discovery

drug on human body across the globe which would supplement the fine tuning of the trial. Phase III trials are both costliest and longest trials taking somewhere up to 3–5 years to complete and demanding U$50–100 million. The data collected from various sources is carefully analysed which provides the basis for labelling instructions containing information on potential interactions with other medicines and safe dosage. This phase finally confirms various parameters studied in phase II clinical trials. After the completion of this stage, 'new drug application (NDA)' is filed to the FDA by the pharmaceutical companies. Once the drug is approved by FDA, it is launched in the market.

5.6.6 Phase IV Clinical Trial

This stage is marked by post marketing surveillance studies once the drug has entered the market. Careful monitoring is done to review the performance of the drug and to detect any adverse or undesirable reaction which might have been failed to be encountered in pre-stages of drug development. If any serious adverse reaction is detected in the patients, the drug is withdrawn from the market (Fig. 5.5).

5.7 Challenges Before In Silico Drug Designing

Although with the aid of drug designing, several potential drugs are being discovered in a short span of time, but there are certain issues which are yet to be solved. The main problem faced during initiating drug designing is the lack of structural, biological and chemical information of the identified target molecule. Therefore,

the chemical libraries containing the structures of various lead molecules need to be more advanced with structures of more and more chemical entities included. More reliable experimental data related to ADME, drug toxicity, natural leads and physicochemical properties also needs to be made available online. But, even with the above-mentioned limitations, drug designing is an effective technology, continuously striving towards reducing the present time taken to discover a drug molecule with intervention of computers at every possible step making it more rational and successful in the future. It is being used intensively for the discovery of drugs to cure fatal diseases which cost lives of millions of people.

5.8 Conclusion

The traditional drug discovery is a long, complex and costly process, suffering from limitations like lack of accuracy and absence of high-throughput techniques. These drawbacks have resulted into a shift from the traditional methods of drug discovery to in silico drug designing which is an economical and time-saving process, thus making it commercially more viable as compared to the traditional method. Emergence of various in silico approaches, such as vHTS, QSAR and homology modelling which are of supreme significance in accurate and reliable drug identification, have helped in silico drug designing to play a central role in the field of drug discovery. In the recent past, significant achievements have been made in in silico drug designing, which in turn have opened new gateways for the pharmaceutical industries all over the world. Hence, it can conclusively be said that drug designing is evolving at a very rapid pace; however, it is still a budding field yet to be explored completely but holds great potential for several significant achievements in the future.

References

Bharath EN, Manjula SN, Vijaychand A (2011) In-silico drug design tool for overcoming the innovation deficit in the drug discovery process. Int J Pharm Sci 3(2):812

Chhabra N, Bihani SGL, Bhardwaj S (2012) Introduction to various stages in process of drug. Development 3(1)

Dutta S, Sutradhar S, Sachan K (2010) Computer-aided drug design-a new approach in drug design and discovery. Int J Pharm Sci Rev Res 4(5):146–151

Gmuender H (2002) Perspectives and challenges for DNA microarrays in drug discovery and development. Drug Discov Genomic Technol 32:152–158

Gupta PK, Agarwal P, Shivakumar N, Hiremath SB (2011) In-silico modelling and drug design. IRJP 2(9):15–17

Hajduk PJ (2006) Fragment-based drug design: how big is too big. J Med Chem 49:6972–6976

Khan A, Firoz N (2015) Drug discovery and development, a new hope. Int J Adv Res Comput Sci Softw Eng 5(1):337–345

Kore PP, Mutha MM, Antre RV, Oswal RJ, Kshirsagar SS (2012) Computer-aided drug design: an innovative tool for modeling. OJMC 2:139–148

Kumar CS (2013) An insight to drug designing by in-silico approach in biomedical research. J Pub Health Med Res 1(2):63–65

Laeeq S, Sirbaiya AK, Siddiqui HH, Zaidi SMH (2014) An overview of the computer aided drug designing. World J Pharm Pharm Sci 3(5):963–994

Lee CH, Huang HC, Juan HF (2011) Reviewing ligand-based rational drug design: the search for an ATP synthase inhibitor. Int J Mol Sci 12:5304–5318

Mandal S, Moudgil M, Mandal SK (2009) Rational drug design. Eur J Pharmacol 625(1–3):90–100

Martis EA, Somani RR 2012 Drug designing, discovery and development techniques, promising pharmaceuticals. In Basnet P (ed). InTech, pp 19–36. doi:https://doi.org/10.5772/38948

Rahman MM, Karim MMR, Ahsan MQ, Bashar A, Khalipha R, Raihan M, Chowdhury, Saifuzzaman M 2012 Use of computer in drug design and drug discovery. Int J Pharm Life Sci 1(2), Serial 5

Roy K, Sengupta C, De AU (2001) Theoretical aspects of rational drug design- an overview. J Sci Ind Res 60:699–716

Singh DB (2014) Success, limitation and future of computer aided drug designing. Transl Med (Sunnyvale) 4:E-127

Yang SY (2010) Pharmacophore modelling and applications in drug discovery: challenges and recent advances. Drug Discov Today 15(11–12):444–450

Nanocarriers in Drug and Gene Delivery

6

Deepti Pandita, Aman Munjal, Sandeep Godara,
and Viney Lather

6.1 Introduction

Nanotechnology and nanoscience are widely seen as having a tremendous promise to revolutionize the scientific landscape in terms of research and applications. Among various approaches, nanocarriers as a tool of nanotechnology offer great advantages for biomedical applications, viz., drug delivery, sensing, and imaging (Singh and Lillard 2009). Concurrently, the applications of nanotechnology in target-specific delivery of drug and gene have opened up new areas of research in nanomedicine.

Nanotechnology is the design of objects with dimensions conveniently described in units of nanometers (10^{-9} m) (Thassu et al. 2007). The dimensions of nanoparticles (NPs) lie between 10 and 1000 nm. Objects in the nanometer size range often exhibit properties that are not found in bulk materials of the same composition. However, particles >200 nm are not widely used, and nanomedicine holds interest in particles <200 nm as physicochemical properties of the particles in this range make them attractive for commercial and medical development (Singh and Lillard 2009). Nanocarriers for drug and gene delivery can be classified as lipid-based (solid lipid NPs, liposomes), polymer-based (polymeric micelles, dendrimers or polymeric NPs), and inorganic NPs (metallic NPs, silica NPs (SiNPs), carbon nanotubes (CNTs)) (Estanqueiro et al. 2015). The selection of material for development of nanocarrier is highly dependent on encapsulated payload/bioactives, therapeutic or diagnostic goal, and route of administration (Jabr-Milane et al. 2008).

Nowadays nanosystems focus primarily on the development of target-specific and slow but controlled drug release systems. A major milestone has been achieved in drug delivery systems with the development of technologies that can mask the

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nanocarriers from the immune system. Specific decoration of nanocarriers has significantly increased the half-life of the drug. The introduction of synthetic lipid derivatives of polyethylene glycol (PEG) confers “stealth” capability on nanocarrier system, due to the hydrophilicity of the PEG chains. This PEG stealth avoids opsonization and reduces fast blood clearance by immune recognition, hence helping in passive accumulation in the tumors via an enhanced permeation and retention (EPR) effect (Gref et al. 1995, 2000; Mosqueira et al. 1999). The first generation of nanosystems was nanometric liposomes. They are able to encapsulate both hydrophilic and hydrophobic drugs (Patel et al. 2015). The next generation were polymeric NPs precipitated with drug molecules and surface functionalized that prevented the immune recognition and nowadays even having targeting moieties in form of antibodies or folic acid. They have potential to enhance therapeutic benefit while reducing side effects as compared to free drug. Furthermore, inorganic NPs are being widely explored because of their chemical, physical and structural characteristics. This chapter considers current status and possible future directions of conventional and engineered pharmaceutical nanocarriers used for drug and gene delivery.

6.2 Lipid-Based Nanoparticles

Among the group of several nanoformulations, lipid nanoformulations have received much attention due to its biocompatible and biodegradable nature. They have huge potential for delivery of drugs and genes in several diseases. In the field of nanomedicine, they offer interesting benefits, viz., enhancing drug efficacy and providing controlled and convenient drug release. The performance of lipid nanoformulations greatly depends upon the composition and structure of formulations. Lipid-based nanocarriers may be further categorized as liposomes, solid lipid nanoparticles (SLNs), and nanostructured lipid carriers (NLCs) (Lim et al. 2012) (Fig. 6.1). Several lipid-based formulations have already entered in the market, and others are in preclinical and clinical phase (Table 6.1).

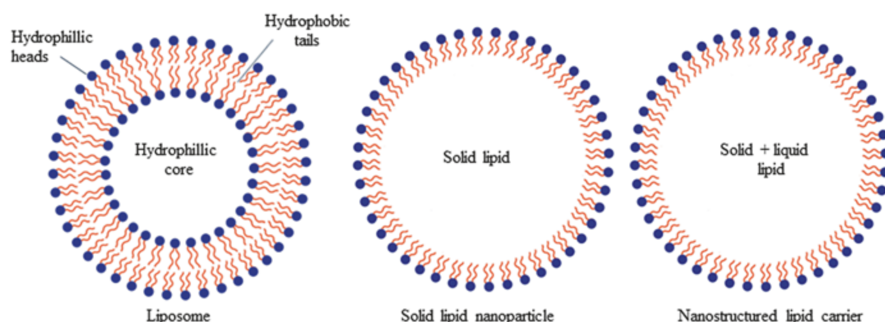

Fig. 6.1 Structure of lipid-based nanocarriers

Table 6.1 Clinical status of liposomal formulations

Brand name	Drug	Indication	Status	References
Mepact® [Takeda]	Mifamurtide MTE-PE	Osteosarcoma	Marketed	Venkatakrishnan et al. (2014)
AmBisome™ [Astellas Pharma/ Gilead Sciences]	Amphotericin B	Serious fungal infections	Marketed	Pagano et al. (2013)
Marqibo® [Talon Therapeutics, Inc.]	Vincristine sulfate	Acute lymphoblastic leukemia	Marketed	Rodriguez et al. (2009)
Doxisome®/ Lipo-Dox® [Taiwan Liposome Company, Ltd]	Doxorubicin	Kaposi's sarcoma, ovarian cancer, breast cancer	Marketed	Rivera (2003)
Myocet®50 [Cephalon]	Doxorubicin	Metastatic breast cancer	Marketed	Batist et al. (2001)
Visudyne® [Novartis AG/QLT Inc.]	Verteporfin	Age-related macular degeneration	Marketed	Huber and Levy (2001)
DepoCyt ® [Pacira Pharmaceuticals, Inc.]	Cytarabine	Lymphomatous meningitis	Marketed	Glantz et al. (1999)
Doxil®/Caelyx® [Sequus Pharmaceutical, Inc./ Schering-Plough]	Doxorubicin	Kaposi's sarcoma, ovarian cancer, breast cancer	Marketed	Muggia et al. (1997)
LEP-ETU [Liposomal Insys Therapeutics]	Paclitaxel	Metastatic pancreatic cancer	Phase II	Slingerland et al. (2013)
EndoTAG®-1 [MediGene AG]	Paclitaxel	HER2-negative breast cancer	Phase II	Fasol et al. (2012)
OSI-211 [Astellas Pharma Inc.]	Lurtotecan	Metastatic or recurrent head and neck cancer	Phase II	Duffaud et al. (2004)
L-Annamycin [Callisto Pharmaceutical, Inc.]	Annamycin	Acute lymphoblastic leukemia	Phase II	Booser et al. (2002)
MBP-426 [Mebiopharm Co., Ltd.]	Oxaliplatin	Gastric, gastroesophageal, esophageal adenocarcinomas	Phase II	Higashihara et al. (1991)
TKM-PLK1 [Tekmira Pharmaceuticals]	siRNA	Gastrointestinal neuroendocrine tumors, adrenocortical carcinoma, hepatocellular carcinoma	Phase II	Stevens et al. (1991)

(continued)

Table 6.1 (continued)

Brand name	Drug	Indication	Status	References
NL CPT-11 [University of California, San Francisco]	Irinotecan	Solid tumor	Phase I/ II	Prados et al. (2006)
ATI-1123[Azaya Therapeutics]	Docetaxel	Advanced solid tumors	Phase I	Mahalingam et al. (2014)
ALN-VSP02[Alnylam Pharmaceuticals]	Liposomal RNAi	Solid tumors with liver involvement	Phase I	Tabernero et al. (2013)
ALN-TTRsc [Alnylam Pharmaceuticals]	Liposomal RNAi	Subcutaneous, TTR-mediated amyloidosis	Phase I	Kanasty et al. (2013)
Anti-EGFR immunoliposome [University Hospital, Switzerland]	Doxorubicin	Solid tumor	Phase I	Mamot et al. (2012)
BikDD nanoparticles [MD Anderson Cancer Center]	Proapoptotic Bik gene (BikDD)	Pancreatic cancer	Phase I	Xie et al. (2007)

6.2.1 Liposomes

Almost from the time of their first report in the 1960s by Bangham and coworkers, liposomes have been extensively used by researchers as prospective carriers for various bioactive molecules. Liposomes are self-closed spherical phospholipid vesicular system having size ranges of 20–200 nm. These lipid vesicles are derived by the dispersion of phospholipids in an aqueous media. Various techniques pursued to disperse the lipids into the aqueous media include thin lipid film hydration, extrusion, sonication, etc. The basic component of liposome is formed by phospholipids or any similar amphipathic lipids (having both hydrophilic head and hydrophobic tail) (Onyuksel et al. 2006; Rawat et al. 2008). The phospholipid molecules used are either naturally occurring and/or derived from synthetic sources such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, polyethylene glycol, cholesterol, distearoyl-sn-glycero-3-phosphoglycerol, and dipalmitoyl-sn-glycero-3-phosphothioethanol (Arias 2013).

Liposomes have been broadly classified on the basis of their size, number of bilayers, composition, and method of preparation employed, viz., multilamellar vesicles (MLV), large unilamellar vesicles (LUV), small unilamellar vesicles (SUV), conventional liposomes (CL), pH-sensitive liposomes, cationic liposomes, long circulating liposomes (LCL), reverse phase evaporation vesicles (REV), French press vesicles (FPV), ether injection vesicles (EIV), etc. (Samad et al. 2007; Pattni et al. 2015; Matougui et al. 2016).

Liposomes possess superior capability of delivering drugs to target site and thus overcomes the adverse effect and resistance of drug due to reduced non-targeted circulation of the drug. Zhang et al. (2016a) prepared oxaliplatin and irinotecan hydrochloride co-loaded liposomes for the treatment of colorectal cancer. In vitro cytotoxicity results demonstrated that co-loaded liposomes showed higher cytotoxicity as compared to single loaded liposomes in both CT-26 and HCT-116 cells. Also, co-loaded liposomes exhibited greater antitumor therapeutic activity in CT-26-bearing BALB/c mice. In vivo safety evaluation showed that liposomes had less toxicity as compared to free drug solution (Zhang et al. 2016a). Recently, a liposome-based theranostic system was developed for delivery of docetaxel and quantum dots (QD) simultaneously. In this study, liposomes were conjugated with arginine-glycine-aspartic acid (RGD) to target them effectively in brain for theranostic applications. Brain distribution study confirmed higher accumulation of targeted liposomes in comparison to Docel™ and free QD. Results of brain histopathology were also in concordance to above results (Sonali et al. 2016). More recently, the effectiveness and safety profile of trastuzumab and liposome-loaded doxorubicin and paclitaxel combination was evaluated for early or locally advanced breast cancer therapy, clinically. The study was carried out on 30 breast cancer patients, and treatment was found to be well tolerated with very few adverse effects suggesting good therapeutic option for treatment of cancer patients (Uriarte-Pinto et al. 2016). Owing to the several beneficial properties, several liposome-based formulations has been approved by the FDA for various anticancer agents such as daunorubicin (DaunoXome®, Doxil®/Caelyx®), cytarabine (DepoCyt®), vincristine (Marqibo®), etc. (Lian and Ho 2001).

Liposome-mediated gene transfer has also been found as a promising approach not only in the treatment of diseases with genetic disorders but also in the development of strategies for treatment of several fatal diseases, viz., cancer, degenerative disorders, and AIDS. For instance, Landen et al. (2005) developed 1, 2-dioleoylsn-glycero-3-phosphatidylcholine (DOPC)-encapsulated siRNA liposomes using tertiary-butanol and nonionic detergent Tween 20. Developed system was found to be effective in reducing EphA2 expression 48 h after administration of a single dose in an orthotopic model of ovarian carcinoma suggesting its applicability (Landen et al. 2005). Bubble liposomes (containing perfluoropropane gas) possess specific advantage of high gene loading with improved intracellular penetration. Sugii and coworkers formulated the plasmid DNA-loaded bubble liposomes. The developed system was specifically targeted to neovessels via conjugating cyclic RGD peptides on their surface and exhibited high gene transfection efficiency to human umbilical vein endothelial cells (HUVECs) (Sugii et al. 2016). Similarly, folic acid modified liposomes were also loaded with plasmid DNA and binding affinity of gene was modified by agarose gel electrophoresis assays. Gene transfection efficiency was studied against NCI-H460 cells. The results showed improvement in gene transfection efficiency after attaching the targeting moieties (Cui et al. 2016).

6.2.2 Solid lipid Nanoparticles

SLNs were first developed in 1991 and are used as alternative carrier systems to traditional colloidal carriers, such as emulsions, liposomes, and polymeric NPs (Muller et al. 2002). SLNs consist of 0.1–30% (w/w) lipid, dispersed in an aqueous solution of 0.5–5% (w/w) of surfactant and are solid at both room and body temperature (Rawat et al. 2008). These particles are prepared by using number of solid lipid materials such as mono-, di-, and triglycerides; fatty acids; waxes; and steroids. The lipid materials employed should possess favorable properties such as biocompatibility, biodegradability, and low toxicity. Various surfactants employed for steric stabilization includes phospholipids, poloxamers, and polysorbates. Most commonly, SLNs are fabricated using hot or cold homogenization technique. The average diameter of the SLNs ranges from approximately 40–1000 nm (Mehnert and Mader 2001).

SLNs have gained popularity as these combine advantages of various "soft" drug carriers such as emulsions, liposomes, and polymeric NPs and at the same time avoid or minimize some of their drawbacks. They have progressively been explored for the encapsulation of labile hydrophilic and hydrophobic drugs protecting them from degradation in the body and for sustained release. Some of the drug classes that are being investigated with this system includes antibacterial, antiparasitic, antioxidant, anticancer, antiviral, antiandrogenic, antihypertensive, anti-inflammatory, antipsychotic agents, vitamins, and various bioactive compounds having multipotential (polyphenols, flavonoids, carotenoids). Numerous studies have shown that bioavailability of drugs loaded in SLNs can be extensively improved and also provide site specificity. For example, Bhandari and Kaur prepared isoniazid-loaded SLNs to improve its oral bioavailability. The study showed 6 and 4 times higher relative bioavailability of the drug in plasma and brain, respectively, when compared to the free drug solution at the same dose in rats. The high plasma drug concentration reported was attributed to the lipidic transport of SLNs through lymphatics and thus bypassing first pass metabolism of isoniazid (Bhandari and Kaur 2013). Pandita et al. successfully developed resveratrol loaded stearic acid-based SLNs using a mixture of surfactants (lecithin/poloxamer 188) as stabilizers. Pharmacokinetic results demonstrated that oral bioavailability of resveratrol was 8.035-fold high in male Wistar rats when compared to its pure suspension (Pandita et al. 2014). Also, in a study, paclitaxel loaded SLNs when administered orally in male Swiss albino mice exhibited tenfold high bioavailability as compared to paclitaxel solution, and the toxicity studies confirmed the relatively safe nature of the SLNs carrier systems with or without drug (Pandita et al. 2011). In another study, curcumin-loaded SLNs prepared with liquid lipid Sefsol-218® showed higher bioavailability and prolonged inhibitory activity in cancer cells. After the i.v. administration to rat, SLNs were capable of enhancing the bioavailability of curcumin to 1.25-fold compared to free drug (Sun et al. 2013).

Although, exhilarating results have been obtained with SLNs as mentioned above, various biological barriers, viz., rapid clearance, serum instability, and nonspecific uptake by the mononuclear phagocytic system, are associated with

conventional SLNs. PEGylation was introduced for surface functionalization of SLNs to provide a hydrophilic layer resulting in increased circulation time via overcoming of opsonization process (Uner and Yener 2007). In a study, Madan and colleagues prepared noscapine-loaded SLNs and noscapine-loaded PEG conjugated SLNs. After i.v. administration to mice, the plasma half-life was significantly enhanced up to ~11-fold and ~5-fold in case of noscapine-PEG-SLNs and noscapine-SLN, respectively, in comparison to free drug suggesting applicability of surface modification (Madan et al. 2013).

Since early 2000, SLNs have been investigated for delivery of genetic material in several disease treatments (Olbrich et al. 2001). Radaic et al. formulated SLNs for gene delivery using factorial design approach wherein different formulation parameters were varied w.r.t. DNA load, colloidal stability, in vitro cytotoxicity, and transfection efficiency in prostate cancer cells. Results suggested that the concentration of lipids and surfactant employed affected the size, stability, and transfection efficiency (Radaic et al. 2015). Yu et al. prepared paclitaxel-loaded cationic SLNs and fabricated its complex with human MCL1-specific siRNA. They showed that co-delivery of paclitaxel and MCL1-specific siRNA using cationic SLNs enhanced anticancer efficacy both in vitro and in vivo when compared to each agent alone (Yu et al. 2012). In spite of several beneficial properties as drug-carrier, SLNs have lots of drawbacks such as limited drug loading and leakage of drug during storage. Further, NLCs were developed as next generation of lipid drug carrier systems at the end of 1990s to overcome the drawbacks of SLNs.

6.2.3 Nanostructured Lipid Carriers

NLCs may overcome the limitations associated with SLNs such as low drug payloads, leakage of drug during storage, and stability (Weber et al. 2014). They are mainly produced by mixing of different lipids, i.e., solid lipids (glyceryl behenate, glyceryl palmitostearate, glyceryl monostearate/monostearin, cetyl palmitate, and stearic acid) with liquid a lipid (caprylic and capric fatty acids with a minor level of caproic, lauric, myristic, corn oil, coconut or palm kernel oils, etc.) which remains solid at room temperature (Naseri et al. 2015). NLCs show higher entrapment efficiency because solid lipids provide higher space for hold of drug and higher solubility of drugs in liquid lipids in comparison to solid lipids (Poonia et al. 2016). NLCs have been investigated for parenteral, oral, pulmonary, and topical delivery of drugs and have also presented good potential in gene delivery.

The attractive properties of NLCs on skin offer increase in drug penetration mainly through occlusion effect. They transfer drugs into deeper layers of the skin with reservoir action and provide sustained release (Muller et al. 2007). Sweety and coworkers formulated NLCs of an anti-acne drug, azelaic acid, using solvent diffusion-solvent evaporation method for enhancing its dermal retention and overcoming its adverse effects. Results of skin retention study showed high retention of azelaic acid from NLCs gel (63.96 ± 4.45%) followed by plain azelaic acid gel (15.12 ± 3.2%) and drug solution (4.78 ± 1.1%) in rat skin (Sweety et al. 2015).

NLCs are also employed for the treatment of the damaged or inflamed skin due to nonirritant and nontoxic properties of NLCs. Valdecoxib-loaded NLC carbopol gel demonstrated no skin irritation while the marketed gel of valdecoxib caused slight irritation after 48 h (Joshi and Patravale 2006). NLC-based gel also showed prolonged release activity as compared to market gel. Further, chitosan-coated NLCs showed 7.7-fold higher flurbiprofen residence time on the cornea when compared to uncoated NLCs. Also, transcorneal penetrations were increased up to 2.4-fold compared to uncoated NLCs (Luo et al. 2011). In a short period of time, NLC technology has come out as a big boon for topical delivery and two NLC-based products are available in the market (Cutanova Nanorepair Q10 and FloraGLO®).

Another widely employed route of administration for NLCs is the oral route. Due to the increased drug loading capacity of NLCs, most of these studies have focused on the ability of NLCs to improve the oral bioavailability of poorly water-soluble drugs. For example, lovastatin-loaded NLCs made from mixtures of precirol and squalene were able to promote the oral absorption and led to increased bioavailability of lovastatin. More than 70% of lovastatin was entrapped in the NLCs, which was significantly higher compared to the SLNs. Results of in vivo studies on rats showed that NLCs produced a significant improvement in the bioavailability compared to the free solution (Chen et al. 2010). In another study, saquinavir, a P-gp substrate, loaded NLCs were prepared and transport mechanisms across Caco-2 cells were studied. A 3.5-fold increase in drug permeability was reported in case of NLCs than the drug suspension (Beloqui et al. 2013). In another study, a ~2.5- and ~3-fold higher bioavailability was achieved with silymarin-loaded NLCs than the marketed formulation (Legalon®) and free drug (Zhai and Zhai 2014). Yang et al. (2013) prepared hyaluronic acid-coated NLCs using electrostatic attraction for targeted delivery of paclitaxel at tumors' site and investigated the in vitro cytotoxicity and in vivo antitumor efficacy against three CD44-overexpressing cell lines. The result of hyaluronic-coated NLCs showed better antitumor efficacy in B16-bearing Kunming mice when compared to Taxol®. Further, hyaluronic-coated NLCs showed higher accumulation in tumor and enhanced the circulation time of paclitaxel in blood.

NLCs as gene carriers can easily penetrate through the biological membranes effectively because lipids are the main components of cell membranes boosting the uptake of genetic compounds (Pathak et al. 2009). In a study, Zhang and coworkers prepared a novel system by modifying NLCs using cetylated polyethylenimine (PNLC). PNLCs showed high transfection efficiency in human lung adenocarcinoma cell line SPC-A1 and Chinese hamster ovary (CHO) cells as compared to free drug. The transfection efficiency of the optimized PNLC was similar to marketed formulation (Lipofectamine™2000) (Zhang et al. 2008). More recently, Han and coworkers (2016) developed a modified plasmid-containing enhanced green fluorescence protein (pEGFP)-loaded NLCs with transferrin (Tf) as an excellent active targeting ligand for improving the A549 cell targeting ability of the carriers. Tf-NLC/pEGFP showed higher gene expression efficiency in cancer cells both in vitro and in vivo than unmodified NLC/pEGFP suggesting enhanced activity after loading into targeted NLCs.

6.3 Polymer-Based Nanoparticles

A long chain that is made up with a repeated subunit by joining together larger macromolecules or micromolecules is called a polymer. Mainly two types of polymers have been used for the development of polymer-based nanocarriers, i.e., natural and synthetic. Some examples of natural and synthetic polymers include albumin, dextran, hyaluronate, chitosan, and poly(lactic-co-glycolic acid) (PLGA), poly(lactic acid) (PLA), poly(cyanoacrylate) (PCA), poly(N-(2-hydroxypropyl) methacrylamide) (PHPMA), PEG, polyethyleneimine (PEI), etc., respectively (Haag and Kratz 2006). On the basis of method of preparation, various types of polymeric-based nanoformulations have been obtained such as polymeric NPs, nanospheres or nanocapsules, micelles, polymer drug conjugates, etc. (Fig. 6.2). The average size ranges of such nanocarriers are from 10 to 1000 nm (Peer et al. 2007).

6.3.1 Polymeric Micelles

Polymeric micelles are spherical, colloidal, and nanoscopic core/shell structures and usually have narrow size distributions with diameters ranging from 10 to 100 nm (Jhaveri and Torchilin 2014). They are formed via self-assembly of amphiphilic copolymer chain in aqueous milieu. They present a core and shell architecture; the inner core is hydrophobic part which serves as a microenvironment for poorly water-soluble drugs, whereas the outer hydrophilic corona protects drug from aqueous environment and stabilizes interface between the core and the external medium (Kedar et al. 2010). This self-assembly monomer provides the main driving force behind the micellization process. Polyion complex micelles are subclass of polymeric micelles and are obtained from electrostatic interactions between oppositely charged copolymer (one is charged segment and other is neutral polymer chain) and drug followed by self-assembly of charge-neutralized blocks (Harada and Kataoka 1995; Kataoka et al. 1996). Both polymeric micelles and polyion complex micelles can serve as reservoir for drugs, which may be loaded by different methods, i.e., chemically, physically, or electrostatically, depending on the chemistry of drug and core forming block.

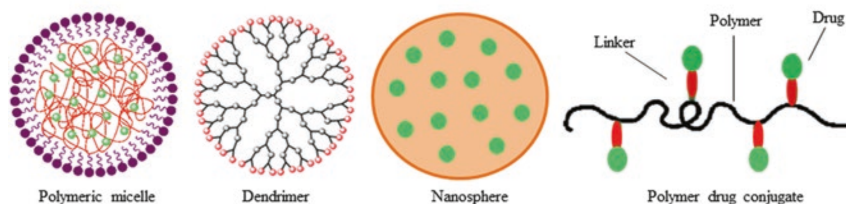

Fig. 6.2 Structure of polymer-based nanocarriers

Many drugs present low aqueous solubility and poor accumulation at their target site which lower their efficacy and can promote systemic adverse effects. In addition, macromolecular drugs such as peptides, DNA, and RNA suffer from premature degradation upon administration, low bioavailability, and inefficient cellular entry, compromising their therapeutic outcome. The versatile attributes of polymeric micelles make these systems an attractive nanocarrier for the delivery of these therapeutics. Due to the nonionic hydrophilic corona, polymeric micelles can prevent the adsorption of opsonins, thereby limiting the rapid uptake by the mononuclear phagocyte system (MPS) and prolonging the circulation half-life of the encapsulated drug (Jones and Leroux 1999). Hamaguchi et al. (2005) showed that the incorporation of paclitaxel in PEG-*b*-P(Asp) polymeric micelle resulted in about 90- and 25-fold increase in plasma concentration and tumor area, respectively, compared to the free drug. This remarkable increase in the AUC, in spite of using equivalent doses, can be ascribed to the greater stability conferred by the micelles which permits long circulation and minimizes the drug leakage (four to six times longer elimination half-life vs. the free drug) and thus allow the passage and accumulation of the drug in the tumor. Polymeric micelles can be functionalized with targeting ligands in order to achieve active targeting. Perche et al. (2012) demonstrated superior cellular uptake and cytotoxicity of doxorubicin when loaded in antibody-conjugated (anti-nucleosome 2C5) poly(ethylene)glycol-phosphatidylethanolamine micelles in comparison to free drug and conjugated micelle system. Also, developed system was found to be efficient in drug-resistant ovarian cancer cells.

In addition to modifying the pharmacokinetics and biodistribution, and decreasing the systemic toxicity of drugs, polymeric micelles have been also found to stabilize their incorporated drug, as in the case of amphotericin B. Amphotericin B is a membrane disruptive drug used to treat systemic fungal diseases in its monomeric form. Its direct administration, however, is associated with self-aggregation, leading to loss of selectivity and systemic toxicity. Adams et al. have shown that when amphotericin B was incorporated into the core of PEG-*b*-poly(*N*-hexyl-laspartamide-stearic acid) micelles, it was stabilized in its monomeric form, preventing the non-selective hemolysis of mammalian red blood cells in vitro (Adams et al. 2003). Moreover, the drug-loaded polymeric micelle retained in vivo antifungal activity as compared to the standard clinical Fungizone® formulation (Barratt and Bretagne 2007). In another study, Ge et al. formulated polyplex micelle modified with PEG-polycation block copolymer for gene delivery. Furthermore, in order to enhance targeted delivery and to promote cellular uptake, cyclic RGD peptide was attached at distal end of PEG. It was found that circulation time was successfully increased through PEG conjugation. Thus, constructed cyclic RGD conjugated polyplex micelle with the prominent PEG shielding resulted in both improved accumulation at tumor site and provided efficient gene expression of antiangiogenic protein (sFlt-1) through intracellular trafficking (Ge et al. 2014).

6.3.2 Dendrimers

Dendrimers are highly branched, three-dimensional polymeric architecture with many arms originating from a central core (Gillies and Frechet 2005). They have an internal cavity, in which drug can be incorporated and also large number of functional groups present on the outer surface can be used to bind variety of drugs and other functional groups. Dendrimers can be prepared by iterative synthetic methodology, i.e., divergent synthesis or convergent synthesis (Nanjwade et al. 2009). The void area within dendrimer and its ease of modification makes it applicable for drug and gene delivery, imaging, and boron neutron capture therapy. Dendrimers generally have a uniform structure, with the prospective to create an isolated “active site” core area through chemical functionalization. Modification of branching may allow for incorporation of molecule within this system. For example, dendrimers may become water soluble when outer surface is functionalized with hydrophilic groups, such as carboxylic acids (Faraji and Wipf 2009). Cytotoxicity of dendrimers strongly depends on its surface modification and core material. For example, cytotoxicity can be reduced by changing the surface amine groups with hydroxyl groups (Wilczewska and Niemirowicz 2012). It was found by Singh et al. that doxorubicin dendrimers conjugated with biodegradable polymer were ten times less toxic than free doxorubicin toward colon carcinoma cells (Singh and Lillard 2009).

Among many different types of dendrimers, poly(amidoamine) (PAMAM) has been one of the commonly used systems and shown significant applications in pharmaceutical and biomedical fields (Madaan et al. 2014). Although it has many advantages in drug delivery, it has several issues which limit its clinical applications. PAMAM is cytotoxic and rapidly removed from systemic circulation due to presence of amine groups on the surface. This drawback is one of the key barriers for the clinical use of dendrimers. PAMAM surface has been modified using different techniques such as PEGylation, carbohydrate conjugation, acetylation, and amino acid or peptide conjugation for enhancing the biocompatibility and drug encapsulation as well as targeting ability. Various studies have been investigated w.r.t. the low cytotoxicity and enhanced transfection efficiency of modified PAMAM dendrimers (Bae et al. 2016; Luong et al. 2016). Yu et al. developed PAMAM dendrimers modified with histidine and arginine, which resulted in higher gene transfection efficiency when compared to unmodified PAMAM dendrimers. They also found that histidine unit attached with PAMAM dendrimers improved its proton buffering capacity in the range of 3.5–6. The histidine and arginine modified dendrimers also showed less cytotoxicity as compared to PEI modified dendrimers. This proves that PAMAM dendrimers modified with histidine and arginine may provide a promising gene carrier system (Yu et al. 2011). Dutta et al. studied toxicity profile of conventional and surface engineered fifth generation poly(propyleneimine) (PPI) dendrimers. They were evaluated after administering 2.5 mg/kg, 25 mg/kg, and 250 mg/kg doses via i.v. route to Wister rats. Hemolytic studies depicted a decrease in RBCs

and hemoglobin content in conventional PPI dendrimers. Biochemical analysis also showed an increase in level of serum biochemical parameters indicative of the hepatotoxic effect of PPI. However, functionalized PPI dendrimers showed no signs of toxicity suggesting reduction of toxicity followed by functionalization (Dutta et al. 2008). In another study, fifth generation PAMAM dendrimer was functionalized with hydrophobic chains (12–16 C-alkyl chain) for delivery of plasmid DNA in mesenchymal stem cells. In vitro results demonstrated that functionalized dendrimers had low level of cytotoxicity due to presence of alkyl group on the outer surface. Further, it was found that smallest carbon chain of alkyl group showed higher efficiency for gene delivery (Santos et al. 2010).

6.3.3 Polymeric Nanoparticles

Polymeric NPs have great potential to enhance therapeutic benefit while reducing side effects of the free drug. These NPs are generally aqueous dispersions or dry powders obtained by freeze/spray drying. They overcome the stability and premature drug release issues associated with liposomes and emulsions and also facilitate prolonged drug release (Kumari et al. 2010). Polymeric NPs are prepared from PLA, PLGA, etc., possess excellent biodegradability and low toxicity, and consist of drug dispersed in an amorphous form within a polymer matrix; such particles could be prepared as nanosphere, wherein the drug is dispersed uniformly throughout the matrix of the particle or as nanocapsules. They have been studied for localized as well as controlled drug delivery. For example, carmustine-loaded polymeric implant disc made by polyanhydride polymer was used for the treatment of brain cancer. Such disc is placed into the brain to follow controlled release pattern while preventing tumor regrowth (Domb et al. 1999).

6.3.3.1 PLGA Nanoparticles

Among various polymeric NPs, PLGA-based NPs are particularly attractive in biomedical applications. PLGA is a frequently used biodegradable polymer as its hydrolytic degradation produces the original monomers, lactic acid, and glycolic acid which are side-products of various metabolic pathways. The degradation time of PLGA is controlled by the ratio of lactic acid and glycolic acid: the higher the content of glycolide units, the less is the time required. However, the copolymer with 50:50 monomers ratio reveals the fastest degradation. Because of its minimal toxicity and ability for providing greater control over the drug release profile, PLGA is suitable for drug delivery (Astete and Sabliov 2006). PLGA NPs are versatile among various polymeric NPs systems because of their biocompatibility for drug targeting at the cellular level (Mohamed and van der Walle 2008). Mathew et al. (2012) developed curcumin-loaded PLGA NPs conjugated with Tet-1 peptide for the treatment of Alzheimer's disease. Due to antioxidant and anti-amyloid activity of curcumin, curcumin-loaded NPs were successfully used in the treatment of

Alzheimer's disease. In vitro cytotoxicity studies toward LAG cell lines demonstrated that Tet-1 conjugated or unconjugated NPs showed no cytotoxicity while exhibiting antioxidant property. Furthermore, cell uptake was performed by using GI-1 glioma cells for Tet-1 conjugated and unconjugated NPs. It was found that Tet-1-conjugated PLGA NPs showed multiple-fold increased uptake than the unconjugated NPs. Moreover, confocal microscopy studies showed that Tet-1 targeted NPs distributed more around the cell soma and nucleus compared to unconjugated NPs. These results demonstrated that Tet-1 conjugated PLGA NPs were proved to have great potential as therapeutic carrier of curcumin for Alzheimer's disease treatment (Mathew et al. 2012). Yu et al. (2015) developed paclitaxel-loaded PLGA NPs complexed with PEI conjugated with Herceptin onto the surface of NPs through electrostatic interaction. In vitro cell line study toward HER-2 cancer cells showed that Herceptin-loaded polymeric NPs have high degree of cytotoxicity when compared with unmodified polymeric NPs. Furthermore, they also fabricated Herceptin-loaded polymeric NPs through chemical conjugation. It was found that chemically conjugated Herceptin polymeric NPs showed less cytotoxicity and cellular uptake efficiency in comparison with electrostatically bound Herceptin. These results demonstrated that electrostatic interaction provided an appropriate method for the synthesis of protein-loaded NPs (Yu et al. 2015).

6.3.3.2 PLA Nanoparticles

PLA is linear aliphatic polyester derived from lactic acid monomers and is widely used in micro- and nanoparticulate drug delivery systems because of its biocompatibility and biodegradation properties. It possesses the Generally Recognized as Safe (GRAS) status of the FDA (Kumari et al. 2010; Athanasiou et al. 1996). Among all biopolymers, PLA has been widely investigated in drug delivery systems since the 1980s due to its extensive biodegradability nature. For instance, Zhu et al. successfully developed D-alpha-tocopherol polyethylene glycol 1000 succinate (TPGS)-PLA NPs modified with polydopamine for the treatment of liver cancer. In this study, docetaxel was used as a model drug and galactosamine was conjugated on the surface of modified polymeric NPs to enhance the targeted delivery of drug to cancer cells. In vitro cellular uptake and cytotoxicity studies showed that galactosamine-conjugated polymeric NPs target HepG2 cells and inhibit the growth of cancer cells in comparison to Taxotere®. Furthermore, in vivo results demonstrated that drug-loaded polymeric NPs reduced the tumor size most extensively on hepatoma-bearing mice (Zhu et al. 2015). In another study, Tan et al. (2014) fabricated PLA-TPGS NPs to reduce the antagonist effect of docetaxel and tamoxifen. It was found that co-delivery of both anticancer drugs in PLA-TPGS NPs can significantly enhance the cytotoxicity toward MCF7 cancer cells. Moreover, decrease in IC_{50} in MCF7 cell line showed that polymeric NPs have the potential to enhance the synergistic effects by reducing antagonistic effect.

In gene delivery studies, DNA is coupled to NPs to create non-viral vectors protecting the DNA in body conditions. A size of around 100 nm and the positive charge of the particles facilitate the delivery and interactions with the cell membrane, respectively. Qian et al. (2014) developed different shaped PLA-polydimethylaminoethyl methacrylate (PDMAEMA) nanoarchitectures for the co-delivery of miR-21 and doxorubicin to treat glioma. It was found that star-shaped copolymer formed nanosized micelles and had positive charge and low cytotoxicity toward GES-1 cells in comparison to control PEI 25 kDa. Also, they had higher gene transfection efficiency (2.5 times) than the control PEI. The results depicted that star-shaped copolymers are promising candidate for the gene delivery and hydrophobic therapeutics.

6.3.3.3 Chitosan Nanoparticles

Chitosan NPs are also one of the most accepted polymer-based NPs used in drug delivery and targeting applications. Due to its hydroxyl and amine functional groups, the chemical modifications of chitosan have been extensively investigated in literature in order to improve its physicochemical properties, without changing its fundamental skeleton. The main goals of chemically modifying chitosan are to provide derivatives that are soluble at neutral and basic pH values, to control hydrophobic, cationic, and anionic properties as well as to attach various functional groups and ligands and optimize the process of drug release. Furthermore, the hydrophobic modification of cationic polymers has shown facilitated dissociation of polymer/DNA, enhancing the release of DNA to cytoplasm that would otherwise remain strongly bound through ionic interactions between phosphate groups of DNA and cationic units of polymers (Maximilien et al. 2015). The chemical modification of this cationic natural polymer, without changing its main properties, could be a huge approach for enhancing the efficiency of transfection. Recently, Rudzinski et al. (2016) formulated PEGylated chitosan NPs for delivery of siRNA with improved transfection efficacy in colon cancer cells. Confocal studies depicted high level of fluorescent tagged RNAs into cells showing better effect in treatment of colon cancer.

Hybrid lipid-based polymeric NPs provide several opportunities in drug delivery and gene delivery due to their physicochemical properties in terms of drug encapsulation, size as well as low immunogenicity, and no risk of transmission of infectious diseases (Pandita et al. 2015a). The versatility of this lipid-polymer hybrid NP platform allows for surface chemistry modifications. Ewe and Aiqner (2016) used cationic lipids in their hybrid NPs in order to form a DNA complex for gene delivery. In a study by Morales et al. (2009), the end group on the PEG that makes up the corona can be changed from a carboxyl group to an amine or a methoxy group in order to change the surface zeta potential. It was also shown that the surface chemistry of the hybrid NPs affects human plasma and serum absorption patterns by inducing different levels of compliment activation. The compliment and coagulant

activation studies exhibited the potential for the lipid-polymer hybrid NPs to be a viable immunocompatible delivery option. Another type of surface chemistry modification is the addition of targeting ligands, which are used to increase cellular uptake and accumulation in the tumor sites. Different types of ligands are used to target hybrid NPs to cancer cells such as antibodies, proteins, small molecules, aptamers, and peptides (Wang and Thanou 2010). Zhao et al. (2015) synthesized lipid-polymer hybrid NPs to co-deliver HIF1α and gemcitabine for the treatment of pancreatic cancer. It was found that lipid-polymer hybrid NPs have higher stability and longer circulation time than polymer NPs. Furthermore, drug-loaded lipid-polymer hybrid NPs exhibited synergistic antitumor effect in vivo than drug-loaded polymer NPs.

6.3.4 Polymer-Drug Conjugates

Ringsdorf proposed the idea of polymer-drug conjugates in the mid-1970 that could enhance the delivery of an anticancer drug to a tumor. Polymer-drug conjugate is a drug delivery technology in which a drug is covalently attached to a polymeric carrier, mostly via biodegradable linker (Haag and Kratz 2006). These linkages might be cleaved to release the pharmacologically active moieties in a particular condition such as pH, temperature, and osmolality around the tumor. Polymers such as PEG, poly-styrene-maleic anhydride copolymer (SMA), *N*-(2-hydroxypropyl)-methacrylamide copolymer (HPMA), and poly(a, L-glutamic acid) (PG) have been investigated with different cytotoxic drugs (doxorubicin, paclitaxel, gemcitabine, camptothecin, irinotecan, cisplatin, etc.). More than 15 polymer-drug conjugates have entered in clinical trials till now (Table 6.2). The main benefits of polymer-drug conjugates compared to the free drug include EPR effect, reduced toxicity, improved solubility in biological fluids, ability to overpass some mechanisms of drug resistance, and increased half-life of the drug (Duncan 2006). For instance, Vasey et al. (1999) reported the conjugation of HPMA copolymer with doxorubicin (PK1). They found that PK1 demonstrated antitumor activity with significantly lower cardiotoxicity and alopecia when compared to unconjugated doxorubicin. Chytil et al. (2006) developed water-soluble polymer-drug conjugate for cancer therapy. This agent consisted of anticancer drug doxorubicin conjugated to a HPMA copolymer through the hydrolytically degradable hydrazone bond. The system was incubated at pH 7.4 and 5.0 in order to enable pH-controlled release of the drug. At pH 7.4 conjugates were found to be stable and sustain the drug release while at pH 5.0 burst release of drug was observed. Moreover, in vitro cell line study showed higher cytotoxicity of polymer-drug conjugate system when compared to the free drug suspension.

Table 6.2 Clinical status of polymer-based nanocarriers

Product (company)	Nanoplatform	Drug	Indication	Status	References
Genexol-PM® [Samyang Biopharm]	PEG-PLA polymeric micelle	Paclitaxel	Breast cancer, lung cancer	Approved	Lee et al. (2008)
Paclical [Oasmia Pharmaceutical]	Polymeric micelle	Paclitaxel	Ovarian cancer	Phase III	Oasmia Pharmaceutical (2014)
NKTR-102 [Nektar]	PEG drug conjugate	Irinotecan	Breast cancer, ovarian cancer	Phase III	Awada et al. (2013)
Nanotax [CritiTech]	Polymeric micelle	Paclitaxel	Peritoneal neoplasms	Phase III	Roby et al. (2008)
Transdrug BA-003 [BioAlliance Pharma]	Polymeric micelle	Doxorubicin	Hepatocellular carcinoma	Phase III	Barraud et al. (2005)
Xyotax, Opaxio (CT-2103) [Cell Therapeutics]	Polyglutamic acid (polyglumex) drug conjugate	Paclitaxel	Lung cancer, ovarian cancer	Phase III	Sabbatini et al. (2004)
NK-105 [Nanocarrier Nippon Kayaku]	PEG-PAA polymeric micelle	Paclitaxel	Gastric cancer, breast cancer	Phase II/III	Kato et al. (2012)
NC-6004 [Nanocarrier]	PEG-PGA polymeric micelle	Cisplatin	Pancreas cancer	Phase II/III	Plummer et al. (2011)
Taxoprexin [Protagra]	Docosahexaenoic acid drug conjugate	Paclitaxel	Melanoma, liver cancer, kidney cancer	Phase II/III	Bedikian et al. (2011)
SP1049C [Supratek Pharma]	Polymeric micelle	Doxorubicin	Advanced adenocarcinoma	Phase II/III	Morris (2003
PEG-SN38 [Belrose Pharma/Enzone]	PEG drug conjugate	SN 38 (irinotecan derivative)	Solid tumors, breast cancer, colorectal cancer	Phase II	Patnaik et al. (2013)
Pegasys [Genentech]/ PegIntron [Merck]	PEG drug conjugate	IFNα2a/-IFNα2b	Melanoma, leukemia	Phase II	Egusquiaguirre et al. (2012)
NK-012 [Nippon Kayaku]	PEG-PAA polymeric micelle	SN-38 (active metabolite of irinotecan)	Solid tumors, small cell lung cancer, breast cancer	Phase II	Hamaguchi et al. (2010)
Pegamotecan [Enzon]	PEG drug conjugate	Camptothecin	Gastric cancer	Phase II	Scott et al. (2009)
DHAD-PBCA-NP	Polymeric micelle	Mitoxantrone	Hepatocellular carcinoma	Phase II	Zhou et al. (2009)
PK 2 (FCE28069) [UK Cancer Research/Pfizer]	HPMA drug conjugate	Doxorubicin	Hepatocellular carcinoma	Phase II	Seymour et al. (2002)

PK 1 (FCE28068) [UK Cancer Research/Pfizer]	HPMA drug conjugate	Doxorubicin	Hepatocellular carcinoma	Phase II	Vasey et al. (1999)
Lipotecan [Taiwan liposome]	Polymeric micelle	TLC388 (Camptothecin derivative)	Liver cancer, renal cancer	Phase I/II	Ghamande et al. (2014)
NKTR-105 [Nektar]	PEG drug conjugate	Docetaxel	Solid tumors, ovarian cancer	Phase I/II	Awada et al. (2013)
CT-2106 [CTI Biopharma]	Polyglutamic acid drug conjugate	Camptothecin	Colon cancer, ovarian cancer	Phase I/II	Homsi et al. (2007)
AP5280 [Access Pharmaceutical]	HPMA drug conjugate	Platinum	Solid tumors	Phase I/II	Rademaker-Lakhai et al. (2004)
NC-4016 [Nanocarrier]	Polymeric micelle	Oxaliplatin	Solid tumors, lymphoma	Phase I	Ueno et al. (2014)
Nanoxel [Fresenius Kabi Oncology]	Polymeric micelle	Paclitaxel	Advanced breast cancer	Phase I	Madaan et al. (2013)
Docetaxel-PNP [Samyang Biopharm]	Polymeric micelle	Docetaxel	Solid tumors	Phase I	Jung et al. (2012)
NC-6300 [Nanocarrier]	pH-sensitive polymeric micelle	Epirubicin	Solid tumors	Phase I (Japan)	Harada et al. (2011)
XMT-1001 [Mersana]	Fleximer drug conjugate	Camptothecin	Gastric cancer, lung cancer	Phase I	Yurkovetskiy and Fram (2009)
DE-310 [Daiichi Pharmaceutical]	Carboxymethyldextran polyalcohol drug conjugate	DX-8951 (camptothecin derivative)	Solid tumors	Phase I	Soepenberg et al. (2005)
NK-911 [National Cancer Institute Japan/Nippon Kayaku]	PEG-PAA polymeric micelle	Doxorubicin	Solid tumors	Phase I	Matsumura et al. (2004)
MAG-CPT [Pfizer]	HPMA drug conjugate	Camptothecin	Solid tumors	Phase I	Bissett et al. (2004)
PNU166945 [Pfizer]	HPMA drug conjugate	Paclitaxel	Solid tumors	Phase I	Meerum et al. (2001)

6.4 Inorganic Nanocarriers

Inorganic nanocarriers are emerging nanosystems utilizing inorganic nanomaterials to achieve medical breakthroughs in therapeutics and advanced diagnostics (Sekhon and Kamboj 2014; Huang et al. 2011; Malmsten 2013). These include metallic NPs, SiNPs, CNTs, QD, etc. (Fig. 6.3). Due to large size and anionic nature, these systems can reduce problems associated with chemical stability and poor gene transfection efficacy (Malmsten 2013). By varying size and surface composition, smart engineered nanocarriers can avoid the RES and can improve drug targeting to the specific area of the body. These nanocarriers have strong affinity to thiol, amine, and carboxyl groups. Such affinity enables surface-modified nanocarriers as promising material for targeting drugs and/or chemotherapeutics. For example, gold nanoparticles modified with albumin do not show any hemolytic response and are used as a carrier for drug release in systemic circulation (Khullar et al. 2012).

6.4.1 Metallic Nanoparticles

Numerous efforts have been made to fabricate various noble metal NPs and to investigate their optical properties. The use of gold (Au) and silver (Ag) NPs has been explored broadly in biological imaging and gene and drug delivery owing to their unique dimensions, tunable functionalities, and controlled drug release (Yang et al. 2012)

The strong plasmon resonance, surface-enhanced Raman scattering, and photothermal conversion of metallic AuNPs have been exploited for imaging and therapy of cancerous cells (Sekhon and Kamboj 2014). The scattering from a single AuNP or AgNPs has been found to be of many orders of magnitude than the signal from single fluorophores or QD. AuNPs are good candidates for drug and gene delivery. Andres and coworkers synthesized AuNPs and grafted them with thioctic acid-PEG-folic acid. The resulting folate-PEG-coated NPs were soluble in aqueous solution over pH range 2–12 and at electrolyte concentration of up to 0.5 M NaCl. They also demonstrated the specific uptake of folate-conjugated AuNPs by folate-receptor positive tumor cells (Dixit et al. 2006). Brown et al. (2010) functionalized gold NPs

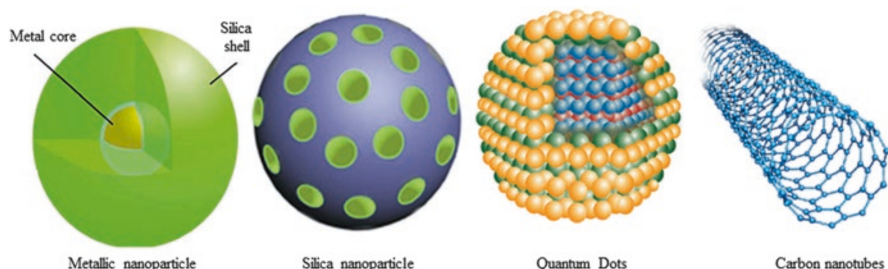

Fig. 6.3 Structure of inorganic nanocarriers

with a thiolated PEG capped with a carboxylate group for improved drug delivery of oxaliplatin anticancer drug (Brown et al. 2010). In an another attempt, doxorubicin-loaded PEGylated AuNPs were successfully delivered for overcoming multidrug resistance of MCF-7/ADR cancer cells (Wang et al. 2011). Recently, Manivasagan et al. (2016) synthesized fucoidan (Fu)-capped AuNPs for delivery of doxorubicin and photoacoustic imaging. Fu was used as a capping and reducing agent. The in vitro cytotoxicity effect of Fu, Fu AuNPs, and DOX-Fu AuNPs were studied on human breast cancer cells (MDA-MB-231). It was found that in vitro cytotoxicity of DOX-Fu AuNPs can be recognized to the greater uptake potential of Fu AuNPs, thus establishing the role of AuNPs as capable carriers for the delivery of anticancer drugs (Manivasagan et al. 2016). AuNPs have been used in gene therapy by using viral and non-viral vectors to transport foreign genes to somatic cells. Mirkin and coworkers demonstrated functionalized AuNPs with single-stranded thiolated oligonucleotide for gene therapy. They found that modified AuNPs have higher binding affinity for complementary nucleic acids and exhibited greater cellular uptake than unmodified NPs (Rosi et al. 2006). Niidome et al. (2006) synthesized spherical AuNPs to investigate the release of plasmid DNA through pulsed-laser-induced fragmentation and dissociation. The result indicated that laser power could release plasmid DNA without significant degradation. Braun et al. (2009) reported novel lipid-based gold NPs for delivery of siRNA that provided temporally and spatially controlled cellular delivery for gene silencing, with the help of direct endosomal release mechanism activated via pulsed laser treatment.

6.4.2 Silica Nanoparticles

Humans are introduced to silica, the most generous substance on earth surface and widely used in our daily life from various sources such as clays, rocks, and sand (Barik et al. 2008). Fumed silica, colloidal silica, etc. are industrial silica extensively used in microelectronics, optical communication, and food additives. SiNPs have attracted key attention for tumor-targeted drug delivery due to their enormous biomedical applications. There are two major types of SiNPs for tumor-targeted drug delivery, i.e., core/shell SiNPs (C/S-SiNPs) and mesoporous silica nanoparticles (MSNs) (Vallet-Regi et al. 2007; Vivero-Escoto et al. 2010; Malmsten 2013). But MSNs have served its groundbreaking potential in drug and gene delivery due to good physical and chemical stability, high loading capacity, zero premature release, and controlled release of drug molecules with proper rate of release to achieve an effective local concentration. MSNs possess unique features of tunable particle size/pore size/morphology, high surface area and pore volume, biocompatibility, and facile surface functionalization (Yang et al. 2012; Malmsten 2013; Pandita et al. 2015b). Recently, researchers have made several efforts to synthesize hybrid MSNs (AuNPs and magnetic NPs as functional components) loaded with drug molecules (Sekhon and Kamboj 2014). These multifunctional nanosystems have potential applications like targeted drug delivery, in vivo imaging, and theranostic applications (Pandita and Lather 2015). For example, researchers

successfully loaded camptothecin in fluorescent MSNs and confirmed the release of drug to various types of human cancer cells to induce cell death (Lu et al. 2007). MSNs are promising vehicle for drug delivery, on account of their successful encapsulation and delivery to the target sites, for drugs such as quercetin, paclitaxel, and doxorubicin (Sarkar et al. 2016; Jia et al. 2013; Fu et al. 2016; Zhang et al. 2016b). Suwalski and coworkers developed a MSN-based gene transfer system that allows an acceleration of tendon regeneration by PDGF-B gene transfer. The MSNs were modified with amino and carboxyl group and in vitro and in vivo transfection efficiency were evaluated. The result showed that modified MSNs did not have signs of inflammation or necrosis in the tendon, kidney heart, and liver of rat during a period of 1.5 months (Suwalski et al. 2010). Roy et al. (2005) used organically modified MSNs to deliver the non-viral DNA sequence for gene delivery. Pan et al. (2012) synthesized peptide-MSN conjugates for targeting cell nuclei and delivering the anticancer drug doxorubicin into targeted nucleus, killing cancer cells with high efficiency. This study provided an effective strategy for design and development of cell-nuclear-targeted drug delivery systems.

6.4.3 Quantum Dots

QD are semiconductor nanocrystals whose optical and electronic properties are strongly size-dependent (Alivisatos 1996). Larger QD have a smaller bandgap and emit lower energy photons (toward the red side of the spectrum) and smaller QD have a larger bandgap and emit higher energy photons (toward the blue side of the spectrum). QD of various semiconductor compositions, such as ZnS, CdS, ZnSe, CdTe, and PbS, have size-tunable fluorescence emission that range between the UV and infrared range (Medintz et al. 2005). QD can replace organic dyes in many applications and offer several advantages over organic dyes, such as photo stability, long fluorescence lifetime, broad absorption, and narrow, tunable emission spectra (Chan et al. 2002). These properties make QD well suited for applications such as single molecule tracking (Dahan et al. 2003), time-gated imaging (Dahan et al. 2001), and multiplexed imaging where organic dyes would be of limited use due to their photo bleaching, short fluorescence lifetime, and narrow absorption spectra (Ghazani et al. 2006). Their photo stability and tunable emission spectra also enable long-term multicolor imaging, which can offer real-time insights into how cells and proteins interact (Jaiswal et al. 2003). They have also been used to track metastasis. The ability to tune their fluorescence into the near infrared window has also enabled their use in optically guided surgery and in vivo cancer imaging (Gao et al. 2004). The surface of QD can be coated or conjugated with polymers in order to enhance its biocompatibility and extend stability against biochemical reactions. Gao et al. (2004) successfully coated triblock amphiphilic copolymer over CdSe/ZnS QD which protected QD from hydrolysis and enzymatic degradation. They also found that in vivo imaging results of QD probes can be targeted to tumor site through active and passive mechanisms but active targeting is much efficient than passive targeting (Gao et al. 2004). In another study, Johari-Ahar et al. (2016) synthesized L-cysteine-capped CdSe QD conjugated with methotrexate (MTX) and evaluated

their internalization and cytotoxicity in the KB cells. Based on cytotoxicity assessment results, drug-QD conjugate was found to efficiently internalize into cancer cells and eradicate MTX-resistant KB cells with IC_{50} of 12.0 μg/ml as compared to free drug molecules, i.e., 105.0 μg/ml. These findings proposed MTX-QD as promising material for targeted therapy of MTX-resistant cells (Johari-Ahar et al. 2016). Further, QD has been found as promising approache in gene delivery. For example, Yang et al. (2014) functionalized PEI on QD to study the effect of particle size and PEI coating on the efficiency of gene delivery into human mesenchymal stem cells. QD of several sizes (5, 10, 15, and 20 nm) were used in the study. It was found that PEI-coated QD exhibited high gene transfection efficacy as compared to unmodified QD. Particularly, QD with largest particle size (20 nm) exhibited much higher uptake capability and greater gene transfection efficacy.

6.4.4 Carbon Nanotubes

The rapid development of carbon nanotube (CNT)-based technology in many different fields rendered this material not as much unknown as few years ago for scientific community. CNTs are classified into single-walled carbon nanotubes (SWNTs) and multiwalled carbon nanotubes (MWNTs). These exhibit unique physicochemical properties like high aseptic ratio, high mechanical strength, ultralight weight, and high electrical and thermal conductivity (Yang et al. 2012). They have been demonstrated to be potential carriers for a wide variety of agents such as drugs, DNA, proteins, and genes. Functionalization of CNTs is receiving attention in biomedical applications. With the help of covalent functionalization of CNTs through side-walls and tips, these can be soluble in wide range of solvents (Madani et al. 2011). Also, functionalization of CNTs is responsible to determine its biocompatibility and cytotoxic effect. It was reported that the higher the degree of CNTs functionalization, the safer the material is, thus offering the potential exploitation of nanotubes for drug administration (Prato et al. 2008). Although the clearance mechanism of CNTs is still not clear, numerous studies have reported that accumulation of functionalized CNTs takes place in the RES after i.v. administration. Kang et al. (2010) reported that endocytosis of larger SWNTs (100–200 nm) takes place mainly through clathrin-mediated pathway, whereas smaller SWNTs having size 50–100 nm are internalized employing both clathrin- and caveolae-mediated pathway.

Although amine functionalized CNTs have good binding efficiency for DNA, their transfection efficiency is low (Singh et al. 2005). PEI was reported to be employed on the surface of CNTs to enhance DNA binding and cell uptake (Liu et al. 2005). Later, Liu et al. (2008) developed SWNTs functionalized with phospholipid branched PEG via a cleavable ester bond to deliver the paclitaxel drug at tumor site. They found that functionalized SWNTs owing to prolong blood circulation and tenfold higher tumor PTX uptake by SWNTs through EPR presented higher tumor suppression efficacy compared to clinical Taxol in a murine 4T1 breast cancer model. They also found that drug molecules were excreted via biliary pathway without causing toxic effect to normal cells. The clinical status of different inorganic nanocarriers is listed in Table 6.3.

Table 6.3 Clinical status of inorganic nanocarriers

Product (company)	Nanoplatform	Application	Indication	Status	References
Feridex i.v. [AMAG]	Iron oxide nanoparticles	Magnetic resonance imaging	Liver cancer	FDA approved	Wang (2011)
Resovist [Bayer]	Iron oxide nanoparticles	Magnetic resonance imaging	Liver cancer	FDA approved	Wang (2011)
Ferumoxtran-10 [Guerbet/AMAG]	Iron oxide nanoparticles	Magnetic resonance imaging	Prostate cancer	Phase III	Fortuin et al. (2013)
Aurimune (CYT-6091) [Cytimmune Sciences]	Colloidal gold	Tumor necrosis factor delivery	Solid tumors	Phase I/II	Libutti et al. (2010)
Ferumoxytol [MD Anderson/AMAG]	Iron oxide nanoparticles	Magnetic resonance imaging	Head and neck cancer, lymph node cancer	Phase I	Hedgire et al. (2014)
AuroLase [Nanospectra]	Gold-coated silica nanoparticles	Photothermal ablation	Head and neck cancer	Phase I	Li et al. (2013)
Targeted SNP [Memorial Sloan Kettering cancer Centre]	Silica nanoparticles	Lymph node imaging	Head and neck cancer	Phase 0	Benezra et al. (2011)

6.5 Conclusion

A variety of nanocarriers are being investigated for the drug and gene delivery. The size of these nanocarriers is responsible for the wide range of promising properties. The ideal nanocarrier can be used to achieve the suitable kinetic properties such as long circulation time, excellent biocompatibility, selective targeting to tumor sites, etc. Nanocarriers are developed to enhance the pharmacological effect and therapeutic properties of drugs. Several nanocarriers are being developed and under preclinical stages with few of them already being used in clinical cancer care like liposomal-based formulation Doxil® (doxorubicin), DepoCyt® (cytarabine), Myocet® (doxorubicin), etc. Nanoscale-based delivery strategies have begun to make a significant impact on global pharmaceutical market. NPs in gene therapy have also drawn significant attention as a potential method for treating both acute illnesses and chronic diseases.

The near future may cling to the appearance of new commercial nanocarrier-based products. However, this revolution of technology in terms of NPs is a challenge, since challenges are high in terms of investments, but due to increasing competition and industrial occurrence, the reimbursement can be greater.

References

Adams ML, Andes DR, Kwon GS (2003) Amphotericin B encapsulated in micelles based on poly(ethylene oxide)-block-poly(L-amino acid) derivatives exerts reduced in vitro hemolysis but maintains potent in vivo antifungal activity. Biomacromolecules 4(3):750–757

Alivisatos AP (1996) Semiconductor clusters, nanocrystals, and quantum dots. Science 271(5251):933–937

Arias JL (2013) Liposomes in drug delivery: a patent review (2007–present). Expert Opin Ther Pat 23(11):1399–1414

Astete CE, Sabliov CM (2006) Synthesis and characterization of PLGA nanoparticles. J Biomater Sci Polym Ed 17(3):247–289

Athanasiou KA, Niederauer GG, Agrawal CM (1996) Sterilization, toxicity, biocompatibility and clinical applications of polylactic acid/polyglycolic acid copolymers. Biomaterials 17:93–102

Awada A, Garcia AA, Chan S, Jerusalem HM, Coleman RE, Huizing MT, Mehdi A (2013) Two schedules of etirinotecan pegol (NKTR-102) in patients with previously treated metastatic breast cancer: a randomised phase 2 study. Lancet Oncol 14(12):1216–1225

Bae Y, Lee S, Green ES, Park JH, Ko KS, Han J, Choi JS (2016) Characterization of basic amino acids-conjugated PAMAM dendrimers as gene carriers for human adipose-derived mesenchymal stem cells. Int J Pharm 501(1–2):75–86

Barik TK, Sahu B, Swain V (2008) Nanosilica-from medicine to pest control. Parasitol Res 103(2):253–258

Barratt G, Bretagne S (2007) Optimizing efficacy of Amphotericin B through nanomodification. Int J Nanomedicine 2(3):301–313

Barraud L, Merle P, Soma E, Lefrancois L, Guerret S, Chevallier M, Dubernet C, Couvreur P (2005) Increase of doxorubicin sensitivity by doxorubicin-loading into nanoparticles for hepatocellular carcinoma cells in vitro and in vivo. J Hepatol 42(5):736–743

Batist G, Ramakrishnan G, Rao CS, Chandrasekharan A, Gutheil J, Guthrie T, Shah P, Khojasteh A, Nair MK, Hoelzer K, Tkaczuk K, Park YC, Lee LW (2001) Reduced cardiotoxicity and preserved antitumor efficacy of liposome-encapsulated doxorubicin and cyclophosphamide compared with conventional doxorubicin and cyclophosphamide in a randomized, multicenter trial of metastatic breast cancer. J Clin Oncol 19(5):1444–1454

Bedikian AY, DeConti RC, Conry R, Agarwala S, Papadopoulos N, Kim KB, Ernstoff M (2011) Phase 3 study of docosahexaenoic acid–paclitaxel versus dacarbazine in patients with metastatic malignant melanoma. Ann Oncol 22(4):787–793

Beloqui A, Solinís MÁ, Gascón AR, del Pozo-Rodríguez A, des Rieux A, Préat V (2013) Mechanism of transport of saquinavir-loaded nanostructured lipid carriers across the intestinal barrier. J Control Release 166:115–123

Benezra M, Penate-Medina O, Zanzonico PB, Schaer D, Ow H, Burns A, DeStanchina E, Longo V, Herz E, Iyer S, Wolchok J, Larson SM, Wiesner U, Bradbury MS (2011) Multimodal silica nanoparticles are effective cancer-targeted probes in a model of human melanoma. J Clin Investig 121(7):2768–2780

Bhandari R, Kaur IP (2013) Pharmacokinetics, tissue distribution and relative bioavailability of isoniazid-solid lipid nanoparticles. Int J Pharm 441(1–2):202–212

Bissett D, Cassidy J, de Bono JS, Muirhead F, Main M, Robson L, Fraier D, Magnè ML, Pellizzoni C, Porro MG, Spinelli R, Speed W, Twelves C (2004) Phase I and pharmacokinetic (PK) study of MAG-CPT (PNU 166148): a polymeric derivative of camptothecin (CPT). Br J Cancer 91(1):50–55

Booser DJ, Esteva FJ, Rivera E, Valero V, Esparza-Guerra L, Priebe W, Hortobagyi GN (2002) Phase II study of liposomal annamycin in the treatment of doxorubicin-resistant breast cancer. Cancer Chemother Pharmacol 50(1):6–8

Braun GB, Pallaoro A, Wu G, Missirlis D, Zasadzinski JA, Tirrell M, Reich NO (2009) Laser-activated gene silencing via gold nanoshell-siRNA conjugates. ACS Nano 3(7):2007–2015

Brown SD, Nativo P, Smith JA, Stirling D, Edwards PR, Venugopal B, Flint DJ, Plumb JA, Graham D, Wheate NJ (2010) Gold nanoparticles for the improved anticancer drug delivery of the active component of oxaliplatin. J Am Chem Soc 132(13):4678–4684

Chan WC, Maxwell DJ, Gao X, Bailey RE, Han M, Nie S (2002) Luminescent quantum dots for multiplexed biological detection and imaging. Curr Opin Biotechnol 13(1):40–46

Chen CC, Tsai TH, Huang ZR, Fang JY (2010) Effects of lipophilic emulsifiers on the oral administration of lovastatin from nanostructured lipid carriers: physicochemical characterization and pharmacokinetics. Eur J Pharm Biopharm 74(3):474–482

Chytil P, Etrych T, Konák C, Sírová M, Mrkvan T, Ríhová B, Ulbrich K (2006) Properties of HPMA copolymer-doxorubicin conjugates with pH-controlled activation: effect of polymer chain modification. J Control Release 115(1):26–36

Cui SH, Zhi DF, Zhao YN, Chen HY, Meng Y, Zhang CM, Zhang SB (2016) Cationic lioposomes with folic acid as targeting ligand for gene delivery. Bioorg Med Chem Lett 26(16):4025–4029

Dahan M, Laurence T, Pinaud F, Chemla DS, Alivisatos AP, Sauer M, Weiss S (2001) Time-gated biological imaging by use of colloidal quantum dots. Opt Lett 26(11):825–827

Dahan M, Lévi S, Luccardini C, Rostaing P, Riveau B, Triller A (2003) Diffusion dynamics of glycine receptors revealed by single-quantum dot tracking. Science 302(5644):442–445

Dixit V, Van den Bossche J, Sherman DM, Thompson DH, Andres RP (2006) Synthesis and grafting of thioctic acid-PEG-folate conjugates onto Au nanoparticles for selective targeting of folate receptor-positive tumor cells. Bioconjug Chem 17(3):603–609

Domb AJ, Israel ZH, Elmalak O, Teomim D, Bentolila A (1999) Preparation and characterization of carmustine loaded polyanhydride wafers for treating brain tumors. Pharm Res 16(5):762–765

Duffaud F, Borner M, Chollet P, Vermorken JB, Bloch J, Degardin M, Rolland F, Dittrich C, Baron B, Lacombe D, Fumoleau P (2004) Phase II study of OSI-211 (liposomal lurtotecan) in patients with metastatic or loco-regional recurrent squamous cell carcinoma of the head and neck. An EORTC New Drug Development Group study. Eur J Cancer 40(18):2748–2752

Duncan R (2006) Polymer conjugates as anticancer nanomedicines. Nat Rev Cancer 6(9):688–701

Dutta T, Garg M, Dubey V, Mishra D, Singh K, Pandita D, Singh AK, Ravi AK, Velpandian T, Jain NK (2008) Toxicological investigation of surface engineered fifth generation poly (propyleneimine) dendrimers in vivo. Nanotoxicology 2(2):62–70

Egusquiaguirre SP, Igartua M, Hernández RM, Pedraz JL (2012) Nanoparticle delivery systems for cancer therapy: advances in clinical and preclinical research. Clin Transl Oncol 14(2):83–93

Estanqueiro M, Amaral MH, Conceição J, Sousa Lobo JM (2015) Nanotechnological carriers for cancer chemotherapy: the state of the art. Colloids Surf B Biointerf 126:631–648

Ewe A, Aiqner A (2016) Cationic lipid-coated polyplexes (Lipopolyplexes) for DNA and small RNA delivery. Methods Mol Biol 1445:187–200

Faraji AH, Wipf P (2009) Nanoparticles in cellular drug delivery. Bioorg Med Chem 17(8):2950–2962

Fasol U, Frost A, Büchert M, Arends J, Fiedler U, Scharr D, Scheuenpflug J, Mross K (2012) Vascular and pharmacokinetic effects of EndoTAG-1 in patients with advanced cancer and liver metastasis. Ann Oncol 23(4):1030–1036

Fortuin AS, Meijer H, Thompson LC, Witjes JA, Barentsz JO (2013) Ferumoxtran-10 ultrasmall superparamagnetic iron oxide–enhanced diffusion-weighted imaging magnetic resonance imaging for detection of metastases in normal-sized lymph nodes in patients with bladder and prostate cancer: do we enter the era after extended pelvic lymph node dissection? Eur Urol 64(6):961–963

Fu Q, Hargrove D, Lu X (2016) Improving paclitaxel pharmacokinetics by using tumor-specific mesoporous silica nanoparticles with intraperitoneal delivery. Nanomedicine 12(7):1951–1959

Gao X, Cui Y, Levenson RM, Chung LWK, Nie S (2004) In vivo cancer targeting and imaging with semiconductor quantum dots. Nat Biotechnol 22(8):969–976

Ge Z, Chen Q, Osada K, Liu X, Tockary TA, Uchida S, Dirisala A, Ishii T, Nomoto T, Toh K, Matsumoto Y, Oba M, Kano MR, Itaka K, Kataoka K (2014) Targeted gene delivery by polyplex micelles with crowded PEG palisade and cRGD moiety for systemic treatment of pancreatic tumors. Biomaterials 35(10):3416–3426

Ghamande S, Lin CC, Cho DC, Shapiro GI, Kwak EL, Silverman MH, Tseng Y, Kuo MW, Mach WB, Hsu SC, Coleman T, Yang JC, Cheng AL, Ghalib MH, Chuadhary I, Goel S (2014) A phase 1 open-label, sequential dose-escalation study investigating the safety, tolerability, and pharmacokinetics of intravenous TLC388 administered to patients with advanced solid tumors. Investig New Drug 32(3):445–451

Ghazani AA, Lee JA, Klostranec J, Xiang Q, Dacosta RS, Wilson BC, Tsao MS, Chan WC (2006) High throughput quantification of protein expression of cancer antigens in tissue microarray using quantum dot nanocrystals. Nano Lett 6(12):2881–2886

Gillies ER, Frechet JM (2005) Dendrimers and dendritic polymers in drug delivery. Drug Discov Today 10(1):35–43

Glantz MJ, Jaeckle KA, Chamberlain MC, Phuphanich S, Recht L, Swinnen LJ, Maria B, LaFollette S, Schumann GB, Cole BF, Howell SB (1999) A randomized controlled trial comparing intrathecal sustained-release cytarabine (DepoCyt) to intrathecal methotrexate in patients with neoplastic meningitis from solid tumors. Clin Cancer Res 5(11):3394–3402

Gref R, Domb A, Quellec P, Blunk T, Muller RH, Verbavatz JM, Langer VR (1995) The controlled intravenous delivery of drugs using PEG-coated sterically stabilized nanospheres. Adv Drug Deliv Rev 16(2):215–233

Gref R, Lück M, Quellec P, Marchand M, Dellacherie E, Harnisch S, Blunk T, Müller RH (2000) Stealth' corona-core nanoparticles surface modified by polyethylene glycol (PEG): influences of the corona (PEG chain length and surface density) and of the core composition on phagocytic uptake and plasma protein adsorption. Colloids Surf B Biointerf 18(3–4):301–313

Haag R, Kratz F (2006) Polymer therapeutics: concepts and applications. Angew Chem Int Ed Eng 45(8):1198–1215

Hamaguchi T, Matsumura Y, Suzuki M, Shimizu K, Goda R, Nakamura I, Nakatomi I, Yokoyama M, Kataoka K, Kakizoe T (2005) NK105, a paclitaxel-incorporating micellar nanoparticle formulation, can extend in vivo antitumour activity and reduce the neurotoxicity of paclitaxel. Br J Cancer 92(7):1240–1246

Hamaguchi T, Doi T, Eguchi-Nakajima T, Kato K, Yamada Y, Shimada Y, Fuse N, Ohtsu A, Matsumoto S, Takanashi M, Matsumura Y (2010) Phase I study of NK012, a novel SN-38–incorporating micellar nanoparticle, in adult patients with solid tumors. Am Ass Cancer Res 16(20):5058–5066

Han Y, Li Y, Zhang P, Sun J, Li X, Sun X, Kong F (2016) Nanostructured lipid carriers as novel drug delivery system for lung cancer gene therapy. Pharm Dev Technol 21(3):277–281

Harada A, Kataoka K (1995) Formation of polyion complex micelles in an aqueous milieu from a pair of oppositely-charged block copolymers with poly(ethylene glycol) segments. Macromolecules 28(15):5294–5299

Harada M, Bobe I, Saito H, Shibata N, Tanaka R, Hayashi T, Kato Y (2011) Improved anti-tumor activity of stabilized anthracycline polymeric micelle formulation, NC-6300. Cancer Sci 102(1):192–199

Hedgire SS, Mino-Kenudson M, Elmi A, Thayer S, Fernandez-del Castillo C, Harisinghani MG (2014) Enhanced primary tumor delineation in pancreatic adenocarcinoma using ultrasmall super paramagnetic iron oxide nanoparticle-ferumoxytol: an initial experience with histopathologic correlation. Int J Nanomedicine 9:1891–1896

Higashihara M, Kinoshita M, Kume S, Teramoto T, Kurokawa K (1991) Inhibition of platelet function by high-density lipoprotein from a patient with apolipoprotein E deficiency. Biochem Biophys Res Commun 181(3):1331–1336

Homsi J, Simon GR, Garrett CR, Springett G, De Conti R, Chiappori AA, Munster PN, Burton MK, Stromatt S, Allievi C, Angiuli P, Eisenfeld A, Sullivan DM, Daud AI (2007) Phase I trial of poly-l-glutamate camptothecin (CT-2106) administered weekly in patients with advanced solid malignancies. Am Ass Cancer Res 13(19):5855–5861

Huang HC, Barua S, Sharma G, Dey SK, Rege K (2011) Inorganic nanoparticles for cancer imaging and therapy. J Control Release 155(3):344–357

Huber G, Levy J (2001) Development of verteporfin therapy: a collaboration between pharmaceutical companies, device manufacturers and clinical investigators. Semin Ophthalmol 16(4):213–217

Jabr-Milane L, van Vlerken L, Devalapally H, Shenoy D, Komareddy S, Bhavsar M, Amiji M (2008) Multi-functional nanocarriers for targeted delivery of drugs and genes. J Control Release 130(2):121–128

Jaiswal JK, Mattoussi H, Mauro JM, Simon SM (2003) Long-term multiple color imaging of live cells using quantum dot bioconjugates. Nat Biotechnol 21(1):47–51

Jhaveri AM, Torchilin VP (2014) Multifunctional polymeric micelles for delivery of drugs and siRNA. Front Pharmacol 5:77

Jia LJ, Shen J, Li Z, Zhang D, Zhang Q, Liu G, Zheng D, Tian X (2013) In vitro and in vivo evaluation of paclitaxel-loaded mesoporous silica nanoparticles with three pore sizes. Int J Pharm 445(1–2):12–19

Johari-Ahar M, Barar J, Alizadeh AM, Davaran S, Omidi Y, Rashidi MR (2016) Methotrexate-conjugated quantum dots: synthesis, characterisation and cytotoxicity in drug resistant cancer cells. J Drug Target 24(2):120–133

Jones M, Leroux J (1999) Polymeric micelles – a new generation of colloidal drug carriers. Eur J Pharm Biopharm 48(2):101–111

Joshi M, Patravale V (2006) Formulation and evaluation of nanostructured lipid carrier (NLC)-based gel of valdecoxib. Drug Dev Ind Pharm 32(8):911–918

Jung J, Park SJ, Chung HK, Kang HW, Lee SW, Seo MH, Park HJ, Song SY, Jeong SY, Choi EK (2012) Polymeric nanoparticles containing taxanes enhance chemoradiotherapeutic efficacy in non-small cell lung cancer. Int J Radiat Oncol Biol Phys 84(1):77–83

Kanasty R, Dorkin JR, Vegas A, Anderson D (2013) Delivery materials for siRNA therapeutics. Nat Mater 12(11):967–977

Kang B, Chang S, Dai Y, Yu D, Chen D (2010) Cell response to carbon nanotubes: size-dependent intracellular uptake mechanism and subcellular fate. Small 6(21):2362–2366

Kataoka K, Togawa H, Harada A, Yasungi K, Matsumoto T, Katayose S (1996) Spontaneous formation of polyion complex micelles with narrow distribution from antisense oligonucleotide and cationic block copolymer in physiological saline. Macromolecules 29(26):8556–8557

Kato K, Chin K, Yoshikawa T, Yamaguchi K, Tsuji Y, Esaki T, Sakai K, Kimura M, Hamaguchi T, Shimada Y, Matsumura Y, Ikeda R (2012) Phase II study of NK105, a paclitaxel-incorporating micellar nanoparticle, for previously treated advanced or recurrent gastric cancer. Investig New Drug 30(4):1621–1627

Kedar U, Phutane P, Shidhaye S, Kadam V (2010) Advances in polymeric micelles for drug delivery and tumor targeting. Nanomed Nanotech Biol Med 6(6):714–729

Khullar P, Singh V, Mahal A, Dave PN, Thakur S, Kaur G, Singh J, Kamboj SS, Bakshi MS (2012) Bovine serum albumin bioconjugated gold nanoparticles: synthesis, hemolysis, and cytotoxicity toward cancer cell lines. J Phys C C 116(15):8834–8843

Kumari A, Yadav SK, Yadav SC (2010) Biodegradable polymeric nanoparticles based drug delivery systems. Colloid Surf B Biointerf 75:1–18

Landen CN, Chavez-Reyes A, Bucana C, Schmandt R, Deavers MT, Lopez-Berestein G, Sood AK (2005) Therapeutic EphA2 gene targeting in vivo using neutral liposomal small interfering RNA delivery. Cancer Res 65(15):6910–6918

Lee KS, Chung HC, Im SA, Park YH, Kim CS, Kim SB, Rha SY, Lee MY, Ro J (2008) Multicenter phase II trial of Genexol-PM, a cremophor-free, polymeric micelle formulation of paclitaxel, in patients with metastatic breast cancer. Breast Cancer Res Treat 108(2):241–250

Li J, Gupta S, Li C (2013) Research perspectives: gold nanoparticles in cancer theranostics. Quant Imaging Med Surg 3(6):284–291

Lian T, Ho RJ (2001) Trends and developments in liposome drug delivery systems. J Pharm Sci 90(6):667–680

Libutti SK, Paciotti GF, Byrnes AA, Alexander HR Jr, Gannon WE, Walker M, Seidel GD, Yuldasheva N, Tamarkin L (2010) Phase I and pharmacokinetic studies of CYT-6091, a novel PEGylated colloidal gold-rhTNF nanomedicine. Am Ass Cancer Res 16(24):6139–6149

Lim SB, Banerjee A, Önyüksel H (2012) Improvement of drug safety by the use of lipid-based nanocarriers. J Control Release 163(1):34–45
Liu Y, Wu DC, Zhang WD, Jiang X, He CB, Chung TS, Goh SH, Leong KW (2005) Polyethylenimine-grafted multiwalled carbon nanotubes for secure noncovalent immobilization and efficient delivery of DNA. Angew Chem Int Ed 44(30):4782–4785
Liu Z, Chen K, Davis C, Sherlock S, Cao Q, Chen X, Dai H (2008) Drug delivery with carbon nanotubes for in vivo cancer treatment. Cancer Res 68(16):6652–6660
Lu J, Liong M, Zink JI, Tamanoi F (2007) Mesoporous silica nanoparticles as a delivery system for hydrophobic anticancer drugs. Small 3(8):1341–1346
Luo Q, Zhao J, Zhang X, Pan W (2011) Nanostructured lipid carrier (NLC) coated with chitosan oligosaccharides and its potential use in ocular drug delivery system. Int J Pharm 403(1–2):185–191
Luong D, Kesharwani P, Deshmukh R, Mohd Amin MC, Gupta U, Greish K, Iyer AK (2016) PEGylated PAMAM dendrimers: enhancing efficacy and mitigating toxicity for effective anticancer drug and gene delivery. Acta Biomater 43:14–29
Madaan A, Singh P, Awasthi A, Verma R, Singh AT, Jaggi M, Mishra SK, Kulkarni S, Kulkarni H (2013) Efficiency and mechanism of intracellular paclitaxel delivery by novel nanopolymer-based tumor-targeted delivery system Nanoxel TM. Clin Transl Oncol 15(1):26–32
Madaan K, Kumar S, Poonia N, Lather V, Pandita D (2014) Dendrimers in drug delivery and targeting: drug-dendrimer interactions and toxicity issues. J Pharm Bioallied Sci 6(3):139–150
Madan J, Pandey RS, Jain V, Katare OP, Chandra R, Katyal A (2013) Poly (ethylene)-glycol conjugated solid lipid nanoparticles of noscapine improve biological half-life, brain delivery and efficacy in glioblastoma cells. Nanomedicine 9(4):492–503
Madani SY, Naderi N, Dissanayake O, Tan A, Seifalian AM (2011) A new era of cancer treatment: carbon nanotubes as drug delivery tools. Int J Nanomedicine 6:2963–2979
Mahalingam D, Nemunaitis JJ, Malik L, Sarantopoulos J, Weitman S, Sankhala K, Hart J, Kousba A, Gallegos NS, Anderson G, Charles J, Rogers JM, Senzer NN, Mita AC (2014) Phase I study of intravenously administered ATI-1123, a liposomal docetaxel formulation in patients with advanced solid tumors. Cancer Chemother Pharmacol 74(6):1241–1250
Malmsten M (2013) Inorganic nanomaterials as delivery systems for proteins, peptides, DNA, and siRNA. Curr Opin Colloid Interface Sci 18(5):468–480
Mamot C, Ritschard R, Wicki A, Stehle G, Dieterle T, Bubendorf L, Hilker C, Deuster S, Herrmann R, Rochlitz C (2012) Tolerability, safety, pharmacokinetics, and efficacy of doxorubicin-loaded anti-EGFR immunoliposomes in advanced solid tumours: a phase 1 dose-escalation study. Lancet Oncol 13(12):1234–1241
Manivasagan P, Bharathiraja S, Bui NQ, Jang B, Oh YO, Lim IG, Oh J (2016) Doxorubicin-loaded fucoidan capped gold nanoparticles for drug delivery and photoacoustic imaging. Int J Biol Macromol 91:578–588
Mathew A, Fukuda T, Nagaoka Y, Hasumura T, Morimoto H, Yoshida Y, Maekawa T, Venugopal K, Kumar DS (2012) Curcumin loaded-PLGA nanoparticles conjugated with Tet-1 peptide for potential use in Alzheimer's disease. PLoS One 7(3):32616
Matougui N, Boge L, Groo AC, Umerska A, Ringstad L, Bysell H, Saulnier P (2016) Lipid-based nanoformulations for peptide delivery. Int J Pharm 502(1–2):80–97
Matsumura Y, Hamaguchi T, Ura T, Muro K, Yamada Y, Shimada Y, Shirao K, Okusaka T, Ueno H, Ikeda M, Watanabe N (2004) Phase I clinical trial and pharmacokinetic evaluation of NK911, a micelle-encapsulated doxorubicin. Br J Cancer 91(10):1775–1781
Maximilien J, Beyazit S, Rossi C, Bui BTS (2015) Nanoparticles in biomedical applications. In: Wegener J (ed) Bioanalytical reviews. Springer, Cham
Medintz IL, Uyeda HT, Goldman ER, Mattoussi H (2005) Quantum dot bioconjugates for imaging, labelling and sensing. Nat Mater 4(6):435–446
Meerum Terwogt JM, ten Bokkel Huinink WW, Schellens JH, Schot M, Mandjes IA, Zurlo MG, Rocchetti M, Rosing H, Koopman FJ, Beijnen JH (2001) Phase I clinical and pharmacokinetic study of PNU166945, a novel water-soluble polymer-conjugated prodrug of paclitaxel. Anti-Cancer Drugs 12(4):315–323

Mehnert W, Mader K (2001) Solid lipid nanoparticles: production, characterization and applications. Adv Drug Deliv Rev 47(2–3):165–196
Mohamed F, van der Walle CF (2008) Engineering biodegradable polyester particles with specific drug targeting and drug release properties. J Pharm Sci 97(1):71–87
Morales CS, Zhang L, Langer R, Farokhzad OC (2009) Immunocompatibility properties of lipid-polymer hybrid nanoparticles with heterogeneous surface functional groups. Biomaterials 30:2231–2240
Morris B (2003) The components of the wired spanning forest are recurrent. Probab Theory Relat Fields 125(2):259–265
Mosqueira VC, Legrand P, Gref R, Heurtault B, Appel M, Barratt G (1999) Interactions between a macrophage cell line (J774A1) and surface-modified poly (D, L-lactide) nanocapsules bearing poly(ethylene glycol). J Drug Target 7(1):65–78
Muggia FM, Hainsworth JD, Jeffers S, Miller P, Groshen S, Tan M, Roman L, Uziely B, Muderspach L, Garcia A, Burnett A, Greco FA, Morrow CP, Paradiso LJ, Liang LJ (1997) Phase II study of liposomal doxorubicin in refractory ovarian cancer: antitumor activity and toxicity modification by liposomal encapsulation. J Clin Oncol 15(3):987–993
Muller RH, Radtke M, Wissing SA (2002) Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations. Adv Drug Deliv Rev 54(Suppl 1):S131–S155
Muller RH, Petersen RD, Hommoss A, Pardeike J (2007) Nanostructured lipid carriers (NLC) in cosmetic dermal products. Adv Drug Deliv Rev 59(6):522–530
Nanjwade BK, Bechra HM, Derkar GK, Manvi FV, Nanjwade VK (2009) Dendrimers: emerging polymers for drug-delivery systems. Eur J Pharm Sci 38(3):185–196
Naseri N, Valizadeh H, Milani PZ (2015) Solid lipid nanoparticles and nanostructured lipid carriers: structure, preparation and application. Ad Pharm Bull 5(3):305–313
Niidome Y, Niidome T, Yamada S, Nakashima (2006) Pulsed-laser induced fragmentation and dissociation of DNA immobilized on gold nanoparticles. Mol Cryst Liq Cryst 445(1):201–206
Oasmia Pharmaceutical AB (2014) Study of paclitaxel in patients with ovarian cancer. Retrieved. https://clinicaltrials.gov/ct2/show/NCT00989131
Olbrich C, Bakowsky U, Lehr CM, Müller RH, Kneuer C (2001) Cationic solid-lipid nanoparticles can efficiently bind and transfect plasmid DNA. J Control Release 77(3):345–355
Onyuksel H, Séjourné F, Suzuki H, Rubinstein I (2006) Human VIP-alpha: a long-acting, biocompatible and biodegradable peptide nanomedicine for essential hypertension. Peptides 27(9):2271–2275
Pagano L, Cornely OA, Busca A, Caira M, Cesaro S, Gasbarrino C, Girmenia C, Heinz WJ, Herbrecht R, Florl CL, Nosari A, Potenza L (2013) Combined antifungal approach for the treatment of invasive mucormycosis in patients with hematologic diseases: a report from the SEIFEM and FUNGISCOPE registries. Haematologica 98(10):127–130
Pan L, He Q, Liu J, Chen Y, Ma M, Zhang L, Shi J (2012) Nuclear-targeted drug delivery of TAT peptide-conjugated monodisperse mesoporous silica nanoparticles. J Am Chem Soc 134(13):5722–5725
Pandita D, Lather V (2015) Silica based nanoparticles for theranostic applications. In: Singh B (ed) NanoBioMedicine vol 3 nanotheranostics. Studium Press LLC, Houston
Pandita D, Ahuja A, Lather V, Benjamin B, Dutta T, Velpandian T, Khar RK (2011) Development of lipid-based nanoparticles for enhancing the oral bioavailability of paclitaxel. AAPS PharmSciTech 12(2):712–722
Pandita D, Kumar S, Poonia N, Lather V (2014) Solid lipid nanoparticles enhance oral bioavailability of resveratrol, a natural polyphenol. Food Res Int 62:1165–1174
Pandita D, Kumar S, Lather V (2015a) Hybrid poly(lactic-co-glycolic acid) nanoparticles: design and delivery prospectives. Drug Discov Today 20(1):95–104
Pandita D, Poonia N, Lather V (2015b) Mesoporous silica nanoparticles in drug delivery and targeting. In: Singh B (ed) NanoBioMedicine vol 1 nanomedicine. Studium Press LLC, Houston

Patel KD, Mahapatra C, Jin GZ, Singh RK, Kim HW (2015) Biocompatible mesoporous nanotubular structured surface to control cell behaviors and deliver bioactive molecules. ACS Appl Mater Interfaces 7(48):26850–26859
Pathak A, Patnaik S, Gupta KC (2009) Recent trends in non-viral vector-mediated gene delivery. Biotechnol J 4(11):1559–1572
Patnaik A, Papadopoulos KP, Tolcher AW, Beeram M, Urien S, Schaaf LJ, Tahiri S, Bekaii-Saab T, Lokiec FM, Rezaï K, Buchbinder A (2013) Phase I dose-escalation study of EZN-2208 (PEG-SN38), a novel conjugate of poly(ethylene) glycol and SN38, administered weekly in patients with advanced cancer. Cancer Chemother Pharmacol 71(6):1499–1506
Pattni BS, Chupin VV, Torchilin VP (2015) New developments in liposomal drug delivery. Chem Rev 115(19):10938–10966
Peer D, Karp JM, Hong S, Farokhzad OC, Margalit R, Langer R (2007) Nanocarriers as an emerging platform for cancer therapy. Nat Nanotechnol 2(12):751–760
Perche F, Patel NR, Torchilin VP (2012) Accumulation and toxicity of antibody-targeted doxorubicin-loaded PEG-PE micelles in ovarian cancer cell spheroid model. J Control Release 164(1):95–102
Plummer R, Wilson RH, Calvert H, Boddy AV, Griffin M, Sludden J, Tilby MJ, Eatock M, Pearson DG, Ottley CJ, Matsumura Y, Kataoka K, Nishiya T (2011) A phase I clinical study of cisplatin-incorporated polymeric micelles (NC-6004) in patients with solid tumours. Br J Cancer 104(4):593–598
Poonia N, Kharb R, Lather V, Deepti P (2016) Nanostructured lipid carriers: versatile oral delivery vehicle. Future Sci OA 2(3):FSO135
Prados MD, Lamborn K, Yung WK, Jaeckle K, Robins HI, Mehta M, Fine HA, Wen PY, Cloughesy T, Chang S, Nicholas MK, Schiff D, Greenberg H, Junck L, Fink K, Hess K, Kuhn J, North American Brain Tumor Consortium (2006) A phase 2 trial of irinotecan (CPT-11) in patients with recurrent malignant glioma: a North American brain tumor consortium study. Neuro-Oncol 8(2):189–193
Prato M, Kostarelos K, Bianco A (2008) Functionalized carbon nanotubes in drug design and discovery. Acc Chem Res 41(1):60–68
Qian X, Long L, Shi Z, Liu C, Qiu M, Sheng J, Pu P, Yuan X, Ren Y, Kang C (2014) Star-branched amphiphilic PLA-b-PDMAEMA copolymers for co-delivery of miR-21 inhibitor and doxorubicin to treat glioma. Biomaterials 35(2322):2335
Radaic A, de Paula E, de Jesus MB (2015) Factorial design and development of solid lipid nanoparticles (SLN) for gene delivery. J Nanosci Nanotechnol 15(2):1793–1800
Rademaker-Lakhai JM, Terret C, Howell SB, Baud CM, De Boer RF, Pluim D, Beijnen JH, Schellens JH, Droz JP (2004) A phase I and pharmacological study of the platinum polymer AP5280 given as an intravenous infusion once every 3 weeks in patients with solid tumors. Am Ass Cancer Res 10(10):3386–3395
Rawat M, Singh D, Saraf S, Saraf S (2008) Lipid carriers: a versatile delivery vehicle for proteins and peptides. Yakugaku Zasshi 128(2):269–280
Rivera E (2003) Liposomal anthracyclines in metastatic breast cancer: clinical update. Oncologist 8(Suppl 2):3–9
Roby KF, Niu F, Rajewski RA, Terranova PF (2008) In: Coukos G, Berchuck A, Ozols R (eds) Syngeneic mouse model of epithelial ovarian cancer: effects of nanoparticulate paclitaxel, Nanotax®. Ovarian cancer: state of the art and future directions in translational research. Springer New York, New York, pp 169–181
Rodriguez MA, Pytlik R, Kozak T, Chhanabhai M, Gascoyne R, Lu B, Deitcher SR, Winter JN, Marqibo Investigators (2009) Vincristine sulfate liposomes injection (Marqibo) in heavily pretreated patients with refractory aggressive non-Hodgkin lymphoma: report of the pivotal phase 2 study. Cancer 115(15):3475–3482
Rosi NL, Giljohann DA, Thaxton CS, Lytton-Jean AKR, Han MS, Mirkin CA (2006) Oligonucleotide-modified gold nanoparticles for intracellular gene regulation. Science 312(5776):1027–1030

Roy I, Ohulchanskyy TY, Bharali DJ, Pudavar HE, Mistretta RA, Kaur N, Prasad PN (2005) Optical tracking of organically modified silica nanoparticles as DNA carriers: a nonviral, nanomedicine approach for gene delivery. Proc Natl Acad Sci U S A 102(2):279–284

Rudzinski WE, Palacios A, Ahmed A, Lane MA, Aminabhavi TM (2016) Targeted delivery of small interfering RNA to colon cancer cells using chitosan and PEGylated chitosan nanoparticles. Carbohydr Polym 147:323–332

Sabbatini P, Aghajanian C, Dizon D, Anderson S, Dupont J, Brown JV, Peters WA, Jacobs A, Mehdi A, Rivkin S, Eisenfeld AJ, Spriggs D (2004) Phase II study of CT-2103 in patients with recurrent epithelial ovarian, fallopian tube, or primary peritoneal carcinoma. J Clin Oncol 22(22):4523–4531

Samad A, Sultana Y, Aqil M (2007) Liposomal drug delivery systems: an update review. Curr Drug Deliv 4(4):297–305

Santos JL, Oliveira H, Pandita D, Rodrigues J, Pêgo AP, Granja PL, Tomás H (2010) Functionalization of poly(amidoamine) dendrimers with hydrophobic chains for improved gene delivery in mesenchymal stem cells. J Control Release 144(1):55–64

Sarkar A, Ghosh S, Chowdhury S, Pandey B, Sil PC (2016) Targeted delivery of quercetin loaded mesoporous silica nanoparticles to the breast cancer cells. Biochim Biophys Acta 1860(10):2065–2075

Scott LC, Yao JC, Thomas AL, Falk S, Mena RR, Picus J, Wright J, Mulcahy MF, Ajani JA, Evans TR (2009) A phase II study of pegylated-camptothecin (pegamotecan) in the treatment of locally advanced and metastatic gastric and gastro-oesophageal junction adenocarcinoma. Cancer Chemother Pharmacol 63(2):363–370

Sekhon BS, Kamboj SR (2014) Inorganic nanomedicine-Part 2. Nanomed Nanotechnol Biol Med 6(5):612–618

Seymour LW, Ferry DR, Anderson D, Hesslewood S, Julyan PJ, Poyner R, Doran J, Young AM, Burtles S, Kerr DJ (2002) Hepatic drug targeting: phase I evaluation of polymer-bound doxorubicin. J Clin Oncol 20(6):1668–1676

Singh R, Lillard JW (2009) Nanoparticle-based targeted drug delivery. Exp Mol Pathol 86(3):215–223

Singh R, Pantarotto D, McCarthy D, Chaloin O, Hoebeke J, Partidos CD, Briand JP, Prato M, Bianco A, Kostarelos K (2005) Binding and condensation of plasmid DNA onto functionalized carbon nanotubes: toward the construction of nanotube-based gene delivery vectors. J Am Chem Soc 127(12):4388–4396

Slingerland M, Guchelaar HJ, Rosing H, Scheulen ME, van Warmerdam LJ, Beijnen JH, Gelderblom H (2013) Bioequivalence of Liposome-Entrapped Paclitaxel Easy-To-Use (LEP-ETU) formulation and paclitaxel in polyethoxylated castor oil: a randomized, two-period crossover study in patients with advanced cancer. Clin Ther 35(12):1946–1954

Soepenberg O, de Jonge MJ, Sparreboom A, de Bruin P, Eskens FA, de Heus G, Wanders J, Cheverton P, Ducharme MP, Verweij J (2005) Phase I and pharmacokinetic study of DE-310 in patients with advanced solid tumors. Am Ass Cancer Res 11(2):703–711

Sonali SRP, Sharma G, Kumari L, Koch B, Singh S, Bharti S, Rajinikanth PS, Pandey BL, Muthu MS (2016) RGD-TPGS decorated theranostic liposomes for brain targeted delivery. Colloids Surf B Biointerf 147:129–141

Stevens JM, Auer J, Strong CA, Hughes RT, Oliver DO, Winearls CG, Cotes PM (1991) Stepwise correction of anaemia by subcutaneous administration of human recombinant erythropoietin in patients with chronic renal failure maintained by continuous ambulatory peritoneal dialysis. Nephrol Dial Transplant 6(7):487–494

Sugii M, Suzuki R, Oda Y, Unga J, Omata D, Urunga H, Negishi Y, Maruyama K (2016) Development of cell-targeting bubble liposome for effective gene delivery with ultrasound. Asian J Pharm Sci 11(1):146–147

Sun J, Bi C, Chan HM, Sun S, Zhang Q, Zheng Y (2013) Curcumin-loaded solid lipid nanoparticles have prolonged in vitro antitumour activity, cellular uptake and improved in vivo bioavailability. Colloids Surf B Biointerf 111:367–375

Suwalski A, Dabboue H, Delalande A, Bensamoun SF, Canon F, Midoux P, Saillant G, Klatzmann D, Salvetat JP, Pichon C (2010) Accelerated Achilles tendon healing by PDGF gene delivery with mesoporous silica nanoparticles. Biomaterials 31(19):5237–5245

Sweety K, Deepti P, Poonia N, Lather V (2015) Nanostructured lipid carriers for topical delivery of an anti-acne drug: characterization and *ex vivo* evaluation. Pharm Nanotechnol 3(2):122–133

Tabernero J, Shapiro GI, LoRusso PM, Cervantes A, Schwartz GK et al (2013) First-in-humans trial of an RNA interference therapeutic targeting VEGF and KSP in cancer patients with liver involvement. Cancer Discov 3(4):406–417

Tan GR, Fenq SS, Leong DT (2014) The reduction of anti-cancer drug antagonism by the spatial protection of drugs with. PLA-TPGS Nanopart Biomater 35(9):3044–3051

Thassu D, Deleers M, Pathak YV (2007) Nanoparticulate drug delivery systems. CRC Press, Boca Raton

Ueno T, Endo K, Hori K, Ozaki N, Tsuji A, Kondo S, Wakisaka N, Murono S, Kataoka K, Kato Y, Yoshizaki T (2014) Assessment of antitumor activity and acute peripheral neuropathy of 1, 2-diaminocyclohexane platinum (II)-incorporating micelles (NC-4016). Int J Nanomedicine 9:3005–3012

Uner M, Yener G (2007) Importance of solid lipid nanoparticles (SLN) in various administration routes and future perspectives. Int J Nanomedicine 2(3):289–300

Uriarte-Pinto MÁ, Escolano-Pueyo Á, Gimeno-Ballester V, Pascual-Martínez O, Abad-Sazatornil MR, Agustín-Ferrández MJ (2016) Trastuzumab, non-pegylated liposomal-encapsulated doxorubicin and paclitaxel in the neoadjuvant setting of HER-2 positive breast cancer. Int J Clin Pharm 38(2):446–453

Vallet-Regi MF, Balas F, Arcos D (2007) Mesoporous materials for drug delivery. Angew Chem Int Ed Eng l46(40):7548–7558

Vasey PA, Kaye SB, Morrison R, Twelves C, Wilson P, Duncan R, Thomson AH, Murray LS, Hilditch TE, Murray T, Burtles S, Fraier D, Frigerio E, Cassidy J (1999) Phase I clinical and pharmacokinetic study of PK1 [N-(2-hydroxypropyl)methacrylamide copolymer doxorubicin]: first member of a new class of chemotherapeutic agents-drug-polymer conjugates. Cancer Research Campaign Phase I/II Committee. Clin Cancer Res 5(1):83–94

Venkatakrishnan K, Liu Y, Noe D, Mertz J, Bargfrede M, Marbury T, Farbakhsh K, Oliva C, Milton A (2014) Pharmacokinetics and pharmacodynamics of liposomal mifamurtide in adult volunteers with mild or moderate hepatic impairment. Br J Clin Pharmacol 77(6):998–1010

Vivero-Escoto JL, Slowing II, Trewyn BG, Lin VS (2010) Mesoporous silica nanoparticles for intracellular controlled drug delivery. Small 6(18):1952–1967

Wang YXJ (2011) Superparamagnetic iron oxide based MRI contrast agents: current status of clinical application. Quant Imaging Med Surg 1(1):35–40

Wang M, Thanou M (2010) Targeting nanoparticles to cancer. Pharmacol Res 62(2):90–99

Wang F, Wang YC, Dou S, Xiong MH, Sun TM, Wang J (2011) Doxorubicin-tethered responsive gold nanoparticles facilitate intracellular drug delivery for overcoming multidrug resistance in cancer cells. ACS Nano 5(5):3679–3692

Weber S, Niemirowicz K, Markiewicz KH, Car H (2014) Solid Lipid Nanoparticles (SLN) and Nanostructured Lipid Carriers (NLC) for pulmonary application: a review of the state of the art. Eur J Pharm Biopharm 86(1):7–22

Wilczewska AZ, Niemirowicz K (2012) Nanoparticles as drug delivery systems. Pharmacol Rep 64(5):1020–1037

Xie X, Xia W, Li Z, Kuo HP, Liu Y, Li Z, Ding Q, Zhang S, Spohn B, Yang Y, Wei Y, Lang JY, Evans DB, Chiao PJ, Abbruzzese JL, Hung MC (2007) Targeted expression of BikDD eradicates pancreatic tumors in noninvasive imaging models. Cancer Cell 12(1):52–65

Yang F, Jin C, Subedi S, Lee CL, Wang Q, Jiang Y, Li J, Di Y, Fu D (2012) Emerging inorganic nanomaterials for pancreatic cancer diagnosis and treatment. Cancer Treat Rev 38(6):566–579

Yang XY, Li YX, Li M, Zhang L, Feng LX, Zhang N (2013) Hyaluronic acid-coated nanostructured lipid carriers for targeting paclitaxel to cancer. Cancer Lett 334(2):338–345

Yang HN, Park JS, Jeon SY, Park W, Na K, Park KH (2014) The effect of quantum dot size and poly(ethylenimine) coating on the efficiency of gene delivery into human mesenchymal stem cells. Biomaterials 35(29):8439–8449

Yu GS, Bae YM, Choi H, Kong B, Choi IS, Choi JS (2011) Synthesis of PAMAM dendrimer derivatives with enhanced buffering capacity and remarkable gene transfection efficiency. Bioconjug Chem 22(6):1046–1055

Yu YH, Kim E, Park DE, Shim G, Lee S, Kim YB, Kim CW, Oh YK (2012) Cationic solid lipid nanoparticles for co-delivery of paclitaxel and siRNA. Eur J Pharm Biopharm 80(2):268–273

Yu K, Zhao J, Zunkai Z, Yin G, Yulin Z, Lesheng T, Li Y (2015) Enhanced delivery of paclitaxel using electrostatically-conjugated Herceptin-bearing PEI/PLGA nanoparticles against HER-positive breast cancer cells. Int J Pharm 497(1–2):78–87

Yurkovetskiy AV, Fram RJ (2009) XMT-1001, a novel polymeric camptothecin pro-drug in clinical development for patients with advanced cancer. Adv Drug Deliv Rev 61(13):1193–1202

Zhai Y, Zhai G (2014) Advances in lipid-based colloid systems as drug carrier for topic delivery. J Control Release 193:90–99

Zhang Chen Z, Song X, Si J, Tang G (2008) Low generation polypropylenimine dendrimer graft beta-cyclodextrin: an efficient vector for gene delivery system. Technol Cancer Res Treat 7(2):103–108

Zhang B, Wang T, Yang S, Xiao Y, Song Y, Zhang N, Garg S (2016a) Development and evaluation of oxaliplatin and irinotecan co-loaded liposomes for enhanced colorectal cancer therapy. J Control Release 238:10–21

Zhang J, Sun Y, Tian B, Li K, Wang L, Liang Y, Han J (2016b) Multifunctional mesoporous silica nanoparticles modified with tumor-shedable hyaluronic acid as carriers for doxorubicin. Colloids Surf B Biointerf 144:293–302

Zhao X, Sun X, Zeng L, Liu J, Zhang Z (2015) Co-delivery of HIF1α siRNA and gemcitabine via biocompatible lipidpolymer hybrid nanoparticles for effective treatment of pancreatic cancer. Biomaterials 46:13–25

Zhou Q, Sun X, Zheng L, Liu J, Zhang Z (2009) A randomized multicenter phase II clinical trial of mitoxantrone-loaded nanoparticles in the treatment of 108 patients with unresected hepatocellular carcinoma. Nanomed Nanotechnol Biol Med 5(4):419–423

Zhu D, Tao W, Zhang H, Liu G, Wang T, Zhang L, Zeng X, Mei L (2015) Docetaxel (DTX)-loaded polydopamine-modified TPGS-PLA nanoparticles as a targeted drug delivery system for the treatment of liver cancer. Acta Biomater 30:144–154

7 Nano-based Delivery System for Nutraceuticals: The Potential Future

Manju Bernela, Pawan Kaur, Munish Ahuja, and Rajesh Thakur

7.1 Introduction

Drug delivery field is currently being revolutionized by nanoencapsulation of bioactives. Main sources for bioactives are the natural products which are believed to be the most important discovery of modern medicine (Molinski 1993; Grabley and Thiericke 1999). Identification of novel pharmacophores and active compounds has always been in demand because still synthetic medicinal chemistry has yet to find substitutes to many of the natural compounds (Leach et al. 2010). But several phytochemicals have low solubility leading to their poor bioavailability. Encapsulation of such bioactives in appropriate carrier matrix can enhance their bioavailability because of altered pharmacokinetics and biodistribution (Huang et al. 2013). Therapeutic index of drugs has been shown to increase using controlled drug delivery systems due to increase in their localization to specific tissues (Riehemann et al. 2009; Ferrari 2005). Recent trends in medical field have shown that nanoparticle-based therapeutic products have the potential of being commercialized and there is an upsurge in number of commercially available nanoparticle-based products. These days not only researchers but ordinary people are also attracted towards natural dietary agents due to their proven benefits in healthcare and fitness (Amin et al. 2009). Recently taurine, a major constituent of cactus pear, has become an active constituent of nutraceuticals after the reported health benefit spectrum of cactus plant such as anticancer, antiviral and antidiabetic properties. Due to the increasing interest of scientists and researchers in traditional medicines, the World Health Organization has initiated a global strategy to deal with the concerns related to traditional medicine. The European Commission has also resolved to put the disease

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risk reduction at priority in their coming plans. Researches on nanoencapsulation of nutraceuticals have been in recent trends to eliminate the limitations associated with them and maximize their health benefits. The present review focuses on different nanocarriers for nutraceuticals and major challenges in the way of commercialization of nano-nutraceuticals.

7.2 Nutraceuticals

The term nutraceuticals arises from the combination of nutrition and pharmaceuticals. These are a part of food or foods which offers nutritional and pharmaceutical benefits, that is, give nutrients to the body, provide resistance against several diseases and also help in curing of some diseases (Trottier et al. 2010). In ancient time, the knowledgeable people working in the field of medicine thought of developing such food which could be used as medicine to prevent and cure diseases. Those brilliant ideas gave birth to the field of nutraceuticals. Nutraceuticals can be divided into three main categories—dietary supplements, functional foods and functional beverages. Further the dietary supplements can be subdivided into vitamin and mineral supplements, herbal supplements, plant extracts and protein supplements. Functional foods include omega fatty acid foods and probiotics, whereas functional beverages can be subsegmented into energy drinks, sports drinks and fortified juices. Some common words related to nutraceuticals or used as synonym for nutraceuticals are functional food, multifunctional food, dietary supplements, etc. Functional foods are just the same as basic foods providing nutritions, incorporated with special and specific ingredients which provide health benefits to the body (Kalra 2003). The recent advancements in the field of food technology have opened gates for the development of functional foods exclusively produced to promote good health for human being. Some basic steps taken into consideration while scrutinizing are identification, isolation, purification and characterization of the properties of incorporated food components, that is, the nutritional value, medicinal value, etc. Primary food elements comprise of carbohydrates, proteins and lipids which are the basic necessity for proper functioning of the body and its normal energy requirements. Vitamins are secondary food elements which are commonly not synthesized within the human body, so these must be taken in food diet for proper functioning of the body. Nutraceuticals are also minor food elements which improve the body functioning by fighting against some persistent diseases (McClements 2012b).

Efficacy of any nutraceutical product depends on its bioavailability. In terms of nutritional concept, bioavailability means that some nutrients in food are partially available, whereas in terms of pharmacology it refers to rate and extent to which a drug reaches to its site of action. With increasing popularity of nutraceuticals as preventive medicine, their bioavailability has become a major issue to the regulators and manufacturers of health-related products (Rapaka and Coates 2006). When administered orally, various parameters such as insufficient gastric residence time, low permeability and/or solubility within the gut and instability under conditions encountered in food processing or in the gastrointestinal (GI) tract limit the activity

and hence health benefits of various nutraceuticals (Bell 2001). Apart from their low bioavailability and poor water solubility, other challenges such as chemical instability and crystallization need to be overcome before the incorporation of these bioactive molecules into commercial food products (Augustin and Sanguansri 2012; McClements et al. 2012a, c). Patenting of new delivery systems like nanotechnology has been in trend for improving the efficacy of nutraceuticals.

7.3 Nanocarriers as Delivery Platforms

There has been a boom of nanotechnology in most of the sectors with major impact in health and food industry. Encapsulation of active food ingredients in nano-based drug delivery system is an important application of this technology, in the field of food and nutrition (Fig. 7.1).

Nanoformulations of nutraceuticals also follow the fundamental principles of nanotechnology. The nanotechnology platforms are mainly used to prepare delivery systems for nutraceuticals with poor water solubility. The technology possesses a great potential for commercialization of the bioactives by overcoming the limitations associated with them and therefore a multifold increase for this technology is eminent in the coming years (Augustin and Hemar 2009). However before its use in commercial food products, it is a must to ensure that these newly developed nanoscale delivery systems are safe to be incorporated in commercialized food products. There are several desirable characteristics of nanoscale delivery systems which need to be taken into consideration. First of all, as reduced to nanoscale, they will behave differently within gastrointestinal tract in comparison to conventional particulate matter (Tiede et al. 2008; Bouwmeester et al. 1998). If the digestion product of nanoscale delivery system is the same as that of conventional particulate

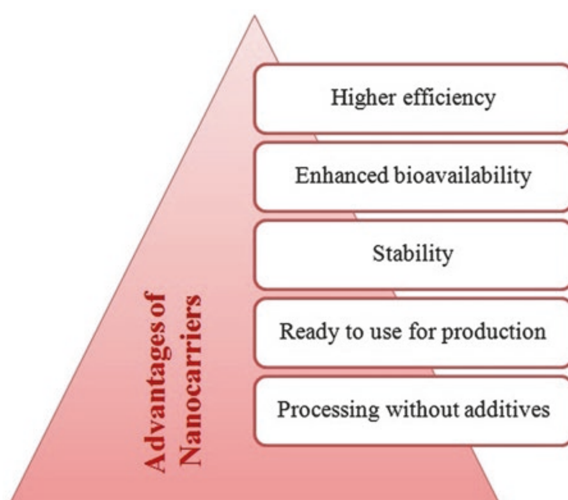

Fig. 7.1 Advantages of nanocarriers over the conventional methods of encapsulation (McClements 2012a)

matter, then they can't be expected to be more toxic; otherwise toxicity concern may arise. So it is very important to assess potential toxicity of these food-grade nanoscale delivery systems to ensure their safety. Use of food-grade ingredients for fabrication of these delivery systems is preferable. These nanoscale systems should be economically viable, strong enough to confront storage conditions as well as robust enough for practical applications. Further, their incorporation into final food product should not adversely affect its quality aspects.

- Delivery system should be chemically and physically stable to environmental stresses while preserving its functional characteristics (McClements 2012a).
- Delivery system should be able to improve gastric stability of labile bioactive nutrients.
- Should be able to maintain constant dosage level within systemic circulation.
- In case of highly lipophilic compound, it should be capable of facilitating lymphatic transport.
- Should be able to extend gastric retention time (Ting et al. 2014).
- Over the past few years, several researchers have described the use of nanotechnology for nutraceutical compounds. Oral absorption and bioavailability of phenolic compounds have been reported to increase thereby promoting their nutraceutic effect (Munin and Edwards-Levy 2011; Rein et al. 2013). The nanoparticles have mainly been prepared using lipids, polysaccharides or proteins, loaded with different bioactive compounds. Some of the nanodelivery systems used for nutraceuticals are discussed below.

7.3.1 Lipid-Based Nanostructures

Lipid-based structures such as solid lipid nanoparticles, liposomes, nanoemulsions and self-emulsifying systems hold a great potential for encapsulation and delivery of sensitive bioactives and may serve as bioreactors for release of aroma compound and flavours. They are advantageous on the part that most of the natural bioactives can be encapsulated in them. Moreover they can incorporate high loads of different molecules and target them on specific sites via active or passive mechanisms (Mozafari et al. 2006; Taylor et al. 2005; Mozafari and Mortazavi 2005; Mozafari et al. 2009).

7.3.1.1 Solid Lipid Nanoparticles (SLN)

Solid lipid nanoparticles are colloidal dispersions of lipid in water or aqueous surfactant solution (Weber et al. 2014). They have many advantages such as protection of incorporated molecule from external environment, better stability of the encapsulated compound, ability to carry both lipophilic as well as hydrophilic drugs, controlled release and easy to scale up. These lipidic systems may also help to improve organoleptic and functional properties. Moreover these systems include the compounds with GRAS (generally recognized as safe) status (Severino

et al. 2011). Disadvantages including relatively high water content and increased particle size, flocculation, aggregation and compound release may occur during storage (Das and Chaudhury 2010).

7.3.1.2 Nanoliposomes

Liposomes are lipid bilayer membrane structures with hydrophilic heads and hydrophobic fatty acid tails (Lasic 1998). They can carry both hydrophobic and hydrophilic bioactives, hydrophilic in aqueous core whereas hydrophobic component in lipid bilayers (Langer 1990). Nanoliposomes specifically refer to liposomes with nanometric size (Mozafari and Mortazavi 2005). Route of administration for nanoliposomes could be parenteral, oral, topical or nasal (Shoji and Nakashima 2004; Li et al. 2013). Their disadvantage is that they are considered as foreign particles by circulatory system and hence rapidly get cleared by reticuloendothelial system (Tang et al. 2013). Furthermore they can get disintegrated by various forces such as electrostatic, hydrophobic and van der Waals forces that can disintegrate nanoliposomes (Lasic et al. 1991). Hence some stability mechanism is required such as steric stabilization using inert polymers (Momekova et al. 2007).

7.3.1.3 Nanoemulsions

Emulsions are basically biphasic systems which are composed of an inner phase, i.e. dispersed phase, and an outer phase, i.e. continuous phase. There is an interphase made up of surfactant molecule. Nanoemulsions are emulsions which are extremely small in size and appear transparent or translucent. Their size range is usually 50–200 nm which is much smaller than the conventional emulsions (Solans et al. 2005). In general, the size of a surfactant molecule is 2 nm long, and therefore a micelle is usually 5 nm or more in diameter. But incorporation of oil phase into micellar core can cause increases in its size sometimes to a large extent (Huang et al. 2013). Nanoemulsion is a better option to incorporate poorly soluble nutraceuticals into food matrix, and it is well known that most of the bioactive phytochemicals are either poorly soluble or lipophilic in nature. Systemic bioavailability of these active components is considerably influenced by their poor solubility in water or oil because their properties, such as solubility, partition coefficient, lipophilicity, etc., decide their route of administration, transport and target sites. Entrapment of such bioactives into nanoemulsions can prove advantageous as the small particle size of nanoemulsions will increase their surface area thereby resulting in enhanced digestion rates, rapid diffusion across mucus membrane and increased epithelium cell permeability (Sivakumar et al. 2014; Ting et al. 2014; Yu and Huang 2013). Moreover, nanoemulsions may protect the chemically labile bioactives from oxidation, thereby resulting in increased shelf life and reduced degradation in the gastrointestinal tract (GIT) (Augustin et al. 2011; Frede et al. 2014). There are a large number of reports on entrapment of bioactives into nanoemulsions, and recent trends have shown the use of food-grade nanoemulsions (Sun et al. 2015). Carrier oil is an important component in preparation of food-grade nanoemulsions as it determines the bioavailability of encapsulated components (Qian et al. 2012;

Zheng et al. 2014). The carrier oil should be able to form mixed micelles with a high solubilization capacity for active component and should be fully digestible (Li et al. 2012).

7.3.2 Polysaccharide-Based Nanoparticles

Most of the naturally occurring polysaccharides are considered to be cheap and adequate raw materials for nanoencapsulation of different bioactives. Nanoparticles of different shape and sizes have been prepared using various methods. Selection of method and polysaccharides depends on factors such as safety, economic and environmental considerations, etc. Different processes can be used for nanoencapsulation of bioactives depending upon physical and chemical properties of bioactives and polysaccharides. Polysaccharides are able to encapsulate both hydrophilic and hydrophobic compounds (Renard et al. 2002). Their structural versatility and site-specific digestion properties project them as suitable carriers for the targeted and controlled delivery of nutraceuticals along the human gastrointestinal tract (GIT) (Sinha and Kumria 2001). Their non-toxic, biocompatible, stable structure, low-cost, hydrophilic nature along with availability of reactive sites for chemical modifications makes them the material of choice (Sinha and Kumria 2001). Commonly used polysaccharides include starch, pectin and guar gum, chitosan, chondroitin sulphate, alginate, etc. (Kosaraju 2005; Augustin and Hemar 2009). They are advantageous on the part that they can be used for delivery of synergistic combinations, but when considered for food applications, high molecular weight of polysaccharides sometimes limits its application, e.g. for delivery in clear drinks, because it is difficult to prepare transparent system based on biopolymeric nanoparticles (Livney 2008).

Vast research has been conducted to encapsulate wide variety of bioactive components in nutraceuticals and functional foods (McClements et al. 2009) as shown in Table 7.1, but clear in vitro or in vivo evidences of their biological efficacies are still limited.

7.4 Nanotechnology-Enabled Food Products

According to the Food Standards Agency, fumed silica, nanosilver, nanoclay and titanium nitride are the nanomaterials that are permitted to be used in food provided they follow the relevant legislation. Centre for Food Safety has released a database which enlists near about 300 food products/food contact products that use nanotechnology. Those with food supplement and additives category are summarized in Table 7.2.

Apart from these, Chinese Nano tea, Nano silver and Nano gold have been used as mineral supplements; carotenoid nanoparticles have been used in fruit drinks; patented “Nano drop” delivery systems have been used for encapsulation of materials such as vitamins; and Nano cages or Nano clusters have been used in

Table 7.1 Recent works on nanoencapsulation of nutraceuticals

Sr. No.	Nanodelivery system used	Material used	Achievements	References
1. Allicin				
a.	Nanocapsules	Alginate, chitosan	–	Fakoor Yazdan Abad et al. (2016)
2. Anthocyanins				
a.	SLNs	Palmitic acid, span 85 and egg lecithin	↑ the stability against high pH	Ravanfar et al. (2016)
3. Bromelain				
a.	Nanoparticles	Katira gum	↑ anti-inflammatory activity	Bernela et al. (2016a)
4.Caffeine				
a.	SLNs	Softisan, pluronic F68	↑skin permeation through skin	Puglia et al. (2016)
b.	Nanohydrogels	Lactoferrin-glycomacropeptide	↑ antimicrobial	Bourbon et al. (2016)
c.	Nanoparticles	PLGA	Pronounced increase in the endurance of dopaminergic neurons, fibre outgrowth and expression of tyrosine hydroxylase (TH)	Singhal et al. (2015)
5. Capsaicin				
a.	Polymeric micelles	Phospholipid, sodium cholate and PVP K30	Prolonged plasma circulation with ↑ oral bioavailability	Zhu et al. (2014)
6. Curcumin				
a.	Hydrogel nanoparticles	Hydrolyzed tetramethyl orthosilicate, chitosan, polyethylene glycol 400	↑antimicrobial and wound healing	Krausz et al. (2015)
b.	SLNs	Tween 80, curcumin, cholesterol	Effective oxygen scavenging activity	Jourghanian et al. (2016)
c.	Nanoemulsion		Controlled lipid digestion rate and free fatty acid adsorption	Joung et al. (2016)
7. Glycyrrhizic acid				
a.	Polymeric nanoparticles	Chitosan, katira gum	↑ anti-inflammatory activity	Bernela et al. (2016)
b.	Polymeric nanoparticles	Chitosan	↑ anti-bacterial activity	Rani et al. (2015)
8. Green tea extract				
a.	Nanostructured lipid carriers	Cetyl palmitate, glyceryl stearate, grape seed oil, St. John's wort oil (*Hypericum perforatum* oil), sea buckthorn oil	↑ antioxidant activity	Manea et al. (2014)

(continued)

Table 7.1 (continued)

Sr. No.	Nanodelivery system used	Material used	Achievements	References
9. Hesperetin				
a.	SLNs	Glycerol monostearate, stearic acid, glyceryl behenate, oleic acid, Tween 80	Could well mask the bitter taste, after taste, and obviate poor solubility of hesperetin	Fathi et al. (2013)
10. Lutein				
a.	Polymeric nanoparticles	Poly-γ-glutamic acid, chitosan	↑ solubility	Hong et al. (2015)
b.	Polymeric nanoparticles	Chitosan and dextran sulphate	↑ chemical stability	Chaiyasan et al. (2016)
11. Melatonin				
a.	Polymeric nanoparticles	Poly(D,L-lactide-co-glycolide), polyvinyl alcohol	Sustained release	Altındal and Gümüşderelioglu (2015)
b.	Polymeric nanoparticles	Lecithin, chitosan	Improved wound epithelialization	Blazevic et al. (2016)
12. Quercetin				
a.	Polymeric nanoparticles	Polyhydroxybutyrate-*co*-hydroxyvalerate, polyvinyl alcohol	–	Vidal et al. (2016)
b.	Nanoparticles	Bovine serum albumin (BSA), 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS)	Prolonged quercetin release and improved antioxidant activity	Antônio et al. (2016)
c.	Polymeric nanoparticles	Poly(lactic-*co*-glycolic acid)-d-α-tocopheryl polyethylene glycol 1000 succinate	Enhanced pharmacological effects of quercetin with increased liver targeting	Guan et al. (2016)
13. Resveratrol				
a.	Solid lipid nanoparticles (SLNs) and nanostructured lipid carriers	Cetyl palmitate, polysorbate 60, miglyol-812	Validated for trans-resveratrol protection, stabilization and intestinal permeability	Neves et al. (2016)
b.	Solid lipid nanoparticles	Phosphatidylcholine from soybean, D-α-tocopheryl polyethylene glycol 1000 succinate	Significantly higher cytotoxicity than resveratrol against C6 glioma cells	Vijayakumar et al. (2016)
c.	Nanoparticles	Beta-lactoglobulin	Improvement in treatment of oxidative stress	Kim et al. (2016)
14. Thymoquinone				
a.	Nanoparticles	Poly(styrene-b-ethylene oxide)	Enhanced antitumor activity	Fakhoury et al. (2014)

Table 7.2 List of companies which produced food supplement and additives with nanomaterials

Nanomaterial	Product name	Company
Calcium and magnesium	24Hr Microactive® CoQ10, CoQ10, rejuvenate, rejuvenate for children nano calcium/magnesium	Genceutic Naturals, Cell Direct, Mag-I-Cal.com
Cobalt	CoQ10 Q-Avail VS softgel	Design for Health
Copper	MesoCopper	Purest Colloids Inc.
Corosolic acid	Nano slim	
Curcuminoid	Nano curcuminoids	Life Enhancement
Gold	MesoGold, Ultra Colloidal Gold™	Colloids for Life LLC., Source Naturals
Iridium	Meso-Iridium	Purest Colloids Inc.
Magnesium	Nano-Ionic Calcium and Magnesium, Nano-Magnesium	MMI Labs Inc., Arrowhead Health Works
Nanoencapsulation	Anabolic Vitakic, Gakic Hardcore, Nitro-Tech Hardcore, Bionic Joint Support, DHLA Nano-Plex, humic and fulmic acids, LifePak Nano, Liposomal Can-Help, Lypo-Spheric Vitamin C, Mucolyxir® Nanotech Nutrients® 12 ml liquid #75360, MuscleTech Nano Vapor Performance, nano green tea drops, Nano Vapor, Nanoceuticals Artichoke Nanoclusters/Cleanse, Nutri-Nano CoQ-10 3.1x softgels, Resveratrol VESIsorb, Solgar CQ10, Spray For Life vitamin supplements, summit vitamins vita-SEDDS multipacks, Sunshine Mist Vitamin D Spray, Taurox 6X Nanotech Nutrients® 0.45 fl. oz. (13.5 mL) # 75090, Taurox High Potency 80 Pellets Nanotech Nutrients® net 0.14 oz. (4 g) # 75250, Ubiquinol VESIsorb, VESIsorb®CoQ10 Plus Omega-3 30S/G, Vitamin D3 VESIsorb	Muscletech Sports Nutrition Supplements, Life enhancement, Quantum Nutrition Labs, Nano Health Solutions, Pharmanex, HealthyItems.co, LivOn Labs, Allergy Research Group, Julian Bakery LLC., Iovate Health Sciences Research Inc., RBC Life Sciences Inc., Solgar, Pure Encapsulation, Nutrition Centre Ltd., Summit Medical Group, Mercola Advanced Nutrition, Doctor's Best
Nano polymeric film	LiquiGlide	Liqui Glide Inc.
Palladium	MesoPalladium	Purest Colloids Inc.
Platinum	MesoPlatinum	Purest Colloids Inc.

(continued)

Table 7.2 (continued)

Nanomaterial	Product name	Company
Resveratrol	Nano resveratrol	Life Enhancement
Selenium	Nano ionic selenium, nano selenium	The Wellness Centre, JST (sold by Alibaba.com)
Silica/silicon dioxide	Advanced Cell Life X-1 and X- 2, nano-2 bio-slim, Microhydrin products, Nanosiliceo Kapseln,	Maat Shop, RBC Life Sciences Inc., Neosino Nanotech
Silver	ASAP Double Strength 8oz, Colloidal Silver, Colloidal Silver Cream, Colloidal Silver Liquid, Colloidal Silver Spray, MaatShop Nano-2+, MesoSilver, Nano Health Silver, Nano Silver (1 and 2), Nano Sil-10, Silver (8 oz), Silver (16 oz), Silver-22, Silver Biotics, Silvix3, Sovereign Silver, Ultra Colloidal Silver™, Ultra Colloidal Silver™ Spray, Ultra Colloidal™ Silver Salve™, Utopia Silver Supplements Colloidal Silver, Wellness Colloidal Silver Nasal Spray, Wellness Colloidal Silver™	American Biotech Labs, Fair Vital, Skybright Natural health, Spirit of Maat, Purest Colloids Inc., Nano Health Solutions, Maat Shop, Greenwood Research/ Consumer Products, Activz, RBC Life Sciences Inc., American Biotech Labs, Natural Care Products, Natural-Immunogenics Corp., Source Naturals, Utopia Silver Supplements
Zeolite	ACZ nano® Advanced Cellular Zeolite Extra Strength	Vitality Products CO Inc.
Zinc	MesoZinc	Purest Colloids Inc.

nanoceutical products such as chocolate drink thereby imparting sweetness without addition of any sugar or sweeteners (Paul and Dewangan 2016).

7.5 Regulatory Issues

Nutraceuticals products claiming medicinal benefits would be required to abide by regulatory requirements for medicinal products regarding safety, efficacy, quality testing and marketing authorization procedures (Pandey et al. 2010). Use of nanodelivery systems for designing not only fresh food but also healthier food has been in trend, but however many of them can cause serious threats to safety of people (Pradhan et al. 2015). A number of regulatory bodies such as the European Food and Safety Authority (EFSA), Environmental Protection Agency (EPA), Food and Drug

Administration (FDA), National Institute for Occupational Safety and Health (NIOSH), Occupational Safety and Health Administration (OSHA), US Department of Agriculture (USDA), Consumer Product Safety Commission (CPSC) and US Patent and Trademark Office (USPTO) govern application of nanosystems in food (Qi et al. 2004). In 2012, FDA released two draft guidance documents regarding nanotechnology. The documents cover the category of food and cosmetics, but none of them points out to dietary supplements specifically because as per FDA dietary supplements are considered to be a category of food. As per FDA guidance document, bioavailability of a food substance is changed if its physical or chemical properties are changed. Further, such changes can also have an effect on its toxic levels. In its new dietary ingredient (NDI) draft guidance, the FDA considered nanotechnology as an example of a process that creates a new dietary ingredient and hence requires a notification to the FDA. But nanoceuticals are not regulated and therefore can be launched to the market with little or no verification of safety. The FDA anticipates that these nanotechnology products should come under the jurisdiction of the Office of Combination Products (Javeri 2016). As per EC Food Law Regulation, several points need to be considered, while designing nanomaterials for food applications such as the nanomaterials use should be free of toxic and heavy metals and mycotoxins (Scampicchio et al. 2008). Directive 89/107/EEC states that nanomaterials intended for use in food packaging should be initially evaluated as a direct food additive (Sondi and Salopek-Sondi 2004).

7.6 Conclusion

Production of NPs using the environment-friendly processes is quite a promising area of research to develop the various food products. Although many important goals have been reached in achieving controlled release of food products, cost is the overriding factor that has hindered the introduction of sophisticated controlled release technologies in food products. We all are aware about the potential health benefits of nutraceuticals and probiotics. So the added value of nutraceutical ingredients justifies the additional cost of nanoencapsulation technology used to maintain the stability of these ingredients. The published literature indicates that in the near future, nanoparticle-based delivery systems will have more commercial status in the market than in the past. It seems these new technologies are viable and promising strategy for the food product industries and provoke various manufacturers to introduce nano-based ingredients into their food products and as a part of their marketing strategy. Nanoparticles can minimize some of these food products unique problems by safeguarding stability and preserving their safety, low cost, appeal (taste, odour, colour and texture), stability and nutritional value. The published literature indicates that in the near future, nanoparticles based delivery systems will have more commercial status in the market than in the past.

References

Altındal DC, Gümüşderelioglu M (2015) Melatonin releasing PLGA micro/nanoparticles and their effect on osteosarcoma cells. J Microencapsul 33(1):1–12
Amin AR, Kucuk O, Khuri FR, Shin DM (2009) Perspectives for cancer prevention with natural compounds. J Clin Oncol 27:2712–2725
Antônio E, Khalil NM, Mainardes RM (2016) Bovine serum albumin nanoparticles containing quercetin: characterization and antioxidant activity. J Nanosci Nanotechnol 16:1346–1353
Augustin MA, Hemar Y (2009) Nano- and micro-structured assemblies for encapsulation of food ingredients. Chem Soc Rev 38(4):902–912
Augustin MA, Sanguansri L (2012) Challenges in developing delivery systems for food additives, nutraceuticals, and dietary supplements. In: Garti N, McClements DJ (eds) Encapsulation technologies and delivery systems for food ingredients and nutraceuticals. Woodhead Publishing, Cambridge, UK, pp 19–48
Augustin MA, Abeywardena MY, Patten G, Head R et al (2011) Effects of microencapsulation on the gastrointestinal transit and tissue distribution of a bioactive mixture of fish oil, tributyrin and resveratrol. J Funct Foods 3:25–37
Bell LN (2001) Stability testing of nutraceuticals and functional foods. In: Wildman REC (ed) Handbook of nutraceuticals and functional foods. CRC Press, New York, pp 501–516
Bernela M, Ahuja M, Thakur R (2016a) Enhancement of anti-inflammatory activity of bromelain by its encapsulation in katira gum nanoparticles. Carbohydr Polym 143:18–24
Bernela M, Ahuja M, Thakur R (2016b) Enhancement of anti-inflammatory activity of glycyrrhizic acid by encapsulation in chitosan-katira gum nanoparticles. Eur J Pharm Biopharm 105:141–147
Blazevic F, Milekic T, Romic MD, Juretic M et al (2016) Nanoparticle-mediated interplay of chitosan and melatonin for improved wound epithelialisation. Carbohydr Polym 146:445–454
Bourbon AI, Cerqueira MA, Vicente AA (2016) Encapsulation and controlled release of bioactive compounds in lactoferrin-glycomacropeptide nanohydrogels: curcumin and caffeine as model compounds. J Food Eng 180:110–119
Bouwmeester H, Dekkers S, Noordam MY, Hagens WI et al (1998) Nutraceuticals: poised for a healthy slice of the healthcare market? Nat Biotechnol 16:728–731
Chaiyasan W, Srinivas SP, Tiyaboonchai W (2016) Development and characterization of topical ophthalmic formulations containing lutein-loaded mucoadhesive nanoparticles. Int J Pharm Pharm Sci 8(3):261–266
Das S, Chaudhury A (2010) Recent advances in lipid nanoparticle formulations with solid matrix for oral drug delivery. AAPS Pharm Sci Tech 12:62–76
Fakhoury IH, Saad WS, Gali-Muhtasib HU, Schneider-Stock R (2014) Thymoquinone nanoparticle formulation and in vitro efficacy in: materials for drug & gene delivery & cancer nanotech. Nanotech (2014) 2:367–370
Fakoor Yazdan Abad M, Rajabzadeh G, Taghvaei Ganjali S, Tavakoli R (2016) Preparing allicin nanocapsules and determining the factors controlling their particle size through artificial intelligence. Int J Food Eng 12(3):257–264
Fathi M, Varshosaz J, Mohebbi M, Shahidi F (2013) Hesperetin-loaded solid lipid nanoparticles and nanostructure lipid carriers for food fortification: preparation, characterization, and modeling. Food Bioprocess Technol 6:1464–1475
Ferrari M (2005) Cancer nanotechnology: opportunities and challenges. Nat Rev Cancer 5:161–171
Frede K, Henze A, Khalil M, Baldermann S, Schweigert FJ, Rawel H (2014) Stability and cellular uptake of lutein-loaded emulsions. J Funct Foods 8:118–127
Grabley S, Thiericke R (1999) Bioactive agents from natural sources: trends in discovery and application. Adv Biochem Eng Biotechnol 64:101–154
Guan X, Gao M, Xu H, Zhang C et al (2016) Quercetin-loaded poly (lactic-co-glycolic acid)-d-α-tocopheryl polyethylene glycol 1000 succinate nanoparticles for the targeted treatment of liver cancer. Drug Deliv 23(9):3307–3318

Hong DY, Lee J-S, Lee HG (2015) Chitosan/poly-*rmgamma*-glutamic acid nanoparticles improve the solubility of lutein. Int J Biol Macromol 85:9–15

Huang Q, Yu H, Ru Q (2013) Bioavailability and delivery of nutraceuticals using nanotechnology. J Food Sci 75:50–57

Javeri I (2016) Application of “nano” nutraceuticals in medicine. In: Nutraceuticals: efficacy, safety and toxicity. Elsevier, Amsterdam, pp 189–192

Joung HJ, Choi M-J, Kim JT, Park SH, Park HJ, Shin GH (2016) Development of food-grade curcumin nanoemulsion and its potential application to food beverage system: antioxidant property and in vitro digestion. J Food Sci 81:745–753

Jourghanian P, Ghaffari S, Ardjmand M, Haghighat S, Mohammadnejad M (2016) Sustained release curcumin loaded solid lipid nanoparticles. Advan Pharma Bulletin 6:17–21

Kalra EK (2003) Nutraceutical – definition and introduction. AAPS Pharm Sci 5:27–28

Kim JH, Park EY, Ha HK, Jo CM et al (2016) Resveratrol-loaded nanoparticles induce antioxidant activity against oxidative stress. Asian-Aust J Anim Sci 29(2):288–298

Kosaraju SL (2005) Colon targeted delivery systems: review of polysaccharides for encapsulation and delivery. Crit Rev Food Sci Nutr 45(4):251–258

Krausz AE, Adler BL, Cabral V, Navati M et al (2015) Curcumin-encapsulated nanoparticles as innovative antimicrobial and wound healing agent. Nanomed: Nanotechnol Biol Med 11(1):195–206

Langer R (1990) New methods of drug delivery. Science 249:1527–1533

Lasic DD (1998) Novel applications of liposomes. Trends Biotechnol 16:307–321

Lasic DD, Martin FJ, Gabizon A, Huang SK, Papahadjopoulos D (1991) Sterically stabilized liposomes: a hypothesis on the molecular origin of the extended circulation times. Biochim Biophys Acta 1070:187–192

Leach AR, Gillet VJ, Lewis RA, Taylor R (2010) Three-dimensional pharmacophore methods in drug discovery. J Med Chem 53:539–558

Li Y, Xiao H, McClements DJ (2012) Encapsulation and delivery of crystalline hydrophobic nutraceuticals using nanoemulsions: factors affecting polymethoxyflavone solubility. Food Biophys 7(4):341–353

Li C, Zhang X, Huang X, Wang X, Liao G, Chen Z (2013) Preparation and characterization of flexible nanoliposomes loaded with daptomycin, a novel antibiotic for topical skin therapy. Int J Nanomedicine 8:1285–1292

Livney YD (2008) Complexes and conjugates of biopolymers for delivery of bioactive ingredients via food. In: Delivery and controlled release of bioactives in foods and nutraceuticals, Woodhead Publishing series in Food Science, Technology and Nutrition. Woodhead, Cambridge, pp 234–242

Manea A-M, Vasile BS, Meghea A (2014) Antioxidant and antimicrobial activities of green tea extract loaded into nanostructured lipid carriers. C R Chim 17:331–341

McClements DJ, Decker EA, Park Y, Weiss J (2009) Structural design principles for delivery of bioactive components in nutraceuticals and functional foods. Crit Rev Food Sci Nutr 49(6):577–606

McClements DJ (2012a) Advances in fabrication of emulsions with enhanced functionality using structural design principles. Curr Opin Colloid Interface Sci 17(5):235–245

McClements DJ (2012b) Requirements for food ingredient and nutraceutical delivery systems. In: Encapsulation technologies and delivery systems for food ingredients and nutraceuticals. Woodhead Publishing, Cambridge, pp 3–18

McClements DJ (2012c) Nanoemulsions versus microemulsions: clarification of differences, similarities and terminology. Soft Matter 8(6):1719–1729

Molinski TF (1993) Developments in marine natural products, receptor-specific bioactive compounds. J Nat Prod 56:1–8

Momekova D, Rangelov S, Yanev S, Nikolova E et al (2007) Long-circulating, pH-sensitive liposomes sterically stabilized by copolymers bearing short blocks of lipid-mimetic units. Eur J Pharm Sci 32:308–317

Mozafari MR, Mortazavi SM (2005) Nanoliposomes: from fundamentals to recent developments. Trafford, Pub Ltd, Oxford. UK
Mozafari MR, Flanagan J, Matia-Merino L et al (2006) Recent trends in the lipid-based nanoencapsulation of antioxidants and their role in foods. J Sci Food Agric 86(13):2038–2045
Mozafari MR, Pardakhty A, Azarmi S, Jazayeri JA et al (2009) Role of nanocarrier systems in cancer nanotherapy. J Liposome Res 19:310–321
Munin A, Edwards-Levy F (2011) Encapsulation of natural polyphenolic compounds: a review. Pharmaceutics 3:793–829
Neves AR, Martins S, Segundo MA, Reis S (2016) Nanoscale delivery of resveratrol towards enhancement of supplements and nutraceuticals. Forum Nutr 8:131
Pandey M, Verma RK, Saraf SA (2010) Nutraceuticals: new era of medicine and health. Asian J Pharm Clin Res 3:11–15
Paul SD, Dewangan D (2016) Nanotechnology and nutraceuticals. Int J Nanomater Nanotechnol Nanomed 2(1):9–12
Pradhan N, Singh S, Ojha N, Shrivastava A et al (2015) Facets of nanotechnology as seen in food processing, packaging, and preservation industry. Bio Med Res Int 2015:365672
Puglia C, Offerta A, Tirendi GG, Tarico MS et al (2016) Design of solid lipid nanoparticles for caffeine topical administration. Drug Deliv 23(1):36–40
Qi LF, Xu ZR, Jiang X, Hu C, Zou X (2004) Preparation and antibacterial activity of chitosan nanoparticles. Carbohydr Res 339(16):2693–2700
Qian C, Decker EA, Xiao H, McClements DJ (2012) Nanoemulsion delivery systems: influence of carrier oil on beta-carotene bioaccessibility. Food Chem 135:1440–1447
Rani R, Dilbaghi N, Dhingra D, Kumar S (2015) Optimization and evaluation of bioactive drug-loaded polymeric nanoparticles for drug delivery. Int J Biol Macromol 78:173–179
Rapaka RS, Coates PM (2006) Dietary supplements and related products: a brief summary. Life Sci 78:2026–2032
Ravanfar R, Tamaddon AM, Niakousari M, Moein MR (2016) Preservation of anthocyanins in solid lipid nanoparticles: optimization of a microemulsion dilution method using the Plackett–Burman and Box–Behnken designs. Food Chem 199(15):573–580
Rein MJ, Renouf M, Cruz-Hernandez C, Actis-Goretta L, Thakkar SK, da Silva MP (2013) Bioavailability of bioactive food compounds: a challenging journey to bioefficacy. Br J Clin Pharmacol 75:588–602
Renard D, Robert P, Lavenant L, Melcion D et al (2002) Biopolymeric colloidal carriers for encapsulation or controlled release applications. Int J Pharm 242(1–2):163–166
Riehemann K, Schneider SW, Luger TA, Godin B (2009) Nanomedicine-challenge and perspectives. Angew Chem Int Ed Eng 48:872–897
Scampicchio M, Ballabio D, Arecchi A, Cosio SM, Mannino S (2008) Amperometric electronic tongue for food analysis. Microchim Acta 163:11–21
Severino P, Andreani T, Macedo AS, Fangueiro JF et al (2011) Current state-of-art and new trends on lipid nanoparticles (SLN and NLC) for oral drug delivery. J Drug Deliv 12:1–10
Shoji Y, Nakashima H (2004) Nutraceutics and delivery systems. J Drug Target 12:385–391
Singhal NK, Agarwal S, Bhatnagar P, Tiwari MN et al (2015) Mechanism of nanotization-mediated improvement in the efficacy of caffeine against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced parkinsonism. J Biomed Nanotechnol 11(12):2211–2222
Sinha VR, Kumria R (2001) Polysaccharides in colon-specific drug delivery. Int J Pharm 224:19–38
Sivakumar M, Tang SY, Tan KW (2014) Cavitation technology – a greener processing technique for the generation of pharmaceutical nanoemulsions. Ultrason Sonochem 21:2069–2083
Solans C, Izquierdo P, Nolla J, Azemar N, Garcia-Celma MJ (2005) Nanoemulsions. Curr Opin Colloid Interface Sci 10(3–4):102–110
Sondi I, Salopek-Sondi B (2004) Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gramnegative bacteria. J Colloid Interface Sci 275(1):177–182
Sun Y, Xia Z, Zheng J, Qiu P, Zhang L, McClements DJ, Xiao H (2015) Nanoemulsion-based delivery systems for nutraceuticals: influence of carrier oil type on bioavailability of pterostilbene. J Funct Foods 13:61–70

Tang S, Gao D, Zhao T, Zhou J, Zhao X (2013) An evaluation of the anti-tumor efficacy of oleanolic acid-loaded PEGylated liposomes. Nanotechnology 24:235102

Taylor TM, Davidson PM, Bruce BD, Weiss J (2005) Liposomal nanocapsules in food science and agriculture. Crit Rev Food Sci Nutr 45(7–8):587–605

Tiede K, Boxall ABA, Tear SP, Lewis J, David H, Hassellov M (2008) Detection and characterization of engineered nanoparticles in food and the environment. Food Addit Contam Part A-Chem Anal Control Expo Risk Assess 25(7):795–821

Ting YW, Jiang Y, Ho CT, Huang QR (2014) Common delivery systems for enhancing in vivo bioavailability and biological efficacy of nutraceuticals. J Funct Foods 7:112–128

Trottier G, Boström PJ, Lawrentschuk N, Fleshner NE (2010) Nutraceuticals and prostate cancer prevention. A Curr Rev Nat Rev Urol 7:21–30

Vidal SL, Rojas C, Padin RB, Rivera MP, Haensgen A, Gonzalez M, Rodriguez-Llamazares S (2016) Synthesis and characterization of polyhydroxybutyrate-cohydroxyvalerate nanoparticles for encapsulation of quercetin. J Bioact Compat Polym 31(5):1–14

Vijayakumar MR, Kumari L, Patel KK, Vuddanda PR et al (2016) Intravenous administration of trans-resveratrol-loaded TPGS-coated solid lipid nanoparticles for prolonged systemic circulation, passive brain targeting and improved in vitro cytotoxicity against C6 glioma cell lines. RSC Adv 6:50336–50348

Wagner V, Dullaart A, Bock AK, Zweck A (2006) The emerging nanomedicine landscape. Nat Biotechnol 24:1211–1217

Weber S, Zimmer A, Pardeike J (2014) Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) for pulmonary application: a review of the state of the art. Eur J Pharma Biopharm 86:7–22

Yu H, Huang Q (2013) Bioavailability and delivery of nutraceuticals and functional foods using nanotechnology. In: Di B, Bagchi M, Moriyama H, Shahidi F (eds) Bio-nanotechnology: a revolution in food, biomedical and health sciences, 1st edn. Blackwell Publishing Ltd., Oxford. https://doi.org/10.1002/9781118451915. ch35

Zheng J, Li Y, Song M, Fang X et al (2014) Improving intracellular uptake of 5-demethyltangeretin by food grade nanoemulsions. Food Res Int 62:98–103

Zhu Y, Peng W, Zhang J, Wang M et al (2014) Enhanced oral bioavailability of capsaicin in mixed polymeric micelles: preparation, in vitro and in vivo evaluation. J Funct Foods 8:358–366

Chitosan Nanoparticles as Carrier for Anticancer Drugs: An Overview

8

Raj Kumar Salar and Naresh Kumar

8.1 Introduction

Today cancer is the major cause of morbidity and mortality in Western and industrialized countries (De Rosa et al. 2013). Cancer is a condition of the cell in which the normal mechanisms of cell growth and proliferation are disturbed (Shakeri-Zadeh et al. 2013). The cancerous cells show no response to antiproliferative signals, and growth becomes abnormal. These cells invade and destroy nearby tissues and also invade into other parts of the body by lymphatic or circulatory system. A total of 1,660,290 new cancer cases and 580,350 cancer deaths were expected in the United States in 2013 (Siegel et al. 2013). Various treatment methods have been developed for cancer, such as chemotherapy and radiotherapy. Chemotherapy is an important therapeutic option for different malignancies, especially for primary, advanced, and metastatic tumors (Rebucci and Michiels 2013). But the major disadvantage of chemotherapy is the destruction of normal cells of the body along with cancer cells (Shakeri-Zadeh et al. 2013). Thoracic radiotherapy is administered to patients with lung cancer, but it also has toxic effects. Nanoparticle-based drug delivery systems provide newer approaches for cancer treatment. Drug delivery systems based on nanoparticles include organic and inorganic materials, liposomal, polymer-encapsulating drug conjugates, and micellar formulations (Alexis et al. 2010).

Among various polymeric nanoparticles, chitosan and chitosan derivatives have been used for targeted drug delivery against different types of cancers. Chitosan nanoparticles have been used as carriers of different anticancer drugs and are biocompatible and biodegradable. Most importantly, they can be readily modified which help in their release of drug inside the body (Wang et al. 2011). Chitosan nanoparticles can be taken up by the endosomes and allow drug to overcome the permeability barrier by the epithelium of cancer tissue (Pan et al. 2002; Sakuma et al. 2001). Additionally, chitosan nanoparticles provide protection against

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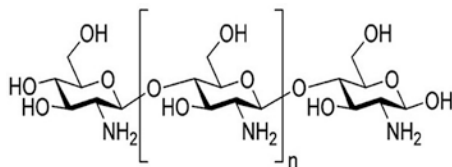


Fig. 8.1 Chemical structure of chitosan. (Adapted from Mahapatro and Singh 2011)

enzymatic degradation and ensure that encapsulated drugs or genes can be delivered at target sites (Mao et al. 2001; Ozbas-Turan et al. 2003).

Chitosan has unique physicochemical and biological properties that make it ideal as an anticancer drug carrier (Patel et al. 2008). Chitosan [β-1–4-linked polymer of 2-amino-2-deoxy-β-D-glucose] (Fig. 8.1) is prepared by the N-deacetylation of chitin, the second most abundant natural biopolymer after cellulose (Jari et al. 2004). Chitosan has cationic characteristics and reaction with polyanions giving rise to polyelectrolyte complexes. Its cationic character also provides unique properties of controlled drug release, mucoadhesion, and efflux pump inhibitory properties with β-(1,4)-linked-D-glucosamine residue with the amine groups randomly acetylated (Sevda and McClureb 2004). Amine and hydroxyl groups provide chitosan distinctive properties, which make it applicable in several areas. Moreover, chitosan is safe and nontoxic and, with polyanions, forms complexes and gels (Sunil et al. 2004). Physical and chemical properties of chitosan depend mainly on the molecular weight and degree of deacetylation (Jin and Hu 2008). Chitosan nanoparticles have the general characteristics of chitosan itself and also have nanomaterial characteristics such as the small size effect, surface and interface effect, and quantum size effects (Shi et al. 2005). Chitosan and its derivatives have been tried against different types of cancer to improve their safety and efficacy. Anticancer drugs such as doxorubicin (Mitra et al. 2001), 5-flourouracil (Anitha et al. 2012), paclitaxel, (Huang et al. 2014), etc. have been studied in vitro against different cancer cell lines and have shown promising results. Based on these studies, chitosan is considered as a good candidate for site-specific drug delivery (Kas 1997).

This chapter provides an overview of the current research activities on various methods of characterization, synthesis, and characteristics of chitosan nanoparticles as an anticancer drug carrier. Further, current applications of chitosan nanoparticles in various biomedical fields are discussed.

8.2 Characterization of Nanoparticles

Nanoparticles have a diameter between 1 and 1000 nm and have unique physicochemical, optical, and electrical properties. There are several techniques for characterization of chitosan nanoparticles such as UV-visible spectrophotometry (UV-Vis), dynamic light scattering (DLS), Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), scanning electron microscopy (SEM), and transmission electron microscopy (TEM).

8.2.1 UV-Visible Spectrophotometry (UV-Vis)

UV-visible spectrophotometry is the most prevalent technique used for characterization of nanoparticles (Salar et al. 2015; Zimbone et al. 2011; Bhui et al. 2009; Hao et al. 2004; Creighton and Eadon 1991). Researchers use this technique for determination of encapsulation efficiency and percent loading of anticancer drugs in chitosan nanoparticles. This technique shows characteristic peaks at a particular wavelength. Shi et al. (2012) determined 83.7% encapsulation efficiency of paclitaxel in glycyrrhizin-modified O-carboxymethyl chitosan nanoparticles against hepatocellular carcinoma at 227 nm. Maximum encapsulation efficiency (71.8 ± 8.8%) of doxorubicin at 480 nm in bovine serum albumin (BSA)-dextran-chitosan nanoparticles has been determined (Qi et al. 2010). Encapsulation efficiency (63.41 ± 0.65%) of methotrexate in pH-sensitive chitosan nanoparticles at 306 nm has been reported (Nogueira et al. 2013). Spectrophotometric absorption measurements in the wavelength of 285 nm have been used in the characterization of catechin-loaded chitosan nanoparticles (Manikkam and Pitchai 2014). Chitosan conjugated with other compounds have also been characterized by this technique. Capecitabine-loaded polycaprolactone-chitosan nanospheres have been analyzed spectrophotometrically at 240 nm.

8.2.2 Dynamic Light Scattering (DLS)

Cellular uptake of nanoparticles is usually a prerequisite for targeted drug delivery and is governed by size and surface characteristics such as hydrophobicity and charge (Fröhlich 2012). The dynamic light scattering is used to characterize the surface charge and size distribution of the nanoparticles suspended in liquid (Jiang et al. 2009). This technique was used in 1972 first time for measurement of particle size (Lee et al. 1972). Particle size measurement (77.14 nm) and surface charge (Zeta potential, +5.17 mV) determination of folic acid-conjugated chitosan nanoparticles by DLS are shown in Figs. 8.2 and 8.3, respectively. Curcumin-loaded dextran sulfate-chitosan nanoparticles showed average size of 200–220 nm and zeta potential value of −30 mV using DLS (Anitha et al. 2011). Snima et al. (2012) determined the zeta potential (−18.32 mV) at pH 7.4 and size (230 ± 50 nm) of metformin-loaded O-carboxymethyl chitosan (O-CMC) nanoparticles. Koo et al. (2013) determined the zeta-potential value of paclitaxel-loaded glycol chitosan nanoparticles with hydrotropic oligomers of 13.09 ± 0.42 mV in phosphate-buffered saline (pH 7.4).

Paclitaxel was loaded in different ratios, and average particle size of glycol chitosan nanoparticles with hydrotropic paclitaxel-loaded glycol chitosan nanoparticles with hydrotropic oligomers 10 wt. % was 28 ± 18 nm, 20 wt. % was 343 ± 12 nm, and 30 wt. % was 358 ± 21 nm. Duan et al. (2010) determined the size and zeta potential of curcumin-loaded chitosan/poly(butyl cyanoacrylate) nanoparticles of about 200 nm and +29.11 mV, respectively, whereas the empty poly(butyl

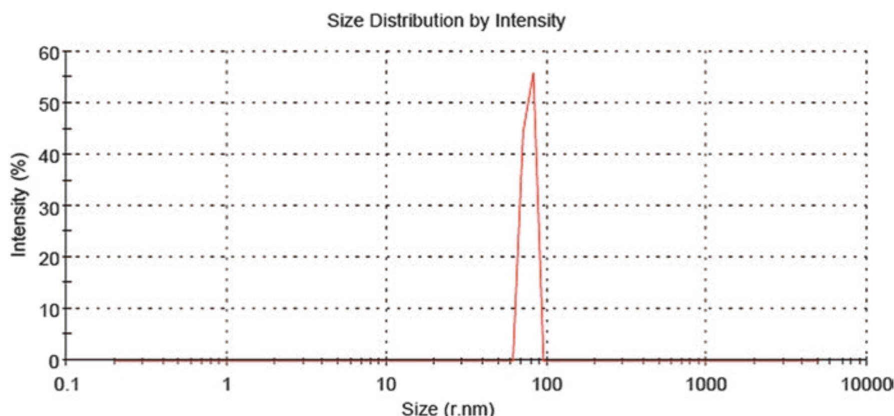


Fig. 8.2 Particle size measurement of folic acid-conjugated chitosan nanoparticles by DLS

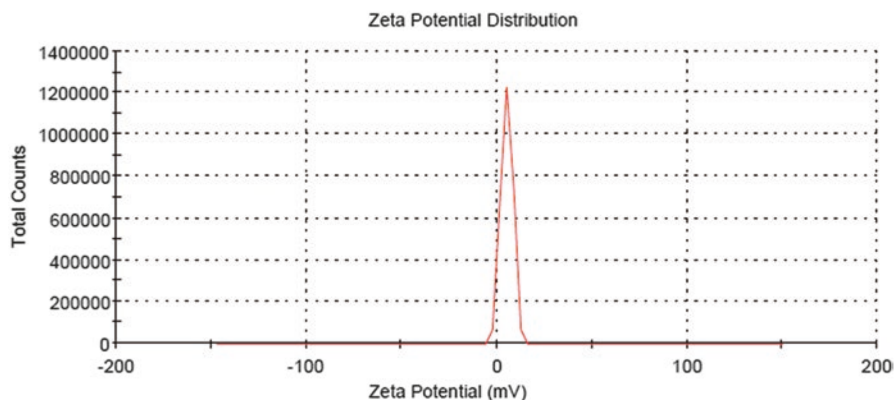


Fig. 8.3 Surface charge (zeta potential) determination of folic acid-conjugated chitosan nanoparticles by DLS

cyanoacrylate) nanoparticles coated with chitosan had a little positive charge of 1.46 ± 0.03 mV on the surface.

8.2.3 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR is used to determine the nature of the chemical surface species as well as the reactive sites (Baraton et al. 1996). It is commonly used for determination of functional groups and interactions of anticancer drug with chitosan. Results are obtained in the form of characteristic peaks for different functional groups. FTIR spectrum of folic acid-conjugated chitosan nanoparticles is shown in Fig. 8.4.

Duan et al. (2012) recorded FTIR spectra of doxorubicin-loaded chitosan-coated poly(butyl cyanoacrylate), pure folic acid, and folic acid-modified doxorubicin

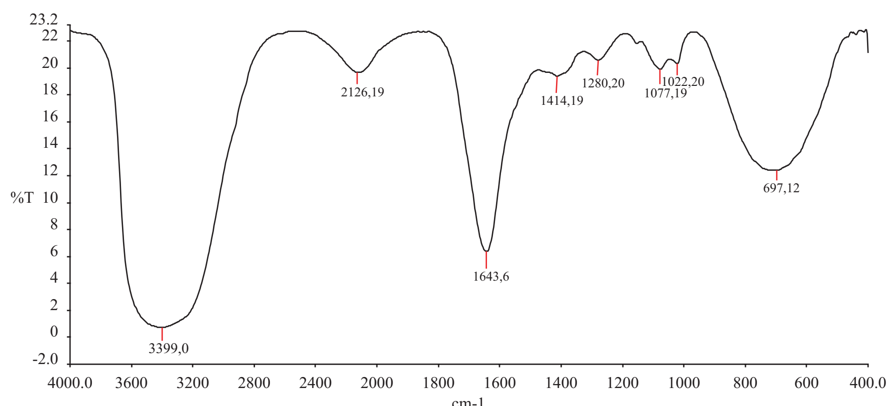


Fig. 8.4 FTIR spectroscopic image of folic acid-conjugated chitosan nanoparticles

poly(butyl cyanoacrylate) nanoparticles. The IR spectrum for pure folic acid was characterized by a number of characteristic bands at a wave length of 3413.94, 1608.02, 1480.77, and 1196.92 cm^{-1}. The bands between 3600 and 3000 cm^{-1} were due to the hydroxyl (OH) stretching and NH stretching vibration bands. The band at 1608.02 cm^{-1} was related to the bending mode of NH vibration, and the band at 1480.77 cm^{-1} was attributed to a characteristic absorption band of the phenyl ring. The residue still possessed a –CN stretch in the blank poly(butyl cyanoacrylate) nanoparticles (2252.18 cm^{-1}), doxorubicin-poly(butyl cyanoacrylate) nanoparticles (2252.18 cm^{-1}), and folic acid-modified doxorubicin-poly(butyl cyanoacrylate) nanoparticles (2247.08 cm^{-1}). The wavelength 1624.90 cm^{-1} in the blank poly(butyl cyanoacrylate) and doxorubicin-poly(butyl cyanoacrylate) nanoparticles represented the amide band in the chitosan. The band at 1650.40 cm^{-1} belonged to the C=O bond stretching vibration of $–CONH_2$ group. Significant peak broadening at 1650.40 cm^{-1} was also attributable to the amide linkage formed between the chitosan and folate. This demonstrated the successful folic acid conjugation on the surface of the doxorubicin-poly(butyl cyanoacrylate) nanoparticles. Wang et al. (2015) observed the combination of folic acid and chitosan using FTIR while loading mitoxantrone. Folic acid-chitosan conjugate had the characteristic peak of benzene (1606 cm^{-1}) due to folic acid group; while compared with that of IR spectrum of chitosan and folic acid, the absorption peak of chitosan $-NH_2$ at 1640 $cm-^{1}$ and the absorption peak of folate −COOH at 1692 $cm-^{1}$ disappeared. The results indicated that −COOH of folic acid reacted with $-NH_2$ of chitosan on the formation of the amide bond between the carboxyl groups, and FA-CS was obtained.

8.2.4 X-Ray Diffraction (XRD)

X-ray diffraction is used for phase identification and characterization of the crystal structure of the nanoparticles (Sun et al. 2000). Shi et al. (2014) determined the XRD patterns of chitosan, deoxycholic acid, chitosan-deoxycholic acid,

methoxypoly(ethylene glycol), chitosan-deoxycholic acid-methoxypoly(ethylene glycol), folic acid, and chitosan-deoxycholic acid-methoxypoly(ethylene glycol)-folic acid while synthesizing nanoparticles of deoxycholic acid, polyethylene glycol, and folic acid-modified chitosan for targeted delivery of anticancer drug, doxorubicin. A strong peak in the diffractogram of chitosan appears at 2θ values of 20°, indicating the crystalline nature of chitosan. Intense peaks were also observed in the scattering pattern of deoxycholic acid, methoxypoly(ethylene glycol), and folic acid. For chitosan-deoxycholic acid, chitosan-deoxycholic acid-methoxypoly(ethylene glycol), and chitosan-deoxycholic acid-methoxypoly(ethylene glycol)-folic acid conjugates, only a slight hump peak was observed at 2θ = 20°, which indicated that crystalline structure of chitosan was greatly destroyed. From these results, it can be concluded that the deoxycholic acid, methoxypoly(ethylene glycol), and folic acid were successfully coupled to the chitosan.

Ji et al. (2012b) determined powder X-ray diffraction patterns of chitosan, folic acid-chitosan conjugate, methotrexate, and methotrexate-loaded folic acid-chitosan-conjugated nanoparticles. There were two strong peaks in the diffractogram of chitosan at 2θ values of 11° and 20°, and several strong peaks in the diffractogram of methotrexate indicated the high degree of crystallinity of chitosan and methotrexate. They noted that the peaks at 2θ = 11°and 20° decreased greatly in the newly prepared polymer folic acid-chitosan conjugate as the crystalline nature was found to decrease than that of chitosan itself due to the deformation of the strong hydrogen bonds between chitosan and folic acid. No peak was found in the diffractogram of the methotrexate-loaded folic acid-chitosan-conjugated nanoparticles. X-ray diffractogram of the folic acid-chitosan-conjugated nanoparticles was characteristic of an amorphous polymer. They concluded that the nanoparticles were composed of a dense network structure of interpenetrating polymer chains cross-linked to one another by sodium tripolyphosphate counterions. In addition, the characteristic peaks of methotrexate disappeared in those corresponding to drug-loaded nanoparticles. It indicated that methotrexate might exist as molecular dispersion in the polymeric nanoparticles.

8.2.5 Scanning Electron Microscopy (SEM)

Scanning electron microscopy is used for determination of surface morphology and size of nanoparticles. Chitosan nanoparticles synthesized by the ionic gelation method have spherical structures with varying sizes (187.14–556.34 nm) as shown in Fig. 8.5. Anitha et al. (2011) determined smooth surfaced with spherical morphology of the curcumin-loaded dextran sulfate-chitosan nanoparticles by SEM. Cheng et al. (2012) observed smooth-surfaced spherical-shaped 5-fluorouracil-loaded galactosylated chitosan nanoparticles with no adhesion between nanoparticles. Spherical-shaped methotrexate-loaded folic acid-conjugated O-carboxymethyl chitosan nanoparticles have been reported (Ji et al. 2012a).

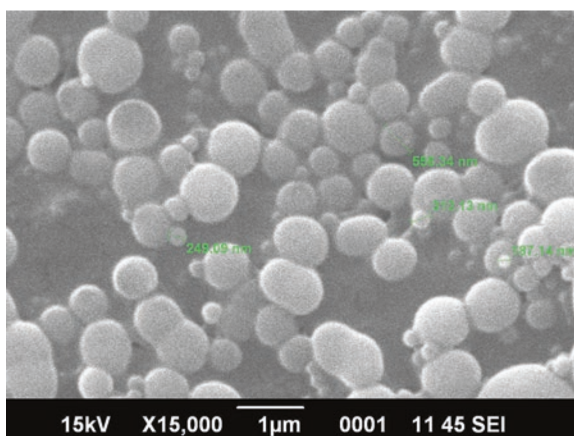


Fig. 8.5 Scanning electron microscopic image of blank folic acid-conjugated chitosan nanoparticles

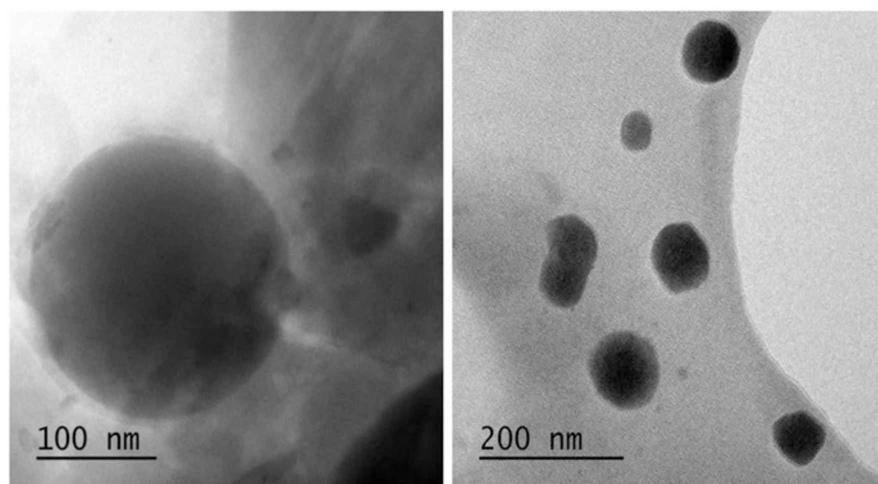


Fig. 8.6 Transmission electron microscopic images of blank folic acid-conjugated chitosan nanoparticles

8.2.6 Transmission Electron Microscopy (TEM)

Transmission electron microscopy shows a 1000-fold higher resolution compared with the SEM and is mainly used for determination of size of nanoparticles (Eppler et al. 2000). Figure 8.6 shows spherical images of different sized chitosan nanoparticles (50–200 nm) in transmission electron microscope. Min et al. (2008) determined the spherical-shaped camptothecin-loaded hydrophobically modified glycol chitosan nanoparticles using transmission electron microscopy while treating human breast cancer cell line (MDA-MB231). Uniform geometrical-shaped 5-fluorouracil-loaded ferrocene-modified chitosan oligosaccharide (FcCOS) nanoparticles were observed by TEM (Xu et al. 2014). Duan et al. (2010) reported

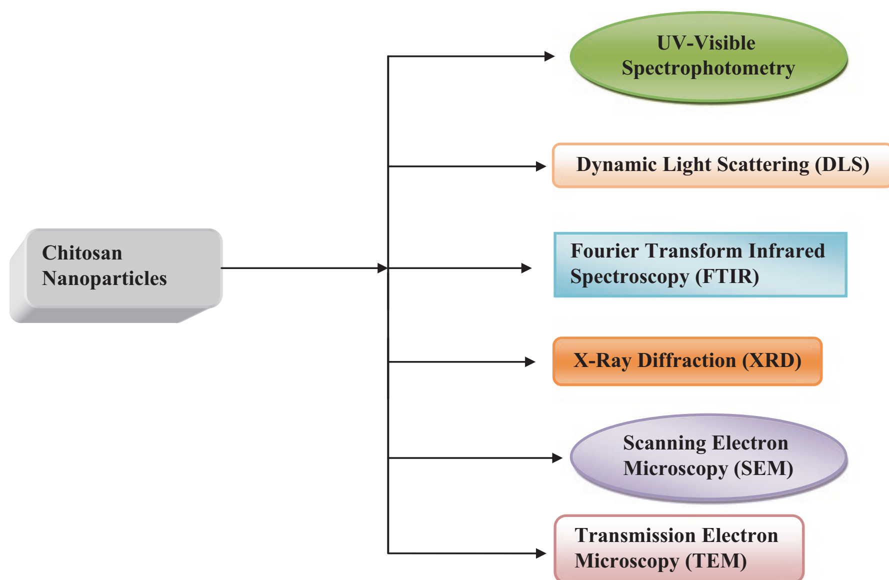


Fig. 8.7 Techniques for characterization of chitosan nanoparticles

well-separated, spherical-shaped curcumin-loaded chitosan/poly(butyl cyanoacrylate) nanoparticles with smaller than 250 nm in size by TEM. Termsarasab et al. (2013) observed spherical-shaped doxorubicin-loaded chitosan oligosaccharide-arachidic acid-based nanoparticles against head and neck cancer cell line (FaDu). Li et al. (2009) observed smoot-surfaced, spherical-shaped paclitaxel-loaded chitosan nanoparticles without aggregation. A schematic representation of various techniques of characterization of nanoparticles is presented in Fig. 8.7.

8.3 Synthesis of Chitosan Nanoparticles

There are different methods of preparation of anticancer drug-loaded chitosan nanoparticles, i.e., ionic gelation, emulsion polymerization, nanoprecipitation, dialysis, and covalent cross-linking method. These are discussed as follows.

8.3.1 Ionic Gelation Method

Ionic gelation is most widely used method for preparation of chitosan nanoparticles. In this method, a stock solution of chitosan is prepared by dissolving chitosan in varying concentration of dilute acetic acid in the presence of cross-linking agent such as sodium tripolyphosphate. Sodium tripolyphosphate is added to a solution of chitosan with continuous stirring under a suitable temperature. The positive charge of chitosan and negative charge of sodium tripolyphosphate combine to form

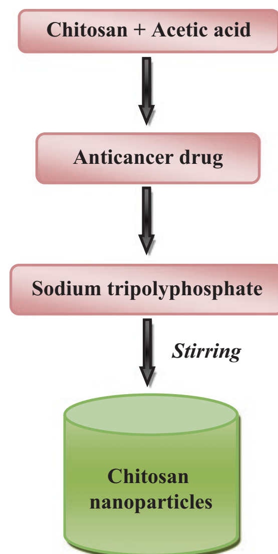


Fig. 8.8 Ionic gelation method

nanoparticles (Amidi et al. 2010) as shown in Fig. 8.8. This method was first reported by Calvo et al. (1997) for preparation of novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein bovine serum albumin as carrier.

With the attachment of a number of ligands like folic acid, chitosan nanoparticles prepared by ionic gelation carrying anticancer drug can be made target specific. Song et al. (2013) prepared folic acid-conjugated chitosan nanoparticles loaded with doxorubicin hydrochloride for improving targeted drug delivery to tumor. Compared to unmodified chitosan nanoparticles, folate-chitosan nanoparticles conjugated with acid showed much higher cell uptake ability in vitro against human liver cancer SMMC-7221 cell line due to folate receptor-mediated endocytosis. Trapani et al. (2011) evaluated methotrexate-loaded chitosan or modified glycol chitosan nanoparticles in the presence and absence of Tween 80 for brain delivery to cross MDCKII-MDR1 monolayer and to check cytotoxicity studies against C6 glioma cell line. These particles were reported to be cytotoxic against C6 cells line and were able to cross the MDCKII-MDR1 cell barrier. It was also concluded that low concentration of Tween 80 could be used for enhancing the transport of methotrexate from the chitosan nanoparticles across MDCKII-MDR1 cells. Sahoo et al. (2012) synthesized chitosan nanoparticles loaded with paclitaxel with different concentrations for controlled release and in vitro studied different parameters such as changing time, pH, and drug concentrations. Debnath et al. (2010) prepared cytarabine loaded with chitosan nanoparticles and also studied their in vivo biodistribution. Results showed that the nanoparticles loaded with cytarabine had better distribution as compared to free drug in different organs such as the spleen, lungs, kidney, etc.

Chitosan nanoparticles loaded with anticancer drugs have also been studied to check in vitro behavior of anticancer drugs prepared by the ionic gelation method. In vitro release study helps to predict release pattern of anticancer drugs over a

period of time. Prameela Rani et al. (2014) studied in vitro release of anastrozole-loaded chitosan nanoparticles with different formulations. Similarly, Kumari et al. (2013) studied percentage drug release of methotrexate in sodium alginate-chitosan nanoparticles in vitro, and the results indicated that sodium alginate-chitosan-loaded nanoparticles could be used for controlled release of methotrexate for prolonged time. Stability of nanoparticles is a very important parameter. Chitosan nanoparticles loaded with anticancer drugs have also been studied for their stability. Othayoth et al. (2013) studied tamoxifen-loaded chitosan-pluronic nanoparticles for blood compatibility and found good results.

8.3.2 Emulsion Polymerization Method

Though ionic gelation method is most common, emulsion polymerization method has also been used for synthesis of anticancer drug-loaded chitosan nanoparticles. This method can be divided into two categories, i.e., organic and aqueous continuous phase. The organic phase is not used nowadays because this method requires toxic surfactants and solvents like n-pentane and toluene. In aqueous continuous phase, the monomer is dissolved in an aqueous solution with addition of surfactants and emulsifiers as shown in Fig. 8.9. Initiation of nanoparticles synthesis occurs when dissolved monomer molecule collides with initiator molecules which could be an ion or a free radical. When initiated monomer ions or monomer radicals collide with other monomer molecules, chain growth starts according to anionic polymerization mechanism (Reis et al. 2006). Anticancer drugs have been encapsulated in chitosan nanoparticles and tested in vitro and in vivo against cancer cell lines.

Saremi et al. (2011) loaded docetaxel into thiolated spherical chitosan nanoparticles using cerium ammonium nitrate as an initiator, and their effect on cancer cell lines against Caco-2 (heterogeneous human epithelial colorectal adenocarcinoma cell) and MCF-7 (human breast cancer cell line) was evaluated in vitro and ex vivo. Results showed that if thiolated polymers and colloidal particles were combined, it can be used as a better drug carrier system for mucosal delivery of hydrophobic drugs. Saremi et al. (2013) also reported enhanced oral delivery of the docetaxel using core of polymethyl methacrylate surrounded by a shell of thiolated chitosan against Caco-2 (heterogeneous human epithelial colorectal adenocarcinoma cell). Docetaxel like other taxans has been reported to have low oral absorption from gastrointestinal (GI) tract (Kuppens et al. 2005). Results showed that docetaxel-loaded nanoparticles had more permeation across the Caco-2 cell monolayer in comparison to the docetaxel alone and could be used for development of an oral drug delivery system for anticancer drugs.

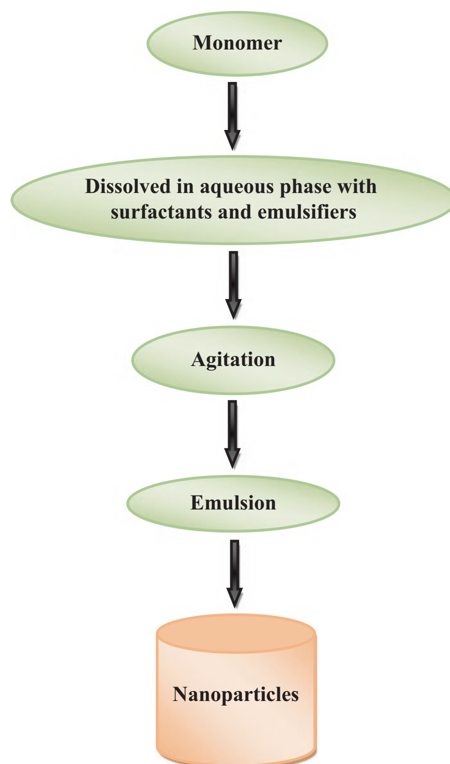


Fig. 8.9 Emulsion polymerization method (aqueous)

8.3.3 Nanoprecipitation Method

This method is used for encapsulation of hydrophobic and hydrophilic drugs. There are two approaches for nanoparticles preparation by this method: desolvation and diffusion of emulsified solvent. In first method, sodium sulfate as flocculant is added to a water solution of chitosan. As a result of this, solubility of chitosan is decreased by the combination of water and sodium sulfate, which leads to precipitation of nanoparticles due to hydrogen bonding between molecules (Berthold et al. 1996) as shown in Fig. 8.10. In the second method, chitosan in the water phase under the action of emulsified solvent is dispersed in the organic phase, which then encapsulates the drug. Appearance of turbulence between the interfaces of the two phases indicates precipitation of chitosan and production of nanoparticles (El-Shabouri 2002) as shown in Fig. 8.11. Using different molar masses, concentrations of chitosan, and triblock surfactant poloxamer, chitosan containing curcumin decorated with polycaprolactone nanoparticles has been tested for buccal delivery (Mazzarino et al. 2012). Luque-Alcaraz et al. (2012) prepared nobiletin-loaded chitosan nanoparticles and tested in vitro against cancerous cells, i.e., RAW

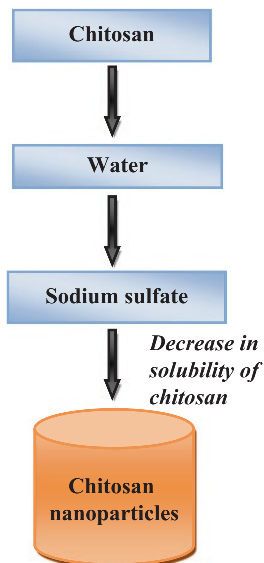


Fig. 8.10 Nanoprecipitation method (desolvation)

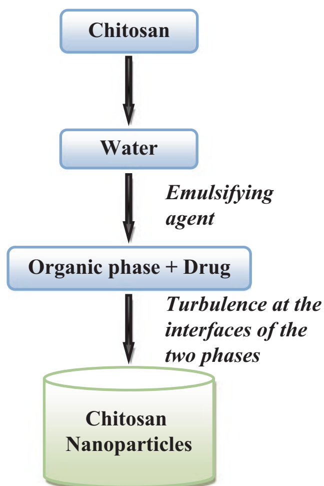
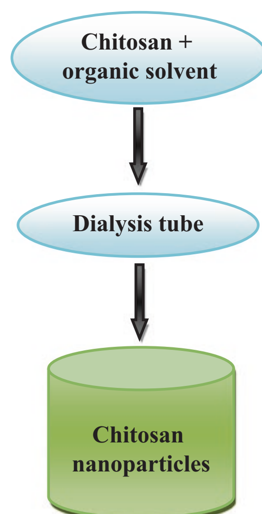


Fig. 8.11 Nanoprecipitation method (diffusion of emulsified solvent)

Fig. 8.12 Dialysis method



264.7 (Abelson murine leukemia virus-induced tumor) and L-929 (normal subcutaneous connective tissue) cell line. Nobiletin-loaded chitosan nanoparticles had shown considerable effect on these cancerous cells and could be used for applications in cancer chemotherapy.

8.3.4 Dialysis Method

In this method, chitosan is dissolved in a suitable organic solvent like acetic acid. This solution is then placed in a dialysis tube of proper molecular weight cutoff. Dialysis is then performed, resulting in displacement of solvent in dialysis tube and the formation of nanoparticles due to loss of solubility of polymer as shown in Fig. 8.12. Wang et al. (2008) produced self-assembled cholesterol-modified O-carboxymethyl chitosan nanoparticles for delivery of paclitaxel. The biodistribution pattern of paclitaxel-loaded cholesterol-modified O-carboxymethyl chitosan-6.9 self-assembled nanoparticles was also studied in female BALB/c mice. Compared with paclitaxel in the solution of Cremophor EL (polyethoxylated castor oil)/ethanol (PTX-Cre), cholesterol-modified O-carboxymethyl chitosan-6.9 self-assembled nanoparticles significantly increased the uptake of paclitaxel in the liver, spleen, and plasma, but there was decrease in the uptake in the heart and kidney, which might be helpful in enhancing the therapeutic index and reducing the toxicity

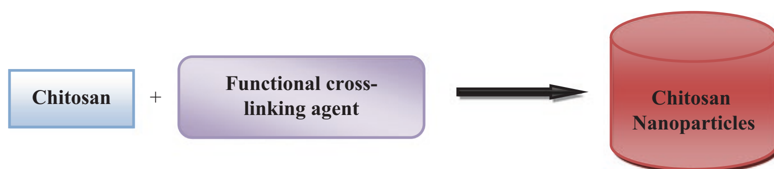


Fig. 8.13 Cross-linking method

of paclitaxel (Wang et al. 2008). Qiu et al. (2013) synthesized self-assembled phytosterol-fructose-chitosan nanoparticles encapsulated with doxorubicin and reported release profiles of doxorubicin in vitro over 48 h. Their study indicated that phytosterol-fructose-chitosan nanoparticles had the potential as a carrier for targeted delivery of hydrophobic anticancer drugs with declined cytotoxicity to normal tissues (Qiu et al. 2013).

8.3.5 Covalent Cross-Linking

Chitosan nanoparticles can be prepared by covalent cross-linking as shown in Fig. 8.13. This method involves the formation of covalent bonds between the chitosan and a functional cross-linking agent (Prabaharan and Mano 2004). Commonly used cross-linking agents are polyethylene glycol dicarboxylic acid, glutaraldehyde, and epichlorohydrin (Goldberg et al. 2007; Bodnar et al. 2005). Small-sized chitosan-gel nanospheres with an average diameter of 250 nm containing 5-fluorouracil or immobilizing 5-fluorouracil derivatives (aminopentyl-carbamoyl-5-fluorouracil or aminopentyl-ester-methylene-5-fluorouracil) were prepared using glutaraldehyde cross-linking agent and the emulsion method (Ohya et al. 1994). The release rate of 5-fluorouracil from the chitosan-gel nanospheres could be controlled by immobilization of 5-fluorouracil, degree of deacetylation of chitosan used, and coating with polysaccharides (Ohya et al. 1994). Wang et al. (2015) synthesized folic acid-conjugated chitosan by cross-linking reaction with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and then prepared mitoxantrone folic acid-chitosan-conjugated nanoparticles. The anticancer activity of nanoparticles was evaluated against human nasopharyngeal carcinoma cell lines (HONE1) in artificial gastric fluid and intestinal fluid. This study supported that folic acid helps in targeting chitosan nanoparticles and releases mitoxantrone at a sustained rate. Covalent cross-linking method can be combined with another method to get better results. Li et al. (2009) prepared paclitaxel chitosan nanoparticles by solvent evaporation and emulsification cross-linking method. They reported sustained release of paclitaxel and higher cell toxicity than the paclitaxel alone. In addition to above-mentioned methods, some methods and information on chitosan loaded anticancer drugs are given in Table 8.1.

Table 8.1 Methods of preparation and applications of anticancer drug-loaded chitosan nanoparticles

S. No.	Anticancer drug	Polymer	Methods of preparation	Applications	References
1	Lomustine	Chitosan	Ionic gelation	Lung cancer (L132 cancer cell line)	Mehrotra et al. (2011)
2	Fluorouracil	Chitosan		Cervical cancer (HeLa cells)	Saravanabhavan et al. (2013)
3	Gemcitabine	Chitosan and Pluronic® F-127		Colon cancer (HT-29 cell line)	Hosseinzadeh et al. (2012)
4	Gemcitabine	Chitosan		Hematological malignancies	Derakhshandeh and Fathi (2012)
5	5-Aminolaevulinic acid	Succinate-modified chitosan		Oral cancer	Yang et al. (2013)
6	Doxorubicin	Thiolated chitosan		Breast cancer (T47D cell)	Talaei et al. (2011)
7	Docetaxel	Polylactic acid-grafted chitosan	Dialysis method	Lung cancer (A549 cell line)	Hwang et al. (2008)
8	Methotrexate	Chitosan	Complexation process	Breast and neck cancer (HeLa and MCF-7 cell lines)	Nogueira et al. (2013)
9	Flutamide	Chitosan and dextran sulfate	Poly electrolyte complex	Prostate cancer	Anitha et al. (2013)
10	Doxorubicin	Chondroitin sulfate/ chitosan		Epidermal carcinoma (KB cells) and lung cancer	Tsai et al. (2011)
11	Oxaliplatin	Solvent evaporation	Chitosan and Eudragit S100	Colorectal cancer	Jain et al. (2010)

8.4 Characteristics of Chitosan Nanoparticles as Anticancer Drug Carrier

8.4.1 Site-Specific Targeting

Chitosan has positive charge which helps in selective adsorption and neutralizing effects on the tumor cell surface. Chitosan can be targeted to the liver, spleen, lung, and colon (Park et al. 2007). Moreover, chitosan nanoparticles can be made target specific to cancer cell by attaching various ligands on its surface. Since, cancer cell expresses folate receptors on its surface. Hence folic acid can be attached on the

surface of chitosan nanoparticles by making some modifications. Recently, we (Salar and Kumar 2016) have synthesized vincristine-loaded folic acid-chitosan-conjugated nanoparticles using ionic gelation method. The in vitro drug release study showed slow and sustained release of vincristine in phosphate-buffered saline at pH 6.7. Since cancer cells express folate receptors on their surface, such vincristine-loaded folic acid-chitosan-conjugated nanoparticles could be used for targeted delivery against resistant cancer with some modifications. Folic acid-chitosan-conjugated nanoparticles have also been targeted against human liver cancer cell line SMMC-7721 in vitro by folate receptor-mediated endocytosis (Song et al. 2013). Xu et al. (2008) prepared doxorubicin-loaded N-octyl-N′-succinyl chitosan polymeric micelle and studied their biodistribution in mice. These nanoparticles were suitable for targeting the spleen and liver and significantly reduced drug toxicity to the heart and kidney. Zu et al. (2011) optimized methods for synthesis of oligomycin A-loaded folate-conjugated chitosan nanoparticles as a tumor-targeted drug delivery system using a two-level factorial design method. The two-level factorial design was composed of five independent variables, viz., folic acid-conjugated chitosan, oligomycin A and sodium tripolyphosphate concentration, the mass ratio of folate-conjugated chitosan to sodium tripolyphosphate, and cross-linking time. The mean particle size and the drug loading rate of resulting oligomycin-loaded folate-conjugated chitosan nanoparticles were used as response variables. The results indicated that folate-oligomycin-chitosan nanoparticles obtained as a targeted drug delivery system could be used effectively in the therapy of leukemia in the future. Doxorubicin and antisense oligonucleotide-loaded thiolated chitosan nanoparticles were prepared and evaluated against epidermal growth factor receptor (EGFR) in breast cancer cell line (T47D). This study facilitated functionality of synthetic polymers N-acetyl cysteine-chitosan and N-acetyl penicillamine-chitosan polymers as an efficient antisense oligonucleotide delivery system in vitro and in vivo (Talaei et al. 2011).

Development and characterization of colchicine-loaded methotrexate-loaded chitosan and glycol chitosan-based nanoparticles have been reported for administration of the anticancer drug to brain tumors. Transport of methotrexate across MDCKII-MDR1 monolayer and cytotoxicity studies against C6 glioma cell line were also performed. These nanocarriers represented a promising strategy for the administration of methotrexate to brain tumors which merits further investigations under in vivo conditions (Sahoo et al. 2012).

8.4.2 Size

Chitosan nanoparticles can be prepared in the particle size range from 1 to 1000 nm. Due to small size, these nanoparticles can enter the barrier across the plasma membrane of cancer cells. This might be due to the small size of nanoparticles as compared to the plasma membrane or by receptor-mediated endocytosis process. Chitosan-based nanospheres as a drug delivery system for anticancer drug cytarabine in the size range of 466.45 ± 5.32 nm have been synthesized by the ionic

gelation method (Sangeetha et al. 2010). Anticancer efficacy of cisplatin-loaded glycol chitosan nanoparticles in the size range of 300–500 nm in tumor-bearing mice has also been studied (Kim et al. 2008). Le et al. (2013) reported curcumin-loaded pluronic F127/chitosan nanoparticles of average size of 150.9 nm for cancer therapy against human embryonic kidney 293 (HEK293) cells. Chitosan nanoparticles for the controlled release of anticancer drug paclitaxel with an average particle size diameter of 250 nm and 800 nm have been prepared and characterized (Sahoo et al. 2012).

8.4.3 Biodegradability

Biodegradability is an important parameter for effective drug delivery system. Biodegradability also ensures less toxicity of drug delivery systems. The higher the degree of deacetylation of chitosan, the higher will be the degradation rate (Kean and Thanou 2010). Enzyme catalysis degradation also depends on the availability of the amino group of chitosan. Chitosan is degraded mainly by enzyme catalysis in vivo process (Kean and Thanou 2010).

8.4.4 Adhesivity

Chitosan has amino and carboxyl groups which can be combined with glycoprotein present in mucus to form a hydrogen bond. This leads to an adhesive effect which is helpful to formulate bioadhesive dosage forms through different routes such as buccal, oral, etc. Mucoprotein in the mucus is positively charged; hence, chitosan and mucus are attracted to each other. This results in enhancement of the retention time of drugs and continuous drug release in vivo along with improvement in drug bioavailability (Jin and Hu 2008). However, exact mechanism is not clearly understood. Under neutral and acidic conditions, adhesivity of chitosan gets strengthened. The higher the degree of deacetylation and the greater the molecular weight of chitosan, the stronger will be its adhesivity and the amount of adhesion (Feng et al. 2013).

8.4.5 Safety

Chitosan is nontoxic and biocompatible polymer and is approved by the Food and Drug Administration (USA) as a wound dressing material (Chaudhury and Das 2011). However anticancer drugs loaded in chitosan nanoparticles are safe, non-toxic, and biodegradable; more research is still needed in this field. Chitosan/O--carboxymethyl chitosan nanoparticles have been evaluated for efficient and safe oral anticancer drug delivery of doxorubicin hydrochloride both in vitro as well as in vivo. Feng et al. (2013) investigated the ability of a polyelectrolyte complex consisting of chitosan and O-carboxymethyl chitosan as a pH-responsive carrier for

oral delivery of doxorubicin hydrochloride. Results showed that polyelectrolyte complex were highly efficient and safe as an oral delivery system for doxorubicin hydrochloride. Tamoxifen citrate-loaded chitosan nanoparticles have been developed and tested (Patel et al. 2011).

8.4.6 Sustained/Controlled Release of Anticancer Drug

Anticancer drug encapsulated in chitosan nanoparticles is released through degradation. By varying molecular weight and degree of deacetylation of chitosan, different types of nanoparticles of varied sizes can be prepared which helps in sustainability and controlled release of drugs. Chitosan can be readily modified by attaching various ligands like folic acid to it. Anticancer drug paclitaxel have been prepared and characterized in chitosan nanoparticles for the controlled release (Sahoo et al. 2012). Thiolated chitosan-modified polycaprolactone, unmodified chitosan-modified unmodified polylactic acid-polycaprolactone-d-α-tocopheryl polyethylene glycol 1000 succinate, and thiolated chitosan-modified polylactic acid-polycaprolactone-d-α-tocopheryl polyethylene glycol 1000 succinate nanoparticles have been evaluated against Taxol® for oral chemotherapy of lung cancer (A549 cells). The thiolated chitosan-modified polylactic acid-polycaprolactone-d-α-tocopheryl polyethylene glycol 1000 succinate nanoparticles were found to have higher levels of the cell uptake as compared to others (Jiang et al. 2013). Doxorubicin delivery by chitosan-g-TPGS nanoparticles against human hepatocarcinoma cells (HepG2 and BEL-7402) and human breast adenocarcinoma cells (MCF-7) has been evaluated and compared with Adriamycin for overcoming multidrug resistance (Guo et al. 2014).

8.5 Applications

Due to the unique properties of chitosan nanoparticles or modified forms, they have applications in the treatment of different types of cancer as shown in Fig. 8.14 and Table 8.1. Nogueira et al. (2013) reported in vitro antitumor activity of methotrexate via pH-sensitive chitosan nanoparticles with average size of 301 nm against HeLa and breast cancer cell lines (MCF-7). Results suggested that pH-sensitive methotrexate-loaded chitosan nanoparticles could be potentially useful as a carrier system for tumor and intracellular drug delivery in cancer therapy. Doxorubicin-encapsulated nanoparticles composed of poly(ethylene glycol)-grafted carboxymethyl chitosan have been synthesized, and their anticancer activity against C6 glioma cells was tested in vitro. Nanoparticles showed increased cytotoxicity compared to doxorubicin alone. The results also suggested that doxorubicin was unable to penetrate into cells and did not effectively inhibit cell proliferation. In contrast, nanoparticles could penetrate into cells and effectively inhibit cell proliferation (Jeong et al. 2010). A novel nanoparticle drug delivery system consisting of chitosan and glyceryl monooleate for paclitaxel with a hydrophobic core and a

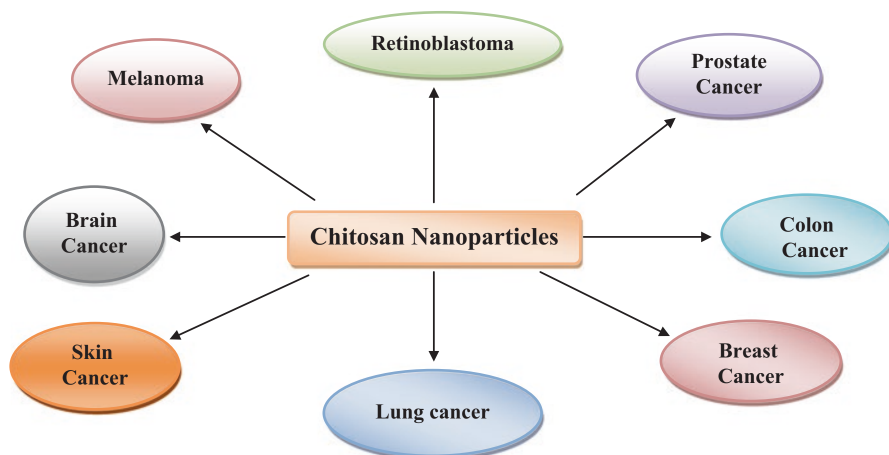


Fig. 8.14 Applications of chitosan nanoparticles

hydrophilic shell has been developed by multiple emulsion (o/w/o) solvent evaporation method. This novel nanoparticle formulation showed evidence of mucoadhesive properties, a fourfold increase in cellular uptake, and a 1000-fold reduction in the IC_{50} of paclitaxel (Trickler et al. 2008). Li et al. (2014) prepared doxorubicin glucose-conjugated chitosan nanoparticles for target-specific delivery against glucose transporters (Gluts) overexpressed by tumor cells. Results suggested that antitumor activity of doxorubicin-/glucose-conjugated nanoparticles was four to five times effective in 4T1 cell killing than that of doxorubicin/chitosan nanoparticles. This study supported the Gluts-targeted glucose-conjugated chitosan nanoparticles may be promising delivery agents in cancer therapies.

8.6 Conclusion

Chitosan nanoparticles could offer various advantages as an effective drug delivery system. Being biodegradable, bioavailable, and less toxic as compared to other polymers, chitosan polymers can be administered through different routes such as oral, nasal, etc. These nanoparticles can be easily modified with various ligands such as folic and can be targeted to cancer cells, which express folate receptors. By varying degree of deacetylation of chitosan, chitosan nanoparticles of different sizes can be prepared. There are some limitations of chitosan being poor soluble. Also biocompatibility is main question when hydrophobic drugs are encapsulated in chitosan. One of the biggest challenges for researchers is approval of drugs from the Food and Drug Administration (FDA) based on chitosan nanoparticles since most of the studies have been done in vitro. Chitosan materials hold much promise for treatment of different types of cancers. Although research is going on worldwide, more research is needed in the area of anticancer drug-loaded chitosan nanoparticles, so that chitosan nanoparticles can be used in efficient ways to save lives of millions of cancer.

References

Alexis F, Pridgen EM, Langer R, Farokhzad OC (2010) Nanoparticle technologies for cancer therapy. In: Schäfer-Korting M (ed) Drug delivery. Springer, Berlin/Heidelberg, pp 55–86
Amidi M, Mastrobattista E, Jiskoot W, Hennink WE (2010) Chitosan-based delivery systems for protein therapeutics and antigens. Adv Drug Deliv Rev 62:59–82
Anitha A, Deepagan VG, Divya Rani VV, Menon D, Nair SV, Jayakumar R (2011) Preparation, characterization, in vitro drug release and biological studies of curcumin loaded dextran sulphate–chitosan nanoparticles. Carbohydr Polym 84:1158–1164
Anitha A, Chennazhi KP, Nair SV, Jayakumar R (2012) 5-Flourouracil loaded N,O –carboxymethyl CSNPs as an anticancer nanomedicine for breast cancer. J Biomed Nanotechnol 8:1–14
Anitha A, Uthaman S, Nair SV, Jayakumar R, Lakshmanan VK (2013) Enhanced delivery system of flutamide loaded chitosan-dextran sulphate nanoparticles for prostate cancer. J Biomed Nanotechnol 9:335–340
Baraton MI, Chen X, Gonsalves KE (1996) Application of fourier transform infrared spectroscopy to nanostructured materials surface characterization. ACS Symp Ser 622:312–333
Berthold A, Cremer K, Kreuter J (1996) Preparation and characterization of chitosan microspheres as drug carrier for prednisolone sodium phosphate as model for anti-inflammatory drugs. J Control Release 39:17–25
Bhui DK, Bar H, Sarkar P, Sahoo GP et al (2009) Synthesis and UV-vis spectroscopic study of silver nanoparticles in aqueous SDS solution. J Mol Liq 145:33–37
Bodnar M, Hartmann J, Borbely J (2005) Preparation and characterization of chitosan-based nanoparticles. Biomacromolecules 6:2521–2527
Calvo P, Remunan-Lopez C, Vila-Jato JL, Alonso MJ (1997) Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers. J Appl Polym Sci 63:125–132
Chaudhury A, Das S (2011) Recent advancement of chitosan-based nanoparticles for oral controlled delivery of insulin and other therapeutic agents. AAPS Pharm Sci Tech 12:10–20
Cheng M, Han J, Li Q, He B, Zha B et al (2012) Synthesis of galactosylated chitosan/5-fluorouracil nanoparticles and its characteristics, in vitro and in vivo release studies. J Biomed Mater Res Part B Appl Biomater 100:2035–2043
Creighton JA, Eadon DG (1991) Ultraviolet-visible absorption spectra of the colloidal metallic elements. J Chem Soc Faraday Trans 87:3881–3891
De Rosa G, Caraglia M, Salmaso S, Elbayoumi T (2013) Nanotechnologies in Cancer. J Drug Deliv. https://doi.org/10.1155/2013/604293
Debnath S, Datta D, Babu MN et al (2010) Studies on the preparation and evaluation of chitosan nanoparticles containing cytarabine. Int J Pharm Sci Nanotechnol 3:957–964
Derakhshandeh K, Fathi S (2012) Role of chitosan nanoparticles in the oral absorption of Gemcitabine. Int J Pharm 437:172–177
Duan J, Zhang Y, Han S, Chen Y, Li B et al (2010) Synthesis and in vitro/in vivo anti-cancer evaluation of curcumin-loaded chitosan/poly(butyl cyanoacrylate) nanoparticles. Int J Pharm 400:211–220
Duan J, Liu M, Zhang Y, Zhao J, Pan Y, Yang X (2012) Folate-decorated chitosan/doxorubicin poly(butyl)cyanoacrylate nanoparticles for tumor-targeted drug delivery. J Nanopart Res 14:761. https://doi.org/10.1007/s11051-012-0761-4
El-Shabouri M (2002) Positively charged nanoparticles for improving the oral bioavailability of cyclosporin-A. Int J Pharm 249:101–108
Eppler AS, Rupprechter G, Anderson EA, Somorjai GA (2000) Thermal and chemical stability and adhesion strength of Pt nanoparticle arrays supported on silica studied by transmission electron microscopy and atomic force microscopy. J Phys Chem B 104:7286–7292
Feng C, Wang Z, Jiang C, Kong M, Zhou X et al (2013) Chitosan/o-carboxymethyl chitosan nanoparticles for efficient and safe oral anticancer drug delivery: in vitro and in vivo evaluation. Int J Pharm 457:158–167

Fröhlich E (2012) The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles. Int J Nanomedicine 7:5577–5591

Goldberg M, Langer R, Jia X (2007) Nanostructured materials for applications in drug delivery and tissue engineering. J Biomater Sci Polym Ed 18:241–268

Guo Y, Chu M, Tan S, Zhao S, Liu H et al (2014) Chitosan-g-TPGS nanoparticles for anticancer drug delivery and overcoming multidrug resistance. Mol Pharm 11:59–70

Hao E, Schatz GC, Hupp JT (2004) Synthesis and optical properties of anisotropic metal nanoparticles. J Fluoresc 14:331–341

Hosseinzadeh H, Atyabi F, Dinarvand R, Ostad SN (2012) Chitosan–Pluronic nanoparticles as oral delivery of anticancer gemcitabine: preparation and in vitro study. Int J Nanomedicine 7:1851–1863

Huang P, Yang C, Liu J, Wang W, Guo S et al (2014) Improving the oral delivery efficiency of anticancer drugs by chitosan coated polycaprolactone grafted hyaluronic acid nanoparticles. J Mater Chem 2:4021–4033

Hwang HY, Kimb IS, Kwon IC, Kim YH (2008) Tumor targetability and antitumor effect of docetaxel-loaded hydrophobically modified glycol chitosan nanoparticles. J Control Release 128:23–31

Jain A, Jain SK, Ganesh N, Barve J, Beg AM (2010) Design and development of ligand-appended polysaccharidic nanoparticles for the delivery of oxaliplatin in colorectal cancer. J Nanotechonol 6:179–190

Jari V, Rose M, Heikki K, Marjaana R, Eija S, Raija A (2004) Chitosan-coated paper: Effects of nisin and different acids on the antimicrobial activity. J Appl Polym Sci 94:986–993

Jeong YI, Jin SG, Kim IY, Pei J, Wen M et al (2010) Doxorubicin-incorporated nanoparticles composed of poly(ethylene glycol)-grafted carboxymethyl chitosan and antitumor activity against glioma cells in vitro. Colloids Surf B 79:149–155

Ji J, Wu D, Liu L, Chen J, Xu Y (2012a) Preparation, evaluation, and in vitro release of folic acid conjugated O-Carboxymethyl chitosan nanoparticles loaded with methotrexate. J Appl Polym Sci 125:E208–E215

Ji J, Wu D, Liu L, Chen J, Xu Y (2012b) Preparation, characterization, and in vitro release of folic acid-conjugated chitosan nanoparticles loaded with methotrexate for targeted delivery. Polym Bull 68:1707–1720

Jiang J, Oberdörster G, Biswas P (2009) Characterization of size, surface charge, and agglomeration state of nanoparticle dispersions for toxicological studies. J Nanopart Res 11:77–89

Jiang L, Li X, Liu L, Zhang Q (2013) Thiolated chitosan-modified PLA-PCL-TPGS nanoparticles for oral chemotherapy of lung cancer. Nanoscale Res Lett 8:66. https://doi.org/10.1186/1556-276X-8-66

Jin MX, Hu QH (2008) Characterization and application in bioadhesive drug delivery system of chitosan. Centr South Pharm 6:324–327

Kas HS (1997) Chitosan: properties, preparations and application to microparticulate systems. J Microencapsul 14:689–711

Kean T, Thanou M (2010) Biodegradation, biodistribution and toxicity of chitosan. Adv Drug Deliv Rev 62:3–11

Kim JH, Kim YS, Park K, Lee S et al (2008) Antitumor efficacy of cisplatin-loaded glycol chitosan nanoparticles in tumor-bearing mice. J Control Release 127:41–49

Koo H, Min KH, Lee SC, Park JH, Park K et al (2013) Enhanced drug-loading and therapeutic efficacy of hydrotropic oligomer-conjugated glycol chitosan nanoparticles for tumor-targeted paclitaxel delivery. J Control Release 172:823–831

Kumari SDC, Tharani CB, Narayanan N, Kumar SC (2013) Formulation and characterization of methotrexate loaded sodium alginate chitosan nanoparticles. Ind J Res Pharm Biotechnol 1:915–921

Kuppens IE, Bosch TM, van Maanen MJ et al (2005) Oral bioavailability of docetaxel in combination with OC144-093 (ONT-093). Cancer Chemother Pharmacol 55:72–78

Le TMP, Pham VP, Dang TML, La TH et al (2013) Preparation of curcumin-loaded pluronic F127/chitosan nanoparticles for cancer therapy. Adv Nat Sci Nanosci Nanotechnol 4:025001–025004

Lee SP, Tscharnuter W, Chu B (1972) Calibration of an optical self-beating spectrometer by polystyrene latex spheres, and confirmation of the Stokes-Einstein formula. J Polym Sci 10:2453–2459

Li F, Li J, Wen X, Zhoub S, Tong X et al (2009) Anti-tumor activity of paclitaxel-loaded chitosan nanoparticles: an in vitro study. Mater Sci Eng C 29:2392–2397

Li J, Ma FK, Dang QF, Liang XG, Chen XG (2014) Glucose-conjugated chitosan nanoparticles for targeted drug delivery and their specific interaction with tumor cells. Front Mater Sci 8:363–372

Luque-Alcaraz AG, Lizardi J, Goycoolea FM, Valdez MA et al (2012) Characterization and antiproliferative activity of nobiletin-loaded chitosan nanoparticles. J Nanomater. https://doi.org/10.1155/2012/265161

Mahapatro A, Singh DK (2011) Biodegradable nanoparticles are excellent vehicle for site directed in vivo delivery of drugs and vaccines. J Nanobiotechnol 9:55. https://doi.org/10.1186/1477-3155-9-55

Manikkam R, Pitchai D (2014) Catechin loaded chitosan nanoparticles as a novel drug delivery system for cancer – synthesis and in vitro and in vivo characterization. World J Pharm Pharm Sci 3:1553–1577

Mao HQ, Roy K, Troung-Le VL, Janes KA, Lin KY et al (2001) Chitosan-DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency. J Control Release 70:399–421

Mazzarino L, Travelet C, Murillo SO et al (2012) Elaboration of chitosan-coated nanoparticles loaded with curcumin for mucoadhesive applications. J Colloid Interface 370:58–66

Mehrotra A, Nagarwal RC, Kumar J (2011) Lomustine loaded chitosan nanoparticles: characterization and in vitro cytotoxicity on human lung cancer cell line L132. Chem Pharm Bull 59:315–320

Min KH, Park K, Kim YS, Bae SM, Lee S et al (2008) Hydrophobically modified glycol chitosan nanoparticles-encapsulated camptothecin enhance the drug stability and tumor targeting in cancer therapy. J Control Release 127:208–218

Mitra S, Gaur U, Ghosh PC, Maitra AN (2001) Tumour targeted delivery of encapsulated dextran–doxorubicin conjugate using chitosan nanoparticles as carrier. J Control Release 74:317–323

Nogueira DR, Tavano L, Mitjans M, Perez L et al (2013) In vitro antitumor activity of methotrexate via pH-sensitive chitosan nanoparticles. Biomaterials 4:2758–2772

Ohya Y, Shiratani M, Kobayashi H, Ouchi T (1994) Release behavior of 5-fluorouracil from chitosan-gel nanospheres immobilizing 5-fluorouracil coated with polysaccharides and their cell specific cytotoxicity. J Macromol Sci Part A 31:629–642

Othayoth R, Santhosh Kumar K, Karthik V (2013) Development and characterization of chitosan-pluronic polymeric nanoparticles for the breast cancer treatment. Int J Mech Eng Robotics 1:71–79

Ozbas-Turan S, Aral C, Kabasakal L et al (2003) Co-encapsulation of two plasmids in chitosan microspheres as a non-viral gene delivery vehicle. J Pharm Pharm Sci 6:27–32

Pan Y, Li YJ, Zhao HY, Zheng JM, Xu H et al (2002) Bioadhesive polysaccharide in protein delivery system: chitosan nanoparticles improve the intestinal absorption of insulin in vivo. Int J Pharm 249:139–147

Park K, Kim JH, Nam YS, Lee S et al (2007) Effect of polymer molecular weight on the tumor targeting characteristics of self-assembled glycol chitosan nanoparticles. J Control Release 122:305–314

Patel MP, Patel RR, Patel JK (2008) New drug delivery systems based on chitosan. Curr Drug Discovery Technol 5:333–341

Patel PN, Patel LJ, Patel JK (2011) Development and testing of novel temoxifen citrate loaded chitosan nanoparticles using ionic gelation method. Der Pharmacia Sinica 2:17–25

Prabaharan M, Mano J (2004) Chitosan based particles as controlled drug delivery systems. Drug Deliv 12:41–57

Prameela Rani A, Sivannarayana P, Abbulu K, Saikishore V (2014) Design and characterisation of Anastrazole loaded chitosan nanoparticles by ionotropic gelation method. Res J Pharm, Biol Chem Sci 5:2069–2078

Qi J, Yao P, He F, Yu C, Huang C (2010) Nanoparticles with dextran/chitosan shell and BSA/chitosan core-Doxorubicin loading and delivery. Int J Pharm 393:176–184

Qiu Y, Zhu J, Wang J, Gong R et al (2013) Self-assembled phytosterol-fructose-chitosan nanoparticles as a carrier of anticancer drug. J Nanosci Nanotechnol 13:5935–5941

Rebucci M, Michiels C (2013) Molecular aspects of cancer cell resistance to chemotherapy. Biochem Pharmacol 85:1219–1226

Reis CP, Neufeld RJ, Ribeiro AJ, Veiga F (2006) Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles. Nanomedicine 2:8–21

Sahoo D, Parida UK, Rout N, Bindhani BK (2012) Preparation and characterization of chitosan nanoparticles for the controlled release of anticancer drug paclitaxel. Int J Pharm Bio Sci 11:1687–1698

Sakuma S, Hayashi M, Akashi M (2001) Design of nanoparticles composed of graft copolymers for oral peptide delivery. Adv Drug Deliv Rev 47:21–37

Salar RK, Kumar N (2016) Synthesis and characterization of vincristine loaded folic acid–chitosan conjugated nanoparticles. Resour-Efficient Technol 2(4):199–214

Salar RK, Sharma P, Kumar N (2015) Enhanced antibacterial activity of streptomycin against some human pathogens using green synthesized silver nanoparticles. Resour-Efficient Technol 1:106–115

Sangeetha S, Harish G, Samanta MK (2010) Chitosan-based nanospheres as drug delivery system for cytarabine. Int J Pharm Bio Sci 1:1–8

Saravanabhavan SS, Bose R, Skylab S, Dharmalingam S (2013) Fabrication of chitosan/TPP nanoparticles as a carrier towards the treatment of cancer. Int J Drug Deliv 5:35–42

Saremi S, Atyabi F, Akhlaghi SP et al (2011) Thiolated chitosan nanoparticles for enhancing oral absorption of docetaxel: preparation, in vitro and ex vivo evaluation. Int J Nanomedicine 12:119–128

Saremi S, Dinarvand R, Kebriaeezadeh A et al (2013) Enhanced oral delivery of docetaxel using thiolated chitosan nanoparticles: preparation, in vitro and in vivo studies. Bio Med Res Int. https://doi.org/10.1155/2013/150478

Sevda S, McClureb SJ (2004) Potential applications of chitosan in veterinary medicine. Adv Drug Deliv Rev 56:1467–1480

Shakeri-Zadeh A, Khoei S, Khoee S, Sharifi AM, Shiran MB (2013) Targeted, monitored and controlled chemotherapy: a multimodal nanotechnology-based approach against cancer. ISRN Nanotech. https://doi.org/10.1155/2013/629510

Shi XW, Du YM, Yang JH (2005) Effect of degree of substitution and molecular weight of carboxymethyl chitosan nanoparticles on doxorubicin delivery. J Appl Polym Sci 100:4689–4696

Shi L, Tang C, Yin C (2012) Glycyrrhizin-modified O-carboxymethyl chitosan nanoparticles as drug vehicles targeting hepatocellular carcinoma. Biomaterials 33:7594–7604

Shi Z, Guo R, Li W, Zhang Y, Xue W et al (2014) Nanoparticles of deoxycholic acid, polyethylene glycol and folic acid-modified chitosan for targeted delivery of doxorubicin. J Mater Sci Mater Med 25:723–731

Siegel R, Naishadham D, Jemal A (2013) Cancer statistics. CA-Cancer J Clin 63:11–30

Snima KS, Jayakumar R, Unnikrishnan AG, Nair SV, Lakshmanan VK (2012) O-carboxymethyl chitosan nanoparticles for metformin delivery to pancreatic cancer cells. Carbohydr Polym 89:1003–1007

Song H, Su C, Cui W, Zhu B et al (2013) Folic acid-chitosan conjugated nanoparticles for improving tumor-targeted drug delivery. Biomed Res Int. https://doi.org/10.1155/2013/723158

Sun S, Murray C, Weller D, Folks L, Moser A (2000) Monodisperse FePt nanoparticles and ferromagnetic FePt nanocrystal superlattices. Science 287:1989–1992

Sunil AA, Nadagouda NM, Tejraj M (2004) Recent advances on chitosan-based micro- and nanoparticles in drug delivery. J Control Release 100:5–28

Talaei F, Azizi E, Dinarvand R, Atyabi F (2011) Thiolated chitosan nanoparticles as a delivery system for antisense therapy: evaluation against EGFR in T47D breast cancer cells. Int J Nanomed 6:1963–1975

Termsarasab U, Cho HJ, Kim DH, Chong S et al (2013) Chitosan oligosaccharide-arachidic acid-based nanoparticles for anti-cancer drug delivery. Int J Pharm 441:373–380

Trapani A, Denora N, Iacobellis G, Sitterberg J et al (2011) Methotrexate-loaded chitosan- and glycol chitosan-based nanoparticles: A promising strategy for the administration of the anticancer drug to brain tumors. AAPS Pharm Sci Tech 12:1302–1311

Trickler WJ, Nagvekar AA, Dash AK (2008) A novel nanoparticle formulation for sustained paclitaxel delivery. AAPS Pharm Sci Tech 9:486–493

Tsai HY, Chiu CC, Lin PC et al (2011) Antitumor efficacy of doxorubicin released from cross-linked nanoparticulate chondroitin sulfate/chitosan polyelectrolyte complexes. Macromol Biosci 11:680–688

Wang YS, Jiang Q, Li RS, Liu LL, Zhang QQ et al (2008) Self-assembled nanoparticles of cholesterol-modified O-carboxymethyl chitosan as a novel carrier for paclitaxel. Nanotechnology 19:145101. https://doi.org/10.1088/0957-4484/19/14/145101

Wang JJ, Zeng ZW, Xiao RZ, Xie T et al (2011) Recent advances of chitosan nanoparticles as drug carriers. Int J Nanomedicine 6:765–774

Wang W, Tong CY, Liu XY, Li T et al (2015) Preparation and functional characterization of tumor-targeted folic acid-chitosan conjugated nanoparticles loaded with mitoxantrone. J Cent South Univ 22:3311–3317

Xu XY, Zhou JP, Li L et al (2008) Preparation of doxorubicin-loaded chitosan polymeric micelle and study on its tissue biodistribution in mice. Acta Pharm Sin 43:743–748

Xu Y, Wang L, Li YK, Wang CQ (2014) Oxidation and pH responsive nanoparticles based on ferrocene-modified chitosan oligosaccharide for 5-fluorouracil delivery. Carbohydr Polym 114:27–35

Yang SJ, Lin CF, Kuo ML, Tan CT (2013) Photodynamic detection of oral cancers with high-performance chitosan-based nanoparticles. Biomacromolecules 14:3183–3191

Zimbone M, Calcagno L, Messina G, Baeri P, Compagnini G (2011) Dynamic light scattering and UV-vis spectroscopy of gold nanoparticles solution. Mater Lett 65:2906–2909

Zu Y, Zhao Q, Zhao X, Zu S, Meng L (2011) Process optimization for the preparation of oligomycin-loaded folate-conjugated chitosan nanoparticles as a tumor-targeted drug delivery system using a two-level factorial design method. Int J Nanomedicine 6:3429–3441

Metallic Nanoparticles: Potential Antimicrobial and Therapeutic Agents

9

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9.1 Introduction

As the year 1959 approached its ending, Nobel Laureate Richard Feynman marked the beginning of a new era in the field of science with his lecture entitled "There's Plenty of Room at the Bottom," which he delivered at the American Physical Society at the California Institute of Technology. The lecture had a subtitle stating "An invitation to enter a new field of physics" (Feynman 1960). However, the series of events that followed were no longer exclusive to physics but engulfed almost every branch of science. One such event was the emergence of a new field in science, "nanotechnology." In the year 1974, Norio Taniguchi first coined the term "nanotechnology" combining the ultrafine size dimensions of nanometers with technology. Nanometer (nm) refers to the billionth part of a meter, i.e., 10^{-9} m, and the science dealing with particles or structures of this size is what we call nanotechnology (Taniguchi 1974). Moving on, another Nobel laureate Richard Smalley (1992) stated that "the impact of nanotechnology on health, wealth, and the standard of living for people will be at least the equivalent of the combined influences of microelectronics, medical imaging, computer-aided engineering, and man-made polymers in this century" (Mnyusiwalla et al. 2003). Since then, nanotechnology has become one of the most important, interesting, and rapidly expanding fields existing as the fusion product of various fields such as physics, chemistry, biology, engineering, etc. In terms of its applications in medicine and therapeutics, nanostructures are well researched owing to their unique behavior as compared to their bulk counterparts.

Medical professionals all over the world are facing major challenges in the field of microbial infections. While timely and correct diagnosis is one of the issues, the development of resistance in pathogenic bacteria and fungi against routinely used

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antibiotics and other antimicrobial agents is another area that requires intensive research. One major cause of concern is the emergence of multiple drug-resistant bacteria that account for high mortality rates (Kolar et al. 2001; Walker et al. 2009). Nanoparticles can offer their services in all these areas, with nanoparticle-recognition element conjugates helping in fast and reliable diagnosis (Syed 2014; Jamdagni et al. 2016) and nanoparticles in their naive form as antimicrobial agents to fight away the infection. The current chapter deals with various aspects of nanoparticles that make them an attractive choice for antimicrobial therapy and in therapeutics. Beginning with the basic properties of nanoparticles, the chapter gives a detailed account of the approaches prominently used for their synthesis, various mechanisms utilized by nanoparticles for effecting their antimicrobial action, the use of nanoparticles as platforms for drug delivery, and other therapeutic applications of nanoparticles. The chapter finally concludes with a brief account on the need for realizing the toxic effects of nanoparticles on nontarget population for their more effective utilization in the field of medicine.

9.2 Nanostructures: Properties and Characterization

The size of nanostructures ranges between 1 and 100 nm. Such small size allows for very different physical and chemical properties of nanostructures when compared to their bulk counterparts. This is because when the material is confined in such a small space, the mechanisms do not satisfy the crucial length parameters for displaying a particular property. The modified optical, magnetic, and electrical properties have proved their worth in medicine, electronics, and various other fields. All types of nanostructures possess three common features, “atomic domains spatially confined to less than 100 nm, significant atom fractions associated with interfacial environments, and interactions between the constituent domains.” These are the major governing factors responsible for specific properties of nanostructures (Siegel 1993). The nanostructures can thus exhibit properties very different from their larger in size counterparts (Shah and Tokeer 2010). Additionally, as the size of materials is reduced, it improves their biocompatibility (Kaur et al. 2014; Mirkin and Taton 2000; Kim et al. 2007). These properties make them an attractive choice to be worked upon as biocompatible therapeutic agents.

Nanostructures are primarily characterized on the basis of their dimensionality, the concept of which was first raised by Richard Siegel in 1993. He stated that any structure that has at least one dimension in the scale of nanometer can be called a nanostructure. While having one dimension in nanoscale is a prerequisite to be classified as a nanostructure, nanostructures can also possess all three dimensions in nanoscale. They can be regularly shaped such as triangles and hexagons, or be irregular in shape, can occur as single individual particles, or be present in the form of clusters and aggregates. Based on this dimensionality, nanostructures can be classified as zero- dimensional, one-dimensional, two-dimensional, and three-dimensional (Table 9.1) (Siegel 1993; Vollath 2008).

Table 9.1 Dimensional classification of nanostructures

Dimensionality (D)	Description	Nanostructures	Example
Zero-dimensional (0-D)	All dimensions in nanoscale	Atom clusters and assemblies	Nanoparticles
One-dimensional (1-D)	One dimension not in nanoscale	Modulated multilayers	Nanorods, nanotubes
Two-dimensional (2-D)	Two dimensions not in nanoscale	Ultrafine-grained overlayers	Thin nanolayers
Three-dimensional (3-D)	Three dimensions not in nanoscale	Nanophase materials having approximately equal dimensions in all the directions	Nanocomposites

While most materials form nanostructures of one or two sets of dimensionalities, some have been known to yield nanostructures covering all the dimensionalities, such as carbon. Carbon can yield nanostructures of various dimensionalities in the form of fullerenes (0-D), nanotubes (1-D), graphene (2-D), and nanocrystalline diamond (3-D). The studies on carbon nanostructures are primarily focused on harnessing their mechanical properties and in nanoelectronic applications including potential applications in the field of medical sciences, probe cantilevers, and drug delivery (Shenderova et al. 2002).

On the other hand, metals in pure or oxidized form most commonly assume nanoparticle structures. A number of metals have been reported to yield nanoparticle structures, some of them being gold, silver, zinc, iron, titanium, magnesium, copper, etc. While gold and silver form nanoparticles as pure metals, others yield nanoparticles in their oxidized form.

9.3 Synthesis of Nanoparticles

Nanoparticles (NPs) can be synthesized using various physical, chemical, and biological processes. Whatever the method used, there are two basic approaches for the synthesis of nanoparticles, namely, top-down and bottom-up approach (Fig. 9.1). Top-down approaches can be summed up as “subtractive” technologies, while bottom-up approaches are described as “additive” processes (Vollath 2008).

The top-down approach refers to mechanical processing (physical approach), such as grinding and milling of bulk materials to yield nanostructures of desired shape and size. For this, various techniques, namely, microfluidization, milling, high-pressure homogenization, etc., have been employed. However, such methods not only involve energy inputs but also generate considerable amount of heat which renders them futile when dealing with thermolabile materials (Verma et al. 2009). Bottom-up approach, on the other hand, involves nanoparticle fabrication using chemical and biological processes. These methods rely upon assembling atomic or

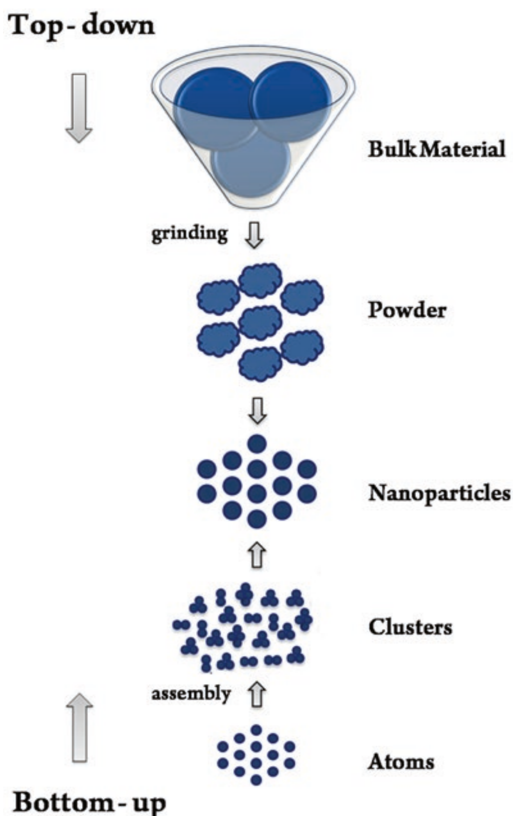

Fig. 9.1 Schematic showing approaches followed for nanoparticle synthesis

molecular units into more complex multilevel nanostructures eliminating any waste generation (Biswas et al. 2012). The courses followed in bottom-up approaches are sol-gel-mediated synthesis, precipitation, and preparation of nanodispersions using biological reducing agents.

The physical and chemical methods employed for NP synthesis suffer from various drawbacks and require multistep processing for reduction and stabilization. Biogenic synthesis, on the other hand, eliminates all impending hazards and problems associated, thus leading to an eco-friendly and biocompatible synthesis, also making it the method of choice for synthesis nowadays. Biogenic synthesis or green synthesis can be executed using filtrate of microorganism suspensions plant extracts (Kaur et al. 2016; Bansal et al. 2014) and even purified macromolecules, such as lipids, carbohydrates, and peptides. These methods circumvent large inputs of energy and harsh chemicals and prove cost-effective over various physical and chemical methods. Another advantage of biogenic synthesis is that reduction, capping, and stabilization all can be achieved in a single step, and the procedure can be easily controlled by manipulating synthesis conditions like temperature, pH, etc. The simplification of NP synthesis using biological methods has initiated a surge in research related to their exploitation in various fields, a major focus being on the probable use of NPs as antimicrobial and therapeutic agents.

9.4 Nanoparticles as Antimicrobial Agents

Antimicrobial activity can be defined as the potential to retard the growth or kill microorganisms without adversely affecting the host tissues. While bacterial structures are very different from their eukaryotic hosts, fungal pathogens share biochemical structures and biosynthetic pathways very similar to that of their hosts. Hence, it becomes a difficult task to design therapies that selectively affect fungal growth without interfering with normal functioning of the host cells. But, nanoparticles, being selectively and equally toxic to both pathogenic bacteria and fungi (Kaur et al. 2012, 2013) and showing good biocompatibility (Kaur et al. 2014) with various cell lines, offer a solution in this regard.

9.4.1 Antimicrobial Action of Nanoparticles: Mechanisms

Although the exact mechanism of action for antimicrobial potential of NPs against microbial pathogens is not completely known, but it has been reported that NPs may exert their antimicrobial effect either through a direct action or by producing secondary active agents. Major causes for growth inhibition of microbes include cell wall/plasma membrane damage, interruption of metabolic pathways, oxidation of cellular components, or DNA damage (Kaur et al. 2011; Li et al. 2008). The mechanism of antimicrobial action of NPs depends on the size, shape, concentration, and interaction of NPs with the target pathogens. It has been reported that the smaller the size of NPs, the better is their capability of penetrating the cell surface, and consequently the better is their antimicrobial potential (Buzea et al. 2007; Padmavathy and Vijayaraghavan 2008; Bera et al. 2014). The different possible mechanisms affected by NPs have been summarized in Fig. 9.2 and are explained in detail in the subsequent sections.

9.4.1.1 Membrane Damage

One of the primary mechanisms responsible for the antimicrobial effects of NPs is cell lysis which in turn is mediated through cell wall and cell membrane damage. Cell walls and cell membranes are the most important structural entities that maintain the integrity of cellular systems. While cell wall provides rigidity, shape, and strength to the cells and protects against mechanical damage and osmotic rupture (Singleton 2004), plasma membrane harbors various receptor molecules to aid in cellular signaling and is responsible for nutrient uptake too. Any interference with the proper functioning of the plasma membrane leads to imbalances in-and-out of the cell which may be severe enough to cause lysis.

NPs mediate cell lysis by disrupting the balance around the membranes. Silver NPs adhere to the bacterial membranes via electrostatic interactions, thereby disrupting their integrity (Thill et al. 2006). Nanosilver is also reported to degrade the lipopolysaccharide molecules of the cell wall and resulting into its accumulation in the membranes. This accumulation leads to the formation of pits altering the membrane permeability and causing cell fragmentation (Sondi and Sondi 2004; Iavicoli

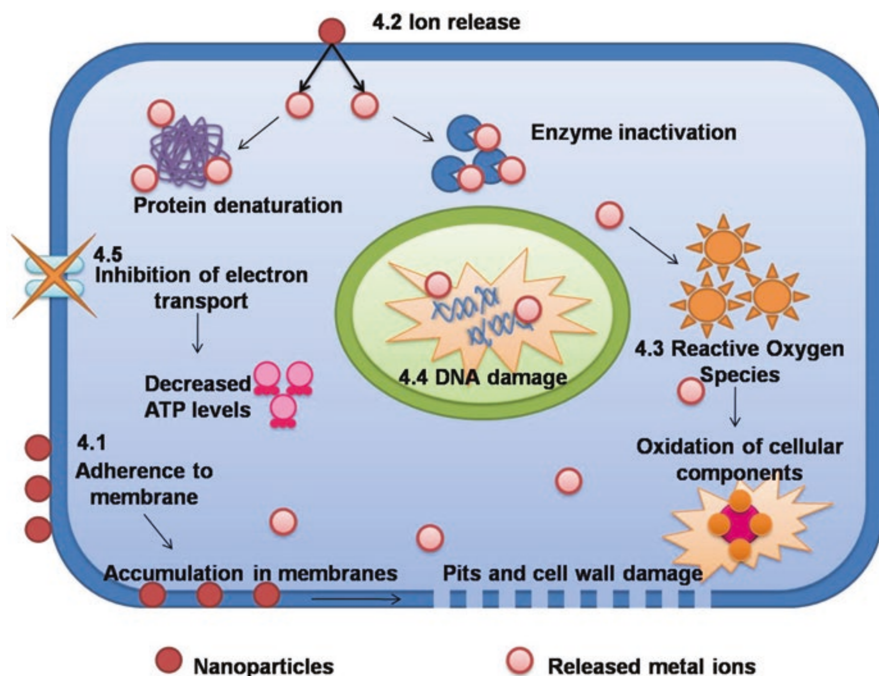

Fig. 9.2 Different mechanisms of antimicrobial action of nanoparticles

et al. 2013; Yun et al. 2013). Also, ZnO NPs accumulate on the surface, penetrate the cell wall leading to the disruption of membrane structure, and accumulate in the cytoplasm and interfere with cell growth (Brayner et al. 2006; Huang et al. 2008; Zhang et al. 2008; Sinha et al. 2011). Copper NPs are also reported to interfere with the cellular membranes, hamper their integrity, and cause cell death (Midander et al. 2009).

9.4.1.2 Ion Release

NPs are known to dissolute into toxic ions that contribute to their antimicrobial effects. Nanosilver releases Ag^+ ions that contribute to their antimicrobial potential and are believed to be major contributors for their cytotoxicity (Kaur et al. 2011; Morones et al. 2005; Maurer-Jones et al. 2013). These silver ions cause inactivation of cellular proteins and enzymes by reacting with their thiol groups, disturbing the metabolic processes and finally leading to the formation of reactive oxygen species (ROS) (Matsumura et al. 2003; Egger et al. 2009). Furthermore, silver ions penetrate the cell surface, interact with DNA, prevent its replication, and induce DNA damage (Feng et al. 2000). Gold NPs dissociate into gold ions which also react with thiol groups present in various vital enzymes such as NADH dehydrogenase. Interference with the functioning of this enzyme adversely affects the oxidative phosphorylation pathway, assisting in oxidative stress and release of ROS. The cumulative effect of these effectors ultimately becomes fatal for the bacterial cells

(Ahmad et al. 2013). Zinc oxide NPs are also known to release Zn^{2+} ions into the medium in the surroundings of the microorganisms. These ions have been reported as a major factor of toxicity toward *Saccharomyces cerevisiae* (Kasemets et al. 2009), *E. coli*, and *S. aureus* (Kaur et al. 2011). However, it has also been stated that the presence of low levels of solubilized Zn^{2+} ions into the medium can induce tolerance in various microorganisms and can, in fact, act as a nutrient source for microbial growth. Responses toward zinc ions are inherent to the target organism and differ in accordance to the target.

9.4.1.3 Generation of Reactive Oxygen Species (ROS)

ROS are basically the reactive species of oxygen which are oxidative in nature, namely, hydroxyl radicals ($OH^{\bullet}$), superoxide ions ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl). ROS are inevitably released during the aerobic metabolic processes of biological cells. Since these are reactive in nature, ROS can severely damage essential biomolecules, such as DNA, proteins, and lipid molecules of the cells by oxidizing their constituents (Apel and Hirt 2004). In case of lipid damage, ROS mutilate polyunsaturated fatty acids; while in proteins, they oxidize amino acid constituents and cofactors leading to enzyme deactivation (Brooker 2011).

Generation of ROS in the cells has been shown to affect the viability of bacterial cultures. It is one of the primary modes of action adopted by ZnO NPs to mediate their cytotoxicity. Among the various ROS generation pathways, the most favored pathway adopted by the nanoparticles is the photocatalytic generation of hydrogen peroxide (Sawai 2003; Zhang et al. 2008). However, light-induced generation of reactive oxygen radicals also contribute to their antimicrobial potential (Sawai et al. 1998; Yamamoto 2001; Padmavathy and Vijayaraghavan 2008; Zhang et al. 2008; Jalal et al. 2010; Gordon et al. 2011; Lipovsky et al. 2011). Dissociation of ZnO NPs for release of metal ions aids in ROS-mediated effects (Knaapen et al. 2004).

Among other NPs that follow, the ROS-mediated antimicrobial activity are iron oxide and copper oxide NPs. Iron oxide NPs release ferrous (Fe^{2+}) ions that react with hydrogen peroxide and result in the evolution of hydroxyl ions (Thannickal and Fanburg 2000). Fenton-type reactions are responsible for the generation of superoxide and hydroxyl radicals (Valko et al. 2006). Hydroxyl radicals can also be released as a result of interaction between oxidized metal and hydrogen peroxide via Haber-Weiss-type reactions (Aust et al. 1993; Thannickal and Fanburg 2000). Copper oxide NPs induce stress and genotoxicity in the target cell populations. Posttreatment, microbial cells show a marked increase in ROS release and enzymes associated with oxidative stress such as catalase and superoxide dismutase. This upregulation in the expression of stress-responsive enzymes has also been observed at the genetic level (Karlsson et al. 2008; Ahamed et al. 2010; Hanagata et al. 2011).

9.4.1.4 DNA Damage

DNA or deoxyribonucleic acid is the basic biomolecule of every living entity. It stores and transmits genetic information indispensable for the growth and development of any organism in the cell nucleus or nucleoid (in case of prokaryotes). The

basic structure of DNA consists of a double-stranded phosphodiester backbone onto which genetic information is written in the form of four bases, adenine (A), thymine (T), guanine (G), and cytosine (C), which is same in all organisms. Various combinations of these four bases constitute the huge diversity observed in all living forms. Any deviation from the normal structure, such as single- or double-strand breaks, missing bases, or modified bases, is termed as DNA damage. DNA damage may be natural or induced. Naturally occurring DNA damage is quite common, and every cell has developed specialized mechanisms to repair this damage and counteract its effects on cell survival. Induced DNA damage, on the other hand, is caused by various agents, such as chemicals or radiations, which can prove fatal to the cell, if on a hefty side.

NP-mediated DNA damage is a type of induced DNA damage. The metallic silver ions released from NPs are known to bind bacterial DNA and RNA molecules leading to their denaturation which prevents DNA replication and eventually halts cell division (Lansdown 2002; Castellano et al. 2007). One of the major triggers in initiating DNA damage is the release of ROS (Kumar et al. 2011). The amalgamation of silver ion-mediated DNA condensation and interaction with thiol groups of proteins results in bacterial cell death (Feng et al. 2000). However, upon comparing DNA damage in gram-positive and gram-negative bacteria, it has been observed that the thicker cell wall of gram-positive bacteria provides a small amount of defense against penetration by silver ions and, hence, results into a lesser extent of damage. Other metallic NPs such as ZnO and TiO_2 have also been reported to induce DNA damage in *E. coli* cells.

9.4.1.5 Interaction with Vital Proteins

Proteins are one of the major structural, functional, and regulatory components of living systems. They are complex units synthesized by polymerization of hundreds and thousands of amino acids. Proteins not only help in providing structure to living cells (such as tubulin) but also perform vital biochemical functions (such as respiratory enzymes). Enzymes are classes of proteins that help catalyze various biochemical processes by increasing the rate of reaction. The action of regulatory proteins and enzymes is one of the major control elements in maintaining the biological homeostasis of living cells. Any disturbance in the balance of these macromolecules can prove fatal for the parent organism. NPs interact with cellular proteins, hampering their destined physiochemical functions, and hence disturb the intracellular balances.

Gold NPs primarily interfere with the functioning of proton pumping ATPase which in turn is controlled by F-type ATP synthase gene. This leads to decreased levels of ATP molecules in treated bacterial cells and hence dwindled metabolism (Cui et al. 2012). Sulfur- and phosphorus-containing soft bases are the chosen targets for gold and silver NPs. These interact primarily with sulfur-containing proteins and phosphorous-containing DNA molecules. These interactions hamper the growth and integrity of bacterial cells and prove lethal (Sharma et al. 2012; Ahmad et al. 2013). ROS are the prime effectors in the inactivating proteins by inducing structural modifications in the constituent amino acids such as cysteine, histidine, tryptophan, etc. (Lushchak 2001).

9.5 Potential Applications of Nanoparticles in Therapeutics

In addition to the use of nanoparticles as antimicrobial agents, they have also been gaining significant importance in prophylaxis and therapeutics (Rai and Bai 2011). NPs are finding immense applications in the area of medicine, especially in drug delivery. Delivery of therapeutic agents mediated by NPs has resulted into the introduction of new opportunities for the expansion of medical treatment. In the recent past, various therapeutics relying on nanoparticles has been effectively introduced for dealing with cancer and several other diseases. Extended half-life, improved therapeutic index, and solubility have been achieved with little immunogenicity in NP-coupled drug delivery systems (Davis et al. 2008; Zhang et al. 2008; Park et al. 2009; Wang et al. 2009). At present many nanoparticles are under study for their role in drug delivery, particularly for cancer therapy. The worldwide survey carried out in 2006 by the European Science and Technology Observatory (ESTO) unveiled the involvement of 150 companies in development of nanoscale therapeutics, and currently 24 nanoparticle therapeutics have been used to treat a wide range of diseases, for instance, fungal or bacterial infections, HIV, diabetes, and cancers (Wagner et al. 2006; Emerich and Thanos 2007). Some of the therapeutic applications of different nanoparticles have been summarized in Table 9.2.

9.5.1 Nanoparticles as a Platform for Drug Delivery

One of the major necessities in the treatment of diseases is the targeted delivery of drugs, which can be achieved by cell-specific targeting using individually designed drug carriers. Current advances in the field of nanotechnology have revealed that nanoparticles have an immense potential as drug carriers. The potential of nanoparticles as the most favorable drug delivery vehicles has augmented the interest in developing novel targeted nanoparticles for therapeutic applications. There are numerous nanoparticle platforms that have been studied, but the most dominant nanoparticles used for such applications and which have also been approved for clinical use are based on liposomes and polymer-drug conjugates (Wang et al. 2008a).

9.5.1.1 Liposome-Based Nanoparticle Drug Delivery Platforms

Liposomes are sphere-shaped vesicles that include a bilayered membrane structure which is composed of natural or synthetic amphiphilic lipid molecules. They are used as drug delivery nanoparticles usually with a size limit of about 80–300 nm. Liposomes have been considered as excellent therapeutic nanocarriers due to their unique ability to encapsulate hydrophilic and hydrophobic therapeutic agents, to be coated with biocompatible polymers such as polyethylene glycol (PEG) which prolong their circulation half-life in vivo, and to be functionalized with specific ligands for targeting ligand conjugation (Zhang and Granick 2006; Torchilin 2005). In this system, the drug is incorporated into the liposomes by the process of encapsulation; once the liposome-drug conjugates reach the desired tissues, the therapeutic agents

Table 9.2 Applications of various metallic nanoparticles in therapeutics

Nanoparticles	Applications	References
Silver	Excellent candidate for use as anti-inflammatory agents that can be used for various therapies	Shin et al. (2007)
	Excellent candidates for bioimaging and as anticancer agents	Zhou et al. (2011) and Braun et al. (2014)
	Used as bone cement, which in turn are being used as artificial joint replacements	Alt et al. (2004)
Gold	Inhibit angiogenesis and act as a promising candidate for drug delivery systems and in cancer therapeutics	Arvizo et al. (2010)
	Used in the treatment of rheumatic diseases including psoriasis, juvenile arthritis, palindromic rheumatism, and discoid lupus erythematosus	Felson et al. (1990)
	Increase apoptosis in B-chronic lymphocytic leukemia (BCLL) treatment-suffering patients	Mukherjee et al. (2007)
	Used as surface coating in various biomedicine applications such as implants, dressing fabrics, glass surfaces, etc.	Das (2009)
Zinc oxide	ZnO NPs have been regarded as a possible treatment for cancer and autoimmune diseases and have been found to be involved in preferential killing of cancer cells and activated human T cells	Hanley et al. (2008) and Tian et al. (2015)
	Used in bioimaging, gene delivery, drug delivery, and as zinc-based biosensors	Zhang et al. (2013)
Titanium dioxide	Used in pharmaceuticals and biomedical sciences such as in bone tissue engineering	Gerhardt et al. (2007)
	Used in cell imaging and as biosensors for biological assay	Prokop and Davidson (2008), Etrych et al. (2008), and Li et al. (2009)
	Used as efficient drug delivery systems and in photodynamic therapy (PDT) of tumors	Miyoshi et al. (2011), Uddin et al. (2011) and Sortino 2012
Iron oxide	Used as contrast agents for MRI, in targeted tumor imaging and therapy, and imaging of human atherosclerotic plaques	Babes et al. (1999), Kooi et al. (2003), Morales et al. (2003), Chertok et al. (2008), Peng et al. (2008), and Elias and Tsourkas (2009)
	Involved in targeted cellular uptake to tissues, organs, or tumors: targeted probes have already been developed for in vivo mapping of tissues	Mazooz et al. (2005) and Mody et al. (2009)
	Used as gene therapy agents for cancer; iron chelators conjugated with nanoparticles are being used for Alzheimer's disease treatment	Wei et al. (2006) and Liu et al. (2009)

get released. The first liposome-based therapeutic was liposome-encapsulated doxorubicin, which was accepted in 1995 by the US Food and Drug Administration (FDA) for HIV-related Kaposi's sarcoma treatment and was shortly approved for the ovarian cancer and multiple myeloma treatment (Farokhzad and Langer 2006; Lian and Ho 2001). Since then, several other liposome-based therapeutic nanocarriers have been developed and are being used in the treatment of various diseases.

9.5.1.2 Polymer-Drug Conjugates as Drug Delivery Platforms

Polymer-drug conjugates, the other drug delivery platform system, has also been extensively studied (Duncan 2006). The conjugation of drugs to polymeric nanocarriers helps in reducing the undesirable adverse effects caused by small-molecule therapeutic agents, especially anticancer chemotherapeutic agents having two major unfavorable properties: short circulation half-life and non-site-specific targeting, hence leading to prolongation of in vivo circulation time from several minutes to several hours. This trait enhances the passive delivery of drugs to tissues having leaky blood vessels, such as tumors and atherosclerotic plaques (Tanaka et al. 2004; Deguchi et al. 2006). In this system, after a polymerization reaction, drugs can be immobilized on the surface of polymers (Luo et al. 2011), and drugs may be released by desorption, diffusion, or nanoparticle erosion in the target tissue (Torchilin 2008). The polymers used in the formulation of polymer-drug conjugates can be utilized for targeting ligand conjugation, in order to create biologically targeted therapeutics (Wang et al. 2008a).

9.5.2 Toxicity Effects of Nanoparticles

Despite the potential benefits of nanoparticles, there is a growing concern about the interaction of nanoparticles with the environment. Although metal nanoparticles have far-reaching industrial and medical applications, there is a lack of information about the effect of prolonged exposure to nanoparticles on human health and environment. The implications of using nanoparticles in therapeutics need to be assessed wholly before their large-scale production and application in this field (Finney and O'Halloran 2003; Canesi et al. 2008; Sharma et al. 2009).

Although every person gets exposed to nanometer-sized foreign particles which are present in our environment and get inhaled with every breath, a vast majority cause little ill-effect; however, occasionally, an intruder can cause considerable harm to the organism. Studies on the NP-induced toxicity have discovered that metal-based nanoparticles can affect the biological behavior at cellular, subcellular, organ, tissue, and protein levels. However, toxicity of nanoparticles depends on a number of factors, including size, aggregation, composition, crystallinity, surface functionalization, etc. Some nanoparticles can break through lung or dermal (skin) barriers and get entry in the circulatory and lymphatic systems of humans and animals, unsettling cellular processes and resulting into diseases (Braydich-Stolle et al. 2005; Huster et al. 2007; Kennedy et al. 2009; Lanone et al. 2009). Nanoparticles that enter the circulatory system are connected to incidences of arteriosclerosis, ultimately leading

to cardiac death. Exposure to a number of nanoparticles has also been found to be related to the occurrence of autoimmune diseases (Buzea et al. 2007).

Metal-based nanoparticles have been found to have different toxic effects on the environment and human health (Moreno-Garrido et al. 2015). Silver is found to have adverse toxic effects in the environment; its toxicity is largely believed to be due to free silver ions present in the aqueous phase. Free silver ions can cause adverse effects on all living beings which include permanent bluish gray discoloration of the skin or the eyes; exposure to soluble silver compounds may also lead to liver and kidney damage (Panyala et al. 2008). On the contrary, in comparison to silver, gold nanoparticles are inherently nontoxic. Further, copper oxide NPs are usually used in lower quantities and unlike other NPs, their hazardous effects are not well studied (Kahru and Savolainen 2010). Copper oxide NPs have been found to be more toxic to crustaceans and algae but only at somewhat higher concentrations. It has been reported that liver is one of the target organs for copper oxide nanoparticles once they get entry in the body via any of the possible routes (Nishimori et al. 2009; Xie et al. 2010). Similarly, toxic effects of ZnO NPs have also been found to be higher to aquatic animals than to their effect to bacteria. Reports have shown that ZnO NPs can reach various organs after systemic distribution and manifest hazardous effects on the lungs, kidney, liver, stomach, pancreas, tests, thymus, heart, spleen, brain, and blood (Wang et al. 2008b; Cho et al. 2011; Li et al. 2012; Vandebriel and De Jong 2012).

Although the toxic effects of nanoparticles have been found to affect living organisms, but still data on their toxicity remains scarce and scattered (Moreno-Garrido et al. 2015). Therefore, it is also very important to be familiar with the fact that not all nanoparticles are toxic; toxicity relies on chemical composition and shape of NPs, in addition to their size and particle aging. In fact, several types of nanoparticles have been found to be nontoxic, others can be rendered nontoxic, while some others emerge to have advantageous health effects (Buzea et al. 2007).

9.6 Future Prospects

Over the past few years, research on nanoparticles has grown tremendously, and they have become a part of our day-to-day lives. Owing to their unique properties, nanoparticles have shown immense potential both as antimicrobials and in therapeutics. The diversity in the antimicrobial effector mechanisms exhibited against microorganisms seems to offer the advantage of hindered to failed development of resistance making them superior to their conventional counterparts. Furthermore, they are known to offer excellent biocompatibility making them preferred research subjects in the field of nanomedicine. However, nanomedicine studies are still in their infancy, and the behavior of nanoparticles inside human body along with the long-term effects of their release in the environment is yet to be deciphered. This is one of the major challenges that need to be addressed before we start using nanoparticles as routine therapeutic agents. But still, nanotechnology is a budding science in the field of alternative medicines and can find massive applications at consumer ends.

References

Ahamed M, Siddiqui MA, Akhtar MJ, Ahmad I, Pant AB, Alhadlaq HA (2010) Genotoxic potential of copper oxide nanoparticles in human lung epithelial cells. Biochem Biophys Res Commun 396:578–583

Ahmad T, Wani IA, Manzoor N, Ahmed J, Asiri AM (2013) Biosynthesis, structural characterization and antimicrobial activity of gold and silver nanoparticles. Colloids Surf B: Biointerfaces 107:227–234

Alt V, Bechert T, Steinrücke P, Wagener M, Seidel P, Dingeldein E, Domann E, Schnettler R (2004) An in vitro assessment of the antibacterial properties and cytotoxicity of nanoparticulate silver bone cement. Biomaterials 25:4383–4391

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55:373–399

Arvizo R, Bhattacharya R, Mukherjee P (2010) Gold nanoparticles: opportunities and challenges in nanomedicine. Expert Opin Drug Deliv 7(6):753–763

Aust SD, Chignell CF, Bray TM, Kalyanaraman B, Mason RP (1993) Free radicals in toxicology. Toxicol Appl Pharm 120(2):168–178

Babes L, Denizot B, Tanguy G, Le Jeune JJ, Jallet P (1999) Synthesis of iron oxide nanoparticles used as MRI contrast agents: a parametric study. J Colloid Interface Sci 212:474–482

Bansal P, Duhan JS, Gahlawat SK (2014) Biogenesis of nanoparticles. Afr J Biotechnol 13(28):2778–2785

Bera RK, Mandal SM, Raj CR (2014) Antimicrobial activity of fluorescent Ag nanoparticles. Lett Appl Microbiol 58:520–526

Biswas A, Bayer IS, Biris AS, Wang T, Dervishi E, Faupel F (2012) Advances in top-down and bottom-up surface nanofabrication: techniques, applications & future prospects. Adv Colloid Interf Sci 170(1–2):2–27

Braun GB, Friman T, Pang H-B, Pallaoro A, de Mendoza TH, Willmore A-MA, Kotamraju VR, Mann AP, She Z-G, Sugahara KN, Reich NO, Teesalu T, Ruoslahti E (2014) Etchable plasmonic nanoparticle probes to image and quantify cellular internalization. Nat Mater 13(9):904–911

Braydich-Stolle L, Hussain SM, Schlager J, Hofmann MC (2005) In vitro cytotoxicity of nanoparticles in mammalian germline stem cells. Toxicol Sci 88:412–419

Brayner R, Ferrari-Iliou R, Brivois N, Djediat S, Benedetti MF, Fiévet F (2006) Toxicological impact studies based on Escherichia coli bacteria in ultrafine ZnO nanoparticles colloidal medium. Nano Lett 6(4):866–870

Brooker RJ (2011) Genetics: analysis and principles, 4th edn. McGraw-Hill Science, New York. ISBN 978-0-07-352528-0

Buzea C, Pacheco II, Robbie K (2007) Nanomaterials and nanoparticles: sources and toxicity. Biointerphases 2:MR17–MR71s

Canesi L, Ciacci C, Betti M, Fabbri R, Canonico B, Fantinati A, Marcomini A, Poiana G (2008) Immunotoxicity of carbon black nanoparticles to blue mussel hemocytes. Environ Int 34:1114–1118

Castellano JJ, Shafii SM, Ko F, Donate G, Wright TE, Mannari RJ, Payne WG, Smith DJ, Robson MC (2007) Comparative evaluation of silver-containing antimicrobial dressings and drugs. Int Wound J 4(2):114–122

Chertok B, Moffat BA, David AE, Yu F, Bergemann C, Ross BD (2008) Iron oxide nanoparticles as a drug delivery vehicle for MRI monitored magnetic targeting of brain tumors. Biomaterials 29:487–496

Cho WS, Duffin R, Howie SE, Scotton CJ, Wallace WA, Macnee W, Bradley M, Megson IL, Donaldson K (2011) Progressive severe lung injury by zinc oxide nanoparticles; the role of Zn^{2+} dissolution inside lysosomes. Part Fibre Toxicol 8:27–42

Cui Y, Zhao Y, Tian Y, Zhang W, Lü X, Jiang X (2012) The molecular mechanism of action of bactericidal gold nanoparticles on *Escherichia coli*. Biomaterials 33(7):2327–2333

Das SK (2009) Gold nanoparticles: microbial synthesis and application in water hygiene management. Langmuir 25:8192–8199

Davis ME, Chen Z, Shin DM (2008) Nanoparticle therapeutics: an emerging treatment modality for cancer. Nat Rev Drug Discov 7:771–782

Deguchi J-O, Aikawa M, Tung C-H, Aikawa E, Kim D-E, Ntziachristos V, Weissleder R, Libby P (2006) Inflammation in atherosclerosis–visualizing matrix metalloproteinase action in macrophages in vivo. Circulation 114:55–62

Duncan R (2006) Polymer conjugates as anticancer nanomedicines. Nat Rev Cancer 6:688–701

Egger S, Lehmann RP, Height MJ, Loessner MJ, Schuppler M (2009) Antimicrobial properties of a novel silver-silica nanocomposite material. Appl Environ Microbiol 75:2973–2976

Elias A, Tsourkas A (2009) Imaging circulating cells and lymphoid tissues with iron oxide nanoparticles. Hematol Am Soc Hematol Educ Program 2009:720–726

Emerich DF, Thanos CG (2007) Targeted nanoparticle-based drug delivery and diagnosis. J Drug Target 15(3):163–183

Etrych T, Chytil P, Mrkvan T, Sırova, Rihova B, Ulbrich K (2008) Conjugates of doxorubicin with graft HPMA copolymers for passive tumor targeting. J Control Release 132(3):184–192

Farokhzad OC, Langer R (2006) Nanomedicine: developing smarter therapeutic and diagnostic modalities. Adv Drug Deliv Rev 58:1456–1459

Felson DT, Anderson JJ, Meenan RF (1990) The comparative efficacy and toxicity of second-line drugs in rheumatoid arthritis. Arthritis Rheum 33:1449–1461

Feng QL, Wu J, Chen GQ, Cui FZ, Kim TN, Kim JO (2000) A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*. J Biomed Mater Res 52(4):662–668

Feynman RP (1960) There's plenty of room at the bottom. Eng Sci 23(5):22–36

Finney LA, O'Halloran TV (2003) Transition metal speciation in the cell: insights from the chemistry of metal ion receptors. Science 300:931–936

Gerhardt LC, Jell GMR, Boccaccini AR (2007) Titanium dioxide (TiO_2) nanoparticles filled poly (D, L lactic acid) (PDLLA) matrix composites for bone tissue engineering. J Mater Sci Mater Med 95:69–80

Gordon T, Perlstein B, Houbara O, Felner I, Banin E, Margel S (2011) Synthesis and characterization of zinc/iron oxide composite nanoparticles and their antibacterial properties. Colloids Surf A Physicochem Eng Aspect 374(1–3):1–8

Hanagata N, Zhuang F, Connolly S, Li J, Ogawa N, Xu M (2011) Molecular responses of human lung epithelial cells to the toxicity of copper oxide nanoparticles inferred from whole genome expression analysis. ASC Nano 5(12):9326–9338

Hanley C, Layne J, Punnoose A, Reddy KM, Coombs I, Coombs A, Feris K, Wingett D (2008) Preferential killing of cancer cells and activated human T cells using ZnO nanoparticles. Nanotechnology 19(29):295103

Huang Z, Zheng X, Yan D, Yin G, Liao X, Kang Y, Yao Y, Huang D, Hao B (2008) Toxicological effect of ZnO nanoparticles based on bacteria. Langmuir 24(8):4140–4144

Huster D, Purnat TD, Burkhead LL, Ralle M, Fiehn O, Stuckert F, Olson NE, Teupser D, Lutsekno SJ (2007) High copper selectively alters lipid metabolism and cell cycle machinery in the mouse model of Wilson disease. J Biol Chem 282:8343–8355

Iavicoli I, Fontana L, Leso V, Bergamaschi A (2013) The effects of nanomaterials as endocrine disruptors. Int J Mol Sci 14:16732–16801

Jalal R, Goharshadi EK, Abareshi M, Moosavi M, Yousefi A, Nancarrow P (2010) ZnO nanofluids: green synthesis, characterization, and antibacterial activity. Mater Chem Phys 121(1–2):198–201

Jamdagni P, Khatri P, Rana JS (2016) Nanoparticles based DNA conjugates for detection of pathogenic microorganisms. Int Nano Lett. https://doi.org/10.1007/s40089-015-0177-0

Kahru A, Savolainen K (2010) Potential hazard of nanoparticles: from properties to biological and environmental effects. Toxicology 269(2–3):89–91

Karlsson HL, Cronholm P, Gustafsson J, Möller L (2008) Copper oxide nanoparticles are highly toxic: a comparison between metal oxide nanoparticles and carbon nanotubes. Chem Res Toxicol 21:1726–1732

Kasemets K, Ivask A, Dubourguier HC, Kahru A (2009) Toxicity of nanoparticles of ZnO, CuO and TiO_2 to yeast *Saccharomyces cerevisiae*. Toxicol In Vitro 23(6):1116–1122

Kaur P, Thakur R, Kumar S, Dilbaghi N (2011) Interaction of ZnO nanoparticles with food borne pathogens *Escherichia coli* DH5α and *Staphylococcus aureus* 5021 & their bactericidal efficacy. AIP Conf Proc 1393:153–154

Kaur P, Thakur R, Chaudhury A (2012) An in vitro study of the antifungal activity of silver/chitosan nanoformulations against important seed borne pathogens. Int J Sci Technol Res 1(6):83–86

Kaur P, Chaudhury A, Thakur R (2013) Synthesis of chitosan-silver nanocomposites and their antibacterial activity. Int J Sci Eng Res 4(4):869

Kaur P, Thakur R, Barnela M, Chopra M, Manuja A, Chaudhury A (2014) Synthesis, characterization and in vitro evaluation of cytotoxicity and antimicrobial activity of chitosan–metal nanocomposites. J Chem Technol Biotechnol. https://doi.org/10.1002/jctb.4383

Kaur P, Thakur R, Chaudhury A (2016) Biogenesis of copper nanoparticles using peel extract of *Punica granatum* and their antimicrobial activity against opportunistic pathogens. Green Chem Letters Rev 9(1):33–38

Kennedy DC, Lyn RK, Pezacki JP (2009) Cellular lipid metabolism is influenced by the coordination environment of copper. J Am Chem Soc 131:2444–2445

Kim JS, Kuk E, Yu KN, Kim JH, Park SJ, Lee HJ, Kim SH, Park YK, Park YH, Hwang CY, Kim YK, Lee YS, Jeong DH, Cho MH (2007) Antimicrobial effects of silver nanoparticles. Nanomedicine Nanotechnol Biol Med 3:95–101

Knaapen M, Borm PJA, Albrecht C, Schins RPF (2004) Inhaled particles and lung cancer, part A: mechanisms. Int J Cancer 109(6):799–809

Kolar M, Urbanek K, Latal T (2001) Antibiotic selective pressure and development of bacterial resistance. Int J Antimicrob Ag 17:357–363

Kooi ME, Cappendijk VC, Cleutjens KB, Kessels AG, Kitslaar PJ, Borgers M (2003) Accumulation of ultrasmall superparamagnetic particles of iron oxide in human atherosclerotic plaques can be detected by in vivo magnetic resonance imaging. Circulation 107:2453–2458

Kumar A, Pandey AK, Singh SS, Shanker R, Dhawan A (2011) Engineered ZnO and TiO_2 nanoparticles induce oxidative stress and DNA damage leading to reduced viability of *Escherichia coli*. Free Radic Biol Med 51(10):1872–1881

Lanone S, Rogerieux F, Geys F, Dupont A, Maillot-Marechal E, Boczkowski J, Lacroix G, Hoet P (2009) Comparative toxicity of 24 manufactured nanoparticles in human alveolar epithelial and macrophage cell lines. Part Fibre Toxicol 6:14–25

Lansdown ABG (2002) Silver I: its antibacterial properties and mechanism of action. J Wound Care 11:125–138

Li Q, Mahendra S, Lyon DY, Brunet L, Liga MV, Li D, Alvarez PJ (2008) Antimicrobial nanomaterials for water disinfection and microbial control: potential applications and implications. Water Res 42(18):4591–4602

Li J, Han T, Wei N, Du J, Zhao X (2009) Three-dimensionally ordered macroporous (3DOM) gold-nanoparticle-doped titanium dioxide (GTD) photonic crystals modified electrodes for hydrogen peroxide biosensor. Biosens Bioelectron 25(4):773–777

Li CH, Shen CC, Cheng YW, Huang SH, Wu CC, Kao CC, Liao JW, Kang JJ (2012) Organ biodistribution, clearance, and genotoxicity of orally administered zinc oxide nanoparticles in mice. Nanotoxicology 6:746–756

Lian T, Ho RJ (2001) Trends and developments in liposome drug delivery systems. J Pharm Sci 90:667–680

Lipovsky A, Nitzan Y, Gedanken A, Lubart R (2011) Antifungal activity of ZnO nanoparticles-the role of ROS mediated cell injury. Nanotechnology 22(10):105101

Liu G, Mena P, Perry G, Smith MA (2009) Metal chelators coupled with nanoparticles as potential therapeutic agents for Alzheimer's disease. Nanoneuroscience 1(1):42–55

Luo X, Matranga C, Tan S, Alba N, Cui XT (2011) Carbon nanotube nanoreservior for controlled release of anti-inflammatory dexamethasone. Biomaterials 32:6316–6323

Lushchak VI (2001) Oxidative stress and mechanisms of protection against it in bacteria. Biochemistry (Moscow) 66:476–489

Matsumura Y, Yoshikata K, Kunisaki S, Tsuchido T (2003) Mode of bactericidal action of silver zeolite and its comparison with that of silver nitrate. Appl Environ Microbiol 69(7):4278–4281

Maurer-Jones MA, Mousavi MPS, Chen LD, Bühlmann P, Haynes CL (2013) Characterization of silver ion dissolution from silver nanoparticles using fluorous-phase ion-selective electrodes and assessment of resultant toxicity to *Shewanella oneidensis*. Chem Sci 4:2564–2572

Mazooz G, Mehlman T, Lai TS, Greenberg CS, Dewhirst MW, Neeman M (2005) Development of magnetic resonance imaging contrast material for in vivo mapping of tissue transglutaminase activity. Cancer Res 65:1369–1375

Midander K, Cronholm P, Karlsson HL, Elihn K, Moller L, Leygraf C, Wallinder IO (2009) Surface characteristics, copper release, and toxicity of nanoand micrometer-sized copper and copper (II) oxide particles: a cross-disciplinary study. Small 5:389–399

Mirkin CA, Taton TA (2000) Semiconductors meet biology. Nature 40:626–627

Miyoshi N, Kume K, Tsutumi K, Fukunaga Y, Ito S, Imamura Y, Bibin AB (2011) Application of titanium dioxide (TiO_2) nanoparticles in photodynamic therapy (PDT) of an experimental tumor. AIP Conf Proc 1415:21. https://doi.org/10.1063/1.3667210

Mnyusiwalla A, Daar AS, Singer PA (2003) 'Mind the gap': science and ethics in nanotechnology. Nanotechnology 14:R9–R13

Mody VV, Nounou MI, Bikram M (2009) Novel nanomedicine-based MRI contrast agents for gynecological malignancies. Adv Drug Deliv Rev 61:795–807

Morales MP, Bomati-Miguel O, de Alejo RP, Ruiz-Cabello J, Veintemillas-Verdaguer S, O'Grady K (2003) Contrast agents for MRI based on iron oxide nanoparticles prepared by laser pyrolysis. J Magn Magn Mater 266:102–109

Moreno-Garrido I, Pérez S, Blasco J (2015) Toxicity of silver and gold nanoparticles on marine microalga. Mar Environ Res 111:60–73

Morones JR, Elechiguerra JL, CamachoA HK, Kouri JB, Ramirez JT, Yacaman MJ (2005) The bactericidal effect of silver nanoparticles. Nanotechnology 16(10):2346–2353

Mukherjee P, Bhattacharya R, Bone N, Lee YK, Patra CR, Wang S, Lu L, Secreto C, Banerjee PC, Yaszemski MJ, Kay NE, Mukhopadhyay D (2007) Potential therapeutic application of gold nanoparticles in B-chronic lymphocytic leukemia (BCLL): enhancing apoptosis. J Nanobiotechnol 5:4–16

Nishimori H, Kondoh M, Isoda K, Tsunoda S, Tsutsumi Y, Yagi K (2009) Silica nanoparticles as hepatotoxicants. Eur J Pharm Biopharm 72:496–501

Padmavathy N, Vijayaraghavan R (2008) Enhanced bioactivity of ZnO nanoparticles-an antimicrobial study. Sci Technol Adv Mater 9:1–7

Panyala NR, Pena-Mendez EM, Havel J (2008) Silver or silver nanoparticles: a hazardous threat to the environment and human health? J Appl Biomed 6:117–129

Park J, Fong PM, Lu J, Russell KS, Booth CJ, Saltzman WM, Fahmy TM (2009) PEGylated PLGA nanoparticles for the improved delivery of doxorubicin. Nanomedicine 5:410–418

Peng XH, Qian X, Mao H, Wang AY, Chen ZG, Nie S (2008) Targeted magnetic iron oxide nanoparticles for tumor imaging and therapy. Int J Nanomedicine 3:311–321

Prokop A, Davidson JM (2008) Nanovehicular intracellular delivery systems. J Pharm Sci 97:3518–3590

Rai RV, Bai JA (2011) Nanoparticles and their potential application as antimicrobials. In: Méndez-Vilas A (ed) Science against microbial pathogens: communicating current research and technological advances. Formatex Research Center, Spain, pp 197–209

Sawai J (2003) Quantitative evaluation of antibacterial activities of metallic oxide powders (ZnO, MgO and CaO) by conductimetric assay. J Microbiol Methods 54(2):177–182

Sawai J, Shoji S, Igarashi H, Hashimoto A, Kokugan T, Shimizu M, Kojima H (1998) Hydrogen peroxide as an antibacterial factor in zinc oxide powder slurry. J Ferment Bioeng 86(5):521–522

Shah MA, Tokeer A (2010) Principles of nanoscience and nanotechnology. Narosa Publishing House, New Delhi
Sharma VK, Yngard RA, Lin Y (2009) Silver nanoparticles: green synthesis and their antimicrobial activities. Adv Colloid Interf Sci 145:83–96
Sharma TK, Chopra A, Sapra M, Kumawat D, Patil SD, Pathania R, Navani NK (2012) Green synthesis and antimicrobial potential of silver nanoparticles. Int J Green Nanotechnol 4(1):1–16
Shenderova OA, Zhirnov VV, Brenner DW (2002) Carbon nanostructures. Crit Rev Solid State Mater Sci 27(3–4):227–356
Shin SH, Ye MK, Kim HS, Kang HS (2007) The effects of nano-silver on the proliferation and cytokine expression by peripheral blood mononuclear cells. Int Immunopharmacol 7:1813–1818
Siegel RW (1993) Nanostructured materials- mind over matter. Nanostruct Mater 3:1–18
Singleton P (2004) Bacteria, in biology, biotechnology and medicine, 6th edn. Wiley, West Sussex
Sinha R, Karan R, Sinha A, Khare SK (2011) Interaction and nanotoxic effect of ZnO and Ag nanoparticles on mesophilic and halophilic bacterial cells. Bioresour Technol 102:1516–1520
Smalley RE (1999) U.S. Congress Testimony, p2. http://www.sc.doe.gov//bes/Senate/smalley.pdf
Sondi I, Sondi BS (2004) Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for gram-negative bacteria. J Colloid Interface Sci 275(1):177–182
Sortino S (2012) Photoactivated nanomaterials for biomedical release applications. J Mater Chem 22:301–318
Syed MA (2014) Advances in nanodiagnostic techniques for microbial agents. Biosens Bioelectron 51:391–400
Tanaka T, Shiramoto S, Miyashita M, Fujishima Y, Kaneo Y (2004) Tumor targeting based on the effect of enhanced permeability and retention (EPR) and the mechanism of receptor-mediated endocytosis (RME). Int J Pharm 277(1–2):39–61
Taniguchi N (1974) On the basic concept of nanotechnology. In: Proceedings of the international conference production engineering, Tokyo, Part II, Japan Society of Precision Engineering, pp 18–23
Thannickal VJ, Fanburg BL (2000) Reactive oxygen species in cell signaling. Am J Physiol Lung Cell Mol Physiol 279(6):L1005–L1028
Thill A, Zeyons O, Spalla O, Chauvat F, Rose J, Auffan M, Flank AM (2006) Cytotoxicity of CeO_2 nanoparticles for *Escherichia coli*. Physico-chemical insight of the cytotoxicity mechanism. Environ Sci Technol 40:6151–6156
Tian L, Lin B, Wu L, Li K, Liu H, Yan J, Liu X, Xi Z (2015) Neurotoxicity induced by zinc oxide nanoparticles: age-related differences and interaction. Sci Rep 5:16117
Torchilin VP (2005) Recent advances with liposomes as pharmaceutical carriers. Nat Rev Drug Discov 4:145–159
Torchilin V (2008) Multifunctional pharmaceutical nanocarriers. Springer, New York
Uddin MJ, Mondal D, Morris CA, Lopez T, Diebold U, Gonzalez RD (2011) An in vitro controlled release study of valproic acid encapsulated in a titania ceramic matrix. Appl Surf Sci 257:7920–7927
Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M (2006) Free radicals, metals and antioxidants in oxidative stress induced cancer. Chem Biol Interact 160(1):1–40
Vandebriel RJ, De Jong WH (2012) A review of mammalian toxicity of ZnO nanoparticles. Nanotechnol Sci Appl 5:61–71
Verma S, Gokhale R, Burgess DJ (2009) A comparative study of top-down and bottom-up approaches for the preparation of micro/nanosuspensions. Int J Pharm 380(1–2):216–222
Vollath D (2008) Nanomaterials: an introduction to synthesis, properties and application. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, pp 1–20
Wagner V, Dullaart A, Bock A-K, Zweck A (2006) The emerging nanomedicine landscape. Nat Biotechnol 24:1211–1217
Walker B, Barrett S, Polasky S, Galaz V, Folke C, Engström G, Ackerman F, Arrow K, Carpenter S, Chopra K, Daily G, Ehrlich P, Hughes T, Kautsky N, Levin S, Mäler KG, Shogren J, Vincent J, Xepapadeas T, de Zeeuw A (2009) Environment. Looming global-scale failures and missing institutions. Science 325:1345–1346

Wang AZ, Gu F, Zhang L, Chan JM, Radovic-Moreno A, Shaikh MR, Farokhzad OC (2008a) Biofunctionalized targeted nanoparticles for therapeutic applications. Expert Opin Biol Ther 8(8):1063–1070

Wang B, Feng W, Wang M, Wang T, Gu Y, Zhu M, Ouyang H, Shi J, Zhang F, Zhao Y, Chai Z, Wang H, Wang J (2008b) Acute toxicological impact of nano and submicro scaled zinc oxide powder on healthy adult mice. J Nanopart Res 10:263–276

Wang X, Li J, Wang Y, Cho KJ, Kim G, Gjyrezi A, Koenig L, Giannakakou P, Shin HJ, Tighiouart M, Nie S, Chen ZG, Shin DM (2009) HFT-T, a targeting nanoparticle, enhances specific delivery of paclitaxel to folate receptor-positive tumors. ACS Nano 3:3165–3174

Wei W, Xu C, Wu H (2006) Magnetic iron oxide nanoparticles mediated gene therapy for breast cancer – an in vitro study. J Huazhong Univ Sci Technol Med Sci 26:728–730

Xie G, Sun J, Zhong G, Shi L, Zhang D (2010) Biodistribution and toxicity of intravenously administered silica nanoparticles in mice. Arch Toxicol 84:183–190

Yamamoto O (2001) Influence of particle size on the antibacterial activity of zinc oxide. Int J Inorg Mater 3(7):643–646

Yun H, Kim JD, Choi HC, Lee CW (2013) Antibacterial activity of CNT-Ag and GO-Ag nanocomposites against Gram-negative and Gram-positive bacteria. Bull Kor Chem Soc 34:3261–3264

Zhang L, Granick S (2006) How to stabilize phospholipid liposome (using nanoparticles). Nano Lett 6:694–698

Zhang L, Ding Y, Povey M, York D (2008) ZnO nanofluids–a potential antibacterial agent. Prog Nat Sci 18(8):939–944

Zhang Y, Nayak TR, Hong H, Cai W (2013) Biomedical applications of zinc oxide nanomaterials. Curr Mol Med 13(10):1633–1645

Zhou JC, Yang ZL, Dong W, Tang RJ, Sun LD, Yan CH (2011) Bioimaging and toxicity assessments of near-infrared up conversion luminescent $NaYF_4$: Yb, Tm nanocrystals. Biomaterials 32(34):9059–9067

Application of Nanotechnology for Cancer Treatment

10

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10.1 Introduction

Cancer is a multifactorial, heterogeneous disease affecting mankind. Cancer is defined as the uncontrolled proliferation of cells in which apoptosis doesn't play its part well. It's a complex process at the genetic and phenotypic level which creates major issues with therapeutic resistance. Cancer has become a big threat to mankind worldwide. Despite the progress in chemotherapeutic treatment, a major challenge to the available therapeutic regimes is the significant metastatic property of the cancer cells. So the alternative therapies that can alter the metastatic potential of cancer cells are now becoming the major focus of researchers worldwide. The efficacy of many drugs is often limited by their potential to reach the site of therapeutic action due to various problems such as – bioavailability, stability and solubility, reach to site of action, increases the chances of side effects.

Shikonin has a major role in multiple therapeutic areas including diabetes and cancer; its mechanistic on pain modulation via sodium channel modulation has been reported from our laboratory by Gupta et al. (2016). The role of nanotechnology has come in many aspects and is radically changing the treatment pattern of various diseases and cancer in particular. It recognizes the cancerous cells very selectively and targets the drug delivery. It ensures enhanced permeability and retention to localize in solid tumors and not in healthy tissues. Cancer nanotechnology is rising very enthusiastically as a major advancement in detection, diagnosis, and treatment of the disease. Drug delivery systems include liposomes, polymeric micelles, dendrimers, nanospheres, nanocapsules, and nanotubes as reported by Praetorius and Mandal (2007) and Park (2007). Nanotechnology-based constructs are available in the market, including liposomal doxorubicin (Doxil, Centocor Ortho Biotech Products LP) and albumin-bound paclitaxel (Abraxane, Abraxis BioScience).

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Recently, significant advancement has been made in the development of biosensor-based protocols toward the detection of microbes as well as synthesis of nanoparticles possessing antimicrobial activity which have been reported from our laboratory by Arora et al. (2011, 2012, 2013). Furthermore, several nanoformulations have been reported from our laboratory such as Ag/chitosan having significant antifungal activity against *A. flavus*, *A. alternata*, and *R. solani*, by Kaur et al. (2012). Chitosan/silver nanocomposites exhibited high antimicrobial activity against *Micrococcus luteus* MTCC 1809, *Pseudomonas aeruginosa* MTCC 424, and *Salmonella enterica* MTCC 1253 from our laboratory by Kaur et al. (2013). Similarly, chitosan-metal oxide nanoparticles showed antimicrobial activity against *Staphylococcus aureus* MTCC 1809, *Pseudomonas aeruginosa* MTCC 424, and *Salmonella enterica* MTCC 1253 by Kaur et al. (2014). Similarly, green biosynthesis of nanoparticles (Bansal et al. 2014) is a very environment-friendly approach in the field of synthesis of nanoparticles. Biogenesis of copper nanoparticles from peel extracts from *Punica granatum* is also reported from our laboratory (Kaur et al. 2016) which showed high antibacterial activity against opportunistic pathogens such as *Micrococcus luteus* MTCC 1809, *Pseudomonas aeruginosa* MTCC 424, *Salmonella enterica* MTCC 1253, and *Enterobactor aerogenes* MTCC 2823. Concerted efforts are being devoted for developing strategies for early detection, diagnosis, and treatment of cancer worldwide (Kulhari et al. 2013). Great strides have been made in early detection of metastatic tumor in local and distant lymph nodes in humans by Wunderbaldinger et al. (2002) and application of SPIO nanoparticle-aptamer bioconjugates for combined prostate cancer imaging and therapy by Wang et al. (2008) and Mousa and Bharali (2011). Daunorubicin, doxorubicin, and vincristine active against Kaposi's sarcoma, breast cancer, and non-Hodgkin's lymphoma are **approved drugs available in the market** (Vanna et al. 2014)**.** Albumin-bound paclitaxel ABI-007 nanoparticles have shown promising results against patients with metastatic breast cancer in Phase III trial as reported by Gradishar et al. (2005).

10.2 Magnitude of the Problem

According to the GLOBOCAN project of IARC, nearly 14 million new cases of cancer in 184 countries were recorded worldwide in 2012. The cancer statistics indicates, in the USA alone, 1.6 million people are diagnosed and 585,000 are expected to die. It's an emerging major public health problem depicting a great threat to the world population. As per Indian population census data, the rate of mortality due to cancer is very high and alarming. According to recent reports from ICMR (Indian Council of Medical Research, New Delhi), 1300 people die every day in India due to cancer. It is the second most common disease with maximum mortality with approximately 0.3 million deaths annually accounting for about 23% and 7% deaths in the USA and India, respectively. The burden of cancer to Indian society is much greater than in developed countries and is expected to grow faster over the period of time. In one estimate, the world's population is going to become

7.5 billion by 2020, and approximations predict that about 15.0 million new cancer cases will be diagnosed, with deaths of about 12.0 million cancer patients anticipated. Even after having good advancements in diagnosis and treatment, cancer is still a big threat to our society (Kulhari et al. 2013). The National Cancer Registry Program has provided a picture of the cancer pattern in India. Over 800,000 new cancer cases have been registered in India every year out of which 35–50% of all cancer cases are associated in men and 17% in women. Therefore, cancer poses a big threat which can be controlled to a large extent by various treatments like surgery, radiation, chemotherapy, and other recent biotechnological and nanotechnological interventions.

10.3 Treatment

Currently cancer therapies available are surgery, radiation, and chemotherapy, but all these have risk to normal tissues, or incomplete eradication of cancer cells is not certain. Chemotherapy is a standard treatment for most disseminated cancers, but its clinical benefits are reduced by dose-limiting toxicities and the emergence of cancer cells which are resistant to chemotherapeutic drugs. Various therapeutic strategies like blockage of cell signaling, prostaglandin production, inflammation, cell-cycle proteins, invasion, anti-apoptosis, cellular proliferation, and drug resistance gene (Aggarwal et al. 2006) are available, but still there is no single best treatment or any therapeutic strategy that can deal with all different types of cancer. The impact of many drugs is often restricted by their potential to reach the site of therapeutic action. The best result can be obtained by utilizing combinatorial therapies as compared to single one; designing chemotherapeutics drugs with combination of several drugs is the strategy used to deal with multiple drug-resistant cells in advance stages of cancers (Kulhari et al. 2013). In order to overcome this problem, development of new therapy against tumor cells is today's requirement to deal with this deadly disease. However, application of nanotechnology in drug delivery system has opened up new possibilities in sustained and targeted release of drugs.

10.4 Nanotechnology

Nanotechnology is an interdisciplinary research field that involves chemistry, engineering, biology, as well as medicine and has great potential for early detection, accurate diagnosis, and personalized treatment of cancer (Cai and Chen 2007). Nanoparticles are typically smaller in size, which is below a hundred nanometer in diameter as compared to large biological molecules such as enzymes, receptors, and antibodies. It's an upcoming field with extensive applications. According to the US National Cancer Institute (Office of Technology and Industrial Research 2006) https://www.cancer.gov/sites/nano, "Nanotechnology will change the very foundations of cancer diagnosis, treatment, and prevention." Nanotechnology helps in chemotherapies directly as well as selectively to target cancerous cells and neoplasms,

helps in the surgical removal, and enhances the therapeutic efficacy of radiation treatment. This will help the patient with decreased risk to cancer and increased survival period. Nanotechnology helps to provide the technical power and tools in developing new diagnostics, therapeutics, and preventive measures for cancer treatment with the following advancements described below:

- Research tools have been identified to target cancer cells.
- Agent to observe the predictive molecular changes and prevent precancerous cells to become malignant cells.
- Imaging agents and diagnostics to detect cancer at the early stage.
- Actual systems to provide real-time assessments of therapeutic and surgical efficacy.
- Novel methods to manage with the quality of life of patient.

Applications of nanotechnology in the field of medical science have enhanced multiple times in several directions in the past decade, thereby nanotechnology definition has been widened. Depending on definitions, four ingredients are important in identification of a nanotechnology tool: these are (1) the characteristic size of the tool has to be nano, (2) the tool should be man-made, (3) the device has to depict attributes that can only appear because of the nanoscopic dimensions, and (4) construction of appropriate mathematical models should predict the typical behavior of the device. It comes as an emerging field in medical science which has been the interdisciplinary research of biology, medicine, pharmacology, toxicology, chemistry, engineering, and material science along with mathematics, and it is anticipated to make a major breakthrough to solve unsolved medical issues.

10.4.1 Role of Nanotechnology in Detection of Cancer

Detection of cancer is of paramount importance using nanotechnology-based approach. It generates a special approach with comprehensive technology aim for directing against cancer via early diagnosis, prediction, prevention, and personalized therapy encompassing medicine. Nanotechnology would play an important role in target-specific drug therapy and designing ways for early diagnosis of diseases. Nanotechnology can provide fast and sensitive identification of cancer-related molecules, to observe molecular changes even if they are present only in a low percentage of cells. A more useful method called “tagging” is required which can preferentially attach to cancerous cells and can enhance their visibility scans several fold. Metal oxide nanoparticles coated with antibodies which bind to a certain receptors are used which generate strong signal in cancerous cells than in normal cells through CT and MRI scans. Nanomaterials like SPIO and USPIO are widely used under various trademarks for imaging for different types of cancers (Mousa and Bharali 2011).

Reimer and Tombach (1998) showed that Kupffer cells can efficiently uptake supermagnetic iron oxide (SPIO) nanoparticles and increase the contrast between

healthy and tumor cells. By using this technique, liver tumors or metastases which are as small as 2–3 mm can be detected. Wang et al. (2008) showed synthesized TCL-SPION-Apt bioconjugate used for combined prostate cancer imaging and therapy in vitro. Ultra-small supermagnetic iron oxide (USPIO) brand name Combidex® is used for imaging. Lymphotropic superparamagnetic nanoparticles along with MRI can detect metastatic tumor in local and distant lymph nodes in humans (Wunderbaldinger et al. 2002). Iron oxides improve the two-dimensional MR angiography technique quality by decreasing background signal and increasing intravascular signal. Cancer cells would be surrounded by nanoparticles which will allow cancerous growth to be recognized very easily. The presence of strong signal indicates that the very initial stage of tumors could be identified by this technique. The joint effort of "The Center of Cancer Nanotechnology Excellence at Stanford University" and "National Cancer Institute's (NCI) Alliance for Nanotechnology in Cancer" has devised a process in which nanoparticles of gold are used for detection and attachment to cancer cells. Nanoparticle has a multifunctional role in targeted drug delivery, observation of therapeutic delivery, and detection of the drug response (Wang et al. 2008). When rays thrown from a device that is embedded inside a standard endoscope (e.g., a colonoscope) reach tumor cells that have attached to the gold nanoparticles, they differentiate from the normal cells and can be separated, and this can provide solution to the problem. Researchers are looking forward to find out new methods to improve the ways to detect cancer before it gets metastasize.

Nanowires have incredible properties of selectivity and specificity that detects the presence of altered genes which are associated with cancer that help researchers in pinpointing the exact location of those changes. Nano-sized sensing wires are laid down across a microfluidic channel. When the particles move via microfluidic channel, then nanowire sensors choose the molecular signatures of these particles and immediately pass them to electrodes. Nanoscale cantilevers are microscopic, flexible beams which resemble a row of diving boards and are developed with the help of semiconductor lithographic techniques. These can be coated with particles which have the capability of binding to specific substrates, for example, DNA complementary to a unique gene sequence. Such micron-sized instruments, consisting of many nanometer-sized cantilevers, are capable of detecting even single molecules of DNA or protein. As a tumor cell releases its molecular products, the antibodies present on the cantilever fingers selectively combine to these released proteins. These antibodies have been specialized to choose one or more unique and specific molecular expressions from a tumor cell. Binding event will change the physical properties of cantilevers. This change can be observed in real time and provide information not only about the presence or absence but also about the concentration of different molecular expressions.

Nanoshells can be designed in such a way to absorb light of different frequencies and generate heat (hyperthermia). Nanoshells have a core of silica and a metallic outer layer and can be given safely in animal model, and they will preferentially accumulate in cancer lesion sites because of their characteristic size. As the cancer cells take up the nanoshells via active targeting, now scientists can apply

near-infrared light which is absorbed by the nanoshells and create an intense heat inside the tumor which will selectively kill tumor cells without meddling with neighboring healthy cells. In the same way, new targeted magnetic nanoparticles are in development process that will be visible through magnetic resonance imaging (MRI) and can destruct cells by hyperthermia.

10.4.2 Broad Classification of Nanocarriers

Nanotechnology is an important field of interest in science and technology and has been developing fast with the advent of several carriers having vesicular and particulate systems like liposomes, dendrimers, niosomes, ethosomes, transfersomes, micelles, polymeric protein lipid nanoparticles, carbon nanotubes, and nanoshell as shown in Table 10.1.

10.4.2.1 Liposome

Liposomes are defined as spherical lipid colloid, with a bilayer membrane surrounded with central aqueous space and composed of phospholipids, and are most widely studied. Doxil or Caelyx, a PEGylated liposomal doxorubicin, was the first nanoparticle formulation approved for application in the clinic by the US Food and Drug Administration (FDA) in 1995 (Barenholz 2012). Liposomes are vesicles that are lipid based and have the capability to carry payloads in either an aqueous compartment or embedded in the lipid bilayer. The delivery of these liposomes to cancer cells often depends on passive targeting and is based on the enhanced permeability and retention (EPR) effect, and a leaky tumor vasculature is necessary for this. 5-Fluorouracil has been prepared by the film hydration method by varying the lipid phase and hydration conditions of dry lipid. Lyophilized liposomes' drug release acted as a reservoir systems for continuous delivery of the encapsulated drug (Glavas-Dodov et al. 2003). Melatonin-loaded liposomes were prepared having low polydispersity and nanometric size range (Dubey et al. 2007). Metformin hydrochloride was prepared using varying concentrations of phosphatidylcholine and cholesterol. The in vitro drug release studies showed that it sustained for >12 h (Divakar et al. 2013).

10.4.2.2 Transfersomes

Transfersomes are used for the poorly soluble drugs, which have the problem with oral drug delivery. It was first introduced in the early 1990s and later became increasingly apparent in drug target. Its formulation varies with different concentration of nonionic surfactant, soya lecithin, and carbopol 940 (Gupta et al. 2012). It is widely accepted in pharmaceutical and established compounds which penetrate under the skin barrier along the naturally occurring transcutaneous moisture gradient. Low and high molecular weight drugs like anesthetic, analgesic, corticosteroids, anticancer, sex hormone, insulin, gap junction protein, and albumin use this as carrier (Walve et al. 2011). Meloxicam (MX)-loaded cationic transfersomes as skin delivery carriers have greater MX skin permeation as compared to

Table 10.1 Broad classification of nanocarriers

Nanocarriers	Drugs	Attributes	References
Liposomes	Doxil or Caelyx	First nanoparticle formulation approved for application in the clinic by the US Food and Drug Administration (FDA) in 1995	Barenholz (2012)
	5-Fluorouracil	5-Fluorouracil has been prepared by the film hydration method by varying the lipid phase and hydration conditions of dry lipid. For the continuous delivery of the encapsulated drug, lyophilized liposomes act as reservoir	Glavas-Dodov et al. (2003)
	Melatonin	Melatonin-loaded liposomes were prepared having low polydispersity and nanometric size range	Dubey et al. (2007)
	Metformin hydrochloride	Metformin hydrochloride was prepared using varying concentrations of phosphatidylcholine and cholesterol. The in vitro drug release studies showed that it sustained for >12 h	Divakar et al. (2013)
Transfersomes	Meloxicam (MX)	First introduced in the early 1990s and later became increasingly apparent in drug target. Its formulation varies with different concentration of nonionic surfactant, soya lecithin, and carbopol 940	Gupta et al. (2012)
		Low and high molecular weight drugs like sex hormone, anticancer, gap junction protein, corticosteroids, analgesic insulin, anesthetic, and albumin use this as carrier	Walve et al. (2011)
		Meloxicam (MX)-loaded cationic transfersomes act as skin delivery carriers; compared to conventional, MX has greater skin permeation due to the vesicle adsorption to and/or fusion with the stratum corneum. Meloxicam (MX)-loaded vesicles were prepared exhibiting from 40% to 70% EE. Skin permeation of MX is significantly greater compared to liposomes	Duangjit et al. (2013) Duangjit et al. (2011)
	Ibuprofen	Using various ratios of soya Span 80, Tween 80, and phosphatidylcholine and using lipid film hydration by rotary evaporation method of ibuprofen, transfersomes were prepared. Entrapment efficiency and the elasticity increase. In vitro skin permeation studies showed diffusion cell and release of the drug and flux even after 24 h	Irfan et al. (2012)
Ethosomes	Propranolol and trihexyphenidyl	Ethosomes are noninvasive delivery carriers that can make drugs reach in the deep layers of the skin and/or the systemic circulation	Godin and Touitou (2003)
	Cannabinoids, testosterone, and minoxidil	Encapsulated drug delivery of the highly lipophilic molecules like cationic drugs, e.g., propranolol and trihexyphenidyl, through the skin	Singh et al. (2015)

(continued)

Table 10.1 (continued)

Nanocarriers	Drugs	Attributes	References
Micelles		Polymeric micelles fall within 10–100 nm range that enhanced permeability and retention	Mohamed et al. (2014)
		Physiological changes on tumor, such as overexpressing the receptors on cancerous cell surface and enhancing the permeability and prolonging the retention	Fang et al. (2011)
	Daunomycin	Accumulation of daunomycin in OX26-immunoliposomes in rat brain to higher levels as compared to brain uptake of free daunomycin	Schnyder et al. (2005)
Dendrimers	Paclitaxel	Dendrimers are globular structures having polymers with a central core, repeating units of branches, and multivalent functional groups at the outer layer	Bakers (2009)
		The paclitaxel solubility was increased with the higher generation due to the dendritic architecture	Ooya et al. (2004)
Polymer-based platforms	Doxorubicin	Doxorubicin showed in vivo cytotoxicity against solid tumor with low toxicity	Estanqueiro et al. (2015)
	Tamoxifen Paclitaxel	Polymer-based doxorubicin showed in vivo cytotoxicity against solid tumor with low toxicity	Wong et al. (2007) and Ooya et al. (2004)
	Retinoic acid	Retinoic acid-loaded nanoparticles were more potent stimulators of hepatocyte DNA synthesis than its free form	Gao et al. (2002) and Cho et al. (2001)
Carbon nanotubes	Paclitaxel	Enhanced permeability and retention of cancer cells	Zhuang et al. (2016)
		Emission computed tomography	
Nanoshells		Gold nanoparticles which are labeled with various radionuclides are widely explored for single-photon emission computed tomography (SPECT)	Zhao et al. (2016)
	Doxorubicin	Mn-porphyrin-conjugated Au nanoshells encapsulating doxorubicin for potential magnetic resonance imaging and light-triggered synergistic therapy of cancer	Jing et al. (2014)
	10-Hydroxycamptothecin	HCPT-AuNPs showed more cytotoxic effects on the MDA-MB-231 cell line	Wu et al. (2011)
		AuNPs were not only acting as a carrier but are also playing an active role in mediating biological effects	Bao et al. (2016)

conventional liposomes and MX suspensions. The skin permeation capability by the vesicles prepared in this study may be due to the vesicle adsorption to and/or fusion with the stratum corneum. Meloxicam (MX)-loaded vesicles were prepared exhibiting from 40 to 70% EE. It provided a significantly higher skin permeation of MX compared to liposomes (Duangjit et al. 2011, 2013). Ibuprofen transfersomes were prepared by using various ratios of soya phosphatidylcholine, Span 80, and Tween 80, using lipid film hydration by rotary evaporation method. Entrapment efficiency and the elasticity increase. In vitro skin permeation studies showed diffusion cell and release of the drug and flux even after 24 h (Irfan et al. 2012).

10.4.2.3 Ethosomal

Ethosomal systems are highly sophisticated which are characterized by the simplicity in their preparation, safety, and efficacy in the expansion of their application (Touitou et al. 2001). It can be tailored made for greater delivery of active agents. Ethosomes are noninvasive delivery carriers that can make drugs reach in the deep layers of the skin and/or the systemic circulation. It enabled the drug to be encapsulated and deliver through the skin the highly lipophilic molecules like cannabinoids, testosterone, and minoxidil, as well as cationic drugs e.g., propranolol and trihexyphenidyl. It consists of phospholipid bilayers along with ethanol, and this gives efficient permeation and penetration of the drugs. It delivers large-sized molecules such as peptides and protein molecules (Singh et al. 2015).

10.4.2.4 Micelles

Micelles are among the most aspiring delivery systems which can be used in nanomedicine. The size range of most polymeric micelles falls within 10–100 nm, which ensures that they can preferentially exit from the circulation at tumor site through the enhanced permeability and retention effect. Their unique structure allows them to dissolve hydrophobic drugs and prolongs the circulatory half-life which ultimately increase therapeutic efficacy (Mohamed et al. 2014). Polymeric micelles are formed by the self-assembling of amphiphilic copolymers with certain conditions in aqueous environments. Polymeric micelles of 20–200 nm are used for therapeutic delivery. It is found that the polymeric micelles could be associated with physiological changes on tumor, such as overexpressing the receptors on cancerous cell surface and enhancing the permeability and prolonging the retention (Fang et al. 2011). Higher levels of accumulation of daunomycin in OX26-immunoliposomes in rat brain as compared to brain uptake of free daunomycin (Schnyder et al. 2005).

10.4.2.5 Dendrimers

Dendrimers are globular structures having polymers with a central core, repeating units of branches, and multivalent functional groups at the outer layer. In recent years, dendrimers have begun to be applied to a variety of cancer treatments to improve the safety and effectiveness of convectional therapeutics. The new approach has been successfully tested in preclinical in animal model as cancer therapeutic

platform and will hopefully enter clinical trials soon (Bakers 2009). The paclitaxel solubility was increased with the higher generation due to the dendritic architecture (Ooya et al. 2004)

10.4.2.6 Polymeric Nanoparticles

Polymeric nanoparticles can either attach covalently or by encapsulation. By mixing the drug with the polymers, capsules may be formed. Currently, polylactide (PLA) and poly (lactideco-glycolide) (PLGA) are polymeric biodegradable nanoplatforms that are used for synthesis of FDA-approved nanomedicine. Polymer-based doxorubicin showed in vivo cytotoxicity against solid tumor with low toxicity (Estanqueiro et al. 2015). Various drugs like retinoic acid-, tamoxifen-, paclitaxel-, and doxorubicin-loaded nanoparticles were more potent stimulators of hepatocyte DNA synthesis than its free form (Wong et al. 2007; Ooya et al. 2004; Gao et al. 2002; Cho et al. 2001).

10.4.2.7 Carbon Nanotubes

The advantage of carbon nanotubes is that they have the capability to get into cells and deliver its molecules in cytoplasm. Nanoparticles have a lot of attachment sites for ligands and internal cavity that is used for therapeutic or diagnostic purposes. It can be highly useful in the future as it has electrical and thermal conductivity. The length and diameter of carbon nanotubes are more important to make desirable smaller and thicker and biodegradable access. Paclitaxel carbon nanoshell which enhanced the permeability and retention of cells has been reported by Zhuang et al. (2016).

10.4.2.8 Nanoshells

Nanoshells are more effective against small molecules like RNA, DNA, and proteins. The gold core used in nanoshell is nontoxic and can be released from the conjugate due to its photo-physical properties. Drugs attached to nanoshell either by covalent or ionic bonds or by adhesion. In the context of cancer diagnosis, gold nanoparticles which are labeled with various radionuclides are widely explored for single-photon emission computed tomography (SPECT) (Zhao et al. 2016). 10-Hydroxycamptothecin (HCPT)-AuNP conjugates showed cytotoxic effect in vitro and antitumor effect in vivo. In vitro it showed drug release continuously for 120 h. It has more cytotoxic effects on the MDA-MB-231 cell line as compared to free HCPT. More importantly, HCPT-AuNPs of an average diameter of 50 nm (HCPT-AuNPs-50) had the greatest effect. HCPT-AuNPs-50 administration showed the highest tumor-suppressing activity against MDA-MB-231 tumor compared to all treated groups. Overall AuNPs played as carrier as well as an active role in mediating biological effects (Bao et al. 2016). HCPT-AuNPs which showed more cytotoxic effects on the MDA-MB-231 cell line have been reported by Wuet al. (2011). DOX-loaded Au nanoshells were more effective to kill the cancer cells than the Au nanoshells without DOX or DOX alone (Mohammad and Yusof 2014).

10.4.3 Role of Nanotechnology in Treatment of Cancer and Clinical Outcome

Currently the cancer therapies are limited to radiation, chemotherapy, and surgery, but all these therapies risk damage to normal tissues or can result in incomplete removal of the cancer. Nanotechnology offers the means to direct therapies at cancerous cells directly and selectively. Conventionally cytotoxic drugs destroy healthy cells as well as tumor cells, which will lead to adverse side effects like fatigue, nausea, hair loss, neuropathy, as well as compromised immune function (Kulhari et al. 2013). Nanoparticles can possibly be used as drug carriers to deliver directly to the tumor while passing to healthy tissue. Nanocarriers offer many advantages over conventional chemotherapy. They can not only protect drugs from being degraded in the body before reaching to their target, they also help in increasing the absorption of drugs into tumors as well as into the cancerous cells themselves. It makes easier for oncologists to assess how they work and allows better control over the timing and distribution of drugs to the tissue. Furthermore it also helps in avoiding side effects by preventing drugs from interacting with normal cells.

Nanocarrier-based drugs which depend upon passive targeting via the process called “enhanced permeability and retention” are available in the market. Due to their size and surface properties, some nanoparticles can escape through blood vessel walls into tissues. Besides, tumors are likely to have leaky blood vessels and defective lymphatic drainage, causing nanoparticles to accumulate there and thus accumulating the attached cytotoxic drug where it’s required. This protects healthy tissue and so greatly reduces adverse side effects. In active targeting, based on the molecules that they express on their cell surface, nanoparticles actively target drugs to the cancer cells. Molecules binding particular cellular receptors can be attached to a nanoparticle for actively target cells expressing the receptor. Active targeting can also be used to load drugs into the cancerous cell by inducing the cell to absorb the nanocarrier (Byrne et al. 2008). Synthesis of a ternary conjugate heparin-folic acid-paclitaxel (HFT), loaded with additional paclitaxel, showed increase in the chemotherapeutics effect against human head and neck cancer cell line KB-3-1 with higher cytotoxicity compared to the free form of paclitaxel. It also helps in preventing tumors from developing drug resistance Wang et al. (2009). Combination of active targeting and passive targeting further diminishes the interaction of drugs with healthy tissue. Nanotechnology-enabled active and passive targeting enhances the effectiveness of a chemotherapeutics thus achieving greater tumor reduction along with lower doses of the drug.

10.4.3.1 Nanodrugs for Cancer Therapeutics

The application of nanotechnology provides significantly novel, effective treatments and perspectives in healthcare system. Nanomedicine can be defined as molecules like cisplatin, carboplatin, bleomycin, 5-fluorouracil, doxorubicin, dactinomycin, 6-mercaptopurine, paclitaxel, topotecan, vinblastine, and etoposide which are important molecules for synthesis of nanodrugs (Ali et al. 2011). The materials used most commonly for preparing nanoparticle carriers are dendrimers, polymer,

liposome, micelles, and inorganic and organic nanoparticles. The nanoparticle-based drug development appears to be effective, provided they have low side effects and targeted action on cancer cells. During the last few years, nanotechnology has witnessed great groundbreaking discoveries in different fields of medicine. Synthesis of nanoparticles has added a new dimension in the field of cancer therapy because of its unique and novel properties such as the small size, controlled release of drugs, and reduced toxic side effects (Jana et al. 2001; Sun and Xia 2002). Polymeric nanoparticles have a very important role to play in delivering such kinds of chemotherapeutic agents in a controlled manner.

Drug delivery through the nanoparticles makes it possible to have the desired concentration of drug in the particular location, thus minimizing the side effects and lessening the toxicity. The potential advantages of making nanoparticles will help in developing strategies as alternative drug delivery systems for cancer therapy. Recent development in using microfluidic devices for the preparation of drug-loaded nanoparticles with specific characteristics (such as size, composite, surface modification, structure, and rigidity) for better cancer treatment and diagnosis as well as to investigate the bio-nanoparticle interaction is reported as stated by Feng et al. (2016). Metallic nanoparticles can be prepared by using physical, chemical, and biological methods. However, biological synthesis is more reliable and eco-friendly and has received particular attention as compared to other methods. Biosynthesized silver nanoparticles from the red seaweed *Pterocladiella capillacea* also showed cytotoxicity on the liver cell cancer (HepG2) cell line (El-Kassas and Attia 2014). When chemotherapy is no longer effective in treating, then triple-negative breast cancer has a very poor prognosis. To counteract drug resistance, new drug delivery systems depending on nanoparticles have had significant success. Common chemotherapeutic drugs, such as paclitaxel, docetaxel and doxorubicin, are being designed using various nanotechnological approaches to improve drug delivery, while nanoparticle (NP)-based imaging technologies can monitor the tumor microenvironment and facilitate molecular targeting for effective lung cancer therapy (Hofferberth et al. 2016). Hayashi et al. (2015) prepared a novel nanoparticle "MDC" from milk-derived colloid and conjugated with doxorubicin (Dox) and found that there is an increase in cancer cell growth inhibition than MDC alone especially in cell lines which have high EGFR expression. It was reported that in mouse melanoma model, MDC-Dox significantly suppressed tumor growth when compared with free Dox.

Zhang et al. (2015) reported that paclitaxel (PTX) nanocrystals (PTX-NCs) and PEGylated PTX nanocrystals (PEG-PTX-NCs) were prepared and injected in breast cancer xenografted mice; results show that PEG-PTX-NCs showed significantly better tumor inhibition as compared to saline and PTX-NC groups after intravenous administration. In a model of lung tumor metastasis quantified by the luciferase activity, the PEG-PTX-NC group showed higher anticancer efficacy not only than saline and PTX-NC groups but also more than Taxol, achieving an overall 82% reduction. Katiyar et al. (2015) reported the formulation and evaluation of nanoparticles of rapamycin (RPM), along with a chemosensitizer (piperine, PIP), and showed improved bioavailability of 4.8-folds in combination with a chemosensitizer. In vitro

cell line study also showed higher efficacy of nanoparticles compared to free drug solution. Combinatorial use of PIP with RPM nanoparticles would be a promising approach in the treatment of breast cancer leading to improved oral bioavailability and efficacy of the drug (Martins et al. 2015). Polyelectrolyte complex beads/silver nanoparticles (beads/AgNPs) showed in vitro cytotoxicity significantly on the growth of colon cancer cells (Caco-2). Mittal et al. (2015) synthesized silver nanoparticles of *Potentilla fulgens* and evaluated for anticancer and antimicrobial properties. It showed cytotoxicity against various cancer cell lines from 0.2 to 12 $\mu g ml^{-1}$. Nanoparticles have the capability to kill cancer cells compared to normal cells. It also showed antimicrobial activity against both Gram-positive and Gram-negative bacteria. Patra et al. (2015) experiment the delivery of anticancer drug to the cancer cells by biosynthesis of gold and silver nanoparticles (b-AuNP and b-AgNP). Nanoparticles from *Butea monosperma* (BM) leaf extract were synthesized. Both b-AuNP and b-AgNP were stable in biological buffers and have biocompatibility toward normal endothelial cells (HUVEC, ECV-304) as well as toward cancer cell lines (B16F10, MCF-7, HNGC2, and A549). Administration of nanoparticle-based drug delivery systems (DDSs) using doxorubicin (DOX) showed significant inhibition of cancer cell proliferation (B16F10, MCF-7) as compared to pristine drug.

Therefore, in the near future, biosynthesized nanoparticles will be effective for the development of cancer therapy using nanotechnology approach. Cerium oxide nanoparticles act as a therapeutic agent both in vitro and in vivo OvCa cells. The result demonstrates that nanoceria (NCe) that is a nanoparticle of cerium oxide was able to suppress growth factor, migration and invasion of SKOV3 cells, VEGF165-induced proliferation, capillary tube formation, and activation of VEGFR2 and MMP2 in HUVEC cells (Giri et al. 2013). When nanoceria conjugates with folic acid (NCe), its concentration increases internally and inhibited cell proliferation. In vivo assay also showed less tumor burden compared to NCe alone, without any harm done to normal cells. Combination of NCe-FA with cisplatinum further decreases the tumor burden more significantly. Therefore, it has proven that NCe-FA showed marked inhibition of proliferation and angiogenesis in the xenograft mouse model. Thus, specific targeting of ovarian cancer cells by NCe-FA holds great potential as an effective therapeutic alone or in combination with standard chemotherapy. NPs can be made by using a wide variety of synthetic strategies of chemical composition, size, shape, and surface decoration. Furthermore, if radio-labeling is done with positron or gamma emitters, it results in potential diagnostic agents which target the organ or tissue with more selectivity and/or specificity enabling the acquisition of images with higher signal-to-contrast ratio. The use of of alpha or beta emitters leads to therapeutic agents with application in the field of radiotherapy (Koziorowski et al. 2016). Hou et al. (2016) showed that nanocarriers can achieve active targeting site that help in increasing the potential of chemotherapeutic drugs and decrease its side effects. Designing new drug delivery systems for the welfare of human beings to overcome the limitations of conventional formulations is being continually classified (Malam et al. 2009). Current nanotechnology-based drug delivery systems for cancer treatment, which include liposomes, polymeric micelles,

dendrimers, nanospheres, nanocapsules, and nanotubes, are already marketed (Praetorius and Mandal 2007; Park 2007).

Nanotechnology-based formulations are Doxil (liposomal doxorubicin) and Abraxane (albumin-bound paclitaxel) which are available in the market. Conventional chemotherapeutic agents attack on fast growing cells which are the property of neoplastic cells that is the reason it damages normal healthy cells such as cells in the bone marrow, macrophages, digestive tract, and hair follicles. In brain drug delivery system, the nanoparticles used should be biocompatible and rapidly biodegradable. Therefore, nanocarriers fabricated from natural polymers including polysaccharides and proteins are mainly used; this helps in low cytotoxicity, abundant surface functional groups, increased drug-binding capacity, and significant uptake into the targeted cells; natural polymer-based nanocarriers represent promising candidates for efficient drug and gene delivery to the brain (Elzoghby et al. 2016). Nanotechnology provides the vision to the researchers for studying and manipulating macromolecules in real time and most importantly the early detection of cancer progression which helps in saving number of lives. Various researches are trying to come up with more accurate nanotechnology-based cancer treatments in order to minimize the side effects of the conventional ones (Peer et al. 2007).

Nanotechnology also has the potential to produce entirely novel and highly effective therapeutic agents. It also helps in drug delivery system which has opened up new possibilities in sustained and targeted release of drugs. Cancer nanotechnology comes and raises new hopes in cancer treatment particularly in detection, diagnosis, and treatment of the disease. Its emergence has created vast opportunities for the development of nanoparticles in diagnostic and therapeutic areas.

10.4.3.2 Clinically Approved Drugs

Liposome-based bioactive compound daunorubicin (brand name DaunoXome), doxorubicin (Myocet), and vincristine (Onco TCS) active against Kaposi's sarcoma, breast cancer, and non-Hodgkin's lymphoma are **approved drugs available in the market** (Vanna et al. 2014)**.** Cytarabine (DepoCyt) and irinotecan (NL CPT) are active against glioblastoma and glioma, respectively, in Phase I/II trial, while cytarabine (DepoCyt) and cisplatin (SPI-77) active against leukemia and ovarian cancer are in Phase I/II trial. Doxorubicin (ThermoDox) active against liver cancer and breast cancer is in Phase III trial. Polymeric nanoparticle-based drugs like paclitaxel (Genexol-PM, NK105, Opaxi, NK911) are in Phase II and Phase III trials against various cancer cells. Doxorubicin (NK(11), camptothecin (CRLX101), and cisplatin (NC-6004) are in Phase II and Phase III trials. Gradishar et al. (2005) reported that albumin-bound paclitaxel ABI-007 nanoparticles exhibited greater efficacy against patients with metastatic breast cancer in Phase III trial. A few nanomedicines which are in clinical development are oxaliplatin (MBP-426), p53 gene (SGT53), and doxorubicin (MCC-465) and are in Phase I/II trial. Furthermore, a lot compounds have to be evaluated which are currently under intensive research worldwide.

10.5 Conclusions and Future Prospects

Cancer is one of the most fatal diseases, caused by the uncontrolled proliferation of malignant cells. In order to eradicate the cancer, various treatment approaches are available like surgical removal, chemotherapy, radiation, and hormone therapy, but all have some significant limitations and side effects. The main problem in conventional chemotherapy is that it fails to differentiate between cancerous cells and the normal body cells which cause organ damage and ultimately low survival rates. Cancer nanotechnology is emerging to play a vital role in detection, diagnosis, and treatment of disease. Drug delivery system includes liposomes, polymeric micelles, dendrimers, nanospheres, nanocapsules, and nanotubes. It potentially contributes to a positive move in clinical practice for a lifesaving approach. Some nanotechnology-based formulations have already been launched in the market, and many more are undergoing research and clinical trials. This cutting-edge technology has already given Abraxane, a nanomedicine against metastasis breast cancer in the current pharmaceutical industry. Paclitaxel an aluminum-bound nanoparticle is also available in the market. Many other nanomaterials like SPIO and USPIO nanoparticles are widely used for the imaging of various types of cancers under various trademarks. It has been revealed that over 70 nanomedicines are currently in clinical trials for cancer treatment and imaging. The contribution of nanotechnology is a positive move for saving precious lives of people worldwide, and the future appears bright.

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References

Aggarwal BB, Shishodia S, Sandur SK, Pandey MK, Sethi G (2006) Inflammation and cancer: how hot is the link? Biochem Pharmacol 72:1605–1621

Ali I, Rahis-Uddin SK, Rather MA, Wani WA, Haque A (2011) Advances in nano drugs for cancer chemotherapy. Curr Cancer Drug Targets 11(2):135–146

Arora P, Sindhu A, Dilbaghi N, Chaudhury A (2011) Biosensors as innovative tools for the detection of food borne pathogens. Biosens Bioelectron 28(1):1–12

Arora P, Sindhu A, Dilbaghi N, Chaudhury A, Rajakumar G, Rahuman AA (2012) Nano-regenerative medicine towards clinical outcome of stem cell and tissue engineering in humans. J Cell Mol Med 16(9):1991–2000

Arora P, Sindhu A, Kaur H, Dilbaghi N, Chaudhury A (2013) An overview of transducers as platform for the rapid detection of foodborne pathogens. Appl Microbiol Biotechnol 97(5):1829

Bakers RJ (2009) Dendrimer-based nanoparticles for cancer therapy. Hematology 1:708–719

Bansal P, Duhan JS, Gahlawat SK (2014) Biogenesis of nanoparticles. Afr J Biotechnol 13(28):2778–2785

Bao H, Zhang Q, Xu H, Yan Z (2016) Effects of nanoparticle size on antitumor activity of 10-hydroxycamptothecin-conjugated gold nanoparticles: in vitro and in vivo studies. Int J Nanomedicine 7(11):929–940

Barenholz Y (2012) Doxil -the first FDA-approved nano-drug: lessons learned. J Control Release 160(2):117–134

Byrne J, Betancourt T, Brannon-Peppas L (2008) Active targeting schemes for nanoparticle systems in cancer therapeutics. Adv Drug Deliv Rev 60(14):1615–1626

Cai W, Chen X (2007) Nano platforms for targeted molecular imaging in living subjects. Small 3:1840–1854

Cho CS, Cho KY, Park IK, Kim SH, Sasagawa T, Uchiyama M, Akaike T (2001) Receptor mediated delivery of all trans-retinoic acid to hepatocyte using poly(L-lactic acid) nanoparticles coated with galactose-carrying polystyrene. J Control Release 77(1–2):7–15

Divakar P, Kumar D, Praveen C, Sowmya C, Suryaprakash Reddy C (2013) Formulation and in vitro evaluation of liposomes containing metformin hydrochloride. Int J Res Pharm Biomed Sci 4(2):479–485

Duangjit S, Opanasopit P, Rojanarata T, Ngawhirunpat T (2011) Characterization and in vitro skin permeation of meloxicam-loaded liposomes versus transfersomes. J Drug Deliv 2011:418316

Duangjit S, Opanasopit P, Rojanarata T, Ngawhirunpat T (2013) Evaluation of meloxicam-loaded cationic transfersomes as transdermal drug delivery carriers. AAPS Pharm Sci Tech 14:133–140

Dubey V, Mishra D, Jain NK (2007) Melatonin loaded ethanolic liposomes: physicochemical characterization and enhanced transdermal delivery. Eur J Pharm Biopharm 67(2):398–405

El-Kassas H, Attia AA (2014) Bactericidal application and cytotoxic activity of biosynthesized silver nanoparticles with an extract of the red seaweed *Pterocladiella capillacea* on the HepG2 cell line. Asian Pac J Cancer Prev 15:1299–1306

Elzoghby AO, Abd-Elwakil MM, Abd-Elsalam K, Elsayed MT, Hashem Y, Mohamed O (2016) Natural polymeric nanoparticles for brain-targeting: implications on drug and gene delivery. Curr Pharm Des 22:3305–3323

Estanqueiro M, Amaral MH, Conceiçao J, Sousa Lobo JM (2015) Nanotechnological carriers for cancer chemotherapy: the state of the art. Colloids Surf B Biointerfaces 126:631–648

Fang J, Nakamura H, Maeda H (2011) The EPR effect: unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect. Adv Drug Deliv Rev 63(3):136–151

Feng Q, Sun J, Jiang X (2016) Microfluidics-mediated assembly of functional nanoparticles for cancer-related pharmaceutical applications. Nano Scale. https://doi.org/10.1039/C5NR07964K

Gao Z, Lukyanov A, Singhal A, Torchilin V (2002) Diacyllipid-polymer micelles as nano- carriers for poorly soluble anticancer drugs. Nano Lett 2(9):979–982

Giri S, Karakoti A, Graham RP, Maguire JL, Reilly CM, Seal S, Rattan R, Shridhar V (2013) Nanoceria: a rare-earth nanoparticle as a novel anti-angiogenic therapeutic agent in ovarian cancer. PLoS One 81:545–578

Glavas-Dodov M, Fredro-Kumbaradzi E, Goracinova K, Calis S, Simonoska M, Hincal AA (2003) 5-Fluorouracil in topical liposome gels for anticancer treatment-formulation and evaluation. Acta Pharma 53(4):241–250

Godin B, Touitou E (2003) Ethosomes: new prospects in transdermal delivery. Crit Rev Ther Drug Carrier Syst 20(1):63–102

Gradishar WJ, Tjulandin S, Davidson N et al (2005) Phase III trial of nanoparticle albumin-bound paclitaxel compared with polyethylated castor oil-based paclitaxel in women with breast cancer. J Clin Oncol 23(31):7794–7803

Gupta A, Aggarwal G, Singla S, Arora R (2012) Transfersomes: a novel vesicular carrier for enhanced transdermal delivery of sertraline: development, characterization, and performance evaluation. Sci Pharm 80(4):1061–1080

Gupta B, Chakraborty S, Saha S, Chandel S, Baranwal A, Banerjee M, Chatterjee M, Chaudhury A (2016) Antinociceptive properties of shikonin: in vitro and in vivo studies. Canad J Physiol Pharmacol. https://doi.org/10.1139/cjpp-2015-0465

Hayashi M, Silanikove N, Chang X, Ravi R, Pham V, Baia G, Paz K, Brait M, Sidransky D (2015) Milk derived colloid as a novel drug delivery carrier for breast cancer. Cancer Biol Ther 16(8):1184–1193

Hofferberth SC, Grinstaff MW, Colson YL (2016) Nanotechnology applications in thoracic surgery. Eur J Cardiothorac Surg 50(1):6–16. PMID 26843431

Hou XY, Jiang G, Yang CS, Tang JQ, Wei ZP, Liu YQ (2016) Application of nanotechnology in the diagnosis and therapy of hepatocellular carcinoma. Recent Pat Anticancer Drug Discov 11(3):322–331. PMID:26955964

Irfan M, Verma S, Ram A (2012) Preparation and characterization of Ibuprofen loaded transferosome as a novel carrier for transdermal drug delivery system. Asian J Pharm Clin Res 5:162–165

Jana NR, Gearheart L, Murphy CJ (2001) Wet chemical synthesis of high aspect ratio cylindrical gold nanorods. J Phys Chem B 105:4065–4067

Jing L, Liang X, Li X, Lin L, Yang Y, Yue X, Dai Z (2014) Mn-porphyrin conjugated au nanoshells encapsulating doxorubicin for potential magnetic resonance imaging and light triggered synergistic therapy of cancer. Theranostics. 2014 4(9):858–871. https://doi.org/10.7150/thno.8818

Katiyar SS, Muntimadugu E, Rafeeqi TA, Domb AJ, Khan W (2015) Co-delivery of rapamycin- and piperine-loaded polymeric nanoparticles for breast cancer treatment. Drug Deliv 2:1–9

Kaur P, Thakur R, Chaudhury A (2012) An in vitro study of the antifungal activity of silver/chitosan nanoformulations against important seed borne pathogens. Int J Sci Technol Res 1(6):83–86

Kaur P, Chaudhury A, Thakur R (2013) Synthesis of chitosan-silver nanocomposites and their antibacterial activity. Int J Sci Eng Res 4(4):869

Kaur P, Thakur R, Barnela M, Chopra M, Manuja A, Chaudhury A (2014) Synthesis, characterization and in vitro evaluation of cytotoxicity and antimicrobial activity of chitosan–metal nanocomposites. J Chem Technol Biotech. https://doi.org/10.1002/jctb.4383

Kaur P, Thakur R, Chaudhury A (2016) Biogenesis of copper nanoparticles using peel extract of *Punica granatum* and their antimicrobial activity against opportunistic pathogens. Green Chem Lett Rev 9(1):33–38

Koziorowski J, Stanciu AE, Gómez-Vallejo V, Llop J (2016) Radiolabeled nanoparticles for cancer diagnosis and therapy. Anticancer Agents Med Chem. 17 333–354 PMID:26899184

Kulhari A, Sheorayan A, Chaudhury A (2013) Targeted chemotherapeutics: an overview of recent progress in effectual cancer treatment. Pharmacologia 4(9):535–552

Malam Y, Loizidou M, Seifalian AM (2009) Liposomes and nanoparticles: nanosized vehicles for drug delivery in cancer. Trends Pharmacol Sci 30(11):592–599

Martins AF, Follmann HD, Monteiro JP, Bonafé EG, Nocchi S, Silva CT, Nakamura CV, Girotto EM, Rubira AF, Muniz EC (2015) Polyelectrolyte complex containing silver nanoparticles with antitumor property on Caco-2 colon cancer cells. Int J Biol Macromol 79:748–755 S0141-8130(15)00377-3

Mittal AK, Tripathy D, Choudhary A, Aili PK, Chatterjee A, Singh IP, Banerjee UC (2015) Biosynthesis of silver nanoparticles using *Potentilla fulgens* Wall. ex Hook and its therapeutic evaluation as anticancer and antimicrobial agent. Mater Sci Eng C Mater Biol Appl 1(53):120–127

Mohamed S, Parayath NN, Taurin S, Greish K (2014) Polymeric nano-micelles: versatile platform for targeted delivery in cancer. Ther Deliv 5(10):1101–1121

Mohammad F, Yusof NA (2014) Doxorubicin-loaded magnetic gold nanoshells for a combination therapy of hyperthermia and drug delivery. J Colloid Interface Sci 434:89–97

Mousa SA, Bharali DJ (2011) Nanotechnology-based detection and targeted therapy in cancer. Nano-Bio Paradigms Appl Cancers (Basel) 3(3):2888–2903

Office of Technology and Industrial Relations of the US National Cancer Institute (OTIR) (2006). https://www.cancer.gov/sites/nano

Ooya T, Lee J, Park K (2004) Hydrotropic dendrimers of generations 4 and 5: synthesis, characterization, and hydrotropic solubilization of paclitaxel. Bioconjug Chem 15:1221–1229

Park K (2007) Nanotechnology: what it can do for drug delivery. J Control Release 120:1–3

Patra S, Mukherjee S, Barui AK, Ganguly A, Sreedhar B, Patra CR (2015) Green synthesis, characterization of gold and silver nanoparticles and their potential application for cancer therapeutics. Mater Sci Eng C Mater Biol Appl 53:298–309

Peer D, Karp JM, Hong S, Farokhzad OC, Margalit R, Langer R (2007) Nanocarriers as an emerging platform for cancer therapy. Nat Nanotechnol 2(12):751–760

Praetorius NP, Mandal TK (2007) Engineered nanoparticles in cancer therapy. Recent Pat Drug Deliv Formul 1(1):37–51

Reimer P, Tombach B (1998) Hepatic MRI with SPIO: detection and characterization of focal liver lesions. Eur Radiol 8(7):1198–1204

Schnyder A, Krahenbuhl S, Drewe J, Huwyler J (2005) Targeting of daunomycin using biotinylated immunoliposomes: pharmacokinetics, tissue distribution and in vitro pharmacological effects. J Drug Target 13(5):325–335

Singh SY, Aher SS, Saudagar RB (2015) Ethosomes-novel drug delivery system. Res J Top Cosmet Sci 6(1):7–14

Sun Y, Xia Y (2002) Shape-controlled synthesis of gold and silver nanoparticles. Science 298:2176–2179

Touitou E, Godin B, Dayan N, Weiss C, Piliponsky A, Levi-Schaffer F (2001) Intracellular delivery mediated by an ethosomal carrier. Biomaterials 22:3053–3059

Vanna S, Nicolino P, Mario S (2014) Targeted therapy using nanotechnology: focus on cancer. Int J Nanomedicine 9:467–483

Walve JR, Bakliwal SR, Rane BR, Pawar SP (2011) Transferomes: a surrogated carrier for transdermal drug delivery system. Int J Appl Biol Pharm Tech 2:1. ISSN 0976-4550

Wang AZ, Bagalkot V, Vasilliou C, Gu F, Alexis F, Zhang L, Shaikh M, Yuet K, Cima M, Langer R, Kantoff P, Bander N, Jon S, Farokhzad O (2008) Superparamagnetic iron oxide nanoparticle-aptamer bioconjugates for combined prostate cancer imaging and therapy. Chem Med Chem 3:1311–1315

Wang X, Li J, Wang Y et al (2009) HFT-T, a targeting nanoparticle, enhances specific delivery of paclitaxel to folate receptor-positive tumors. ACS Nano 3:3165–3174

Wong HL, Rauth AM, Bendayan R, Wu XY (2007) In vivo evaluation of a new polymer– lipid hybrid nanoparticle (Pln) formulation of doxorubicin in a murine solid tumor model. Eur J Pharm Biopharm 65(3):300–308

Wu C, Yu C, Chu M (2011) A gold nanoshell with a silica inner shell synthesized using liposome templates for doxorubicin loading and near-infrared photothermal therapy. Int J Nanomedicine 6:807–813

Wunderbaldinger P, Josephson L, Bremer C, Moore A, Weissleder R (2002) Detection of lymph node metastases by contrast-enhanced MRI in an experimental model. Magn Reson Med 47:292–297

Zhang H, Hu H, Zhang H, Dai W, Wang X, Wang X, Zhang Q (2015) Effects of PEGylated paclitaxel nanocrystals on breast cancer and its lung metastasis. Nanoscale 7(24):10790–10800

Zhao Y, Pang B, Luehmann H, Detering L, Yang X, Sultan D, Harpstrite S, Sharma V, Cutler CS, Xia Y, Liu Y (2016) Gold nanoparticles doped with 199 Au atoms and their use for targeted cancer imaging by SPECT. Adv Healthc Mater 5(8):928–935

Zhuang L, Kai C, Davis C, Sherlock S, Cao Q, Chen X, Dai H (2016) Drug delivery with carbon nanotubes for in vivo cancer treatment. 68 6652–6660 PMID:18701489

Part III

Biotechnology in Diagnostics & Therapeutics

Molecular Approaches for Identification of Lactobacilli from Traditional Dairy Products

11

Dhiraj Kumar Nanda, Reeti Chaudhary, and Dinesh Kumar

11.1 Introduction

Lactic acid bacteria isolated from traditional fermented dairy products contribute to unique taste and flavour of the product. In this way traditional dairy products can prove to be an interesting niche for getting indigenous *Lactobacillus* strains. Therefore, indigenous isolates isolated from these food samples after characterization can be used as a starter culture. With the use of advanced molecular biology techniques, it is easy to characterize the 'new *Lactobacillus*' strains, though any specific approach for molecular characterization of the indigenous strains associated with the traditional products has been suggested. Thus the objective of this chapter would be to make an intuition towards various methods used for molecular characterization of indigenous lactobacillus from traditional dairy products.

Traditional fermented foodstuffs are in part or whole the products of fermentation carried out by a class of microbes known as lactic acid bacteria (LAB), LAB comprises of a group of number of genera (Holzapfel et al. 2001), these groups are mostly present in foods and fermented products (O'sullivan et al. 2002), and these LAB take part in fermentation of milk (Beresford et al. 2001). Since many species of LAB and other bacteria associated with food got a long historical association in our foods, thus they have been accepted as 'generally regarded as safe' (GRAS)

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bacteria (Maassen et al. 2003). This GRAS status inspires accumulative use of LAB in food products with nutritional or health benefits (van den Berg et al. 1993; Bianchi-Salvadori et al. 1997).

The most important genera of LAB can be well-defined as group of gram-positive microaerophilic organisms that can ferment hexose sugars and produce lactic acid primarily. LAB groups mainly comprise of industrially important genera, including *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Enterococcus*, *Pediococcus*, *Leuconostoc*, *Weissella*, *Carnobacterium*, *Tetragenococcus*, and *Bifidobacterium* (Teuber 1993; Klein et al. 1998; Axelsson and Ahrné 2000; König and Fröhlich 2009).

Isolation and characterization of LAB have tremendously contributed in the production of fermented milk products. Keeping the view in mind, substantial efforts have been given towards increasing the information about the physiology, biochemistry, and genetics of LAB. In addition, the studies on metabolism and genetics of LAB have allowed researchers to select superior strains and improve the productivity, value, and safety of the end products. Thus, it needs consideration to explore new LAB from traditional foods.

11.2 Historical Background of *Lactobacillus*

11.2.1 Lactic Acid Bacteria (LAB)

Milk is known to be a complete food for newborn mammal as it has high nutritive values; we humans are very smart and use milk produced by various milking species as our food, but microbes are far clever, and they use this milk very smartly. If we keep raw as such at room temperature, there is perfect chance that it will be spoiled by microbes. Varieties of fermented milk products and derived products have been developed in all parts of the world, and every such product has its peculiar characteristic and own historical background. The most natural way for the preparation of such dairy products is spontaneous acidification of milk, which depends on lactic acid bacteria (LAB) naturally present in milk as adventitious contaminants; they propagate and make lactic acid which is required for coagulation of milk. These normal cultures are further used to inoculate fresh milk the next day. In this manner, several tribes around the world have developed traditional fermented dairy products (Wouters et al. 2002).

LAB are native to food-related habitats that include plant products (fruits, vegetables, and cereal grains) and milk. Besides this, LAB is also naturally linked to mucosal surfaces of animals, e.g., the small intestine, colon, and vagina, and LAB are often isolated from these environments (Makarova et al. 2006).

11.2.2 *Lactobacillus*

The generic name *Lactobacillus* and the family name *Lactobacillaceae* were assigned to rod-shaped organisms (*Bacillus*) who were recognized by peculiarity of high acid-forming gram-positive bacteria, having the ability to produce and tolerate

lactic acid (Winslow et al. 1917). The genus is characterized by gram-positive, catalase-negative, nonmotile, non-sporulating, facultative anaerobes, growing under microaerophilic to strictly anaerobic conditions (Klein et al. 1998). The genus *Lactobacillus* is the largest group among the *Lactobacteriaceae*; presently, it contains a diverse assemblage of more than 187 species (Euzéby 1997; Singh et al. 2009). They are usually thin slender rods, although they can also attain spiral or coccobacillus forms under certain conditions. Lactobacilli are considered as one of the most important LAB which are friendly bacteria found in our digestive tract and are often considered to be commensal or beneficial participants in human microbial ecology, together with a number of 'generally recognized as safe' (GRAS) species whose major role is in producing fermented foods, fermented milk, yogurt, and cheeses.

Considerable researches have been performed worldwide for the effective application of *Lactobacillus* in the form of condiments in human and animal diets. Specific attention imposed in lactobacilli is mainly because of the following: (a) they improve the quality and nutrition of food in a number of foods as curd; (b) *Lactobacillus* is associated with promoting health as curd has been found to cure intestinal disorders; and (c) *Lactobacillus* strains are used in industries and have a good commercial value which leads to patenting and commercialization of specific strains of *Lactobacillus* (Charteris et al. 1998; Prasad et al. 1998; Holzapfel et al. 2001).

11.2.3 Application of Lactobacilli

Lactobacillus is esteemed as an important member of LAB with a significant role in the food industry. A range of diverse metabolic properties exhibited by the members of genus *Lactobacillus* make them useful for a number of fermented food products like yogurt and cheeses, beverages, fruits, vegetables, and fermented meats. In these products, lactobacilli contribute to their preservation, nutrient availability, and flavour. Besides these properties, a concern has been derived towards their possible application as probiotics as these are the natural inhabitant of GI tract as well as the urogenital tract and are considered important to maintain their health. The probiotic effects have been attributed to definite strains of *Lactobacillus* and have been intensively investigated. *Lactobacillus* strains which are capable to colonize body cavities appear to have a significant role in keeping a secure microflora or in stopping or pulling down the occurrence of regular urinary or intestinal tract infections (Tannock 1999). A fermented dairy product named Yakult drink (Yakult, Tokyo, Japan) containing *L. casei shirota* strain has been found with tremendous health beneficial effects (Almeida et al. 2012; Thomson et al. 2012). Thus, a number of probiotic drinks are making market worldwide.

In addition, lactobacilli also represent attractive candidates for mucosal vaccination which aim at causing an immune response that neutralizes pathogenic viruses or bacteria at their primary point of entrance to the system (Wells et al. 1996; Wells and Mercenier 2008). Another appreciable thing of probiotic lactobacilli is the

ability to produce bacteriocins (Duhan et el. 2013), and it acts as unusual antibiotics to destroy unwanted organisms.

Thus, the genus *Lactobacillus* is an important group in LAB, and isolation and detailed characterization of *Lactobacillus* strains is essential which provides complete knowledge of the species, their physiological and metabolic properties, and also the technological properties of industrial interest.

11.2.4 Habitat and Sources of *Lactobacillus*

Screening of sample used for any study is of crucial importance. Traditional fermented food has been found to be a good source for harbouring strains genetic diversity outlined in the traditional cheese produced without pasteurization have been studied by Corroler et al. (1998).

11.2.5 Traditional Dairy Products

The traditional dairy products exist to be one of the tools to provide food and nutrition security in developing word since ancient times (Prajapati 2011). In most of the countries, conservation and prevention of milk from spoilage remains an important activity, and preparation of traditional milk products is found to be the best for preserving milk. Fermentation is found as one of the best options to improve food security, besides this fermentation can improve flavour and appearance of food. Fermented products can play an important role in contributing livelihoods of rural and semiurban residents as an option of small-scale enterprise (Marshall and Mejia 2011). Fermentation activities are highly combinable with a variety of traditional and domestic activities, but comparatively very few operations are carried out at an industrial level (Holzapfel et al. 2001).

Many of traditional milk products include a fermentation stage which not only affects the shelf-life of the product but it also affects the quality and characteristics of the product. This is mainly prepared by immediate boiling of milk after procurement and the use of lactose fermentation by lactic acid bacteria. It is an important means of preventing or limiting milk spoilage due to the growth of contaminating bacteria and their enzymatic activity (Singh et al. 2009).

Fermentation not only conserves vital nutrients of milk in such foods but also modifies certain milk constituents to enhance their functional and nutritional status. Culturing in this way generates live and active culture in significant numbers to provide health benefits beyond conventional nutrition to the consumer (Chandan 2011).

There are a number of examples of such traditional fermented milk products (Food and Agriculture Organization of the United 1990) which include *Irgo* (fermented milk), *hard fermented milk curd*, and *Arrera* (sour butter milk) in Southern and Eastern Africa, Ethiopia; *Kadam* (fermented milk) and *sour butter milk* in West Africa, Mali; *yogurt* in the Southern Cone Countries of Latin America; and *Laban*, *Labaneh* (*labanmousafa*), *Shenineh*, *Shenglish* (*Sorke*), and *Keshkeh* in the Middle East, Syria.

11.3 Identification of Lactobacilli

The genus *Lactobacillus* is the biggest assemblage among the *Lactobacteriaceae*, currently known to possess a diverse assemblage of more than 187 species (Euzéby 1997; Singh et al. 2009). They are genetically diverse; with their G+C content ranging from 52 to 64 mol% indicating that *Lactobacillus* is not a distinct genus (Vandamme et al. 1996). The classical division of lactobacilli based on their fermentation characteristics places them under three major groups: the obligate homofermentative lactobacilli, which ferment hexose sugar and produce lactic acid; the facultative heterofermentative lactobacilli, which ferment hexose sugar for producing only lactic acid or for producing lactic acid along with acetic acid, ethanol, and formic acid in limited glucose; and, on the other side, obligate heterofermentative lactobacilli, which ferment hexose sugar to lactic acid, acetic acid, ethanol, and CO_2. These groups, however, do not reflect the phylogenetic relationships between species.

In most case accurate identification is the necessity of the study, but the occurrence of a large number of closely resembling species similar in morphology as well as physiology imposes a serious limitation for their unambiguous identification.

11.3.1 Conventional Methods of Identification

Classical identification methods traditionally used for differentiation between *Lactobacillus* species are based on morphology (both cellular and colonial) and a range of physiological features, for example, aerobic-anaerobic respiratory type, motility, temperature, and salt concentration at which they grow, and biochemical characteristics as homo-/heterofermentative, ability to produce lactic acid isomers, metabolism of carbohydrate substrates, coagulation of milk, and the presence of specific enzymes (e.g., arginine dihydrolase, antibiotic susceptibilities, etc.) (Kandler and Weiss 1986; Coeuret et al. 2003). Besides, cell wall protein analysis, electrophoretic profile of peptidoglycan hydrolases (Lortal et al. 1997), serology, and more recently fatty acid methyl ester (FAME) analysis (Klein et al. 1998; Giraffa and Neviani 2000) have also been added among the classical techniques. Though these typing methods are simple and easy to perform, they are not completely accurate, and strains, which show intermediate characteristics, are frequently encountered (Williams and Banks 1997). Some of these techniques have also been found useful in identification of lactobacilli such as cell wall protein profiling for thermophilic lactobacilli (Holzapfel et al. 2001) and whole-cell protein profiling for certain lactobacilli at both species and subspecies level (Giraffa and Neviani 2000).

11.3.1.1 Limitations of Conventional Methods

Although these conventional tests provide proof of the metabolic capabilities of the strains, there is a general consciousness that some aspects of phenotypic characterization are principally faulty, and the reflection of an analogous phenotype always does not compare to a similar or closely related genotype. Other drawbacks of

phenotypic methods are poor reproducibility and the vagueness of few techniques, resulting to poor discrimination power (McCartney 2002). They have problems such as non-reproducibility and lack of discriminatory power and are time-consuming too. In overall, these typing approaches are not totally correct, and strains, which show intermediate characteristics, are frequently encountered (Gasser 1970; Williams and Sadler 1971).

Thus, although the traditional phenotypic and biochemical methods are helpful in the identification and characterization of members of *Lactobacillus* genus, they alone are not sufficient for the true interpretation of diversity and inter- and intraspecies differentiation sometimes, which can be easily attained by molecular methods, hence providing them an edge over phenotypic methods.

11.3.2 Molecular Genotyping of *Lactobacillus*

The genetic makeup of this organism is a major attraction among researchers because of the need of the greater understanding of the genes involved in the various probiotic attributes, or fermentation pathways, in order to screen good strains for their maximum utilization in pharmaceutical and food industries. The genetic information obtained from genotyping is also helpful in determining the correct taxonomy of the recently diverged subspecies or to differentiate a new species from other closely resembling species. The importance of genetic information within this genus can be assumed by the fact that as many as 50 lactobacilli genomes of different species have been completely sequenced, genome sequencing of several other lactobacilli are underway this huge success is due to advent of new next-generation sequencing (NGS) techniques (Mardis 2008, 2013). The objective of investigators is to well realize the roles, capabilities, and interactions of lactobacilli. It is now known that the genome of *L. plantarum* is the largest among lactic acid bacteria and is of 3.3 Mbp size with 3052 open-reading frames and a G+C content of 44.5%. In fact, many *Lactobacillus* species have been reclassified on the basis of innovative molecular techniques and their taxonomic status has been rectified. For example, recently, *L. cellobiosus* has now been converted to *L. fermentum* (Dellaglio et al. 2004).

In view of the above, an overview of the common molecular genotyping techniques employed in *Lactobacillus* characterization is discussed as follows.

11.3.3 Multilocus Approaches

11.3.3.1 Pulse-Field Gel Electrophoresis (PFGE)

It works as an alternating field of electrophoresis with increasing the pulse times during run of electrophoresis, to allow separation of the large DNA fragments (5–50) resulted from restriction digestion with uncommon restriction enzymes (O'Sullivan and Fitzgerald 1998; Holzapfel et al. 2001; Dong et al. 2011; Xu et al. 2012). An essential requirement for PFGE is the extraction of intact chromosomal

DNA to prevent it from mechanical shearing in aqueous solutions, which is reached by implanting bacterial culture in low-melting-point agarose gel before its lysis. After lysis, the restricted fragments are embedded in the agarose gel, which is sliced and put into a well of agarose gel and is further resolved by PFGE, and the resulting DNA banding pattern on gel is the restriction fragment length polymorphism (RFLP) which has a specific characteristic unique for the particular organism. As PFGE fingerprint signifies the complete genome of the organism, a minute change over time in genome as deletion, insertion, or mutation can be detected on the fingerprint. This feature makes the technique one of the most biased and reliable among other techniques (O'Sullivan and Fitzgerald 1998). The utility of PFGE has been verified by McCartney et al. (1996). PFGE shows large alterations in the genome sizes due to insertions or deletions, but even a point mutation that affects a restriction site may also result in a diverse PFGE profile. Thus PFGE is suitable for displaying minute differences among two strains. Many times PFGE can resolve up to species level only, by exceptional subspecies diversity, has been confirmed by using PFGE for a number of organisms, including lactobacilli and bifidobacteria (McCartney et al. 1996; Kimura et al. 1997; Xu et al. 2012).

11.3.3.2 Amplified Fragment Length Polymorphism (AFLP)

Amplified fragment length polymorphism or AFLP is a variation of RAPD that can identify restriction site polymorphisms even if we don't have knowledge of the sequence to be amplified, just we have to digest the genomic DNA by restriction enzyme and use specific primer sequences that recognize restriction sites with additional nucleotides that can extend outwards for PCR amplification and analyse the resulting DNA banding pattern (Zabeau and Vos 1993; Vos et al. 1995; Blears et al. 1998; Mueller and Wolfenbarger 1999; Nguyen et al. 2013b). The fixed part in primer provides stability, and the random portion lets it to identify multiple loci. Now further the amplified DNA is run on polyacrylamide gel electrophoresis. This technique is considered as a highly sensitive method for fingerprinting DNA of any origin and complexity. AFLP is proved to be a good multiple-locus fingerprinting techniques used for genotypic characterization (Babalola 2004).

11.3.3.3 Randomly Amplified Polymorphic DNA (RAPD)

It is a rapid fingerprinting method, generously used for the intra- and interspecies differentiation within genus *Lactobacillus* (Du Plessis and Dicks 1995; Johansson et al. 1995; Björkroth et al. 1996; Daud Khaled et al. 1997; Tynkkynen et al. 1999). The major attractions behind its wide popularity are: it is rapid, simple, and easy and is free from the earlier knowledge of the sequence information from the genome of source organism; also it is universally applicable on any genome. In RAPD, single short arbitrary primer (10–12 bp) is used under low stringency conditions to provide multiple random amplifications of the variable length sequences in PCR. Thus, a complex pattern of multiple amplification products from loci distributed throughout the genome is generated. Variation is based on the position and orientation of primer-annealing sites and the interval they span. The DNA fragments obtained can be used for similarity analysis and to build a phylogenetic tree.

RAPD is a rapid and discriminative technique but is likely to have poor reproducibility, since any minute changes in reaction conditions as *Taq* polymerase, primers, Mg^{2+}, buffer concentration, efficiency of cell lysis, and DNA extraction may change the results (Marilley and Casey 2004). RAPDs are also extremely liable to error by user unless the most steady conditions are strictly followed while RAPD profiling. Thus, it is difficult to reproduce results between laboratories, which may be different from one PCR machine to another or use different set of reagents as polymerase, buffer, primer, etc.; even the results from the same person can vary from 1 day to the other. Due to these limitations, RAPD often is a laborious technique, and the time saved by RAPD is not fruitful, and it is difficult to reproduce the results. In view of the above limitations, RAPD is slowly being replaced by other fast methods, where possible.

11.3.3.4 REP-PCR and ERIC-PCR

REP-PCR is amplification of repetitive extragenic palindromic (REP) elements that are scattered extragenic sequences present in a number of regions in bacterial genome and can be used for microbial diversity studies (Versalovic et al. 1994). REP and ERIC primers are mostly used for fingerprinting of gram-positive bacteria (Versalovic et al. 1991; Song 2005). Nevertheless, they have been used by several authors. REP-PCR have also been extensively used for molecular profiling of LAB. The population structure of lactobacilli in Comte cheese has been studied using ERIC and REP-PCR fingerprinting.

The high discriminatory power of multilocus amplification in RAPD can be more reliable if the target loci are known. The repetitive extragenic palindromic elements (REP) and enterobacterial repetitive intergenic consensus (ERIC) sequences can also be targeted in PCR. These techniques have advantages of defined targets, high discriminatory power, low cost, and suitability for high throughput of strains and are also reliable for classification and typing of a wide range of gram-negative and several gram-positive bacteria (Versalovic et al. 1994; Olive and Bean 1999). Gevers et al. (2001) employed BOX and REP-PCR on 30 lactobacilli strains including both reference strains and natural isolates from dry sausages and found that the number of DNA fragments produced by BOX and REP-PCR was comprised between 0 and 6 and between 1 and 10, respectively. These workers amplified the average number of bands per PCR profile to 16.5 by using the primer (GTG) 5. Their results showed that (GTG) 5 primer based REP-PCR fingerprinting can be used for identification and possibly for intraspecies differentiation and is especially useful for screening a huge number of strains. The high discriminatory power of (GTG) 5 PCR fingerprinting was evident from the clear differentiation of closely resembling *L. pentosus*, *L. plantarum*, *L. paraplantarum*, and *L. alimentarius* as well as *L. paralimentarius.* Recently, both of these methods were found highly reliable in the rapid characterization of *L. johnsonii* strains (Ventura and Zink 2002). REP-PCR has been found suitable for grouping of lactic acid bacteria provided the most discriminative, reproducible, and complex fingerprints and has been recently used by many researchers (Gevers et al. 2001; Van Hoorde et al. 2008; Ouoba et al. 2009; Švec et al. 2009; Golic et al. 2012; Nguyen et al. 2013a).

11.3.4 Single Locus Approaches

For the selection of a single gene sequence to be used as a phylogenetic marker, it should have two important characteristics: first, it must be universally present in bacteria and second, it should have high sequence conservation, which demonstrates that sequence changes are less subjective to temporary environmental alterations.

11.3.4.1 16S rRNA Sequence

Small-subunit rRNA (16S rRNA in prokaryotes) sequencing is a potent means to detect, identify, and classify prokaryotic organisms, and there is currently an explosion of SSU rRNA sequencing in the microbiology community (Gutell 1993; Olsen and Woese 1993; Clayton et al. 1995). It shows a higher degree of functional fidelity, diverse locations in their sequences change at very different rates, and they are universally spread. Functional constraints on the translational tool limit unevenness in the 16S rRNA molecule, which results in a high degree of sequence conservation. These genes are part of *rrn* operon (Acinas et al. 2004) that has the subsequent components in the order (5–3′) in most bacteria (Gürtler and Stanisich 1996) with some exclusions (Gürtler 1999) 16S rRNA, spacer, tRNA, spacer, 23S rRNA, spacer, and 5S rRNA sequences. The tDNA spacer (De Gheldre et al. 1999) and tDNA sequences have been used in a limited extent straight though they may have significant phylogenetic data. The 23S rRNA gene sequence is double sized to 16S rRNA gene sequence but is not as extensively in use. Hence, the 16S rRNA gene is utilized as the major phylogenetic marker in bacterial taxonomy. The ribosomal RNA genes are coordinately transcribed as a whole *rrn* operon to produce equimolar quantities of each gene product. The polymerase chain reaction (PCR) is used for amplifying the 16S rRNA gene directly from colonies grown on agar medium by using universal primers which are conserved regions at both ends of the gene.

The whole PCR amplicon is of ~1.5 kb and can subsequently be sequenced, and the data generated can be compared with rRNA database. The main 16S rRNA public databases are NCBI GenBank and the Ribosomal Database Project, which work as ‘composite’ sequences retrieved from sequencing of PCR amplicons obtained by simultaneous amplification of all 16S rRNA gene copies on a genome (Clayton et al. 1995). However, the intergenic (16S-23S rDNA) spacer region (ISR) is presently being used most frequently for typing of isolates (Gürtler and Stanisich 1996), evolutionary studies (Gürtler and Stanisich 1996; Garcia-Martinez et al. 1999), and microbial diversity studies (Fisher and Triplett 1999). All molecular identification techniques target either 16S rRNA gene, 23SrRNA gene, or 16S-23S spacer in designing specific probes, in PCR, or in RFLP generated fingerprints either through southern hybridization or directly in ARDRA. However, despite their taxonomic importance there, reliability in determining the true phylogeny and taxonomic status appears to be questionable, since, due to the extremely slow evolution of rRNA genes, organisms may belong to different species as judged by DNA-DNA hybridization even with higher than 98% 16S rRNA gene sequence similarities (Stackebrandt and Goebel 1994). The DNA reassociation values below 70% threshold value are known to share 99.8 or even 100% rDNA similarity. For

discriminating highly related organisms, the resolution power of DNA-DNA reassociation is, therefore, significantly higher than that of 16S rDNA sequences. Below 16S rDNA similarity value of 97%, the corresponding DNA-DNA reassociation value has never been shown to be higher than 60% (Stackebrandt and Goebel 1994) from which follows that below a sequence similarity of 97% strains do not belong to the same species (Stackebrandt 2003). Another factor that influences the quality of 16S rRNA gene sequences is the occurrence of microheterogeneity within multiple *rrn* operons (Rainey et al. 1996; Acinas et al. 2004). Length heterogeneity and the presence of different nucleotides at the same position in different operons will lead to undeterminable sequences and positions, respectively, thus to low-quality sequences.

11.3.4.2 Constitutive Gene Sequence

The gene sequence encoding protein has been found to be better to 16S rRNA gene sequence for classifying microbial diversity. The DNA sequence of housekeeping or constitutive gene coding for important proteins is effective than 16S rRNA genes for distinction of closely related populations (Palys et al. 2000).

Conserved or constitutive genes provide an alternate to the 16S rRNA gene for bacterial diversity analysis. As these genes are found as a single copy only, this provides a significant advantage over 16s rRNA that are mostly found in multiple copies (up to 15 copies). High or moderately conserved genes commonly found are bacteria that are listed below:

(i) *rpoB* gene: This gene is 1300 bp in size that encodes the RNA polymerase β-subunit. Resolution goes below the species level (Mollet et al. 1997; Drancourt and Raoult 2005).
(ii) *gyrB* gene: The gyrB gene is of size 2200 bp; it encodes the DNA gyrase (topoisomerase II) B subunit. A database of gyrB gene is maintained at Marine Biotechnology Institute in Japan. By using this marker, the resolution goes below the species level (Fukushima et al. 2002).
(iii) *gyrA* gene: Its size is 550 bp, and the gene encodes DNA gyrase (topoisomerase II) A subunit (Brisse and Verhoef 2001).
(iv) *tmRNA:* The gene tmRNA is of size 300 bp, this is also known as 10Sa RNA, and it acts as a catalytic RNA and takes part in *trans*-translation for addition of C-terminal peptide tag to incomplete protein product of a broken mRNA (Zwieb et al. 2003). A *tmRNA* database has been managed by the University of Texas Health Science Center.
(v) *recA:* recA is 1600 bp gene that encodes a 352 amino acid protein RecA, which implicates in homologous DNA recombination, SOS induction, and DNA damage-induced mutagenesis. By using this marker, the diversity can be resolved below species level (Felis et al. 2001).
(vi) EF-Tu (*tuf*) gene: This 300 bp gene encodes bacterial elongation factor EF-Tu, and the gene can resolve identity up to species level (Ludwig et al. 1993; Baldauf et al. 1996).

(vii) *groEL* (*cnp60*, *hsp60*) gene: This is a 1800 bp gene that encodes protein GroEL/Hsp60, and it can resolve identity of microbes below species level (Wong and Houry 2004).

(viii) *atpD* gene: atpD gene is of 150 bp size and encodes β-subunit of bacterial F1F0 type ATP-synthetases, and the resolution power is almost similar to 16S rDNA (Ludwig et al. 1993).

(ix) *ompA* gene: This 350 bp gene encodes outer membrane protein A (OmpA). Resolution is below species level (Wertz et al. 2003).

(x) *gapA* gene: It is of 150 bp, and it codes for glyceraldehyde-phosphate dehydrogenase. The level of resolution is high and can discriminate below species level (Wertz et al. 2003).

(xi) *gpi* gene: The 350 bp gene encodes for glucose-6-phosphate isomerase (Wertz et al. 2003). It can resolve identity of microbial cultures below species level.

11.3.5 Other Molecular Techniques

Besides these techniques, there are other techniques as terminal restriction fragment length polymorphism (T-RFLP) and multilocus sequence typing (MLST) not used for identification of *Lactobacillus* from food and gastrointestinal tract. For T-RFLP, PCR amplification product of defined sequence is synthesized with fluorescently tagged primers. Subsequently, the amplicon is digested by restriction enzyme, and the product is run on gel with high resolution, and fluorescently labelled terminal is analysed under automated analyser which gives the T-RFLP pattern. T-RFLP has been used for studying diversity in environmental samples comprising of soil. These techniques are advantageous above other methods such as DGGE/TGGE and improve resolution. Multilocus sequence typing (MLST) is an alteration to multilocus enzyme electrophoresis (MLEE), where nucleic acid sequence of housekeeping genes is done in comparison to electrophoretic mobilities of gene product (Enright and Spratt 1999; Ramachandran et al. 2013). Mainly MLST is an identification tool for differentiation of isolates with highly related genotypes. MLST analysis using seven loci is proved to be exceptionally refinement for discriminating bacterial isolates (McCartney et al. 1996).

11.4 Conclusion

Isolation, identification, and characterization of microbes from new habitats have promoted the technology of developing defined bacterial strains, which are in demand and potentially used in industry. Traditional dairy products harbour a good population of microbes consisting of a good number of *Lactobacillus*; these strains are responsible for unique flavour and identity of the product. For better use of these strains, their proper identification and characterization is important. After culturing microbes in test tubes for decades, new molecular techniques have been found better in the field for characterization of lactobacilli from traditional dairy products.

References

Acinas SG, Marcelino LA, Klepac-Ceraj V, Polz MF (2004) Divergence and redundancy of 16S rRNA sequences in genomes with multiple rrn operons. J Bacteriol 186:2629–2635

Almeida CC, Lorena SLS, Pavan CR, Akasaka HMI, Mesquita MA (2012) Beneficial effects of long-term consumption of a probiotic combination of Lactobacillus casei Shirota and Bifidobacterium breve Yakult may persist after suspension of therapy in lactose-intolerant patients. Nutr Clin Pract 27:247–251

Axelsson L, Ahrné S (2000) Lactic acid bacteria. Appl microbial systematics: Springer, The Netherlands, pp 367–388

Babalola OO (2004) Molecular techniques: an overview of methods for the detection of bacteria. Afri J Biotech 2:710–713

Baldauf SL, Palmer JD, Doolittle WF (1996) The root of the universal tree and the origin of eukaryotes based on elongation factor phylogeny. Proc Natl Acad Sci 93:7749–7754

Beresford TP, Fitzsimons NA, Brennan NL, Cogan TM (2001) Recent advances in cheese microbiology. Int Dairy J 11:259–274

Bianchi-Salvadori B, Cocconcelli PS, Fernandes I, Gomezr J, Gomez R et al (1997) Characterization of the lactic acid bacteria in artisanal dairy products. J Dairy Res 64:409–421

Björkroth J, Ridell J, Korkeala H (1996) Characterization of *Lactobacillus sake* strains associating with production of ropy slime by randomly amplified polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE) patterns. Int J Food Microbiol 31:59–68

Blears MJ, De Grandis SA, Lee H, Trevors JT (1998) Amplified fragment length polymorphism (AFLP): a review of the procedure and its applications. J Industria Microbiol Biotechn 21:99–114

Brisse S, Verhoef J (2001) Phylogenetic diversity of *Klebsiella pneumoniae* and *Klebsiella oxytoca* clinical isolates revealed by randomly amplified polymorphic DNA, gyrA and parC genes sequencing and automated ribotyping. Int J Syst Evol Microbiol 51:915–924

Chandan RC (2011) Dairy ingredients for food processing: an overview. Wiley Online Library

Charteris WP, Kelly PM, Morelli L, Collins JK (1998) Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. J Appl Microbiol 84:759–768

Clayton RA, Sutton G, Hinkle PS, Bult C, Fields C (1995) Intraspecific variation in small-subunit rRNA sequences in GenBank: why single sequences may not adequately represent prokaryotic taxa. Int J Syst Bacteriol 45:595–599

Coeuret V, Dubernet S, Bernardeau M, Gueguen M, Vernoux JP (2003) Isolation, characterisation and identification of Lactobacilli focusing mainly on cheeses and other dairy products. Lait 83:269–306

Corroler D, Mangin I, Desmasures N, Gueguen M (1998) An ecological study of lactococci isolated from raw milk in the Camembert cheese registered designation of origin area. Appl Environ Microbiol 64:4729–4735

Daud Khaled AK, Neilan BA, Henriksson A, Conway PL (1997) Identification and phylogenetic analysis of *Lactobacillus* using multiplex RAPD-PCR. FEMS Microbiol Lett 153:191–197

De Gheldre Y, Vandamme P, Goossens H, Struelens MJ (1999) Identification of clinically relevant viridans streptococci by analysis of transfer DNA intergenic spacer length polymorphism. Int J Syst Evol Microbiol 49:1591–1598

Dellaglio F, Torriani S, Felis GE (2004) Reclassification of *Lactobacillus cellobiosus* Rogosa et al. 1953 as a later synonym of *Lactobacillus fermentum* Beijerinck 1901. Int J Syst Evol Microbiol 54:809–812

Dong YP, Cui SH, Yu HX, Li FQ (2011) Development of pulsed field gel electrophoresis and application for characterization and identification of *Lactobacillus* and *Streptococcus thermophilus*. Chi J Prev Med 45:1086

Drancourt M, Raoult D (2005) Sequence-based identification of new bacteria: a proposition for creation of an orphan bacterium repository. J Clin Microbiol 43:4311–4315

Du Plessis EM, Dicks LMT (1995) Evaluation of random amplified polymorphic DNA (RAPD)-PCR as a method to differentiate *Lactobacillus acidophilus, Lactobacillus crispatus, Lactobacillus amylovorus, Lactobacillus gallinarum, Lactobacillus gasseri, and Lactobacillus johnsoni*i. Curr Microbiol 31:114–118

Duhan JS, Nehra K, Gahlawat SK, Saharan P, Surekha (2013) Bacteriocins from lactic acid bacteria. In: Salar RK, Gahlawat SK, Siwach P, Duhan JS (eds) Biotechnology: prospects and applications. Springer, New Delhi, pp 127–142. ISBN 978-81-322-1682-7 ISBN 978-81-322-1683-4 (eBook). https://doi.org/10.1007/978-81-322-1683-4

Enright MC, Spratt BG (1999) Multilocus sequence typing. Trends Microbiol 7:482–487

Euzéby JP (1997) List of bacterial names with standing in nomenclature: a folder available on the Internet. Int J Syst Bacteriol 47:590–592

Felis GE, Dellaglio F, Mizzi L, Torriani S (2001) Comparative sequence analysis of a recA gene fragment brings new evidence for a change in the taxonomy of the *Lactobacillus casei* group. Int J Syst Evol Microbiol 51:2113–2117

Fisher MM, Triplett EW (1999) Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. Appl Environ Microbiol 65:4630–4636

Food, Agriculture Organization of the United Nations (1990) The technology of traditional milk products in developing countries. Food and Agriculture Organization of the United Nations, Rome

Fukushima M, Kakinuma K, Kawaguchi R (2002) Phylogenetic analysis of *Salmonella*, *Shigella*, and *Escherichia coli* strains on the basis of the gyrB gene sequence. J Clin Microbiol 40:2779–2785

Garcia-Martinez J, Acinas SG, Anton AI, Rodriguez-Valera F (1999) Use of the 16S-23S ribosomal genes spacer region in studies of prokaryotic diversity. J Microbiologica Method 36:55–64

Gasser F (1970) Electrophoretic characterization of lactic dehydrogenases in the genus *Lactobacillus*. J Gen Microbiol 62:223–239

Gevers D, Huys G, Swings J (2001) Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. FEMS Microbiol Lett 205:31–36

Giraffa G, Neviani E (2000) Molecular identification and characterization of food-associated Lactobacilli. Italian J food Sci 12:403–423

Golic N, Strahinic I, Terzic-Vidojevic A, Begovic J, Nikolic M et al (2012) Molecular diversity among natural populations of *Lactobacillus paracasei* and *Lactobacillus plantarum/paraplantarum* strains isolated from autochthonous dairy products. Eur Food Res Technol 234:627–638

Gürtler V (1999) The role of recombination and mutation in 16S-23S rDNA spacer rearrangements. Gene 238:241–252

Gürtler V, Stanisich VA (1996) New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. Microbiologica 142:3–16

Gutell RR (1993) Collection of small subunit (16S-and 16S-like) ribosomal RNA structures. Nucleic Acids Res 21:3051

Holzapfel WH, Haberer P, Geisen R, Bjorkroth J, Schillinger U (2001) Taxonomy and important features of probiotic microorganisms in food and nutrition. Am J Clin Nutr 73:365s–373s

Johansson ML, Quednau M, Molin G, Ahrn S (1995) Randomly amplified polymorphic DNA (RAPD) for rapid typing of *Lactobacillus plantarum* strains. Lett Appl Microbiol 21:155–159

Kandler O, Weiss N (1986) Genus lactobacillus. In: Bergey's manual of systematic bacteriology, vol 2. Springer, Berlin, pp 1209–1234

Kimura K, McCartney AL, McConnell MA, Tannock GW (1997) Analysis of fecal populations of bifidobacteria and lactobacilli and investigation of the immunological responses of their human hosts to the predominant strains. Appl Environ Microbiol 63:3394–3398

Klein G, Pack A, Bonaparte C, Reuter G (1998) Taxonomy and physiology of probiotic lactic acid bacteria. Int J Food Microbiol 41:103–125

König H, Fröhlich J (2009) Lactic acid bacteria. Biology of microorganisms on grapes, in must and in wine. Springer, Heidelberg, pp 3–29

Lortal S, Valence F, Bizet C, Maubois JL (1997) Electrophoretic pattern of peptidoglycan hydrolases, a new tool for bacterial species identification: application to 10 *Lactobacillus* species. Res Microbiol 148:461–474

Ludwig W, Neumaier J, Klugbauer N, Brockmann E, Roller C et al (1993) Phylogenetic relationships of bacteria based on comparative sequence analysis of elongation factor Tu and ATP-synthase Î²-subunit genes. Anton Leeuw 64:285–305

Maassen C, Boersma WJA, van Holten-Neelen C, Claassen E, Laman JD (2003) Growth phase of orally administered *Lactobacillus* strains differentially affects IgG1/IgG2a ratio for soluble antigens: implications for vaccine development. Vaccine 21:2751–2757

Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B et al (2006) Comparative genomics of the lactic acid bacteria. Proc Natl Acad Sci 103:15611–15616

Mardis ER (2008) The impact of next-generation sequencing technology on genetics. Trend Geneti 24:133–141

Mardis ER (2013) Next-generation sequencing platforms. Ann Rev Anal Chem 6:287–303

Marilley L, Casey MG (2004) Flavours of cheese products: metabolic pathways, analytical tools and identification of producing strains. Int J Food Microbiol 90:139–159

Marshall E, Mejia D (2011) Traditional fermented food and beverages for improved livelihoods. FAO

McCartney AL (2002) Application of molecular biological methods for studying probiotics and the gut flora. British J Nutri 88:S29–S37

McCartney AL, Wenzhi W, Tannock GW (1996) Molecular analysis of the composition of the bifidobacterial and *Lactobacillus* microflora of humans. Appl Environ Microbiol 62:4608–4613

Mollet C, Drancourt M, Raoult D (1997) rpoB sequence analysis as a novel basis for bacterial identification. Mol Microbiol 26:1005–1011

Mueller UG, Wolfenbarger LL (1999) AFLP genotyping and fingerprinting. Trends Ecol Evol 14:389–394

Nguyen DTL, Cnockaert M, Van Hoorde K, De Brandt E, Snauwaert I et al (2013a) *Lactobacillus porcinae* sp. nov., isolated from traditional Vietnamese nem chua. Int J Syst Evol Microbiol 63:1754–1759

Nguyen HKT, Ha DL, Doan TTV, Quach TT, Nguyen HK (2013b) Study of Lactobacillus acidophilus by Restriction Fragment Length Polymorphism (RFLP) analysis. Springer, Berlin, pp 195–197

O'Sullivan TF, Fitzgerald GF (1998) Comparison of *Streptococcus thermophilus* strains by pulse field gel electrophoresis of genomic DNA. FEMS Microbiol Lett 168:213–219

O'sullivan L, Ross RP, Hill C (2002) Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. Biochimie 84:593–604

Olive DM, Bean P (1999) Principles and applications of methods for DNA-based typing of microbial organisms. J Clin Microbiol 37:1661–1669

Olsen GJ, Woese CR (1993) Ribosomal RNA: a key to phylogeny. FASEB J 7:113–123

Ouoba LII, Nyanga-Koumou CAG, Parkouda C, Sawadogo H, Kobawila SC et al (2009) Genotypic diversity of lactic acid bacteria isolated from African traditional alkaline-fermented foods. J Appl Microbiol 108:2019–2029

Palys T, Berger E, Mitrica I, Nakamura LK, Cohan FM (2000) Protein-coding genes as molecular markers for ecologically distinct populations: the case of two *Bacillus* species. Int J Syst Evol Microbiol 50:1021–1028

Prajapati JB (2011) Traditional dairy products in developing countries. Invited paper presented at World Dairy Summit organized by International Dairy Federation. p 16–19

Prasad J, Gill H, Smart J, Gopal PK (1998) Selection and characterisation of *Lactobacillus* and *Bifidobacterium* strains for use as probiotics. Int Dairy J 18:993–1002

Rainey FA, Ward-Rainey NL, Janssen PH, Hippe H, Stackebrandt E (1996) *Clostridium paradoxum* DSM 7308T contains multiple 16S rRNA genes with heterogeneous intervening sequences. Microbiologica 142:2087–2095

Ramachandran P, Lacher DW, Pfeiler EA, Elkins CA (2013) Development of a tiered multilocus sequence typing scheme for members of the *Lactobacillus acidophilus* complex. Appl Environ Microbiol 79:7220–7228

Singh S, Goswami P, Singh R, Heller KJ (2009) Application of molecular identification tools for *Lactobacillus,* with a focus on discrimination between closely related species: a review. LWT-Food Sci Tech 42:448–457

Song Y (2005) PCR-based diagnostics for anaerobic infections. Anaerobe 11:79–91

Stackebrandt E (2003) The richness of prokaryotic diversity: there must be a species somewhere. Food Technol Biotechnol 41:17–22

Stackebrandt E, Goebel BM (1994) Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 44:846–849

Švec P, Sedláček I, Žáčková L, Nováková D, Kukletová M (2009) *Lactobacillus* spp. associated with early childhood caries. Folia Microbiol 54:53–58

Tannock GW (1999) Identification of lactobacilli and bifidobacteria. Curr Issues Mol Biol 1:53–64

Teuber M (1993) Lactic acid bacteria. In: Biotechnology set, 2nd edn. Weinheim: Wiley p 325–366

Thomson CH, Hassan I, Dunn K (2012) Yakult: a role in combating multi-drug resistant Pseudomonas aeruginosa? J Wound Care 21:566–569

Tynkkynen S, Satokari R, Saarela M, Mattila-Sandholm T, Saxelin M (1999) Comparison of ribotyping, randomly amplified polymorphic DNA analysis, and pulsed-field gel electrophoresis in typing of *Lactobacillus rhamnosus* and *L. casei* strains. Appl Environ Microbiol 65:3908–3914

van den Berg DJC, Smits A, Pot B, Ledeboer AM, Kersters K et al (1993) Isolation, screening and identification of lactic acid bacteria from traditional food fermentation processes and culture collections. Food Biotechnol 7:189–205

Van Hoorde K, Verstraete T, Vandamme P, Huys G (2008) Diversity of lactic acid bacteria in two Flemish artisan raw milk Gouda-type cheeses. Food Microbiol 25:929–935

Vandamme P, Pot B, Gillis M, De Vos P, Kersters K et al (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol Rev 60:407–438

Ventura M, Zink R (2002) Rapid identification, differentiation, and proposed new taxonomic classification of *Bifidobacterium lactis*. Appl Environ Microbiol 68:6429–6434

Versalovic J, Koeuth T, Lupski R (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res 19:6823–6831

Versalovic J, Schneider M, De Bruijn FJ, Lupski JR (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mole Cellular Biol 5:25–40

Zabeau M, Vos P (1993) Selective restriction fragment amplification: a general method for DNA fingerprinting. CA Patent CA2,119,557

Vos P, Hogers R, Bleeker M, Reijans M, van De Lee T et al (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414

Wells JM, Mercenier A (2008) Mucosal delivery of therapeutic and prophylactic molecules using lactic acid bacteria. Nat Rev Microbiol 6:349–362

Wells JM, Robinson K, Chamberlain LM, Schofield KM, Page RWF (1996) Lactic acid bacteria as vaccine delivery vehicles. Anton Leeuw 70:317–330

Wertz JE, Goldstone C, Gordon DM, Riley MA (2003) A molecular phylogeny of enteric bacteria and implications for a bacterial species concept. J Evol Biol 16:1236–1248

Williams AG, Banks JM (1997) Proteolytic and other hydrolytic enzyme activities in non-starter lactic acid bacteria (NSLAB) isolated from cheddar cheese manufactured in the United Kingdom. Int Dairy J 7:763–774

Williams RAD, Sadler SA (1971) Electrophoresis of glucose-6-phosphate dehydrogenase, cell wall composition and the taxonomy of heterofermentative lactobacilli. J Gen Microbiol 65:351–358

Winslow CEA, Broadhurst J, Buchanan RE, Krumwiede C Jr, Rogers LA et al (1917) The families and genera of the bacteria preliminary report of the Committee of the Society of American bacteriologists on characterization and classification of bacterial types. J Bacteriol 2:505–566

Wong P, Houry WA (2004) Chaperone networks in bacteria: analysis of protein homeostasis in minimal cells. J Struct Biol 146:79–89

Wouters J, Ayad EHE, Hugenholtz J, Smit G (2002) Microbes from raw milk for fermented dairy products. Int Dairy J 12:91–109

Xu F-L, Guo Y-C, Wang H-X, Fu P, Zeng H-W et al (2012) PFGE genotyping and antibiotic resistance of *Lactobacillus* distributed strains in the fermented dairy products. Ann Microbiol 62:255–262

Zwieb C, Gorodkin J, Knudsen B, Burks J, Wower J (2003) tmRDB (tmRNA database). Nucleic Acids Res 31:446–447

12 New Approaches for Improvement of Diva Vaccines and Their Companion Diagnostic Tests for Foot-and-Mouth Disease and Bluetongue Disease

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12.1 Bluetongue Disease

Bluetongue (BT) disease is caused by the bluetongue virus (BTV) of family *Reoviridae*.BT is economically important, non-contagious but infectious disease of ruminants and camels. It is transmitted by biting midges *Culicoides*. Globally it causes both direct and indirect economic losses. The direct losses include reduced milk production, abortions, reduced meat production and death. Indirect losses are export restrictions of live animals and their by-products. Clinical manifestation of disease is mainly observed in sheep (fine wool breeds) and white-tailed deer. Clinical signs are characterized by facial oedema, coronitis and haemorrhages in the oral mucosa.

Till date, 27 BTV serotypes have been identified globally (Roy and Noad 2006; Schwartz-Cornil et al. 2008; Hofmann et al. 2008; Chaignat et al. 2009; Maan et al. 2011). The genome of BTV consists of double-stranded RNA having ten segments which codes for four

nonstructural proteins (NS1–NS3 and NS3A) and seven structural proteins (VP1–VP7) (Kar et al. 2007; Roy 2008). Sequence of NS3 is found conserved amongst all serotypes of BTV. Therefore, NS3 is a good candidate against which DIVA can be developed for all serotypes.

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Due to segmented genome of BTV, reassortment of ds-RNA segments may happen if the host cell is concurrently infected by several different serotypes or strains (Oberst et al. 1987; Samal et al. 1987; Belyaev and Roy 1993; Batten et al. 2008). The reassortment event plays an important role in the development of viral diversity (Carpi et al. 2010) and gives rise to changes in virulence and serological characteristics of the virus (Cowley and Gorman 1989; Mertens et al. 1989; Nuttall et al. 1992; O'Hara et al. 1998; Batten et al. 2008).

BT vaccination can prevent clinical disease and can minimize the economic losses (Savini et al. 2008; Bhanuprakash et al. 2009; Caporale and Giovannini 2010). For vaccination, it is important to know the prevalent serotypes in a given area because the BT vaccines are serotype specific (Bhanuprakash et al. 2009).

12.2 Inactivated Vaccine

In Northern Europe, after 2008 outbreak use of inactivated vaccines has prevented the reoccurrence of the disease. Inactivated vaccines prevent occurrence of the disease. These vaccines require booster doses to attain protective immune response (Savini et al. 2008, 2009) and can prevent the clinical disease in animals, thus, facilitating economically. Vaccination by inactivated vaccines requires large amounts of inactivated antigen. The first inactivated vaccine against BTV was prepared against BTV-2 after Southern Europe witnessed outbreak of BT in 1998 (Savini et al. 2008). Monovalent vaccines are available against BTV-1, BTV-8 and BTV-9 (Zientara et al. 2010). The inactivated vaccines have advantage that they can be used for DIVA. NS3 nonstructural protein of BTV has been used for development of DIVA test (ELISA) and has shown promising results (Barros et al. 2009).

12.3 Live Attenuated Vaccines

Live vaccines are useful to prevent the clinical disease against BTV; however, it has been found to show teratogenicity (Veronesi et al. 2010). There is possibility that the vaccine virus, after reaching to blood, can be transmitted to other infected hosts by the bite of *Culicoides* (Elia et al. 2008). This can lead to reassortment of gene segments between vaccine and field virus, hence, resulting into development of novel strains (Batten et al. 2008).

Recombinant vaccine using poxviruses as vectors expressing VP2 and VP5 proteins of BTV has been developed. This vaccine is suitable to be used for DIVA purpose (Lobato et al. 1997; Boone et al. 2007).

12.4 New-Generation Vaccines

New vaccines like, virus-like particles (VLPs), subunit vaccines, DISC (disabled infectious single cycle) vaccines and gene reassortant vaccines are the next-generation vaccines which can have promising results. These vaccines have several added benefits like they can be used as polyvalent vaccines, suitability for DIVA, no virus transmission risk and rapid generation of immune response (Savini et al. 2008; Bhanuprakash et al. 2009; Roy et al. 2009).

12.4.1 Subunit Vaccines

For subunit vaccines, VP2 protein expressed in baculovirus (Van Dijk 1993) and yeast (*Pichia pastoris*) (Athmaram et al. 2007) has been used. It has been found that it can induce immune response capable to protect sheep against homologous virus challenge. It has been observed that core-like particles (CLPs) are also immunogenic. CLPs consisting of VP7 and VP3 can inhibit manifestation of clinical signs (Van Dijk 1993). These subunit vaccines have been found to be as safe as the inactivated vaccines and can be considered as the promising DIVA vaccines.

12.4.2 VLPs

VLPs consist of only capsid proteins and lack the viral genome. They share all immunological properties with the parent virus and are safe to handle. They do not show reassortment, recombination and reversion. The most common expression system used for VLP expression is baculovirus, due to its higher recombinant protein production. VP2, VP3, VP5 and VP7 protein of BTV have been expressed in baculovirus (French et al. 1990; Pearson and Roy 1993), and their use with adjuvants (aluminium hydroxide, incomplete Freund's adjuvant or Montanide ISA) was found to elicit protective immune response in sheep for 15 months. Moreover, VLPs expressing VP2 protein from various BTV serotypes were found to be protective not only against challenge from the BTV serotypes whose VP2 was included in VLPS (Roy et al. 1994) but also partial protection against heterologous serotypes (Roy et al. 1994). As these VLPs lack NSPs, hence, it can be used for DIVA purpose.

12.4.3 DISC (Disabled Infectious Single Cycle) Vaccines

These vaccines have gene deletions/mutations of one or more genes so that only single cycle of replication can be achieved (Matsuo et al. 2011). These vaccines are compatible with DIVA tests. The rescue of DISC viruses can be achieved by reverse genetics methodology. Experimentally, DISC vaccine has shown protective immune

response against all serotypes present in vaccine (Celma et al. 2013). Based on this strategy, DISC vaccine with negatively marked NS3 protein has been developed along with DIVA test which can detect antibodies against NS3 protein (Feenstra et al. 2014).

12.4.4 Gene Segment Reassortant Vaccines

Using reverse genetics system reassortant vaccine has been developed. Segments 2 (VP2) and 6 (VP5) of the live attenuated BTV-6 vaccine were exchanged for those of BTV-1 and BTV-8, resulting in the generation of BTVac-1 and BTVac-8, respectively (van Gennip et al. 2012). After vaccination, single dose of these vaccines was found to induce a virus-neutralizing antibody response against the homologous serotype. On further experimentation these vaccines have shown a promising result. Further work is needed to explore any potential biosafety risk linked with the exchange of gene segments between vaccine and field viruses.

Important advantages of new-generation vaccines compared to conventional vaccines are the inherent safety and the possibility to distinguish serologically infected from vaccinated animals, which allow the control of the disease and surveillance. For both reasons, development of recombinant vaccines against BTV is still essential nowadays.

DIVA tests for BTV are still in progress. DIVA test to detect antibodies against NS3 has been developed using inactivated vaccine and DISC vaccine. However, due to contamination of inactivated vaccines by NSPs, inactivated vaccines may not be a suitable approach. BTV VLPs or DISC vaccines may be a suitable alternative to provide complete protection against the disease and subsequently developing DIVA against NS3 protein of BTV.

12.5 Foot-and-Mouth Disease

Foot-and-mouth disease (FMD) is a highly contagious and economically important disease of domestic and wild cloven-hoofed animals. Causative agent of FMD is foot-and-mouth disease virus (FMDV) of genus *Aphthovirus* and family *Picornaviridae* (Belsham 1993). FMDV exists as seven distinct serotypes [O, A, C, Asia1 and South African Territories (SAT-1, SAT-2 and SAT-3)], and each serotype contains multiple subtypes. FMDV spreads by air, fomites and direct contact between infected and susceptible animals/animal products like milk, meat and semen (Alexandersen et al. 2002; Barnett and Cox 1999; Callens et al. 1998).An asymptomatic FMDV persistent infection (carrier state) can be established in ruminants beyond 28 days postinfection and can last to several years, irrespective of vaccination status (Salt 1993). Animals immunized with inactivated FMD vaccines may be protected against clinical disease but may not be protected against subclinical infection as the vaccine does not provide sterile immunity (Cox et al. 2006). Therefore, in the case of an outbreak controlled by vaccination, serosurveillance is

necessary to substantiate absence of infection and declare freedom from the disease. If the vaccine is purified from nonstructural proteins (NSP), vaccinated animals will only elicit antibodies against FMDV structural proteins (SP), whereas infected animals will elicit antibodies against both NSP and SP. Hence, detection of serum antibodies elicited against NSPs of FMDV in vaccinated, and subsequently infected animals is used to indicate infection. Though some commercial and in-house tests have been developed based on the detection of antibodies to NSPs, their specificity and sensitivity are not sufficient to demonstrate the absence of infection with a high level of confidence (Paton et al. 2006).

At an international workshop held under the EU Improcon project at Brescia, Italy, four commercial tests (PrioCHECK FMDV NS, CHEKIT FMD 3ABC, UBI FMDV NS ELISA, SVANOVIR FMDV 3ABC-Ab ELISA), one in-house test (3ABC trapping-ELISA from IZS Brescia) and the OIE index test NCPanaftosa were validated. The specificity of the tests ranged from 97.2% to 98.5% at the first round of screening, and the specificity was further increased to 98.3–99.7% after retesting the nonspecific samples. Sensitivity to detect vaccinated cattle that became carriers ranged from 68% to 94% depending on the test used. A suggestion was made to increase the specificity and sensitivity of the tests by using more than one NSP antibody assay to differentiate infection in vaccinated animals (DIVA) (Brocchi et al. 2006; Paton et al. 2006). Moreover, it has been suggested that the confidence to detect infection may be augmented by using multiple FMDV antigens in a multiplex ELISA (Perkins et al. 2006, 2007).

12.6 Vaccine and DIVA Tests

FMD vaccines were produced on large scale since 1950s by Frenkel method. In this method, the tongue epithelium from healthy slaughtered animals was cultured and infected with FMDV. After getting sufficient titre, the virus was collected from infected cells and subsequently inactivated by formaldehyde (Lombard et al. 2007). The current FMD vaccine is produced in baby hamster kidney (BHK-21) cell culture and inactivating live virus using binary ethyleneimine. They are then devoid of nonstructural proteins by chromatographic purification and formulated with adjuvants (Rodriguez and Gay 2011). The two types of adjuvants used are aluminium hydroxide gel [$Al(OH)_3$] supplemented with saponin and oil emulsion (Doel 1999). $Al(OH)_3$/saponin vaccines are incorporated in vaccine for their use in ruminants and oil-based adjuvants for use in ruminants and pigs. The most widely used vaccine is oil-adjuvanted vaccine as it can maintain sufficient level of immunity and can prevent reoccurrence of clinical disease. However, these vaccines require revaccination after every 6 months of first vaccination. It has been shown that oil adjuvant vaccines of $3PD_{50}$ can generate protective immune response in cattle, swine and sheep within 7 days of vaccination (Golde et al. 2005). The emergency vaccines are of higher potency [usually ≥6 protective dose 50(PD50)] than the conventional vaccines (≥3 PD50) and provide rapid protection by inducing innate immunity. FMD vaccines are also stored as frozen antigen reserves or vaccine banks, ready for rapid

formulation and emergency use in FMD-free countries (Bhanuprakash et al. 2009; Caporale and Giovannini 2010). There are 40 million doses of FMD vaccine in the European vaccine bank which comprises of various FMDV serotypes and strains.

There are certain drawbacks associated with conventional vaccines. The major shortcomings are that they do not induce long-term protection and require multiple vaccinations and periodic inclusion of new viral strains into the vaccine formulations. Other shortcomings are short shelf life, requirement of cold chain and difficulties in growth of certain serotypes/subtypes in cell culture for vaccine production. Hence, in order to rectify these problems, several attempts have been made to advent new vaccine platforms (Rodriguez and Grubman 2009) such as DNA vaccines, subunit vaccines, empty capsid vaccines and viral vectored vaccines.

In mouse models, the protective immunity has been found to get induced after vaccinating with plasmid DNA having FMD sequences (Yang et al. 2005; Ward et al. 1997). In contrast, to induce partial or full protection in cattle, sheep and pigs, DNA vaccine requires multiple doses along with adjuvants and cytokines (Fowler et al. 2012). Attempts to make subunit vaccine have been made targeting VP1-GH loop. VP1 has been used after producing from recombinant techniques or isolating from purified virus (Bachrach et al. 1975; Kleid et al. 1981), VP1-derived peptide (Strohmaier et al. 1982), chemically synthesized VP1 peptides (Bittle et al. 1982; Francis et al. 1991; Nargi et al. 1999; Pfaff et al. 1982), live vectors expressing VP1 fusion proteins (Kit et al. 1991; Kitson et al. 1991), inoculation of DNA expressing VP1 epitopes (Kit et al. 1991; Kitson et al. 1991), co-administration with DNA encoding IL2 (Wong et al. 2002) and plants infected with recombinant tobacco mosaic virus expressing VP1 (Wigdorovitz et al. 1999). Due to limited presentation of viral immunogens by subunit vaccine, animals are not always protected against virus challenge (DiMarchi et al. 1986; Mulcahy et al. 1992).

Another strategy adopted is to use virus-like particles/empty capsids which are non-replicating and non-pathogenic and resemble to parent virus in structure and antigenicity. These VLPs contain entire repertoires of immunogenic sites present on the intact virus particles. A number of attempts have been made to construct plasmid with genes encoding for capsid and 3C. Several expression systems such as mammalian cell line transfected with viral expression vectors (Ren et al. 2009; Li et al. 2008a), baculovirus/insect cell and larvae systems (Li et al. 2011, 2008b; Cao et al. 2009), yeasts like *Saccharomyces cerevisiae* and *Pichia pastoris*, *E. coli* and other bacteria (Li et al. 2007; Bachrach et al. 1975; Vidal et al. 1991) and viral vectored vaccine platforms like herpes virus, poxvirus, alpha virus and Replication-defective human adenovirus type 5 (Ad5) have been used.

Human replication defective adenovirus 5 (hAd5) is a successful platform that can be used to deliver FMDV capsids to the animals. It has been shown that cattle and swine receiving one hAd5–FMD vaccine dose and challenged as early as 7 days postvaccination (dpv) showed complete protection (Moraes et al. 2002; Pacheco et al. 2005). This vaccine consists of all FMDV structural proteins and nonstructural proteins 2A and 3C. Thus 3B and 3DNSPs can be used to develop serological tests for FMD DIVA.

Adenovirus expressing porcine IFN λ3 has proven to be a potent therapeutic agent in swine (Perez-Martin et al. 2014). Also, a baculovirus-expressed capsid stable vaccine has been designed by irrationally engineering a disulphide bond by mutation (Porta et al. 2013). Cattles vaccinated using this vaccine have shown sustained virus neutralization titre and protective immunity (Porta et al. 2013). Recently, the use of RNA interference (RNA-i) for FMDV therapy has been demonstrated in mice and PK15 cell lines by the knockout of αv receptors, subsequently resulting into higher survival rate of mice and less FMDV infection in PK15 cells (Du et al. 2014).

DIVA tests based on detection of antibodies against various NSPs (3ABC, 3AB, 3A, 3B, 2A, 2B and 2C) of FMDV have been developed to discriminate FMDV-vaccinated uninfected animals from vaccinated infected animals (Bruderer et al. 2004; De Diego et al. 1997; Hema et al. 2007; Inoue et al. 2006; Oem et al. 2007; Parida et al. 2005; Perkins et al. 2006, 2007; Shen et al. 1999; Mahajan et al. 2013; Sharma et al. 2012; Mohapatra et al. 2014; Srisombundit et al. 2013). To date, detection of antibodies to polyprotein 3ABC has been the most successful NSP antibody test (Brocchi et al. 2006). The 3ABC NCPanaftosa test is considered as the OIE index method supported by a confirmatory immunoblot test for antibodies against the 3A, 3B, 2C, 3D and 3ABC NSPs. Fu and colleagues (Fu et al. 2011) have developed a dot immunoblot method using 3A, 3B, 2C, 3D and 3ABC nonstructural proteins. Apart from these in-house tests, commercial kits from PrioCHECK®FMDV-NS, Chekit FMD-3ABC ELISA, UBI® FMD NS ELISA and SVANOVIR™ FMDV 3ABC-Ab are available and validated (Brocchi et al. 2006).

In PrioCHECK® FMDV-NS test, the serum antibodies compete with NSP 3B-specific monoclonal antibody for binding to baculovirus-derived 3ABC NSP of FMDV (Chung et al. 2002; Sorensen et al. 1998). Chekit FMD-3ABC ELISA (Bommeli Diagnostics, Switzerland) and the UBI® FMD NS ELISA (United Biomedical Inc., New York, USA) are both indirect ELISAs that measure the binding of antibodies to 3ABC NSP expressed in *E. coli* (Bruderer et al. 2004) and to 3B peptide (Shen et al. 1999), respectively. SVANOVIR™ FMDV 3ABC-Ab (Svanova Biotech, Sweden) is also an indirect ELISA measuring antibodies against 3ABC NSP of FMD (Persson et al. 2004).

These four commercial NSP tests and one in-house NS assay, i.e. 3ABC-trapping ELISA, IZS, Brescia (De Diego et al. 1997), were validated in Brescia, Italy, and compared to the PANAFTOSA index assay (Malirat et al. 1998) under the scope of an EU-funded international project (Brocchi et al. 2006). The specificity of the tests ranged between 97% and 98% and was not affected by a single dose of European manufactured vaccine. All these tests detected infection in unvaccinated cattle with high sensitivity. However the sensitivity to detect FMDV carriers in vaccinated subsequently infected cattle was ranged 68–94% depending on the tests used. The workshop concluded that two tests (PrioCHECK FMDV NStest® FMDV-NS and 3ABC trapping-ELISA, IZS-Brescia) performed as good as the OIE index method. This validation suggested that the commercially available PrioCHECK FMDV NS test has good sensitivity and specificity and can be used as a screening test for European countries (Paton et al. 2006). However, this large validation at Brescia

concluded that the specificity and sensitivity of these tests are not absolutely sufficient to demonstrate the absence of infection with a high level of confidence and needs to develop and validate confirmatory tests to support the above suggested screening tests (Paton et al. 2006). Alternatively, by using multiple antigens in liquid array, the sensitivity and specificity to detect infection can be increased.

In liquid array technology multiple analytes can be measured (Kellar and Oliver 2004) and have several applications in serological assays (Balasuriya et al. 2006; Feng et al. 2004; Khan et al. 2005; Komatsu et al. 2004), antigen and nucleic acid-based detection (McBride et al. 2003a, b). The main advantage of this test is to increase the confidence of detection of infection with less variation by using more than one analytes in single well. Using this technique, Clavijo and colleagues (2006) have developed a multiplex assay for FMD by using four recombinant nonstructural proteins (3ABC, 3A, 3B and 3D). The assay was able to differentiate between positive and negative sera, and further validation with large number of samples was suggested. Following this, Perkins and colleagues (2007) improved this multiplex assay using three peptides (3A, 3B, 3D) and one nonstructural protein (3ABC). In this assay 3ABC signature in multiplex format showed good sensitivity and specificity that was similar to PrioCHECK® FMDV NS. Using Luminex technology, multiplexing of antigens to develop DIVA test is going on in many labs, worldwide, so as to increase the confidence to detect infection.

12.7 Conclusion

The new-generation vaccines developed for BTV and FMD can serve as important tools for serosurveillance. The DISC vaccine for BTV and adenovirus vector-based empty capsid vaccine for FMD are important from DIVA point of view. These vaccines are safe, effective and have the ability to be used as a marker vaccine which can be used along with a companion diagnostic test for DIVA purpose.

References

Alexandersen S, Zhang Z, Reid SM et al (2002) Quantities of infectious virus and viral RNA recovered from sheep and cattle experimentally infected with foot-and-mouth disease virus O UK 2001. J Gen Virol 83:1915–1923

Athmaram TN, Bali G, Kahng GG, Dwarakanath S (2007) Heterologous expression of bluetongue VP2 viral protein fragment in *Pichia pastoris*. Virus Genes 35(2):265–271

Bachrach HL, Moore DM, McKercher PD, Polatnick J (1975) Immune and antibody responses to an isolated capsid protein of foot-and-mouth disease virus. J Immunol (Baltimore, Md.: 1950) 115:1636–1641

Balasuriya UB, Shi PY, Wong SJ, Demarest VL, Gardner IA, Hullinger, PJ, Ferraro GL, Boone JD, De Cino CL, Glaser AL, Renshaw RW, Ledizet M, Koski RA, MacLachlan NJ (2006) Detection of antibodies to West Nile virus in equine sera using microsphere immunoassay. J Vet Diagn Invest: official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 18, 392–395

Barnett PV, Cox SJ (1999) The role of small ruminants in the epidemiology and transmission of foot-and-mouth disease. Vet J 158:6–13

Barros SC, Cruz B, Luis TM et al (2009) A DIVA system based on the detection of antibodies to non-structural protein 3 (NS3) of blue tongue virus. Vet Microbiol 137:252–259

Batten CA, Maan S, Shaw AE et al (2008) A European field strain of bluetongue virus derived from two parenteral vaccine strains by genome segment reassortment. Vir Res 137(1):56–63

Belsham GJ (1993) Distinctive features of foot-and-mouth disease virus, a member of the picornavirus family; aspects of virus protein synthesis, protein processing and structure. Prog Biophys Mol Biol 60:241–260

Belyaev AS, Roy P (1993) Development of baculovirus triple and quadruple expression vectors: co-expression of three or four bluetongue virus proteins and the synthesis of bluetongue virus-like particles in insect cells. Nuc Acids Res 21:1219–1223

Bhanuprakash V, Indrani BK, Hosamani M et al (2009) Bluetongue vaccines: the past, present and future. Exp Rev Vacc 8:191–204

Bittle JL, Houghten RA, Alexander H, Shinnick TM, Sutcliffe JG, Lerner RA, Rowlands DJ, Brown F (1982) Protection against foot-and-mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence. Nature 298:30–33

Boone JD, Balasuriya UB, Karaca K, Audonnet JC et al (2007) Recombinant canary-pox virus vaccine co-expressing genes encoding the VP2 and VP5 outer capsid proteins of bluetongue virus induces high level protection in sheep. Vaccine 25(4):672–678

Brocchi E, Bergmann IE, Dekker A, Paton DJ et al (2006) Comparative evaluation of six ELISAs for the detection of antibodies to the non-structural proteins of foot-and-mouth disease virus. Vaccine 24:6966–6979

Bruderer U, Swam H, Haas B, Visser N, Brocchi E, Grazioli S, Esterhuysen JJ, Vosloo W, Forsyth M, Aggarwal N, Cox S, Armstrong R, Anderson J (2004) Differentiating infection from vaccination in foot-andmouth-disease: evaluation of an ELISA based on recombinant 3ABC. Vet Microbiol 101:187–197

Callens M, de Clercq K, Gruia M, Danes M (1998) Detection of foot-and-mouth disease by reverse transcription polymerase chain reaction and virus isolation in contact sheep without clinical signs of foot-and-mouth disease. Vet Q 20(Suppl 2):37–40

Cao Y, Lu Z, Sun J, Bai X, Sun P, Bao H, Chen Y, Guo J, Li D, Liu X, Liu Z (2009) Synthesis of empty capsid-like particles of Asia I foot-and-mouth disease virus in insect cells and their immunogenicity in guinea pigs. Vet Microbiol 137:10–17

Caporale V, Giovannini A (2010) Bluetongue control strategy, including recourse to vaccine: a critical review. Rev Sci Tech Off Int des Epizoot 29:573–591

Carpi G, Holmes EC, Kitchen A (2010) The evolutionary dynamics of bluetongue virus. J Mol Evol 70:583–592

Celma CC, Boyce M, van Rijn PA et al (2013) Rapid generation of replication-deficient monovalent and multivalent vaccines for bluetongue virus: protection against virulent virus challenge in cattle and sheep. J Virol 87(17):9856–9864

Chaignat V, Worwa G, Scherrer N, Hilbe M et al (2009) Toggenburg orbivirus, a new bluetongue virus: initial detection, first observations in field and experimental infection of goats and sheep. Vet Microbiol 138:11–19

Chung WB, Sorensen KJ, Liao PC, Yang PC, Jong MH (2002) Differentiation of foot-and-mouth disease virus-infected from vaccinated pigs by enzyme-linked immunosorbent assay using nonstructural protein 3AB as the antigen and application to an eradication program. J Clin Microbiol 40:2843–2848

Clavijo A, Hole K, Li M, Collignon B (2006) Simultaneous detection of antibodies to foot-and-mouth disease non-structural proteins 3ABC, 3D, 3A and 3B by a multiplexed Luminex assay to differentiate infected from vaccinated cattle. Vaccine 24:1693–1704

Cowley JA, Gorman BM (1989) Cross-neutralization of genetic reassortants of bluetongue virus serotypes 20 and 21. Vet Microbiol 19:37–51

Cox E, Verdonck F, Vanrompay D, Goddeeris B (2006) Adjuvants modulating mucosal immune responses or directing systemic responses towards the mucosa. Vet Res 37:511–539

De Diego M, Brocchi E, Mackay D, De Simone F (1997) The non-structural polyprotein 3ABC of foot-andmouth disease virus as a diagnostic antigen in ELISA to differentiate infected from vaccinated cattle. Arch Virol 142:2021–2033

DiMarchi R, Brooke G, Gale C, Cracknell V, Doel T, Mowat N (1986) Protection of cattle against foot-andmouth disease by a synthetic peptide. Science 232:639–641

Doel TR (1999) Optimisation of the immune response to foot-and-mouth disease vaccines. Vaccine 17:1767–1771

Du J, Guo X, Gao S, Luo J, Gong X, Hao C, Yang B, Lin T, Shao J, Cong G, Chang H (2014) Induction of protection against foot-and-mouth disease virus in cell culture and transgenic suckling mice by miRNA targeting integrin αv receptor. J Biotechnol 187:154–161

Elia G, Savini G, Decaro N, Martella V et al (2008) Use of real-time RT-PCR as a rapid molecular approach for differentiation of field and vaccine strains of bluetongue virus serotypes 2 and 9. Mol Cell Probes 22(1):38–46

Feenstra F, Pap JS, van Rijn PA (2014) Application of blue tongue Disabled Infectious Single Animal (DISA) vaccine for different serotypes by VP2 exchange or incorporation of chimeric VP2. Vaccine 33(6):812–818

Feng Q, Yu H, Liu Y, He C, Hu J, Sang H, Ding N, Ding M, Fung YW, Lau LT, Yu AC, Chen J (2004) Genome comparison of a novel foot-and-mouth disease virus with other FMDV strains. Biochem Biophys Res Commun 323:254–263

Fowler V, Robinson L, Bankowski B, Cox S, Parida S, Lawlor C, Gibson D, O'brien F, Ellefsen B, Hannaman D, Takamatsu HH, Barnett PV (2012) A DNA vaccination regime including protein boost and electroporation protects cattle against foot-and-mouth disease. Antiviral Res 94:25–34

Francis MJ, Hastings GZ, Brown F, McDermed J, Lu YA, Tam JP (1991) Immunological evaluation of the multiple antigen peptide (MAP) system using the major immunogenic site of foot-and-mouth disease virus. Immunology 73:249–254

French TJ, Marshall JJ, Roy P (1990) Assembly of double-shelled, virus like particles of bluetongue virus by the simultaneous expression of four structural proteins. J Virol 64(12):5695–5700

Fu Y, Cao Y, Sun P, Bao H, Bai X, Li P, Li D, Lu Z, Liu Z (2011 January) Development of a dot immunoblot method for differentiation of animals infected with foot-and-mouth disease virus from vaccinated animals using non-structural proteins expressed prokaryotically. J Virol Methods 171(1):234–240

Golde WT, Pacheco JM, Duque H et al (2005) Vaccination against foot-and-mouth disease virus confers complete clinical protection in 7 days and partial protection in 4 days: use in emergency outbreak response. Vaccine 23:5775–5782

Hema M, Nagendrakumar SB, Yamini R, Chandran D, Rajendra L, Thiagarajan D, Parida S, Paton DJ, Srinivasan VA (2007) Chimeric tymovirus-like particles displaying foot-and-mouth disease virus non-structural protein epitopes and its use for detection of FMDV-NSP antibodies. Vaccine 25:4784–4794

Hofmann MA, Renzullo S, Mader M et al (2008) Genetic characterization of Toggenburg orbivirus, a new bluetongue virus from goats, Switzerland. Emerg Infect Dis 14:1855–1861

Inoue T, Parida S, Paton DJ, Linchongsubongkoch W, Mackay D, Oh Y, Aunpomma D, Gubbins S, Saeki T (2006) Development and evaluation of an indirect enzyme-linked immunosorbent assay for detection of foot-and-mouth disease virus nonstructural protein antibody using a chemically synthesized 2B peptide as antigen. J Vet Diagn Invest 18:545–552

Kar AK, Bhattacharya B, Roy P (2007) Bluetongue virus RNA binding protein NS2 is a modulator of viral replication and assembly. BMC Mol Bio 8:4

Kellar KL, Oliver KG (2004) Multiplexed microsphere assays for protein and DNA binding reactions. Methods Cell Biol 75:409–429

Kit M, Kit S, Little SP, Di Marchi RD, Gale C (1991) Bovine herpesvirus-1 (infectious bovine rhinotracheitis virus)-based viral vector which expresses foot-and-mouth disease epitopes. Vaccine 9:564–572

Kitson JD, Burke KL, Pullen LA, Belsham GJ, Almond JW (1991) Chimeric polioviruses that include sequences derived from two independent antigenic sites of foot-and-mouth disease

virus (FMDV) induce neutralizing antibodies against FMDV in guinea pigs. J Virol 65:3068–3075

Khan IH, Kendall LV, Ziman M, Wong S, Mendoza S, Fahey J, Griffey SM, Barthold SW, Luciw PA (2005) Simultaneous serodetection of 10 highly prevalent mouse infectious pathogens in a single reaction by multiplex analysis. Clin Diagn Lab Immunol 12:513–519

Kleid DG, Yansura D, Small B, Dowbenko D, Moore DM, Grubman MJ, McKercher PD, Morgan DO, Robertson BH, Bachrach HL (1981) Cloned viral protein vaccine for foot-and-mouth disease: responses in cattle and swine. Science 214:1125–1129

Komatsu N, Shichijo S, Nakagawa M, Itoh K (2004) New multiplexed flow cytometric assay to measure antipeptide antibody: a novel tool for monitoring immune responses to peptides used for immunization. Scand J Clin Lab Invest 64:535–545

Li J, Liu Y, Liu X, Shang Y, Liu J, An F, Yin H (2008a) [Screening and stability of Madin-Darby bovine kidney cell strain co-expressing the capsid precursor protein P1-2A gene and the protease 3C gene of foot-andmouth disease virus]. Wei sheng wu xue bao = Acta Microbiol Sin 48:1520–1525

Li Z, Yi Y, Yin X, Zhang Z, Liu J (2008b) Expression of foot-and-mouth disease virus capsid proteins in silkworm-baculovirus expression system and its utilization as a subunit vaccine. PLoS One 3:e2273

Li YG, Tian FL, Gao FS et al (2007) Immune responses generated by *Lactobacillus* as a carrier in DNA immunization against foot-and-mouth disease virus. Vaccine 25:902–911

Li Z, Yin X, Yi Y, Li X, Li B, Lan X, Zhang Z, Liu J (2011) FMD subunit vaccine produced using a silkwormbaculovirus expression system: protective efficacy against two type Asia1 isolates in cattle. Vet Microbiol 149:99–103

Lobato ZI, Coupar BE, Gray CP et al (1997) Antibody responses and protective immunity to recombinant vaccinia virus-expressed bluetongue virus antigens. Vet Immunol Immunopathol 59(3–4):293–309

Lombard M, Pastoret PP, Moulin AM (2007) A brief history of vaccines and vaccination. Rev Sci Tech 26:29–48

Maan S, Maan NS, Nomikou K, Batten C et al (2011) Novel bluetongue virus serotype from Kuwait. Emergy Infect Dis 17:886–889

Mahajan S, Mohapatra JK, Pandey LK, Sharma GK, Pattnaik B (2013) Truncated recombinant nonstructural protein 2C-based indirect ELISA for FMD sero-surveillance. J Virol Methods 193:405–414

Malirat V, Neitzert E, Bergmann IE, Maradei E, Beck E (1998) Detection of cattle exposed to foot-and-mouth disease virus by means of an indirect ELISA test using bioengineered nonstructural polyprotein 3ABC. Vet Q 20(Suppl 2):S24–S26

Matsuo E, Celma CC, Boyce M, Viarouge C et al (2011) Generation of replication-defective virus-based vaccines that confer full protection in sheep against virulent bluetongue virus challenge. J Virol 85:10213–10221

McBride N, Shichijo S, Nakagawa M, Itoh K (2004) New multiplexed flow cytometric assay to measure antipeptide antibody: a novel tool for monitoring immune responses to peptides used for immunization. Scand J Clin Lab Invest 64:535–545

McBride MT, Gammon S, Pitesky M, O'Brien TW, Smith T, Aldrich J, Langlois RG, Colston B, Venkateswaran KS (2003a) Multiplexed liquid arrays for simultaneous detection of simulants of biological warfare agents. Anal Chem 75:1924–1930

McBride MT, Masquelier D, Hindson BJ, Makarewicz AJ, Brown S, Burris K, Metz T, Langlois RG, Tsang KW, Bryan R, Anderson DA, Venkateswaran KS, Milanovich FP, Colston BW Jr (2003b) Autonomous detection of aerosolized Bacillus anthracis and Yersinia pestis. Anal Chem 75:5293–5299

Mertens PPC, Pedley S, Cowley J et al (1989) Analysis of the roles of bluetongue virus outer capsid proteins VP2 and VP5 in determination of virus serotype. Virology 170:561–565

Mohapatra AK, Mohapatra JK, Pandey LK, Sanyal A, Pattnaik B (2014) Diagnostic potential of recombinant nonstructural protein 3B to detect antibodies induced by foot-and-mouth disease virus infection in bovines. Arch Virol 159:2359–2369

Moraes MP, Mayr GA, Mason PW, Grubman MJ (2002) Early protection against homologous challenge after a single dose of replication-defective human adenovirus type 5 expressing capsid proteins of foot-and-mouth disease virus (FMDV) strain A24. Vaccine 20:1631–1639

Mulcahy G, Reid E, Dimarchi RD, Gale C, Doel TR (1992) Maturation of functional antibody affinity in animals immunised with synthetic foot-and-mouth disease virus. Res Vet Sci 52:133–140

Nargi F, Kramer E, Mezencio J, Zamparo J, Whetstone C, Van Regenmortel MH, Briand JP, Muller S, Brown F (1999) Protection of swine from foot-and-mouth disease with one dose of an all-D retro peptide. Vaccine 17:2888–2893

Nuttall PA, Jacobs SC, Jones LD, Carey D, Moss SR (1992) Enhanced neurovirulence of tick-borne orbiviruses resulting from genetic modulation. Virology 187:407–412

O'Hara RS, Meyer AJ, Burroughs JN et al (1998) Development of a mouse model system and identification of individual genome segments of African horse sickness virus, serotypes 3 and 8 involved in determination of virulence. Arch Virol 14:259–279

Oberst RD, Stott JL, Blanchard-Channell M, Osburn BI (1987) Genetic reassortment of bluetongue virus serotype 11 strains in the bovine. Vet Microbiol 15:11–18

Oem JK, Chang BS, Joo HD, Yang MY, Kim GJ, Park JY, Ko YJ, Kim YJ, Park JH, Joo YS (2007) Development of an epitope-blocking-enzyme-linked immunosorbent assay to differentiate between animals infected with and vaccinated against foot-and-mouth disease virus. J Virol Methods 142:174–181

Pacheco JM, Brum MC, Moraes MP, Golde WT, Grubman MJ (2005) Rapid protection of cattle from direct challenge with foot-and-mouth disease virus (FMDV) by a single inoculation with an adenovirus-vectored FMDV subunit vaccine. Virology 337:205–209

Parida S, Cox SJ, Reid SM, Hamblin P, Barnett PV, Inoue T, Anderson J, Paton DJ (2005) The application of new techniques to the improved detection of persistently infected cattle after vaccination and contact exposure to foot-and-mouth disease. Vaccine 23:5186–5195

Paton DJ, de Clercq K, Greiner M, Dekker A et al (2006) Application of non-structural protein antibody tests in substantiating freedom from foot-and-mouth disease virus infection after emergency vaccination of cattle. Vaccine 24:6503–6512

Pearson LD, Roy P (1993) Genetically engineered multi-component virus-like particles as veterinary vaccines. Immunol Cell Biol 71(5):381–389

Perez-Martin E, Diaz-San Segundo F, Weiss M, Sturza DF, Dias CC, Ramirez-Medina E, Grubman MJ, de Los Santos T (2014) Type III interferon protects swine against foot-and-mouth disease. J Interf Cytokine Res: the official journal of the international society for interferon and cytokine research

Perkins J, Clavijo A, Hindson BJ et al (2006) Multiplexed detection of antibodies to nonstructural proteins of foot-and-mouth disease virus. Anal Chem 78:5462–5468

Perkins J, Parida S, Clavijo A (2007) Use of a standardized bovine serum panel to evaluate a multiplexed nonstructural protein antibody assay for serological surveillance of foot-and-mouth disease. Clin Vaccine Immunol 14:1472–1482

Persson K, Nordengrahn A, Decker C, Merza M. (2004, October 11–15) The development of an indirect ELISA for the detection of antibodies to the non-structural protein 3ABC of the foot-and-mouth disease virus; the use of a polyclonal conjugate that allows a multi-species detection of antibodies. Report of the Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and- Mouth Disease. FAO, Rome, Chania, Crete, Greece, p. 479–80.

Pfaff E, Mussgay M, Bohm HO, Schulz GE, Schaller H (1982) Antibodies against a preselected peptide recognize and neutralize foot and mouth disease virus. EMBO J 1:869–874

Porta C, Kotecha A, Burman A, Jackson T, Ren J, Loureiro S, Jones IM, Fry EE, Stuart DI, Charleston B (2013) Rational engineering of recombinant picornavirus capsids to produce safe, protective vaccine antigen. PLoS pathogens 9:e1003255

Ren X, Xue F, Zhu Y, Tong G, Wang Y, Feng J, Zu L, Li J, Shi H, Gao Y, (2009). [Construction and identification of recombinant BHV-1 expressing foot and mouth disease virus VP1 gene]. Wei sheng wu xue bao = Acta Microbiol Sin 49:677–682

Rodriguez LL, Gay CG (2011) Development of vaccines toward the global control and eradication of foot-and-mouth disease. Expert Rev Vaccines 10:377–387

Rodriguez LL, Grubman MJ (2009) Foot and mouth disease virus vaccines. Vaccine 27(Suppl 4):D90–D94

Roy P (2008) Functional mapping of bluetongue virus proteins and their interactions with host proteins during virus replication. Cell Biochem Biophy 50:143–157

Roy P, Noad R (2006) Bluetongue virus assembly and morphogenesis. In: Roy P (ed) Reoviruses: entry, assembly and morphogenesis, current topics in microbiology and immunology. Springer, Berlin, pp 87–116

Roy P, Bishop DH, Le Blois H, Erasmus BJ (1994) Long-lasting protection of sheep against bluetongue challenge after vaccination with virus-like particles: evidence for homologous and partial heterologous protection. Vaccine 12(9):805–811

Roy P, Boyce M, Noad R (2009) Prospects for improved bluetongue vaccines. Nat Rev Microbiol 7:120–128

Salt JS (1993) The carrier state in foot and mouth disease-an immunological review. Br Vet J 149:207–223

Samal SK, El-Hussein A, Holbrook FR, Beaty BJ, Famig RF (1987) Mixed infection of *Culicoides variipennis* with bluetongue virus serotypes 10 and 17: evidence for high frequency reassortment in the vector. J Gen Virol 68:2319–2329

Savini G, MacLachlan NJ, Sanchez-Vizcaino JM, Zientar S (2008) Vaccines against blue tongue in Europe. Comp Immunol Microbiol Infect Dis 31(2–3):101–120

Savini G, Hamers C, Conte A, Migliaccio P et al (2009) Assessment of efficacy of a bivalent BTV-2 and BTV-4 inactivated vaccine by vaccination and challenge in cattle. Vet Microbiol 133:1–8

Sharma GK, Mohapatra JK, Pandey LK, Mahajan S, Mathapati BS, Sanyal A, Pattnaik B (2012) Immunodiagnosis of foot-and-mouth disease using mutated recombinant 3ABC polyprotein in a competitive ELISA. J Virol Methods 185:52–60

Schwartz-Cornil I, Mertens PPC, Contreras V et al (2008) Bluetongue virus: virology, pathogenesis and immunity. Vet Res 39:46

Shen F, Chen PD, Walfield AM, Ye J, House J, Brown F, Wang CY (1999) Differentiation of convalescent animals from those vaccinated against foot-and-mouth disease by a peptide ELISA. Vaccine 17:3039–3049

Sorensen KJ, Madsen KG, Madsen ES, Salt JS, Nqindi J, Mackay DK (1998) Differentiation of infection from vaccination in foot-and-mouth disease by the detection of antibodies to the non-structural proteins 3D, 3AB and 3ABC in ELISA using antigens expressed in baculovirus. Arch Virol 143:1461–1476

Srisombundit V, Tungthumniyom N, Linchongsubongkoch W, Lekcharoensuk C, Sariya L, Ramasoota P, Lekcharoensuk P (2013) Development of an inactivated 3C(pro)-3ABC (mu3ABC) ELISA to differentiate cattle infected with foot and mouth disease virus from vaccinated cattle. J Virol Methods 188(1–2):161–167

Strohmaier K, Franze R, Adam KH (1982) Location and characterization of the antigenic portion of the FMDV immunizing protein. J Gen Virol 59:295–306

Van Dijk AA (1993) Development of recombinant vaccines against blue tongue. Biotechnol Adv 11(1):1–12

van Gennip RGP, van de Water SGP, Potgieter CA et al (2012) Rescue of recent virulent and avirulent field strains of bluetongue virus by reverse genetics. PLoS One 7(2):1–10

Veronesi E, Darpel KE, Hamblin C, Carpenter S et al (2010) Viraemia and clinical disease in Dorset Poll sheep following vaccination with live attenuated bluetongue virus vaccines serotypes 16 and 4. Vaccine 28(5):1397–1403

Vidal M, Cairo J, Mateu MG, Villaverde A (1991) Molecular cloning and expression of the VP1 gene of footand-mouth disease virus C1 in E. coli: effect on bacterial cell viability. Appl Microbiol Biotechnol 35:788–792

Ward G, Rieder E, Mason PW (1997) Plasmid DNA encoding replicating foot-and-mouth disease virus genomes induces antiviral immune responses in swine. J Virol 71:7442–7447

Wigdorovitz A, Perez Filgueira DM, Robertson N, Carrillo C, Sadir AM, Morris TJ, Borca MV (1999) Protection of mice against challenge with foot and mouth disease virus (FMDV) by immunization with foliar extracts from plants infected with recombinant tobacco mosaic virus expressing the FMDV structural protein VP1. Virology 264:85–91

Wong HT, Cheng SC, Sin FW, Chan EW, Sheng ZT, Xie Y (2002) A DNA vaccine against foot-and-mouth disease elicits an immune response in swine which is enhanced by co-administration with interleukin-2. Vaccine 20:2641–2647

Yang NS, Wang JH, Lin KF, Wang CY et al (2005) Comparative studies of the capsid precursor polypeptide P1 and the capsid protein VP1 cDNA vectors for DNA vaccination against foot-and-mouth disease virus. J Gene Med 7:708–717

Zientara S, MacLachlan NJ, Calistri P et al (2010) Bluetongue vaccination in Europe. Exp Rev Vacc 9:989–991

Anthrax Bacterium: Its Etiology and Possible Therapeutics Against Cancer

13

Rekha Khandia and Ashok Munjal

13.1 Introduction

Anthrax is an oldest recorded disease of grazing animals and is known through several synonyms, viz. ragpickers' disease, charbon, milzbrand, black bain, tanner's disease and splenic fever; the disease among cattle is also known as murrain (Latin *morire* meaning to die). Mainly it infects grazing domesticated animals; however it has also been reported in exotic wildlife animals such as hippos, elephants and Cape buffalo (Bhatnagar and Batra 2001). It causes zoonosis among human due to occupational, accidental, or agricultural exposure.

The *B. anthracis*, causative agent of anthrax, keeps very important place in the history of bacteriology. It is credited to be the first bacterium to be observed under a microscope. It was also the first bacterium known to cause the disease and revealed to be transmitted by inoculation of infected blood. Koch, in 1877, isolated it in pure culture and revealed that it possesses spores. Koch gave his famous postulates about germs based on his study on anthrax bacillus. It was also the first bacterium which was attenuated to use as vaccine (Pasteur 1881).

13.1.1 Morphological Characteristics

B. anthracis belongs to the *Bacillus cereus* group comprising of *B. cereus*, *B. anthracis*, *B. thuringiensis* and *B. mycoides*. It exhibits differential morphological features on a blood-agar plate. It has characteristic flat, greyish-white, mucoid, irregularly "medusa head-shaped" curly margined colony (Parry et al. 1983) and a large encapsulated Gram-positive rod. It grows well aerobically at a temperature ranging from 12 to 45 °C (optimum 35 °C) on sheep or horse blood agar, and most

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B. anthracis isolates produce non-haemolytic colonies, whereas Turnbull (1999) reported some haemolytic colonies.

13.1.2 Gamma Phage Sensitivity

Brown and Cherry (1955) isolated a variant of the phage W, designated as gamma phage, which is able to lyse both the encapsulated smooth and rough forms of *B. anthracis*. Owing to higher specificity towards *B. anthracis* strains, gamma phage is routinely being used as a diagnostic tool (Sneath 1986). Approximately 98% anthrax isolates show lytic behaviour (Abshire et al. 2005).

13.1.3 Penicillin Resistance

Except for *B. anthracis*, all the members of *B. cereus* group produce β-lactamase; hence, they are penicillin resistant (Turnbull 1996). In the presence of penicillin, anthrax cells become swollen and resemble like a string of pearls, and this test is also used often as diagnostic test. However few penicillin-resistant clinical isolates of *B. anthracis* also have been reported (Jayachandran 2001). This might be due to horizontal transfer of β-lactamase gene among related species (Lalitha and Thomas 1997).

13.1.4 Microscopic Characteristics

B. anthracis exists in two forms, as a spore (dormant form) and as a growing bacterium (vegetative form). Vegetative form can be observed under light microscope by staining with Giemsa. In culture, the bacilli are arranged in long chains appeared as "joined bamboo-rod" with sharp squared-off ends. It exists as long chains in vitro but present as single organisms or short chain in vivo. Capsules of *B. anthracis* can be stained with polychrome methylene blue (McFadyean's reaction). The *Bacilli* stains blue and capsule stains as an amorphous red material surrounding the *Bacilli* (Parry et al. 1983).

When all the nutrients are exhausted, spores are developed. These are central/subterminal and non-bulging and do not take stain with Gram stain. Spores take green stain with malachite green staining. A well-defined capsule surrounds the bacilli, which are often lost in normal culture. In the media containing serum and 5–20% carbon dioxide in atmosphere, the capsule may be induced. The vegetative form grows in body tissues where anthrax spores are cosmopolitan in soil. Spores are very hardy in nature and may tolerate extreme heat, drought, radiations and several disinfectants (Driks 2002) and can remain viable for decades.

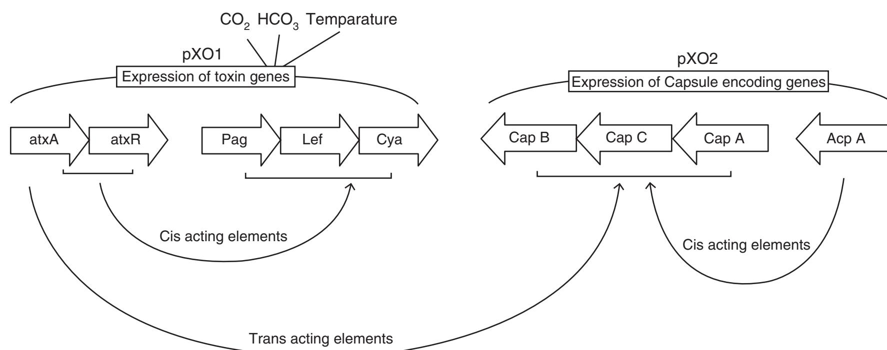

Fig. 13.1 Regulation of anthrax toxin expression (expression of toxin-encoding genes *pag*, *lef* and *cya* present on pXO1 is controlled by both environmental factors (by quantity of bicarbonate, CO_2 and temperature) and genetically by *atx*A and *atx*R. Expressions of *Cap*B, *Cap*C, *Cap*A genes present on pXO2 are regulated by *atx*A and *Acp*A genes)

13.2 Virulence Factor and Its Regulation

The presence of two large plasmids, pXO1 and pXO2, is essential to full virulence of *B. anthracis*. These encode for the two binary exotoxins and capsule, respectively. The plasmid pXO1 encodes for protective antigen (PA), lethal factor (LF) and edema factor (EF) proteins, respectively. The PA is central to the toxin moiety and with EF and/or LF forms the edema and lethal toxins (Bhatnagar and Batra 2001; Khandia et al. 2013). The *cap*B, *cap*C and *cap*A genes are present on pXO2 and synthesize poly-γ-D-glutamic acid capsule. pXO2-cured strains are only toxigenic and thus attenuated with vaccine properties. Several physical factors including bicarbonate, CO_2 and temperature and genetic factors like *atx*A and *atx*R (*trans*-acting genes) regulate the expression of toxin genes (Little and Ivins 1999). Capsule gene expression is controlled by *cis*-acting transcriptional regulator, *Acp*A present on pXO2 (Dixon et al. 1999; Bhatnagar and Batra 2001) and a trans-acting regulator *atx*A present on pXO1 (Guignot et al. 1997) as shown in Fig. 13.1.

13.3 Structure and Function of Protective Antigen (PA)

The PA is a protein molecule composing 735 amino acids (83 kDa) and also called as PA83 (Welkos et al. 1988). The PA is named protective antigen as it elicits protective antibodies against lethal toxin and edema toxin. Protective immunity against lethal spore challenge is achieved by immunizing animals with PA. Also it is the major immunogen present in anthrax vaccines.

It is composed of four domains (Petosa and Liddington 1996; Petosa et al. 1997). Domain I constitutes of residues 1–258 including PA20 (20 kDa; residues 1–167), which is generally proteolytically cleaved by cellular proteases leaving behind residues 168–258 of domain I and provides the binding site for LF and EF (Petosa et al.

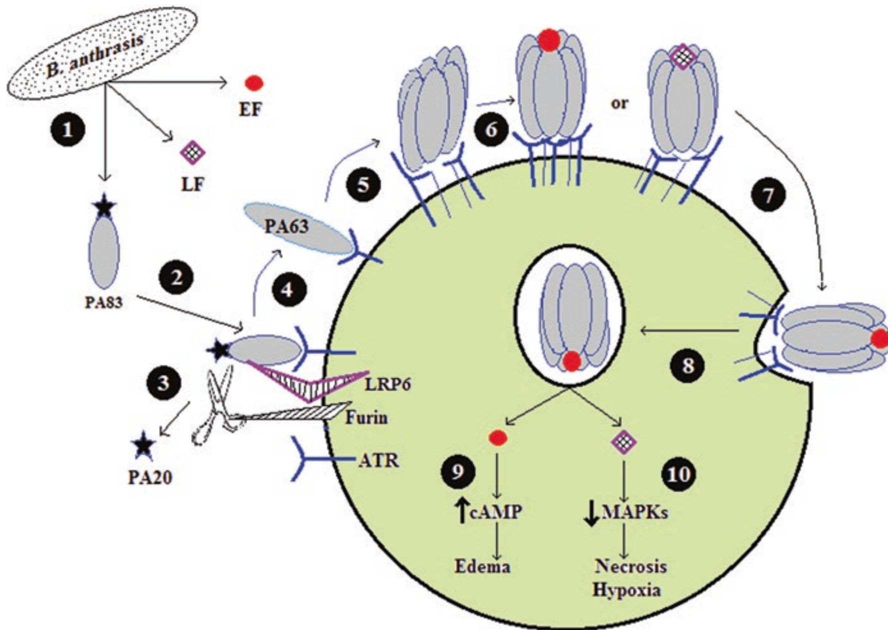

Fig. 13.2 Mechanism for anthrax toxin action. (*1*. *B. anthracis* encoding for PA, LF and EF proteins. *2* The PA binds to the ATR (CMG2/TEM8) and additional receptor (LRP6) present on the host cell surface. *3* PA20 is cleaved by the furin and separated from the 83 kDa form of PA. *4* 63-kDa (PA63) monomer remained attached to ATR. *5* PA63 molecules oligomerize to form either a heptamer or octamer. *6* To this heptamer/octamer, either LF or EF binds competitively. *7* and *8* The toxin-receptor complex is internalized by clathrin-mediated endocytosis. *9* EF produces edema through increased cAMP. *10* LF induces necrosis and hypoxia due to cleavage of MAPKs)

1997). By furins the PA is proteolytically cleaved at site RKKR, which is present between amino acids 164–167 (Gordon et al. 1995); the 20 kDa fragment (PA20) is clipped off leaving behind biologically active carboxy-terminal fragment, PA63 attached with ATR (Allured et al. 1985; Elliott et al. 2000). Both the PA83 and processed PA63 are able to bind with the cellular receptors.

Proteolytic cleavage of PA83 accelerates the formation of heptamers/octamers of PA63 (Uchida et al. 1986) to which LF or EF competitively binds. PA63 serves as a trafficking molecule to carry LF and EF inside the host cell (Fig. 13.2). The LF and EF binding site is located between the interfaces of two PA63 molecules, where one LF/EF molecule binds (Singh et al. 1999). After assembly, the toxin complex is trafficked through receptor-mediated endocytosis (Abrami et al. 2003).

Domain II (residues 259–487) probably participates in PA63 oligomer formation and membrane insertion (Milne et al. 1994; Petosa et al. 1997). Domain III (residues 488–595) is the smallest domain and participates in binding of LF or EF. The domain IV present at carboxyl terminus (residues 596–735) is responsible for binding to the target cells (Petosa et al. 1997), and its deletion mutants fail to bind the target cells (Singh et al. 1991).

13.4 Structure and Function of Lethal Factor (LF)

LF is 776 amino acid-long zinc metalloprotease (Klimpel et al. 1994), and its 3D structure reveals that it evolved from a metal-dependent enzyme which later gained a higher degree of specificity. The zinc-binding motif, HEXXH, is characteristic of LF (Klimpel et al. 1994; Kochi et al. 1994). Metal ion chelators eliminate the effect of lethal toxin on macrophage cell lines and protect Fischer 344 rats (Klimpel et al. 1994). The substrates for LF are MEKs (Duesbery et al. 1998; Vitale et al. 1998). It cleaves the N-termini of several intracellular MEK members, viz. MEK1, MEK2, MKK3, MKK4, MKK6 and MKK7 (Duesbery et al. 1998; Pellizzari et al. 1999). Several signalling molecules including ERK (extracellular signal-regulated kinase), p38 and JNK (c-Jun N-terminal kinase), which play imperative role in cell cycle progression, are blocked when MEKs are cleaved (Duesbery and Woude 1999; Vitale et al. 1999).

The amino terminal domain of LF binds with PA. The central domain (amino acid 307–383) is consisting of five imperfect repeats of 19 amino acids. Deletions in this region result in unstable protein with reduced ability to cause cytotoxicity to mouse macrophage cell. Possibly this region is essential to maintain the stable conformation of LF (Quinn et al. 1991). The C-terminal domain of the protein is having catalytic function (Pannifer et al. 2001).

Various levels of sensitivity towards lethal toxin are present in different animals (Dixon et al. 2000). Evidences are growing which indicates that lethal toxin also targets endothelial cells, causing vascular leakage (similar to haemorrhage), and ultimately, septic shock and internal bleeding cause patient's death (Klimpel et al. 1994).

13.5 Structure and Function of Edema Factor (EF)

Edema factor (a protein of 89 kDa) is a secretory adenylate cyclase exotoxin composing three functionally distinct domains. The first 250 amino acids of EF present at amino terminal participate in binding with PA (Bragg and Robertson 1989), while carboxy-terminal residues (291–767) have two distinct regions which bind to ATP and calmodulin. There is a stretch of seven amino acids (^{147}VYYEIGK^{153}), which is common in LF and EF both (Lacy et al. 2002). The consensus sequence, GxxxxGKS, located between amino acids 314–321, functions to bind ATP (Labruyere et al. 1990). Its adenylyl cyclase activity converts ATP into cAMP, which abnormally reaches quickly to a very high level inside the cells and upsets water homeostasis. cAMP, being an intracellular signalling molecule, disturbs other intracellular signalling pathways. In response to EF, the accumulation of intracellular cAMP leads to the induction of interleukin (IL)-6 and inhibition of tumour necrosis factor (TNF)-α (Hoover et al. 1994). Alongside, EF impairs neutrophil functions (Merka and Patocka 2002; Vale et al. 2016). In such conditions, immune system is failed against bacterial invasion (Welkos et al. 1988). Since cAMP is not cytotoxic, their elevated level does not result in lethality.

13.5.1 Capsule

The poly-γ-D-glutamic acid capsule of *B. anthracis* inhibits phagocytosis by neutrophils and also prevents opsonization due to presence of negative charge present on it (Zwartouw and Smith 1956). Elevated (5% or greater) quantities of CO_2 and added bicarbonate or serum help in increasing capsule production. Capsule is present outside of S-layer, and this layer is not essential for attachment of capsule to cell surface (Fouet et al. 1999). In absence of capsule, the virulence of *B. anthracis* isolates is reduced, and such isolates are often used as vaccine candidates to elicit immunity in livestock and humans in some countries.

13.5.2 Other Virulence Factors

RNA polymerase sigma factor, sigma H, is another important virulence factor that is essential to toxin gene expression. The mutants devoid of the sigma H gene are asporogenous and toxin deficient (Hadjifrangiskou et al. 2007). Virulence of *B. anthracis* also is associated with the numbers of toxin-encoding pXO1 and pXO2 plasmids. The cells containing more numbers of plasmids within will be more virulent (Coker et al. 2003; Read et al. 2003). Some virulence factors are chromosomally encoded. These include extracellular proteases (Stepanov et al. 1996), haemolysin, phospholipases (i.e. cereolysin), iron acquisition proteins (Zawadzka et al. 2009) and S-layer proteins (Etienne-Toumelin et al. 1995).

Iron is obtained by two siderophores: bacillibactin (BB) and petrobactin (PB). These siderophores help in evading the immune system and increase virulence. However, out of the two, only petrobactin is needed to ensure full virulence (Abergel et al. 2006). Introduction of mutation in petrobactin genes leads to limited growth of bacterium in culture. Supplementation of exogenous petrobactin helps in restoring growth (Lee et al. 2007).

Another toxin, anthrolysin O (ALO), is a cholesterol-dependent cytolysin. The treatment of human neutrophils, monocytes and macrophages cells with native or recombinant ALO results in a dose- and time-dependent cytotoxicity (Mosser and Rest 2006).

13.6 Mode of Anthrax Toxin Action

Two receptors, tumour endothelium marker 8 (TEM8) and capillary morphogenesis gene 2 (CMG2), exist for binding of toxins, known as anthrax toxin receptors (ATRs). The affinity of PA towards CMG2 is tenfold (cell culture) to 1000-fold (in vitro) higher than TEM8 (Wigelsworth et al. 2004; Liu et al. 2009). CMG2/TEM8 are present on the surface of host cells, and PA binds to it. The 83-kDa PA is processed by furin to give a 63-kDa (PA63) monomer, and PA20 is separated from it. PA63 molecules make a heptamer or octamer. To this heptamer/octamer, either LF or EF binds competitively. LF and EF can bind to PA, present in oligomeric form,

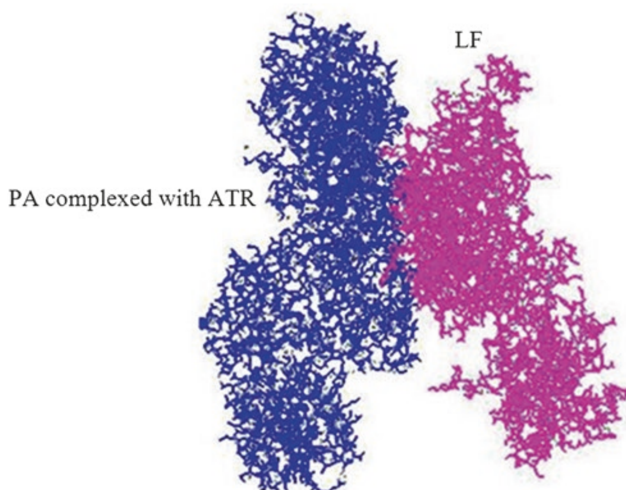

Fig. 13.3 *HEX*-8 (protein docking software; http://hex.loria.fr) generated docking model of interactions between LF and PA complexed with ATR

with a 3:1 or 4:1 stoichiometry. The toxin-receptor complex is trafficked inside the cell through LRP6 (Wei et al. 2006) and clathrin-mediated endocytosis (Boll et al. 2004; Abrami et al. 2010).

In order to understand the interaction of LF (pdb-1JKY) PA bound to anthrax toxin receptor (ATR) (pdb-1T6B), in silico analysis was carried out (Fig. 13.3) using *HEX*-8 (protein docking software; http://hex.loria.fr). A number of hydrogen bonds involved in the interactions between the docked proteins and the total free energy for each interaction revealed a stronger interaction between these proteins due to the presence of higher number of H bonds and lower free energy (Khandia et al. 2017).

After binding to toxin, Src-like kinases phosphorylate the receptors (Src or Fyn – a family of nonreceptor tyrosine kinases), and TEM8 and CMG2 are ubiquitinated by Cbl protein (E3 ubiquitin-protein ligase involved in cell signalling and protein ubiquitination). The receptor-toxin complex is now internalized through the process of clathrin, dynamin, AP-1 (a transcription factor) and actin-dependent endocytosis. Actin cytoskeleton is having important role in enabling the entry of toxin inside the cells, and the receptors are preorganized on surface by actin cytoskeleton. Actin cytoskeleton is reorganized by calpain to mediate toxin entry (Jeong et al. 2013). After trafficking to the early endosome, under the low pH of endosome, enzymatic subunits are translocated across the membrane of endosome. This translocation is either directly from PA prepore to cytoplasm (Zheng et al. 2014) or through intraluminal vesicles (ILV) and back fusion of ILVs to endosomes (Abrami et al. 2004). Intraluminal presence of enzymatic moiety is advantageous for it as it remains protected from cellular proteases for long-term action, and they can be expelled out of cell through exosomes and can be transmitted to naive cells.

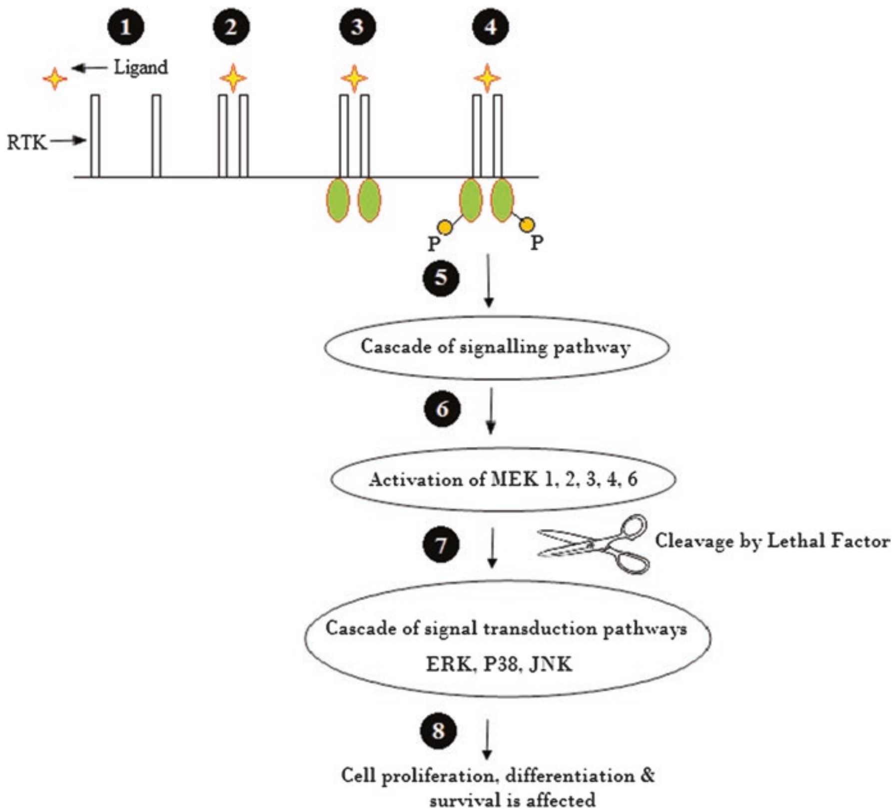

Fig. 13.4 Schematic representation of receptor tyrosine kinase (RTK)-mediated activation of MEKs, and cleavage by LF results in affected cell proliferation, differentiation and survival. (Upon binding with ligands, like nerve growth factor, human epidermal growth factor, human platelet-derived growth factor and fibroblast growth factor, the tyrosine kinase receptor dimerizes and autophosphorylates to further activate MEKs. These MEKs are involved in cell proliferation, differentiation and survival. Cleavage of MEKs by lethal factor blocks several signalling pathways involved in cell cycle progression like ERK (extracellular signal-regulated kinase), p38 and JNK (c-Jun N-terminal kinase) pathways leading to an inhibitory effect on tumour growth. *1* Ligand and individual RTK. *2* Binding of ligand results in dimerization of RTKs. *3* and *4* Autophosphorylation of RTK dimer. *5* Initiating the signalling cascade. *6* MEK activation. *7* Cleavage of MEK by LF prevents the cellular signalling pathways ERK, p38 and JNK (c-Jun N-terminal kinase). *8* Cell proliferation, differentiation and further cell survival are affected)

The LF, being the primary effecter molecule, inactivates MEKs (Fig. 13.4), thereby promoting apoptosis of the cells (Park et al. 2002; Reig et al. 2008). Apart from MEK, NLRP1 (NACHT, LRR and PYD domain-containing protein 1), a member of apoptosis protein, is also cleaved by LF leading to a rapid, caspase-1-dependent apoptosis (Fink et al. 2008). EF is adenylyl cyclase; by elevating cAMP, it activates the transcription factor CREB via PKA (Park et al. 2005).

13.7 Anthrax Toxin Receptors (ATRs)

There are two main receptors for the binding of anthrax toxin, TEM8 and CMG2. Both contain von Willebrand factor A (vWA) domain (Lacy et al. 2004) which is having an α-helical transmembrane region of 23 amino acid and a cytosolic tail of 148 residues. vWA domains are commonly present in extracellular matrix and are responsible for cell adhesion (Whittaker and Hynes 2002). Both ATR receptors bind to the PA through vWA site (Bradley and Young 2003). The extracellular domain of ATR contains a metal ion-dependent adhesion site (MIDAS) motif (DxSxS...T...D, where x can be any amino acid). The MIDAS site is absolutely required for ligand binding. Transmembrane region of both the TEM8 and CMG2 is an essential component. The variants which lack transmembrane region cannot serve as receptor for PA and found intracellularly in soluble state. Cytosolic tail is not essential to function as receptor, but its post-translational palmitoylation and ubiquitination have positive effect on its receptor function. Low-density lipoprotein receptor-related protein 6 (LRP6) is a ubiquitously expressed cell surface protein, complexed with TEM8 and CMG2 which aid to the toxin internalization. It is considered that during evolution anthrax toxins used other receptors which are essential for physiological processes.

13.7.1 TEM 8

TEM8 protein is a product of *tem*8 gene (a gene of 190 kb). Three different splice variants of 564, 368 and 333 amino acids have been observed (Abrami et al. 2006). TEM-564 and TEM-368 are identical in extracellular and transmembrane domain; however, TEM-564 has proline-rich cytoplasmic tail, whereas TEM-368 is having last four dissimilar amino acids at C-terminal end. Shortest isoform TEM-333 is devoid of transmembrane region and extracellular region, thus present in soluble form in cytosol and cannot act as receptor (Bell et al. 2001). TEM8 receptor is highly conserved among different species (Yang et al. 2010). Gloria et al. (2005) reported the selective upregulation of TEM8 in endothelial cells of newly forming blood vessels and neoplastic tissue blood vessels. Higher occurrence of TEM8, on tumour vessels as well as on normal vasculature, indicates its role in process of angiogenesis (Nanda et al. 2004).

13.7.2 CMG2

Capillary morphogenesis gene 2 (CMG2) is product of *cmg2* gene of 80 kb. It is the main receptor of the anthrax toxin, and mice knockouts were anthrax infection resistant. Out of many isoforms, result of alternative splicing, four major isoforms are identified possessing 489, 488, 386 and 322 amino acid residues. CMG-489 and CMG-488 act as receptor and are only dissimilar in carboxy terminal amino acids, where CMG-322 can’t act as receptor due to absence of anchoring transmembrane

region (Bell et al. 2001). Intracellular localization of CMG2-386 isoform in endoplasmic reticulum renders it impossible to act as receptor. CMG2 expression was detected in the heart, lung, liver, skeletal muscle, peripheral blood leukocyte, placenta, small intestine, kidney, colon, spleen, brain and thymus (Scobie et al. 2003).

13.8 Physiological Functions of ATR

Omnipresence of TEM8/CMG2 receptor on various cell types is indicative of its dual role in anthrax pathogenesis as well as in physiological processes. Functions of this receptor were understood by carrying out experiments on knockout mice (Liu et al. 2009). Transmembrane regions of both the receptors were deleted to create TEM8 and CMG2 null mice ($CMG2^{-/-}$ and $TEM8^{-/-}$, respectively). Various studies indicated that the toxicity is mainly due to CMG2, as upon challenge $CMG2^{-/-}$ mice survived; however $TEM8^{-/-}$ mice were susceptible. CMG2 is having 11 times higher affinity towards PA in comparison to CMG2.

TEM8 is highly expressed in vessels of developing embryos and tumour; hence, its imperative function during angiogenesis is augmented. Endothelial cell migration and tubule formation are controlled by TEM8, where endothelial proliferation and morphogenesis are controlled by CMG2 (Reeves et al. 2012).

The studies to determine the physiological functions of TEM8 revealed that TEM8 has role in migration of endothelial cells (Hotchkiss et al. 2005; Nagase et al. 2006). Extracellular matrix is excessively deposited in ovaries and uteri of TEM8- and CMG2-deficient female mice (Cullen et al. 2009; Reeves et al. 2012). These mice were incompetent to reproduce indicative of involvement of both receptors in embryo development although these receptors are nonessential for life (Liu et al. 2009).

13.9 Therapeutic Potential of Different Components of *Anthrax* Bacterium

13.9.1 Therapeutic Uses of Engineered PA Protein

LT has been observed to be selectively toxic against many human melanoma cell lines (Koo et al. 2002). About 70% of human melanomas and few other human cancer types comprise valine to glutamic acid substitution (V600E) in proto-oncogene B-Raf (Davies et al. 2002). B-Raf is a serine/threonine kinase, and melanoma cell lines with B-Raf mutations are dependent on constitutive expression of MEKs for survival and proliferation and lethal toxin cleaves MEKs (Abi-Habib et al. 2005). The cell lines without BRAF mutations are resistant to lethal toxin. Because lethal toxin as such can't be used for human treatment, and protective antigen is absolutely required for the action of the toxin, modification in the amino acid sequence of the PA cleavage site can create tumour-selective PA variants, which are activated only when tumour-associated proteases are present.

Protective antigen is engineered in such a way that instead of furin (a ubiquitously expressed protease), it is cleaved and activated by matrix metalloproteases (MMPs). The MMPs have imperative role in tumour progression, metastasis and angiogenesis. A mutant PA is constructed into which furin cleavage site, RKKR, was replaced with MMP-susceptible cleavage sequence GPL-GMLSQ to selectively target LF to tumour cell (Liu et al. 2000). In comparison to wild PA/LF treatment, mutant PA/LF showed higher in vivo antitumour activity due to higher bioavailability of it in plasma. Also it is having less immunogenicity which makes it an ideal choice for repeated regimens of treatment without compromising therapeutic activity (Liu et al. 2008). Likewise furin cleavage site replaced with urokinase plasminogen activator (uPA) cleavable sequences, which are overexpressed on various tumours and tumour cell lines, also provides a way to combat several tumours (Liu et al. 2001).

Similarly PA variants were prepared which are activated when both MMPs and uPA are present, therefore offering more specific tumour targeting (Liu et al. 2005). When LF binds to PA, the binding site is comprised of three sites present on two adjacent molecules of PA. Two mutated PA molecules were constructed. In one construct PA-U2-R200A, at LF-binding subsite II, residue Arg mutated to Ala, with cleavage site converted for urokinase activation. In the other construct PA-L1-I210A, at subsite III, residue Ile mutated to Ala with cleavage site converted for matrix metalloproteinase activation. Individual usage of the mutated PA resulted in reduced toxicity to tumour cells, but when they were used in combination, they formed heterogeneous oligomers of PA, however competent for LF binding with higher degree of specificity in tumour targeting (Wein et al. 2015).

In the presence of PA-U2-R200A or PA-L1-I210A, there is still a possibility of forming PA homo-oligomers and residual toxicity. However introducing the D512K and GN or NS mutations into PA-U2 (PA-U2-D512K) and PA-L1 (PA-L1-GN or PA-L1-NS) causes formation of PA hetero-octamer instead of heptamer, enabling binding to LF. Domain IV of PA can have two different orientations, and alternate arrangements of these result in formation of functional octamer. By introducing these mutations, such a targeting system can be produced specifically and requires both the proteases to intoxicate a cancer target cell (Phillips et al. 2013) making tumour targeting more precise.

13.9.2 Therapeutic Uses of LF

Specific targeting to tumour cells can be achieved by fusing initial 254 residues of LF, which are crucial for binding to the PA heptamer, to the toxin enzymatic moiety. Arora et al. (1992) fused anthrax toxin lethal factor residues 1–254 (LFn) to the ADP-ribosylation domain of *Pseudomonas* exotoxin A (designated FP59). This domain is responsible for transferring ADP-ribose to eukaryotic elongation factor 2 (eEF2). The transfer results in inhibition of protein synthesis and cell death. Another toxin which has been fused to LF is cytolethal distending toxin (Cdt). The Cdt is a tripartite bacterial protein toxin, having CdtA, CdtB and CdtC subunits. Several

Gram-negative human pathogens including *Haemophilus ducreyi*, *Escherichia coli*, *Aggregatibacter actinomycetemcomitans*, *Campylobacter jejuni* and *Shigella dysenteriae* secrete Cdt. CdtB is an enzymatic moiety having high sequence homology to DNase I and degrades nuclear DNA of host cells (Elwell and Dreyfus 2000). It leads to the arrested G2/M phase and apoptosis in several mammalian cell types (Jinadasa et al. 2011). PA-LFn-CdtB may not be sufficient for efficient killing of targeted cell because of delivery of fused toxin in cytosol, so additional nuclear localization signals may be added in the construct to direct it to the nucleus.

Another approach to deliver LF specifically to tumour cells is to clone under human telomerase reverse transcriptase promoter (hTERTp), a promoter having high GC content. This promoter is highly tumour specific and can be an imperative tool in cancer-targeted therapy (Deng et al. 2007). Upon transfecting the construct in a human lung adenocarcinoma cell line A549, expression of LF caused apoptosis in cancer cells where normal cells remained unaffected (Zhuo et al. 2013).

To further improve the efficacy of delivered therapeutic proteins to the cytosol of tumour cells, they must be prevented from host cell-mediated ubiquitination and subsequent proteasome degradation. Varshavsky (2011) described the N-end rule which correlates with in vivo half-life of a protein to its N-terminal residues. The presence of amino acid residues like Met, Gly or Ala at N-terminus increases the stability of proteins, whereas presence of residues like Arg, Lys or Asp result in rapid degradation. To improve the cytosolic stability of toxic fusion protein FP59 (anthrax fusion toxin contains LFn at the N terminus and *Pseudomonas* exotoxin A catalytic domain III at its C terminus), all lysine residues were reductively methylated in a two-step process. In the first process, between the amino group and aldehydic group of formaldehyde, reversible imine adducts are formed. Then the complex is stabilized by treating it with reducing agent like borane compounds. The resulting mono-methylated protein amino acid undergoes another such round of reaction to give dimethylated amino acid (Rayment 1997). The dimethylated amino acids are not susceptible to ubiquitination pathways and are with improved stability. The study of Bachran et al. (2014) revealed that reductive methylation of lysine residue had no apparent adverse effect on the activity of FP59 and offers a potential method for modifying therapeutic proteins.

13.9.3 Therapeutic Uses of Anthrax Receptor

In tumour endothelium, TEM8 protein is selectively upregulated (Werner et al. 2006). Its selective upregulation on tumour vasculature offers a therapeutic approach against cancer. Recombinant TEM8-based vaccines and soluble TEM8-Fc trap (Duan et al. 2007) resulted in slowed down tumour growth and angiogenesis inhibition and provided prolonged survival (Ruan et al. 2009; Cryan and Roger 2011).

In immunocompromised mice, tumour growth was inhibited using recombinant F*ab* against TEM8 without effect on wound healing. Anti-TEM8 antibodies are proven to be non-hazardous. It has been observed that cancer therapeutic molecules are often encountered with the problem of non-specificity. Along with cancer cells,

these molecules kill normal cells also. TEM8 exhibited uniqueness in a manner that in the developmental angiogenesis and normal angiogenic corpus luteum of human ovaries (Nanda et al. 2004), it is undetectable.

Also in TEM8 knockout mice, wound healing process was undisturbed (Cullen et al. 2009), indicating preferential targeting of pathological angiogenesis (Khandia et al. 2014). In TEM8 knockout mice, human tumour xenograft growth is impaired which further strengthen the fact that TEM8 is overexpressed in pathological angiogenesis (Chaudhary et al. 2012). Antibodies against the TEM8 block anthrax toxin entry in cell and prevent tumour-induced angiogenesis. During the course normal healing processes remain unaffected. Therefore it can be used as therapeutic candidate.

13.10 Conclusion

Anthrax is an ancient zoonotic disease, affecting mainly animals and occasionally humans and leads to death if not treated timely. It causes tissue necrosis in case of cutaneous anthrax. Modernization in science has opened several new avenues to treatment. The bacteria encompass two megaplasmids, encoding for secretary exotoxins and capsule. Lethal factor, the component of lethal toxin, cleaves MEKs which is an integral part of cell signalling pathway for growth and differentiation. This cleavage of MEKs promotes apoptosis of targeted cells. Elevated level of MEK is associated with deregulated cellular signalling cascade which is associated with several kinds of cancers of the lung, pancreas, and kidney, epithelial origin tumour, etc. Apart from MEKs, NLRP1 is another protein, leading to rapid caspase-dependent apoptosis. LF, by blocking the signalling cascade, offers an excellent approach to obstruct cancer progression. As lethal toxin is cytotoxic to target cells, protective antigen is manipulated in several ways to deliver it precisely to cancer cells only, by its selective targeting to urokinase plasminogen activator (uPA) and matrix metalloproteases (MMPs) overexpressing tumour cells. Also antibodies and vaccine against anthrax toxin receptor provided a strategy to combat tumours. Summarily, diverse components of anthrax bacterium, using different molecular techniques to modify and manipulate components, offer an excellent approach to cure or at least reduce the amplitude of a life-threatening disease cancer and turning a monster bacterium to a life-saving bacterium.

References

Abergel RJ, Wilson MK, Arceneaux JE et al (2006) Anthrax pathogen evades the mammalian immune system through stealth siderophore production. Proc Natl Acad Sci U S A 103:18499–18503

Abi-Habib RJ, Urieto JO, Liu S, Leppla SH et al (2005) BRAF status and mitogen-activated protein/extracellular signal-regulated kinase kinase 1/2 activity indicate sensitivity of melanoma cells to anthrax lethal toxin. Mol Cancer Ther 4:1303–1310

Abrami L, Liu S, Cosson P et al (2003) Anthrax toxin triggers endocytosis of its receptor via a lipid raft-mediated clathrin-dependent process. J Cell Biol 160:321–328
Abrami L, Lindsay M, Parton RG et al (2004) Membrane insertion of anthrax protective antigen and cytoplasmic delivery of lethal factor occur at different stages of the endocytic pathway. J Cell Biol 166:645–651
Abrami L, Leppla SH, Van der Goot FG (2006) Receptor palmitoylation and ubiquitination regulate anthrax toxin endocytosis. J Cell Biol 172(2):309–320
Abrami L, Bischofberger M, Kunz B, Groux R, van der Goot FG (2010) Endocytosis of the anthrax toxin is mediated by clathrin, actin and unconventional adaptors. PLoS Pathog 6:e1000792. https://doi.org/10.1371/journal.ppat.1000792
Abshire TG, Brown JE, Ezzell JW (2005) Production and validation of the use of gamma phage for identification of *Bacillus anthracis*. J Clin Microbiol 43:4780–4788
Allured VS, Case LM, Leppla SH, McKay DB (1985) Crystallization of the protective antigen protein of *Bacillus anthracis*. J Biol Chem 260:5012–5013
Arora N, Klimpel KR, Singh Y, Leppla SH (1992) Fusions of anthrax toxin lethal factor to the adp-ribosylation domain of *pseudomonas* exotoxin A are potent cytotoxins which are translocated to the cytosol of mammalian cells. J Biol Chem 267(22):15542–15548
Bachran C, Gupta PK, Bachran S, Leysath CE et al (2014) Reductive methylation and mutation of an anthrax toxin fusion protein modulates its stability and cytotoxicity. Sci Rep 4:4754. https://doi.org/10.1038/srep04754
Bell SE, Mavila A, Salazar R, Bayless KJ et al (2001) Differential gene expression during capillary morphogenesis in 3D collagen matrices: regulated expression of genes involved in basement membrane matrix assembly, cell cycle progression, cellular differentiation and G-protein signalling. J Cell Sci 114:2755–2773
Bhatnagar R, Batra S (2001) Anthrax toxins. Crit Rev Microb 27:167–200
Boll W, Ehrlich M, Collier RJ, Kirchhausen T (2004) Effects of dynamin inactivation on pathways of anthrax toxin uptake. Eur J Cell Biol 83:281–288
Bradley KA, Young JA (2003) Anthrax toxin receptor proteins. Biochem Pharmacol 65:309–314
Bragg TS, Robertson DL (1989) Nucleotide sequence and analysis of the lethal factor gene (*lef*) from *Bacillus anthracis*. Gene 81:45–54
Brown ER, Cherry WB (1955) Specific identification of *Bacillus anthracis* by means of a variant bacteriophage. J Infect Dis 96:34–39
Chaudhary A, Hilton MB, Seaman S, Haines DC et al (2012) TEM8/ANTXR1 blockade inhibits pathological angiogenesis and potentiates tumoricidal responses against multiple cancer types. Cancer Cell 21:212–226
Coker PR, Smith KL, Fellows PF et al (2003) *Bacillus anthracis* virulence in guinea pigs vaccinated with anthrax vaccine adsorbed is linked to plasmid quantities and clonality. J Clin Microbiol 41:1212–1218
Cryan LM, Roger MS (2011) Targeting the anthrax receptors, TEM-8 and CMG-2, for anti-angiogenic therapy. Front Biosci 16:1574–1588
Cullen M, Seaman S, Chaudhary A, Yang MY et al (2009) Host-derived tumor endothelial marker 8 promotes the growth of melanoma. Cancer Res 69(15):6021–6026
Davies H, Bignell GR, Cox C, Stephens P et al (2002) Mutations of the *BRAF* gene in human cancer. Nature 417:949–954
Deng WG, Jayachandran G, Wu G, Xu K et al (2007) Tumor-specific activation of human telomerase reverses transcriptase promoter activity by activating enhancer-binding protein-2beta in human lung cancer cells. J Biol Chem 282(36):26460–26470
Dixon TC, Meselson M, Guillemin J, Hanna PC (1999) Anthrax. N Engl J Med 341:815–826
Dixon TC, Fadl AA, Koehler TM et al (2000) Early *Bacillus anthracis*–macrophage interactions: intracellular survival and escape. Cell Microbiol 2:453–463
Driks A (2002) Overview: development in bacteria: spore formation in *Bacillus subtilis*. Cell Mol Life Sci 59:389–391
Duan HF, Hu XW, Chen JL, Gao LH et al (2007) Antitumor activities of TEM8-Fc: an engineered antibody-like molecule targeting tumor endothelial marker 8. J Natl Cancer Inst 99:1551–1555

Duesbery NS, Woude GFV (1999) Anthrax toxins. Cell Mol Life Sci 55:1599–1609
Duesbery NS, Webb CP, Leppla SH, Gordon VM et al (1998) Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. Science 280:734–737
Elliott JL, Jeremy M, Collier RJ (2000) A quantitative study of the interactions of *Bacillus anthracis* edema factor and lethal factor with activated protective antigen. Biochemistry 39(22):6706–6713
Elwell CA, Dreyfus LA (2000) DNase I homologous residues in CdtB are critical for cytolethal distending toxin-mediated cell cycle arrest. Mol Microbiol 37:952–963
Etienne-Toumelin I, Sirard JC, Duflot E et al (1995) Characterisation of the *Bacillus anthracis* S-layer: cloning and sequencing of the structural gene. J Bacteriol 177:614–620
Fink SL, Bergsbaken T, Cookson BT (2008) Anthrax lethal toxin and *Salmonella* elicit the common cell death pathway of caspase-1-dependent pyroptosis via distinct mechanisms. Proc Natl Acad Sci U S A 105(11):4312–4317
Fouet A, Mesnage S, Tosi-Couture E, Gounon P, Mock M (1999) *Bacillus anthracis* surface: capsule and S-layer. J Appl Microbiol 87:251–255
Gloria B, Federica S, Philippe G, Terence M et al (2005) ATR/TEM8 is highly expressed in epithelial cells lining *Bacillus anthracis*' three sites of entry: implications for the pathogenesis of anthrax infection. Am J Physiol 288:1402–1410
Gordon VM, Klimpel KR, Arora N et al (1995) Proteolytic activation of bacterial toxins by eukaryotic cells is performed by furin and by additional cellular proteases. Infect Immun 63(1):82–87
Guignot J, Mock M, Fouet A (1997) AtxA activates the transcription of genes harboured by both *Bacillus anthracis* virulence plasmids. FEMS Microbiol Lett 147:203–207
Hadjifrangiskou M, Chen Y, Koehler TM (2007) The alternative sigma factor sigma H is required for toxin gene expression by *Bacillus anthracis*. J Bacteriol 189:1874–1883
Hoover DL, Friedlander AM, Rogers LC et al (1994) Anthrax edema toxin differentially regulates lipopolysaccharide-induced monocyte production of tumor necrosis factor alpha and interleukin-6 by increasing intracellular cyclic AMP. Infect Immun 62:4432–4439
Hotchkiss KA, Basile CM, Spring SC, Bonuccelli G et al (2005) TEM8 expression stimulates endothelial cell adhesion and migration by regulating cell–matrix interactions on collagen. Exp Cell Res 305:133–144
Jayachandran R (2001) Anthrax: biology of *Bacillus anthracis*. Science 294:1810–1812
Jeong SY, Martchenko M, Cohen SN (2013) Calpain-dependent cytoskeletal rearrangement exploited for anthrax toxin endocytosis. Proc Natl Acad Sci U S A 110(42):E4007–E4015. https://doi.org/10.1073/pnas.1316852110
Jinadasa RN, Bloom SE, Weiss RS, Duhamel GE (2011) Cytolethal distending toxin: a conserved bacterial genotoxin that blocks cell cycle progression, leading to apoptosis of a broad range of mammalian cell lineages. Microbiology 157:1851–1875
Khandia R, Pattnaik B, Rajukumar K, Pateriya AK et al (2013) Evaluation of a protective antigen gene based SYBR green I real time PCR for detection of Bacillus anthracis in field samples. Indian J Anim Sci 83:118–123
Khandia R, Bhatia S, Victoria C, Sood R, Dhama K (2014) Anthrax toxin receptors, functions and their possible use in therapeutics: a review. Asian J Anim Vet Adv 9(10):599–609
Khandia R, Pattnaik B, Rajukumar K, Pateriya A et al (2017) Anti-proliferative role of recombinant lethal toxin of Bacillus anthracis on primary mammary ductal carcinoma cells revealing its therapeutic potential. Oncotarget 8(22):35835–35847
Klimpel KR, Arora N, Leppla SH (1994) Anthrax toxin lethal factor contains a zinc metalloprotease consensus sequence which is required for lethal toxin activity. Mol Microbiol 13(6):1093–1100
Kochi SK, Schaiva G, Mock M, Montecucco C (1994) Zn content of *Bacillus anthracis* lethal factor. FEMS Microbiol Lett 124:343–348
Koo HM, VanBrocklin M, McWilliams MJ et al (2002) Apoptosis and melanogenesis in human melanoma cells induced by anthrax lethal factor inactivation of mitogen-activated protein kinase kinase. Proc Natl Acad Sci U S A 99(5):3052–3057

Labruyere E, Mock M, Ladant D, Michelson S et al (1990) Characterization of ATP and calmodulin-binding properties of a truncated form of *Bacillus anthracis* adenylate cyclase. Biochemistry 29:4922–4928

Lacy DB, Mourez M, Fouassier A, Collier RJ (2002) Mapping the anthrax protective antigen binding site on the lethal and edema factors. J Biol Chem 277(4):3006–3010

Lacy DB, Wigelsworth DJ, Scobie HM, Young JA, Collie RJ (2004) Crystal structure of the von Willebrand factor A domain of human capillary morphogenesis protein 2: an anthrax toxin receptor. Proc Natl Acad Sci U S A 101:6367–6372

Lalitha MK, Thomas MK (1997) Penicillin resistance in *Bacillus anthracis*. Lancet 349:1522–1532

Lee JY, Janes BK, Passalacqua KD et al (2007) Biosynthetic analysis of the petrobactin siderophore pathway from *Bacillus anthracis*. J Bacteriol 189:1698–1710

Little SF, Ivins BE (1999) Molecular pathogenesis of *Bacillus anthracis* infection. Microbes Infect 1(2):131–139

Liu S, Netzel AS, Birkedal HH, Leppla SH (2000) Tumor cell-selective cytotoxicity of matrix metalloproteinase-activated anthrax toxin. Cancer Res 60(21):6061–6067

Liu S, Bugge TH, Leppla SH (2001) Targeting of tumor cells by cell surface urokinase plasminogen activator-dependent anthrax toxin. J Biol Chem 276(21):17976–17984

Liu S, Redeye V, Kuremsky JG, Kuhnen M et al (2005) Intermolecular complementation achieves high-specificity tumor targeting by anthrax toxin. Nat Biotechnol 23(6):725–730

Liu S, Wang H, Currie BM, Molinolo A et al (2008) Matrix metalloproteinase-activated anthrax lethal toxin demonstrates high potency in targeting tumor vasculature. J Biol Chem 283(1):529–540

Liu S, Crown D, Miller RS, Moayeri M et al (2009) Capillary morphogenesis protein-2 is the major receptor mediating lethality of anthrax toxin in vivo. Proc Natl Acad Sci U S A 106:12424–12429

Merka V, Patocka J (2002) Anthrax: an important agent of biological terrorism. Nederl Milit Geneesk 55:142–145

Milne JC, Furlong D, Hanna PC et al (1994) Anthrax protective antigen forms oligomers during intoxication of mammalian cells. J Biol Chem 269:20607–20612

Mosser EM, Rest RF (2006) The *Bacillus anthracis* cholesterol-dependent cytolysin, anthrolysin O, kills human neutrophils, monocytes and macrophages. BMC Microbiol 6:56

Nagase H, Visse R, Murphy G (2006) Structure and function of matrix metalloproteinases and TIMPs. Cardiovasc Res 69(3):562–573

Nanda A, Carson-Walter EB, Seaman S, Barber TD et al (2004) TEM8 interacts with the cleaved C5 domain of collagen alpha 3(VI). Cancer Res 64(3):817–820

Pannifer AD, Wong TY, Schwarzenbacher R, Renatus M et al (2001) Crystal structure of the anthrax lethal factor. Nature 414:229–233

Park JM, Greten FR, Li ZW, Karin M (2002) Macrophage apoptosis by anthrax lethal factor through p38 MAP kinase inhibition. Science 297:2048–2051

Park JM, Greten FR, Wong A, Westrick RJ et al (2005) Signaling pathways and genes that inhibit pathogen-induced macrophage apoptosis –CREB and NF-κB as key regulators. Immunity 23(3):19–329

Parry JM, Turnbull PCB, Gibson JR (1983) A color atlas of *Bacillus* species. Wolfe Medical, London, p 272

Pasteur L (1881) De l'attenuation des virus et de leur retour a la virulence. C R Acad Sci Bulg 92:429–435

Pellizzari R, Recchi C, Napolitani G, Mock M, Montecucco C (1999) Anthrax lethal factor cleaves the N-terminus of MAPKKS and induces tyrosine/threonine phosphorylation of MAPKS in cultured macrophages. J Appl Microbiol 87:288

Petosa C, Liddington RC (1996) The anthrax toxin. In: Parker MW (ed) Protein toxin structure. R.G. Landes Company Austin, Texas, pp 97–121

Petosa C, Collier RJ, Klimpel KR et al (1997) Crystal structure of the anthrax toxin protective antigen. Nature 385:8833–8838

Phillips DD, Fattah RJ, Crown D et al (2013) Engineering anthrax toxin variants that exclusively form octamers and their application to targeting tumors. J Biol Chem 288:9058–9065
Quinn CP, Chaudhary VK, Leppla SH (1991) The carboxyl-terminal end of protective antigen is required for receptor binding and anthrax toxin activity. J Biol Chem 266:15493–15497
Rayment I (1997) Reductive alkylation of lysine residues to alter crystallization properties of proteins. Methods Enzymol 276:171–179
Read TD, Peterson SN, Tourasse N, Baillie LW et al (2003) The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. Nature 423:81–86
Reeves CV, Wang X, Charles-Horvath PC, Vink JY et al (2012) Anthrax toxin receptor 2 functions in ECM homeostasis of the murine reproductive tract and promotes MMP activity. PLoS One 7:e34862
Reig N, Jiang A, Couture R et al (2008) Maturation modulates caspase-1-independent responses of dendritic cells to anthrax lethal toxin. Cell Microbiol 10:1190–1207
Ruan Z, Yang Z, Wang Y, Wang H et al (2009) DNA vaccine against tumor endothelial marker 8 inhibits tumor angiogenesis and growth. J Immunother 32(5):486–491
Scobie HM, Rainey GJ, Bradley KA, Young JA (2003) Human capillary morphogenesis protein 2 functions as an anthrax toxin receptor. Proc Natl Acad Sci U S A 100:5170–5174
Singh Y, Klimpel KR, Quinn CP et al (1991) The carboxyl-terminal end of protective antigen is required for receptor binding and anthrax toxin activity. J Biol Chem 266:15493–15497
Singh Y, Klimpel KR, Goel S, Swain PK, Leppla SH (1999) Oligomerization of anthrax toxin protective antigen and binding of lethal factor during endocytic uptake into mammalian cells. Infect Immun 67:1853–1859
Sneath PHA (1986) Endospore-forming gram-positive rods and cocci. Bergey's manual of systematic bacteriology, vol 2. Williams & Wilkins, Baltimore, p 1131
Stepanov AV, Marinin LI, Pomerantsev AP, Staritsin NA (1996) Development of novel vaccines against anthrax in man. J Biotechnol 44:155–160
Turnbull PCB (1996) Bacillus. In: Baron S (ed) Medical Microbiology, 4th edn. The University of Texas Medical Branch at Galveston, Galveston, pp 233–246
Turnbull PC (1999) Definitive identification of *Bacillus anthracis*—review. J Appl Microbiol 87:237–240
Uchida I, Hashimoto K, Terakado N (1986) Virulence and immunogenicity in experimental animals of Bacillus anthracis strains harbouring or lacking 110 MDa and 60 MDa plasmids. J Gen Microbiol 132:557–559
Vale AD, Cabanes D, Sousa S (2016) Bacterial toxins as pathogen weapons against phagocytes. Front Microbiol 7:42
Varshavsky A (2011) The N-end rule pathway and regulation by proteolysis. Protein Sci 20(8):1298–1345
Vitale G, Pellizzari R, Recchi C, Napolitani G et al (1998) Anthrax lethal factor cleaves the N-terminus of MAPKKs and induces tyrosine/threonine phosphorylation of MAPKs in cultured macrophages. Biochem Biophys Res Commun 248:706–711
Vitale G, Pellizzari R, Recchi C, Napolitani G et al (1999) Anthrax lethal factor cleaves the N-terminus of MAPKKS and induces tyrosine/threonine phosphorylation of MAPKS in cultured macrophages. J Appl Microbiol 248(3):706–711
Wei W, Lu Q, Chaudry GJ et al (2006) The LDL receptor-related protein LRP6 mediates internalization and lethality of anthrax toxin. Cell 124(6):1141–1154
Wein AN, Peters DE, Valivullah Z, Hoover BJ et al (2015) An anthrax toxin variant with an improved activity in tumor targeting. Sci Rep 5:16267. https://doi.org/10.1038/srep16267
Welkos SL, Lowe JR, Eden-Mccutchan F, Vodkin M et al (1988) Sequence and analysis of the DNA encoding protective antigen of *Bacillus anthracis*. Gene 69:287–300
Werner E, Kowalczyk AP, Faundez V (2006) Anthrax toxin receptor 1/tumor endothelium marker 8 mediates cell spreading by coupling extracellular ligands to the actin cytoskeleton. J Biol Chem 281:23227–23236

Whittaker CA, Hynes RO (2002) Distribution and evolution of von Willebrand/integrin A domains: widely dispersed domains with roles in cell adhesion and elsewhere. Mol Biol Cell 13(10):3369–3387

Wigelsworth DJ, Krantz BA, Christensen KA et al (2004) Binding stoichiometry and kinetics of the interaction of a human anthrax toxin receptor, CMG2, with protective antigen. J Biol Chem 279:23349–23356

Yang MY, Chaudhary A, Seaman S et al (2010) The cell surface structure of tumor endothelial marker 8 (TEM8) is regulated by the actin cytoskeleton. Biochem Biophys Acta 1813(1):39–49

Zawadzka AM, Abergel RJ, Nichiporuk R et al (2009) Siderophore-mediated iron acquisition systems in *Bacillus cereus*: identification of receptors for anthrax virulence-associated petrobactin. Biochemistry 48:3645–3657

Zheng S, Zhang G, Li J, Chen PR (2014) Monitoring endocytic trafficking of anthrax lethal factor by precise and quantitative protein labelling. Angew Chem Int Ed Eng 53:6449–6453

Zhuo W, Tao G, Zhang L, Chen Z (2013) Vector-mediated selective expression of lethal factor, a toxic element of *Bacillus Anthracis*, damages A549 cells via inhibition of MAPK and AKT pathways. Int J Med Sci 10(3):292–298

Zwartouw HT, Smith H (1956) Polyglutamic acid from *Bacillus anthracis* grown in vivo: structure and aggressin activity. Biochem J 63:437–442

Novel Molecular Diagnostics and Therapeutic Tools for Livestock Diseases

14

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14.1 Introduction

Increasing human population, urbanization and income sources are raising the demand of animal origin food (milk, meat, eggs) for fulfilment of protein deficit in developing countries. All over the world, the livestock production is gaining higher speed than other agricultural sectors, and by 2020, the livestock sector will be the most significant agricultural sector in terms of value addition.

However, the health of animals is under the constant threat of various dreaded infectious diseases, which may result in significant economic losses to livestock

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owners. Maintenance of good health status of animals by effective control and treatment of diseases lies in the access to various standard diagnostic tests which are rapid, reliable, precise and highly sensitive and therefore helps in confirming early detection of causative agent. The conventional diagnostic methods are too much demanding in terms of time and labour. Latest advances in the molecular biology and biotechnology have opened new avenues in disease diagnosis and therapeutics, but these technologies are in budding stage so need to be further explored to their full potential to safeguard the health of human in addition to their companion animals. Along with this there is a need to build up qualified, trained and competent manpower for bringing the existing disease diagnostic methods in laboratories to field of action. Because of the lack of skilled manpower in different institutions of public undertakings, it is highly desirable that the gained expertise and knowledge be disseminated to users of these techniques directly so benefits can be harnessed more efficiently and effectively.

Veterinary pathogens have been traditionally diagnosed by detecting the pathogens by culture isolation or serological detection of antigen or antibody such as neutralization tests, enzyme-linked immunosorbent assays, agar gel immunodiffusion and complement fixation tests. In the past diagnostic capabilities have been enhanced by complementing conventional assays with molecular diagnostics. Incredible progress in the molecular diagnosis and characterization of avian influenza virus infections in the past two decades is the true example of this. Moreover, recent advancements in the field of molecular diagnostics have made the speedy identification of group A influenza and H5 and H7 subtype viruses feasible, which is a major global cause for the spread of avian influenza from poultry to humans. The innovative, reliable, rapid and appropriate DNA-based diagnostic techniques have the potential to assist international efforts to check the incursion of exotic diseases into new geographic areas.

This write-up focuses on recent advancements in molecular diagnostic tools which can help clinicians in promoting human and animal health within 'One Health' concept.

14.2 Pathogen Genome-Based Diagnostics

The use of molecular diagnostics has been rampant in recent years for the detection of veterinary pathogens. These involves detection of pathogen either directly by sensing the presence of DNA or RNA in the host or indirectly by prior amplification of genome of infectious agent. These techniques have not only helped in earlier disease diagnosis but also animal disease control programmes. Further, modifications in PCR-based molecular detection techniques have generated a vast array of fast, reliable and specific assays which have widespread applications in veterinary diagnostics.

The sensitivity of any genome detection-based method can be enhanced to a very high degree by manipulating any of the three pillars of the assay, i.e. by amplification of target, signal and probe.

1. Target amplification: different assays as polymerase chain reaction (PCR), transcription-based amplification (TBA)/nucleic acid sequence-based amplification (NASBA)/self-sustained sequence replication (SSSR/3SR), strand displacement amplification (SDA) and loop-mediated isothermal amplification (LAMP) come under target amplification.
2. Signal amplification.
3. Probe amplification: ligase chain reaction (LCR).

14.3 Target Amplification

14.3.1 Polymerase Chain Reaction

The revolutionary, very sensitive and specific PCR method was developed by Kary Mullis in the 1980s. It has allowed scientists to detect and amplify a unique region of DNA multiple folds under strict thermal conditions (Mullis 1990). The specificity of the assay is determined by complementarity of short stretches of synthetic DNA oligonucleotides or primers to the target sequence followed by amplification of the sequences between these primers by the thermostable DNA-dependent DNA polymerase enzyme. It is a faster technique than other methods of detection of pathogens (bacteria, fungi or viruses), which require initial isolation in culture. PCR has been used in veterinary diagnostics for specific genomic detection, e.g. infectious bovine rhinotracheitis virus, foot-and-mouth disease virus, bovine viral diarrhoea virus, buffalopox virus, ephemeral fever virus and many others. Mass level detection of avian influenza and Newcastle disease poultry pathogens in recent outbreaks in the USA has been made with PCR. The following is the list of the different types of PCR being used in the diagnosis of animal diseases:

1. Reverse transcription PCR (RT-PCR)
2. Semi-quantitative PCR
3. Nested/semi-nested PCR (nPCR)
4. Asymmetric PCR
5. Quantitative PCR (qPCR)/real-time PCR
6. Linear after the exponential PCR (LATE-PCR)
7. Multiplex PCR
8. Other variants of PCR

14.3.2 Reverse Transcription (RT)-PCR

Ever since the discovery of PCR, various changes in the PCR protocol have been developed (Erlich et al. 1991). Some of them effectively increased the diagnostic capabilities of PCR and enhanced its uses in the molecular diagnostics. Reverse transcription PCR (RT-PCR) is particularly designed for the amplification of RNA sequences by converting into complementary DNA (cDNA). After reverse

transcription, cDNA is amplified using PCR. The RT-PCR has played a vital role in diseases diagnosis especially RNA viral infections, for example, influenza viruses, rotavirus, bluetongue virus, foot-and-mouth disease virus, etc. (Erlich et al. 1991; Persing 1991).

Furthermore, in vitro gene expression study can be done by application of RT-PCR as the acquired cDNA retains the original complementary RNA sequences. The main confrontation in RT-PCR is the difficulties in handling low level of target RNA/mRNA. Moreover, low stability of RNA/mRNA and its susceptibility to be degraded by ribonucleases and pH change poses many problems to combat.

14.3.3 Semi-quantitative PCR

This technique helps in the approximate estimation of nucleic acids present in a given sample. For RNA quantitation, firstly, the RNA sample is converted into cDNA by RT-PCR. The internal controls/markers (e.g. Apo A1 and β-actin) are also amplified simultaneously. Amplified products are separated by agarose gel electrophoresis that are photographed on gel documentation system. The amount of ethidium bromide bound visible DNA is calculated by measuring its optical density using densitometer. The drawback of this method is the possibility of nonspecific annealing, producing false positive/negative results. The specificity can be enhanced by using highly specific primers.

14.3.4 Nested PCR

This PCR uses two sets of primer pairs for two rounds of amplification for increased sensitivity and specificity. The product of the first set of outer primer pair is used as template for the second PCR using the second set of inner primer pairs. The latter are specific to the amplified product sequence of the first PCR thereby verifying the specificity of the first round of PCR with the specific product availability of second PCR. Nested PCR (nPCR) has been used to detect a number of etiological agents of veterinary importance such as canine corona virus, West Nile virus and orf virus (Pratelli et al. 2004; Bora et al. 2015). The drawback of nested PCR is the possibility of contamination of the first amplified product. This problem can be overcome by using the primer pairs annealing at different temperatures. Secondly, ultrapure oil or wax can be used to make physical separation between two amplification mixtures as a contamination control measure (Erlich et al. 1991).

14.3.5 Asymmetric PCR

In a symmetric PCR, there is exponential growth of double-stranded DNA, whereas an asymmetric PCR generates a single-stranded DNA predominantly as it is governed by the concentration ratio of the primers (i.e. one of the strands of DNA by

the primer in excess) by linear amplification due to the use of unequal concentrations of two primers in a primer pair and a fraction of dsDNA. In the asymmetric PCR, the primer in lower concentration is quantitatively incorporated into dsDNA. Thus the diagnostic technique provides lower intensity signal in agarose gel leading to lesser sensitivity than symmetric PCR. Asymmetric PCR has been used in detection of gene mutations (Zhang et al. 2008).

14.3.6 Real-Time PCR

Real-time PCR is a modification of conventional PCR to address the need for robust quantification of nucleic acids. During this type of PCR, fluorescent signals arise due to the use of fluorescent dyes. These signals are directly proportional to the number of amplified products produced. The amount of product is measured after each cycle of PCR. The exponential phase data represent reaction yield or quantitative information based on the starting quantity of the target. Common real-time PCRs include (1) SYBR green method where the fluorescent dye SYBR green binds to random dsDNA and can also give nonspecific amplification and (2) dual dye-labelled probe method which involves the use of sequence-specific DNA probes that are labelled with a fluorescent reporter, permitting specific detection after hybridization of the probe with its complementary sequence.

There are several advantages of real-time PCR that include:

(1) Its ability to monitor the reaction progress in real time.
(2) It is helpful for accurate quantification of samples.
(3) It has a wide dynamic range of detection, and being a single-tube method, post-PCR manipulations and contamination is not a problem.

RT-PCR is also extensively used for the genotyping and phylogenetic analysis (relatedness) of veterinary pathogens. Real-time RT-PCR is the most sensitive, informative technique yielding rapid results, with the only drawback of high cost of start-up and of reagents. It has been employed for detection of bluetongue virus (De Leeuw et al. 2015; Maan et al. 2015), foot-and-mouth disease virus and bovine piroplasmids (Criado-Fornelio 2007) etc.

The quantification of nucleic acids in RT-PCR is performed by two methods, i.e. the relative and absolute quantitation. In absolute quantification, exact numbers of target DNA molecules are calculated by using a calibration curve. The calibration curve is built with DNA standards. Limitation of this method is that sample and standard should have the same amplification efficiency. The relative quantification is depending on fold differences in the expression of target gene as compared to internal reference genes. The result is expressed in different expression levels of mRNA as cDNA (reverse transcription). It is an easy approach for quantification because there no need of making calibration curves.

14.3.7 Linear After the Exponential (LATE)-PCR

There are multiple reasons for considering conventional asymmetric PCR inefficient, as there are difficulties in optimization and it tends to promote nonspecific amplification and restricted concentration of one primer lowers the Tm (melting temperature) below the reaction annealing temperature. To overcome these limitations and to increase efficiency comparable to symmetric PCRs, linear after the exponential (LATE)-PCR was developed based on primer pairs purposely designed for use at unequal concentrations to yield specific single-stranded DNA products in a robust way (Pierce et al. 2005). In the LATE-PCR, detection step is distinct from the annealing and extension step, and it improves allele discrimination and increases signal strength significantly as compared to symmetric PCR (Sanchez et al. 2004). LATE-PCR does not reach the characteristic plateau like conventional PCR but ends in a nearly linear phase (Johann et al. 2015). LATE-PCR is highly appropriate for high-throughput field applications, e.g. clinical analysis, biodefense, forensics and DNA sequencing.

14.3.8 Multiplex Polymerase Chain Reaction (mPCR)

Multiplex PCR (mPCR) is a modification of PCR for concurrent amplification of many sequences by inclusion of different sets of genome sequence-specific primer pairs for different targets. mPCR is used for diagnosis of different disease pathogens in a single reaction. It can also be used to identify exonic and intronic sequences in specific genes. In multiplex PCR the design of various primer pairs is crucial so that they complementarily anneal to specific DNA sequences at more or less similar temperatures, i.e. annealing temperature should be the same for different primer pairs used in combination. A multiplex PCR assay to detect H5N1 and other human respiratory pathogens (Lam et al. 2007; Rheem et al. 2012) and mastitis in animals has been developed (Shome et al. 2011). Other variant and combination of this technique is multiplex one-step real-time PCR kits that are available with all plus points inherited (Gautam et al. 2016).

14.3.9 Other Variants of PCR

14.3.9.1 Hot-Start PCR

During the amplification of target DNA, sometimes amplification of nontarget sequences also takes place which are nonbeneficial, and their presence in huge amount decreases the amount of desired product that leads to complication in data analysis. For getting higher reaction specificity and yield, 'hot-start PCR' was developed, where the Taq polymerase enzyme stays inactivate/blocked by specific antibodies at temperatures below 72 °C, the optimal temperature of primer extension by Taq polymerase, thus avoiding non-specific amplification. The blocking

antibodies get denatured and removed at an initial step at 95 °C allowing the specific reaction to proceed (Kellogg et al. 1994).

14.3.9.2 Touchdown PCR

In this type of PCR, the annealing temperature (Ta) is deliberately brought down during the cycling process. At initial stage, Ta is set 5–10 °C higher than the Tm of the primers, while in the follow-up cycles, Ta is slowly decreased, and by the end of the amplification, Ta is below 2–5 °C from Tm (Don et al. 1991). The high temperature increases the specificity of primer and template binding leading to amplification of only target sequences.

14.3.9.3 Assembly PCR/Polymerase Cycling Assembly (PCA)

PCA involves two sets of PCR. In the first set of PCR, overlap primers are used. The product of the first PCR is used as a template for the second PCR, which amplifies final full-length product. PCA synthesizes DNA from a pool of long oligonucleotides having short overlapping segments and can be used as an alternative for ligation-based assembly (Bang and Church 2008).

14.3.9.4 Colony PCR

This PCR is specifically to screen the bacterial colonies. The bacterial colonies are transferred into a PCR master mix aseptically. The cellular DNA is released either by incubation at 95 °C (with standard polymerase) or by shortened denaturation step at 100 °C with a recombinant DNA polymerase (Pavlov et al. 2006). This technique is used for an established Fung's double-tube method for rapid detection and confirmation of *Clostridium perfringens* (Ruengwilysup et al. 2009).

14.3.9.5 Digital PCR/Emulsion PCR

The digital PCR performs simultaneous amplification for a large number of samples. These samples are present in an emulsion in a separate droplet, e.g. 454 sequencing platforms and Ion Torrent technology.

14.3.9.6 Suicide PCR

Suicide PCR is performed where very high specificity of the desired product is required, e.g. palaeogenetics. The target of this PCR is the genomic region, which has never been amplified in the laboratory with any sets of primers. This is to avoid false positive results from contaminating DNA from previous PCR reactions (Raoult et al. 2000).

14.4 Non-PCR Methods of Nucleic Acid Detection

Nucleic acid techniques (NATs) are sensitive, rapid and reliable diagnostics that are based on amplification of specific regions of pathogen genome. Though quantitative in nature, the non-amplification nucleic acid detection methods are not so commonly used because of their lower sensitivity compared to amplification methods.

These methods have less specific requirements for performing enzymatic process and also less dependency on good reagents. The property of less sensitivity of these methods is favoured to reduce carryover product contamination.

The different methods of nucleic acid amplification have been developed, e.g. rolling circle amplification technique and direct signal amplification system. In human medicine these techniques are presently being used for the detection of cytomegalovirus (CMV) and human immunodeficiency virus (HIV). These methods can be useful in the diagnosis of livestock diseases.

14.5 Signal Amplification

14.5.1 Branched DNA (bDNA) Assay

To minimize the chances of contamination during amplification, various molecular technologies have been developed. One alternate to enzymatic amplification of target nucleic acid is hybridized probe-based signal amplification. The most common signal amplification technologies are branched DNA (bDNA) and hybrid capture (HC) assays (Datar and Joshi 2001).

In bDNA technology, the presence of specific nucleic acid is detected by measuring the signal generated by specific hybridization of many branched labelled DNA probes on an immobilized target nucleic acid. One end of bDNA binds to the immobilized target DNA, and the other end of it has many branches of DNA which amplify the detection signals. The amplification of signals is linear and is achieved by sequential or simultaneous hybridization of synthetic oligonucleotides. Final detection uses alkaline phosphatase (AP) as it generates chemiluminescence. bDNA is a quantitative technology and is used in the determination of viral or pathogen load (Cao et al. 1995; Kern et al. 1996; Collins et al. 1997). This technology is a highly sensitive, specific, less labour-intensive, less contamination prone and reliable tool in the diagnosis of viral and bacterial infections and for monitoring disease status during the course of therapy. This technology has been proved versatile since it helped in the detection of infections by a wide range of microorganisms (Tsongalis 2006).

14.5.2 Nucleic Acid Sequence-Based Amplification (NASBA)

NASBA, introduced in 1989, is an isothermal amplification method which is carried out at a constant temperature of 41 °C. It is also called self-sustained sequence replication and transcription-mediated amplification. Three enzymes, viz. avian myeloblastosis reverse transcriptase, RNase H and T7 DNA-dependent RNA polymerase, are involved in the process (Fakruddin et al. 2012). It is a sensitive, isothermal, transcription-based amplification system which is specifically designed for the detection of RNA targets (Van Gemen et al. 1995; Deiman et al. 2002).

The major advantage of NASBA is the production of single-stranded RNA amplicons. These amplified RNA products can be sequenced directly with a dideoxy method using RT and a labelled oligonucleotide primer. Additionally, the ssRNA amplicons can either be amplified or probed for direct detection. The technique can be easily adopted for the development of various pathogen detection kits (Fakruddin et al. 2012).

14.5.3 Transcription-Based Amplification System (TAS)

TAS was developed by Kwoh and his coworkers in 1989. In TAS, RNA is the target molecule as well as primary product (Kwoh et al. 1989). A copy of DNA is made from RNA, and then transcription produces million copies of RNA. The variations of TAS are transcription-mediated amplification (TMA), nucleic acid sequence-based amplification (NASBA) and self-sustaining sequence replication (3SR). In the process, the primer complementary to the sequences that has the binding site for RNA polymerase is added to the targeted RNA molecule. Three enzymes, reverse transcriptase (RT), RNase H and T7 DNA-dependent RNA polymerase, are used in the amplification reaction. The primer anneals and then RT enzyme is used to produce cDNA copy of target RNA. RNase H then degrades the initial RNA from DNA-RNA hybrid. The second primer set binds to newly synthesized cDNA which is extended to produce double-stranded cDNA. Both the strands of this ds cDNA can act as a template for RNA polymerase for transcription.

TAS has few advantages over various amplification processes: (1) TAS is an isothermal process, negating the requirement of thermal cycling to drive reactions due to which it helps to minimize contamination risks (Guatelli et al. 1990). Apart from direct detection of RNA-containing viruses, e.g. hepatitis C virus, this technique can be used for detection of low amount of certain bacterial and fungal pathogens (Compton 1991). The only demerit of TAS is heating step which denatures the enzyme due to which enzyme is needed after every denaturation step.

14.5.4 Strand Displacement Amplification (SDA): Loop-Mediated Isothermal Amplification (LAMP)

14.5.4.1 Strand Displacement Amplification (SDA)

SDA, a non-PCR nucleic acid amplification technique, developed in 1991, involves DNA polymerase-initiated DNA synthesis. It works on a single-stranded nick followed by the displacement of the nicked strand during the process. The displaced strand is used as a substrate for further simultaneous nicking and displacement reactions. SDA uses specific primers, a DNA polymerase and a restriction endonuclease, to achieve exponential amplification of target. In brief, this method makes copies of the target sequence flanked by nickable restriction sites, allowing the exponential increase of these modified target sequences by recurrent nicking, strand dislocation and further priming of banished strands (Spargo et al. 2000).

SDA has two important advantages: (a) SDA is isothermal with the exception of the initial denaturation step and (b) it can be applied to both single- and double-stranded DNA (Kim and Easley 2011).

Loop-mediated isothermal amplification (LAMP) assay, initially described by Notomi et al. 2000, is an important method for disease diagnosis especially in the developing countries (Abdullahi et al. 2015). LAMP is a simple, quick, highly specific and cheap single-tube technique for the amplification of DNA. LAMP can be used as a simple test in the field or at the point of disease outbreak.

In LAMP test, four different types of primers, a forward inner primer (FIP) and a backward inner primer (BIP) set and a forward and backward outer primer set, are used. These primers recognize six distinct binding sites on target DNA. LAMP uses Bst DNA polymerase having strand displacement activity. Both primers and enzymes work together for LAMP cycling initiation and continuation of DNA synthesis with auto-strand displacement leading to the accumulation of 10^9 copies of target in less than an hour. DNA synthesis is primed by FIP and BIP, in combination with initial strand displacement mediated by the extension of forward and backward outer primers, resulting in the successive formation of a DNA molecule. This DNA molecule is looped out at one end, further by a dumb-bell DNA structure, which is rapidly transformed to a stem-loop structure which is the substrate for the elongation and recycling step of LAMP.

LAMP-based commercial detection kits for bacterial and viral pathogens are available. LAMP has also been developed for important animal pathogens, e.g. foot-and-mouth disease virus (Yamazaki et al. 2013), bluetongue virus (Maan et al. 2016), capripox viruses (Batra et al. 2015) and peste des petits ruminants virus (Zhan et al. 2009). The process demands the use of multiple primers where a major disadvantage of LAMP over PCR lies, that is, the need of frequently updated primer sequences in order to detect the prevalent virus strains with adequate sensitivity and specificity.

14.6 Probe Amplification

14.6.1 Ligase Chain Reaction

Ligase chain reaction (LCR) is a probe-based amplification technique and was first described by Wu and Wallace (1989) to detect point mutations. In this technique one probe is formed by ligation of two adjacent probes. The main characteristic of LCR is the second set of primer which is complementary to the first pair, designed with the nucleotide at the 3′ end of the upstream primer indicating the sequence difference. When target DNA is present, the two adjacent probes are ligated by DNA ligase. If ligated product is absent, that means there is at least a single base-pair change in the target sequence. In the following steps, the ligated products can serve as templates and can be amplified by thermal cycling in an exponential manner (Wiedmann et al. 1994). In LCR contamination risk and variation in copy number of the plasmid containing the target is a problem (Umesha and Manukumar 2016).

14.6.2 RNA Aptamers

RNA aptamers are defined as RNA oligonucleotides (56–120 long) having variable and constant region that bind to a specific target with high affinity and specificity (Ellington and Szostak 1990). The method for isolation of aptamers is called systematic evolution of ligands by exponential enrichment (SELEX) (Tuerk and Gold 1990).

RNA aptamers are an important tool in RNA nanotechnology as compared to DNA and protein aptamers (Germer et al. 2013). They are easy to synthesize in huge quantity under controlled way having well-defined structure and stereochemistry. Similar to antibodies, RNA aptamers have low immunogenicity as compared to other macromolecule such as proteins. RNA aptamers are more thermodynamically stable and its unique tertiary structure leads to specific binding. It is small in size due to its single-stranded nature so it can easily enter into the cell.

RNA aptamers can be used in different fields of science, i.e. diagnostic, prognostic and therapeutic (Germer et al. 2013).

14.7 Rapid Whole-Genome Sequencing Technologies

Nowadays whole-genome sequencing techniques are being extensively utilized to study a wide variety of infectious agents. With the advent of high-throughput 'next-generation' sequencing technologies, detailed analyses of entire pathogen genomes have been made possible within a few days which previously took many years. Different rapid whole-genome sequencing platforms as Illumina, 454 and Ion Torrent have tremendously revolutionized the world of disease diagnostics by enhancing the ability to rapidly screen complex mixtures of genomes. It has become easier to make comparison between diseased and apparently normal/healthy states of patients. No prior knowledge is required about the infectious agent to be sequenced, so it plays an important role in the detection and identification of new and emerging pathogens. The latest sequencing methods hold promises in increasing the present knowledge regarding the evolution of pathogenic microorganisms especially with respect to the development of antimicrobial resistance. Expensiveness is always a matter of consideration when any diagnostic assay or kit is developed. At present whole-genome sequencing technologies particularly the next-generation sequencing (NGS) are very costly, but as per the increasing demand and use in different field of scientific research, diagnostics and many others, it can be expected to become cheaper in the future. Some of the NGS platforms are briefly described below.

14.7.1 454 Genome Sequencer FLX (Roche Applied Science)

The very first next-generation system brought in the market was Genome Sequencer (GS) instrument based on the principle of pyrosequencing by 454 Life Science in

2005 (Margulies et al. 2005). Traditional sequencing methods required cloning of the gene to be sequenced, but this demerit was removed by 454 GS pyrosequencing system. Briefly, the procedure involved initial random shearing of genome (may be enzymatic or physical by ultrasonication), followed by ligation of both the ends of each fragmented DNA genome with specific adaptors. These adaptors allowed the DNA molecule capturing on the surface of beads. Now these ligated fragments were amplified (emulsion PCR) and captured in emulsion droplet. Individual bead with amplified fragment arrayed in a well of a fibre-optic slide. The 454 GS followed the same sequencing principle as of most primitive Sanger sequencing method. In the 454 method, when a new base was incorporated in the growing chain, a pyrophosphate group was released and detected as emitted light by a CCD imager coupled to the fibre-optic array.

The main disadvantage of this system was high error rates as insertion and deletion mutations (Indels) were produced because of the false reading of template by misjudging the homopolymers. To increase the read lengths, a new GS FLX Titanium XL+ has been developed which is able to generate 700 Mb of sequence data with 99.997% accuracy. The system involves a relatively high cost and lower throughput than other developed next-generation sequencing methods and therefore has decreased its preferences.

14.7.2 Ion Torrent

Ion Torrent is known by different synonyms as ion semiconductor sequencing or pH-mediated sequencing or silicon sequencing. It is based on the principle of detection of hydrogen ions by ion-sensitive field-effect transistor (ISFET) ion sensor that is released during polymerization reaction of new DNA strand synthesis. This is a method of sequencing by synthesis, i.e. sequence data of template is generated, while complementary strand is synthesized. The labelled or modified nucleotides and optical instruments are not employed as done in other sequencing methods. This system also has various limitations like difficult enumeration of long homopolymer repeats. Signals obtained from high repeats of different nucleotides create obstacles as differentiation could not be made from that of similar but different number homopolymer repeats. It gives an average read length of 400 nucleotides per read, which is a shorter read length compared to pyrosequencing and Sanger sequencing methods. Thus this is best suited for small-scale applications as microbial genome/transcriptome sequencing, amplicon sequencing or quality testing of sequencing libraries.

14.7.3 Illumina

Illumina NGS technology was developed by Canard and Sarfati in 1994 as a second next-generation sequencing technology, which uses the reversible termination chemistry concept (Canard and Sarfati 1994). This concept allows the identification

of single-nucleotide base as it is polymerized into new DNA strand, i.e. sequencing by synthesis. It is multipurpose solving as has been used in whole-genome sequencing, transcriptome analysis, metagenomics, small RNA and methylation profiling and also helpful in the analysis of protein and nucleic acid interaction. It is also known as Solexa, released in 2006 as Solexa sequencing platform. Solexa platform uses sequencing by synthesis (SBS) technology where fluorescently labelled nucleotides are used as terminating base. The latter when removed leave an unblocked 3′ terminus making the process of chain termination reversible.

The Illumina technology involves DNA fragmentation, ligation with specific adapters and followed by denaturation. The single-stranded denatured templates are then immobilized on one end on a flow cell surface which is already laid with complementary adapters. Immobilization of every fragmented template strand on flow cell surface occurs by hybridization of free end to the complementary adapter. It is followed by amplification, which results up to 1000 identical copies of every ssDNA template molecule named as DNA ‘polonies’. During the cycling process of sequencing, as a single fluorescent dye-labelled dNTP is incorporated, the fluorescence of dye is imaged by the CCD camera for identification of the base, and then the dye is enzymatically cleaved, which allows incorporation of the subsequent nucleotide.

14.7.4 Microarray Technology

Microarray technology is very powerful and high throughput which can be used for expression studies, transcriptome analysis, detection and characterization of genetic variants, DNA-protein interaction study and detecting genome methylation. The important feature of this method is immobilization of different molecules (oligonucleotides, proteins, small drug-like compounds) onto a solid and activated surface as matrix (Schena et al. 1998). This matrix arrangement is called microarray. Microarray plate has specific point at which high concentration of immobilized molecules are present to interact with their targets. These are named as microchips, biochips, DNA chips or gene chips to detect DNA in diverse biological samples. The advantages of microarray technology are the following: the expression of the entire gene content of a genome of interest can be monitored and it also provides data analysis in field conditions because detection is simple and rapid and also provides real-time data analysis (Zhou and Tompson 2004).

14.7.5 Nanotechnology

The term ‘nanotechnology’ was given for the first time by Norio Tangiuch in 1974. This technology includes manipulation in atoms and molecules at very small scale that is nano in size (Savage et al. 2007; Medina et al. 2007). Till date so many types of nanoparticles are obtained from transition metals, silicon, carbon and metal oxides (Torres-Sangiao et al. 2016). This nanomaterial shows different type of

physico-chemical properties, thereby widening its application area. This technology is very helpful in designing molecular diagnostic assays, pen-side test or chip-based diagnostics for medical and veterinary fields. The very small size also increases its use in nanomedicine (Jos et al. 2009).

14.7.5.1 Gold Nanoparticles for Use in Diagnostics

Gold nanoparticles (AuNPs) are very flexible nanostructures and can be used in different field of biomedical science (Mirkin et al. 1996). AuNPs show so many useful properties like more flexible structure, defined size, shape, structure and better optical properties. These particles are being used for diseases diagnosis as biosensors (Dilbaghi et al. 2013; Mirkin et al. 1996; Shah et al. 2014).

14.7.6 Proteomics

The whole set of proteins expressed by a genome, cell, tissue or organism is termed as 'proteome'. The study and characterization of complete set of proteins is called as 'proteomics' (Anderson and Anderson 1998). Proteomics recently in the last two decades has emerged as a field of research and has developed rapidly (Ceciliani et al. 2014a, b).

Proteomics is a better approach than genomics for studying changes in metabolism under stress conditions. Various methods in proteomics, viz. 2-D gel electrophoresis (2DGE), MALDI-TOF/MS, etc., play a significant role in the analysis of novel proteins and in disease diagnosis. The expression of proteins depends on the status of the body and different environmental factors. This field of study has an important role in novel drug discovery and in the early-stage disease diagnosis (OIE 2016). Proteome maps are being derived from a range of veterinary pathogens (Mujer et al. 2002; Rout and Field 2001; Yatsuda et al. 2003). Proteomics-based diagnosis may be used to identify known or unknown disease markers in the future. Proteomics may have applications in the diagnosis of disease pattern development in combination with biochip technology, bead technology, mass spectrometry and other separation chromatographic methods. This type of combination of technologies can be useful for identification of pathogens that do not induce predictable serological reactions, i.e. bovine tuberculosis.

14.8 Conclusion and Future Applications

Molecular diagnostic assays are becoming more popular for confirmation of field-based diagnosis of livestock diseases on the basis of clinical signs and symptoms. Recent advancements of diverse biotechnological tools promise for improvement in the speed and accuracy of diagnostics for both human and veterinary medicine. This can also support the development of advance epidemiological tools and data for various animal and human pathogens, thereby allowing prioritization and implementation of appropriate biosecurity measures during outbreaks more effectively and saving valuable national funds.

References

Abdullahi UF, Naim R, Aliyu S et al (2015) Loop-mediated isothermal amplification (LAMP), an innovation in gene amplification: bridging the gap in molecular diagnostics; a review. Indian J Sci Technol 8:557–567

Anderson NL, Anderson NG (1998) Proteome and proteomics: new technologies, new concepts, and new words. Electrophoresis 19:1853–1861

Bang D, Church GM (2008) Gene synthesis by circular assembly amplification. Nat Methods 5:37–39

Batra K, Kumar A, Kumar V et al (2015) Development and evaluation of loop-mediated isothermal amplification assay for rapid detection of Capripox virus. Vet World 8:1286–1292

Bora M, Bora DP, Barman NN et al (2015) Isolation and molecular characterization of Orf virus from natural outbreaks in goats of Assam. Virus 26:82–88

Canard B, Sarfati RS (1994) DNA polymerase fluorescent substrates with reversible 3′-tags. Gene 148:1–6

Cao Y, Ho DD, Todd J et al (1995) Clinical evaluation of branched DNA signal amplification for quantifying HIV type 1 in human plasma. AIDS Res Hum Retrovir 11:353–361

Ceciliani F, Eckersall D, Burchmore R et al (2014a) Proteomics in veterinary medicine: applications and trends in disease pathogenesis and diagnostics. Vet Pathol 51:351–362

Ceciliani F, Restelli L, Lecchi C (2014b) Proteomics in farm animals models of human diseases. Proteomics Clin Appl 8:677–688

Collins ML, Irvine B, Tyner D et al (1997) A branched DNA signal amplification assay for quantification of nucleic acid targets below 100 molecules/ml. Nucleic Acids Res 25:2979–2984

Compton J (1991) Nucleic acid sequence-based amplification. Nature 350:91–92

Criado-Fornelio A (2007) A review of nucleic-acid-based diagnostic tests for Babesia and Theileria, with emphasis on bovine piroplasms. Parassitologia 49(Suppl 1):39–44

Datar RH, Joshi NN (2001) Nucleic acids in diagnosis. Natl Med J India 14:34–42

De Leeuw I, Garigliany M, Bertels G et al (2015) Bluetongue virus RNA detection by real-time rt-PCR in post-vaccination samples from cattle. Transbound Emerg Dis 62:157–162

Deiman B, van Aarle P, Sillekens P (2002) Characteristics and applications of nucleic acid sequence-based amplification (NASBA). Mol Biotechnol 20:163–179

Dilbaghi N, Kaur H, Kumar R et al (2013) Nanoscale device for veterinary technology: trends and future prospective. Adv Mater Lett 4:175–184

Don RH, Cox PT, Wainwright BJ et al (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res 19:4008

Ellington AD, Szostak JW (1990) In vitro selection of RNA molecules that bind specific ligands. Nature 346:818–822

Erlich HA, Gelfand D, Sninsky JJ (1991) Recent advances in the polymerase chain reaction. Science 252:1643–1651

Fakruddin M, Mazumdar RM, Chowdhury A et al (2012) Nucleic acid sequence based amplification (NASBA)-prospects and applications. Int J Life Sci Pharma Res 2:106–121

Gautam R, Mijatovic-Rustempasic S, Esona MD et al (2016) One-step multiplex real-time RT-PCR assay for detecting and genotyping wild-type group A rotavirus strains and vaccine strains (Rotarix(R) and RotaTeq(R)) in stool samples. Peer J 4:e1560

Germer K, Leonard M, Zhang X (2013) RNA aptamers and their therapeutic and diagnostic applications. Int J Biochem Mol Biol 4:27–40

Guatelli JC, Whitfield KM, Kwoh DY et al (1990) Isothermal, in vitro amplification of nucleic acids by a multienzyme reaction modeled after retroviral replication. Proc Natl Acad Sci U S A 87:1874–1878

Johann KS, Schürenkamp M, Sibbing U et al (2015) Linear-after-the-exponential (LATE)-PCR: improved asymmetric PCR for quantitative DNA-analysis. Forensic Sci Int Genet Suppl Ser 5:e659–e661

Jos A, Pichardo S, Puerto M et al (2009) Cytotoxicity of carboxylic acid functionalized single wall carbon nanotubes on the human intestinal cell line Caco-2. Toxicol in Vitro 23:1491–1496

Kellogg DE, Rybalkin I, Chen S et al (1994) TaqStart antibody: "hot start" PCR facilitated by a neutralizing monoclonal antibody directed against Taq DNA polymerase. BioTechniques 16:1134–1137

Kern D, Collins M, Fultz T et al (1996) An enhanced-sensitivity branched-DNA assay for quantification of human immunodeficiency virus type 1 RNA in plasma. J Clin Microbiol 34:3196–3202

Kim J, Easley CJ (2011) Isothermal DNA amplification in bioanalysis: strategies and applications. Bioanalysis 3:227–239

Kwoh DY, Davis GR, Whitfield KM et al (1989) Transcription based amplification system and detection of amplified human immunodeficiency virus type-1 with a bead based sandwich hybridisation format. Proc Natl Acad Sci U S A 86:1173–1177

Lam WY, Yeung AC, Tang JW et al (2007) Rapid multiplex nested PCR for detection of respiratory viruses. J Clin Microbiol 45:3631–3640

Maan NS, Maan S, Belaganahalli M et al (2015) A quantitative real-time reverse transcription PCR (qRT-PCR) assay to detect genome segment 9 of all 26 bluetongue virus serotypes. J Virol Methods 213:118–126

Maan S, Maan NS, Batra K et al (2016) Reverse transcription loop-mediated isothermal amplification assays for rapid identification of eastern and western strains of bluetongue virus in India. J Virol Methods 234:65–74

Margulies M, Egholm M, Altman WE et al (2005) Genome sequencing in microfabricated high-density picolitre reactors. Nature 437:376–380

Medina C, Santos-Martinez MJ, Radomski A et al (2007) Nanoparticles: pharmacological and toxicological significance. Br J Pharmacol 150:552–558

Mirkin CA, Letsinger RL, Mucic RC et al (1996) A DNA-based method for rationally assembling nanoparticles into macroscopic materials. Nature 382:607–609

Mujer CV, Wagner MA, Eschenbrenner M et al (2002) Global analysis of Brucella melitensis proteomes. Ann N Y Acad Sci 969:97–101

Mullis KB (1990) The unusual origin of the polymerase chain reaction. Sci Am 262(56–61):64–55

Notomi T, Okayama H, Masubuchi H et al (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28:E63

OIE (2016) Manual of standards for diagnostic tests and vaccines. Organization International des Epizootics, Paris, pp 178–188

Pavlov AR, Pavlova NV, Kozyavkin SA, Slesarev AI (2006) Thermostable DNA Polymerases for aWide Spectrum of Applications: comparison of a Robust Hybrid TopoTaq to other enzymes. In: Kieleczawa J (ed) DNA Sequencing II: optimizing preparation and cleanup. Jones and Bartlett, Sudbury, p 241–257. ISBN 0-7637-3383-0

Persing DH (1991) Polymerase chain reaction: trenches to benches. J Clin Microbiol 29:1281–1285

Pierce KE, Sanchez JA, Rice JE et al (2005) Linear-after-the-exponential (LATE)-PCR: primer design criteria for high yields of specific single-stranded DNA and improved real-time detection. Proc Natl Acad Sci U S A 102:8609–8614

Pratelli A, Decaro N, Tinelli A et al (2004) Two genotypes of canine coronavirus simultaneously detected in the fecal samples of dogs with diarrhea. J Clin Microbiol 42:1797–1799

Raoult D, Aboudharam G, Crubezy E et al (2000) Molecular identification by "suicide PCR" of Yersinia pestis as the agent of medieval black death. Proc Natl Acad Sci U S A 97:12800–12803

Rheem I, Park J, Kim TH et al (2012) Evaluation of a multiplex real-time PCR assay for the detection of respiratory viruses in clinical specimens. Ann Lab Med 32:399–406

Rout MP, Field MC (2001) Isolation and characterization of subnuclear compartments from Trypanosoma brucei. Identification of a major repetitive nuclear lamina component. J Biol Chem 276:38261–38271

Ruengwilysup C, Detvisitsakun C, Aumyat N et al (2009) Application of a colony pcr technique with fung's double tube method for rapid detection and confirmation of clostridium perfringens. J Rapid Method Autom Microbiol 17:280–290

Sanchez JA, Pierce KE, Rice JE et al (2004) Linear-after-the-exponential (LATE)-PCR: an advanced method of asymmetric PCR and its uses in quantitative real-time analysis. Proc Natl Acad Sci 101:1933–1938

Savage N, Thomas T, Duncan J (2007) Nanotechnology applications and implications research supported by the US environmental protection agency star grants program. J Environ Monit 9:1046–1054

Schena M, Heller RA, Theriault TP et al (1998) Microarrays: biotechnology's discovery platform for functional genomics. Trends Biotechnol 16:301–306

Shah M, Badwaik VD, Dakshinamurthy R (2014) Biological applications of gold nanoparticles. J Nanosci Nanotechnol 14:344–362

Shome BR, Das Mitra S, Bhuvana M et al (2011) Multiplex PCR assay for species identification of bovine mastitis pathogens. J Appl Microbiol 111:1349–1356

Spargo CA, Dean CH, Nycz CM et al (2000) SDA target amplification. Springer, Berlin

Torres-Sangiao E, Holban AM, Gestal MC (2016) Advanced nanobiomaterials: vaccines, diagnosis and treatment of infectious diseases. Molecules 21:E867

Tsongalis GJ (2006) Branched DNA technology in molecular diagnostics. Am J Clin Pathol 126:448–453

Tuerk C, Gold L (1990) Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science 249:505–510

Umesha S, Manukumar HM (2016) Advanced molecular diagnostic techniques for detection of food-borne pathogens: current applications and future challenges. Crit Rev Food Sci Nutr 58:1–21

Van Gemen B, Kievits T, Romano J (1995) Transcription based nucleic acid amplification methods like nasba and 3sr applied to viral diagnosis. Rev Med Virol 5:205–211

Wiedmann M, Wilson WJ, Czajka J et al (1994) Ligase chain reaction (LCR) – overview and applications. PCR Methods Appl 3:S51–S64

Wu DY, Wallace RB (1989) The ligation amplification reaction (LAR) – amplification of specific DNA sequences using sequential rounds of template-dependent ligation. Genomics 4:560–569

Yamazaki W, Mioulet V, Murray L et al (2013) Development and evaluation of multiplex RT-LAMP assays for rapid and sensitive detection of foot-and-mouth disease virus. J Virol Methods 192:18–24

Yatsuda AP, Krijgsveld J, Cornelissen AW et al (2003) Comprehensive analysis of the secreted proteins of the parasite Haemonchus contortus reveals extensive sequence variation and differential immune recognition. J Biol Chem 278:16941–16951

Zhan Z, Wei G, Zhu X et al (2009) Development of loop-mediated isothermal amplification (LAMP) method for the detection of peste des petits ruminants virus. J Pathog Biol 4:241–246

Zhang X, Xu S, Gao X et al (2008) The application of asymmetric PCR-SSCP in gene mutation detecting. Front Agric China 2:361–364

Zhou J, Tompson DK (2004) Microarray technology and applications in environmental microbiology. Adv Agron 82:183–270

Part IV

Food and Environmental Biotechnology

Antiproliferative Effects of Probiotics

15

Tejinder Kaur and Praveen P. Balgir

15.1 Introduction

15.1.1 Probiotics

The concept of "probiotics," used to designate the natural defense against the growth of pathogens and antibiotic-resistant bacteria, has with time been expanded to encompass broad health benefits. According to FAO (Food and Agriculture Organization of United Nations) and WHO (World Health Organization), probiotics are defined as "live microorganisms which confer a health benefits on the host when administered in adequate amounts" (FAO/WHO 2002). Most commonly used probiotics in humans include *Lactobacillus acidophilus*, *L. casei*, *Bifidobacterium bifidum*, *B. longum*, and the yeast *Saccharomyces boulardii* which are natural residents of human gastrointestinal tract acquired as natural food constituents. Probiotic bacteria have been observed to establish microbiome homeostasis by correcting the disturbed intestinal microfloral imbalance and related dysfunctions of the gastrointestinal tract (Kailasapathy and Chin 2000). Furthermore, they are known to exert a number of other beneficial health effects on the host, such as enhancement of host immune response, reduction in incidence of colon cancer, lowering serum cholesterol, and production of antimicrobial compounds, such as bacteriocins which inhibit the growth and colonization of pathogenic microorganisms in the intestine. However, the applications are not limited to these effects only but are also proven to suppress allergies, modulate immune function, and prevent cancer initiation (Commane et al. 2005; Lee et al. 2008; Tiptiri-Kourpeti et al. 2016).

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15.1.2 Antiproliferative Effects of Probiotics

A number of studies using various strains of already known probiotic bacteria and their cellular components have displayed anticarcinogenic activity and were found to inhibit metastasis (Lee et al. 2004). Anticarcinogenic effects exerted by probiotic strains have also been demonstrated in vivo in animal and human subjects, followed by confirmation in vitro studies with cancer cell lines and anti-mutagenicity assays (Commane et al. 2005).

15.1.2.1 In Vitro Studies

Studies have reported that human intestinal LAB strains can bind mutagenic heterocyclic amines produced in meat-based diet during cooking, markedly reducing their mutagenicity. A significant reduction in the growth and viability of HT-29 cells was observed along with a parallel increase in dipeptidyl peptidase IV and brush border enzymes. These enzymes mark the beginning of differentiation of cancer cells into enterocytes (Baricault et al. 1995; Hiryama and Rafter 2000).

Antiproliferative activity was also confirmed in homogenates derived from *Lactobacillus rhamnosus* GG with and without heat treatment. Homogenate with cell wall extract suppressed the proliferation of mononuclear cells induced by phytohemagglutinin, irrespective of heat treatment, due to the presence of heat-stable components with antiproliferative properties. Further, a dose-dependent decrease in growth and proliferation of CT26 cancer cells derived from the colon of BALB/C mice was observed after treatment with LAB peptidoglycan which promoted apoptosis in cancer cells (Pessi et al. 1999; Sun et al. 2005).

Lee et al. (2008) listed antitumor activity of glycoproteins secreted in the culture media and polysaccharide fractions of *Lactobacillus* cultures. Antitumor activity was reported to be detected in peptidoglycans extracted from *B. infantis* ATCC 15697. LAB strains, *L. acidophilus*, *L. casei*, *L. rhamnosus* GG, *B. adolescentis*, *B. breve*, *B. infantis*, and *B. longum*, were also observed to suppress experimental colon tumor. A dose-dependent inhibition of cancer cell proliferation has been observed against human colon cancer cell lines, namely, Caco-2, HT-29, and SW480, after exposure to butanol extract of *B. adolescentis* isolates.

Chang et al. (2010) screened a LAB strain cultured in commercial as well as traditional types of kimchi revealing that *Lactobacillus acidophilus* KFRI342 displayed the highest ability to reduce the growth of cancer cell line SNU-C4. Grimoud et al. (2010), working with HT-29 cells mimicking colorectal cancer (CRC) and inflammatory activated transgenic Caco-2 cells, observed that treatment with a synbiotic constituted of *Bifidobacterium breve*, *Lactococcus lactis*, and oligoalternan leads to a decrease in proliferation, by upregulation of differentiation biomarker alkaline phosphatase.

Sadeghi-Aliabadi et al. (2014) reported antiproliferative effect of heat-inactivated cells and cell-free supernatants of *L. plantarum* A7 and *L. rhamnosus* GG against cancerous (Caco-2 and HT-29) as well as noncancerous normal cell lines (L-929). This nonspecific antiproliferative effect was observed on cancerous as well as normal cell lines.

Antiproliferative potential of supernatants of *L. plantarum* strains has been reported against Caco-2, melanoma, and TLT human cell lines, mainly attributable to non-proteinaceous less than 10KD metabolite present in the supernatant (Dimitrovski et al. 2014). Li et al. (2014) investigated in vitro antiproliferative effect of three purified exopolysaccharide fractions (LHEPS-1, LHEPS-2, and LHEPS-3) as well as the crude LHEPS from *Lactobacillus helveticus* MB2-1. The exopolysaccharides inhibited the cell division of human gastric cancer BGC-823 cells in concentration and time-dependent manner. LHEPS-2 exhibited higher antiproliferative activity than LHEPS-1, LHEPS-3, and crude LHEPS. Similarly, the cell-bound exopolysaccharides (c-EPS) of *Lactobacillus plantarum* 70810, identified as a galactan, significantly inhibited cell division of HepG-2, BGC-823, and HT-29 tumor cells in vitro (Wang et al. 2014).

In the gut, bacteria are known to produce short-chain fatty acids (SCFAs) by fermentation that exert anti-inflammatory and antitumorigenic effects. Kahouli et al. (2015) reported production of SCFAs as the pro-apoptotic agents, by orally administered probiotic strain, *L. fermentum* NCIMB 5221 against Caco-2 cells. Apart from SCFAs, these bacteria also generated metabolites like organic acids, peptides, etc. in the gut that influence cellular proliferation, differentiation, apoptosis, and inflammation and play a role in reduction of CRC risk.

Tiptiri-Kourpeti et al. (2016) used *Lactobacillus casei* ATCC 393 co-incubation with murine (CT26) and human (HT29) colon carcinoma cell lines and detected their antiproliferative property with the underlying mechanism being induction of apoptosis in cell lines as indicated by upregulation of two apoptotic markers the Annexin V and TRAIL proteins. In vivo study corroborated the in vitro results.

15.1.2.2 In Vivo Studies

Animal Studies

Antitumor properties of LAB are also demonstrated against pre-implanted tumor cells in mice model where administration of LAB cultures or fermented milk has an inhibitory effect on the growth of tumor cells (Kato et al. 1981; Friend et al. 1982). Supplementing diet of DMH-induced colon cancer model of rats with *L. acidophilus* not only suppressed the incidence of carcinogenesis but also increased the latency period. Further it was found that feeding fermented milk to deliver LAB improved the survival rate among the rats (Goldin and Gorbach 1980). Further, intralesional injections of *Bifidobacterium* cells suppressed the growth of tumor cells in mice (Kohwi et al. 1978). Dietary supplementation of freeze-dried *B. longum* cultures resulted in suppression of liver, colon, and mammary tumors, caused due to food mutagens. Oral administration of LAB effectively reduced DNA damage, induced by chemical carcinogens, in gastric and colonic mucosa in rats. Feeding of freeze-dried culture of *B. longum* to rats in which mutagen AOM was used to induce colon cancer resulted in significant reduction of occurrence, multiplicity, and tumor volume (Singh et al. 1997; Rowland et al. 1998).

The incidence of adenocarcinoma in the colon of IL-10 knockout mice treated with probiotic *Lactobacillus salivarius* ssp. *salivarius* was found to reduce

gradually after the treatment (O'Mahony et al. 2001). Lee et al. (2004) reported inhibitory effect of *Lactobacillus acidophilus*, *L. casei*, and *Bifidobacterium longum* on growth and proliferation of chemically induced as well as implanted tumors in rodents**.**

Direct antiproliferative and immunostimulatory effects of cytoplasmic fraction of LABs on tumor cell lines and tumor-bearing Balb/c mice have been observed. Kumar et al. (2012b) reported decrease in tumor growth after supplementation of probiotic *L. plantarum* AS1 in rats.

Human Studies

The efficacy of probiotics vis-à-vis their antiproliferative potential has also been assessed among human subjects, but to a limited extent. The dietary administration of large amounts of dairy products like yogurt and fermented milk containing *Lactobacillus* or *Bifidobacterium* reduced the incidence of colon cancer in volunteers (Shahani and Ayebo 1980).

Biasco et al. (1991) reported that there was a significant decrease in mucosal cell proliferation in upper colonic crypts of patients with colon adenomas after the administration of probiotic cultures of *L. acidophilus* and *B. bifidus*. These bacteria when consumed reduce the mutagenicity of urine and feces caused due to ingestion of carcinogens in cooked meat (Lidbeck et al. 1992). Hayatsu and Hayatsu (1993) also demonstrated a marked inhibitory effect of orally delivered *L. casei* on mutagenic compounds present in urine of human subjects originating from consumption of fried beef.

15.1.3 Mechanism of Antiproliferative Action

The antitumor action of probiotics observed so far may be broadly attributed to (i) binding and degradation of carcinogens, (ii) modulation of intestinal microflora, (iii) modulation of the host's immune system, (iv) alteration of physicochemical conditions in the colon, and (v) induction of apoptosis.

15.1.3.1 Binding and Degradation of Carcinogens

LABs have been shown to bind and degrade carcinogenic compounds in GI tract and may be used to estimate the ratio of bound to free toxins in the colon. Zhang and Ohta (1993) showed that lyophilized cells of LAB, intestinal bacteria, and yeast resulted in significant reduction in the absorption of 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1) from the small intestine in rats, along with a decrease in its blood levels. Probiotic lactobacilli and bifidobacterial strains show a marked ability to metabolically inactivate mutagens under in vitro conditions.

Geier et al. (2006) has reported reduction in levels of carcinogenic compounds such as N-nitroso compounds and heterocyclic aromatic amines after binding to probiotic strains, thus reducing DNA damage. There are claims for anticancer activity of probiotics *Lactobacillus*, *Bifidobacterium*, and *E. coli* Nissle 1917 strains by metabolizing and inactivating mutagenic compounds (Lindner et al. 2010).

15.1.3.2 Modulation of Intestinal Microflora

Probiotic LABs exert a beneficial effect on the intestinal microecology by suppressing the bacteria that convert procarcinogens to carcinogens. Such putrefactive microbes are capable of producing tumor promoters and presumed pre-carcinogens. Oral administration of fermented milk with *L. acidophilus* was proven to reduce significantly the numbers of fecal putrefactive bacteria like coliforms with a parallel rise in numbers of lactobacilli in the intestine. LABs are capable of generating antimicrobial compounds, such as bacteriocins, to regulate the growth of other species. Fermented milk products have the ability to alter the metabolism of the intestinal flora in human subjects (Commane et al. 2005).

Bacterial enzymes have also been known to be involved in the carcinogenic process, by releasing carcinogens in the gut. Some reports suggested that feeding lactic acid bacteria supplements in the food of rodents significantly lowered fecal enzymes activities which lead to postulation of the role of these bacteria as possible anticancer agents. These microbes also decreased the specific activities of fecal enzymes among human volunteers. Nitroreductase, azoreductase, and β-glucuronidase are the microbial enzymes which are found to be associated with carcinogen production in the gut. Studies have reported decreased fecal concentrations of these microbial enzymes after oral consumption of a fermented milk product containing *L. acidophilus* A1, *B. bifidum* B1, *S. lactis*, and *S. cremoris* in human subjects (Marteau et al. 1990; Burns and Rowland 2000; Commane et al. 2005).

15.1.3.3 Modulation of Host's Immune Response

Modulation of immune response of host by probiotic LAB is another explanation for observed antitumor activity. Studies have reported the immunoprotective and antitumor action of LAB on host by promoting specific and nonspecific mechanisms, such as induction of inflammatory cytokines (IL-6, TNF-α) in mouse peritoneal cells (De Simone et al. 1993; Schiffrin et al. 1995). *B. infantis* has been found to stimulate the host-mediated response, resulting in tumor suppression or regression. Regular consumption of immunogenic probiotic strains may prime the immune system against infection. Double-blind clinical trials on human cancer patients fed *L. casei* preparations resulted in inhibition of the recurrence of bladder tumors, indicating anticarcinogenic effects exerted by LABs beyond the local environment of the colon (Commane et al. 2005).

Lee et al. (2008) reported dose-dependent antiproliferative activity of butanol extract of *B. adolescentis* isolates on three human colon cancer cell lines (Caco-2, HT-29, and SW480). The cytotoxicity can be attributed to macrophage activation and increased production of TNF-α and NO induced by *B. adolescentis.* Although the potential mechanisms of probiotic-induced immune suppression of carcinogenesis are complex, it was recently proposed that in response to inflammation, cytokine activated monocytes, and macrophages are produced, which release cytotoxic molecules that cause lysis of tumor cells in vitro. Some probiotics such as *Streptococcus thermophilus* strain TH-4 have the ability to interfere with chronic recurrent inflammation of gastrointestinal tract and can be applied in prevention of colon carcinoma (Oelschlaeger 2010).

15.1.3.4 Alteration of Physicochemical Conditions in the Colon

Probiotic LABs are capable of modifying the environmental conditions of host GI tract affecting the colonization of invading pathogenic microorganisms. Bile acids produced by gut microflora have been reported to exert a cytotoxic effect on the colonic epithelium in colon carcinogenesis, thereby increasing proliferation of cells in the intestine. Studies have reported that large bowel cancer could be affected directly by decreasing intestinal pH, thereby inhibiting the growth of putrefactive bacteria. In a 3-month trial involving patients with colonic adenomas, administered with *L. acidophilus* and *B. bifidum*, a reduction in fecal pH and proliferative activity in the upper colonic crypts was reduced significantly after therapy with LAB (Biasco et al. 1991; Lidbeck et al. 1991).

LABs produce antimicrobial substances such as bacteriocins which result in formation of pores in cell membrane and inhibition of cell wall synthesis in pathogenic bacterial strains (Hassan et al. 2012; Duhan et al. 2013).

15.1.3.5 Induction of Apoptosis

Probiotics have been found to regulate cell signaling pathways affecting cell cycle and cell proliferation in a strain-specific manner. Pro-inflammatory stimuli, such as tumor necrosis factor (TNF), through a series of highly regulated steps including phosphorylation, ubiquitination, and proteolysis, activate NF-kB. The probiotic-secreted factors have been found to inhibit the ubiquitination of IkBα which leads to its degradation by the 26S proteasome, facilitating the nuclear translocation of NF-kB, and sequence-specific identification of target promoters. Commensal bacteria such as *Lactobacillus* spp. affect the regulatory pathways of the intestinal epithelium in mammals via direct modulation of the ubiquitin-proteasome system. Direct contact of a nonpathogenic strain of *Salmonella typhimurium* with intestinal epithelial cells leads to inhibition of IkBa degradation. *Bacteroides thetaiotaomicron*, a commensal bacterium, has been reported to decrease the level of pro-inflammatory cytokine expression due to induction of nuclear export of complexes formed by NF-kB and peroxisome proliferator-activated receptor-g. Another mechanism that contributes to TNF-induced apoptotic signaling pathways involves MAPKs (Iyer et al. 2008).

Probiotic *L. reuteri* promotes apoptosis in human myeloid cells in a contact-independent manner via modulation of NF-kB and MAPK signaling by secreting 6475 factor (Lr-S 6475). TNF treatment induced the expression of antiproliferative (Cox-2 and cyclin D1) and antiapoptotic (Bcl-2 and Bcl-xL) proteins in human myeloid cells. Probiotic factors downregulate NF-kB signaling and expression of related genes in a dose- and time-dependent manner, leading to TNF-induced apoptosis. In TNF-treated KBM-5 cells, administration of *L. reuteri* increased TNF-induced phosphorylation of JNK and p38 proteins along with suppression of ERK1/2 phosphorylation. The latter pathway is involved in regulation of cyclin D1 activity, which is a key molecule affecting cell proliferation. Since NF-kB-mediated inhibition is negatively correlated to JNK activation, JNK promoted TNF-induced apoptosis only in the absence of NF-kB activation. Bacterial cells prolonged JNK activation via suppression of NF-kB activation, thereby potentiating TNF-induced

apoptosis. Furthermore, p38 and JNK signaling pathways have antagonistic effects on ERK signaling. These may act as additional means of regulating apoptosis via enhanced p38 and JNK phosphorylation (Iyer et al. 2008).

Antimicrobial compounds such as bacteriocins are capable of interacting with the cell surface without cell penetration affecting both cell division and DNA synthesis. They are highly precise in their interaction with different types of membranes mainly due to the presence of unique receptors found in different species or strains. Studies have reported inhibition of cancer cell growth by antimicrobial peptides or bacteriocins produced by LAB (Cornut et al. 2008). Kumar et al. (2012a) observed antiproliferative and cytotoxic effect of native and rec-pediocin CP2 against diverse neoplastic cell lines, namely, HepG2 (a hepatocarcinoma cell line), HeLa (a cervical adenocarcinoma), MCF7 (a mammary gland adenocarcinoma), and Sp2/0-Ag14 (a spleen lymphoblast). Results indicated a significantly higher cytotoxicity of rec-pediocin that has the ability to induce apoptosis leading to genomic DNA fragmentation in bacteriocin-exposed cell lines.

15.2 Conclusion

Many lactic acid cultures have been attributed with the antitumor activities. As tumorigenesis involves varied molecular mechanisms, so the mechanisms of antiproliferative activity are also found to display strain-specific variation. The multiple mechanisms effective in curbing tumor formation and progression provide for expansion of potential cancer therapeutics. However, for reaching the clinic, research in larger datasets taking into consideration strain specificity of the effective probiotic or derivatives needs to be carried out. Another point to be kept in mind while formulating studies is the influence of live probiotics on the microbiome of the patient, since no set standard microbiome for cancer-free healthy individuals has been established. In order to identify more probiotic LAB strains, further studies need to be carried out which must be focused toward the mechanisms mediating the anticancer effects. Also establishing the precise mechanisms by which LAB inhibit cancer requires carefully designed epidemiological studies. Instead of availability of limited evidence in support of the use of probiotics for human cancer suppression, these bacteria or derivatives thereof hold promise to be applied as novel drug entities with few or no side effects.

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References

Baricault L, Denariaz G, Houri JJ, Bouley C, Sapin C, Trugnan G (1995) Use of HT-29, a cultured human colon cancer cell line, to study the effect of fermented milks on colon cancer cell growth and differentiation. Carcinogenesis 16:245–252

Biasco G, Paganelli GM, Brandi G, Brillanti S et al (1991) Effect of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* on rectal cell kinetics and fecal pH. Ital J Gastroenterol 23:142

Burns A, Rowland I (2000) Anti-carcinogenicity of probiotics and prebiotics. Curr Issues Intest Microbiol 1:13–24

Chang JH, Shim YY, Cha SK, Chee KM (2010) Probiotic characteristics of lactic acid bacteria isolated from kimchi. J Appl Microbiol 109:220–230

Commane D, Hughes R, Shortt C, Rowland I (2005) The potential mechanisms involved in the anti-carcinogenic action of probiotics. Mutat Res 591:276–289

Cornut G, Fortin C, Denis Soulieres D (2008) Antineoplastic properties of bacteriocins: revisiting potential active agents. Am J Clin Oncol 31:399–404

De Simone C, Vesely R, Bianchi Salvadori B, Jirillo E (1993) The role of probiotics in modulation of the immune system in man and in animals. Int J Immunother 9:23–28

Dimitrovski D, Cenci A, Winkelhausen E, Langerholc T (2014) *Lactobacillus plantarum* extracellular metabolites: in vitro assessment of probiotic effects on normal and cancerogenic human cells. Int Dairy J 39:293–300

Duhan JS, Nehra K, Gahlawat SK et al (2013) Bacteriocins from lactic acid bacteria. Salar RK, Gahlawat SK, Siwach P, Duhan JS Biotechnology: prospects and applications, Springer, New Delhi, 127–142. isbn:978-81-322-1682-7. https://doi.org/10.1007/978-81-322-1683-4

FAO/WHO (2002) Guidelines for the evaluation of probiotics in food. Joint FAO/WHO working group report on drafting guide lines for the evaluation of probiotics in food London, Ontario, Canada

Friend BA, Farmer RE, Shahani KM (1982) Effect of feeding and intraperitoneal implantation of yogurt culture cells on *Ehrlich ascites* tumor. Milch Wiss 37:708–710

Geier MS, Butler RN, Howarth GS (2006) Probiotics, prebiotics and synbiotics, a role in chemoprevention of colorectal cancer. Cancer Biol Ther 5:1265–1269

Goldin BR, Gorbach SL (1980) Effect of *Lactobacillus acidophilus* dietary supplements on 1, 2-dimethylhydrazine dihydrochloride-induced intestinal cancer in rats. J Natl Cancer Inst 64:263–265

Grimoud J, Durand H, de Souza S, Monsan P et al (2010) In vitro screening of probiotics and synbiotics according to anti-inflammatory and anti-proliferative effects. Int J Food Microbiol 144:42–50

Hassan M, Kjos M, Nes IF et al (2012) Natural antimicrobial peptides from bacteria: characteristics and potential applications to fight against antibiotic resistance. J Appl Microbiol 113(4):723–736

Hayatsu H, Hayatsu T (1993) Suppressing effect of *Lactobacillus casei* administration on the urinary mutagenicity arising from ingestion of fried ground beef in the human. Cancer Lett 73:173–179

Hirayama K, Rafter J (2000) The role of probiotic bacteria in cancer prevention. Microbes Infect 2(6):681–686

Iyer C, Kosters A, Sethi G, Kunnumakkara AB et al (2008) Probiotic *Lactobacillus reuteri* promotes TNF-induced apoptosis in human myeloid leukemia-derived cells by modulation of NF-kB and MAPK signaling. Cell Microbiol 10(7):1442–1452

Kahouli I, Malhotra M, Alaoui-Jamali M, Prakash S (2015) In vitro characterization of the anti-cancer activity of the probiotic bacterium *Lactobacillus fermentum* NCIMB 5221 and potential against colorectal cancer. J Cancer Sci Ther 7:224–235

Kailasapathy K, Chin J (2000) Survival and therapeutic potential of probiotic organisms with reference to *Lactobacillus acidophilus* and *Bifidobacterium* spp. Immunol Cell Biol 78:80–88

Kato I, Kobayashi S, Yokokura T, Mutai M (1981) Antitumor activity of *Lactobacillus casei* in mice. Gann 72:517–523

Kohwi Y, Imai K, Tamura Z, Hashimoto Y (1978) Antitumor effect of Bifidobacterium infantis in mice. Gann 69:613–618
Kumar B, Balgir PP, Kaur B et al (2012a) In vitro cytotoxicity of native and rec-pediocin CP2 against cancer cell lines: a comparative study. Pharm Anal Acta 3:8
Kumar RS, Kanmani P, Yuvaraj N, Paari KA et al (2012b) *Lactobacillus plantarum* AS1 isolated from South Indian fermented food Kallappamn suppress 1,2-dimethyl hydrazine (DMH)-induced colorectal cancer in male Wistar rats. Appl Biochem Biotechnol 166:620–631
Lee JW, Shin JG, Kim EH, Kang HE et al (2004) Immunomodulatory and antitumor effects *in vivo* by the cytoplasmic fraction of *Lactobacillus casei* and *Bifidobacterium longum*. J Vet Sci 5(1):41–48
Lee DK, Jang S, Kim MJ et al (2008) Anti-proliferative effects of *Bifidobacterium adolescentis* SPM0212 extract on human colon cancer cell lines. BMC Cancer 8:310
Li W, Ji J, Tang W, Rui X et al (2014) Characterization of an antiproliferative exopolysaccharide (LHEPS-2) from *Lactobacillus helveticus* MB2-1. Carbohydr Polym 105:334–340
Lidbeck A, Geltner-Allinger U, Orrhage KM, Ottava L, Brismar B, Gustafsson JA, Rafter JJ, Nord CE (1991) Impact of *Lactobacillus acidophilus* supplements on the faecal microflora and soluble faecal bile acids in colon cancer patients. Microb Ecol Health Dis 4:81–88
Lidbeck A, Overvik E, Rafter J et al (1992) Effect of *Lactobacillus acidophilus* supplements on mutagen excretion in feces and urine in humans. Microb Ecol Health Dis 5:59–67
Lindner DJD, Pandey A, Thomaz-Soccol V (2010) The potential of probiotics: a review. Food Technol Biotechnol 48:413–434
Marteau P, Pochart P, Flourie B, Pellier P et al (1990) Effect of chronic ingestion of a fermented dairy product containing *Lactobacillus acidophilus* and *Bifidobacterium bifidum* on metabolic activities of the colonic flora in humans. Am J Clin Nutr 52:685–688
O'Mahony L, Feeney M, O'Halloran S, Murphy L et al (2001) Probiotic impact on microbial flora, inflammation and tumour development in IL10 knockout mice. Aliment Pharm Therap 15:1219–1225
Oelschlaeger TA (2010) Mechanisms of probiotic actions-a review. Int J Med Microbiol 300:57–62
Pessi T, SuTas Y, Saxelin M et al (1999) Antiproliferative effects of homogenates derived from five strains of candidate probiotic bacteria. Appl Environ Microbiol 65(11):4725–4728
Rowland IR, Rumney CJ, Coutts JT, Lievense LC (1998) Effect of *Bifidobacterium longum* and *inulin* on gut bacteria metabolism and carcinogen-induced aberrant crypt foci in rats. Carcinogenesis 19:281–285
Sadeghi-Aliabadi H, Mohammadi F, Fazeli H, Mirlohi M (2014) Effects of *Lactobacillus plantarum* A7 with probiotic potential on colon cancer and normal cells proliferation in comparison with a commercial strain. Iran J Basic Med Sci 17:815–819
Schiffrin EJ, Rochat F, Link-Amster H, Aeschlimann JM, Donnet-Hughes A (1995) Immunomodulation of human blood cells following the ingestion of lactic acid bacteria. J Dairy Sci 78:491–496
Shahani KM, Ayebo AD (1980) Role of dietary lactobacilli in gastrointestinal microecology. Am J Clin Nutr 33:2448–2457
Singh J, Rivenson A, Tomita M, Shimamura S, Ishibashi N, Reddy BS (1997) *Bifidobacterium longum*, a lactic acid producing intestinal bacterium inhibits colon cancer and modulates the intermediate biomarkers of colon carcinogenesis. Carcinogenesis 18:833–841
Sun J, Shi YH, Le GW, Ma XY (2005) Distinct immune response induced by peptidoglycan derived from *Lactobacillus* sp. World J Gastroenterol 11:6330–6337
Tiptiri-Kourpeti A, Spyridopoulou K, Santarmaki V et al (2016) *Lactobacillus casei* exerts antiproliferative effects accompanied by apoptotic cell death and up-regulation of TRAIL in colon carcinoma cells. PLoS One 11(2):e0147960
Wang K, Li W, Rui X, Chen X et al (2014) Characterization of a novel exopolysaccharide with antitumor activity from *Lactobacillus plantarum* 70810. Int J Biol Macromol 63:133–139
Zhang XB, Ohta Y (1993) Microorganisms in the gastrointestinal tract of the rat prevent absorption of the mutagen carcinogen 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole. Can J Microbiol 39:841–845

16 Technological and Functional Aspects of Yoghurt Cheese

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16.1 Introduction and Scenario of Cheese in India

During the post-independence period, India is witnessing an impressive progress in dairy sector with a growth rate of 10–15% per annum and is anticipated to thrice of its annual production in subsequent 10 years. Moreover, India is producing milk at the lowest cost of 27 cents per litre in comparison with developed countries such as the USA, i.e. 63 cents, and Japan, i.e. 2.8US dollars, respectively. Owing to the fact of increasing demand of milk and milk-based products, investors from multinational companies are always directing Indian dairy sector and market at large extent (Kaushik et al. 2014). Dairy products are integral part of human diet with their health-promoting properties. It is a well-known fact that milk consists of numerous macro- and micronutrients such as casein, whey proteins, calcium, phosphorus, tremendous amount of fat-soluble and vitamin B-complex, magnesium and zinc, respectively (Holland and Coolbear 1996; Kaushik et al. 2015). Converting milk into cheese may preserve surplus milk for a longer period which varies from few days to few years. Milk fat and proteins are significant elements of cheese, and they could be preserved by two approaches, i.e. lactic acid fermentation and decline in water activity (Fox and McSweeney 2004). Even though the significant objective of the manufacturing of cheese is to preserve the foremost elements of milk and amongst milk products, cheese is credited with great importance for its nutrition,

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health benefits and pleasure-giving qualities (Indumathi et al. 2015). The world's production of natural cheese is estimated around 23 million tonnes (IDF 2013; FAO 2013) in which 70–80% of cheese is produced in European and US countries. The cheese market in India is growing with an average CAGR of 17% during 2013–2014 being dominated by Gujarat Cooperative Milk Marketing Federation (60%) under the brand name of Amul followed by Britannia (25%) and the rest by other imported (like Kraft) and regional brands such as Mother Dairy and Vijaya (Jayadevan 2013). At present, more than 900 varieties of cheeses are available in the world market. The quality of final cheese mainly depends on the factors like type, quality and composition of milk, hygienity of surroundings, quality of culture, extent of acid production, extent of moisture retention, curd handling and ripening conditions of cheese (Lucey et al. 2003). One of the most communal norms for the classification of cheese is texture (very hard, hard, semihard, semisoft, soft) which is directly correlated primarily with the moisture content of the cheese (Kaushik et al. 2015).

India is recognized as the world's top producer of buffalo milk followed by Pakistan, China and Italy. Buffalo is the backbone of Indian dairy industry contributing more than 53% of the country's total milk production. Buffalo milk contains comparatively more fat, SNF and TS, and hence yield of buffalo milk Cheddar, Swiss, cottage, mozzarella and many other cheeses is higher. Higher total solids in buffalo milk also provide more calories per unit weight (~100 calories per 100 g for buffalo milk and ~70 calories per 100 g for cow milk). Cheese made from buffalo milk displays typical body and textural characteristics and technologically preferable over cow milk.

Although the hulk of Indian dairy industries such as Amul, Britannia, Nestle, Mother Dairy and Dairy Best, respectively, are currently volunteering into the yoghurt formulation, in India, only 7% of total milk production is utilized for three major fermented products like dahi, i.e. curd; shrikhand, i.e. concentrated curd; and lassi, i.e. stirred curd, which could be deliberated as western dairy products such as yoghurt, quarg and stirred yoghurt, respectively (Aneja et al. 2002). The current market trend demands development of new varieties of dairy products, with incorporation of functional ingredient that keeps well for longer (Gupta et al. 2015). It is well known that despite of acidity of yoghurt, it is more prone to microbial spoilage due to its 85% moisture content. To overcome this problem, attempts are therefore made to prolong its keeping quality through elimination of whey. Moreover, during traditional method yoghurt is strained out through a distinct cloth sack to obtain the desired total solids level. Hence, new techniques such as ultrafiltration and centrifugation have recently been used to produce concentrated form of the yoghurt which is also known as yoghurt cheese (Tamime et al. 1991a). In this context, similar type of several products are produced worldwide such as labneh or lebneh (Middle East), leben zeer (Egypt), skyr (Iceland), chakka and shirkland (India), than or tan (Armenia) and ymer (Denmark), respectively (Tamime et al. 1991b). Yoghurt cheese is always categorized by its pasty and smooth, creamier and thicker texture with a semiviscous mass (Mustafa 1978; Rosenthal 1999; Tamime and Crawford 1984). There is no legal standard to define a minimum protein content or distinct manufacturing process for it. Yoghurt cheese, being fresh curd style cheese, its pleasant mild

flavour will suit the palate of Indian consumers. Fat is one of the major components of cheese that plays an important role in a textural properties and flavour (Tamime 1993). However, with the changing lifestyle, diseases like obesity, diabetes and CVD have become major health issue. Due to this reason, USDA recommended that the energy obtained from fat intake should be less than 30% of the total energy intake. Hence, new products have emerged that allow consumers to limit their fat intake within recommended levels. Yoghurt cheese, being fresh curd style cheese, its pleasant mild flavour and smooth texture will suit the palate of Indian consumers and makes it excellent as a frosting or filling for a diversity of dishes. Yoghurt cheese can often be deputized for sour cream, cream cheese, cottage cheese or ricotta cheese. Furthermore, the most special property is that it can be easily blended with other functional ingredients such as dietary fibres and probiotics.

16.2 Preparation and Technological Aspects of Yoghurt Cheese

To meet the consumer acceptability, various technological aspects and methods of preparation of yoghurt cheese were used by different researchers. Traditionally yoghurt cheese is prepared by straining the set yoghurt in a cloth bag. However, nowadays there are other manufacturing processes involved for the preparation of yoghurt cheese in large volume and at industrial scale. Several authors reviewed widely used preparation methods and technological aspects of yoghurt cheese, and they classified different processes and methods, i.e. traditional method (cloth bag), mechanical separations, membrane processes and method based upon direct recombination. Numerous types of acidic milk can be produced by the lactic acid fermentation, and yoghurt is one of the highly nutritious protein-rich, acidic fermented milk products produced by thermophilic lactic acid bacteria (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*). Several countries are producing huge amount of yoghurt every year, and 15–20% total share of the production of yoghurt worldwide is exhibited only by European countries (EDA 2016). In recent years, there has been an increasing interest on the functionality of these products. According to the report of GAIN (GAIN 2016) in India, only 9–10% milk is used for the purpose of fermented milk product production which is very low as compared to other countries. Some major dairy industries are producing chakka or shrikhand at few extent. The short shelf life of yoghurt, i.e. 1 day under ambient condition (25–30 °C) and around 5 days at 7 °C (Salji et al. 1987; Tirloni et al. 2015), hinders its commercialization. To improve the shelf life of yoghurt, draining of whey is one of the most important factors which give a milky white colour, soft, smooth and spreadable product with a consistency that resembles cultured cream. This yoghurt is known as "yoghurt cheese" or "salted yoghurt". Although there is lack of scientific literature for standards for yoghurt cheese, ideally, it should have smooth and pasty texture with semisolid mass.

Concentrated yoghurt or yoghurt cheese is a type of fresh cheese made by concentration of yoghurt to specific level of total solids. Owing the fact that there is no

legal standard to define a minimum nutritional value or distinct manufacturing process for it, yoghurt cheese exhibited significantly higher nutritional composition as compared to plain yoghurt in terms of protein (i.e. 2.5 times) and minerals (i.e.1.5 times) contents, very low lactose content and fat content. However, yoghurt cheese typically has total solid levels between 23 and 25 g/100 g, of which 8–11 g/100 g is fat and protein contents range from 6% to 12% or about 1.5–4 times that of traditional yoghurt with titratable acidity ranging from 1.8% to 2.0% on the basis of lactic acid, respectively (Rasic and Rasic 1987; Nsabimana et al. 2005). Due to tremendous nutritional aspects of yoghurt cheese, people are taking more interest, and several yoghurt cheese-based products are now produced (Nsabimana et al. 2005). Tamime and Robinson (1978, 1988); Abou-Donia et al. (1992); Al-Kadamany et al. 2002) manufactured yoghurt cheese from cow's milk, and they studied physicochemical attributes of the yoghurt cheese. Also, they revealed nutritional characteristics of the yoghurt cheese. According to them it is also produced at commercial scale by large dairy plants in the Mediterranean and the Middle East countries, and people are consuming this as healthy dairy-based product. For the manufacturing of the yoghurt cheese, several methods are used according to the requirement. All methods exhibit different nutritional aspects to the end product because of the removal process of whey from the yoghurt. In this context the traditional yoghurt cheese is produced by filtration of coagulate on linen cloth. Because of many disadvantages of traditional method such as long production time, contamination, large floor space, labour intensive, low yield and reduction in the nutritive value of product, many attempts have been done to replace the traditional method with new industrial methods including centrifugation and ultrafiltration and reverse osmosis membranes. These processes are simple and require minimal investment in equipment. Furthermore, fat losses during whey separation could also be minimized while manufacturing chakka or shrikhand using homogenizer for milk before fermentation (Desai et al. 1985).

Various authors studied compositional, rheological and organoleptic properties, effect of heat treatments of yoghurt cheese prepared from milk obtained from different animals using various preparation methods. Desouky et al. (2013) prepared yoghurt cheese from camel milk and studied various physicochemical attributes and effect of heat treatment on compositional, rheological and organoleptic properties. Surface characteristics and properties of the yoghurt cheese were revealed by scanning electron microscopy, and it was observed that the protein structures of the high thermally treated labneh samples seemed to be comparatively more intensive than that of control yoghurt. It was clearly displayed that the casein structures of the labneh samples were principally allied with one particle to another particle in the form of protein chains with moderately small interspaced voids instead of particle merging into large aggregates. Whereas, control samples demonstrated weak, open chains, slack and reduced protein network as compared to experimental treatments. Mehaia (2005) prepared fresh labneh from goat's milk using ultrafiltration. During physicochemical characterization he observed the lower protein, fat, ash, pH and total solid content in labneh prepared with UF process in comparison with traditionally prepared labneh. However, an increase in yield and recovery of protein, fat and

total solid was observed in labneh prepared by UF process. Similarly, Shamsia and El-Ghannam (2012) also prepared yoghurt cheese, i.e. labneh from cow's milk by attained retentate after ultrafiltration process with or without addition of permeate concentrate and prepared labneh was exhibited for the physicochemical attributes in comparison with traditionally prepared labneh. From physicochemical parameters synergistic effects of addition of permeate concentrate on coagulation and setting time of the yoghurt was observed. Moreover, ultrafiltration significantly improved the concentration of both organic and inorganic matters such as total solids, proteins, soluble proteins, fat, ash, acidity and pH in comparison with traditionally prepared labneh.

16.3 Functional Aspects of Yoghurt Cheese

Functional characteristics of the food product exhibit significant prominence in terms of improved nutritional status and health benefits. People are gaining more emphasis on the healthy and nutritive food products as they are showing more concern about their health and increased productivity (Chawla et al. 2017a, b; Bhandari et al. 2016). Therefore, with increased consumer awareness about the health benefits of consuming diets low in fat, reduced-fat yoghurt cheese products have emerged on the market (Desouky et al. 2013). Owing to the fact that yoghurt has numerous health beneficial features, these features are directly associated with the physicochemical and biochemical variations occurring during lactic acid fermentation process. Hence, several therapeutic aspects such as lactose intolerance (Adolfsson et al. 2004), antiobesity (Wang et al. 2013), effect on diarrhoeal diseases (Biller et al. 1995), immunity booster (El-Abbadi et al. 2014), effect on colon cancer (Wollowski et al. 1999) and effect on allergic reactions (Adolfsson et al. 2004) attributed the upsurge to consumer's preference for the product's higher-protein content as well as the associated thicker and creamier texture. Moreover, yoghurt cheese is considered appropriate for lactose intolerant individuals than simple yoghurt because of low lactose content which are lost in drained water, whereas the fat content can be diversified according to consumer requirement (Salji et al. 1987). Beside the health benefits of yoghurt cheese, it is an excellent vehicle for the delivery of various functional and health-promoting ingredients and compounds. Various author used yoghurt cheese as a delivery vehicle which increased its functionality. Salem et al. (2013) improved the alimentary and biological aspects of yoghurt cheese by adding dry leaves of *Moringa oleifera* (DLMO). After addition of DLMO, significant improvement was observed in terms of physicochemical characteristics of yoghurt cheese in comparison with control yoghurt cheese. They also revealed that yoghurt cheese enriched with DLMO could be considered as a good source of vital elements such as calcium, iron, zinc and both fat and water soluble vitamins, respectively. Furthermore, yoghurt cheese enriched with DLMO also attributed high quality and digestibility of the protein in terms of high biological value (BV), true protein digestibility (TD) and net protein utilization (NPU), respectively. Seckin and Ozkikine (2011) studied physicochemical, sensory attributes and textural

characteristics of yoghurt cheese prepared from milks which were enriched with some prebiotics such as inulin and oligofructose. They revealed that addition of these prebiotics did not affect the physicochemical characteristics of concentrated yoghurts; however, textural properties showed significant difference. They concluded that prebiotic yoghurt production will play a significant role in nutrition of public and development of dairy industry. In this perspective, Kebary et al. (2007) prepared low-fat labneh containing inulin and bifidobacteria to improve the functional and nutritional value of concentrated yoghurts. Salem et al. (2007) used inulin as symbiotic for the production of functional low-fat labneh. They unveiled that addition of inulin significantly increased the soluble protein content, volatile fatty acids, acetaldehyde and diacetyl content, respectively. Moreover, they also revealed that soluble content of tyrosine and tryptophan was more prominent after the addition inulin. Atamian et al. (2014) characterized the textural characteristics and sensory attributes of full-fat, reduced-fat and low-fat bovine, caprine and ovine Greek yoghurt and revealed the physicochemical properties of the respective samples of the yoghurt cheese. Otaibi and El-Demerdash (2008) improved the keeping quality and shelf life of yoghurt cheese by the enrichment of thyme, marjoram and sage in varied concentrations. Subsequently, improved physicochemical and functional attributes were observed by the addition of these essential oils in concentrated yoghurt cheese. Furthermore, antimicrobial property was also attributed by the yoghurt cheese due to addition of essential oils. Ibrahim et al. (2013) studied the effect of chufa tubers (*Cyperus esculentus*) in Zaraibi goat's diet of the resultant milk and labneh. Higher nonprotein nitrogen content was observed in labneh of G1 (control) and G2 (5% chufa tubers). Total volatile fatty acids were increased with increasing supplementation rate of chufa tubers from 5% (G2) to 15% (G4). The highest score in the assessment of organoleptic characteristics of labneh was for G3 (10% Gufa), followed by G4 (15% chufa tubers) and then G2 (5% chufa tubers). Storage of the food product always led to physicochemical and biological changes in the food. Effect of storage time on the rheology of the yoghurt cheese was evaluated by Mohameed et al. (2004) using a rotational viscometer. Apparent viscosity of the yoghurt cheese sample was measured with increasing shear rate and shear temperature. High shear rate was applied to yoghurt cheese samples to evaluate the flow behaviour of the completely destructed yoghurt cheese sample. During characterization of flow behaviour of fresh yoghurt cheese, significant increase in the apparent viscosity was observed with increasing storage time which further resulted into the development of the gel-like structure. Also, shear thinning was found during flow behaviour evaluation of fresh yoghurt cheese, while thixotropic flow behaviour was observed during storage of the samples. Power-law model was much appropriate for the shear thinning, and the effect of storage time on the power-law characteristics was evaluated. Structural kinetic model was used for the examination of thixotropic behaviour of yoghurt cheese, whereas non-significant difference was observed in flow properties of the completely destructed yoghurt cheese during power-law model experiment.

El-Sanhoty et al. (2009) manufactured low-fat labneh by traditional method containing β-glucan as fat replacer, and yoghurt cheese was evaluated for its functional,

microbiological and sensory properties. They clearly revealed that addition of barley βglucan in yoghurt cheese significantly improved the probiotic sustainability with storage time as compared to the control counterparts of the yoghurt cheese. Proximate composition such as total protein and ash content significantly increase with decrease in fat content of the yoghurt cheese, respectively. Furthermore, barley βglucan-enriched yoghurt cheese showed lower pH values during storage time as compared to the control samples. Also, enrichment with barley βglucan to yoghurt cheese with addition of probiotic bacteria could act as raw material for the production of polyfunctional nutrition.

Ahmed et al. (2013) prepared olive oil-coated labneh mixed with various herb. They analysed the effects of four culinary herbs (i.e. mint, poppy seeds, cinnamon and basil) on physicochemical properties and shelf life of labneh cheese prepared from whole buffalo milk. Significant difference in physicochemical attributes of yoghurt cheese was observed by addition of herbs. Moreover, calcium content was decreased, whereas phosphorus content of labneh cheese was increased due to addition of herbs. Firmness of labneh cheese samples decreased significantly during storage may be due to penetration of oil into the product. Culinary herbs and storage time influenced the organoleptic scores as well. Results indicated that culinary herbs significantly affected the overall quality of labneh cheese during storage, and covering with olive oil after further concentration up to 40% total solids improved its keeping quality by preserving the nutrients.

16.4 Conclusion

From above discussion of technological and functional aspects of yoghurt cheese, it can be concluded that yoghurt cheese is a type of fresh cheese made by concentration of yoghurt to specific level of total solids. Owing to the fact that yoghurt has numerous health beneficial features, these features are directly associated with the physicochemical and biochemical variations occurring during lactic acid fermentation process. Yoghurt cheese can also attribute several therapeutic aspects such as lactose intolerance, antiobesity, effect on diarrhoeal diseases, immunity booster, effect on colon cancer and effect on allergic reactions, respectively. Also, yoghurt cheese is a product with higher-protein content, and the associated thicker and creamier texture could be an excellent vehicle for the delivery of various functional and health-promoting ingredients and compounds.

References

Abou-Donia SA, Attia IA, Khattab AA, El-Khadragy SM (1992) Characteristics of labneh manufactured using different lactic starter cultures. Egyptian J Food Sci 20:1–12

Adolfsson O, Meydani SN, Russell RM (2004) Yoghurt and gut function. Am J Clin Nutr 80:254–256

Ahmed S, Umar H, Anjum FM, Murtaza MA (2013) Quality evaluation of olive oil coated labneh cheese mixed with culinary herbs. Buffalo Bull 32(2):1316–1323

Aneja RP, Mathur BN, Chandan RC Banerjee AK (2002) Technology of Indian milk products: handbook on process technology modernization for professionals, entrepreneurs and scientists. Dairy India Year book 2002

Atamian S, Olabi A, Baghdadi OK, Toufeili I (2014) The characterization of the physicochemical and sensory properties of full-fat, reduced-fat and low-fat bovine, caprine, and ovine Greek yogurt (Labneh). Food Sci Nutr 2(2):164–173

Bhandari L, Sodhi NS, Chawla P (2016) Effect of acidified methanol modification on physico chemical properties of black-eyed pea (Vigna unguiculata) starch. Int J Food Prop 19(12):2635–2648

Biller JA, Katz AJ, Flores AF, Buie TM, Gorbach SL (1995) Treatment of recurrent Clostridium difficile colitis with Lactobacillus GG. J Paediatric Gastroenterol Nutr 21:224–226

Chawla P, Bhandari L, Sadh PK, Kaushik R (2017a) Impact of solid-state fermentation (aspergillus oryzae) on functional properties and mineral bioavailability of black-eyed pea (vigna unguiculata) seed flour. Cereal Chem 94(3):437–442

Chawla P, Bhandari L, Dhull SB, Sadh PK, Sandhu SP, Kaushik R (2017b) Biotechnological aspects for enhancement of mineral bioavailability from cereals and legumes. In: Plant biotechnology: recent advancements and developments. Springer Nature, Singapore, pp 87–100

Desai HK, Vyas SH, Upadhayay KG (1985) Influence of homogenization of milk on the quality of chakka and Shrikhand. Indian J Dairy Sci 38(2):102–106

Desouky MM, Shalaby SM, Soryal KA (2013) Compositional, rheological and organoleptic qualities of camel milk labneh as affected by some milk heat treatments. World J Dairy Food Sci 8(2):118–130

EDA (2016) Yoghurt market in Europe - Important statistics

El-Abbadi NH, Dao MC, Meydani SN (2014) Yogurt: role in healthy and active aging. Am J Clin Nutr 99(1):1263S–1270S

El-Sanhoty R, Zaghlol A, Hassanein AH (2009) The manufacture of low fat labneh containing barley β-glucan-chemical composition, microbiological evaluation and sensory properties. Current Res Dairy Sci 1:1–12

FAO (2013) Statistical yearbook 2013: world food and agriculture. FAO Food Agricultural Organization, UN, Rome

Fox PF, McSweeney PLH (2004) Cheese: an overview. In: Cheese: chemistry physics microbiology. 1:1–18

GAIN (2016) Dairy and products annual. GAIN report number IN6126

Gupta C, Chawla P, Arora S, Tomar SK, Singh AK (2015) Iron microencapsulation with blend of gum arabic, maltodextrin and modified starch using modified solvent evaporation method–milk fortification. Food Hydrocoll 43:622–628

Holland R, Coolbear T (1996) Purification of tributyrin esterase from *Lactococcus lactis* subsp. *cremoris* E8. J Dairy Res 63(01):131–140

Ibrahim FA, Ayad KMK, Ahmed ME, El-Kholeny ME (2013) Effect of using chufa tubers (*Cyprus esculentus* L.) in Zaraibi goats diets on the resultant milk and labneh. Egypt J Sheep Goat Sci 8(1):201–211

IDF Bulletin (2013) The world dairy situation 2013. Bulletin No. 470/2013. Int Dairy Federation, Brussels

Indumathi KP, Kaushik R, Arora S, Wadhwa BK (2015) Evaluation of iron fortified gouda cheese for sensory and physicochemical attributes. J Food Sci Technol 52(1):493–499

Jayadevan GR (2013) A strategic analysis of cheese and cheese products market in India. Indian J Dent Res 2(3):247–250

Kadamany E, Toufeili I, Khattar M, Abou-Jawdeh Y, Harakeh S, Haddad T (2002) Determination of shelf life of concentrated yogurt (labneh) produced by in-bag straining of set yogurt using hazard analysis. J Dairy Sci 85(5):1023–1030

Kaushik R, Sachdeva B, Arora S (2014) Vitamin D_2 stability in fortified milk during processing, packaging and storage. LWT-Food Sci Tech 56:421–426

Kaushik R, Sachdeva B, Arora S, Gupta C (2015) Effect of fat content on sensory and physicochemical properties of calcium and vitamin D fortified milk. Int J Dairy Technol 68(1):135–143

Kebary KMK, Hussein SA, Badawi RM, Badran II (2007) Incorporating *Bifidobacteria* in low fat labneh. In: 10th Egyptian Conf Dairy Science and Technology, pp 359–375
Lucey JA, Johnson ME, Horne DS (2003) Invited review: perspectives on the basis of the rheology and texture properties of cheese. J Dairy Sci 86(9):2725–2743
Mehaia MA (2005) Manufacture of fresh labneh from goat's milk using ultrafiltration process. J Food Technol 3(1):24–29
Mohameed HA, Abu-Jdayil B, Al-Shawabkeh A (2004) Effect of solids concentration on the rheology of labneh (concentrated yogurt) produced from sheep milk. J Food Eng 61(3):347–352
Mustafa SMK (1978) Microbiological studies on labneh, a fermented dairy product in Jordan [lactic acid bacteria; yeasts; spoilage]
Nsabimana C, Jiang BO, Kossah R (2005) Manufacturing, properties and shelf life of labneh: a review. Int J Dairy Technol 58(3):129–137
Otaibi MA, El-Demerdash H (2008) Improvement of the quality and shelf life of concentrated yoghurt (labneh) by the addition of some essential oils. Afr J Microbiol Res 2:156–161
Rasic JL, Rasic JL (1987) Yogurt and yogurt cheese manufacture. Cult Dairy Prod J 22:6–8
Rosenthal AJ (1999) Relation between instrumental and sensory measures of food texture. In: Food texture: measurement and perception. Aspen Publishers, Gaithersburg, pp 1–17
Salem MME, Abd El-Gawal AM, Hassa FAM, Effat BA (2007) Use of symbiotic for production of functional low fat labneh. Polish J Food Nutr Sci 57(2):151–159
Salem AS, Salama WM, Hassanein AM, El-Ghandour HMA (2013) Enhancement of nutritional and biological values of labneh by adding dry leaves of *Moringaoleifera* as innovative dairy products. World Appl Sci J 22(11):1594–1602
Salji JP, Saadi SR, Mashhadi A (1987) Shelf life of plain liquid yogurt manufactured in Saudi Arabia. J Food Prot 50(2):123–126
Seckin AK, Ozkikine AY (2011) Effect of some prebiotics usage on quality properties of concentrated yoghurt. J Anim Vet Adv 10(9):1117–1123
Shamsia SM, El-Ghannam MS (2012) Manufacture of labneh from cow's milk using ultrafiltration retentate with or without addition of permeates concentrate. J Anim Prod Adv 2(3):166–173
Tamime AY (1993) Modern cheesemaking: hard cheeses. In: Modern dairy technology. Springer, New York, pp 49–220
Tamime AY, Crawford RJM (1984) The microbiological quality of yoghurt cheese known in Lebanon as labneh anbaris after one year storage at 20 °C. Egypt J Dairy Sci 12:299–312
Tamime AY, Robinson RK (1978) Some aspects of the production of a concentrated yoghurt (labneh) popular in the Middle East. Milchwissenschaft 33:209–212
Tamime AY, Robinson RK (1988) Fermented milks and their future trends. Part II. Technological aspects. J Dairy Res 55(02):281–307
Tamime AY, Robinson KR (1999a) Processing plants and equipments. In: Background to manufacturing practice, yoghurt: science and technology. Pergamon Press, New York, pp 128–152
Tamime AY, Robinson RK (1999b) Yoghurt: science and technology. Wood head Publishing, Cambridge
Tamime AY Davies G, Chehade AS, Mahdi HA (1991a) The effect of processing temperatures on the quality of labneh made by ultrafiltration. Int J Dairy Technol 44(4):99–103
Tamime AY, Kalab M, Davies G (1991b) The effect of processing temperatures on the microstructure and firmness of Labneh made from cow's milk by the traditional method or by ultrafiltration. Food Struct 10(4):8
Tirloni E, Bernardi C, Colombo F, Stella S (2015) Microbiological shelf life at different temperatures and fate of Listeria monocytogenes and Escherichia coli inoculated in unflavored and strawberry yogurts. J Dairy Sci 98(7):4318–4327
Wang H, Livingston KA, Fox CS, Meigs JB, Jacques PF (2013) Yoghurt consumption is associated with better diet quality and metabolic profile in American men and women. Nutr Res 33:18–26
Wollowski I, Ji S, Bakalinsky AT, Neudecker C, Pool-Zobel BL (1999) Bacteria used for the production of yogurt inactivate carcinogens and prevent DNA damage in the colon of rats. J Nutr 129:77–82

Phytase: The Feed Enzyme, an Overview

17

Namita Singh, Sonia Kuhar, Kanu Priya, Rajneesh Jaryal, and Rakesh Yadav

17.1 Introduction

Mankind uses enzyme since ancient times. The production of cheese, wine and vinegar, brewing of beer and leavening of bread all include enzymatic processes of prehistoric origin. Enzymes have an advantage that the process in which they are used not only becomes highly specific and fast but also eco-friendly. Enzymes replace chemical reactions which control environmental degradation. With the advancement in the field of biotechnology, new applications of industrial enzymes are emerging, and it is expected they will rule catalysing processes in factories and homes in nearby future. Phytic acid, phytin or phytate have been the substrates for phytase enzymes and discovered in 1903 (Mullaney et al. 2007). Phytate term is the widely used for mixed salt of phytic acid (*myo*-inositol hexaphosphate; IP_6). While phytic acid is the free form of IP_6, deposited complex of IP_6 with magnesium, potassium and calcium is known as phytin that exists in plants.

Phytic acid, also known as myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate, is an organic form of phosphorus and chief component of plant-origin food. In plant seeds that are consumed as animal feed element (such as cereal grains, oilseed meals and legumes), it signifies primary source of inositol and the most important storage form of phosphorus (Bedford 2000; Ravindran et al. 1995). Phytic acid is a significant constituent of plant-derived foods which put in 1–5% by weight of edible cereals, legumes, pollens, oil seeds and nuts. The molecular formula of phytic acid as $C_6H_{18}O_{24}P_6$ with molecular weight of 659.86 was given by Posternak in 1965. Generally, 50–80% of total phosphorus of plant-based food exists as phytate (Harland and Morris 1995). Monogastric animals such as swine and poultry carry little or no phytase in their elementary canal; therefore, phytic acid phosphorus confers anti-nutritive values because of its low metabolizing rate

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(Reddy and Pierson 1994). The solubility and digestibility of starch is affected due to binding of phytic acid with starch molecules (Desphande and Cheryan 1984). Phytic acid present in animal excreta is hydrolysed by microorganisms. The phosphorus released out of microbial enzymatic action is eventually pervaded into river and lake water and may cause eutrophication if accumulated in water bodies (Kuhar et al. 2009) (Fig. 17.1).

When one or more acidic hydrogens of the phosphate groups in phytic acid are substituted by a counter ion, the compound is usually known as phytate salt. Based on the pH and divalent metal cations, phytate salt either exists as a metal-phytate complex or a metal-free phytate. The binding magnitude relies on both pH and divalent metal cations to phytate ratios. In addition, at acidic pH and high cation concentration, a metal-phytate complex is formed due to direct electrostatic interaction. According to ionic radii of the metal cations, they explicitly bind to the phosphate groups of phytate. Preferably, metal cations having large ionic radii give rise to the bidentate metal complex (Oh et al. 2004) (Fig. 17.2).

The phytin was earlier considered as a storage product. It was speculated that mostly phosphorus was stored in the seed which was released to be incorporated into ATP on germination. Fifty to eighty percent of total stored phosphate stored in plant seeds exists as phytic acid (Kumar et al. 2012). The role of inositol phosphate is known to mediate the inward transportation of materials of the cell. Berridge and Irvine (1984) reported their function in signal transduction and in cellular transport as secondary messengers. Phytic acid is, in its less complexed state, highly reactive and easily forms complexes with Zn^{2+}, Ca^{2+}, Mg^{2+} and Fe^{2+}, carbohydrates and proteins (Harland and Oberleas 1977). The complexes are sparingly soluble in the small intestine and, hence, least interactive with phytase (Angel et al. 2002). Researchers thought to devise a technique to amputate phytic acid in a way that is economically reasonable with mineral supplementation by considering its values as a phosphorus source and anti-nutritive properties. Catalysing phytic acid is assumed as an effective way of enhancing the nutritional merit of several plant foodstuffs. Bioavailability of the phytate phosphorus was improved by supplementing the diet with phytase enzyme (Ali et al. 2010).

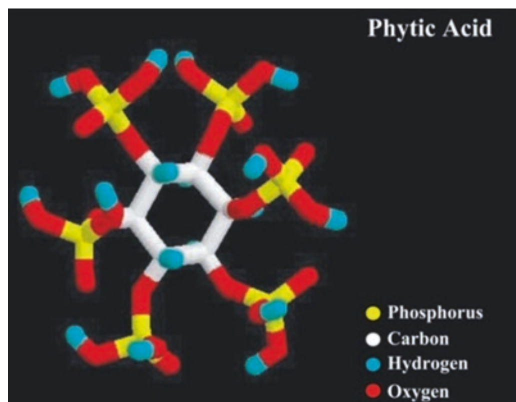

Fig. 17.1 Structure of phytic acid. (Source: W. Schmidt – USDA/ARS)

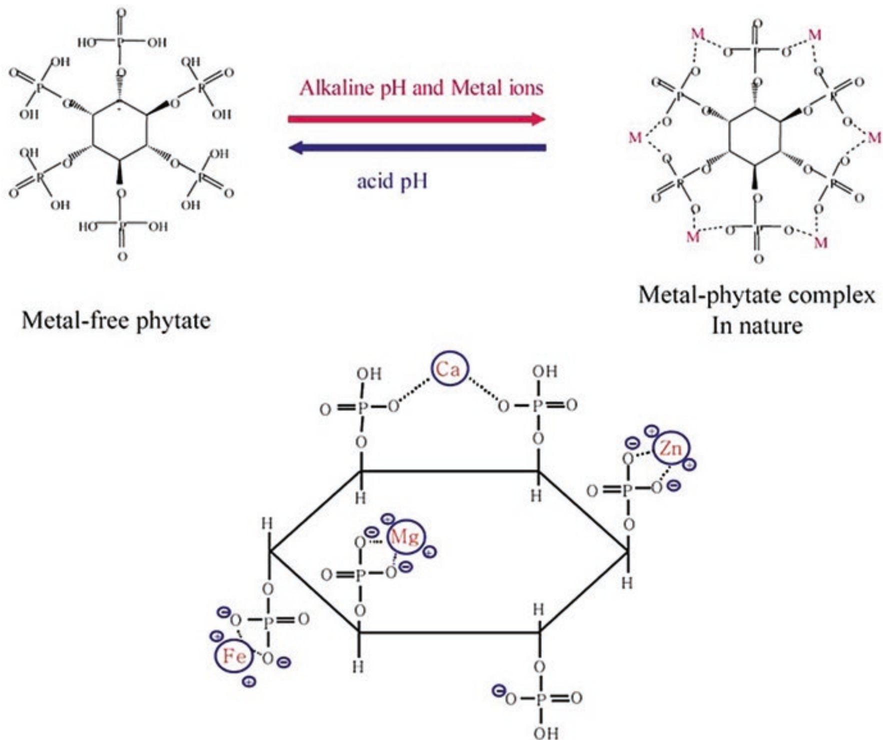

Fig. 17.2 Divalent metal cation complexes. (Source: Oh et al. 2004)

17.2 Phytates in Food Ingredients

Phytates represent 60–90% of the total phosphate and appear during maturation of the seed and its dormancy (Loewus 2002). Hence, phytate is a common component of plant-derived food. The daily intake of phytate may be up to 4500 mg based on the quantity of plant-derived foods in the diet and quality of food processing (Reddy 2002). Normal daily intake of phytate is likely to be 2000–2600 mg in case of herbivores and for the diets of rural people in developing countries and around 150–1400 mg for omnivores (Reddy 2002).

17.2.1 Phytate as an Antinutrient

Research infers that in monogastric animal feed, phytate or phytic acid has a high anti-nutrition effect since monogastric animals are deficient in phytate-degrading enzyme and so unable to utilize the phosphorus part in phytate (Selle and Ravindran 2008). Phytate act in a wide pH range because of having negatively charged ion and a high affinity for food components of cationic nature such as

minerals, proteins and trace elements (Konietzny and Greiner 2003). This association does not only have dietary implications but also affect food ingredients quantitatively as well as qualitatively such as corn steep liquor, starch or plant protein isolates (Desphande and Cheryan 1984; Fredrikson et al. 2001; Kvist et al. 2005). Phytic acid mainly exists as salts of mono- and divalent cations (e.g. calcium-magnesium-potassium salt in soybeans and potassium-magnesium salt in rice) in distinct parts of legumes and cereal grains. Besides other storage matter such as lipids and starch, it accumulates in grains and seeds during maturation. In case of legumes and cereals, it is deposited in the globoid crystals and aleurone particles, respectively (Reddy et al. 1982; Tyagi and Verma 1998). Moreover, non-occurrence of endogenous phytate-degrading enzymes and the limited microflora in the upper part of the digestive tract in human small intestine, only a small fraction of phytate is hydrolysed (Iqbal et al. 1994; Boling et al. 2000). With the decrease in phosphate residues on the myo-inositol ring, the stability and solubility of myo-inositol phosphate-mineral complexes also drop down. Thus, elimination of phosphate residues from phytate leads to lowered uptake of essential dietary minerals in the intestine (Sandberg et al. 1999). The anti-nutritional impact of phytic acid in diets for poultry and pigs was reported by Woyengo and Nyachoti (2013). It reduces mineral digestibility in broilers and pigs.

17.2.2 Phytate-Rich Diets and Health Benefits

Phytate consumption is likely to have positive effect on human health. It was known to slash kidney stone formation (Grases et al. 2001) and to offer defence against coronary heart disease and atherosclerosis (Jariwalla et al. 1990) along with wide type of cancers (Vucenik and Shamsuddin 2003). Dietary deprivation of phytate in the human regulates the levels of phytate and its dephosphorylation products in plasma, urine and other biological fluids (Grases et al. 2001).Therefore, low-phytate diet in developed countries is likely to be responsible for rise in disease incidences in Western societies such as renal lithiasis, diabetes mellitus, coronary heart diseases and atherosclerosis in comparison with the developing countries (Greiner and Konietzny 2006). Phytic acid has been used in diet treatments of rectal and colon cancers (Admassu 2009). Dietary phytate prevents the growth of cancerous cells in the liver and pancreas (Arnarson 2015). It was known to give healthy effects in the gastrointestinal tract and other target tissues through its chelating ability. Phytate is likely to exercise its anticancer impact by influencing cell signalling mechanisms in mammals (Vucenik and Shamsuddin 2003) as a result of occurrence of several *myo*-inositol phosphates, including phytate, as intracellular molecules and the second messenger D-*myo*-inositol (1,4,5)-trisphosphate play diverse pivotal roles in cellular processes such as cell proliferation via mobilizing intracellular Ca^{2+} (Shears 1998). In human erythroleukaemia cells, levels of diverse intracellular *myo*-inositol phosphate esters are affected by extracellular phytate (Shamsuddin et al. 1992). D-*myo*-Inositol (1, 2, 6)-trisphosphate, for example, is known to be related with

impediment of diabetes complications and treatment of cardiovascular diseases as well as chronic inflammations (Claxon et al. 1990), and because of its antitumor and antiangiogenic effects, *myo*-inositol (1, 3, 4, 5, 6)-pentakisphosphate was proposed to offer anticancer therapeutic strategies (Maffucci et al. 2005).

17.2.2.1 Phytate/Protein

Phytate is identified to interact with proteins at both alkaline and acidic pH for complex formation as shown in Fig. 17.3 (Cheryan 1980). Phytic acid is anioinic at acidic, basic and neutral pH (Maenz 2001). Such activity may disrupt protein structure which eventually can lower enzymatic activity, proteolytic digestibility and protein solubility. Conversely, the importance of protein-phytate interaction in nutrition is still under study. Depending upon protein source, phytate-protein interactions depressingly influence protein digestibility in vitro (Cheryan 1980). A harmful effect of phytate on the nutritional value of protein, though, was not evidently established yet in monogastric animals (Sebastian et al. 1998). Few research advocates that phytate does not influence protein digestibility; others have reported an enhanced availability in amino acid along with lower levels of phytate. This variance may arise as a result of use of diverse protein sources. Phytase can inhibit digestive enzymes such as lipase, α-amylase or proteinase including pepsin, chymotrypsin and trypsin (Greiner and Konietzny 2006). The inhibitory activity rises with rising number or levels of phosphate residues in *myo*-inositol molecule. This inhibitory action may be

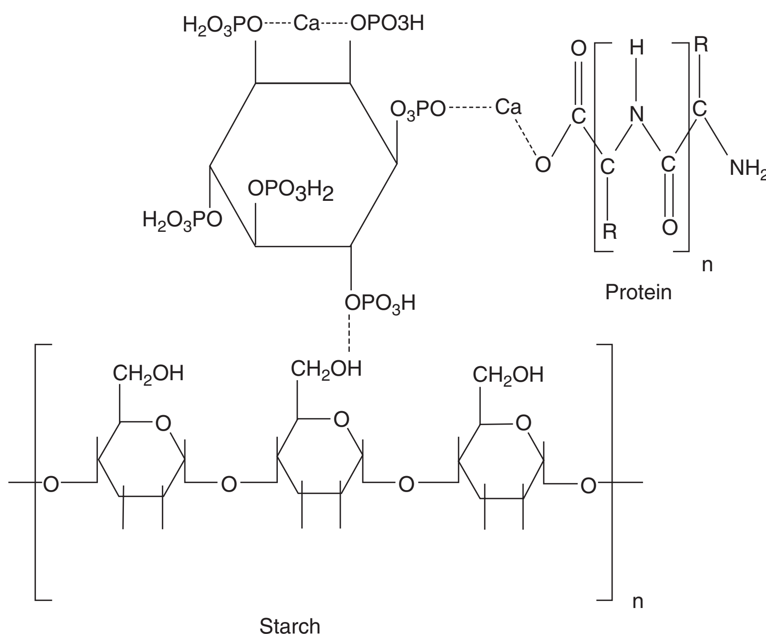

Fig. 17.3 Interaction of phytic acid with metals, proteins and carbohydrate. (Source: Singh et al. 2011)

as a consequence of the non-specific quality of phytate-protein interactions, the chelation of calcium ions which are vital for the activity of trypsin and α-amylase, or due to interaction with substrates of these enzymes. Phytate is known as an inhibitor of α-amylase in vivo in a detrimental relationship between blood glucose response and phytate intake (Jenab and Thompson 2002). Hence, phytate-rich food is assumed to have high nutritional values against diabetes mellitus, which is amongst the universal nutrition-dependent diseases in Western society.

17.2.2.2 Phytate/Mineral

In humans, only isolated form of *myo*-inositol pentakisphosphate can diminish absorption of zinc, iron and calcium, whereas *myo*-inositol tetrakis- and trisphosphates do not affect absorption process of minerals. However, the latter have negative effect of phytate on iron absorption if higher phosphorylated *myo*-inositol phosphates, *myo*-inositol tetrakis- and trisphosphates, exist. Therefore, zinc absorption and the sum of *myo*-inositol tris- through hexakisphosphate from cereal and legume meals exhibit a negative correlation (Sandberg et al. 1999); likewise, it is also true for zinc absorption. Overexpression of phytase at some stage in seed development can lead to lowered phytate quantity in the mature seed (Coello et al. 2001). Upregulated levels of seed phytase may lead to enhancement in mineral absorption by lowering phytate amount in plant-based food at the time of processing and digestion in elementary canal of human. Phytate exerts opposite effect on vitamin absorption; therefore, animals fed with fodder of more phytate content usually show signs of emaciation, off-feed, retarded growth and reproduction failure (Konietzny and Greiner 2003; Lopez et al. 2002; Iqbal et al. 1994). At higher pH than isoelectric point of proteins, it can bind and form protein-mineral-phytate complex that is insoluble, resilient to enzyme hydrolysis and decrease the effectiveness of protein consumption (Kies et al. 2006; Dersjant et al. 2015).

17.3 Phytase

Phytases naturally occurs in plants and microorganisms, particularly in fungi as 3-phytases or 6-phytases. Most of the phytases resides in the family of histidine acid phosphatases. The enzymes hydrolyse phytic acid to phosphoric acid and myo-inositol in a staircase fashion through the formation of myo-inositol phosphate intermediates (Mullaney and Ullah 2003; Yu et al. 2012; Dersjanti et al. 2015). In living systems phosphomonoester hydrolysis is a vital process of metabolic regulation, energy metabolism and signal transduction pathways. Phosphorus is an important element for the growth of all living organisms and also in livestock production. Feed must be augmented with inorganic phosphorus being critical for energy production, forming macromolecular structure and assisting in metabolic regulation; thus, phosphorus in sufficient level is necessary to support the normal growth and development of all living organisms (Carla and Elizabeth 2001).

In case of nutrition, two features of phytic acid are vital: (1) monogastric animals including poultry and pigs cannot consume phytic acid phosphorus, since they have

low phytase activity in their elementary tracts and phytic acid cannot be resorbed. Hence, pigs and poultry feed is augmented either with phytase-enriched feed or inorganic phosphate. (2) Phytic acid is anti-nutritive factor; it develops insoluble complexes with dietary essential metals, viz. zinc, calcium, magnesium and iron, reducing their bioavailability; thus, the enzymatic hydrolysis of phytic acid into less phosphorylated myo-inositol derivatives is desired (Vohra and Satyanarayana 2003). Generally the litter is useful to land as fertilizer. This waste encloses phosphorus that is consumed by diverse plants, though excess phosphorus can surfeit land and come into streams and lakes triggering serious health, environmental and economic problems.

Rigorous animal-based farming led to rise in environmental pollution because of high load of phosphorus excretion by the monogastric animals, due to their inherent inability to digest plant phytate (Vohra et al. 2006; Kuhar et al. 2009). Application of phytase in poultry and pig supplements increases the feed quality, lowers the phytate phosphorus excretion and assists in sustaining the environmental balance of the related areas. Consequently, phytase enzyme can be employed for regulating environmental pollution which arises due to eutrophication and constant chelation of nutrient factors from soil. The research on phytase spans from more than ten decades from its discovery by Suzuki et al. (1907).

All the desirable properties do not exist within single phytase; thus, on the basis of series of the available phytases, an accord phytase could be engineered (Lehmann et al. 2000). Recombinant DNA technology including site-directed mutagenesis could be used for additional remodelling of the features. The approaches employed for the engineering and developing of an ideal phytase are depicted in Fig. 17.4.

17.3.1 Molecular Classification of Phytases

Several alkaline phytases and histidine acid phosphatase (HAP) genes from diverse species were cloned and sequenced. Phylogenetic investigation of the protein sequences of various phytases evidently indicates two major classes, which finely correlate with the classification of phytases related to their electrostatic surface potential and biochemical and catalytic properties (Figs. 17.5 and 17.6; Table 17.1).

17.3.1.1 Histidine Acid Phytases

One of the major classes of phytases (Class I) is the family of HAPs sharing a highly conserved RHGXRXP motif (Van Etten et al. 1991).

The HAP class of phytases can be further subcategorized into three distinct groups (*PhyA*-*PhyC*), on the basis of biochemical properties, such as optimal pH and the position specificity of phytate hydrolysis and amino acid sequence

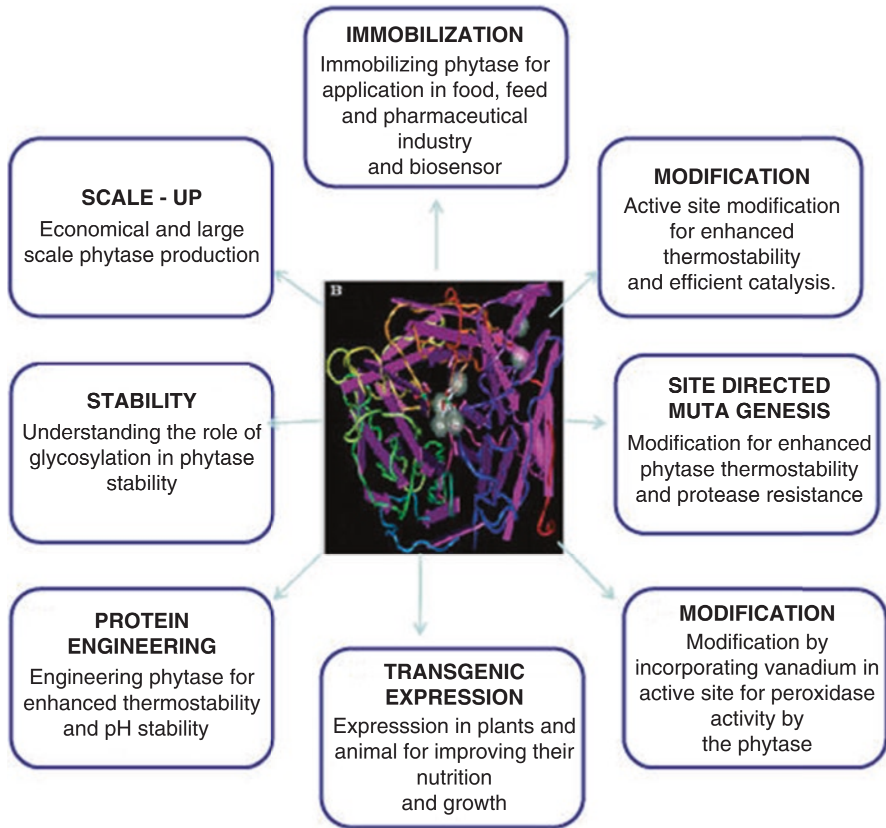

Fig. 17.4 Designing an ideal phytase for biotechnological applications. (Source: Singh et al. 2011)

homology. At acidic pH, HAPs are capable to hydrolyse five phosphate groups from phytate to give rise to myo-inositol monophosphate as the end product. Free from their origin, the acid phytate-degrading enzymes investigated to date in regard with phytate degradation (*A. terreus*, *Emericella nidulans*, *A. niger*, *Myceliophthora thermophila*, *Pseudomonas*, *S. cerevisiae*, *E. coli*, rye, rice, barley P1, barley P2, oat) secrete five amongst the six phosphate groups of phytate, and the end product was known as Ins(2)P (Cosgrove 1970; Wyss et al. 1999; Nakano et al. 2000). This signifies that phytate-degrading enzymes have an intense fondness for equatorial phosphate groups, but they are virtually not capable to slice the axial phosphate group. Rarely, traces of unbound myo-inositol were perceived.

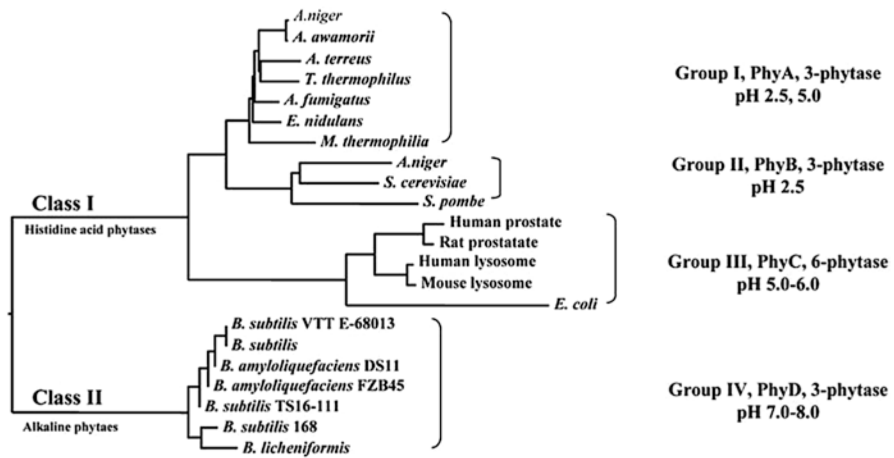

Fig. 17.5 Phylogenetic analyses of various HAPs and alkaline phytases. (Source: Oh et al. 2004)

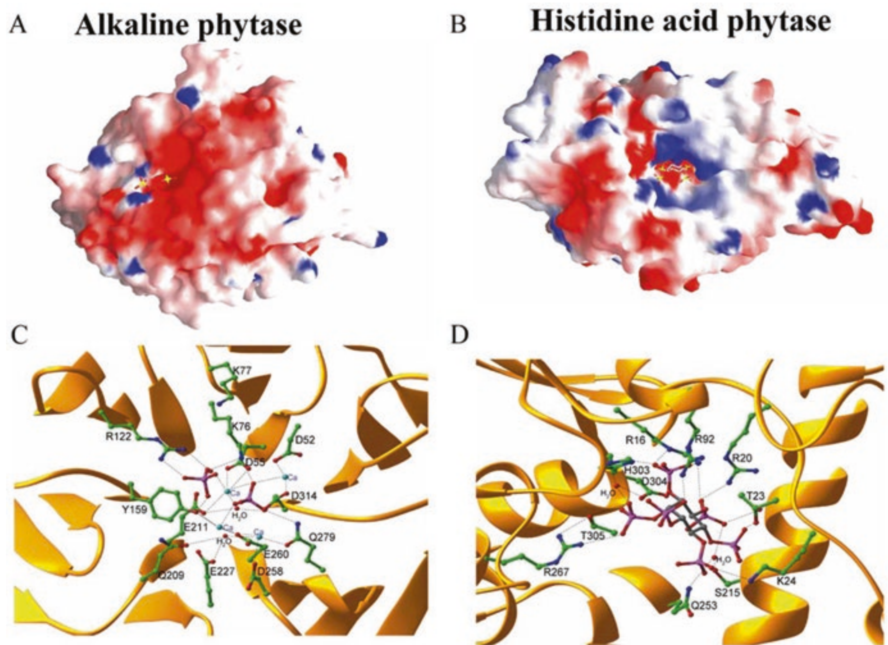

Fig. 17.6 Electrostatic surface potential of alkaline phytase and HAP in the active-site region. (Source: Oh et al. 2004)

Table 17.1 Molecular and biochemical characteristics of HAPs and alkaline phytases

	Histidine acid phosphatases (HAPs)			Alkaline phytase
Characteristics	PhyA	PhyB	PhyC	PhyD
Active site	(+) charged amino acids	(+) charged amino acids	(+) charged amino acids	(−) charged amino acids
Crystal structure	A large α/ß and a small α domain	A large α/ß and a small α domain	A large α/ß and a small α domain	Six-bladed ß propeller
Effect of Ca ions	Inhibition	Inhibition	Inhibition	Stimulation
Effect of EDTA	Stimulation	Stimulation	Stimulation	Inhibition
Final product	IP+5Pi	IP+5Pi	IP+5Pi	IP+3Pi
Glycosylation	Yes	Yes	No	No
Molecular mass (KDa)	62–128	270	42–45	38–45
Nature of phytate	Metal-free phytate	Metal-free phytate	Metal-free phytate	Calcium phytate
Optimum pH	2.5–5.0	2.5–5.0	5.0–6.0	7.0–8.0
Optimum temp.	55–60 °C	55–60 °C	40–60 °C	55–70 °C
Position specificity	D-3 position of phytate	D-3 position of phytate	D-6 position of phytate	D-3 position of phytate
Substrate specificity	Broad	Broad	Broad	Specific
Thermostability	Low (60°)	Low (60°)	Low (60°)	High 85–95 °C

Source: Oh et al. (2004)

17.3.1.2 Alkaline Phytases

Another main class (Class II) includes alkaline phytases that vary from HAPs in several features, such as molecular mass, optimal pH, tertiary structure, calcium ion requirement for enzymatic catalysis and substrate specificity. Based on such biochemical variations and phylogenetic data, alkaline phytases from *Bacillus* sp. and some plant seeds can be categorized as another group: *PhyD* (Oh et al. 2004). These phytases, referred as alkaline phytases, catalyse phytate existing as a metal-phytate complex in plants which have been identified from *Bacillus* sp. (Choi et al. 2001; Gulati et al. 2007a; Kerovuo et al. 1998, 2000) and pollen of certain plants such as *Lilium longiflorum* (Scott and Loewus 1986) and *Typha latifolia* (Hara et al. 1985). Such phytases can likely be employed for remedy of the animal feed before feeding (i.e. during feed mixing, pelleting and storage). In contrast to acid phytases, the alkaline phytate-degrading enzymes from lily pollen (Barrientos et al. 1994), cattail (Hara et al. 1985) and *B. subtilis* (Kerovuo et al. 2000) are incapable of taking a myo-inositol phosphate with three or lesser phosphate residues as a substrate.

Therefore, a myo-inositol trisphosphate isomer is the final product of phytate catalysis by alkaline phytate-degrading enzymes. The end product of phytate catalysis by the lily enzyme was found to be Ins(1,2,3)P_3 (Barrientos et al. 1994), but the *B. subtilis* enzyme was found to produce Ins(1,3,5)P_3 and Ins(2,4,6)P_3 (Kerovuo et al. 2000). Hence, the enzyme from *B. subtilis* is the first phytate-degrading enzyme competent of removing the axial phosphate group at the C-2 position of the myo-inositol ring (Kerovuo et al. 2000).

The surfaces of the substrate-binding sites of *Bacillus amyloliquefaciens* (A) and *Escherichia coli* phytase (B) are coloured according to their local electrostatic potentials ranging from −7 kt/e in red to +7 kt/e in blue, using GRASPP (Honig and Nicholls 1995). Stick models of two phosphates (A, C) and phytate (B, D) are shown in the substrate-binding site.

17.3.2 Structure and Types of Phytases

The basic characters of many phytate-degrading enzymes have been recognized as representatives of formerly identified classes of phosphate (Mullaney and Ullah 2003; Chu et al. 2004; Yao et al. 2012; Dersjant et al. 2015). The International Union of Biochemistry and Molecular Biology (IUBMB) in consultation with the IUPAC-IUB, Joint Commission on Biochemical Nomenclature (JCBN) (1975), on the basis of the position-specific phosphate ester group on the phytate molecule on which hydrolysis is started, listed three types of phytases:

17.3.2.1 3-Phytase (EC 3.1.3.8)

Recommended name, a 3-phytase; systematic name, *myo*-inositol hexakisphosphate-3-phosphohydrolase; hydrolyses the ester bond at the third position of *myo*-inositol hexakisphosphate to d-*myo*-Ins-1,2,4,5,6-pentakisphosphate and orthophosphate (Sajidan et al. 2004).

17.3.2.2 5-Phytase (EC 3.1.3.72)

Recommended name, a 5-phytase; systematic name, *myo*-inositol hexakisphosphate-3-phosphohydrolase; hydrolyses the ester bond at the third position of *myo*-inositol hexakisphosphate to d-*myo*-Ins-1,2,3,4,6-pentakisphosphate and orthophosphate (Chu et al. 2004).

17.3.2.3 6-Phytase (EC 3.1.3.26)

Recommended name, a 6-phytase; systematic name, *myo*-inositol hexakisphosphate-6-phosphohydrolase; hydrolyses the ester bond at the 6th position of *myo*-inositol hexakisphosphate to d-*myo*-Ins-1,2,3,4,5-pentakisphosphate and orthophosphate. Subsequent ester bonds in the substrate were hydrolysed at variable rates (Lassen et al. 2001; Chu et al. 2004).

17.4 Sources of Phytase

17.4.1 Plant Sources

Phytase occurs in seeds of various crops, viz. wheat, rice, maize, soybeans, lettuces, mung beans, faba beans, barley, pea, white mustard, potato, radish, spinach grass, rye and other legumes or oil seeds (Gibson and Ullah 1990). First, Suzuki et al. (1907) have made a preparation of phytase and detected phytase activity in wheat and rice bran. They isolated inositol as a reaction product of phytase. Phytase has been isolated in pure form and characterized from soybean (Gibson and Ullah 1990). Laboure et al. (1993) isolated the phytase in pure form from maize seedlings to characterized it and lateron, cDNA clone was also synthesized for phytase (Maugenest et al. 1997). Roots of several plant species have also been reported for phytases activity (Hayes et al. 2000; Richardson et al. 2000), although the cloning of phytase genes and their characterization in plants are fewer. Primarily, low abundances of phytase genes at transcription and translation levels were reported in the plant species, viz. maize (Maugenest et al. 1997, 1999) and *M. truncatula* (Xiao et al. 2005).

cDNA library was created from the germinated cotyledons of soybean by employing a fragment of *M. truncatula* phytase gene as the probe and used to clone a new phytase gene *Sphy1*. Phytic acids and its derivatives stored in seeds at the time of the seed germination and the early growth of seedlings could be hydrolysed by the activity of *Sphy1*. The activity of phytase rises swiftly during germination which finally decomposed and used in the form of phosphate and inositol; however, seeds confer activities of both constitutive and germination-inducible phytases. Certain feedstuffs hold significant phytase activity (wheat, rye, wheat bran, barley), while others show little or no phytase activity (corn, sorghum, oats and oilseeds) (Eeckhout and de Paepe 1994). Crushed grain diets in both pigs and broilers show a very high correlation with overall phosphorus retention (Barrier-Guillot et al. 1996). The phytase activity was reported to be highly variable (915–1581 U/Kg) in the wheat samples (Eeckhout and de Paepe 1994). Mostly, this deviation can be assumed due to cultivar differences (Barrier-Guillot et al. 1996) and probably due to grain storage time and conditions. It cannot be considered as a consistent source in most commercial swine and poultry operations as a result of this high variability of phytase activity in feedstuffs. Forty-five to 60 °C is the optimal temperature range of plant phytases (Wodzinski and Ullah 1996) which, however, may be partially or totally inactivated due to high steam pelleting temperatures or overheating (Ravindran et al. 1995). Wheat phytase lose significant activity if incubated a proteolytic digestive enzyme, pepsin (Phillippy 1999).

17.4.2 Animal Sources

McCollum and Hart (1908) firstly reported animal phytase in calf liver and blood. It was also detected in the blood of lower vertebrates such as birds, fishes, reptiles and

sea turtle (Rapoport et al. 1941) and in the intestine of pig, cow and sheep (Spitzer and Philips 1972). Phytase was partially purified from rat, calf, chicken and human intestines (Bitar and Reinhold 1972). First phytate hydrolysis was observed by Patwardhan (1937) in the rat intestine. Human intestine shows about 30 times lower phytase activity than rat. Maximum phytase activity was found in the duodenum and minimum in the ileum, but humans have limited capacity to digest undergraded phytases (Iqbal et al. 1994). Microbial flora-based phytase activity was reported in the ruminants. A rat hepatic multiple inositol polyphosphate phosphatase (MIPP) gene showing phytase activity was cloned and expressed by Craxton et al. (1997). The MIPP mRNA was found highly active in the kidney and liver than other tissues. *Paramecium* also contains a phytase-like enzyme activity (Freund et al. 1992).

17.4.3 Microbial Sources

Many bacteria, fungi and yeast show phytase activity; however, it is most frequently detected in fungi. Richardson et al. (2009) reported microbial phytase activity in different types of soil. Microbial phytases are generally cell associated, and they produce only intracellular enzyme, with the exception of *Lactobacillus amylovorus*, *Bacillus subtilis* and *Enterobacter* sp.4. More than 200 fungal isolates of the genera *Aspergillus*, *Mucor*, *Penicillium* and *Rhizopus* have been examined for phytase production. An active extracellular phytase is secreted by all isolates. *Aspergillus niger* was known as the highest active extracellular fungal phytase producer with three different varieties, out of which two are mesoinositol-hexaphosphate phosphohydrolase (E.C. 3.1.3.8.) *PhytA* have two pH optima 2.5 and 5.0 (Shieh et al. 1969; Ullah and Gibson 1987) and a non-specific phosphomonoesterase (E.C. 3.1.3.2.). *PhytB* with a pH optimum of 2.0 (Shieh et al. 1969) to 2.5 (Ullah and Cummins 1987) hydrolyse phytin (Shieh et al. 1969; Ehrlich et al. 1993). A non-specific phosphomonoesterase (Pase) is produced by *A. niger* with a pH optimum of 6.0, which does not hydrolyse phytate (Ullah and Cummins 1988). Neither Pase nor *PhytB* hydrolyses phytate at pH 5.0 (Ullah and Cummins 1987, 1988). *PhytA* is 38% less active at pH 2.5 instead of its Michaelis constant (K_m) at both pH 2.5 and 5.0 is same (Ullah 1988). Both *PhytA* and *PhytB* are repressed by orthophosphate in partially purified form, although the *PhytA* is repressed to a lesser extent (Shieh et al. 1969). A slow-releasing organic phosphate supply such as Hylon starch phosphate can repress *PhytA* production (Gibson 1987).

Amongst yeast, extracellular phytase has been reported in *Schwanniomyces castellii* (Lambrechts et al. 1993), *Arxula adeninivorans* (Sano et al. 1999) and *Pichia anomala* (Vohra and Satyanarayana 2003) and characterized. Phytases from *Schwanniomyces castellii* show the molecular weight of 490,000 Dalton. It is similar to that reported for the phytases from *A. ficuum* being a glycoprotein with an approximate glycosylation rate of 31%. The phytase enzyme bears wide specificity with phytate as its favourite substrate. *Arxula adeninivorans* was found to be one of the rare yeasts competent of synthesizing phytase as a sole source of carbon and phosphate. Nakamura et al. (2000) examined several yeast species for extracellular

phytase activity. *Pichia spartinae* and *Pichia rhodanensis* harboured the greatest levels of phytase activity. An extracellular HAP phytase-producing thermophilic mould which enhances the growth of wheat seedlings was reported by Singh and Satyanarayana (2010).

Microorganisms such as *Klebsiella aerogenes* (Greaves et al. 1967), *Bacillus* sp. (Shimizu 1992; Kim et al. 1998a, b; Kerovuo et al. 2000; Gulati et al. 2007a, b), *Escherichia coli* (Greiner et al. 1993), *Klebsiella ohmeri* BG3 (Li et al. 2009), ruminal bacteria (Yanke et al. 1998), fungal species *A. fumigatus* (Pasamontes et al. 1997) and plants (Houde et al. 1990) were screened for obtaining better phytases. The *E. coli* phytase harbours the greatest specific activity at about eightfold greater than that of the *A. niger* enzyme (Wyss et al. 1999) which is now used as a commercial feed additive. Cost of cell splintering and the separation of cell debris would be too high for industrial enzymes including phytase; therefore, extracellular production is required. Periplasmic proteins are released into the extracellular space through the Kil protein (e.g. *E. coli*). It has been found that the expression level of recombinant proteins can be radically increased by using such system. A significant production of enzyme can be achieved by extracellular system where several heterologous enzymes are naturally expressed at only a very low level (Miksch et al. 2002).

17.5 Production of Phytase

17.5.1 Production Techniques

Phytase production techniques involve submerged fermentation (SmF) and solid-state fermentation (SSF) (Table 17.2). Main factors influencing yield of phytase production taken into consideration for selecting a particular production technique are types of strain, the culture conditions, availability of nutrients and nature of substrate. Phytase production from *Hansenula polymorpha* under oxygen-limited conditions in SmF has been reported by Stockmann et al. (2003).The culture parameters for phytase-producing fungus NSF-7 under SmF conditions and assay conditions using RSM approach for phytase enzyme production under SmF by *Bacillus cereus* MTCC 10072 were optimized (Dahiya et al. 2009, 2010).

The pre-culturing of phytase-producing strains in dextrose medium under limited oxygen supply leads to 25% enhanced production and eradicates 20-h-long lag phase which was practically observed without such limitation. Three different cultivation protocols, namely, submerged fermentations (Ullah and Gibson 1987), solid state (Ebune et al. 1995) and semi-solid (Han and Gallagher 1987), are employed for the production of phytase from *Aspergillus ficuum* NRRL 3135. Culture conditions, especially media composition (wheat bran and full-fat soybean flour), inoculum age and period of SSF affect yield of the phytase production by *A. niger* (Krishna and Nokes 2001). Phytase production by culturing *Rhizopus oligosporus* NRRL 5905, *Mucor racemosus* NRRL1994 and *A. ficuum* NRRL3135 on canola meal, cracked corn, soybean meal and wheat bran in SSF was extensively studied and achieved (Bogar et al. 2003a, b). An enhanced phytase production was

Table 17.2 The biochemical properties of phytases from various organisms

Microbial strain	pH_{opt}	T_{opt}	Fermentation	Carbon source	Nitrogen source	References
Filamentous fungi						
A. fumigatus SRRC 322	5.0	37	SmF	Hylon starch	$NaNO_3$	Mullaney et al. (2000)
Aspergillus niger	5.5	30	SmF	Glucose starch	–	Vats and Banerjee (2005)
Aspergillus ficuum	5.0	30	SmF	Corn starch, glucose	$NaNO_3$	Shieh and Ware (1968)
Aspergillus oryzae	6.4	37	SmF	Glucose	$(NH_4)_2SO_4$	Shimizu (1993)
Rhizopus oligosporus	5.5	27	SmF	Corn starch, glucose	$NaNO_3$	Casey and Walsh (2004)
Rhizopus oryzae	5.5	30	SSF	Glucose	NH_4NO_3	Ramachandaran et al. (2005)
Mucor racemosus	5.5	30	SSF	Starch	$NaNO_3$	Roopesh et al. (2005)
Peniophora lycii	5.5	26	SmF	Maltose, dextrin, soya flour	Peptone	Lassen et al. (2001)
Thermoascus aurantiacus	5.5	45	SmF	Starch, glucose, wheat bran	Peptone	Nampoothiri et al. (2004)
Rhizomucor pusillus	8.0	50	SSF	Wheat bran	Asparagine	Chadha et al. (2004)
Myceliophthora thermophila	5.5	45	SmF	Glucose	$NaNO_3$	Mitchell et al. (1997)
Sporotrichum thermophile	5.0	45	SmF	Starch, glucose	Peptone	Singh and Satyanarayana (2008)
Sporotrichum thermophile	5.0	45	SSF	Sesame oil cake, glucose	$(NH_4)_2SO_4$	Singh and Satyanarayana (2006)
Yeasts						
Pichia anomala	6.0	25	SmF	Glucose	Beef extract	Vohra and Satyanarayana (2001)
Schwanniomyces castellii	4.4	77	SmF	Galactose	$(NH_4)_2SO_4$	Segueilha et al. (1992)
Arxula adeninivorans	5.5	28	SmF	Galactose	Yeast extract	Sano et al. (1999)
Pichia rhodanensis	4.5	70	SmF	Glucose	–	Nakamura et al. (2000)
Pichia spartinae	4.5	75	SmF	Glucose	–	Nakamura et al. (2000)

(continued)

Table 17.2 (continued)

Microbial strain	pH_{opt}	T_{opt}	Fermentation	Carbon source	Nitrogen source	References
Candida krusei	4.6	40	SmF	Glucose	Polypeptone	Quan et al. (2001)
			Bacteria			
Bacillus subtilis	7.0	37	SmF	Glucose	NH_4NO_3	Kerovuo et al. (1998)
Bacillus amyloliquefaciens	6.8	37	SmF	Glucose	Casein, peptone	Idriss et al. (2002)
Escherichia coli	7.0	37	SmF	–	Tryptone	Sunita et al. (2000)
Klebsiella aerogenes	7.0	30	SmF	Sodium phytate	Yeast extract	Tambe et al. (1994)
Lactobacillus sanfranciscensis	5.5	37	SmF	Maltose, glucose	Yeast extract	Angelis et al. (2003)
Lactobacillus fructivorans	5.5	37	SmF	Maltose, glucose	Yeast extract	Angelis et al. (2003)
Lactobacillus lactis subsp. *lactis*	5.5	37	SmF	Maltose, glucose	Yeast extract	Angelis et al. (2003)
Lactobacillus rhamnosus	6.5	37	SmF	Glucose	Yeast extract	Raghavendra and Halami (2009)
Lactobacillus amylovorus	6.5	37	SmF	Glucose	Yeast extract	Raghavendra and Halami (2009)
Pediococcus pentosaceus	6.5	37	SmF	Glucose	Yeast extract	Raghavendra and Halami (2009)

Source: Singh et al. (2011)

optimized on wheat bran supplemented with starch and ammonium sulphate by using Plackett-Burman and central composite designs. Canola oil cake kept on specific fermentation conditions (pH 5.3, 30 °C, 54.5% moisture content) in SSF by using *R. oligosporus* can be used for phytase production without additional nutrients (Sabu et al. 2002). Cultivation of *E. coli* in fed-batch fermentation under constant glucose concentration and low oxygen level in the medium show rapid glucose uptake rate at constant low level of oxygen (5–10%) and lead to high extracellular phytase activity (120 U/ml) in shorter cultivation time (14 h) (Kleist et al. 2003). A thermostable, extracellular phytase can be produced in submerged fermentation (SmF) by using *Bacillus* sp. DS11 in the wheat bran and casein hydrolysate medium at 37 °C (Kim et al. 1998a). Moreover, the phytase gene cloned in *B. subtilis* produces phytase on 100 times enhanced rate in modified Luria broth medium (Kim et al. 1999b). Similarly, cultivation of *Aspergillus* sp. 5990 (higher optimum temperature for catalytic activity than the commercial Natuphos from *A. ficuum* NRRL 3135) by employing SmF at 37 °C, pH 7.0, gives fivefold higher activity of phytase in liquid culture (Kim et al. 1999a). Other researchers also found three to five times

higher phytase activity in SSF than SmF by culturing *Aspergillus niger* CFR 335 and *Aspergillus ficuum* SGA 01 (Shivanna and Venkateshwaran 2014). A qualitative correlation was established amongst medium composition, morphology and phytase production by using *A. niger* (Papagianni et al. 2000). Culturing a new bacterial species from the rumen of cattle, namely, *Mitsuokella jalaludinii*, for phytase production in batch fermentation exhibits glucose suppression in a medium consisted of rice bran-soybean milk (2:1). The finest phytase production requires no surfactant at pH 7.0, 39 °C (Lan et al. 2002). High yield and purity of the enzyme in a relatively low-cost system is likely to be a competitive production. Very low quantity of phytase is obtained from wild-type organisms even after tedious and cost-intensive purification which make them unfit for large-scale/industrial production of the enzyme. Consequently, recombinant DNA technology emerges out to be cost-effective and efficient processes for phytase production.

17.5.2 Physical Parameters

17.5.2.1 Temperature

Rising temperature up to a maximal temperature is accompanied with an enhancement in phytase activity. Rise in temperature beyond limit brings about heat-induced denaturation of the enzymes. Optimal temperature for phytate hydrolysis depends upon the source of enzyme and generally varies from 35 to 80 °C (Table 17.2) (Singh and Satyanarayana 2008; Li et al. 2008, 2009; Tran et al. 2010; Dahiya et al. 2010). Usually, plant-based phytases confer highest activity at lower temperatures than their microbial counterparts (Jog et al. 2005). Application of phytase in food industries finds high suitability of microbial phytase as compared to plant counterpart because of the higher pH and thermal stability of the former. Specific activity has an effect on the economy of the anticipated use; therefore, it is one key factor for commercial exploitation of an enzyme. Phytases characterized from lily pollen, soybean, mung bean, maize, and *Penicillium simplicissimum* exhibit specific activity at <10 U/mg (Kim et al. 2003; Lassen et al. 2001; Ullah et al. 2003), whereas *Citrobacter braakii*, *Candida krusei*, and *Peniophora lycii* exhibit specific activity at >1000 U/mg (Tseng et al. 2000; Hegeman and Grabau 2001; Jog et al. 2005) at 37 °C. Additionally, most plant-based phytases are permanently inactivated at higher temperatures than 70 °C within minutes, but most of the microbial counterparts retain considerable activity even after longer incubation period. Similar treatment of these enzymes at 70 °C for 10 min did not bring about loss of activity, and enzyme isolated from *Pichia anomala* can even endure a prolonged treatment at 70 °C with no loss of activity (Vohra and Satyanarayana 2002).

17.5.2.2 pH

Depending upon pH optimization, phytases can be categorized into acid phytases having pH 3.5–6.0 and alkaline phytases having pH 7.0–8.0 as optimum pH range. Most phytases characterized so far reveal highest phytate-degrading activity under the acidic pH range (Table 17.2). *Bacillus* species (Kerovuo et al. 1998; Shimizu 1992;

Kim et al. 1998a, b), the rat intestine (Yang et al. 1991) and lily pollen (Jog et al. 2005) produce alkaline phytases. Most of the phytases characterized so far bear a single precise pH optimum with the only exception the phytase from *Aspergillus fumigatus*, which shows wide pH optimum with minimum 80% of the maximal activity at pH values from pH 4.0 to 7.3 (Wyss et al. 1999). Majority of microbial enzymes are stable at pH over 8.0 and under 3.0; on the other hand, plant counterparts are least stable under such pH conditions. For instance, an *E. coli* phytase remains active at pH 2.0 and pH 10.0 even exposed to 4 °C for 2 h (Greiner et al. 1993). In addition to high-temperature stability and high specific activity, low pH resistance of the phytase is also essential if it is consumed as feed additives because of occurrence of such acidic conditions and high protease concentration of gastric passages in animals. Two new phytase genes from *Yersinia rohdei* and *Y. pestis* have been cloned to express in *Pichia pastoris* (Huang et al. 2008). Both the recombinant phytases show maximum activity at pH 1.5–6.0 (optimum pH 4.5) at 55 °C. A comparative study of commercial phytases advocates the *Y. rohdei* phytase to be more resilient to pepsin, persist higher activity under gastric conditions and produce two- to tenfold higher inorganic phosphorus from soybean meal. These advanced characters advise that the *Y. rohdei* phytase is suitable supplement to animal feed. Certain new phytase genes harbouring great tolerance against low pH and high temperature have been isolated and cloned by several laboratories (Farhat et al. 2008; Rao et al. 2008). The highest activity of *E. coli* phytases under a pH range of 2–5.5 as compared to *A. niger and P. lycii* makes it more desirable for industrial application (Tran et al. 2011).

17.5.3 Nutritional Parameters

17.5.3.1 Carbon Source

Growth and metabolism of microorganisms depends upon readily available form of carbon sources (Gautam et al. 2002). Selection of carbon source relies on the fundamental structure and digestibility of the organism. Even though fungus possesses same number of carbons as that of sucrose and glucose (Vats and Banerjee 2005), mostly, they do not produce phytase because of absence of cellular mechanisms to convert disaccharides such as cellobiose, lactose and maltose into simpler glucose molecules (Takanobu 2002). A disaccharide like trehalose is highly resistant to acid hydrolysis and quiet stable in solutions under acidic conditions and high temperatures (Takanobu 2002). A trisaccharide like raffinose has higher moles of carbon than other sugars; however, it cannot raise enzyme activity due to repression control mechanism of the enzyme (Vohra and Satyanarayana 2003). *Arxula adeninivorans* produce more phytase molecules on availability of galactose than glucose (Sano et al. 1999). *Aspergillus niger* produce high levels of enzyme on starch followed by a mixture of glucose and starch (Vats and Banerjee 2002). Fructose forms large pellets or clumps which do not trigger phytase production and release of extracellular enzyme (Ullah and Dischinger 1993). *Bacillus licheniformis* LH1 produce higher phytase on beef extract than strain LF1 (Roy et al. 2013). Amongst all sugars,

glucose is all-time favourite carbon source for fungi, yeast and bacteria so far (Vohra and Satyanarayana 2001; Ramachandaran et al. 2005; Kuhar et al. 2009) (Table 17.2).

17.5.3.2 Nitrogen Source

Selection of nitrogen source depends on the type of microorganism employed. In *Aerobacter aerogenes* and *Klebsiella aerogenes*, organic forms of nitrogen such as 1% of each peptone and yeast extract give better results for the production of phytase (Jareonkitmongkol et al. 1997). Potassium nitrate and urea as nitrogen source can lower about 50–70% enzyme activity of *A. niger* in both SmF and SSF (Vats and Banerjee 2004). Comparable inhibition results were obtained when various organic and inorganic nitrogen sources were used for phytase production in *Rhizopus oligosporus* and *Aspergillus ficuum* (Pinky et al. 2002). Generally, ammonium nitrate (Ramachandaran et al. 2005; Kerovuo et al. 1998) acts as sole source of nitrogen followed by yeast extract (Angelis et al. 2003; Raghavendra and Halami 2009). Ammonium sulphate is also efficient nitrogen source for phytase production in *Bacillus licheniformis* LF1 than in LH1 (Roy et al. 2013).

17.5.3.3 Phosphorus Source

In addition to regulatory function, phosphorus (P) is amongst the main constituents in biosynthesis of nucleic acids and cell membranes as well as energy metabolism (Singh and Satyanarayana 2011). Phosphate plays pivotal function in phytase production, and its dose in medium substantially regulates enzyme production rate (Gargova and Sariyska 2003). Strain-dependent phytase repression in the presence of excess orthophosphate can be reversed by supplying a slow-releasing organic phosphate source (Gibson 1987). Inorganic phosphorus in a complex medium usually exerts insignificant suppression rate of phytase synthesis (Fredrikson et al. 2002). A similar phenomenon of phosphate repression on phytase synthesis was also reported in many yeast strains (Nakamura et al. 2000).

Apt dose of inorganic phosphates significantly influences the process of phytase production. High phosphate conditions are repressing, although restrictive phosphate conditions result in increased phytase expression. A comparative study of phytase-producing microorganisms reported that under very low range of inorganic phosphate of about 0.0001–0.005%, A. *ficuum* produced highest amount of phytase (Wodzinski and Ullah 1996). Gibson (1987) also verified the impact of phosphorus dose in the medium and studied the production from many sources and speculated variable cleavage behaviour of phosphoester linkage in some starch sources leads to slow but steady supply of phosphorus. A sudden decline in phytase synthesis by *A. niger* even at 0.05% phosphorus in the medium with complete inhibition of production at 0.1% and above advocates the end product inhibition concept in phytase synthesis (Vats and Banerjee 2002). In phosphorus-exhausted medium, the strain produced 184 nkat/ml phytase with a specific activity of 21,367 nkat/mg protein. Similarly, *A. ficuum* on 10 mg *P*/100 g semi-solid substrate supplemented with soybean meal in the growth medium lead to higher phytase activity of 82.5 U/g substrate instead of 8.0 U/g substrate in control without phosphate (Han and Gallagher 1987). UV irradiation of *A. niger* NRRL 3135 repressed *phyA* production in the

mutant up to 60% under the *Pi* dose of 0.006% (w/v), but phytase production in wild-type strain was not affected considerably by higher concentration of phosphorus (0.006–0.015%, w/v) (Chelius and Wodzinski 1994). 20 mg/dm^3 phosphorus supports maximum secretion of phytase from *Aspergillus* sp. 307 at dual pH optima (pH 5.0 and 2.5), beyond which a regression in production was observed (Gargova et al. 1997). The extracellular phytase production by using *Aspergillus* sp. 5990 was highest at lower phosphate concentration (50 mg/l), whereas by increasing concentration up to 100 mg/l, enzyme production was highly repressed (Kim et al. 1999a). On the other hand, phosphate supplementation in the range from 0.05% to 0.5% to the rice bran-soybean milk medium did not influence phytase production by *M. jalaludinii* (Lan et al. 2002). The repression of phytase synthesis by inorganic phosphorus was insignificant in a medium of increased composition complexity (Fredrikson et al. 2002).

17.5.3.4 Effect of Surfactants on Production

The inclusion of surfactants triggers the development of minor pellets in the broath which lead to enhanced yield of phytase (Boling et al. 2000; Mallin 2000). These are surface active agents who lower surface and interfacial tensions and have wetting and penetration actions, detergency, emulsification, gelling, flocculating actions and microbial growth enhancement, etc. (Elliott et al. 1986). Surfactants enticed researchers to be employed for pellet formation in broath cultures. Pelletization process enhances the extracellular enzyme secretion in submerged fermentation (Kerovuo et al. 1998). Tween-20 is amongst the effective surfactant that causes flocculation and formation of finer pellets for improved yield of the enzyme in submerged cultures. Inclusion of sodium oleate or Tween-80 during the culture of *A. carbonarius* increases phytase production and reduces phytic acid content in canola meal during SSF (Maenz and Classen 1998). Tween-20 triggered phytase synthesis by culturing *A. niger* CFR 335 as a result of increase in cell permeability in SmF. Ethylenediaminetetraacetic acid (EDTA), also known by several other names, which is a chemical used as chelating agent was found to readily inactivate *Bacillus* phytases (Kim et al. 1998a, b), although it promptly stimulates *A. fumigatus* phytase activity (Kim et al. 1999a). EDTA, sometimes, binds to form strong complexes with Mn^{2+}, Fe^{3+}, Cu^{2+}, Co^{2+}, etc., inhibiting the fungal growth and lowering phytase production (Van Etten et al. 1991). Likewise, *A. niger* CFR 335 phytase was repressed by EDTA because of its antifungal activity. Similar antifungal activities of EDTA against *Candida albicans* and in human salivary mucin have already been reported (Kleist et al. 2003). Increased yields of phytase in *A. niger* NCIM 563 with 0.5% Triton X-100 and in *A. niger* NRRL 3135 with 0.5% Triton X-100, sodium oleate and Tween-80 have been shown (Mullaney and Ullah 2003; Wyss et al. 1998).

17.6 Regulation of Phytase Synthesis

17.6.1 Microbial Phytase Regulation

So far, *E. coli* (Greiner et al. 1993) and *Raoultella terrigena* (Zamudio et al. 2002) have been extensively studied amongst bacteria for phytase production. In bacterial system, phytase is an inducible enzyme whose expression is via complex regulation; however, phytase production is not regulated consistently in different bacteria (Liu et al. 1998). There is a robust control inhibition by orthophosphate levels in the culture medium for production of extracellular fungal phytate-degrading enzymes.

17.6.1.1 Factors Affecting Regulation

Media

The production of both the *E. coli* (Greiner et al. 1993) and *Raoultella terrigena* (Zamudio et al. 2002) phytase was switched off in exponentially growing bacteria and turned on as soon as the cultures come into the stationary phase in non-limiting media. Since the formation of the enzymes began with the decreasing growth rate, it was recommended that either energy or a nutrient limitation, known to arise in the stationary phase, could be at the beginning of its induction. Only carbon starvation amongst the nutrient limitation tested was able to trigger an instant synthesis of the *Raoultella terrigena* phytase (Greiner et al. 1997), although in *E. coli*, phytase synthesis was provoked upon starvation of bacteria for inorganic phosphate, while carbon, nitrogen, and sulphur were ineffective factors (Touati and Danchin 1987). The main response to the control of a specific nutrient in *E. coli* was instigation of a certain set of genes which facilitates an improved uptake of the scarce nutrient or the utilization of homologous nutrients belonging to same class. These nutrient-specific systems involve the *NtrB/NtrC/*σ^{54} regulon that is triggered under nitrogen limitation, the cyclic AMP (cAMP) and its receptor the catabolite activator protein (CAP) for the use of substitute carbon sources, and the *PhoB/PhoR* regulon that is triggered under phosphorus limitation (Hengge-Aronis 1996). If the medium is completely exhausted for an essential nutrient, the cells transfer into the stationary phase. The synthesis of many proteins is provoked during transition into stationary phase, and a core set of proteins is triggered irrespective of class of nutrient under depletion. As stated earlier, phytase synthesis in *E. coli* is stimulated in non-limiting media entering into the stationary phase and under anaerobic environment (Greiner et al. 1993). The phytase-encoding gene *appA* expression solely relies on the *rpoS*-encoded sigma factor σ^S, which is known as key regulator for several stationary phase-responsive genes. Very often σ^S-dependent genes are controlled by many promoters, and only one of them is regulated by σ^S. Therefore, all genes are not known to be σ^S controlled for expression. Starvation for phosphate in minimal medium instead for glucose or ammonia leads to strong activation of *rpoS* expression followed by a rise in phytase activity.

Level of Inorganic Phosphate

A strong regulatory inhibition of phytase synthesis by inorganic phosphate dose usually occurs in all microbial phytase producers including moulds, bacteria and yeast, except rumen bacteria (Greiner et al. 1997; Yanke et al. 1998). The downregulation of phytase formation by inorganic phosphate was insignificant with higher medium composition intricacies. The components in the complex media accounting for the lowered repression rate are not known. The proficient activation or repression of phytase synthesis by phosphate starvation in most bacteria (Greiner et al. 1993; Konietzny and Greiner 2002) doubts its putative function in supplying the cell with phosphate hydrolysed from substances such as phytate. This assumption is advocated by the detection of a phytase in the stalk of *Caulobacter crescentus*, an oligotroph gram-negative alpha purple proteobacterium that thrives in aquatic environments with low nutrient availability (Ireland et al. 2002). Phosphate is the regulatory factor in the climate in which *Caulobacte*r exists, and the proposed role of the stalk is phosphate uptake. Stalks get longer for increasing surface area for phosphate uptake when phosphate is limiting as well as the occurrence of a phytase facilitates the uptake of the organic phosphate by the stalk.

Nature of Carbon Source

Besides this, phytase synthesis relies on the nature of the carbon source supplied for growth. Glucose, a catabolite repressor, has been broadly used to increase phytase production. In *E. coli*, the cAMP-CAP instead of the carbon source itself is clearly regulatory in nature (Liu et al. 1998). Phytase production in both *E. coli* and *Raoultella terrigena* was negatively controlled by cAMP (Touati and Danchin 1987; Zamudio et al. 2002), which is assumed to be a part of amphibolic metabolism of glucose and galactose as well as directly or indirectly in regulating the expression of an essential stationary growth regulator.

Substrate Concentration

In *Mitsuokella jalaludinii* substrate, induction mechanism was found (Lan et al. 2002), although phytate had no influence on the synthesis of phytase (Greiner et al. 1993). Therefore, to optimize culture conditions for the synthesis of phytate-degrading enzymes by employing microorganisms is not a simple job; the control of production may vary from microorganism to microorganism. Hence, inability to find phytate-degrading activity does not essentially entail that the experimental microorganism is not a phytate-degrading enzyme producer by any means, but that the growing conditions are not suitable for enzyme production. Substrate dose beyond 300 μM shows inhibitory action for production of the phytase-like enzyme from *Paramecium* (Freund et al. 1992). The *Rhizopus oligosporus* and *Klebsiella* sp. phytases were repressed by the substrate (Sutardi and Buckle 1988; Shah and Parekh 1990), but only under higher substrate concentrations. Fungal phytase activity was also repressed by substrate dose beyond 1 mM (Ullah 1988). Soybean and maize root phytases were inhibited at 20 mM and 300 μM substrate, respectively

(Sutardi and Buckle 1988; Hubel and Beck 1996). Under higher substrate dose, the charge residing over phosphate groups may interfere the local surroundings of the catalytic domain of the enzyme. This might hinder transformation of the enzyme-substrate complex to enzyme and product (Ullah and Phillippy 1994), although repression due to the formation of sparingly soluble protein-phytate complex cannot be affected.

17.6.2 Phytase Regulation in Plants

Cereals, oilseeds, legumes and nuts of higher plants confer phytate-degrading enzymes, although an insignificant phytase activity is also confined to the root part of the plants. The phytate-degrading activity of grains, pollen and seeds mediates phytate degradation during germination to produce phosphate, myo-inositol and minerals for availability during plant growth and development (Reddy and Pierson 1994). Root phytase has been illustrated as mechanisms of plants to increase use of soil phosphate. Organic phosphate usually represents half of the total soil phosphate in which a major constituent ensues in the form of inositol penta- and hexakisphosphate (Richardson et al. 2001). In low phytase activity in roots and the lack of ability of phytase secretion into the rhizosphere, phytate is scantily utilized by plants (Hayes et al. 2000). Therefore, it is advisable that *Bacillus*- and *Enterobacter* sp.-like soil microorganisms colonizing the plant rhizosphere act as plant growth-promoting rhizobacteria (PGPR) by making phytate phosphate absorbable to the plant because of extracellular phytase activity (Idriss et al. 2002). Enabling the plants to utilize phytate phosphate either via activity of extracellular secreted *Aspergillus* phytase in the plant root or via inclusion of purified phytase as well as soil microorganisms secreting extracellular phytase into the rooting medium shows significance of phosphate availability from soil phytate for plant nutrition under phosphate scarcity (Richardson et al. 2001).

Several isoforms of variable controlled phytases are known in a certain plant. Grains, pollen and seeds enclose both germination-inducible and constitutive phytases (Lin et al. 1987; Greiner et al. 2000). The biochemical mechanism undertaking phytase activity is not appropriately identified; however, certain instances are referred, for example, in pollen, it was advised that phytase enzymes activated during germination may be formed from pre-existing, long-lived messenger RNA (mRNA) (Jackson and Linskens 1982; Lin et al. 1987). Rise in phytate-degrading activity during germination in cereals and legumes is also doubtful. Some report favours de novo synthesis (Bianchetti and Sartirana 1967), while others merely propose activation of pre-existing enzymes as consequence of the rise in phytate-degrading activity (Eastwood and Laidman 1971). Phytate regulation process is linked to the presence of gibberellic acid which is assumed to enhance the secretion of phytases to give it access to phytate but does not induce their synthesis (Gabard and Jones 1986); however, few reports suggest that gibberellic acid can induce phytase activity (Srivastava 1964). Two main mechanisms are likely to occur in the

control of phytase activity by phosphate. Acid phytate-degrading enzymes are robustly repressed by phosphate; consequently the enzyme activity itself may be regulated by phosphate. Phosphate added in due course in the germination sequence can suppress the rise in phytate-degrading activity at the transcription level (Sartirana and Bianchetti 1967).

17.6.3 Phytase Regulation in Animals

Animal phytases are poorly studied than the microbial phytases. Though dietary phytate has hostile nutritional effect for animals including man, the occurrence of phytase activity in the gastrointestinal tract of diverse animals was studied. Instead, phytase activity of the mammalian intestine has been endorsed to the action of imprecise intestinal alkaline phosphatases (Davies and Motzok 1972; Davies and Flett 1978); moreover, intestinal alkaline phosphatase and intestinal phytase enzymes are distinctive proteins (Bitar and Reinhold 1972).

17.7 Purification of Phytase

General biochemical techniques such as acetone precipitation, ammonium sulphate fractionation, gel filtration, affinity chromatography, ion-exchange chromatography and hydrophobic interaction are employed for purification of phytase enzymes. Main difficulty during purification of phytases from plant source is the problem in separation of phytases from tainting non-specific acid phosphatases (Konietzny et al. 1995). Besides this, purification of the enzymes from plants requires prolonged germination period. The low stability rate as that of microbial enzymes further makes purification of phytases from plant sources more complicated. Microorganism-based extracellular phytases have been easily achieved from the culture filtrate in rather high amount. A three-step approach has been proposed to purify the phytase from *A. niger* NRRL 3135, including ion-exchange chromatography and chromatofocussing. The phytase was purified about 22-fold with a retrieval of 58% (Ullah and Gibson 1987). Two discrete new extracellular phytases from *A. niger* were first purified by employing phenyl-Sepharose column chromatography, Rotavapor concentration, and Sephacryl S-200 gel filtration (Soni and Khire 2007). *Cladosporium* sp. (Gulati et al. 2007) and *Thermomyces lanuginosus* that yield high amount of phytase were separated from air, and phytase was concentrated to electrophoretic homogeneity by gel filtration and ion-exchange chromatography (Quan et al. 2004). *Rhizopus oligosporus* (DSMZ1964), which is commonly used in tempeh formation, was cultured in rice flour suspension, and intracellular phytases RO1 and RO2 were extracted and purified in a five-step method to give 1.3% (RO1) and 1.6% (RO2) in relation to phytase activity in crude extract. *E. coli*-based two periplasmic phytases have been purified over 16,000-folds with a reclamation of 7% and 18%, respectively, employing a five-step purification method, including ammonium sulphate fractionation, hydrophobic interaction

and ion-exchange chromatography (Greiner et al. 1993). The purification of a phytase from faba beans (Greiner 2001) and oat (Greiner and Larsson Alminger 1999) was attained by employing a seven-step purification process, including acetone precipitation and ammonium sulphate fractionation. The enzyme from faba beans was purified 2190-fold with a recovery of 6% and that of oat 5380-fold with a recovery of 23% and from *A. niger* was purified 51-fold with recovery of 20.3%; *R. oryzae* results in 20.7-fold purification and specific activity of 141.83 U/mg of protein with 26% phytase recovery. The purification of the intestinal phytase of rat can be achieved by a six-step method, involving ethanol precipitation, butanol extraction, ion-exchange chromatography and gel filtration to give 1136-fold purification with a recovery of 19% (Yang et al. 1991). Certain microbial phytate-degrading enzymes have been available by cloning and heterologously expressing the corresponding genes instead of extraction and purification of the enzymes from the wild-type organisms. The recombinant phytases from *Emericella nidulans* (Wyss et al. 1999), *A. fumigatus* (Wyss et al. 1998, 1999), *A. terreus* (Wyss et al. 1999) as well as from the thermophilic fungi *Thermomyces lanuginosus* (Berka et al. 1998) have been characterized biochemically. Measuring the orthophosphate released by the enzymatic action employing a method based upon colorimetric measurements of phosphomolybdate can be used to determine phytase activity (Heinonen and Lahti 1981). The phytate preparation used for revealing phytase activity must devoid of lower myo-inositol phosphates or other phosphorylated compounds. The release of orthophosphate has to be restricted in enzymatic hydrolysis of phytate being a stepwise process in which each lower myo-inositol phosphate may become a substrate for further hydrolysis. The release of orthophosphate is restricted to a maximum 4% of the entirely available phytate phosphate in the assay (Konietzny et al. 1995) by excluding dephosphorylation of lower myo-inositol phosphates in the assay mixtures by the phytase enzyme under study. The separation and quantitative determination of phytate and lower myo-inositol phosphates can be done by reverse-phase C18 high-performance liquid chromatography (HPLC) (Skoglund et al. 1997). This approach is able to detect precisely the decline in phytate during enzymatic hydrolysis, but in regard with revealing phytase activity, the process is too long and considerable extent of lower myo-inositol phosphates in the assay mixtures has been produced until the decline in phytate could be quantified with sufficient precision.

17.8 Biochemical and Molecular Characterization of Enzyme

17.8.1 Effect of Temperature

The activity of purified phytases rises with the temperature increase as evident for most of the cases and was found maximum at its optimum temperature which further declines sharply at temperatures beyond its optimum value. The optimum temperature of phytase activity from *Arxula adeninivorans* is 75 °C at pH range between 4.5 and 5.0 (Sano et al. 1999), while several yeast strains (*Pichia*, *Torulaspora*, *Candida*, *Schwanniomyces* sp., *Kluyveromyces*) and fungus *Thermomyces lanuginosus* are having optimum temperatures in the range of 60–80 °C (Nakamura et al.

Table 17.3 Physico-chemical and kinetic properties of some of the purified phytases

Sources	Mol. Wt. KDa	Isoelectric point pI	Optimum pH	Temp. (°)	Substrate specificity	*Km* (mM)	References
Bacteria							
Bacillus sp. DS11	44	5.3	7.0	70	Specific	0.55	Kim et al. (1998a)
Bacillus amyloliquefaciens							Kim et al. (1999b)
Bacillus subtilis	36–38	6.25	6.0–6.5	60	Specific	0.50	Kerovuo et al. (1998, 2000)
Bacillus licheniformis(168 *phy*A, *phyL*)	44, 47	5.0, 5.1	4.5–6.0	55, 65	Specific		Tye et al. (2002)
Enterobacter sp.			7.0–7.5	50		0.70	Yoon et al. (1996)
Escherichia coli	42	6.3–6.5	4.5	60	Specific	0.13	Greiner et al. (1993)
Klebsiella terrigena	40		5.0	58	Specific		Greiner et al. (1997)
Mitsuokella multiacidus					Specific		Yanke et al. (1998)
Prevotella ruminicola							Yanke et al. (1998) and Cheng et al. (1999)
Pseudomonas syringae	45		5.5	40	Specific	0.38	Cho et al. (2003)
Lactobacillus sanfranciscensis	50		4.0	45			De Angelis et al. (2003)
Citrobacter braakii	47		4.0	50		0.46	Kim et al. (2003)
Yeasts							
Arxula *adeninivorans*			4.5	75	Specific	0.23	Sano et al. (1999)
Pichia pastoris	95		2.5,5.5	60			Han and lei (1999)
Schwanniomyces castellii	490			77			Segueilha et al. (1992)
S. occidentalis					Specific	0.038	Nakamura et al. (2000)
Saccharomyces cerevisiae	120		2.0–2.5	55–60			Han et al. (1999)
Fungi							
Aspergillus ficuum (phy A)	85	4.5	2.5, 5.0	58	Specific	0.027	Ullah and Gibson (1988)
Aspergillus ficuum	68	4.0	2.5	63	Broad	.103	Ehrlich et al. (1993) and Ullah (1987)

(continued)

Table 17.3 (continued)

Sources	Mol. Wt. KDa	Isoelectric point pI	Optimum pH	Optimum Temp. (°)	Substrate specificity	*Km* (mM)	References
Aspergillus oryzae	120	4.15	5.5	50	Broad	.33	Shimizu (1993)
Aspergillus niger SK	57		2.5,5.5	50	Specific	.0187	Nagashima et al. (1999)
Aspergillus Niger ATCC 9142	84		5.0	65	Broad	0.10	Casey and Walsh (2003)
Peniophora lycii (*phyA*)	72	3.61	4–4.5	50–55	Specific		Lassen et al. (2001)

Source: Vats and Banerjee (2004)

2000). *B. laevolacticus*-based phytase production has optimal temperature of 50 °C (Sano et al. 1999). The other bacteria such as *E. coli, Lactobacillus amylovorus*, *M. jalaludinii*, and *Bacillus* sp. yield optimally in mesophilic temperature range of 37–39 °C (Lan et al. 2002; Sreeramulu et al. 1996; Sunitha et al. 1999) optimally at 40 °C. Generally, phytase exhibits high activity in the temperature range of 50–70 °C, whereas optimum temperature is mainly between 45 and 60 °C (Gulati et al. 2007a, b; Singh and Satyanarayana 2008; Dahiya et al. 2010) (Table 17.3). Phytase (PhyC) isolated from *B. subtilis* VTTE-68013 has an optimum temperature at 55 °C (Kerovuo et al. 1998). *Pseudomonas syringae* MOK1-based phytase gives its optimal activity at 40 °C (Cho et al. 2003). *Bacillus* sp. MD2 phytase has optimal temperature for activity between 67 and 73 °C (Tran et al. 2010) and phytase from *B. megaterium*, *B. coagulans*, *B. pumilus* and *B. licheniformis* shows activity at temperature up to 80 °C and also exhibits thermostability and 50% activity at 70 °C (Dechavez et al. 2011). The optimum temperature of enzyme from *Bacillus cereus* isolate MTCC 10072 was 60 °C that is comparable to *A. ficuum* NTG-23 phytase (60 °C) and *A. niger* ATCC 9142 phytase (65 °C) (Casey and Walsh 2003).

Phytase from *A. niger* NW205 (63 °C) (Kostrewa et al. 1999) and *Kodamaea ohmeri* (65 °C) (Li et al. 2008) shows higher optimal temperature than that of *A. niger* van Teighem phytase (52–55 °C) (Vats and Banerjee 2005), *A. niger* NRRL 3135 *phyA* (58 °C) (Kostrewa et al. 1999) and *Bacillus* sp. KHU-10 phytase (40 °C) (Choi et al. 2001).

The *A. fumigatus*-based phytate-degrading enzymes are most resilient to high temperatures as incubation of the enzyme at 90 °C for 20 min resulted in only 10% loss of the initial activity (Pasamontes et al. 1997). It was found that this enzyme is not thermostable, but had a unique property to refold entirely into native-like, fully active conformation by giving heat treatment (Wyss et al. 1998). Practically, the crude extract of phytate-degrading enzymes are highly stable than purified enzyme for a technical application in feed and food processing.

17.8.2 Effect of pH

The histidine acid phosphatases and alkaline phytases are the two broad categories of phytase on the basis of pH of its activity. Acidic phytases are more favourite because of their wide application in animal feeds and broader substrate specificity than those of alkaline phytases (Kaur and Satyanarayana 2010). The potential of a phytase to catalyse phytate in the elementary canal is established by its enzymatic properties. As stomach is the core site of action for the augmented phytase, high tolerance to pepsin and acidic pH is undoubtedly enviable (Selle and Ravindran 2008). The purified phytase of *A. niger* showed significant activity below pH 3.0 in contrast to the *P. lycii* enzyme. Beside this, the purified phytase have high tolerance level to pepsin and acidic pH values than the industrially available *P. lycii* and *A. niger* enzymes. Its pepsin tolerance and resistance of acidic pH values is similar to that of the *E. coli* enzyme which is extraordinarily high under acidic conditions. Moreover, exposure at extremely low pH of 2.0 for longer duration did not cause in a substantial loss of activity (Greiner et al. 1993). A wide pH range of activity has been reported for thermophilic fungi, *Myceliophthora thermophile* and *A. fumigatus* (Wyss et al. 1999). *Rhizopus oryzae* phytases have dual pH optima at 1.5 and 5.5. Phytate purification from *Bacillus subtilis* can be achieved at a pH optimum between 7.0 and 7.5 (Powar and Jagannathan 1982). *B. subtilis* (natto) phytase was prepared to homogeneity and has shown a pH optimum between pH 6.0 and 6.5 (Shimizu 1992). Phytase from *B. subtilis* VTTE-68013 was isolated, purified and characterized at optimum pH 7.0 (Kerovuo et al. 1998). An extracellular phytase from *Bacillus* sp. KHU-10 was isolated, and the optimum pH was determined to be pH 6.5–8.5 at 40 °C without 10 mM $CaCl_2$ whereas pH 6.0–9.5 at 60 °C with 10 mM $CaCl_2$ (Choi et al. 2001). The enzyme was quiet stable from pH 6.5 to 10.0. Gulati et al. (2007a, b) illustrated that the partly purified phytase from *B. laevolacticus* was finest active at 70 °C and pH 7.0–8.0.

pH profile of phytases is also tailored by protein engineering. The pH range for enzyme activity of the *E. coli* phytase (Rodriguez et al. 2000a, b) or the *A. niger* phytase (Mullaney et al. 2002) was widened at acidic pH by mutagenesis. Moreover, phytases with diverse pH optima, ranging from 2.5 (*A. niger PhyB*) to 7.5 (several *Bacillus* sp.), are reported in literature (Oh et al. 2004). Although various potential sites of action such as the crop of poultry with a nearly neutral pH or strong acidic stomach make the focus to an ideal pH profile, the activity and stability of phytases are quite challenging (Lei and Stahl 2001). Successful trials of feeding were carried out with acidic phytase. The phytase having neutral pH optimum exhibits related biological activity. The prospective of these enzymes for industrial applications is yet to be scrutinized for neutral and alkaline phytases.

17.8.3 Catalytic Characterization

Complete degradation of phytic acid by phytases releases free inositol and orthophosphate via several intermediate products, viz. the mono-, di-, tri-, tetra- and

penta-esters of inositol. So, the release of orthophosphate or lower InsPs can determine phytase activity. Typically, inorganic phosphate is estimated by colorimetric technique where phosphomolybdate produced was isolated in organic solvent. Separation and quantitative estimation of phytic acid and lower InsPs is done by reverse-phase C18 HPLC (Burbano et al. 1995). Phytases are discriminated from acid phosphatase that is vulnerable of degrading phytate by its specificity for phytic acid under analysis (Konietzny et al. 1995). Cloning and overexpression of *phy* genes from six new *A. fumigatus* isolates help to examine the innate variability in amino acid sequences and their effect on catalytic activity of phytase (Brugger et al. 2003). Amongst all the proteins, phytase from *Sartorya fumigata* (anamorph of *A. fumigatus*) was highly variable (86% amino acid sequence identity) with more specific activity and discrete variation in catalytic properties.

17.8.4 Effect of Metal Ions

The need for metal ions for enzyme activity depends upon phytases. Metal ions can modify phytate-degrading activity, but it is hard to reveal whether the inhibitory influence of different metal ions is consequence of binding to the enzyme or the appearance of sparingly soluble metal ion-phytate complexes. The formation of a precipitate during addition of Fe^{2+} or Fe^{3+} to the analyte mixtures advises a fall in the active substrate concentration by the appearance of sparingly soluble iron-phytate (Konietzny et al. 1995). Cu^{2+} and Zn^{2+} can highly inhibit most phytate-degrading enzymes. Generally, ethylenediaminetetraacetic acid (EDTA) does not have major effect on the acid phytate-degrading enzymes except the phytases from *A. fumigatus*. The enzyme of *A. fumigatus* was activated up to 50% by EDTA treatment (Wyss et al. 1999), although alkaline phytate-degrading enzymes were highly inhibited by EDTA (Kerovuo et al. 2000) which is evident that a metal ion is required for optimal activity. Ca^{2+}-dependent and EDTA-activated enzymes are produced by *B. amyloliquefaciens* (Kim et al. 1998a), *B. subtilis* (Shimizu 1992; Kerovuo et al. 2000), cattail pollen (Hara et al. 1985) and lily pollen (Scott and Loewus 1986). Reducing agents including reduced glutathione, dithiothreitol and 2-mercaptoethanol do not show key influence on the enzymatic activity of phytase enzymes possibly because of having no any free and accessible sulphydryl group or that free sulphydryl groups play an insignificant role in enzymatic structure and activity. This conclusion is verified by the fact that most mature microbial phytases have an even number of cysteine residues which might be occupied in disulfide bridges as illustrated for the phytases from *E. coli* (Lim et al. 2000). The role of disulphide bonds in the phytate-degrading enzyme from *A. niger* is essential for the structure and activity of the enzyme which contribute in the folding of the protein (Ullah and Mullaney 1996). 2-Mercaptoethanol and dithiothreitol can activate phytases from *Cladosporium* sp. FP-1 which can be inhibited by Pb^{2+}, Ba^{2+}, phenylmethyl sulfonyl fluoride, iodoacetate and P-chloromercuribenzoate (Quan et al. 2004). *Rhizopus oryzae*-derived phytases are resilient to heavy metal ions and proteolysis. Cysteine residues do not appear in the phytases from *B. amyloliquefaciens* (Kim et al. 1998a, b)

and *B. subtilis* (Kerovuo et al. 1998). Fluoride is a robust competitive inhibitor of many acid bacterial, fungal and plant phytates. The inhibition constants range varies from 0.1 to 0.5 mm. However, fluoride cannot inhibit the alkaline phytases from lily pollen (Baldi et al. 1988), *B. subtilis* (Powar and Jagannathan 1982) and *B. amyloliquefaciens* (Kim et al. 1998a, b). Transition metals such as molybdate and vanadate are also recognized as inhibitor of phytases as their oxoanions exert their inhibitory effects by making complexes which mimic the trigonal bipyramidal geometry of the transition state (Zhang et al. 2001). In addition to the hydrolysis product phosphate, the substrate phytate was also known to inhibit various phytases. The modest phytate concentration essential to inhibit phytase activity ranges from 20 mm for the soybean enzyme (Gibson and Ullah 1988) and up to 300 mm for the maize root enzyme (Hubel and Beck 1996). The resident environment of the active site of the enzyme may be affected by the net charge as a result of the phosphate groups present in high substrate concentrations. This might hinder change of the enzyme-substrate complex to enzyme and product along with the formation of sparingly soluble protein-phytate complexes.

17.8.5 Effect of Different Substrates

The efficiency and restrictions of phytase supplementation may also rely on substrate specificity. The phytases with wide substrate specificity voluntarily disintegrate phytate to myo-inositol monophosphate with no main deposition of intermediates, though phytases with restricted substrate specificity lead to myo-inositol tris- and bisphosphate accumulation when phytate degradation linked with a gradual phosphate release rate (Wyss et al. 1999). To date, only the phytases from *Bacillus amyloliquefaciens* (Kim et al. 1998a, b) and *Bacillus subtilis* (Powar and Jagannathan 1982; Shimizu 1992) were known as substrate specific for polyphosphate compounds such as ATP, sodium phytate and sodium tripolyphosphate. The end product of phytate dephosphorylation was recognized as a myo-inositol trisphosphate (Greiner et al. 2002; Kerovuo et al. 2000). The pure phytase though releases five phosphate residues per phytate molecule which insinuate that myo-inositol monophosphate is the end product of enzymatic phytate degradation. Characterization investigation illustrated that the purified enzyme is best suitable for potential commercial interest as an animal feed additive. A wide substrate specificity range is an enviable property in phytase which has earlier been found in *M. thermophila* and *Emericella nidulans* previously (Pasamontes et al. 1997). *Rhizopus oligosporus*-derived phytases show wide affinity for different phosphorylated compounds. Phytases generally exhibit wide substrate specificity. Frequently hydrolysed substrates include guanosine monophosphate (GMP), guanosine trisphosphate (GTP), nicotinamide adenine dinucleotide phosphate (NADP), adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), *p*-nitrophenyl phosphate, 1-naphthyl phosphate, phenyl phosphate, 2-naphthylphosphate, galactose 1-phosphate, fructose 1,6-diphosphate, fructose 6-phosphate, glucose 1-phosphate, glucose 6-phosphate, pyridoxal phosphate, α-glycerophosphate,

ß-glycerophosphate, o-phospho-l-serine and pyrophosphate. Merely a small number of phytases have been illustrated as very specific for phytate, viz. the alkaline phytase enzymes from *B. amyloliquefaciens* (Kim et al. 1998a) and *B. subtilis* (Powar and Jagannathan 1982; Shimizu 1992). The acid phytase enzymes from *A. niger*, *A. terreus* (Wyss et al. 1999) and *E. coli* (Greiner et al. 1993) have also been found to be specific for phytate. All phytase enzymes studied so far adhere to Michaelis-Menten kinetics except phytase enzymes from *Myceliophthora thermophila* and *Emericella nidulans* (Pasamontes et al. 1997). Usually, phytases from microbial sources show the highest turnover number, although the related plant enzymes carry out the maximum relative rates of hydrolysis with ATP and pyrophosphate, even though many phytases characterized so far conferred the maximum affinity to phytate among all phosphorylated compounds studied. The kinetic efficiency of an enzyme is confirmed via the k_{cat}/K_m values for a given substrate. The *E. coli* phytases have a highest value of a k_{cat}/K_m value of 1.34×10^7 M $^{-1}$ s^{-1}min^{-1} reported so far (Golovan et al. 2000). The previously accounted turnover number of 6209 s^{-1} and k_{cat}/K_m value of 4.78×10^7 M^{-1} s^{-1} for the phytase enzyme from *E. coli* (Greiner et al. 1993) was hyped due to a low estimation of enzyme concentration by the Bradford assay employed for protein estimation (Golovan et al. 2000). However, the relative rates of hydrolysis of certain phosphorylated compounds such as pyrophosphate and ATP by plant-based phytases are considerably higher than those of phytate; such phytases gave the highest k_{cat}/K_m values with phytate. Hence, the kinetic parameters illustrate phytate to be the probable substrate for the phytase enzyme from plants under certain physiological circumstances. Microbial phytase-based investigations deduced that enzymes with a wide substrate specificity voluntarily degrade phytate to myo-inositol monophosphate without accumulation of intermediates, but enzymes with restricted substrate specificity produce myo-inositol tris- and bisphosphate accumulation during phytate degradation simultaneous with a growing rate of phosphate release, which conclude that lower myo-inositol phosphates are poorer substrates than phytate (Wyss et al. 1999). Moreover, the variation in the substrate specificity of the two classes of phytate-degrading enzymes reveals a selective difference in the specific activities with phytate to a substantial extent, because phytase enzymes with wide substrate specificity innately had lesser specific activities with phytate as substrate, whereas the phytases with restricted substrate specificity had greater specific activities. Phytase from plants, *B. subtilis* and *B. amyloliquefaciens*, do not adhere to this classification. The plant enzymes have wide substrate specificity and greater specific activity; but the *Bacillus* enzymes seem to be highly specific for phytate instead of apparently low specific activity. Phytate degradation by plant-based phytases is accompanied with a growing rate of phosphate release along with an accumulation of myo-inositol tetrakis-, tris- and bisphosphates (Greiner 2001).

17.8.6 Effect of Calcium Ions

Bacillus phytases show metal ion dependency by requiring calcium for their activity and stability (Kim et al. 1998a, b; Powar and Jagannathan 1982; Shimizu 1992; Oh et al. 2001). Ca^{2+} attaches two oxyanions of the phosphate groups in phytate to produce a precise positively charged calcium-phytate complex. Ca^{2+} works as an important activator to diminish the negative charge around the active site cleft due to the occurrence of three Ca^{2+} binding sites at the active site cleft, whereas excessive quantity of Ca^{2+} acts as a competitive inhibitor (Oh et al. 2001). Additionally, Ca^{2+} exert an essential influence on the stability against temperature and pH (Choi et al. 2001). Removal of metal ions from the enzyme by EDTA leads to absolute inactivation in *B. subtilis* phytase (Kerovuo et al. 2000). The conformational change may lead to the loss of enzymatic activity confer, as the circular dichroism spectra of holoenzyme and metal-depleted enzyme were unrelated. Metal-depleted enzyme was partially capable of restoring the active conformation if incubated by application of calcium. Slighter reactivation was noticed with other divalent metal ions and their combinations. Intake of phytase (and phytate) influences digestibility of calcium along with carbohydrates and amino acids.

17.8.7 Thermostability of Enzyme

Recent focus on the isolation, characterization and engineering of enzyme along with the determinants of thermostability is due to the increasing commercial significance of thermostable enzymes. Thermostability is essential for the profitable application of enzymes in animal feed which are exposed to 60–90 °C during pelleting process. Thus designing phytases for enhancing their catalytic properties under diverse conditions is of much interest. The pH activity profile of phytases (*A. fumigatus* and consensus phytases) was engineered by rational mutagenesis for the probable commercial applications of phytase in animal feed (Tomschy et al. 2002). New consensus method can be used for improving the intrinsic thermostability of fungal phytases (Lehmann et al. 2000). Sequence comparison investigations of 13 phytases from six distinct fungi and selection of highly conserved location of each residue led to the de novo simulation of consensus phytase with improved thermostability. Later, the consensus amino acid sequences of six additional phytase sequences were included and studied 38 amino acid replacements by site-directed mutagenesis. The inclusion of stabilizing amino acids in consensus phytase sequence enhanced the unfolding temperature of phytase 1 and 10, thereby sustaining the general validity of consensus approach for improving thermostability by engineering of proteins (Lehmann et al. 2000). Improvement of thermal resistance and enhancement in specific activity are two significant issues not only for animal feed but also for applications of phytases in food processing. Diverse strategies have been employed to prepare an enzyme capable of tolerating higher temperatures. *E. coli* phytases expressing in the yeast *Pichia pastoris* after introduction of three glycosylation sites into the amino acid sequence by site-directed mutagenesis shift temperature

optimum from 55 to 65 °C and show considerable improvement in its thermal stability at 80 and 90 °C (Rodriguez et al. 2000b). Thermostability is mainly a significant factor since feed pelleting is usually carried out at temperatures between 65 and 95 °C, even though the process is carried out by employing after-spray equipment for pelleted diets and/or by passing heat destruction by chemical coating of phytase to tolerate high temperatures will certainly be superior candidates for feed supplements. Contrast to the commercially available phytases from *P. lycii* and *A. niger*, the pure enzyme shows a greater thermal stability. *E. coli*-based commercially available phytase was found to confer a higher thermal stability than the purified enzyme (Garrett et al. 2004). The purified phytase retained half of the initial activity if exposed to 70 °C for 10 min, although the enzymes from *A. niger* lose 60% activity and the phytase from *P. lycii* was completely inactivated at 70 °C after 15 s (Ullah and Sethumadhavan 2003). The *E. coli* enzyme did not lose any activity after heating at 62 °C for 1 h even 27% residual activity was retained at 85 °C after 10 min (Garrett et al. 2004). The pure phytase lose 88% of the initial activity at 80 °C after 10 min. The *Pichia anomala* and *Schwanniomyces castellii* phytases are highly tolerable to high temperatures reported so far (Segueilha et al. 1992; Vohra and Satyanarayana 2002). Exposure of these enzymes at 70 °C for 10 min did not cause any considerable loss of activity, and even the *Pichia anomala* phytase was reported to tolerate a 70 °C temperature after 30 h treatment without any significant loss of activity (Vohra and Satyanarayana 2002). So far, no phytase was used for commercial application. The pelleting of feed facilitates animals to take a balanced diet and helps preservation of enzyme-containing food and feed, though enzyme is exposed to temperatures around 80–85 °C for 2 min during the pelleting process (Wyss et al. 1998).

17.8.8 Proteolysis Resistance

An effectual phytase must harbour a sharp tolerance to hydrolytic breakdown by proteinases occurring in the elementary canal. Fungal and bacterial phytases exhibit various perception to pepsin and trypsin (Kerovuo et al. 1998; Rodriguez et al. 1999). Bacterial phytases appear to bear more tolerance than fungal counterpart (Igbasan et al. 2000). However phytase from *S. thermophile* is unaffected by trypsin and pepsin and also stay insensitive to EDTA Singh and Satyanarayana (2010) Site-directed mutagenesis promise to engineer the protease- susceptible positions of phytases by blocking or modifying them which generally reside in the exposed loops at the surface of the molecules (Wyss et al. 1999).

17.8.9 Immobilization of Phytase

Phytases perform serial action on *myo*-inositol hexakisphosphate to release numerous lower isomers. Therefore, a proficient immobilized bioreactor can generate several isomers of phytic acid also rendering the molecule non-chelator of metal ions,

proteins, etc. *Candida krusei* cells were immobilized in Ca^{2+}-alginate gel beads for the formation of various InsPs where the pure isomers were separated by ion-exchange chromatography (Quan et al. 2002). Even single isomer of each inositol phosphate was produced by NMR analysis except *myo*-inositol pentakisphosphate. Extracellular phytase were produced by *R. oligosporus* and *A. ficuum* in SSF pH 6.0, 30 °C, 58.3% RH by with polystyrene as inert solid support (Gautam et al. 2002). Ullah and Cummins (1987) designed a packed-bed bioreactor for culturing *A. niger* NRRL 3135 phyA by covalently immobilizing on Fractogel TSK HW-75F. Although no swing in pH optima was found, temperature optima swinged from 58 to 65 °C with a rise of K_m for phytate, and there was increased release of phosphorus up to 50%. HPLC analysis of products only detected $InsP_2$ and InsP in the eluate subsequent to repeated hydrolysis of phytate in bioreactor. Immobilization by using protein backbone can drop the phytase activity and bioreactor's output. Immobilization approach can enhance thermostability of phytases (Pandey et al. 2001). Simple alginate immobilization of *Sporotrichum thermophile* can sustainably produce phytase up to five repeated cycles (Singh et al. 2011). It may be because of the distortion of functioning centre of phytase by widespread cross-linking of enzyme to the protein matrix. Preclusion of extensive cross-linking is tough due to heavily glycosylated native phytase. It should be feasible to amputate glycosyl residues by site-directed mutagenesis to give an enzyme that can be immobilized by a few carbohydrate moieties along with retaining a high level of phytase activity (Dischinger and Ullah 1992). The covalently immobilizing of *E. coli* phytase on NHS-activated Sepharose® can enhance heat resistance of the enzyme (Greiner and Konietzny 1996). Liu et al. (1999) shifted the temperature optima of *A. ficuum* phytase from 50 to 58 °C by immobilizing it in gelatin gels and further hardening with formaldehyde. Noticeably K_m has enhanced to 3.28 mM (K_m = 2.34 mM for free enzyme) and the residual enzyme activity was 34.6% only.

17.8.10 Molecular Characteristics

17.8.10.1 Molecular Weight

Mostly phytases characterized so far act as monomeric proteins with molecular weight between 40 and 70 KDa (Table 17.2), while certain phytases seem to contain multiple protein subunits. The phytase from *Schwanniomyces castellii* was known for its tetrameric protein with three identical small subunits of 70 KDa and one large subunit of 125 KDa (Segueilha et al. 1992), and rat intestine phytase was found to be a heterodimer consisting of 70 and 90 KDa subunit where both subunits were encoded differentially during the development of the rat (Yang et al. 1991), these subunits may signify two separate enzymes. The phytases from maize roots (Hubel and Beck 1996), tomato roots (Li et al. 1997), germinating maize seeds (Laboure et al. 1993), *A. oryzae* (Shimizu 1993) and soybean seeds (Hegeman and Grabau 2001) were found to be homodimeric proteins, but a homohexameric structure was assumed for the *A. terreus* enzyme (Yamamoto et al. 1972). In other reports there was no suggestion of an oligomeric structure of the phytases from soybeans (Gibson

and Ullah 1988) and *A. terreus* (Wyss et al. 1999). This contradictory assessment was advised to be the consequence of using gel filtration or native PAGE (polyacrylamide gel electrophoresis) for determination of molecular mass. These techniques can misjudge the molecular mass, and the inaccuracy intensifies with the level of glycosylation (Wyss et al. 1999). Thus, the assumption of the existence of oligomeric phytate-degrading enzymes should be considered with prudence in support of more credible confirmation that oligomeric forms exist. Two different forms of phytases have been found in *K. aerogenes* (Tambe et al. 1994). One is probably the native enzyme having a remarkably large size (700 KDa), while the other, possibly a fraction of the native enzyme, shows an extremely small molecular mass (10–13 KDa) but a full complement of activity. Phytase from *Sporotrichum thermophile* was reported to be homopentameric glycoprotein with molecular mass 456 KDa (Singh et al. 2011). Fungal and several plant phytases have been noticed to be glycosylated. The galactose and N-linked mannose of native enzyme from *A. niger* NRRL 3135 represent 27.3% of its molecular mass (Ullah 1988). The catalytic properties, isoelectric point and the stability of an enzyme are affected by glycosylation. Amazingly, diverse level of glycosylation does not influence the thermostability and refolding properties of the phytases from *A. niger* and *A. fumigatus*.

17.8.10.2 Amino Acid Sequence and Structural Properties

The amino acid sequences of numerous fungal phytases have been established in recent years. The primary sequences of the phytases from *A. terreus* (Mitchell et al. 1997), *A. niger var. awamori* (Piddington et al. 1993), *Talaromyces thermophilus* (Pasamontes et al. 1997), *A. fumigatus* (Pasamontes et al. 1997), *Emericella nidulans*, *Thermomyces lanuginosus* (Berka et al. 1998) and *Myceliophthora thermophila* (Mitchell et al. 1997) exhibited 60, 97, 61, 66, 48 and 47% homology to the related enzyme from *A. niger* NRRL 3135, while the bacterial phytases from *E. coli* did not exhibit clear sequence similarity (Rodriguez et al. 2000a), although all phytases enjoy the highly conserved sequence motif RHGXRXP known for phosphate acceptor site near the N-terminus. Besides this, they carry a conserved HD motif near the C-end where the aspartate is assumed to be the proton donor for the substrate detaching group. The amino acid sequence of the phytases from maize seedling seems to be entirely distinctive from one of the related enzymes from *A. niger*, but a homologous region of 33 amino acids having the RHGXRXP motif was known (Maugenest et al. 1997). All these phytases come under the subfamily of histidine acid phosphatases (Mitchell et al. 1997). The primary sequence of the phytases from *B. subtilis* (Kerovuo et al. 1998) and *B. amyloliquefaciens* (Kim et al. 1998a) does not have homology to the online databases; moreover, the presumed active-site motifs RHGXRXP and HD found in histidine acid phosphatases are missing. Hence, the *Bacillus* phytase enzymes signify a novel class of phosphatases. Later on, it was found that a phytase enzyme from soybeans was also deficient in the RHGXRXP motif (Hegeman and Grabau 2001). Instead, this enzyme includes characteristic motif of a large group of phosphoesterases, involving purple acid phosphatases. Purple acid phosphatases include binuclear Fe (III)-Me (II) centres where Me may be Fe, Mn or Zn. A phytase (*PhyAsr*) coming under the protein tyrosine phosphatase

(PTP) superfamily has been differentiated from the anaerobic, ruminal bacterium *Selenomonas ruminantium* (Aaron 2006). Investigation reveals that *PhyAsr* has a conserved PTP-like active-site signature sequence (C(X)5R) and a PTP-like fold which assists a standard PTP mechanism of dephosphorylation (Chu et al. 2004). Other thermostable phytase gene of 1404 bp size, expressing for presumed phytases of 468 amino acid residues, has been recognized in *Aspergillus japonicas* BCC18313 (TR86) and BCC18081 (TR170) except for a new phytase gene from *Aspergillus niger* (Promdonkoy et al. 2009). A novel phytase gene, *appA*, of 1302 bp encoding 433 amino acid residues with 27 residues of a putative signal peptide has been cloned by degenerate PCR in *P. wasabiae* (Shao et al. 2008). A phytase gene coming under the β-propeller phytase family and approximately 28.5% identical with *Bacillus subtilis* phytase has been known in *Pedobacter nyackensis* MJ11 CGMCC 2503 (Huang et al. 2009). An extracellular phytase (PHY US417) of 41 KDa molecular weight is extracted to pure form and characterized in *Bacillus subtilis* US417 (Farhat et al. 2008). Besides this, other phytase genes, as a promising ideal feed additive for increasing the phytate phosphorus digestibility in monogastric animals, were cloned and characterized in other species, such as in *Escherichia coli* (*appA*) (Hong et al. 2004), ruminal bacterium *Selenomonas ruminantium*(*SrPf6*) and *Peniophora lycii* (Xiong et al. 2006). HP-HAP-like domain and 24 amino acid signal peptide at N-terminal are present in OSPHY2 gene from rice that produces phytase targeted to cytoplasm.

17.8.10.3 Molecular Biology and Gene Expression

The phytases of the fungi *Talaromyces thermophilus* and *Emericella nidulans* were cloned which encoded 463 and 466 amino acids with a molecular weight of 51,450 and 51,785, respectively (Pasamontes et al. 1997). Both putative amino acid sequences were highly identical up to 48–67% to known phytases. OSPHY2, a histidine acid phosphatase from rice (*Oryza sativa L.*), is 2060-bp-long sequence which codes for polypeptide of 519 amino acids (Li et al. 2011). Lehmann et al. (2000) tried to make consensus phytases by using amino acid sequence comparisons due to non-availability of natural phytases having thermostability for application in animal feeding. An accorded enzyme based on 13 fungal phytase sequences had normal catalytic properties, but exhibited an unpredicted 15–22 °C enhancement in unfolding temperature than parental counterparts. For the first time, the crystal structure of consensus phytase was deduced to compare with that of *A. niger* phytase which helps to gain insight into the molecular basis of increased heat resistance. *A. niger* phytase unfolded at very low temperatures. There is a direct link existing between protein sequence conservation and protein stability for fungal phytases. The expression profile of an *A. niger* phytase gene (*phyA*) was studied in *S. cerevisiae* for determining the effects of glycosylation on the activity of phytase and its thermostability (Han et al. 1999). A 1.4 kb DNA segment harbouring the coding region of the *phyA* gene was incorporated into the expression vector *pYES2* to express in *S. cerevisiae* as an active extracellular phytase. The level of total extracellular phytase activity was influenced by the medium composition and the signal peptide. The expressed phytase had a molecular size of about 120 KDa, a

temperature optimum between 55 and 60 °C and two pH optima at 2.0–2.5 and 5.0–5.5. A 9% loss of activity and 40% of thermostability were reported by deglycosylation of the phytase. The gene (*phyA*) isolated from *A. niger* with optima at pH 5.5 and 2.2 was cloned and expressed in *E. coli* under regulation of T_7*lac* promoter (Phillipy and Mullaney 1997). One hundred sixty-eight *PhyA* phytase genes from *B. subtilis* along with Phy L gene from *B. licheniformis* were cloned to overexpress in *B. subtilis* in ϕ105M0331 prophage vector system (Tye et al. 2002). Han and Lei (1999) investigated the expression profile of a phytase gene (*phyA*) isolated from *A. niger* in *Pichia pastoris*, and Guerrero-Olazarán et al. (2010) expressed *B. subtilis* phytase gene in *Pichia pastoris*. Both host strains yielded high amounts of active phytase (25–65 units/ml of medium) which was mostly released into the medium. Yao et al. (1998) tailored the phytase gene *phyA2* by removing signal peptide encoding sequence and intron sequence. *Yersinia kristensenii* appA gene with 1326-bp-long ORF expressing protein of 441 amino acid includes 24 amino acid signal peptide which was cloned and heterologously expressed in *P. pastoris* to extract a PH-resistant and thermostable phytase (Huang et al. 2008). The *Thermomyces* phytase maintained its activity at 75 °C and exhibited greater catalytic properties than any fungal phytase at 65 °C as optimum temperature (Berka et al. 1998). A large-scale expression of phytase was achieved in *B. subtilis* (Kim et al. 1999b). Kim et al. (1998a) cloned *Bacillus* sp. DS11 phytase gene into *E. coli* which encoded a 2.2 kb fragment.

17.8.10.4 Isoelectric Point (pI)

All phytates from bacteria, plant and fungi have acidic isoelectric point values except *A. fumigatus* enzyme, which has a pI of about 8.6 (Wyss et al. 1999), *B. licheniformis* (168 *phyA*, *phyL*) has a pI of 5.0, 5.1(Tye et al. 2002), *K. terrigena* has a pI of 5.5 (Greiner et al. 1997) and *B. subtilis* has a pI in between 6.3 and 6.5 (Kerovuo et al. 1998). Golovan et al. (2000) purified phytase of 45 kDa size by chromatofocussing from *E. coli* and separated it into two isoforms of identical size with pI of 6.5 and 6.3. The enzyme from *Bacillus* sp. KHU10 was found to have pI value of 6.8 (Choi et al. 2001). The enzyme from other *Bacillus* sp. exhibited an pI value at 6.25 (Shimizu 1992).

17.9 Application of Phytase Enzyme

17.9.1 Animal Feed

Phytases can be included into commercial swine, poultry and fish diets and have broader applications in animal nutrition due to its property to reduce phosphorus excretion of monogastric animals by substituting inorganic phosphates in the animal diet, add considerably for environmental protection, and make increased availability of minerals, amino acids, trace elements and energy (Vats and Banerjee 2004; Haefner et al. 2005). The phosphorus accessibility can be enhanced by using phytase-rich cereal diet or by incorporating microbial phytase to the feed (Nelson

1967). The enzyme diminishes the requirement for augmentation with inorganic phosphorus because of enhancement in the use of organic phosphorus in poultry and hence noticeably dropping the excretion of phosphorus in fertilizer (Kuhar et al. 2009). The inclusion of phytase as a feed supplement is costly approach due to its inactivation by heating required for pelleting. Thermotolerant phytase is desired for animal feed applications (Mullaney et al. 2000). This problem is conquered by producing phytase endogenously in poultry and swine (Selle and Ravindran 2008). This heat resistance feature of phytases led to its cloning from thermophilic fungi such as *T. thermophilus* and *M. thermophila*. Introduction of disulphide bonds and usage of certain compounds like polyols and salts, sorghum liquor wastes (Chen et al. 2001) and calcium (Kim et al. 1998b) can also improve thermostability of enzyme. For commercial applications of phytase as animal feed, it must be optimally active in the natural pH range of digestive tract of animals. Supplementation of fungal phytase in diets for swine and poultry helps in substantial enhancement in phosphorus retention. Inclusion of fungal phytase up to 1000 U/kg in corn/SBM-based diets of pigs and broilers can improve phosphorus retention from 52% to 64% (Kornegay et al. 1999) and from 50% to 60%, respectively (Simons et al. 1990; Kornegay et al. 1996). Augmentation of broiler chickens with industrial phytase has enhanced concentration of NPP, P, albumin and total protein and reduced serum Mg; however, Ca remain unaffected. It can raise activity of aspartate amino transferases and decrease activity of alkaline phosphatase and alanine amino transferase, but did not influence haematological and biochemical parameters of chickens. Industrial phytases are usually tailored by recombinant DNA technology approach. For instance, a bacterial phytase gene has been successfully introduced into yeast genome for its industrial production. Modern genetic engineering has highly improved functional use of phytases by increasing their pH specificity, thermostability and resistance to proteases in the digestive tract of animal (Li et al. 2009).

Efficiency of phytase augmentation, although, relies on the form of the enzyme (coated, size of the particle, etc.), microbial source, pH and temperature optima of the enzyme, diet manufacturing methodology, diet mineral level (Ca, Mg, Fe, Cu and Zn), constituents used in the diet, form of the diet (pelleted, mash or liquid), type and level of vitamin D metabolites, location of addition of phytase (postpelleting or mixer), disease condition of the animal and other factors (Ravindran et al. 1995). Zeng et al. (2001) reported that *Bacillus* phytase augmentation of 300 U/Kg furnishes the same effect as 1000 U/Kg acidic or neutral phytase augmentation.

Microbial phytases encouragingly influenced the pigs' health recital and their daily gain; moreover, the feed conversion ratios were restructured by organic acids (Kim et al. 2005; Pomar et al. 2008; Akinmusire and Adeola 2009; Hill et al. 2009). Dietary modulations to assist the action of exogenous phytases should be taken into account and implemented suitably. *Lactobacillus* and phytase boost up nutrient accessibility by looking after gut microflora and impede digestive diseases which eventually help in pig production and feed (Veum and Ellersieck 2008). Genetically modified *Lactobacillus* for phytase enzyme is advantageous for the gastrointestinal tract and animal health by improving *Lactobacillus* growth and hindering *E. coli* proliferation in pig gut which is related with low diarrhoea rate. Minimal dietary

concentration of calcium and phosphorus and limited Ca/P ratio seem beneficial. The concurrent addition of phytase and xylanase in wheat-based diets can give synergistic gain in digestibility of certain amino acids (Ravindran et al. 1999; Selle et al. 2003). Phytate is accumulated in the aleurone coating of wheat (Ravindran et al. 1995), and xylanase assists contact of phytase to its substrate in the aleurone-like proteolytic enzymes (Parkkonen et al. 1997). The harmonizing use of 3-phytase, 6-phytase and acid phosphatase in broilers may also enhance phytate degradation rates in poultry (Zyla et al. 2004). Amalgamation of phytase enzyme and carbohydrase improves nutrient availability for digestion and eventually feeding value of wheat soybean meal diets having full-fat rapeseed (canola) for poultry. Usually for phytase, the ‘nutrient release or equivalency’ values are legitimately unaffected from raw material employed. The original phytase feed is produced mostly from fungi. But recent developments in genetic engineering in other forms of microorganisms, such as bacteria and yeast, produced new exogenous phytases. It is evident that such bacterial phytases may be more effectual in broiler chickens. For instance, the *E. coli* phytase released more P in broilers than two recombinant fungal phytases based on improvement in tibia ash compared to inorganic P supplementation (Augspurger et al. 2003). Higher body weight, low mortality, better feed utilization and low phosphorus content in faeces were found in broiler fed with test diets with lowered phosphorus level and phytase. *E. coli* phytase is reported to be more tolerant to pepsin activity than fungal counterpart (Rodriguez et al. 2000a; Igbasan et al. 2000), which may account for the enhanced release of phytate-bound phosphorus. Nonetheless, ‘second-generation’ phytase feed enzymes with a naturally higher potential to hydrolyse dietary phytate which can lower phosphorus excretion and produce higher amino acid and energy responses will likely be developed in the future. The ideal enzyme would have good thermostability during feed processing, great specific catalytic activity (per unit of protein), increased activity under broad ranges of gut pH, resistance to proteolysis and better stability under ambient temperatures.

17.9.2 Food Industry

A phytate-rich diet significantly decreases absorption of dietary minerals (Konietzny and Greiner 2002), and the dephosphorylation of phytate at some point in food processing leads to the production of only incompletely phosphorylated myo-inositol phosphate esters with a lower potential to impair with the intestinal uptake of dietary minerals (Sandberg et al. 1999). Occurrence of phytate in plant foodstuffs results in mineral scarcity because of chelation of metal ions. Phytic acid present in rapeseed brings about Mg, Zn and Ca deficiency in chickens. Inclusion of phytase to high phytate holding diet increases assimilation and consumption of phosphorus. Many significant physiological functions in human beings are carried out by individual myo-inositol phosphate esters (Shears 1998). Thus, phytases may obtain application in food processing to make functional foods (Greiner et al. 2002), if such biochemically effective myo-inositol phosphate esters is produced by phytases and

absorbed in the digestive tract of humans. Phytases isolated from *A. niger* decrease IP6 content without distressing quality of brown rice bread during preparation. Mineral uptake is improved by phytase augmentation in food (Kuhar et al. 2009). In addition to increased mineral and trace element bioavailability, inclusion of phytase during food processing can influence cost of the manufacture process with yield and quality of the end products. Practical improvements by including phytase during food processing can be achieved for bread making (Haros et al. 2001), corn wet milling (Antrim et al. 1997), manufacturing of plant protein isolates (Fredrikson et al. 2001) and the fractionation of cereal bran (Kvist et al. 2005).

17.9.3 Preparation of Myo-inositol Phosphates for Other Health Benefits

Metabolic impacts of some myo-inositol phosphates are known such as diabetes mellitus, coronary heart disease, atherosclerosis, HIV and heavy metatoxicity (Kuhar et al. 2009; Claxon et al. 1990; Ruf et al. 1991; Siren et al. 1991; Carrington et al. 1993), improvements in heart disease by regulating atherosclerosis and hypercholesterolaemia (Jariwalla et al. 1990), lowered risk of colon cancer (Yang and Shamsuddin 1995) and deterrence of kidney stone formation (Ohkawa et al. 1984). Besides this, much interest has been paid on lower myo-inositol phosphates, in particular the intracellular second messengers d-myo-inositol (1,3,4,5)-tetrakisphosphate and d-myo-inositol (1,4,5)-trisphosphate which influence cellular metabolism and secretion by activating intracellular secretion of calcium (Greiner and Konietzny 2006). Various phytates may produce several positional isomers of the lower myo-inositol phosphates and hence in diverse physiological impacts (Singh et al. 2008). Phytase intake endorses against several cancers through antioxidation properties, cell cycle inhibition and interruption of cellular signal transduction and enhancement of NK cell activity (Kuhar et al. 2009). By knowing health benefits of the phytases, they may also find use in food processing to supplement foods with enhanced nutritional qualities, health benefits and maintained sensory feature (functional foods) (Singh et al. 2008, 2011).

17.9.4 Potential in Aquaculture

In aquaculture, 70% of total fish production expenses are spent on feed (Rumsey 1993). Attempts were made to utilize soybean meal or other plant meals in aquaculture to replace a more expensive protein source such as menhaden fish meal from low-cost plant protein for considerable reduction of cost (Mullaney et al. 2000). The anti-nutritive impact of phytic acid is highly challenging in fish because of their small gastrointestinal tracts (Richardson et al. 1985), which consecutively hamper the application of plant-origin protein in fish feed. Therefore, phytase has been assessed as a method to support the application of inexpensive plant meals in the aquaculture industry and to sustain tolerable phosphorus levels in water. Phytase

from *Klebsiella sp*. and *Bacillus sp*. due to its optimum pH and excellent thermostability is appropriate for universal carp feed. Phytases may offer the extra benefit in freshwater and marine aquaculture because of their probiotic function. Attempts have also been made on appliance of cell-bound phytase of *Pichia anomala* (Vohra and Satyanarayana 2003) in increasing the growth of the freshwater fish rohu (*Labeo rohita*), the marine seabass (*Lates calcarifer*) and the magur (*Clarias batrachus*) by modifying phosphate absorption. Phytase incorporated yeast biomass were supplied as food to the fish which were monitored for growth, phosphorus absorption, and phosphorus excretion. Thus, phytase has been assessed as a method to both enhance the application of inexpensive plant meals in the aquaculture industry and to sustain suitable phosphorus levels in the water.

17.9.5 Pulp and Paper Industry

Yellowing of papers is one of the major difficulties faced by the pulp and paper industries. A thermo-resistant phytase performs as a new biological agent who decomposes phytic acid at some stage in pulp and paper processing (Vohra and Satyanarayana 2003). The enzymatic decay of phytic acid would not consequence in the formation of toxic and mutagenic by-product, and simultaneously ageing of paper can be precluded. Accordingly, the application of phytases in pulp and paper industry could be recyclable and would assist in the development of hygienic technologies (Liu et al. 1998).

17.9.6 As a Soil Amendment

Phytic acid and its products may signify about 50% of the total organic phosphorus in the soil in the several locations (Dalal 1997). Findenegg and Nelemans (1993) investigated the impact of phytase on the accessibility of phosphorus in the soil from phytic acid for maize plants. An improved rate of phytin degradation was found upon inclusion of phytase to the soil which enhanced growth stimulus Singh and Satyanarayana (2010). This report also advocates that the appearance of phytase in the roots of genetically modified plants may enhance the availability of phosphorus to plant roots (Day 1996).

17.9.7 Semi-synthesis of Peroxidase

Peroxidases are universal enzymes that catalyse various selective oxidations with hydrogen peroxide as the primary oxidant (Correia et al. 2008). The active site of vanadium chloroperoxidase from *Curvularia inaequalis* strongly bears a resemblance to that of the acid phosphatases, and the apoenzyme of vanadium chloroperoxidase confers phosphatase-like activity (Hemrika et al. 1997). The permutation of phytase with vanadate formed an efficient semi-synthetic peroxidase. The influence of pH on the vanadate

phytase-mediated oxidation of thioanisole found that the pH optimum corresponded with that of phytase. Optimization resulted into the highest enantiomeric excess (ee) of 68% attained in formate buffer at 4 °C. The vanadium-integrated phytase was durable for more than 3 days with only an insignificant decline in activity. A cross-linked comprehensive enzyme of 3-phytase was converted into peroxidase by integration of vanadate (Correia et al. 2008). The cross-linked comprehensive phytase exhibited comparable efficacy and uneven activation as the free enzyme. Furthermore, the cross-linked comprehensive phytase can be reclaimed as a minimum three times with no considerable loss of activity. Certain other acids, hydrolases and phosphatases were tested for peroxidase activity upon integration with vanadate ion. Phytases from *A. fumigatus*, *A. ficuum* and *A. nidulans*, phospholipase D from cabbage and sulfatase from *Helix pomatia* mediated the enantioselective oxygen transfer processes by integrating vanadium.

17.10 Market Trends and Manufacture

The phytase enzymes have come into view as big feed augment. Feed enzymes (protease, phytase, xylanase, amylase, lipase, cellulase, β-glucanase) are recently increasing in the animal nutrition market with rapid growth which is estimated to attain $14.1 billion by 2019. Currently, approximately 6% of available animal feeds carry enzymes, about 80–90% for vitamins, which is known as greatest animal nutrition class (Anons 1998). Numerous major animal nutrition industries are emerging in this area very dynamically, and diverse products under discrete trade names are already offered to the marketplace. For instance, 'Cenzyme' is a product from Cenzone, which is an exclusive combination of intense digestive enzymes with phytase from a fungal source which is used as animal feed (Cenzone 1999). The biggest fraction of market (~40%) in feed enzymes is covered by Finnfeeds International, a unit of Finland's Cultor, followed by BASF (Simon and Igbasan 2002; Misset 2003). The latter, which has marketing contract with Dutch-based enzyme producer company Gist-Brocades, is the global leader marketer of phytase. Finnfeeds has currently manufactured a phytase. Novo industry is also marketing a phytase in Europe since 1998. Alltech has founded a production facility in Mexico for the manufacturing of phytase in 1999 (Table 17.4).

Table 17.4 Phytase preparations authorized in the EU as feed additives

Company	Trademark	Phytase source	Production strain	References
BASF	Natuphos	*Aspergillus niger var. ficuum*	*Aspergillus niger*	Simon and Igbasan (2002), Misset (2003), and European Union (2004a)
AB enzymes	Finase	*Aspergillus awamori*	*Trichoderma reesei*	Simon and Igbasan (2002), Misset (2003), and European Union (2004b)
Novozymes	Bio-Feed phytase	*Peniophora lycii*	*Aspergillus oryzae*	Simon and Igbasan (2002), Misset (2003), and European Union (2004c, d)

17.11 Recent Status of Research and Development in the Area

17.11.1 World Scenario

Phytase was isolated from the rice bran (Suzuki et al. 1907), and its production was initiated by *Aspergillus* sp. (Dox and Golden 1911). The first substantial attempt to make phytase an industrial product was begun which dates back to 1962 at the International Minerals and Chemical Corp., Skokie, IL. Shieh and Ware (1968) extracted *Aspergillus ficuum* phytase, which gave the highest production of phytase, and deposited it as NRRL 3135. Shieh et al. (1969) developed the strain *A. niger* NRRL 3135 for production of phytase with two pH optima, i.e. 5.5 and 2.5.

17.11.1.1 US Studies

The technology developed at IMC was transmitted to the Agricultural Research Service (ARS), US Department of Agriculture (USDA), and Southern Regional Research Centre (SRRC), and the researchers at SRRC, over a period of 16 years, studied phytases (*phy A* and *phy B*) and acid phosphatases produced by *A. niger* NRRL 3135. *phy A* gene from *A. niger* NRRL 3135 was cloned to *gt11* expression vector by the N- terminal and intersequences of *A. ficuum* phytases. The entire gene was consequently subcloned and deposited at GenBank. Simultaneously, the *phy A* gene from *A. niger* NRRL 3135 due to the expression of its multiple copies produced up to tenfold enhanced phytase activities than the wild-type strain (Van Hartingsveldt 1993). Another gene, *phy B*, was cloned and expressed to encode amino acid sequence which was validated by the chemically inferred protein sequence (Ehrlich et al. 1993). Phytase is marketed as food additive in the United States since January 1996 as Natuphos receiving approval from many countries and the Food and Drug Administration (FDA) as GRAS (generally recognized as safe) for use in food.

17.11.1.2 Dutch Studies

The scientists at Gist-Brocades in 1994 analysed and overexpressed *phy A* from *A. niger* NRRL 3135 under the control of amyloglucosidase promoter which produced 52-fold enhancement of phytase yield. About 1400-fold enhancement of phytase yield in one of the wild-type nonproducers was obtained by cloning the enzyme along with amyloglucosidase promoter and *A. niger* CBS 513.88.

17.11.1.3 Pan Labs Studies

Another phytase gene *phy B* has been cloned and overexpressed for enhance production of phytases by researchers at Aiko Ltd., Finland, jointly with Pan Labs. The genes for phytase from *A. niger* var. *awamori* were cloned. Miettinen-Oinonen et al. (1997) overexpressed pH 2.5 acid phosphatase in the *Trichoderma reesei* expression

system under the control of main cellobiohydrolase I (cbh1) promoter, and the enzyme is now available in the market as Finase-F.

17.11.1.4 Novo Nordisk Studies

Expression of phytase from a basidiomycete, *Peniophora lycii*, in *A. oryzae* IFO 41779 (WO 9828409 and US 606298) has been reported (Lassen et al. 2001). The preliminary investigation proves its potential of releasing phosphate from phytic acid at a high initial rate joined with high specific activity. Now, the enzyme is available in the marketplace as Bio-Feed phytase by Novo Nordisk.

17.11.2 Current Status in India

The manufacturing and extraction of phytase-specific phosphate were first reported in India from *B. subtilis* (Powar and Jagannathan 1982). A thermostable fungus, *A. niger* NICM 564, for the production of phytase has been isolated (Mandviwala and Khire 2000). The enzyme was found optimally active at 50 °C, pH 5.0, and retained 75% activity at 55 °C after 1 h incubation. Phytase yield from *Pichia anomala* was enhanced from dried flower buds that have a high activity (68 U/g dry biomass) intracellular enzyme at 20 °C (Vohra and Satyanarayana 2003). An overproducing strain of *A. niger* was isolated from rotten wood logs (Vats and Banerjee 2004). The Indian industries are currently applying dicalcium phosphate (DCP) in animal feeds, and it was found that phytase augmentation can substitute 50–60% dicalcium phosphate. About 10 kg DCP can be substituted by 250 g phytase enzyme; hence, perceptive that 50–60% of DCP can be substituted by phytase, the prospective requirement for phytase in cattle and poultry feed will be around 400 tonnes per annum.

17.11.3 Issues Related to Phytase

Regardless of all benefits of phytase, there are problems over its use that necessitates more research. Phytate being a strong chelator of iron and zinc, in plant foods, in fact can function as an antioxidant to decrease free radical formation interceded by these metals. As food-producing animals survive for a comparatively brief period and do not usually obtain high levels of feeding phytase, dietary iron or low phytic acid (Veum et al. 2001; Sands et al. 2003) constituents do not expect to cause any health problem, although low phytic acid grain may have a possible harmful impact on human health, especially in those with high iron stores attributable to high dietary intakes of highly accessible iron from animal foodstuffs or high dietary intakes of fruits that highly improve the absorption of non-heme iron (Fleming et al. 2002). Hence, sufficient carefulness should be taken in encouraging that low phytic acid grain approach from the animal production points to a wide application. The other problem is whether the rate of catalysis of augmented phytases for phytate-phosphorus form is higher than the rate of assimilation in animals which eventually

secrete more free phosphorus to the environment than the control without phytase (Dao 2003). Indeed, total soluble phosphorus excretion is significantly lowered in animals fed phytase, but their comparative percentage of soluble phosphorus in the total excreta phosphorus is marginally enhanced (Xavier et al. 2003). Meanwhile, suitable phytase ample may be titrated with phosphorus needs and dietary conditions (Kemme et al. 1997) of animals. For workforce handling phytase, breathing contact may cause immune reactions such as work-related asthmatic and other respiratory symptoms (Doekes et al. 1999). The hypersensitivity symptoms can be evaded by enhancing local exhaust systems and wearing of all shielding clothing and masks with P_2 filter (Baur et al. 2002).

17.11.4 Environmental Impression

In livestock, phase feeding of phytase can decrease environmental pollution without any impact on production. Certain genetically modified plants like low-phytate corn or soybeans have revealed 1.4% rises in P availability. Transgenic animals also enhance livestock effectiveness in applying P. Hence a combination of lowered P, precision feeding, phase feeding and enzyme can led to sustainable development in livestock production method and environmental protection simultaneously (Kebreab et al. 2012). Phytic acid occurring in the manure of monogastric animals is enzymatically sliced by soil- and water-borne microorganisms which may cause in eutrophication because of extreme algal growth (Bali and Satyanarayana 2001).

Genetically modified plants that produce microbial phytase can be employed in spite of inorganic phosphorus (P) fertilizers to maintain sustainability in agriculture Singh and Satyanarayana (2010). Phytase expressions in genetically modified plants neither get influence by occurrence of rhizosphere microorganisms nor it influence microbial community in the vicinity of the plant. Rising demand of animal products in upcoming years, harmonizing animal productivity with nutrient output, will entail an intensive attempt amongst producers, waste management specialists and nutritionists to lower the menace associated with animal wastes.

17.12 Conclusion

Recombinant DNA technology promises to produce well-customized commercial phytases. Modern genetic engineering technology has significantly enhanced functional use of phytases by making them thermotolerant, pH specific and withstand to protease activity in the digestive tract of animals. Biochemically, phytase is proteinaceous in nature, thus sensitive to denaturation due to excessive temperature, such as during pelleting. Spraying liquid phytase onto the frozen pellets can maintain the thermostability of the enzyme. Besides this, heat-stable phytases are existing. Storage under high-temperature and moisture conditions may also degrade the phytase (Selle et al. 2003). Therefore, appropriate storage measures and frequent replacement of stuffs containing phytase must be practised routinely. Phytase

containing stuffs must be stored only in dark, cool and dry areas. The manufacturer's advices must be precisely be followed, particularly when phytase is incorporated in vitamins and trace mineral premixes.

Knowledge and technology related to the phytase enzyme have emerged to a new exciting field due to increasingly global use of enzyme. Evidently, augmented phytases enhance dietary phytate phosphorus consumption by food-producing animals and lower environmental pollution of phosphorus from animal excreta in region of intensive animal production. Capability of phytase towards enhancing human nutrition and health and in growing specific phytic acid or inositol-derived products is given more attention and will be broaden as a new trend of phytase. Biotechnology will sustainably provide efficient means for developing and improving phytase enzymes and their delivery systems.

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References

Aaron A (2006) Expanding our knowledge of protein tyrosine phosphatase-like phytases: mechanism, substrate specificity and pathways of myo-inositol hexakisphosphate dephosphorylation. Dissertation for the Master's Degree. University of Lethbridge, Lethbridge, p 10–13

Admassu S (2009) Potential health benefits and problems associated with phytochemical in food legumes. East Afr J Sci 3(2):116–133

Akinmusire AS, Adeola O (2009) True digestibility of phosphorus in canola and soybean meals for growing pigs: influence of microbial phytase. J Anim Sci 87:977–983

Ali M, Shuja MN, Zahoor M, Qadri I (2010) Phytic acid:how far have we come? Afr J Biotech 9(11):1551–1554

Angel R, Tamim NM, Applegate TJ, Dhandu AS, Ellestad LE (2002) Phytic acid chemistry: influence on phytin-phosphorus availability and phytase efficacy. J Appl Poult Res 11:471–480

Angelis MD, Gallo G, Corbo MR, McSweeney PLH, Faccia M, Giovine M, Gobbetti M (2003) Phytase activity in sourdough lactic acid bacteria: purification and characterization of a phytase from *Lactobacillus sanfranciscensis* CB1. Int J Food Microbiol 87:259–270

Anons (1998) Enzymes emerge as big feed supplement. Chem Eng News 29–302. 4 May

Antrim RL, Mitchinson C, Solheim LP (1997) Method for liquefying starch. US patent 5652127

Arnarson A (2015) PhD. Phytic acid: 101 everything you need to know authority nutrition. http://authoritynutrition.com/phytic-acid-101/

Augspurger NR, Webel DW, Lei XG, Baker DH (2003) Efficacy of *E. coli* phytase expressedin yeast for releasing phytate-bound phosphorus in young chicks and pigs. J Anim Sci 81:474–483

Baldi BG, Scott JJ, Everard JD, Loewus FA (1988) Localization of constitutive phytases in lily pollen and properties of the pH 8 form. Plant Sci 56:137–147

Bali A, Satyanarayana T (2001) Microbial phytases in nutrition and combating phosphorus pollution. Every Man's Sci 4:207–209

Barrientos L, Scott JJ, Murthy PP (1994) Specificity of hydrolysis of phytic acid by alkaline phytase from lily pollen. Plant Physiol 106:1489–1495

Barrier-Guillot B, Casado P, Maupetit P, Jondreville C, Gatel F (1996) Wheat phosphorus availability: in vitro study; factors affecting endogenous phytasic activity and phytic phosphorus content. J Sci Food Agric 70:62–68

Baur X, Melching-Kollmuss S, Koops F, Straßburger K, Zober A (2002) IgE-mediated allergy to phytase-a new animal feed additive. Allergy 57:943–945

Bedford MR (2000) Exogenous enzymes in monogastric nutrition their current value and future benefits. Anim Feed Sci Technol 86:113

Berka RM, Rey MW, Brown KM, Byun T, Klotz AV (1998) Molecular characterization and expression of a phytase gene from the thermophilic fungus *Thermomyces lanuginosus*. Appl Environ Microbiol 64:4423–4427

Berridge MJ, Irvine ILF (1984) Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature 312:315–321

Bianchetti R, Sartirana ML (1967) The mechanism of the repression by inorganic phosphate of phytase synthesis in the germinating wheat embryo. Biochim Biophys Acta 145:485–490

Bitar K, Reinhold JG (1972) Phytase and alkaline phosphatase activities in intestinal mucose of rat, chicken, calf, and man. Biochim Biophys Acta 268:442–452

Bogar B, Szakacs G, Tengerdy RP, Linden JC, Pandey A (2003a) Production of phytase by *Mucor racemosus* in solid-state fermentation. Biotechnol Prod 19(2):312–319

Bogar B, Szakacs G, Linden JC, Pandey A, Tengerdy RP (2003b) Optimization of phytase production by solid substrate fermentation. J Ind Microbiol Biotech 30(3):183–189

Boling SD, Douglas MW, Johnson ML, Wang X et al (2000) The effects of dietary available phosphorus levels and phytase performance of young and older laying hens. Poult Sci 79:224–230

Brugger R, SimoesNunes C, Hug D, Vogel K, Guggenbuhl P, Mascarello F (2003) Characteristics of fungal phytases from Aspergillus fumigatus and Sartorya fumigata. Appl Microbiol Biotechnol 63:383–389

Burbano C, Muzquiz M, Osagic A, Ayet G, Cuadrado C (1995) Determination of phytate and lower inositol phosphates in Spanish legumes by HPLC methodology. Food Chem 52:321–325

Carla EH, Elizabeth AG (2001) A novel phytase with sequence similarity to purple acid phosphatases is expressed in cotyledons of germinating soybean seedlings. Plant Physiol 126:1598–1608

Carrington AL, Calcutt NA, Ettlinger CB, Gustafsson T, Tomlinson DR (1993) Effects of treatment with myo-inositol or its 1,2,6-trisphosphate (PP56) on nerve conduction in streptozotocin-diabetes. Eur J Pharmacol 237:257–263

Casey A, Walsh G (2003) Purification and characterization of extracellular phytase from *Aspergillus niger* ATCC 9142. Bioresour Technol 86(2):183–188

Casey A, Walsh G (2004) Identification and characterization of a phytase of potential commercial interest. J Biotechnol 110(3):313–322

Cenzone (1999) Cenzyme, http://www.ras-international.com/cenzone/cenzyme.htm

Chadha BS, Gulati H, Minhas M, Saini HS, Singh N (2004) Phytase production by the thermophilic fungus Rhizomucor pusillus. World J Microbiol Biotechnol 20:105–109

Chelius MK, Wodzinski RJ (1994) Strain improvement of *Aspergillus niger* for phytase production. Appl Microbiol Biotechnol 41:79–83

Chen C, Hunag C, Cheng K (2001) Improvement of phytase thermostability by using sorghum liquor wastes supplemented with starch. Biotechnol Lett 23:331–333

Cheng KJ, Selinger LB, Yanke LJ, Bae HD, Zhou L, Forsberg CW (1999) Phytases of rumen micro-organisms, particularly of Selenomonas ruminantium, and uses there of in feed additives and in transgenic plants. US Patent 5(985):605

Cheryan M (1980) Phytic acid interactions in food systems. CRC Crit Rev Food Sci Nutr 13:297–335

Cho JS, Lee CW, Kang SH, Lee JC, Bok JD, Moon YS, Lee HG, Kim SC, Choi YJ (2003) Purification and characterization of a phytase from Pseudomonas syringae MOK1. Curr Microbiol 47:290–294

Choi YM, Suh HJ, Kim JM (2001) Purification and properties of extracellular phytase from *Bacillus sp.* KHU-10. J Protein Chem 20:287–292

Chu HM, Guo RT, Lin TW, Chou CC, Shr HL et al (2004) Structures of *Selenomonas ruminantium* phytase in complex with persulfated phytate: DSP phytase fold and mechanism for sequential substrate hydrolysis. Structure 12:2015–2024

Claxon A, Morris C, Blake D, Siren M et al (1990) The anti-inflammatory effects of D-myo-inositol-1, 2,6-trisphosphate (PP56) on animal models of inflammation. Agents Actions 29:68–70

Coello P, Maughan JP, Mendoza A, Philip R et al (2001) Generation of low phytic acid *Arabidopsis* seeds expressing an *E. coli* phytase during embryo development. Seed Sci Res 11:285–291

Correia I, Aksu S, Adao P, Pessoa JC, Sheldon RA, Arends IWCE (2008) Vanadate substituted phytase: immobilization, structural characterization and performance for sulfoxidations. J Inorg Biochem 102:318–329

Cosgrove DJ (1970) Inositol phosphate phosphatase of microbiological origin, inositol pentaphosphate intermediates in the dephosphorylation of the hexaphosphates of myo-inositol, scyllo-inositol, and D-chiro-inositol, by a bacterial (*Pseudomonas* sp.) phytase. Aust J Biol Sci 23:1207–1220

Craxton A, Caffrey JJ, Burkhart W, Safrany ST, Shears SB (1997) Molecular cloning and expression of a rat hepatic multiple inositol polyphosphate phosphatase. Biochem J 328:75–81

Dahiya S, Kumar K, Kumar N, Singh N (2008) Standardisation of incubation time forphytase enzyme secretion using RSM model international conference on molecular biology and biotechnology. Department of Biosciences and Biotechnology, Banasthali University, p 121. October 19–21

Dahiya S, Singh N, Rana JS (2009) Optimization of growth parameters of phytase producing fungus using RSM. J Sci Ind Res 68(11):955–958

Dahiya S, Kumar K, Kumar N, Singh N, Rana JS (2010) Optimization of assay conditions using RSM approach for phytase enzyme production by *Bacillus cereus* MTCC 10072. An Biol 26(2):101–107

Dalal RC (1997) Soil organic phosphorus. Adv Agron 29:83–117

Dao TH (2003) Polyvalent cation effects on myo-inositol hexakis dihydrogen phosphate enzymatic dephosphorylation in dairy wastewater. J Environ Qual 32:694–701

Davies NT, Flett AA (1978) The similarity between alkaline phosphatase (EC 3.1.3.1) and phytase (EC 3.1.3.8) activities in rat intestine and their importance in phytate-induced zinc deficiency. Br J Nutr 39:307–316

Davies MI, Motzok I (1972) Intestinal alkaline phosphatase and phytase of chicks: separation of isoenzymes, zinc contents and in vitro effects of zinc. Comp Biochem Physiol 42:345–356

Day PL (1996) Genetic modification of plants: significant issues and hurdles to success. Am J Clin Nutr 63:651S–656S

De Angelis M, Gallo G, Corbo MR, McSweeney PLH, Faccia M, Giovine M, Gobbetti M (2003) Phytase activity in sourdough lactic acid bacteria: purification and characterization of a phytase from Lactobacillus 174 sanfranciscensis CB1. Int J Food Microbiol 87:259–270

Dechavez RB, Serrano AE Jr, Nuñal S, Caipang CMA (2011) Production and characterization of phytase from Bacillus spp. as feed additive in aquaculture. AACL Bioflux 4:394–403

Dersjant LY, Awati A, Schulze H, Partridge G (2015) Phytase in non-ruminant animal nutrition: a critical review on phytase activities in the gastrointestinal tract and influencing factors. J Sci Food Agric 95(5):878–896

Desphande SS, Cheryan M (1984) Effects of phytic acid, divalent cations, and their interactions on alpha-amylase activity. J Food Sci 49:516–519

Dischinger HC, Ullah AHJ (1992) Immobilization of *Aspergillus ficuum* phytase by carbohydrate moieties onto cross-linked agarose. Ann N Y Acad Sci 672:583–587

Doekes G, Kamminga N, Helwegen L, Heederik D (1999) Occupational IgE sensitisation to phytase, a phosphate derived from *Aspergillus niger*. Occup Environ Med 56:454–459

Dox AW, Golden R (1911) Phytase in lower fungi. J Biol Chem 10:183–186

Eastwood D, Laidman D (1971) The mobilization of macronutrient elements in the germinating wheat grain. Phytochemistry 10:1275–1284

Ebune A, Alasheh S, Duvnjak Z (1995) Production of phytase during solid-state fermentation using *Aspergillus-ficuum* NRRL-3135 in canola-meal. Bioresour Technol 53(1):7–12

Eeckhout W, de Paepe M (1994) Total phosphorus, phytate-phosphorus and phytase activity in plant feed stuffs. Anim Feed Sci Technol 47:19–29

Ehrlich KC, Montalbano BG, Mullaney EJ, Dischinger HC, Ullah AHJ (1993) Identification and cloning of a second phytase gene (phyB) from *Aspergillus niger* (ficuum). Biochem Biophys Res Commun 195:53–57
Elliott S, Chang CW, Schweingruber ME, Schaller J et al (1986) Isolation and characterization of the structural gene for secreted acid phosphatase from *Schizosaccharomyces pombe*. J Biol Chem 261:2936–2941
European Union (2004a) Official Journal of the European Union C 50/52, published 25/02/2004
European Union (2004b) Official Journal of the European Union C 50/95, published 25/02/2004
European Union (2004c) Official Journal of the European Union C 50/112, published 25/02/2004
European Union (2004d) Official Journal of the European Union L 270/12, published 18/08/2004
Farhat A, Chouayekh H, Ben FM, Bouchaala K, Bejar S (2008) Gene cloning and characterization of a thermostable phytase from *Bacillus subtilis* US417 and assessment of its potential as a feed additive in comparison with a commercial enzyme. Mol Biotechnol 40(2):127–135
Findenegg GR, Nelemans JA (1993) The effect of phytase on availability of P from myo-inositol hexaphosphate (phytate) for maize roots. Plant Soil 154:189–196
Fleming DJ, Tucker KL, Jacques PF, Dallal GE et al (2002) Dietary factors associated with the risk of high iron stores in the elderly Framingham Heart Study cohort. Am J Clin Nutr 76:1375–1384
Fredrikson M, Biot P, Larsson Alminger M, Carlsson NG, Sandberg AS (2001) Production process for high-quality pea-protein isolate with low content of oligosaccharides and phytate. J Agric Food Chem 49:1208–1212
Fredrikson M, Andlid T, Haikara A, Sandberg AS (2002) Phytate degradation by micro-organisms in synthetic media and pea flour. J Appl Microbiol 93:197–204
Freund WD, Mayr GW, Tietz C, Schultz JE (1992) Metabolism of inositol phosphates in the protozoan *Paramecium*. Eur J Biochem 207:359–367
Gabard KA, Jones RL (1986) Localization of phytase and acid phosphatase isoenzymes in aleurone layers of barley. Physiol Plant 67:182–192
Gargova S, Roshkova Z, Vancheva G (1997) Screening of fungi for phytase production. Biotechnol Lett 11:221–224
Gargova S, Sariyska M (2003) Effect of culture conditions on the biosynthesis of *Aspergillus niger* phytase and acid phosphatase. Enzym Microb Technol 32:231–235
Garrett JB, Kretz KA, O'Donoghue E, Kerovuo J et al (2004) Enhancing the thermal tolerance and gastric performance of a microbial phytase for use as a phosphate-mobilizing monogastric-feed supplement. Appl Environ Microbiol 70:3041–3046
Gautam P, Sabu A, Pandey A, Szakacs G, Soccol CR (2002) Microbial production of extra-cellular phytase using polystyrene as inert solid support. Bioresour Technol 83(3):229233
Gibson DM (1987) Production of extracellular phytase from *Aspergillus ficuum* on starch media. Biotechnol Lett 9:305–310
Gibson DM, Ullah AHJ (1988) Purification and characterization of phytase from cotyledons of germinating soybean seeds. Arch Biochem Biophys 260:503–513
Gibson DM, Ullah AB (1990) Phytase and their action on phytic acid in inositol metabolism in plants. Arch Biochem Biophys 262:77–92
Golovan S, Wang G, Zhang J, Forsberg CW (2000) Characterization and overproduction of the *Escherichia coli* appA encoded bifunctional enzyme that exhibits both phytase and acid phosphatase activities. Can J Microbiol 46:59–71
Grases F, Simonet BM, Prieto RM, March JG (2001) Dietary phytate and mineral bioavailability. J Trace Elem Med Biol 15(4):221–228
Greaves MP, Anderson G, Webley DM (1967) The hydrolysis of inositol phosphates by *Aerobacter aerogenes*. Biochim Biophys Acta 132:412–418
Greiner R (2001) Properties of phytate-degrading enzymes from germinated lupine seeds (*Lupinus Albus var.Amiga*). Proceedings of the 4th European Conference on Grain Legumes, Cracow, Poland. p 398–399
Greiner R, Konietzny U (1996) Construction of a bioreactor to produce special breakdown products of phytate. J Biotechnol 48:153–159

Greiner R, Konietzny U (2006) Phytase for food application. Food Technol Biotechnol 44(2):125–140

Greiner R, Larsson Alminger M (1999) Purification and characterization of a phytate-degrading enzyme from germinated oat (*Avena sativa*). J Sci Food Agric 79:1453–1460

Greiner R, Konietzny U, Jany KD (1993) Purification and characterization of two phytases from *Escherichia coli*. Arch Biochem Biophys 303:107–113

Greiner R, Haller E, Konietzny U, Jany KD (1997) Purification and characterization of a phytase from *Klebsiella terrigena*. Arch Biochem Biophys 341:201–206

Greiner R, Jany KD, Larsson Alminger M (2000) Identification and properties of myo-inositol hexakisphosphate phosphohydrolases (phytases) from barley (*Hordeum vulgare*). J Cereal Sci 31:127–139

Greiner R, Larsson Alminger M, Carlsson NG et al (2002) Enzymatic phytate degradation – a possibility to design functional foods? Poult J Food Nutr Sci 11:50–54

Guerrero-Olazarán M, Rodríguez-Blanco L, Carreon-Treviño JG, Gallegos-López JA, Viader-Salvadó JM (2010) Expression of a Bacillus phytase C gene in Pichia pastoris and properties of the recombinant enzyme. Appl Environ Microbiol 76(16):5601–5608

Gulati HK, Chadha BS, Saini HS (2007a) Production and characterization of thermostable alkaline phytase from *Bacillus laevolacticus* isolated from rhizosphere soil. J Ind Microbiol Biotechnol 34:91–98

Gulati HK, Chadha BS, Saini HS (2007b) Production, purification and characterization of thermostable phytase from thermophilic fungus *Thermomyces lanuginosus* TL-7. Acta Microbiol Immunol Hung 54(2):121–138

Haefner S, Knietsch A, Scholten E, Braun J et al (2005) Biotechnological production and applications of phytases. Appl Microbiol Biotechnol 68:588–597

Han YW, Gallagher DJ (1987) Phosphatase production by *Aspergillus ficuum*. J Ind Microbiol 1:295–301

Han Y, Wilson DB, Lei X (1999) Expression of an Aspergillus niger Phytase Gene (phyA) in Saccharomyces cerevisiae. Appl Environ Microbiol 65(5):1915–1918

Hara A, Ebina S, Kondo A, Funagua T (1985) A new type of phytase from *Typhalatifolia*. Agric Biol Chem 49:3539–3544

Harland BF, Morris ER (1995) Phytate: a good or a bad food component. Nutr Res 15:733–754

Harland BF, Oberleas D (1977) A modified method for phytate analysis using an ion-exchange procedure: application to textured proteins. Cereal Chem 54:827–832

Haros M, Rosell CM, Benedito C (2001) Fungal phytase as a potential bread making additive. Eur Food Res Technol 213:317–322

Hayes JE, Simpson RJ, Richardson AE (2000) The growth and phosphorus utilisation of plants in sterile media when supplied with inositol hexaphosphate, glucose 1-phosphate or inorganic phosphate. Plant Soil 220:165–174

Hegeman CE, Grabau EA (2001) A novel phytase with sequence similarity to purple acid phosphatase is expressed in cotyledons of germinating soybean seedling. Plant Physiol 126:1598–1608

Heinonen JK, Lahti RJ (1981) A new and convenient colorimetric determination of inorganic orthophosphate and itsapplication to the assay of inorganic pyrophosphatase. Anal Biochem 113:313–317

Hemrika W, Renirie R, Dekker HL, Barnett P, Wever R (1997) From phosphatases to vanadium peroxidases: a similar architecture of the active site. Proc Natl Acad Sci USA 94:2145–2149

Hengge-Aronis R (1996) Regulation of gene expression during entry into stationary phase. In: Neidhardt (ed) Escherichia coli and Salmonella: cellular and molecular biology, vol 1, 2nd edn. ASM Press, Washington, DC, pp 1497–1512

Hill BE, Sutton AL, Richert BT (2009) Effects of low-phytic acid corn, low-phytic acid soybean meal, and phytase on nutrient digestibility and excretion in growing pigs. J Anim Sci 87:1518–1527

Hong CY, Cheng KJ, Tseng TH, Wang CS, Liu LF, Yu SM (2004) Production of two highly active bacterial phytases with broad pH optima in germinated transgenic rice seeds. Transgenic Res 13:29–39

Honig B, Nicholls A (1995) Classical electrostatics in biology and chemistry. Science 268:1144–1149

Houde RL, Alli I, Kermasha S (1990) Purification and characterization of canola seed (*Brassica sp.*) phytase. J Food Biochem 114:331–351

Huang J, Zhou W, Watson AM, Jan YN, Hong Y (2008) Efficient ends-out gene targeting in *Drosophila*. Genetics 180(1):703–707

Huang HQ, Shao N, Wang YR, Luo HY, Yang PL, Zhou ZG, Zhan ZC, Yao B (2009) A novel beta-propeller phytase from Pedobacter nyackensis MJ11 CGMCC 2503 with potential as an aquatic feed additive. Appl Microbiol Biotechnol 83(2):249–259

Hubel F, Beck E (1996) Maize root phytase. Plant Physiol 112:1429–1436

Idriss EE, Makarewicz O, Farouk A, Rosner K et al (2002) Extracellular phytase activity of *Bacillus amyloliquefaciens* FZB45 contributes to its plant-growth-promoting effect. Microbiology 148:2097–2109

Igbasan FA, Männer K, Miksch G, Borriss R, Farouk A, Simon O (2000) Comparative studies on the in vitro properties of phytases from various microbial origins. Arch Anim Nutr 53:353–373

Iqbal TH, Lewis KO, Cooper BT (1994) Phytase activity in the human and rat small intestine. Gut 35:1233–1236

Ireland MM, Karty JA, Quardokus EM, Reilly JP, Brun YV (2002) Proteomic analysis of the *Caulobacter crescentus* stalk indicates competence for nutrient uptake. Mol Microbiol 45(4):1029–1041

Jackson JF, Linskens HF (1982) Phytic acid in *Petunia hybrida* pollen is hydrolysed during germination by a phytase. Acta Bot Neerlandica 315:441–447

Jareonkitmongkol S, Ohya M, Watanabe R, Takagi H, Nakamori S (1997) Partial purification from a soil isolates bacterium, *Klebsiella oxytoca* MO-3. J Ferment Bioeng 83:393–394

Jariwalla RJ, Sabin R, Lawson S, Herman ZS (1990) Lowering of serum cholesterol and triglycerides and modulation of divalent cations by dietary phytate. J Appl Nutr 42:18–28

Jenab M, Thompson LU (2002) Role of phytic acid in cancer and other diseases. In: Reddy NR, Sathe SK (eds) Food phytates. CRC Press, Boca Raton, pp 225–248

Jog SP, Garchow BG, Mehta BD, Murthy PPN (2005) Alkaline phytase from lily pollen: investigation of biochemical properties. Arch Biochem Biophys 440:133–140

Kaur P, Satyanarayana T (2010) Improvement in cell-bound phytase activity of *Pichia anomala* by permeabilization and applicability of permeabilized cells in soymilk dephytinization. J Appl Microbiol 108:2041–2049

Kebreab E, Hansen AV, Strathe A (2012) Animal production for efficient phosphate utilization: from optimised feed to high efficiency livestock. Curr Opin Biotechnol 23:872–877

Kemme PA, Jongbloed AW, Mroz Z, Beynen AC (1997) The efficacy of *Aspergillus niger* phytase in rendering phytate phosphorus available for absorption in pigs is influenced by pig physiological status. J Anim Sci 75:2129–2138

Kerovuo J, Lauraeus M, Nurminen P, Kalkkinen N, Apajalahti J (1998) Isolation, characterization, molecular gene cloning and sequencing of novel phytase from *Bacillus subtilis*. Appl Environ Microbiol 64:2079–2085

Kerovuo J, Lappalainen I, Reinikainen T (2000) The metal dependence of *Bacillus subtilis* phytase. Biochem Biophys Res Commun 268:365–369

Kies AK, De Jonge LH, Kemme PA, Jongbloed AW (2006) Interaction between protein, phytate, and microbial phytase: in vitro studies. J Agric Food Chem 54:1753–1758

Kim DS, Godber JS, Kim HR (1999a) Culture conditions for a new phytase producing fungus. Biotechnol Lett 21:1077–1081

Kim HW, Kim YO, Lee JH, Kim KK, Kim YJ (2003) Isolation and characterization of a phytase with improved properties from Citrobacter braakii. Biotechnol Lett 25:1231–1234

Kim JC, Simmins PH, Mullan BP, Pluske JR (2005) The effect of wheat phosphorus content and supplemental enzymes on digestibility and growth performance of weaner pigs. Anim Feed Sci Technol 118:139–152

Kim YO, Kim HK, Bae KS, Yu JH, Oh TK (1998a) Purification and properties of thermostable phytase from *Bacillus sp*. DSII. Enzym Microb Technol 22:2–7

Kim YO, Lee JK, Kim HK, Yu JH, Oh TK (1998b) Cloning of the thermostable phytase gene (phy) from *Bacillus sp*. DS11 and its over-expression in *Escherichia coli*. FEMS Microbiol Lett 162:185–191

Kim YO, Lee JK, Oh BC, Oh TK (1999b) High-level expression of a recombinant thermostable phytase in Bacillus subtilis. Biosci Biotechnol Biochem 63:2205–2207

Kleist S, Miksch G, Hitzmann B, Arndt M, Friehs K, Flaschel E (2003) Optimization of the extracellular production of a bacterial phytase with *Escherichia coli* by using different fed-batch fermentation strategies. Appl Microbiol Biotechnol 61:456–462

Konietzny U, Greiner R (2002) Molecular and catalytic properties of phytate-degrading enzymes (phytases). Int J Food Sci Tech 37(7):791–812

Konietzny U, Greiner R (2003) Phytic acid: nutritional impact. In: Caballero B, Trugo L, Finglas P (eds) Encyclopedia of food science and nutrition. Elsevier, London, pp 4555–4563

Konietzny U, Greiner R, Jany KD (1995) Purification and characterization of a phytases from spelt. J Food Biochem 18:165–183

Kornegay ET, Denbow DM, Yi Z, Ravindran V (1996) Response of broilers to graded levels of microbial phytase added to maize–soyabean meal-based-diets containing three levels of non-phytate phosphorus. Br J Nutr 75:839–852

Kornegay ET, Zhang Z, Denbow DM (1999) Influence of microbial phytase supplementation of a low protein/amino acid diet on performance, ileal digestibility of protein and amino acids, and carcass measurements of finishing broilers. In: Phytase in animal nutrition and waste management, 2nd rev edn. BASF Corporation, Mount Olive, pp 557–572

Kostrewa D, Wyss M, D'Arcy A, van Loon APGM (1999) Crystal structure of *Aspergillus niger* pH 2.5 acid phosphatase at 2.4 A resolution. J Mol Biol 288:965–974

Krishna C, Nokes SE (2001) Predicting vegetative inoculum performance to maximize phytase production in solid-state fermentation using response surface methodology. J Ind Microbiol Biotechnol 26:161–170

Kuhar S, Singh N, Rana JS (2009) Isolation and statistical optimization of growth parameters for a phosphate pollution controlling NSB-10 bacteria. Proc Int Conf Changing Environ Trends and Sustainable Development, Department of Environmental Science and Engineering, Guru Jambheshwar University of Science and Technology, Hisar-125001, India, Feb 9–11. p 141–144

Kumar V, Sinha AK, Makkar HPS, De Boeck G, Becker K (2012) Phytate and phytase in fish utrition. J Anim Physiol Anim Nutr 96:335–364

Kvist S, Carlsson T, Lawther JM, DeCastro FB (2005) Process for the fractionation of cereal brans. US patent application US 20050089602

Laboure AM, Gagnon J, Lescure AM (1993) Purification and characterization of a phytase (myo–inositolhexakisphosphate phosphohydrolase) accumulated in maize (*Zea mays*) seedlings during germination. Biochem J 295:413–419

Lambrechts C, Boze H, Segueilha L, Moulin G, Galzy P (1993) Influence of culture conditions on the biosynthesis of *Schwanniomyces castellii* phytase. Biotechnol Lett 15(4):399–404

Lan GQ, Abdullah N, Jalaludin S, Ho YW (2002) Optimization of carbon and nitrogen sources for phytase production by *Mitsuokella jalaludinii*, a new rumen bacterial species. Lett Appl Microbiol 35(2):157–161

Lassen SF, Breinholt J, Østergaard PR, Brugger R et al (2001) Expression, gene cloning, and characterization of five novel phytases from four basidiomycete fungi: *Peniophora lycii*, *Agrocybe pediades*, *Ceriporia sp*., and *Trametes pubescens*. Appl Environ Microbiol 67:4701–4707

Lehmann M, Kostrewa D, Wyss M, Brugger R, D'Arcy A, Pasamontes L (2000) From DNA sequence to improved functionality: using protein sequence comparisons to rapidly design a thermostable consensus phytase. Protein Eng 13(1):49–57

Lei XG, Stahl C (2001) Biotechnological development of effective phytases for mineral nutrition and environmental protection. Appl Microbiol Biotechnol 57:474–481

Li J, Hegemann CE, Hanlon RW, Lacy GH, Denbow DM, Grabau EA (1997) Secretion of active recombinant phytase from soybean cell-suspension cultures. Plant Physiol 114:1103–1111

Li R, Lu W, Gu J, Li X, Guo C, Xiao K (2011) Molecular characterization and functional analysis of OsPHY2, a phytase gene classified in histidine acid phosphatase type in rice (Oryza sativa L.). Afr J Biotechnol 10(54):11110–11123

Li X, Chi Z, Liu Z, Li J, Wang X, Hirimuthugoda NY (2008) Purification and characterization of extracellular phytase from a marine yeast Kodamaea ohmeri BG3. Mar Biotechnol 10:190–197

Li X, Liu Z, Chi Z, Li J, Wang X (2009) Molecular cloning, characterization, and expression of the phytase gene from marine yeast *Kodamaea ohmeri* BG3. Mycol Res 113:24–32

Lin JJ, Dickinson DB, Ho THD (1987) Phytic acid metabolism in lily (*Lilium longiflorum Thunb*) pollen. Plant Physiol 83:408–413

Lim D, Golovan S, Forsberg CW, Jia ZC (2000) Crystal structures of Escherichia coli phytase and its complex with phytate. Nat Struct Biol 7(2):108–113

Liu BL, Jong CH, Tzeng YM (1999) Effect of immobilization on pH and thermal stability of Aspergillus ficuum phytase. Enzym Microb Technol 25:517–521

Liu J, Bollinger DW, Ledoux DR, Veum TL (1998) Lowering the dietary calcium to total phosphorus ratio increased phosphorus utilization in low phosphorus corn–soybean meal diets supplemented with microbial phytase for growing–finishing pigs. J Anim Sci 76:808–813

Loewus F (2002) Biosynthesis of phytate in food grains and seeds. In: Reddy NR, Sathe SK (eds) Food phytates. CRC Press, Boca Raton, pp 53–61

Lopez HW, Leenhardt F, Coudray C, Remesy C (2002) Minerals and phytic acid interactions: is it a real problem for human nutrition? Int J Food Sci Technol 37:727–739

Maenz DD (2001) Enzymatic and other characteristics of phytases as they relate to their use in animal feeds. In: Bedford R, Partridge GG (eds) Enzymes in farm animal nutrition. CABI Publishing, UK, p 6184

Maenz DD, Classen HL (1998) Phytase activity in the small intestinal brush-border membrane of the chicken. Poult Sci 77:557–563

Maffucci T, Piccolo E, Cumashi A, Iezzi M et al (2005) Inhibition of the phosphatidylinositol-3-kinase/Akt pathway by inositol pentakisphosphate results in antiangiogenic and antitumor effects. Cancer Res 65:8339–8349

Mallin MA (2000) Impacts of industrial animal production on rivers and estuaries. Anim Sci 88:26–37

Mandviwala TN, Khire JM (2000) Production of high activity thermostable phytase from thermotolerant phytase from thermotolerant *Aspergillus niger* in solid state fermentation. J Ind Microbiol Biotechnol 24:237–243

Maugenest S, Martinez I, Lescure AM (1997) Cloning and characterization of a cDNA encoding a maize seedling phytase. Biochem J 322:511–517

Maugenest S, Martinez I, Godin B, Perez P, Lescure AM (1999) Structure of two maize phytase genes and their spatio-temporal expression during seedling development. Plant Mol Biol 39:503–514

McCollum EV, Hart EB (1908) On the occurrence of a phytin-splitting enzyme in animal tissue. J Biol Chem 4:497–500

Miettinen-Oinonen A, Torkkeli T, Paloheimo M, Nevalainen H (1997) Overexpression of the Aspergillus niger pH 2.5 acid phosphatase gene in a heterologous host Trichoderma reesei. J Biotechnol 58:13–20

Miksch G, Kleist S, Friehs K, Flaschel E (2002) Overexpression of the phytase from *Escherichia coli* and its extracellular production in bioreactors. Appl Microbiol Biotechnol 59:685–694

Misset O (2003) Phytase. Food Sci Technol 122:687–706

Mitchell DB, Vogel K, Weimann BJ, Pasamontes L, Van Loon APGM (1997) The phytase subfamily of histidine acid phosphatases: isolation of genes for two novel phytases from the fungi *Aspergillus terreus* and *Myceliophthora thermophila*. Microbiology 143:245–252

Mullaney EJ, Ullah AH (2003) The term phytase comprises several different classes of enzymes. Biochem Biophys Res Commun 312(1):179–184

Mullaney EJ, Daly CB, Ullah AHJ (2000) Advances in phytase research. Adv Appl Microbiol 47:157–199

Mullaney EJ, Daly CB, Kim T, Porres JM, Lei XG, Sethumadhavan K, Ullah AHJ (2002) Site-directed mutagenesis of *Aspergillus niger* NRRL 3135 phytase at residue 300 to enhance catalysis at pH 4.0. Biochem Biophys Res Commun 297:1016–1020

Mullaney EJ, Ullah AH, Turner B, Richardson A, Mullaney E (2007) Phytases: attributes, catalytic mechanisms and applications. In: Inositol phosphates: linking agriculture and the environment. p 97–110

Nagashima T, Tange T, Anazawa H (1999) Dephosphorylation of phytate by using the Aspergillus nigerphytase with a high affinity for phytate. Appl Environ Microbiol 65:4682–4684

Nakamura Y, Fukuhara H, Sano L (2000) Secreted phytase activities of yeasts. Biosci Biotechnol Biochem 64:841–844

Nakano T, Joh T, Narita K, Hayakawa T (2000) The pathway of dephosphorylation of myo-inositol hexakisphosphate by phytases from wheat bran of *Triticum aestivum* L. cv. Nourin 61. Biosci Biotechnol Biochem 64:995–1003

Nampoothiri KM, Tomes GJ, Roopesh K, Szakacs G, Nagy V, Soccol CR, Pandey A (2004) Thermostable phytase production by thermoascus aurantiacus in submerged fermentation. Appl Biochem Biotechnol 118:205–214

Nelson TS (1967) The utilization of phytate phosphorus by poultry. Poult Sci 46:862–871

Oh BC, Chang BS, Park KH, Ha NC, Kim HK, Oh BH, Oh TK (2001) Calcium-dependent catalytic activity of a novel phytase from *Bacillus amyloliquefaciens* DS11. Biochemistry 40:9669–9676

Oh BC, Choi WC, Park S, Kim YO, Oh TK (2004) Biochemical properties and substrate specificities of alkaline and histidine acid phytases. Appl Microbiol Biotechnol 63:362–372

Ohkawa T, Ebisuno S, Kitagawa M, Morimoto S, Miyazaki Y, Yasukawa S (1984) Rice bran treatment for patients with hypercalciuric stones: experimental and clinical studies. J Urol 132:1140–1145

Pandey A, Szakacs G, Soccol CR, Rodriguez-Leon JA, Soccol VT (2001) Production, purification and properties of microbial phytases. Bioresour Technol 77:203–214

Papagianni M, Nokes SE, Filer K (2000) Production of phytase by *Aspergillus niger* in submerged and solid-state fermentation. Process Biochem 35(3–4):397–402

Parkkonen T, Tervila-Wilo A, Hopeakoski-Nurminen M, Morgan A, Poutanen K, Autio K (1997) Changes in wheat microstructure following in vitro digestion. Acta Agric Scand Sect B Soil Plant Sci 47:43–47

Pasamontes L, Haiker M, Wyss M, Tessier M, Loon APGM (1997) Gene cloning, purification, and characterization of a heat-stable phytase from the fungus *Aspergillus fumigatus*. Appl Environ Microbiol 63:1696–1700

Patwardhan VN (1937) The occurrence of a phytin splitting enzyme in the intestines of albino rats. Biochem Lett 31:560–564

Phillippy BQ (1999) Susceptibility of wheat and *Aspergillus niger* phytases to inactivation by gastrointestinal enzymes. J Agric Food Chem 47:1385–1388

Phillipy BQ, Mullaney EJ (1997) Expression of an *Aspergillus niger* phytase (phyA) in *Escherichia coli*. J Agric Food Chem 45:3337–3342

Piddington CS, Houston CS, Paloheimo M, Cantrell M, Miettinen-Oinonen A, Nevalanien H (1993) The cloning and sequencing of the genes encoding phytase (*phy*) and pH 25 optimum acid phosphatase (*aph*) from *Aspergillus niger* var. awamori. Gene 133:55–62

Pinky G, Sabu A, Pandey A, Szakacs G, Soccol CR (2002) Microbial production of extra-cellular phytase using polystyrene as inert solid support. Bioresour Technol 83(3):229–233

Pomar C, Gagne F, Matte JJ, Barnett G, Jondreville C (2008) The effect of microbial phytase on true and apparent ileal amino acid digestibilities in growing-finishing pigs. J Anim Sci 86:1598–1608

Posternak T (1965) The cyclilols. Hermann, Paris

Powar VK, Jagannathan V (1982) Purification and properties of phytate-specific phosphatase from *Bacillus subtilis*. J Bacteriol 151:1102–1108

Promdonkoy P, Tang K, Sornlake W, Harnpicharnchai P et al (2009) Expression and characterization of *Aspergillus* thermostable phytases in *Pichia pastoris*. FEMS Microbiol Lett 290(1):18–24

Quan CH, Zhang LH, Wang YJ, Ohta Y (2001) Production of phytase in a low phosphate medium by a novel yeast Candida krusei. J Biosci Bioeng 92:154–160
Quan CH, Zhang LH, Wang YJ, Ohta Y (2002) Purification and properties of a phytase from Candida krusei WZ-001. J Biosci Bioeng 94:111–119
Quan CS, Tian WJ, Fan SD, Kikuchi YI (2004) Purification and properties of a low-molecular-weight phytase from Cladosporium sp. FP-1. J Biosci Bioeng 97:260–266
Raghavendra P, Halami PM (2009) Screening, selection and characterization of phytic acid degrading lactic acid bacteria from chicken intestine. Int J Food Microbiol 133(1–2):129–134
Ramachandaran S, Krishnan R, Nampoothiri KM, Szackacs G, Pandey A (2005) Mixed substrate fermentation for the production of phytase by *Rhizopus* spp. using oil cakes as substrates. Process Biochem 40(5):1749–1754
Rao DE, Rao KV, Reddy VD (2008) Cloning and expression of Bacillus phytase gene (phy) in Escherichia coli and recovery of active enzyme from the inclusion bodies. J Appl Microbiol 105(4):1128–1137
Rapoport S, Leva E, Guest GM (1941) Phytase in plasma and erythrocytes of vertebrates. Biol Chem 139:621–632
Ravindran V, Bryden WL, Kornegay ET (1995) Phytases: occurrence, bioavailability and implications in poultry nutrition. Poult Avian Biol Rev 6:125–143
Ravindran V, Selle PH, Bryden WL (1999) Effects of phytase supplementation, individually and in combination, on the nutritive value of wheat and barley. Poult Sci 78:1588–1595
Reddy NR (2002) Occurrence, distribution, content, and dietary intake of phytate. In: Reddy NR, Sathe SK (eds) Food phytates. CRC Press, Boca Raton, pp 25–51
Reddy NR, Pierson MD (1994) Reduction in antinutritional and toxic components in plant foods by fermentation. Food Res Int 27(3):281–290
Reddy NR, Sathe SK, Salunkhe DK (1982) Phytases in legumes and cereals. Adv Food Res 82:1–92
Richardson AE, Barea J, McNeill AM, Prigent-Combaret C (2009) Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant Soil 339:305–339
Richardson AE, Hadobas PA, Hayes JE (2000) Acid phosphomonoesterase and phytase activities of wheat (*Triticum aestivum* L.) roots and utilization of organic phosphorus substrates by seedlings grown in sterile culture. Plant Cell Environ 23:397–405
Richardson AE, Hadobas PA, Hayes JE (2001) Extracellular secretion of *Aspergillus* phytase from Arabidopsis roots enables plants to obtain phosphorus from phytate. Plant J 25:641–649
Richardson NL, Higgs DA, Beames RM, McBride JR (1985) Influence of dietary calcium, phosphorus, zinc, and sodium phytate level on cataract incidence, growth and histopathology in juvenile Chinook salmon (*Oncorhynchus tshawytscha*). J Nutr 115:553–567
Rodriguez E, Han Y, Lei XG (1999) Cloning, sequencing and expression of an Escherichia coli phosphates/phytase gene (appA2) isolated from pig colon. Biochem Biophys Res Commun 257:117–123
Rodriguez E, Mullaney EJ, Lei XG (2000a) Expression of the *Aspergillus fumigatus* phytase gene in *Pichia pastoris* and characterization of the recombinant enzyme. Biochem Biophys Res Commun 268:373–378
Rodriguez E, Wood ZA, Karplus PA, Lei XG (2000b) Site-directed mutagenesis improves catalytic efficiency and thermostability of *Escherichia coli* pH 2.5 acid phosphatase/phytase expressed in *Pichia pastoris*. Arch Biochem Biophys 382(1):105–112
Roopesh K, Ramachandran S, Nampoothiri KM, Szakacs G, Pandey A (2005) Comparison of phytase production on wheat bran and oilcakes in solid-state fermentation by *Mucor racemosus*. Bioresour Technol 97(3):506–511
Roy T, Banerjee G, Dan SK, Ray AK (2013) Optimization of fermentation conditions for phytase production by two strains of *Bacillus licheniformis* (LF1 and LH1) isolated from the intestine of Rohu, *Labeo rohita* (Hamilton). In: Proceedings of the zoological society, vol 66. Springer-Verlag, p 27–35

Ruf JC, Ciavatti M, Gustafsson T, Renaud S (1991) Effects of PP-56 and vitamin E on platelet hyperaggregability, fatty acid abnormalities, and clinical manifestations in streptozotocin-induced diabetis rats. Diabetis 40:233–239

Rumsey GL (1993) Fish meal and alternate source of protein in fish feeds: update 1993. Fisheries 18:14–19

Sabu A, Sarita S, Pandey A, Bogar B, Szakacs G, Soccol CR (2002) Solid-state fermentation for production of phytase by *Rhizopus oligosporus*. Appl Biochem Biotechnol 102–103:251–260

Sajidan A, Farouk A, Greiner R, Jungblut P, Müller EC, Borriss R (2004) Molecular and physiological characterization of a 3-phytase from soil bacterium *Klebsiella sp.* ASR1. Appl Microbiol Biotechnol 65:110–118

Sandberg AS, Brune M, Carlsson NG, Hallberg L, Skoglund E, Rossander-Hulthen L (1999) Inositol phosphates with different numbers of phosphate groups influence iron absorption in humans. Am J Clin Nutr 70:240–246

Sands JS, Ragland D, Wilcox JR, Adeola O (2003) Relative bioavailability of phosphorus in low-phytate soybean meal for broiler chicks. Can J Anim Sci 83:95–100

Sano K, Fukuhara H, Nakamura Y (1999) Phytase of the yeast *Arxula adeninivorans*. Biotechnol Lett 21:33–38

Sartirana ML, Bianchetti R (1967) The effect of phosphate on the development of phytase in the wheat embryo. Physiol Plant 20:1066–1075

Scott JJ, Loewus FA (1986) A calcium-activated phytase from pollen of *Lilium longiflorum*. Plant Physiol 82:333–335

Sebastian S, Touchburn SP, Chavez ER (1998) Implications of phytic acid and supplemental microbial phytase in poultry nutrition: a review. World Poult Sci J 54:27–47

Segueilha L, Lambrechts C, Boze H, Moulin G, Galzy P (1992) Purification and properties of the phytase from chwanniomyces castellii. J Ferment Bioeng 74:7–11

Selle PH, Ravindran V (2008) Phytate degrading enzymes in pig nutrition. Livest Sci 113:99–122

Selle PH, Ravindran V, Caldwell RA, Bryden WL (2003) Phytate and phytase: consequences for protein utilization. Nutr Res Rev 13:255–278

Shah V, Parekh LJ (1990) Phytase from *Klebsiella* sp. PG-2- purification and properties. Indian J Biochem Biophys 27:98–102

Shamsuddin AM, Baten A, Lalwani ND (1992) Effects of inositol hexaphosphate on growth and differentiation in K-56 erythroleukemia cell line. Cancer Lett 64:195–202

Shao N, Huang H, Meng K (2008) Cloning, expression, and characterization of a new phytase from the phytopathogenic bacterium *Pectobacterium wasabiae* DSMZ 18074. J Microbiol Biotechnol 18(7):1221–1226

Shears SB (1998) The versatility of inositol phosphates as cellular signals. Biochim Biophys Acta 1436:49–67

Shieh TR, Ware JH (1968) Survey of microorganisms for the production of extracellular phytase. Appl Microbiol 16:1348–1351

Shieh TL, Wodzlnski LJ, Ware JH (1969) Regulation of the formation of acid phosphatase by inorganic phosphate in *Aspergillus ficuum*. J Bacteriol 100:1161–1165

Shimizu M (1992) Purification and characterization of phytase from *Bacillus subtilis* (natto) N-77. Biosci Biotechnol Biochem 56:1266–1269

Shimizu M (1993) Purification and characterization of phytase and acid-phosphatase produced by *Aspergillus-oryzae* K1. Biosci Biotechnol Biochem 57(8):1364–1365

Shivanna GB, Venkateshwaran G (2014) Phytase production by *Aspergillus niger* CFR 335 and *Aspergillus ficuum* SGA 01 through submerged and solid-state fermentation. Sci World J 2014:392615

Simon O, Igbasan F (2002) In vitro properties of phytase from various microbial origins. Int J Food Sci Technol 37:813–822

Simons PCM, Versteegh HAJ, Jongbloed AW et al (1990) Improvement of phosphorus availability by microbial phytase in broilers *Schwanniomyces castellii* and pigs. Brit J Nutr 64:525–540

Singh B, Kunze G, Satyanarayana T (2011) Developments in biochemical aspects and biotechnological applications of microbial phytases. Biotechnol Mol Biol Rev 6(3):69–87
Singh B, Satyanarayana T (2006) A marked enhancement in phytase production by a thermophilic mould Sporotrichum thermophile using statistical designs in a cost-effective cane molasses medium. J Appl Microbial 101(2):344–352
Singh B, Satyanarayana T (2008) A marked enhancement in phytase production by a thermophilic mould Sporotrichum thermophile using statistical designs in a cost-effective cane molasses medium. J Appl Microbiol 101(2):344–352
Singh B, Satyanarayana T (2010) Plant growth promotion by an extracellular HAP-phytase of a thermophilic mold *Sporotrichum thermophile*. Appl Biochem Biotechnol 160(5):1267–1276
Singh B, Satyanarayana T (2011) Microbial phytases in phosphorus acquisition and plant growth promotion. Physiol Mol Biol Plants 17(2):93–103
Singh N, Dahiya S, Poonia S (2008) Phytase: application in food and feed industries. Proc. National Seminar on Food Safety and Quality held at Department of Food Technology, Guru Jambheshwar University of Science and Technology, Hisar-125001, Haryana, India. p 161–166
Siren M, Linne L, Persson L (1991) Pharmacological effects of d-myo-inositol-1,2,6-trisphosphate. In: Reitz AB (ed) Inositol phosphates and derivatives. Synthesis, biochemistry and therapeutic potential. American Chemical Society, Washington, DC, pp 103–110
Skoglund E, Carlsson NG, Sandberg AS (1997) Determination of isomers of inositol mono to hexaphosphates in selected foods and intestinal contents using high-performance ion chromatography. J Agric Food Chem 45:431–436
Soni SK, Khire JM (2007) Production and partial characterization of two types of phytase from *Aspergillus niger* NCIM 563 under submerged fermentation conditions. World J Microbiol Biotechnol 23:1585–1593
Spitzer LS, Philips PH (1972) Phytase and alkaline phosphatase activities in intestinal mucosae of rat, chicken, calf and man. Biochim Biophys Acta 268:442–452
Sreeramulu G, Srinivasa DS, Nand K, Joseph R (1996) *Lactobacillus amylovorus* as a phytase producer in submerged culture. Lett Appl Microbiol 23:385–388
Srivastava BIS (1964) The effect of gibberellic acid on ribonuclease and phytase activity of germinating barley seeds. Can J Bot 42:1303–1305
Stockmann C, Losen M, Dahlems U, Knocke C, Gellissen G, Buchs J (2003) Effect of oxygen supply on passaging, stabilising and screening of recombinant *Hansenula polymorpha* production strains in test tube cultures. FEMS Yeast Res 2:195–205
Sunita K, Kim YO, Lee JK, Oh TK (2000) Statistical optimization of seed and induction conditions to enhance phytase production by recombinant Escherichia coli. Biochem Eng J 5:51–56
Sunitha K, Lee JK, Oh TK (1999) Optimization of medium components for phytase production by *E. coli* using response surface methodology. Bioprocess Eng 21:477–481
Sutardi M, Buckle KA (1988) Characterization of extra and intracellular phytase from *Rhizopus oligosporus* used in tempeh production. Int J Food Microbiol 6:67–69
Suzuki U, Yoshimura K, Takaishi M (1907) Ueber ein enzym “Phytase” das “Anhydro-oxy-methylen diphosphorsaure” spaltet. Tokyo Imp Univ Coll Agric Bull 7:503–512
Takanobu H (2002) Novel functions and applications of trehalose. Pure Appl Chem 74:1263–1269
Tambe SM, Kaklij GS, Kelkar SM, Parekh LJ (1994) Two distinct molecular forms of phytase from *Klebsiella aerogenes*: evidence for unusually small active enzyme peptide. J Ferment Bioeng 77:23–27
Tomschy A, Brugger R, Lehmann M, Svendsen A, Vogel K, Kostrewa D (2002) Engineering of phytase for improved activity at low pH. Appl Environ Microbiol 68:1907–1913
Touati E, Danchin A (1987) The structure of the promoter and amino terminal region of the pH 2.5 acid phosphatase structural gene (appA) of *Escherichia coli*-a negative control of transcription mediated by cyclic-AMP. Biochimie 69:215–221
Tran TT, Mamo G, Mattiasson B, Hatti-Kaul R (2010) A thermostable phytase from Bacillus sp. MD2: cloning, expression and high-level production in Escherichia coli. J Ind Microbiol Biotechnol 37:279–287

Tran TT, Hatti-Kaul R, Dalsgaard S, Yu S (2011) A simple and fast kinetic assay for phytases using phytic acid–protein complex as substrate. Anal Biochem 410:177–184

Tseng YH, Fang TJ, Tseng SM (2000) Isolation and characterization of a novel phytase from *Penicillium simplicissimum*. Folia Microbiol 45:121–127

Tyagi PK, Verma SVS (1998) Phytate phosphorus content of some common poultry feed stuffs. Indian J Poult Sci 33:86–88

Tye AJ, Siu FKY, Leung TYC, Lim BL (2002) Molecular cloning and the biochemical characterization of two novel phytases from *B. subtilis* 168 and *B. licheniformis*. Appl Microbiol Biotechnol 59:190–197

Ullah AHJ (1988) Production, rapid purification and catalytic characterization of extracellular phytase from Aspergillus ficuum. Prep Biochem 18:443–458

Ullah AHJ, Cummins BJ (1987) Purification, N-terminal amino acid sequence and characterisation of pH 2.5 optimum acid phosphatases (E.C.3.1.3.2) from *Aspergillus ficuum*. Prep Biochem 17:397–422

Ullah AHJ, Cummins BJ (1988) *Aspergillus ficuum* extracellular pH 6.0 optimum acid phosphatase: purification, N-terminal amino acid sequence and biochemical characterization. Prep Biochem 18:37–65

Ullah AHJ, Dischinger HC (1993) *Aspergillus ficuum* phytase: complete primary structure elucidation by chemical sequencing. Biochem Biophys Res Commun 192:747–753

Ullah AHJ, Gibson DM (1987) Extracellular phytase (E.C.3.1.3.8) from *Aspergillus ficuum* NRRL 3135: purification and characterization. Prep Biochem 17:63–91

Ullah AHJ, Mullaney EJ (1996) Disulfide bonds are necessary for structure and activity in *Aspergillus ficuum* phytase. Biochem Biophys Res Commun 227:311–317

Ullah AHJ, Phillippy BQ (1994) Substrate selectivity in *Aspergillus ficuum* phytase and acid phosphatases using myo-inositol phosphates. J Agric Food Chem 42:423–425

Ullah AHJ, Sethumadhavan K (2003) PhyA gene product of *Aspergillus ficuum* and *Peniophora lycii* produces dissimilar phytases. Biochem Biophy Res Commun 303:463–468

Ullah AHJ, Sethumadhavan K, Mullaney EJ, Ziegelhoffer T, Austin- Phillips S (2003) Fungal phyAgene expressed in potato leaves produces active and stable phytase. Biochem Biophys Res Commun 306:603–609

Van Etten RL, Davidson R, Stevis PE, MacArthur H, Moore DL (1991) Covalent structure, disulfide bonding, and identification of reactive surface and active site residues of human prostatic acid phosphatase. J Biol Chem 266:2313–2319

Van Hartingsveldt W (1993) Cloning, characterization and over expression of the phytase-encoding gene (phyA) of *Aspergillus niger*. Gene 127:87–94

Vats P, Banerjee UC (2002) Studies on the production of phytase by a newly isolated strain of Aspergillus nigervan teighamobtained from rotten wood logs. Process Biochem 38:211–217

Vats P, Banerjee UC (2004) Production studies and catalytic properties of phytases (myo-inositol hexakis phosphate phosphohydrolases): an overview. Enzym Microb Technol 35:3–14

Vats P, Banerjee UC (2005) Biochemical characterisation of extracellular phytase (myo-inositol hexakisphosphate phosphohydrolase) from a hyper-producing strain of *Aspergillus niger* van Teighem. J Ind Microbiol Biotechnol 32:141–147

Veum TL, Ellersieck MR (2008) Effect of low doses of Aspergillus niger phytase on growth performance, bone strength, and nutrient absorption and excretion by growing and finishing swine fed corn-soybean meal diets deficient in available phosphorus and calcium. J Anim Sci 86:858–870

Veum TL, Ledoux DR, Raboy V, Ertl DS (2001) Low-phytic acid corn improves nutrient utilization for growing pigs. J Anim Sci 79:2873–2880

Vohra A, Satyanarayana T (2001) Phytase production by the yeast, Pichia anomala. Biotechnol Lett 23(7):551–554

Vohra A, Satyanarayana T (2002) Purification and characterization of a thermostable and acid-stable phytase from Pichia anomala. World J Microbiol Biotechnol 18:687–691

Vohra A, Satyanarayana T (2003) Phytases: microbial sources, production, purification, and potential biotechnological applications. Crit Rev Biotechnol 23:29–60

Vohra A, Rastogi SK, Satyanarayana T (2006) Amelioration in growth and phosphorus assimilation of poultry birds using cell-bound phytase of *Pichia anomala*. World J Microbiol Biotechnol 22:553–558

Vucenik I, Shamsuddin AM (2003) Cancer inhibition by inositol hexaphosphate (IP6) and inositol: from laboratory to clinic. J Nutr 133:3778–3784

Wodzinski RJ, Ullah AHJ (1996) Phytase. Adv Appl Microbiol 42:263–303

Woyengo TA, Nyachoti CM (2013) Review: anti-nutritional effects of phytic acid in diets for pigs and poultry current knowledge and directions for future research. Can J Anim Sci 93:921

Wyss M, Brugger R, Kronenberger A, Remy R et al (1999) Biochemical characterization of fungal phytases (myo-inositolhexakisphosphate- phosphohydrolases): catalytic properties. Appl Environ Microbiol 65:367–373

Wyss M, Pasamontes L, Remy R, Kohler J, Kusznir E, Gadient M, Muller F, van Loon APGM (1998) Comparison of the thermostability properties of three acid phosphatases from molds: aspergillus fumigatus phytase, A. nigerphytase, and A. nigerpH 2.5 acid phosphatase. Appl Environ Microbiol 64:4446–4451

Xavier EG, Cromwell GL, Lindemann MD (2003) Phytase additions to conventional orlow-phytate corn-soybean meal diets on phosphorus balance in growing pigs. J Anim Sci 81(1):250–258

Xiao K, Harrison M, Wang ZY (2005) Transgenic expression of a novel *M. truncatula* phytase gene results in improving acquisition of organic phosphorus by *Arabidopsis*. Planta 222:27–36

Xiong AS, Yao QH, Peng RH, Zhang Z, Xu F, Liu JG, Han PL, Chen JM (2006) High level expression of a synthetic gene encoding *Peniophora lycii* phytase in methylotrophic yeast *Pichia pastoris*. Appl Microbiol Biotechnol 72(5):1039–1047

Yamamoto S, Minoda Y, Yamada K (1972) Chemical and physicochemical properties of phytase from Aspergillus terreus. Agric Biol Chem 36:2097–2103

Yang G, Shamsuddin AM (1995) IP-6-induced growth inhibition and differentiation of HT-29 human colon cancer cells: involvement of intracellular inositol phosphates. Anticancer Res 15:2479–2488

Yang WJ, Matsuda Y, Sano S, Masutani H, Nakagawa H (1991) Purification and characterization of phytase from rat intestinal mucosa. Biochim Biophys Acta 1075:75–82

Yanke LJ, Bae HD, Selinger LB, Cheng KJ (1998) Phytase activity of anaerobic ruminal bacteria. Microbiology 144:1565–1573

Yao B, Thang C, Wang J, Fan Y (1998) Recombinant *Pichia pastoris* over expressing bioactive phytase. Sci Chin 41:330–336

Yao MZ, Zhang YH, Lu WL, Hu MQ, Wang W, Liang AH (2012) Phytases: crystal structures, protein engineering and potential biotechnological applications. J Appl Microbiol 112(1):1–14

Yoon SJ, Choi YJ, Min HK, Cho KK, Kim JW, Lee SC, Jung YH (1996) Isolation and identification of phytase producing bacterium. Enterobacter sp. 4, and enzymatic properties of phytase enzyme. Enzym Microb Technol 18:449–454

Yu S, Cowieson A, Gilbert C, Plumstead P, Dalsgaard S (2012) Interactions of phytate andmyo-inositol phosphate esters (IP1-5) including IP5 isomers with dietary protein and iron and inhibition of pepsin. J Anim Sci 90:1824–1832

Zamudio M, Gonzalez A, Bastarrachea F (2002) Regulation of *Raoultella terrigena* comb.nov. phytase expression. Can J Microbiol 48:71–81

Zeng H, Yao B, Zhou WH, Fan ZY (2001) Advances in microbial phytase research. J Aqua Chin 15:87

Zhang S, Reddy MS, Kokalis Burelle N, Wells LW (2001) Lack of induced systemic resistance in peanut to late leaf spot disease by plant growth-promoting rhizo bacteria and chemical elicitors. Plant Dis 85:879–884

Zyla M, Mika B, Stodolak A, Wikiera J, Koreleski S, Swiatkiewicz (2004) Towards complete dephosphorylation and total conversion of phytates in poultry feed. Poult Sci 83:1175–1186

Escherichia coli as a Potential Candidate Against Food Borne Bacterial Infections

18

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18.1 Introduction

Regardless of current advances and safety ideas, for example, danger investigation and basic control point framework, the revealed quantities of food-borne diseases and inebriation are still on the rise. As per the Council for Agricultural Science and Technology, bacterial microbes in food products lead to an expected 6.5–33 million instances of human sickness and up to 9000 mortality cases yearly, with the fundamental eatables including meat, poultry, eggs, ocean bottom and dairy items (Tufail et al. 2011). The microbial species that are responsible for these illnesses and mortalities include *Salmonella*, *Vibrio cholerae*, *Bacillus cereus*, *Staphylococcus aureus* and *Clostridium botulinum* (Buzby et al. 1996). In 2004, the Infectious Disease Society of America (IDSA) concluded that most (over 70%) of microbial disease-causing agents that are responsible for fatal infections are probably going to be resistant to no less than one of the drugs generally utilised as a part of the treatment for bacterial diseases (Gillor et al. 2004; Boyle-Vavra and Daum 2007). A few preventive measures have been used to prevent the microbial resistance, yet at the same time, there is a pressing requirement for new antimicrobial drugs to beat risky pathogens. The naturally occurring substances of microbial origin have provided a continuing source of novel antibiotics and medicines since the origin of man. Various investigations have unmistakably exhibited that the ordinary inhabitant intestinal microbes is the major barrier defending animals and humans against flourishment of disease-causing microbes in the intestine (Canny and McCormick 2008). *Escherichia coli* is one of the main bacterial genera, alongside *Streptococcus*, to colonise the digestive tract of human and infants. It has been accounted for that few *E. coli* strains build up a defensive impact against drug-resistant enterotoxigenic *E. coli*. Such beneficial microbes (lactobacilli, bifidobacteria, enterococci, non-pathogenic *E. coli* and yeasts) are found to be synthesising defensive metabolic products and antimicrobial compounds including organic acids and antimicrobial

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proteins (AMPs) (Verna and Lucak 2010). These bacterial AMPs have a few intriguing elements such as narrow range of action, high power and fast killing of microorganisms, properties that have prompted enhanced use as new-generation antibiotics (Rossi et al. 2008; Smith and Hillman 2008). Hence, the central topic of the present examination included investigation of intra- and extracellular proteins of commensal bacterial species against the dangerous food-related pathogens.

18.2 Human Commensal Bacterial Species As Source of Antimicrobial Proteins

The following non-pathogenic bacterial strains belonging to human microbiome were obtained from the Institute of Microbial Technology Chandigarh: *Lactococcus lactis* (MTCC440), *Lactococcus lactis* (MTCC3041), *Escherichia coli* DH5α (MTCC1652), *Escherichia coli* (MTCC1674), *Streptococcus thermophiles* (MTCC1938), *Staphylococcus epidermidis* (MTCC3615), *Staphylococcus epidermidis* (MTCC6810) and *Streptococcus* subsp. *equi* (MTCC3522). Antimicrobial activity of bacterial proteins was investigated against ten registered Microbial Type Culture Collection (MTCC) and American Type Culture Collection (ATCC) pathogenic bacterial isolates, namely, the gram-positive ones, *Staphylococcus aureus* MTCC 3160, *Staphylococcus aureus* ATCC 39315, *Bacillus cereus* MTCC 10085, *Bacillus subtilis* MTCC 441 and *Proteus vulgaris* MTCC 426, and gram negatives including *Salmonella typhi* MTCC 531, *Salmonella typhi* MTCC 733, *Salmonella typhi* ATCC13311, *Vibrio cholerae* MTCC 3904 and *Vibrio cholerae* ATCC 259323.

18.2.1 Methodologies and Techniques for Antimicrobial Evaluation

Various chosen microbial bacterial species were grown in LB Broth at 100 r.p.m. in a BOD incubator shaker at 37 °C for 48 h at medium scale (2–3 l). Bacterial cultures were centrifuged at 8000 r.p.m. for 30 min in a refrigerated centrifuge. The secretory protein fraction was isolated from pellet, concentrated via ammonium sulphate precipitation and dialysed at 4 °C using dialysis membrane with cut-off limit of 12 KDa (Millipore) and optimised as extracellular protein fraction. The pellet was subjected to sonication (Bio Age Sonicator, Mohali India) in a sonication buffer (50 mmol l^{-1}Tris-HCl, 50 mmol l^{-1} EDTA, 5 mmol l^{-1} DTT, 1 mmol l^{-1} PMSF) at 20 s burst at 200 W and 10 s cool period. The sonicate was centrifuged at 12,000 r.p.m. for 1 h and the supernatant was dialysed, lyophilised and used as cytosolic protein. The secretory and cytosolic protein samples were filtered through 0.22 μm syringe filters (Millipore) before performing antimicrobial evaluation experiments (Balhara et al. 2014). In sterilised conditions, a single colony of pathogenic bacterial strain (cultured on LB Agar plates) was transferred and cultured into 250 ml Luria broth in an incubator shaker overnight at 60 r.p.m., 35 °C. After 12 h the culture was centrifuged at 4000 r.p.m. for 5 min with proper sterile environment three to four times till the secretory fraction was transparent. The bacterial pellet obtained

was dissolved in 15 ml of aseptic saline water, and OD of the bacterial culture was calculated at 495 nm. The bacterial suspension was serially diluted under aseptic conditions until the OD for the bacterial culture recorded between 0.5 and 1.0. The accurate quantification of CFUs was evaluated from the survival curve. The dilution factor required to obtain the desired concentration of 5×10^6 cfu/ml was quantified and used for performing various experiments. The antimicrobial activity of bacterial secretory and cytosolic proteins on chosen pathogenic bacterial strains was evaluated by the agar disc diffusion assay. The discs of 0.5 mm-diameter (prepared from Whatmann Number 1 filter paper) were used on pre-inoculated agar plates and surface filled with protein samples at a range of concentrations. The plates were incubated at 37 °C for 24–48 h; zones of antimicrobial activity around the discs were observed and calculated (Dhankhar et al. 2012).

The resazurin solution obtained by homogenously mixing 300 mg resazurin powder in 50 ml of aseptic distilled water was used in antimicrobial microbroth dilution assay performed in 96-well plates under sterile environment (Dhankhar et al. 2013). A 100 μl of secretory and cytosolic proteins in 10% (v/v) sterile water were pipetted into the first row of the plate. All wells of the 96-well plated were inoculated with 50 μl of nutrient broth and 50 μl of sterile saline water. The wells were serially diluted as every well had 100 μl of the antimicrobial proteins in a concentration range of 1000.0–3.90 μg/ml. 10 μl of resazurin indicator solution was added in each well. At the end, 10 μl of pathogenic bacterial cultures was inoculated to each well to achieve a concentration of 5×10^6 CFU/ml. Each plate was covered with parafilm to make sure that bacterial culture remain hydrated. Tetracycline was used as positive control in each set of experiment. The experiment was run in triplicate numbers and the 96-well plates used were incubated at 37 °C for 18–24 h. The change in colour of the resazurin indicator was observed. A transition in colour from purple to pink was taken as positive. The minimum concentration at which this transition happened was calculated as MIC value for the secretory/cytosolic proteins against bacterial strain. The thermostability of most potent secretory/cytosolic proteins in a temperature range of 4–100 °C was evaluated by incubating in 10 mM Tris-HCl buffer (pH 7.5) for 20 min at each temperature. The solutions of proteins were thawed to 4 °C and used for antibacterial activities. The effect of acidic and alkaline stresses was determined by using citrate phosphate buffer (pH 2.5–8.0) and Tris-HCl buffer (pH 7.5–10.5). The potent protein fraction was incubated in each buffer at various pHs at 4 °C for 20 min. The antibacterial activity was assayed after the pH of each protein solution was readjusted to 6.5 using disc diffusion assay. The influence of different hydrolytic enzymes on antimicrobial potential of protein fractions was measured by incubating the concentrated active protein sample with α-amylase, pronase E, lipase and proteinase K at a final concentration of 1.0 mg/ml at 37 °C for 1 h. The preliminary in vitro combination was studied between standard drug tetracycline and most active bacterial protein (*E. coli* DH5α cytosolic protein) by DDA (Ruhil et al. 2013). The discs impregnated with MIC of drug in μg/disc of tetracycline (calculated by DDA) alone and with MIC_{50} of active bacterial protein. The 6 mm zone of inhibition was considered as MIC point and analysed for synergy if any.

18.3 Experimental Data and Future Prospects

The antibacterial activity of secretory and cytosolic proteins of chosen commensal bacteria against medically important food poisoning pathogens was evaluated by resazurin-based microtitre dilution method (Tables 18.1 and 18.2).

Among the cytosolic proteins, *E. coli* DH5α cytosolic fraction was active against all the pathogenic bacterial isolates having MIC in the range of 62.5–250 μg/ml being most active against *B. cereus* and reasonable activity against remaining pathogens. However, the secretory protein fraction of this bacterium did not show significant antagonism against the pathogenic isolates. The cytosolic protein fractions of *Streptococcus* subsp. *equi* depicted inhibition against *B. subtilis* and *P. vulgaris* but did not show any activity against *Salmonella typhi* and *Staphylococcus aureus*. The bacterial proteins of *Staphylococcus epidermidis* (secretory as well as cellular) were devoid of any inhibitory potential. The secretory protein of *Streptococcus thermophilus* showed significant antibacterial activity against majority of the pathogens being most active against *S. aureus*, *B. cereus* and *V. cholerae*. The bacterial supernatant proteins of *Lactococcus lactis* (MTCC 440) were considerably active against *B. cereus*, while moderate inhibition was observed against *S. aureus*, *V. cholerae* and *B. subtilis*. Tetracycline (the standard drug) shows MICs in the range of 5.0–20 μg/ml against different pathogenic bacterial strains. In disc diffusion assay, a positive and low concentration of 25 μg/disc of bacterial proteins used in this study showed potent and broad-spectrum antimicrobial activity against pathogenic strains of microbes. Moreover it was observed that only 4 out of 14 bacterial protein

Table 18.1 Antibacterial evaluation of bacterial cytosolic proteins using resazurin microtitre dilution method

Bacterial species	Bacterial cytosolic proteins MIC in (μg/ml) pathogens						
	S. typhi	*S. aureus*	*B. cereus*	*V. cholerae*	*B. subtilis*	*P. vulgaris*	*S. typhi* (733)
Lactococcus lactis (MTCC 440)	500.0	–	250.0	–	500.0	–	500.0
Lactococcus lactis (MTCC 3041)	500.0	–	500.0	–	–	–	–
E. coli DH5α (MTCC1652)	125.0	250.0	62.5	250.0	125.0	250.0	125.0
E. coli (MTCC 1674)	–	500.0	–	–	250.0	–	–
S. thermophilus (MTCC 1938)	–	500.0	–	–	–	500.0	–
S. epidermidis (MTCC 3615)	–	–	–	–	–	–	–
S. epidermidis (MTCC 6810)	500.0	–	–	500.0	500.0	250.0	–
Streptococcus equi (MTCC 3522)	–	–	500.0	250.0	250.0	500.0	–

– no activity up to tested concentration (1000.00 μg/ml)

S. thermophilus, *Streptococcus thermophilus*, *S. epidermidis*, *Staphylococcus epidermidis*

Table 18.2 Antibacterial evaluation of bacterial secretory proteins using resazurin microtitre dilution method

Bacterial species	Bacterial secretory proteins MIC in (μg/ml) pathogens						
	S. typhi	*S. aureus*	*B. cereus*	*V. cholerae*	*B. subtilis*	*P. vulgaris*	*S. typhi* (733)
Lactococcus lactis (MTCC 440)	500.0	250.0	125.0	250.0	250.0	500.0	500.0
Lactococcus lactis (MTCC 3041)	500.0	–	–	–	–	–	–
E. coli DH5α (MTCC1652)	–	500.0	–	–	–	500	–
E. coli (MTCC 1674)	–	250.0	–	–	500.0	–	–
S. thermophilus (MTCC 1938)	250.0	125.0	125.0	125.0	250.0	500.0	250.0
S. epidermidis (MTCC 3615)	–	–	–	–	–	–	–
S. epidermidis (MTCC 6810)	500.0	–	–	–	–	–	500.0
Streptococcus equi (MTCC 3522)	250.0	250.0	125.0	250.0	250.0	–	250.0

– no activity up to tested concentration (1000 μg/ml)

fractions were found to be endowed with antimicrobial activity at a present concentration of 25 μg/disc (Table 18.3). The cytosolic protein of *E. coli* DH5α had noteworthy zone of inhibition against all the pathogenic strains having maximum inhibition against *B. cereus*. Various concentrations of active protein (*E. coli* DH5α) ranging from 50 to 3.1 μg/disc were used to determine the antimicrobial activity using disc diffusion assay. The MIC was found to be in the range of 12.5–25 μg/disc. The inhibition was strongest against *B. cereus* showing a MIC of 12.5 μg/disc. Therefore cytosolic protein pool of *E. coli* DH5α represents itself as a source of potent antimicrobial agents. Further investigation was carried out to study its antibacterial activity in combination with standard drug tetracycline. An increase in zone of inhibition was observed against *B. cereus* and *B. subtilis* as compared to standard drug alone and in combination with MIC_{50} of *E. coli* DH5α cytosolic protein. However, the activity against remaining pathogens was not enhanced.

The effect of various hydrolytic enzymes (proteinase K, lipase and α-amylase) was studied on inhibitory activity of *E. coli* DH5α cytosolic protein. The treatment with proteinase K led to complete loss of inhibitory potential proving the proteinaceous nature of active substance. However, the activity was not altered due to treatment with lipase, α-amylase which demonstrated that any carbohydrate or lipid moiety is not required for inhibitory activity. The antimicrobial substance appeared to be heat stable as heat treatment up to 80 °C for 10 min did not cause any loss in activity, but the activity was reduced after heat treatment above100 °C. The inhibitory activity of all the bacterial proteins was tested after adjusting the pH to 6.5 to eliminate the possible effect of organic acids. The antimicrobial potential of most active protein fraction (*E. coli* DH5α cytosolic protein) was tested under a wide pH

Table 18.3 Antibacterial potential/zone of inhibition of various potent bacterial proteins shown by disc diffusion assay (25 μg/disc)

Bacterial species	Mean diameter of zone of inhibition (mm) pathogens						
	S. typhi	*S. aureus*	*B. cereus*	*V. cholerae*	*B. subtilis*	*P. vulgaris*	*S. typhi* (733)
Lactococcus lactis (MTCC 440) secretory	–	0.3 ± 0.2	7.1 ± 0.1	6.2 ± 0.3	6.1 ± 0.2	–	–
E. coli DH5α (MTCC1652) cytosolic	0.3 ± 0.4	0.1 ± 0.1	9.3 ± 0.3	6.1 ± 0.1	7.1 ± 0.2	6.4 ± 0.1	7.3 ± 0.2
S. thermophilus (MTCC 1938) secretory	–	0.0 ± 0.2	6.1 ± 0.3	6.0 ± 0.2	–	–	–
Streptococcus equi (MTCC 3522) secretory	0.1 ± 0.2	–	7.1 ± 0.3	–	–	–	6.0 ± 0.1
In vitro combination effect of *E. coli* DH5α cytosolic protein with standard drug tetracycline							
Tetracycline;1.25 μg/disc	0.3 ± 0.2	7.2 ± 0.3	6.4 ± 0.1	6.6 ± 0.2	6.3 ± 0.2	6.5 ± 0.2	6.0 ± 0.2
E. coli protein:tetracycline 6.2 μg(MIC_{50}):1.25 μg/disc	0.2 ± 0.1	7.3 ± 0.2	9.4 ± 0.2	6.5 ± 0.1	8.1 ± 0.2	6.3 ± 0.3	6.3 ± 0.1

range from 2.5 to 10.5. It was most active in pH range of 6–10. The pressing requirement for improved quality 'healthy' foods that are not chemically preserved has generated a home for natural food preservatives. The efficient natural food preservatives possess the qualities (Hill et al. 2012) of negligible toxicity, storage conditions stable, required in minimum amount, economically viable and without any harmful effect on eatables. The bacteriocins belong to the category of ideal natural food preservatives as they possess most of the above-mentioned qualities. Still, to date nisin is the only bacteriocin which is used commercially on a pilot scale, obtaining FDA approval in the USA in 1988. Its efficacy and potency have promoted more research in this arena to identify novel bacteriocins or antimicrobial proteins from commensal bacterial strains which efficiently could be utilised in a commercial way. Various bacteriocins have now been isolated that possess antimicrobial potential towards the common food-borne pathogens. The assumption that LAB (lactic acid bacteria) helps in prevention of disorders of the intestine is almost profound through the ages of the science of microbiology (Molin 2001). LAB found universally among the food products generally belongs to the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*. In the present investigation, the cell-free supernatants (secretory protein) of *Streptococcus thermophilus* (MTCC1938) and *Lactococcus lactis* (MTCC440) were found to be reasonably active against these food-borne pathogens. This antimicrobial potential might be the result of bacteriocin-like substances. However, the cytosolic proteins of *E. coli* DH5α were of immense interest as evidenced by its antibacterial potential against food-borne human pathogens. As the activity resided in cytosolic protein pool, it was not due to bacteriocin (secreted AMPs), but some intracellular protein was responsible for antimicrobial potential. The temperature variations did not impact its antimicrobial potential which gives additional benefit to its activity that would tolerate the storage and processing conditions.

18.4 Conclusion

Such a broad antimicrobial spectra produced by *E. coli DH5α* might make it efficiently usable as biocontrol agent, natural food preservatives and other arenas that focus on eradication of pathogenic microbial species in the food products. Further purification and isolation of the active components might provide novel anti-infective compounds.

References

Balhara M, Ruhil S, Kumar M et al (2014) Inhibition of conidiophore development in *Aspergillus fumigatus* by an *Escherichia coli* DH5alpha strain, a promising antifungal candidate against Aspergillosis. J Mycol Méd 24:1–12

Boyle-Vavra S, Daum SR (2007) Community-acquired methicillin-resistant *Staphylococcus aureus*: the role of Panton–Valentine leukocidin. Lab Investig 87:3–9

Buzby JCT, Roberts CT, Lin J, MacDonald JM (1996) Bacterial food borne diseases: medical costs and productivity losses, Agricultural Economic Report Number 741. USDA Economic Research Service, Washington, DC

Canny OG, McCormick AB (2008) Bacteria in the intestine, helpful residents or enemies from within? Infect Immun 76(8):3360–3373

Dhankhar S, Dhankhar S, Kumar M et al (2012) Analysis toward innovative herbal antibacterial and antifungal drugs. Recent Patents Antiinfect Drug Discov 7(3):242–248

Dhankhar S, Sharma M, Ruhil S, Balhara M et al (2013) Evaluation of antimicrobial and antioxidant activities of Bougainvillea spectabilis. Int J Pharm Sci 5(3):178–182

Gillor O, Kirkup BC, Riley MA (2004) Colicins and microcins: the next generation antimicrobials. Adv Appl Microbiol 54:129–146

Hill C, Keeffe T, Ross RP (2012) Bacteriocins-antimicrobial agents with potential in food preservation and food safety. In: Encyclopaedia of dairy science. Academic, London

Molin G (2001) Probiotics in foods not containing milk or milk constituents, with special reference to lactobacillus plantarum 299v. Am J Clin Nutr 73:380–385

Rossi LM, Rangasamy P, Zhang J et al (2008) Research advances in the development of peptide antibiotics. J Pharm Sci 97:1060–1070

Ruhil S, Balhara M, Dhankhar S et al (2013) Advancement in infection control of opportunistic pathogen (*Aspergillus* spp.): adjunctive agents. Curr Pharm Biotechnol 14:226–232

Smith L, Hillman JD (2008) Therapeutic potential of type A (I) lantibiotics, a group of cationic peptide antibiotics. Curr Opin Microbiol 11(5):401–408

Tufail M, Hussain S, Malik F et al (2011) Isolation and evaluation of antibacterial activity of bacteriocin produced by *Lactobacillus bulgaricus* from yogurt. Afr J Microbiol Res 5(22):3842–3847

Verna EC, Lucak S (2010) Use of probiotics in gastrointestinal disorders: what to recommend? Ther Adv Gastroenterol 3(5):307–319

19 Pharmaceuticals and Personal Care Products (PPCPs) as Emerging Environmental Pollutants: Toxicity and Risk Assessment

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19.1 Introduction

Pharmaceuticals and personal care products (PPCPs), which include diverse groups of organic chemicals, are a class of compounds considered as emerging organic contaminants (EOCs). These products include nutritional supplements, diagnostic agents, antibiotics, hormones, musk fragrances, and non-steroidal anti-inflammatory drugs (NSAIDs) as well as other drugs that are used for veterinary medicine, human health, agricultural practice, and cosmetic care (Farre et al. 2008; Fent et al. 2006). PPCPs in aquatic environments are considered as some of the most critical environmental pollutants (Al-Odaini et al. 2010). PPCPs enter the ecosystem in a number of ways. Effluents from wastewater treatment plants (WWTPs) or sewage treatment plants (STPs) and large farms with many animals are considered as the main sources responsible for the discharging of PPCPs into the environment. These PPCPs are not completely digested by humans and animals, and undigested PPCPs are excreted as waste and washed off into sink drains. PPCPs, like hormones, are naturally excreted by humans and animals, and this poses potential risks both to the ecosystems into which the PPCPs are discharged and to drinking water resources. Many studies performed worldwide have shown that hundreds of PPCPs and their derivatives are usually detected in various environments. Concentrations of PPCPs in groundwater vary from place to place; in incompletely treated water, concentrations are less than 0.1 μgl, and in drinking water and treated water, concentrations are commonly below 0.05 μg/l. PPCP metabolites are also commonly detected in the environment; the lifespan of PPCPs and their metabolites in the environment varies from months to years, depending on their natural degradation in the environment (US EPA, 2013).

Almost all PPCPs seem to be biologically active in nature. These compounds are designed to act in humans and animals according to specific pathways and processes

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to cure diseases. The United States Environmental Protection Agency (US EPA 2013) considers PPCPs as emerging contaminants. There is little knowledge about the impact of these contaminants on human health after they are released into ecosystems. PPCPs are widely detected in many aquatic environments around the world—in rivers, lakes, and groundwater in almost every continent. There is some evidence of PPCPs in groundwater and surface is reported in literature (Table 19.2). PPCPs in aquatic environments have negative effects on aquatic species. Steroid hormones have adverse effects on the environment, and even at μgL^{-1} or ng^{-1}concentrations can inhibit reproduction in aquatic species. The continuous release of huge quantities of PPCPs (including steroid hormones) has led to these compounds being widespread in the environment, and as they exert bio-activity at extremely low concentrations, there is a need to study the possible effects of these compounds in our surroundings on human, aquatic, and ecological environments. PPCPs have unpredictable biochemical interactions with other compounds in the environment, and PPCPs and their metabolites, or the interaction of two or more of these compounds present in the environment, affects both aquatic and terrestrial species. PPCPs are present in water utilized by plants for photosynthesis and photorespiration, and this water is stored in the edible parts of plants. When humans and animals eat the plants, these compounds enter our food cycle. We use water for our daily needs, and wastewater recycled by WWTPs and STPs is often used for irrigation. The use of untreated or treated water obtained from STPs for irrigation will introduce PPCPs into the fields. The uptake of this contaminated water leads to the accumulation of PPCPs that contaminate plants and so these compounds may be further transferred to the food chain (US EPA 2013).

In this chapter the modes of sources of PPCPs that enter the environment and the potential effect of these compounds on the environment and the risks associated with these compounds has been discussed. The biodegradation of PPCPs by microorganisms constitutes an eco-friendly technique to decontaminate the environment and this will also reduce the concentrations of these compounds.

19.1.1 Classification of PPCPs

PPCPs or pharmaceutically active compounds are used to treat various diseases due to their bioactive property. PPCPs consist of two categories: pharmaceuticals and personal care products. The pharmaceuticals include commercially available drugs and agents that are used to treat diseases in both humans and animals. This category includes NSAIDs, other anti-inflammatory agents, antidepressants, tranquilizers, pain killers, antipsychotic agents, anti-cancer drugs, anti-hypertensive medicines, antiseptic agents, lipid regulators, oral contraceptives, antibiotics, synthetic hormones, and many other classes and types of drugs. Personal care products include various compounds; for example, perfumes, deodorants, shampoos, synthetic hair dyes, hair sprays, oral hygiene products, make-up products, sunscreen creams, body lotions, and various other creams (Table 19.1).

Table 19.1 Classification and uses of various pharmaceuticals and personal care products (PPCPs)

Class of PPCP	Generic name	Pharmaceutical use	Scientific name	Chemical formula
A. Pharmaceuticals				
Nonsteroidal anti-inflammatory drugs	Diclofenac	Treat mild to moderate pain, or signs and symptoms of osteoarthritis or rheumatoid arthritis	2-[(2,6-Dichlorophenyl)amino] benzeneacetic acid	$C_{14}H_{11}C_{12}NO_2$
	Ibuprofen	Reduce fever and treat pain or inflammation caused by many conditions, such as headache, toothache, back pain, arthritis, menstrual cramps, and minor injuries	a-Methyl-4-(isobutyl) phenylacetic acid	$C_{13}H_{18}O_2$
	Naproxen	Treat pain or inflammation caused by conditions such as arthritis, ankylosing spondylitis, tendinitis, bursitis, gout, and menstrual cramps	(S)-(+)-2-(6-Methoxy-2-naphthyl) propionic acid	$C_{14}H_{14}O_3$
Antidepressants	Fluoxetine	Treat major depression, obsessive-compulsive disorder, and panic disorder	(±)-N-methyl-c-[4-(trifluoromethyl) phenoxy] benzenepropanamine	$C_{17}H_{18}F_3NO$
	Citalopram	Helps to restore the balance of serotonin (a natural substance) in the brain	1-[3-(Dimethylamino) propyl]-1-(4-fluorophenyl)-1,3-dihydro-5-isobenzofurancarbonitrile	$C_{20}H_{21}FN_2O$
Hormones	Estrone	Manage vaginal menopausal symptoms such as itching, burning, and dryness in or around the vagina, and painful sexual intercourse	1,3,5(10)-Estratrien-3-ol-17-one	$C_{18}H_{22}O_2$
	17b-Estradiol	Progestin therapy, for changes in vaginal bleeding; dysmenorrhea; increases in size of uterine leiomyomata; vaginitis, including vaginal candidiasis; changes in cervical secretion; and cervical ectropion	1,3,5-Estratriene-3,17b-diol	$C_{18}H_{24}O_2$
	17a-Ethinylestradiol	Orally bioactive estrogen used in many formulations of combined oral contraceptive pills	17a-Ethynyl-1,3,5(10)-estratriene-3,17b-diol	$C_{20}H_{24}O_2$

(continued)

Table 19.1 (continued)

Class of PPCP	Generic name	Pharmaceutical use	Scientific name	Chemical formula
Tranquillizers	Diazepam	Treat anxiety disorders, alcohol withdrawal symptoms, and muscle spasms	7-Chloro-1-methyl-5-phenyl-3H-1,4-benzodiazepin-2(1H)-one	$C_{16}H_{13}ClN_2O$
Anti-epileptics	Carbamazepine	Treat seizures and nerve pain such as trigeminal neuralgia and diabetic neuropathy	5H-dibenz[b,f]azepine-5-carboxamide	$C_{15}H_{12}N_2O$
Antibiotics	Sulfamethoxazole	Treat a wide variety of bacterial infections (such as middle ear, urinary, respiratory, and intestinal infections)	4-amino-N-(5-methyl-3-isoxazolyl) benzenesulfonamide	$C_{10}H_{11}N_3O_3S$
	Roxithromycin	Treat respiratory tract, urinary tract, and soft-tissue infections	Erythromycin 9-(-O-[2-methoxyethoxy] methyloxime)	$C_{41}H_{76}N_2O_{15}$
	Erythromycin	Treat bacterial infections, such as bronchitis, diphtheria, legionnaires' disease, pertussis (whooping cough), pneumonia; rheumatic fever; venereal disease (VD); and ear, intestinal, lung, urinary tract, and skin infections	6-(4-Dimethylamino-3-hydroxy-6-methyl-oxan-2-yl)oxy-14-ethyl-7,12,13-trihydroxy-4- (5- hydroxy-4-methoxy-4,6-dimethyl-oxan-2-yl) oxy-3,5,7,9,11,13-hexamethyl-1-oxacyclotetradecane-2,10-dione	C_{37}H67NO13
	Trimethoprim	Treat urinary tract infections caused by certain types of bacteria	2,4-Diamino-5-(3,4,5-trimethoxybenzyl) pyrimidine	$C_{14}H_{18}N_4O_3$
Lipid regulators	Clofibrate (active metabolite: clofibric acid)	Reduce high levels of cholesterol (a type of fat) in the blood. Clofibrate is especially good at reducing levels of triglycerides and very-low-density lipoprotein (VLDL; a form of cholesterol)	Ethyl 2-(4-chlorophenoxy)-2-methylpropanoate	$C_{12}H_{15}ClO_3$
	Gemfibrozil	Reduces levels of fats (triglycerides) and raises levels of "good" cholesterol (high-density lipoprotein; HDL) in the blood. It may also help to reduce levels of "bad" cholesterol (LDL)	5-(2,5-Dimethylphenoxy)-2,2-dimethyl-pentanoic acid	$C_{15}H_{22}O_3$
Stimulants	Caffeine	Stimulate the brain	1,3,7-Trimethylpurine-2,6-dione	$C_8H_{10}N_4O_2$

(continued)

B. Personal care products				
Fragrances (musks)	Galaxolide	Synthetic ingredient with a clean sweet musky floral woody odor, used in fragrances	1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta(g)-2-benzopyran	$C_{18}H_{26}O$
	Tonalide		6-Acetyl-1,1,2,4,4,7-hexamethyl tetralin	$C_{18}H_{26}O$
	Celestolide		4-Acetyl-6-tert-butyl-1,1-dimethylindane	$C_{17}H_{24}O$
	2,6-DMN (2,6-Dimethylnaphthalene)	Commercial importance as a starting material for high-performance polyester fibers and films. Polyethylene naphthalate (PEN) is made using the oxidation product of 2,6- DMN	2,6-dimethylnapthalene	$C_{12}H_{12}$
	Isophorone	Solvent in some printing inks, paints, lacquers, adhesives, copolymers, coatings, finishings, and pesticides. It is also used as a chemical intermediate and as an ingredient in wood preservatives and floor sealants	3,5,5-Trimethyl-2-cyclohexene-1-one	$C_9H_{14}O$
	Benzotriazole	Specific corrosion inhibitor for copper and copper alloys. It is now widely used in industry to reduce the corrosion of these alloys under both atmospheric and immersed conditions	5-Methyl-1H-benzotriazole	$C_6H_5N_3$
Fire reagents	Tri (2-chloroethyl) phosphate	Flame retardant, plasticizer, and viscosity regulator in various types of polymers, including polyurethanes, polyester resins, and polyacrylates fire	Tri(2-chloroethyl) phosphate	$C_6H_{12}C_{13}O_4P$

19.1.2 Sources of PPCPs

Individual households, manufacturing sites, dumping sites, large farms, STPs, and WWTPs are considered to be the main sources of the PPCPs that enter different environmental systems. Studies of these sources have produced information regarding the environmental load of PPCPs. The fate of water coming from STPs and WWTPs also has profound effect in the environment. PPCPs are removed by the treatment processes used in WWTPs ultimately determines the extent of PPCPs still left in the aquatic environment. Various studies have focused on the presence of PPCPs in rivers, lakes, STPs, and WWTPs (Table 19.2). Most of the studies has been conducted on the ways by which these compounds enters into ecological systems and their subsequent fates were carried out in areas having high populations density and with rich ecological resources.

PPCPs enter environmental systems by different pathways, some of which are considered as gateways for the entrance of these compounds to the environment. Examples of gateways are manufacturing sites that release their untreated or less-treated water to surface waters or STPs; domestic waste; STPs and WWTPs; sites of medicine landfills; aquaculture facilities; and biosolids. PPCPs that are present in aquatic systems enter terrestrial systems via the effluent from STPs or WWTPs that is used for irrigation and via river or sewage water and sludge used for agricultural practices. STPs and WWTPs play important roles in the transportation of PPCPs from one place to another. The life cycles of PPCPs and their metabolites can be easily studied in WWTPs and STPs. Owing to the complex structure of PPCPs, traditionally built WWTPs were not able to completely remove these compounds, or else they removed only a fraction of the PPCPs, and transformed them into different metabolites or forms in which two or more compounds were conjugated. The efficiency of STPs in the removal of PPCPs is also affected by the three main types of treatment processes they use; mechanical, chemical, and biological. Advanced processes have been developed to treat these compounds, but in developing countries traditional methods of water treatment have been used. Diverse chemical groups are present in PPCPs, so it is not possible to treat all compounds according to their physicochemical properties. Each compound has different physiochemical properties, such as solubility, absorbance onto sludge, half-life under biotic and abiotic conditions, and tendency to volatilize (Liu et al. 2014). The efficiency of STPs and WWTPs for the removal of PPCPs and their metabolites depends upon the environmental and operational conditions under which they work. Environmental factors such as temperature, redox conditions, and pH affect the degradation kinetics of the compounds. Operational conditions such as hydraulic retention time, biodegradation kinetics, and sludge retention time are important for the degradation of these compounds (Evgenidou et al. 2015). Veterinary pharmaceuticals are an important source of PPCPs, as they are released into the environment directly from the treatment of meadow animals, or indirectly by the application of manure to the land, by the runoff of veterinary medicines from the surfaces of farmyards, by cattle carcasses, and by slurry from livestock facilities. The management and uses of PPCP vary around the world, and the pathways in different geographical areas vary from

Table 19.2 Concentrations of PPCPs in different sources

Name of PPCP	Average concentration	Sample source	References
Diclofenac	29 μg L^{-1}	River Elbe and the mouths of its tributaries (Germany)	Wiegel et al. (2004)
Ibuprofen	35 μg L^{-1}		
Acetaminophen	16 μg L^{-1}		
Bisphenol A	40 μg L^{-1}		
Ibuprofen	28 μg L^{-1}	Han, Nakdong, and Youngsan Rivers (South Korea)	Kim et al. (2007)
Acetaminophen	33 μg L^{-1}		
Diclofenac	3 μg L^{-1}		
Oxybenzone	2 μg L^{-1}		
Acetaminophen	12 μg L^{-1}	Ebro River basin (Spain)	López-Serna (2012)
Triclosan	50 μg L^{-1}	Surface waters of Greenwich Bay (RI, United States)	Katz et al. (2013)
Acetaminophen	5 μg L^{-1}	Near-shore habitats of Lake Michigan	
Ibuprofen	8 μg L^{-1}	River discharges into lakes from predominantly urban watersheds (United States)	Ferguson et al. (2013b)
Carbamazepine	40 μg L^{-1}	Aquifers in the delta area of the Llobregat River (NE Spain)	Teijón et al. (2010)
Ibuprofen	185 μg L^{-1}		
Diclofenac	256 μg L^{-1}		
Acetaminophen	180 μg L^{-1}	Groundwater used for drinking-water supply in California (United States)	Fram and Belitz (2011)
Diclofenac	0.2 μg L^{-1}	Urban groundwater in the district of Poble Sec, Barcelona (Spain)	López-Serna et al. (2012)
Ibuprofen	0.2 μg L^{-1}		
Acetaminophen	<0.1 μg L^{-1}		
Erythromycin	<0.1 μg L^{-1}		
Enrofloxacin	75 μg L^{-1}		
Ibuprofen	23 μg L^{-1}	Groundwater wells in Berlin (Germany)	Heberer (2002)
Diclofenac	34 μg L^{-1}		
Clofibric acid	18 μg L^{-1}		
Antibiotics	360 ng L^{-1}	Mekong Delta (Vietnam)	Managaki et al. (2007)
Antibiotics	544 ng L^{-1}	Seine River (France)	Tamtam et al. (2008)
Antibiotics	183 ng L^{-1}	Taff and Ely Rivers (United Kingdom)	Kasprzyk-Hordern et al. (2009)
Antibiotics	696 ng L^{-1}	Vantaa River (Finland); drinking water sources	Vieno et al. (2007)
Antibiotics	300 ng L^{-1}	Choptank River (United States)	Arikan et al. (2008)
Antibiotics	1900 ng L^{-1}	Streams in Iowa (United States)	Kolpin et al. (2004)

(continued)

Table 19.2 (continued)

Name of PPCP	Average concentration	Sample source	References
Hormones	5 ng L^{-1}	Youngsan River (South Korea)	Kim et al. (2007)
Hormones	5 ng L^{-1}	Llobregat River (NE Spain)	Brix et al. (2009)
Hormones	10 ng L^{-1}	Scheldt estuary (Netherlands)	Arikan et al. (2008
Hormones	18.9 ng L^{-1}	Little River estuary (Victoria, Australia)	Ferguson et al. (2013a)
Pharmaceuticals	749 ng L^{-1}	Tamagawa estuary and 37 rivers in Japan	Nakada et al. (2008)
Pharmaceuticals	500 ng L^{-1}	Rivers in Rio de Janeiro State (Brazil)	Stumpf et al. (1999)
Triclosan	5160 ng L^{-1}	Kaveri, Vellar, and Tamiraparani Rivers (India)	Ramaswamy et al. (2011)

one region to another. Traditional systems of wastewater treatment lead to the incomplete removal of PPCPs from WWTPs. Metabolites produced by pharmaceutical compounds and other PPCPs are designed to resist decomposition and microbial degradation. Newer WWTPs employ specific treatments to remove waste via the degradation of lower-molecular-weight compounds, the physical removal of solids, and the transformation or conjugatation of compounds that are further hydrolyzed when released into the environment.

19.2 Toxicity of PPCPs: Personal Care Products

The everyday use of personal care products such as toothpaste, shampoo, personal soaps, hair products, lotions, and make-up products leads to the release of compounds that are not naturally present in the environment (Lu et al. 2011). Many personal care products show low volatility in nature, so PPCPs are limited in the atmospheric environment. However, some PPCPs, such as siloxanes, are highly volatile so they are found in indoor dust and air. Indoor dust releases these compounds when electrical appliances are used and smoke is present in the house (Lu et al. 2010). The concentrations of parabens in indoor dust particles were found to be in the order of 2320 ng g^{-1} in Korea, 2300 ng g^{-1} in Japan), and 1390 ng g^{-1} in the United States (Wang et al. 2012).

PPCPs and their metabolites follow the same biological pathways and have modes of action similar to those of their parent molecules. Compounds that have been transformed or conjugated are more toxic than their parent compounds and have adverse effects on our aquatic and terrestrial systems. The transformed compounds exist with their parent molecules in the form of mixtures. The eco-toxicological effect of PPCPs and their transformed and conjugated products cannot be ignored. The toxicity of the transformed products differs from that of their parent molecules in two ways: their toxicokinetics and toxicodynamics (mode of action). Transformation creates new toxicophores that have higher toxicity than their parent molecules or that are similar in mode of action. These chemicals are present in low

concentrations, but they form conjugated products with other compounds, resulting in synergistic mixtures with greater toxicity that strongly affect the environment. Therefore, it is essential to evaluate the ecological risk associated with PPCPs and their conjugated products. High exposure to PPCPs and their metabolites leads to a high probability of risk associated with these compounds (Evgenidou et al. 2015).

19.2.1 Human Risk

Humans use water for their day-to-day activities and for drinking; however, the water contains various PPCPs that cause health-related problems. The major potential routes of exposure to PPCPs that entail risks to human health vary. Consumption of marine or territorial fishes exposed to PPCPs is one of the main causes for interaction to these compounds. At present, the concentrations of PPCPs in the environment in some countries are very high, and so people are exposed to these compounds in their drinking water and in bathing/showering. PPCPs are also present in surface and groundwater, and this may also have adverse effects on human health. In drug development and approval, the toxicological properties of drugs in relation to humans and other mammals are studied, and this information is often available from pharmaceutical companies. This data is very important in the evaluation of risks associated with these drugs during human exposure to the EOCs in the environment. The existing data on drugs used to treat human diseases is very satisfactory, whereas data on the prevalence of PPCPs and their derivatives in the environment is limited. Thus, the human risk associated with the intake of these chemicals from the environment is unknown. Because of the daily use and long lifespan of PPCPs, they are found in environmental water and enter food chains. Healthy water and food are primary priorities for humans. But these unwanted compounds are present in human environments. Compounds such as the antibiotic fluoroquinolone have been identified in tap water for drinking in many countries (Wang et al. 2010). Triclosan, a pharmaceutical compound, has been detected not only in tap water but also in packed bottled water, in varying concentrations (9.7–14.5 ng L^{-1}); the concentration was low but has been increasing steadily. The intake of triclosan in adults and infants was shown to be increasing, and it was detected at concentrations of 10 and 5 ng L^{-1}, respectively, in bottled water for adults and in baby bottles (Li et al. 2010). The agricultural use of water containing PPCPs increases health risks to humans. Antibiotics are now used in non-organic farming and for livestock to increase production. Livestock wastes are used as fertilizers in many developing countries, and concentrations of PPCPs in the environment are increasing because of this practice. The accumulation of antibiotics in vegetables probably arises from the transportation and absorption and distribution of water in the plants, with accumulation in the leaves being more than that in the stems and that in the stems being more than that in the roots (Hu et al. 2010).

Owing to their many exposure pathways, PPCPs have been detected in human breast milk, blood, and urine. In human breast milk, synthetic musks have been detected at concentrations of 1.4–917 ng g^{-1}; the synthetic musks enter the body of

the infant during feeding with breast milk, and high concentrations of these compounds may cause physiological or genetic changes in the infant. The main reason for the detection of such compounds in humans is the frequent use of personal care products (Yin et al. 2012). In human breast milk, the four most commonly found synthetic musks are musk xylene (MX); musk ketone; 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-gamma-2-benzopyran (HHCB); and 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene, with HHCB showing the highest concentration. Daily infant intake of milk containing synthetic musks at concentrations of 277–7391 ng g^{-1} increases the potential risk (Zhang et al. 2011). Synthetic musks have been detected in human breast milk in many countries, e.g., in the United States (2–917 ng g^{-1} lipid weight), Denmark (38–422 ng g^{-1} lipid weight), and Sweden (2–268 ng g^{-1} lipid weight) (Duedahl-Olesen et al. 2005; Reiner et al. 2007a, b; Lignell et al. 2008).

Polycyclic compounds contained in musk fragrances and nitro musk fragrances have been commonly detected in human blood (Hu et al. 2010). The synthetic musks HHCB and MX have been reported in human blood plasma. In Austria, HHCB and MX were detected in adult blood at concentrations of 11–450 ng g^{-1} (Hutter et al. 2009). Triclosan wasdetected in urine samples from young children and adults, with the mean concentration in 3- to 24-year-old persons being 3.55 μg g^{-1} (Li et al. 2013). Parabens and their derivatives were detected in urine samples from people in the United States. A pharmacokinetic study reported that PPCPs caused no major risk to human health. However, another study has shown that regular exposure to these compounds in the current environment leads to high risks to human health (Touraud et al. 2011). These studies describe the presence of PPCPs in the environment in various forms as well as describing the accumulation of these compounds in the human body. Regular exposure to PPCPs, whether direct or indirect, and intake of these compounds, are possible reasons for their effect on human health.

19.2.2 Toxicity and Risks to Ecosystems

PPCPs in aquatic environments are mainly discharged into STPs and WWTPs. The concentration of PPCPs in aquatic environments depends upon the source. Water sources near highly populated areas contain high concentrations of PPCPs, while marine water contains low concentrations. Aquatic organisms such as fish and invertebrates, or other species, that are present near sources of aquatic systems, are regularly exposed to PPCPs throughout their life cycles. PPCPs are designed to be biologically active and are intended to react with the metabolic systems of living cells. In general, PPCPs are produced to treat human disease and are used in the protection of larger domesticated animals. Various regulatory agencies and academic institutions are now working on evaluations of the adverse effects of these compounds on aquatic life and the wildlife associated with water bodies.

Currently, data is limited regarding the adverse effects of direct exposure to PPCPs and their derivatives on aquatic organisms. Traditional toxicological tests are

not able to evaluate the potential effect of regular exposure to PPCPs and their derivatives on the lives of organisms in surface waters, marine water, and sediments contaminated by these compounds. Fish tissues collected for study have shown that some PPCPs, such as antidepressants, are present in the cells of fish that live in streams near urban effluent. Large concentrations of antidepressants were detected in the brain and liver tissues of the fish (Liu and Wong 2013). Other studies have focused on evaluating the toxic effects of PPCPs on marine organisms. In many studies, concentrations greater than the concentrations detected in the environment were used to investigate the possible adverse effects of PPCPs on aquatic organisms exposed to PPCPs such as anti-inflammatory agents, antidepressants, and antibiotics. The presence of PPCPs in the ecosystem leads to concern about their probable adverse effects on the ecosystem. The regular use of antibiotics may also increase the risk of antibiotic resistance genes (ARGs) in organisms, and also has the potential to cause adverse effects on the ecosystem and on human health (Kemper 2008). ARGs were universally detected in insects and microbes identified in hospitals and in effluents from livestock farming, while some microbes containing AGR genes were also detected in drinking water, municipal wastewater, and ground water (Pruden et al. 2006). Multiple antibiotic-resistant genes with hundreds of ARG cassettes have been identified in wastewater and drinking water worldwide (Kobayashi et al. 2007).

When artificial and natural hormones reach the marine environment through human excreta and via medical use, this leads to endocrine disruptions in marine organisms. Many studies have reported hormonal reproductive and developmental changes in these organisms, such as reduction of fertility; the presence of intersex organisms; and feminization and vitellogenesis in males (Lai et al. 2002; Khanal et al. 2006). It is assumed that these endogenic compounds reached the ecosystems via sewage systems and were also present in sewage effluents (Jobling et al. 2006). Personal care products such as ultraviolet sunscreens (Gomez et al. 2005) may also be considered as having the potential to cause endocrine disruption in some species. Some compounds, such as triclosan, caffeine, triclocarban (Yang et al. 2008), and some lipid regulators (Rosal et al. 2009) inhibit algal growth, cause endocrine disruption in fish (goldfish), and reduce the viability of eggs (Japanese medaka) (Huggett et al. 2002). Oxidation stress was caused by carbamazepine (an antiepileptic drug) and HHCB in rainbow trout in a marine environment and in goldfish in a freshwater environment. Schwaiger et al, in 2004, reported that NSAIDs such as diclofenac caused gill alterations and renal lesions in rainbow trout. The synergistic effects of NSAIDs in the ecosystem are an issue of concern. Mixtures of various NSAIDs were considered to exert the strongest adverse effects by forming complexes in aquatic organisms (Cleuvers 2003). PPCPs and their derivatives enter the aquatic environment, which is the gateway for entry to the whole food web (Brausch and Rand 2011).

Recent studies found that PPCPs were detected in effluents, wastewater, groundwater, and drinking water too. PPCPs are organic compounds that are designed to be biologically active, and so a risk of toxicity is anticipated for many species exposed to PPCPs in the environment, even when the concentrations of these

compounds are low. The risk to humans from exposure to PPCPs in environmental waters is more disputed than the ecological risks of PPCP exposure. Although the presence of pharmaceuticals at parts-per-million levels in water for daily use does not currently appear to cause a direct adverse threat to humans, indirect impacts from some pharmaceuticals are documented and need to be considered. Indirect impacts of PPCPs other than pharmaceuticals on human health are more difficult to determine. Toxicology studies of chronic exposure to anticonvulsants, antidepressants, anti-hypertensives, endocrine-disrupting chemicals, and cytostatic pharmaceuticals are limited (U.S.EPA (U.S. Environmental Protection Agency) 2013; U.S.FDA (U.S. Food, Drug Administration) 2013).

19.3 Biodegradation and PPCP Removal

In natural conditions, organic compounds are degraded mostly by bacteria and some fungi. These organisms have a rapid growth rate, great metabolic activity, and easily adapt to new substrates that enter the environment. Numerous laboratory experiments have shown positive results for the degradation of xenobiotic compounds by microbes, but the microbial degradation of these compounds does not occur on a large scale. It is necessary to identify microorganisms (bacteria, fungi, etc.) from the environment that are capable of degrading EOCs.

19.3.1 Pure Cultures

Many studies have reported that pure cultures isolated from wastewater, activated sludge, wastewater, or river sediments have the capacity to degrade carbamazepine, sulfamethoxazole, iopromide, paracetamol, ibuprofen, diclofenac, and triclosan. Some pure cultures exhibit a capacity for the degradation of various PPCPs. Sulfamethoxazole is the not only compound degraded by *Achromobacter denitrificans*; this bacterium also degrades other sulfonamides (Reis et al. 2014). Many pure cultures use different mechanisms for the degradation of PPCPs. Some PPCPs providing the sole carbon and energy source for microbial metabolism (Murdoch and Hay 2005; De Gusseme et al. 2011; Zhang et al. 2013). Paracetamol can be degraded by *Delftia tsuruhatensis*, *Pseudomonas aeruginosa*, and *Stenotrophomonas*. Biosorption played a negligible role in the degradation of paracetamol by *Delftia tsuruhatensis* and *Pseudomonas aeruginosa*, while biosorption contributed to the degradation of paracetamol by *Stenotrophomonas*. Various enzymes play important roles in these degradation processes. Some PPCPs that provide the sole carbon and energy source for microbes are barely degraded by pure cultures of microbes. In such cases, supplementary substances will provide the carbon and energy required for the metabolic function of the microbes. For example, carbamazepine shows poor biodegradability, owing to its stable structure. But in the presence of glucose, pure cultures of *Basidiomycetes* and *Streptomyces* MIUG (Santosa et al. 2012; Popa et al. 2014) degraded carbamazepine. Liu et al. (2013) proved that iopromide could

be degraded by *Pseudomonas* sp. I-24 using starch as the primary substrate. The NSAID diclofenac showed high resistance to biodegradation by a pure culture isolated from activated sludge (Alvarino et al. 2014). However, in 2010, Hata et al. reported that the white rot fungus *Phanerochaete sordida* YK-624 almost completely degraded diclofenac.

19.3.2 Mixed Cultures

Mixed cultures are an easier method of degrading PPCPs than pure cultures, because pure cultures are not easily identified. Mixed cultures have a greater capacity than pure cultures for degrading wider ranges of PPCPs, because a mixed culture is a consortium of various strains. 17a-Ethinylestradiol was degraded by a mixed culture of heterotrophic bacteria in mixed media, demonstrating the degradation capacity of mixed strains (Khunjar et al. 2011). Mixed cultures are used most widely in biological activated sludge treatment processes in WWTPs, depending on various strategies for the removal of the PPCPs. However, in some cases, mixed cultures show low removal efficiency for specific PPCPs.

19.3.3 Activated Sludge Process

The removal of PPCPs by biological treatment in WWTPs conventionally uses an activated sludge process. The biological treatment has a mixed effect, causing the adsorption, volatilization, and biodegradation of PPCPs, with the main mechanism of PPCP removal by activated sludge being biodegradation. The limitations of biological treatment can be conquered by the use of bioaccumulation and bioaugmentation (Wang et al. 2002). The modeling framework for xenobiotic trace chemicals developed by Plosz et al. (2012) is used to identify the factors that enhance the efficiency of xenobiotic removal by activated sludge.

19.4 Conclusions

PPCPs are a group of compounds used in medicines and other preparations. Their entry to the environment is mediated by different routes and they enter the environment through different water bodies. In this chapter we have outlined the available information on the adverse effects of PPCPs present in the environment. WWTPs are the major gateway by which PPCPs enter the environment, although there are other sources for PPCP entry to the environment, such as agricultural and veterinary runoff, household waste, dumping sites, and aquaculture. This suggests that WWTPs cannot completely remove PPCPs. So there is a need to develop WWTP technologies that will remove PPCPs efficiently. There is also a need for more studies to be conducted on the persistence of PPCPs and the effects of PPCPs and their metabolites on the environment in developing countries, because the availability of

information about PPCPs in the environment is far behind that in developed countries. Further, there are few studies on the adverse effects of PPCPs and their residues on fish, birds, and mammals. At present, advanced research on the removal of such compounds from the environment is playing the major role in PPCP studies. Biodegradation is one technology that may be used for the removal of PPCPs, with pure cultures, mixed cultures, and activated sludge processes being available for this purpose. Biodegradation is a cost-effective and natural technique for the removal of PPCPs. More studies are needed to enhance these techniques. Toxicity and risk assessment studies employed in environmental management systems may reduce the ecological risks and toxicity of these compounds. New and indigenous technologies can be employed for waste reduction and minimization of the toxicity of different agents that enter the environment. The use of efficient microbial systems and biological treatments will lead to a reduction of the effects of PPCPs on living systems.

References

Al-Odaini NA, Zakaria MP, Yaziz MI, Surif S (2010) Multi-residue analytical method for human pharmaceuticals and synthetic hormones in river water and sewage effluents by solid-phase extraction and liquid chromatography–tandem mass spectrometry. J Chromatogr A 1217:6791–6806

Alvarino T, Suarez S, Lema JM, Omil F (2014) Understanding the removal mechanisms of PPCPs and the influence of main technological parameters in anaerobic UASB and aerobic CAS reactors. J Hazard Mater 278:506–513

Arikan OA, Rice C, Codling E (2008) Occurrence of antibiotics and hormones in a major agricultural watershed. Desalination 226:121–133

Brausch JM, Rand GM (2011) A review of personal care products in the aquatic environment: environmental concentrations and toxicity. Chemosphere 82:1518–1532

Brix R, Postigo C, González S, Villagrasa M, Navarro A, Kuster M (2009) Analysis and occurrence of alkylphenolic compounds and estrogens in a European river basin and an evaluation of their importance as priority pollutants. Anal Bioanal Chem 396:1301–1309

Cleuvers M (2003) Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicol Lett 142:185–194

De Gusseme B, Lynn V, Willy V, Nico B (2011) Degradation of acetaminophen by Delftia tsuruhatensis and Pseudomonas aeruginosa in a membrane bioreactor. Water Res 45:1829–1837

Duedahl-Olesen L, Cederberg T, Pedersen KH, Højgård A (2005) Synthetic musk fragrances in trout from Danish fish farms and human milk. Chemosphere 61:422–431

Evgenidou EN, Konstantinou IK, Lambropoulou DA (2015) Occurrence and removal of transformation products of PPCPs and illicit drugs in wastewaters: a review. Sci Total Environ 505:905–926

Farre M, Perez S, Kantiani L, Barcelo D (2008) Fate and toxicity of emerging pollutants, their metabolites and transformafarretion products in the aquatic environment. Trends Anal Chem 27:991–1007

Fent K, Weston AA, Caminada D (2006) Ecotoxicology of human pharmaceuticals. Aquat Toxicol 76(2):122–159

Ferguson EM, Allinson M, Allinson G, Swearer SE, Hassell KL (2013a) Fluctuations in natural and synthetic estrogen concentrations in a tidal estuary in south-eastern Australia. Water Res 47:1604–1615

Ferguson PJ, Bernot MJ, Doll JC, Lauer TE (2013b) Detection of pharmaceuticals and personal care products (PPCPs) in near-shore habitats of southern Lake Michigan. Sci Total Environ 458–460:187–196

Fram MS, Belitz K (2011) Occurrence and concentrations of pharmaceutical compounds in groundwater used for public drinking-water supply in California. Sci Total Environ 409:3409–3417

Gomez E, Pillon A, Fenet H, Rosain D, Duchesne MJ, Nicolas JC (2005) Estrogenic activity of cosmetic components in reporter cell lines: parabens, UV screens, and musks. J Toxicol Environ Health 68:239–251

Hata T, Kawai S, Okamura H, Nishida T (2010) Removal of diclofenac and mefenamic acid by the white rot fungus Phanerochaete sordida YK-624 and identification of their metabolites after fungal transformation. Biodegradation 21(5):681–689

Heberer T (2002) Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol Lett 131(1–2):5–17

Hu XG, Zhou QX, Luo Y (2010) Occurrence and source analysis of typical veterinary antibiotics in manure, soil, vegetables and groundwater from organic vegetable bases, northern China. Environ Pollut 158:2992–2998

Huggett DB, Brooks BW, Peterson B, Foran CM, Schlenk D (2002) Toxicity of select beta adrenergic receptor-blocking pharmaceuticals (β-blockers) on aquatic organisms. Arch Environ Contam Toxicol 43:229–235

Hutter HP, Wallner P, Moshammer H, Hartl W, Sattelberger R, Lorbeer G (2009) Synthetic musks in blood of healthy young adults: relationship to cosmetics use. Sci Total Environ 407:4821–4825

Jobling S, Williams R, Johnson A, Taylor A, Gross-Sorokin M (2006) Predicted exposures to steroid estrogens in UK rivers correlate with widespread sexual disruption in wild fish populations. Environ Health Perspect 114:32–39

Kasprzyk-Hordern B, Dinsdale RM, Guwy AJ (2009) The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving waters. Water Res 43:363–380

Katz K, Inoue T, Ietsugu H, Koba T, Sasaki H, Miyaji N, Kitagawa K, Sharma PK, Nagasawa T (2013) Performance of six multi-stage hybrid wetland systems for treating high-content wastewater in the cold climate of Hokkaido, Japan. Ecol Eng 51:256–263

Kemper N (2008) Veterinary antibiotics in the aquatic and terrestrial environment. Ecol Indic 8:1–13

Khanal SK, Xie B, Thompson ML, Sung S, Ong SK, van Leeuwen JH (2006) Fate, transport, and biodegradation of natural estrogens in the environment and engineered systems. Environ Sci Technol 40:6537–6546

Khunjar W, Mackintosh S, Skotnicka-Pitak J, Baik S, Aga D, Love N (2011) Elucidating the relative roles of ammonia oxidizing and heterotrophic bacteria during the biotransformation of 17a-ethinylestradiol and trimethoprim. Environ Sci Technol 45(8):3605–3612

Kim SD, Cho J, Kim IS, Vanderford BJ, Snyder SA (2007) Occurrence and removal of pharmaceuticals and endocrine disruptors in South Korean surface, drinking, and waste waters. Water Res 41:1013–1021

Kobayashi T, Suehiro F, Tuyen BC, Suzuki S (2007) Distribution and diversity of tetracycline resistance genes encoding ribosomal protection proteins in Mekong river sediments in Vietnam. FEMS Microbiol Ecol 59:729–737

Kolpin D, Skopec M, Meyer M, Furlong E, Zaugg S (2004) Urban contribution of pharmaceuticals and other organic wastewater contaminants to streams during differing flow conditions. Sci Total Environ 328:119–130

Lai KM, Scrimshaw MD, Lester JN (2002) The effects of natural and synthetic steroid estrogens in relation to their environmental occurrence. Crit Rev Toxicol 32:113–132

Li X, Ying GG, Su HC, Yang XB, Wang L (2010) Simultaneous determination and assessment of 4-nonylphenol, bisphenol A and triclosan in tap water, bottled water and baby bottles. Environ Int 36:557–562

Li X, Ying GG, Zhao JL, Chen ZF, Lai HJ, Su HC (2013) 4-Nonylphenol, bisphenol-A and triclosan levels in human urine of children and students in China, and the effects of drinking these bottled materials on the levels. Environ Int 52:81–86

Lignell S, Darnerud PO, Aune M, Cnattingius S, Hajslova J, Setkova L (2008) Temporal trends of synthetic musk compounds in mother's milk and associations with personal use of perfumed products. Environ Sci Technol 42:6743–6748

Liu, LL and Wong, MH (2013) Pharmaceuticals and personal care products (PPCPs): A review on environmental contamination in China. Environ Int 59:208–224

Liu L, Liu C, Zheng J, Huang X, Wang Z, Liu Y, Zhu G (2013) Elimination of veterinary antibiotics and antibiotic resistance genes from swine wastewater in the vertical flow constructed wetlands. Chemosphere 91:1088–1093

Liu L, Liu Y, Wang Z, Liu C, Huang X, Zhu G (2014) Behavior of tetracycline and sulfamethazine with corresponding resistance genes from swine wastewater in pilot-scale constructed wetlands. J Hazard Mater 278:304–310

López-Serna R, Petrović M, Barceló D (2012) Occurrence and distribution of multi-class pharmaceuticals and their active metabolites and transformation products in the Ebro River basin (NE Spain). Sci Total Environ 440:280–289

Lu Y, Yuan T, Yun SH, Wang WH, Kannan K (2010) Occurrence of synthetic musks in indoor dust from China and implications for human exposure. Arch Environ Contam Toxicol 60:182–189

Lu Y, Yuan T, Wang WH, Kannan K (2011) Concentrations and assessment of exposure to siloxanes and synthetic musks in personal care products from China. Environ Pollut 159:3522–3528

Managaki S, Murata A, Takada H, Tuyen BC, Chiem NH (2007) Distribution of macrolides, sulfonamides, and trimethoprim in tropical waters: ubiquitous occurrence of veterinary antibiotics in the Mekong Delta. Environ Sci Technol 41:8004–8010

Murdoch RW, Hay AG (2005) Formation of catechols via removal of acid side chains from ibuprofen and related aromatic acids. Appl Environ Microbiol 71(10):6121–6125

Nakada N, Kiri K, Shinohara H, Harada A, Kuroda K, Takizawa S (2008) Evaluation of pharmaceuticals and personal care products as water-soluble molecular markers of sewage. Environ Sci Technol 42:6347–6353

Plosz BG, Langford KH, Thomas KV (2012) An activated sludge modeling framework for xenobiotic trace chemicals (ASM-X): assessment of diclofenac and carbamazepine. Biotechnol Bioeng 109:2757–2769

Popa C, Favier L, Dinica R, Semrany S, Djelal H, Amrane A, Bahrim G (2014) Potential of newly isolated wild Streptomyces strains as agents for the biodegradation of a recalcitrant pharmaceutical, carbamazepine. Environ Technol 35(24):3082–3091

Pruden A, Pei RT, Storteboom H, Carlson KH (2006) Antibiotic resistance genes as emerging contaminants: studies in Northern Colorado. Environ Sci Technol 40:7445–7450

Ramaswamy BR, Shanmugam G, Velu G, Rengarajan B, Larsson DGJ (2011) GC–MS analysis and ecotoxicological risk assessment of triclosan, carbamazepine and parabens in Indian rivers. J Hazard Mater 186:1586–1593

Reiner JL, Berset JD, Kannan K (2007a) Mass flow of polycyclic musks in two wastewater treatment plants. Arch Environ Contam Toxicol 52:451–457

Reiner JL, Wong CM, Arcaro KF, Kannan K (2007b) Synthetic musk fragrances in human milk from the United States. Environ Sci Technol 41:3815–3820

Reis PJ, Reis AC, Ricken B, Kolvenbach BA, Manaia CM, Corvini PF, Nunes OC (2014) Biodegradation of sulfamethoxazole and other sulfonamides by Achromobacter denitrificans PR1. J Hazard Mater 280:741–749

Rosal R, Rodea-Palomares I, Boltes K, Fernández-Piñas F, Leganés F, Gonzalo S (2009) Ecotoxicity assessment of lipid regulators in water and biologically treated wastewater using three aquatic organisms. Environ Sci Pollut Res 17:135–144

Santosa IJ, Grossmana MJ, Sartorattob A, Ponezib AN, Durranta LR (2012) Degradation of the recalcitrant pharmaceuticals carbamazepine and 17a-ethinylestradiol by ligninolytic fungi. Chem Eng 27:169–174

Schwaiger J, Ferling H, Mallow U, Wintermayr H, Negele RD (2004) Toxic effects of the non-steroidal anti-inflammatory drug diclofenac: part I: histopathological alterations and bioaccumulation in rainbow trout. Aquat Toxicol 68:141–150
Stumpf M, Ternes TA, Wilken RD, Rodrigues SV, Baumann W (1999) Polar drug residues in sewage and natural waters in the state of Rio de Janeiro, Brazil. Sci Total Environ 225:135–141
Tamtam F, Mercier F, Le Bot B, Eurin J, Tuc Dinh Q, Clément M (2008) Occurrence and fate of antibiotics in the Seine River in various hydrological conditions. Sci Total Environ 393:84–95
Teijón G, Candela L, Tamoh K, Molina-Díaz A, Fernández-Alba AR (2010) Occurrence of emerging contaminants, priority substances (2008/105/CE) and heavy metals in treated wastewater and groundwater at Depurbaix facility (Barcelona, Spain). Sci Total Environ 408:3584–3595
Touraud E, Roig B, Sumpter JP, Coetsier C (2011) Drug residues and endocrine disruptors in drinking water: risk for humans? Int J Hyg Environ Health 214:437–441
U.S. EPA (U.S. Environmental Protection Agency) (2013) Effluent guidelines—pharmaceutical manufacturing point source category—permit guidance document
U.S. FDA (U.S. Food and Drug Administration) (2013) Code of Federal Regulations Title 21, Part 25 Environmental Impact Considerations
Vieno NM, Härkki H, Tuhkanen T, Kronberg L (2007) Occurrence of pharmaceuticals in river water and their elimination in a pilot-scale drinking water treatment plant. Environ Sci Technol 41:5077–5084
Wang JL, Quan XC, Wu LB, Qian Y, Werner H (2002) Bioaugmentation as a tool to enhance the removal of refractory compound in coke plant wastewater. Process Biochem 38(5):777–781
Wang QJ, Mo CH, Li YW, Gao P, Tai YP, Zhang Y (2010) Determination of four fluoroquinolone antibiotics in tap water in Guangzhou and Macao. Environ Pollut 158:2350–2358
Wang L, Liao CY, Liu F, Wu Q, Guo Y, Moon HB (2012) Occurrence and human exposure of p-hydroxybenzoic acid esters (parabens), mbisphenol A diglycidyl Ether (BADGE), and their hydrolysis products in indoor dust from the United States and three East Asian countries. Environ Sci Technol 46:11584–11593
Wiegel S, Aulinger A, Brockmeyer R, Harms H, Löffler J, Reincke H, Schmidt R, Stachel B, von Tümpling W, Wanke A (2004) Pharmaceuticals in the river Elbe and its tributaries. Chemosphere 57:107–126
Yang LH, Ying GG, Su HC, Stauber JL, Adams MS (2008) Growth-inhibiting effects of 12 antibacterial agents and their mixtures on the freshwater microalga Pseudokirchneriella subcapitata. Environ Toxicol Chem 27:1201–1208
Yin J, Wang H, Zhang J, Zhou NY, Gao FD, Wu YN (2012) The occurrence of synthetic musks in human breast milk in Sichuan, China. Chemosphere 87:1018–1023
Zhang Y, He C, Sharma V, Li X, Tian S, Xiong Y (2011) A new reactor coupling heterogeneous Fenton-like catalytic oxidation with membrane separation for degradation of organic pollutants. J Chem Technol Biotechnol 86:1488–1494
Zhang L, Hu J, Zhu R, Zhou Q, Chen J (2013) Degradation of paracetamol by pure bacterial cultures and their microbial consortium. Appl Microbiol Biotechnol 97(8):3687–3698

Part V

Biotechnological Techniques

Materials for Tissue Engineering

20

Pawan Kumar and Anil Sindhu

20.1 Introduction

20.1.1 Tissue Engineering

Tissue designing can be best characterized as the combination of cells, engineered materials, and reasonable biochemical variables to improve or relocate organic capacities. Tissue engineering takes care of issues generated by damaged tissues and organs. Tissue engineering is an integrated field that utilizes engineering and life science's principle for the improvement of biological substitutes and improvement of working of tissues or a whole organ. The animal body is a complex system that consists of different organs and tissues for specific functions. The 2008 census reported that the average world population aged 65 and may be crossed to 506 million till 2040 (Giannoudis et al. 2005). Tissues have limited self-repair capabilities, so it is normal to treat these damaged organs by surgical interventions. The regeneration of damaged tissue in vitro may offer a clinically relevant alternative to the autografts, while an engineered scaffold uses cells derived from the patient. Scaffolds utilized for tissue engineering perform many capacities, and, accordingly, outlining of scaffold with suitable structure and mechanical properties that support cell grip, multiplication, and separation to create practical tissue in vitro is the fundamental prerequisite. The properties (mechanical properties, porosity, biocompatibility, biodegradability, and cytotoxicity) of artificial biomaterials can be tailored for specific applications (Kokubo and Takadama 2006).

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20.2 Scaffold

The scaffold is defined as a "temporary support" used to restore the existing functionality of that organ. It helps in the delivery of matrix, transport of cells for the regeneration of that particular organ or tissue. The bioactive molecules loaded scaffolds are used to promote cells growth and attachments. The properties of scaffolds depend on the intrinsic properties and fabrication process of biomaterials. The functional scaffold should have the following properties (Ma 2004; Salgado et al. 2004):

- Good biocompatibility
- Mechanical properties (tensile strength, toughness)
- Bioresorbable
- Biodegradable
- Proper surface properties
- Optimum porosity

The scaffold should be a temporary support that at the end is replaced by new, regenerated tissue in the body. After implantation of the scaffold in the human body, it should be degraded by molecular fragmentation mechanisms. The disintegration of scaffold can be facilitated by biochemical reactions. The success of a scaffold will depend only on degradation which is completely reabsorbed by the organism (Vert et al. 1992).

20.3 Biomaterials

They are the synthetic material that used for device/scaffold designing to replace/repair a part of the body. In 1986, the most common definition of biomaterial was given at the conference of the European Society for Biomaterials. These are mixture of substances, artificial or natural in source, which can be utilized as a whole part of a framework for the replacement of allogenic bones, and these biomaterials are utilizing to treat and restore any tissue, organ, or capacity of the body. Basically biomaterials must be biocompatible, specifically to achieve the good response from the host body tissues. The biomaterials are used in the fabrication of implants for dental and orthopedic applications. On the basis of chemical nature, biomaterials used for regenerative purpose are classified as (Fig. 20.1 and Table 20.1):

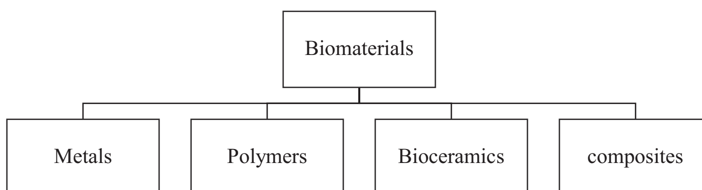

Fig. 20.1 Flow chart for the classification of biomaterials

Table 20.1 Common examples of applications for each class of biomaterial

Materials	Applications
1. Metals	
Tantalum, titanium (Ti) and its alloys, stainless steel, cobalt-chromium alloy, etc.	Bone plates, screw, joint and dental implants, surgical instruments
2. Polymers	
Synthetic polymers	Blood vessels, drug delivery, contact lenses, sutures, catheters
PLA, PGA, PCL, polyphosphazenes, PPF, PGS, etc.	
Natural polymers	
Chitosan, collagen, elastin, fibrinogen, etc.	
3. Bioceramics	
Aluminum oxide, calcium phosphate, hydroxyapatite, bioactive glass, etc.	Dental implants, orthopedic devices, femoral head
4. Composites	
Polymer-coated bioglass, polymer-ceramic, polymer-polymer, HA-7coated metals, etc.	Orthopedic implants

20.3.1 Metals and Alloys

A number of biocompatible metallic implants are generally used as permanent or temporary materials for the fixation of bone fractures and dentine tissues. These are widely used in medical devices such as bone plates and screws for fracture fixation and braces and dental root implants for tooth fixation. The common metal-based implant materials consist of stainless steel, cobalt alloys, and various types of titanium alloys. But, the metallic biomaterials are having a deficiency in biological perception of the material surface. The surface coating/modification is a technique to change or improve the biomechanical properties of metallic implants (Liu et al. 2007). The improvement in implant may enhance the life and compatibility by using cement fixation and surface coating of hydroxyapatite and ceramic coating.

20.3.1.1 Tantalum

It is a porous biomaterial which has special physical and mechanical belongings. The high porosity (>80%) and interconnected pores allow quick bone in growth in the host (Bobyn et al. 1999). Moreover, tantalum proved similar modulus of elasticity to the natural bone. The sufficient strength of porous tantalum permits physiological load-carrying applications. Besides, this material allows better osteoconduction than others. The porous tantalum is used as orthopedic implant, and preliminary clinical data and preclinical studies make it more useful (Adams et al. 2005).

20.3.1.2 Magnesium and Magnesium Alloys

They have great potential for surgical applications, due to high bioresorbable and mechanical properties without inflammatory response. They are osteoconductive, stimulate bone growth, and support cell attachment (Staiger et al. 2006). The second

surgery for the implant removal may be escaped because of its biodegradability. Thus, magnesium is a load-bearing biomaterial with significant potential. Without a doubt, the utilization of Mg is good for the biomedical applications, e.g., for coronary stents (Heublein et al. 2003; Erbel et al. 2007).

20.3.1.3 Titanium and Titanium Alloys

It is an inert and high-cost material and capable of osteointegration with the bone (Davies 2007). The crystallize form of titanium is closed packed and body centered at temperatures below 883 °C. Titanium and its alloys are promising materials and support bone tissues ingrowth. The biological and mechanical properties of titanium are very good, but modulus is low as compared to stainless steel and Co-Cr-Mo alloys. The poor wear resistance of Ti and its alloys make them unsuitable for load bearing (Pilliar 2009). The surface oxidation of titanium makes an oxide layer on its surface that enhances its biocompatibility. This oxide layer also gives the protection to the titanium from corrosion. Titanium-aluminum-vanadium alloys have improved bio and mechanical properties than pure titanium implants (Okazaki 2001). An additional example of titanium alloys consists of ASTM F1295, ASTM F1713, ASTM F1813, and ASTM F2066 and Ti-5Al-2.5Fe (ISO 5832-10).

20.3.1.4 Nickel-Titanium Alloy (Nitinol)

It is a standout material that found different applications because of its novel properties, for example, pseudo-elastic, corrosion resistant, shape memory impact (SME), upgraded biocompatibility, super malleability, comparable young moduli to the bones, and high damping properties (Greiner et al. 2005; Prymak et al. 2005). Nitinol is used to make intramedullary nails, hips, plates, and spinal intervertebral spacers that are useful in scoliosis treatment (Tarnita et al. 2009). Moreover, it is stated that good biocompatibility has been achieved by surface modification of nitinol (Armitage et al. 2002; Michiardi et al. 2006). The surface modification improves biocompatibility and physical properties of alloys that may offer significant application in the hard tissue engineering.

20.3.2 Polymers

Polymers are using for the fabrication of functional and structural medical devices and scaffolds with desired biomechanical properties. The application and selection of polymer depend upon material properties like molecular weight, chemistry, solubility, water absorption capacity, and good degradation. Polymers are chosen as materials for tissue building in view of its porosity, high surface to volume proportion, biodegradation, and mechanical properties. The properties of polymeric implants rely on their composition structure and arrangements of their constituent macromolecules. The better possibility and biodegradability of polymer make them attractive materials for scaffold synthesis. The controlled design and structure of scaffolds should have better compatibility with tissues, rapid biodegradation, well-interconnected porous structure, and mechanical properties (Fig. 20.2).

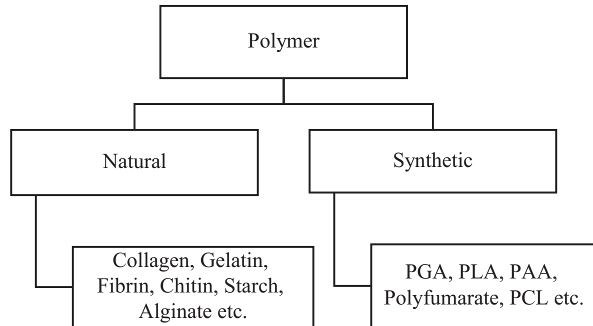

Fig. 20.2 Flow chart for the classification of polymers

20.3.2.1 Natural Polymers

Natural polymers are long-chain monomer, derived from different sources including animals, insects, and aquatic creatures. They degradation rate of natural polymers is fast; it takes a few hours to 6 months. So these are highly biocompatible, but they have low mechanical properties which limit their applications for hard tissue replacement (Bueno and Glowacki 2009). These are bioactive, biocompatible, and biodegradable in nature which are used for tissue engineering. These polymers include proteins (silk, collagen, myosin, fibrinogen, gelatin, actin, and keratin) and polysaccharides (chitin, cellulose, glycosaminoglycans, amylose, and dextran). The natural polymer's scaffolds are effectively applied in soft as well as hard tissue engineering but not for load-bearing applications. Natural polymer scaffold having homogeneous and reproducible structures also causes other problems (Brien 2011). The major natural polymers are:

Collagen

It is also a major part of natural bone extracellular matrix (ECM). In the tissue regeneration, collagens are used to improve strength of scaffolds. In the animal body, collagen provides structural support to the hard and soft tissues (e.g., the skin, bone, tendons, cartilage, blood vessels, and ligaments). About 27 types of collagen have been identified till now (Zohora et al. 2014) in which 80–90% collagen of types 1, 2, and 3 are present in the human body (Yang et al. 2004). The various forms of collagen-based scaffolds like porous sponges and thin sheets are used separately or with other materials for tissue engineering (Dhandayuthapani et al. 2011). Collagen scaffolds have good biocompatibility and poor mechanical properties. Yet, the physical and chemical cross-linking enhances fast degradation, compressive, and tensile strength of scaffold (Zohora et al. 2014).

Chitosan

Chitosan (natural polysaccharide) is derived from chitin and used in tissue regeneration due to its good compatibility with body tissues. It shows easy degradation with antimicrobial and immunogenic properties (Zohora et al. 2014). But pure chitosan scaffold has low mechanical properties (Albanna et al. 2013) and varying behavior

with seeded cells (Madihally and Matthew 1999). But, chitosan can be physically and chemically modified (Michiardi et al. 2006) and produce materials with a widespread range of properties.

Fibrin

It is a blood component which is fibrous and non-globular protein. Fibrin-based scaffolds are easy to biodegrade, biocompatible, easily processable, and nontoxic. The cost of fibrin is lower than synthetic polymers or collagen gels (Ahmed et al. 2008). The integration of bioactive peptides and growth factors improves the function of fibrin scaffold through heparin-binding delivery system (Ahmed et al. 2008). The change in concentration of different component affects the mechanical properties of fibrin-based scaffold. Biodegradation can be accomplished with the help of fibrinolysis inhibitors (Zohora et al. 2014).

20.3.2.2 Synthetic Polymers

A number of synthetic biodegradable polymers have been identified for the tissue engineering application. They enable the reestablishment of structure and function of damaged tissue. These are highly applicable due to their porosity, degradation, compatibility with cells, and mechanical properties. Some synthetic nonbiodegradable polymers are also used in tissue engineering. But these polymers generate high risk of rejection due to their cheap bioactivity. When these polymers start to degrade, they form highly acidic products which create problem with tissues that leads to the necrosis. They are having more improved properties than natural polymers. The common synthetic polymers for tissue engineering are:

Polylactic Acid (PLA)

PLA is biodegradable and thermoplastic polyester. It is produced by polymerization of L, L-lactide. This polymer is semicrystalline in nature. It has varying glass transition temperature (60–65 °C) and melting temperature (170–180 °C). The average tensile strength of PLLA and PDLLA is 15–2300 MPa and 28–1000 MPa, respectively. The elastic modulus of PLLA and PDLLA varies from 1.2–16 GPa to 1.0–3.4 GPa, respectively. Poly-DL-lactic acid with a pretty much irregular appropriation of the stereo arrangements in an amorphous material. The glass transition temperature of PDLLA varies from 50 to 60 °C that depends upon the molecular weight (Zohora et al. 2014).

Polyglycolic Acid (PGA)

It is a biodegradable aliphatic polyester made by polycondensation of glycolic acid. PGA shows a raised degree of crystallinity that varies from 45% to 75% with a glass transition temperature ranging from 25 to 65 °C. The absorption capacity of PGA material is good. It shown better thermal stability and crystallinity. It does not dissolve in the water easily and show fibrous encapsulation. Due to high subatomic weight, PGA is not soluble in the regular natural solvents, but HCL treatment improves cellular attachment (Zohora et al. 2014).

Poly(e-Caprolactone) (PCL)

It is a low-cost, highly elastic polyester which is recognized as biodegradable, biocompatible, but non-osteoinductive. The biocompatibility of PCL was approved by the FDA. It is easy to print and fabricate the scaffolds. After printing it shows good crystallinity and mechanical properties. The slow degradation of PCL was confirmed by the hydrolysis of its aliphatic ester linkages in the different physiological conditions (Zohora et al. 2014). PCL scaffold needs coating or incorporation of calcium precursor that enhances the suitability of this polymer.

Polyphosphazenes

Poly (phosphazenes) is a particular class of versatile polymers having various applications including tissue engineering and drug delivery. These are hybrid and flexible polymers holding a backbone of rotating phosphorus and nitrogen sequence at the interface. Synthesis of bio-stable to biodegradable polyphosphazenes increases its applications. The fusion of phosphorous molecules with the polymer to balance the degradation rate and composition of side substitutes (Amanda and Kibret 2014). High flexibility, biocompatibility, nontoxic degradation, and custom-made mechanical properties make this polymer very suitable for drug delivery and implants fabrication (Laurencin et al. 1996, 2003; Ambrosio et al. 2002; Conconi et al. 2004).

Poly(Propylene Fumarate)

It is unsaturated cross-linked polyester with limited mechanical and biodegradable system. It is one of the promising materials that is utilized for scaffold amalgamation and for the treatment of cervices and deformities. The PPF can be slowly degraded by straightforward hydrolysis of ester linkage into nontoxic items. The mechanical properties of poly (propylene fumarate) change as per the cross-connecting process utilized (Yaszemski et al. 1995; Temeno and Mikos 2000; Payne et al. 2002).

Poly(Glycerol Sebacate)

In 2002, this polymer was reported as a chemically suitable for the different applications in the tissue engineering. It can be synthesized by the condensation of sebacic acid with glycerol. It is a biodegradable, bioresorbable, flexible, and biocompatible polymer with very stimulating elastomeric properties. A 3D tough and biocompatible polyester can be produced from glycerol and sebacic acid by polycondensation process. PGS has wide biomedical applications such as soft tissue replacement including the cardiac muscle, blood, nerve, cartilage, and retina. So PGS becomes an appropriate elastomer that is utilized for the drug delivery and soft and hard tissue regeneration. (Wang et al. 2002; Gao et al. 2006; Rai et al. 2012).

20.3.3 Bioceramics

The ceramic materials which are utilized for the restoration or replacement and regeneration of contaminated or injured tissues. They are amorphous and crystalline in nature such as zirconia, alumina and hydroxyapatite are polycrystalline while

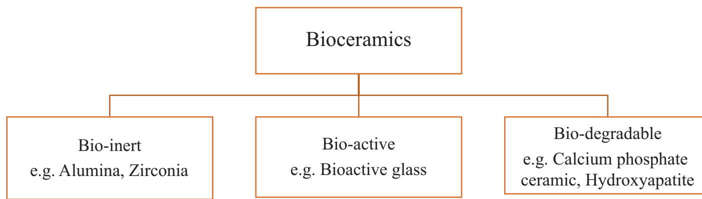

Fig. 20.3 Flow chart for the classification of bioceramics

bioglass is amorphous (Farooq et al. 2012). All these ceramic materials are used for the hard tissue regeneration because of better compatibility with the surrounding tissues (Luz and Mano 2011). The performance of materials used in the medical field is estimated by checking their bio-functionality and biocompatibility. The biocompatibility of a device or material is its ability to perform that particular function throughout the life of the plant and is jointly related to interaction between the tissues and implanted materials (Fig. 20.3).

1. *Bioinert*: Biologically inert materials that give minimal response to the biological tissues. In addition, when the bioinert materials implant into the body, it does not give adverse responses to the body. They keep their physiochemical and biomechanical properties in the host body. They resist corrosion and wear effects and maintain fracture toughness for long time. They are involved in the fabrication of different structural-support scaffolds, e.g., bone plates, nails, and screw. Some examples of bioinert materials are alumina, zirconia, and titanium-aluminum-vanadium alloy which are used in hip replacements and phosphorylcholine (PC) which is used in contact lenses.
2. *Bioactive*: They produce sensible effects on a living organism, tissue, or cell. They do direct interaction with the surrounding matrix and make stable chemical bonding that stimulate bone cell regeneration. When these materials implanted in the body, they promote bond formation with existing materials and bond to bone formation at various rates. Bioactive scaffolds are used for general orthopedic surgery, maxillofacial and periodontal repair, and chronic osteomyelitis. Examples of bioactive materials are hydroxyapatite and calcium phosphate.
3. *Biodegradable*: They are capable of being reacted and broken down rapidly when come in contact with body tissue. These are easily chemically degraded by endogenous tissues. After chemical structure degradation, resorption of materials should be easy by the normal metabolic pathways of the body without producing any toxic effect. Biodegradable scaffolds can be synthesized by different methods by using different resources including synthetic ceramics and natural ceramics (Table 20.2).

20.3.3.1 Alumina (Al_2O_3)

The bioinertness of alumina has proven since 1975. The alumina has great hardness and abrasion resistance. The surface smoothness of alumina increases the wear and friction properties. Hong and his co-authors stated that Al_2O_3 having the hexagonal

Table 20.2 Applications of bioceramic materials (Thamaraiselvi and Rajeshwari 2004)

Biomaterials	Artificial implants	Application
Alumina	Knee, hip, shoulder, elbow, wrist	Reconstruction of fractured part
Bioglass-coated metals		
Bioglass	Spinal fusion	Protect spinal cord
Bioglass-metal composite	Plates, wires, screw, nails	Repair and align fracture
Zirconia	Hip	Reconstruction of fractured part
Hydroxyapatite	Tooth	Replacement of damaged teeth

structure shows interstitial ion position (Hong et al. 2008). The properties like abrasion resistance, antifoaming, highly crystalline, chemically inert make it useful for dental and bone implants. If the alumina implanted in bone marrow, no toxic effect produced in circumferential tissue (Al-khateeb et al. 2012).

20.3.3.2 Zirconia (ZrO_2)

Zirconia has numerous advantages over other materials because it transforms its phase that increases toughness that can be manifested in components made out of them. It is a brittle transition metal oxide which induces the early bone growth and development. It has good mechanical properties with suitable biocompatibility. The specialization on zirconia as biomaterials began since a quarter century, and now zirconia is used as complete hip substitution (THR); however improvements can be done for the betterment of other medicinal applications (Lin et al. 2010).

20.3.3.3 Bioactive Glass

These are an interesting adaptable class of ceramic materials. The bioactive glass becomes a highly suitable material due to its high porosity, osteo-inductivity, and compatibility. The mesoporous structure provides high surface area that allows more cell attachments and proliferation. The high surface area allows fast reaction to generate hydroxyapatite layer. Bioglass-based implants or scaffolds are biocompatible and mesoporous with good mechanical properties. The mica and apatite are two significant crystal phases of bioglass. They formed a calcium phosphate-rich interface layer between glass, ceramic, and the bone by solid-state reaction. The bioglass starts to degrade when comes into contact with tissue fluid, and that reaction was understood as a chemical process, which shows the mineralization of the glass ceramic (Siraparapu et al. 2013).

20.3.3.4 Calcium Phosphate

It shows a unique similarity between the mineral phase of the bone and calcium phosphate. Hydroxyapatite (HAP) and β-tricalcium phosphate (β-TCP) are the common types of calcium phosphate. The bone regeneration also depends upon the crystallinity of the calcium phosphate and Ca/P ratio. Tricalcium phosphate is less soluble than the calcium phosphate. Ca and P ions produced after degradation of calcium phosphate enhance osteogenesis and osseointegration and the development of apatite on their surface. Bone mineral gems are extremely small and have an

Table 20.3 Mechanical properties of bioceramic materials (Thamaraiselvi and Rajeshwari 2004)

Biomaterials	Density (g/cm^3)	Hardness	Bond strength (GPa)	Compressive strength (MPa)	Young modulus (GPa)
Inert Al2O3	>3.9	2000–3000 (HV)	300–400	4000	380
ZrO2 (PS)	6.0	1000–3000 (HV)	200–500	2000	150–200
Graphite	1.5–1.9	NA	NA	138	20–25
Bioglass	2.5	NA	50	1000	75
Bioactive HAP	3.1	350	120	600	73–117
Glass ceramic	2.8	680	215	1080	118
Bone	NA	NA	60–160	130–180	3–30

expansive surface territory. The porosity in the calcium phosphate-based implant allows more entrapment of stem cells that regenerate bone tissue by differentiation.

20.3.3.5 Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$)

It is an alloplastic material. It is bioactive and compatible derivative of calcium phosphate with highly crystallinity. Hence, it shows tremendous stability and similarity with the mineral phase of natural bone. It contains calcium/phosphate atomic ratio of 1:67. The hydroxyapatite has chemical similarity with the inorganic phase of natural bone. It induces bone regeneration by inducing bond formation between HA and surrounding tissues. As compare to allograft and metallic implants, hydroxyapatite produces high biocompatibility and osteo-inductivity. Hydroxyapatite is a superb carrier for transforming growth factors of the bones and drug molecules (Table 20.3).

All the bioceramic materials have different properties so they have no specific biomedical applications. Some metals indicate their wear properties, although, in cell culture test, they show good biocompatibility. The applications of bioceramics depend upon their anticorrosive and biological properties. Bioglass and glass ceramics are nontoxic and easily make bonding with the bone. So the composites are more useful due to their inert and bioactive properties. These composite may consist improved biological and mechanical properties.

20.4 Composites

The composite used for hard tissue engineering is the combination of natural polymer, synthetic polymer, metals, and ceramics. Advancement of composite materials for tissue regeneration is alluring because of their properties which can be engineered to meet the physio-mechanical needs of the host tissue (Rezwan et al. 2006). The ceramic based implant materials can be categorized as inert, bioactive or semi-inert (Hench 1993), and bioresorbable (non-inert) (Hentrich et al. 1971). Hydroxyapatite is semi-inert (bio reactive), and TCP, zirconia, plaster of paris, and

alumina are resorbable materials. To achieve better osteoconductivity, composite fabrication becomes the first choice because composite can be fabricated to get desired characteristics (Park and Lakes 1992). A single material cannot prove all desired properties required for tissue engineering. Some materials are having good mechanical properties but not good biological properties and vice versa. To overcome these problems, the demand of composites comes into existence. The bioceramics and glasses are having high mechanical properties, but they are fragile, so it can be a reason of implant. So, polymer-/ceramics-based composites are synthesized to minimize the said problems. The engineered ceramic/glass-polymer composites were designed to get better biomechanical properties to avoid implant failure. Bioglass® or tricalcium phosphate gives an additional capacity to scaffolds, and they permit the composite to communicate viably with the surrounding bone matrix or tissues by surface degradation and mineralization process that promote a carbonate hydroxyapatite layer formation.

20.5 Discussion

Different fabrication strategies are connected to handle and process biomaterials into 3D polymeric, metallic, bioceramics, and composite scaffolds. The 3D scaffolds need to maintain desired structural, physiochemical, and mechanical integrity after implantation in the body (Sachlos and Czernuszka 2003; Liu and Ma 2004; Rezwan et al. 2006; Guo et al. 2015). Metals are having less porosity, so the cell proliferation becomes very low; this is also another disadvantage of metals. Tantalum is a porous metal with effective mechanical properties which allow better osteoconduction in the body. But the cost of tantalum implant is high as compared to other metal implants. Magnesium is a bioresorbable material which highly support bone growth and cell attachment. It avoids post-surgery implant removal because it degrades in the body. Titanium has good porosity, osteointegration, and biocompatibility. The alloy of titanium or nickel titanium enhances the biological properties of metal implants. The mechanical properties of alloys are more than single metallic, polymeric, or ceramic implants. The metals are replaced by bioceramics because these ceramics are porous and biodegradable. They are having good osteoconductivity and biocompatibility. Due to High porosity and brittleness their applications are limited. The degradation rate and extent of an implant or scaffold are the major factors to study for biomaterials (Engin and Tas 1999; Cama et al. 2009). The degradation rate of an implant controls the regeneration of bone cells and rebuilding process of bone tissue (Sheikh et al. 2015). In some cases a local inflammation is caused by the degraded material at that part of the body (Gooptu and Lomas 2008). A number of polymeric implants have been fabricated and used for the orthopedic and dental applications (Puoci 2015). PGA is considered as an immunologically inactive and contamination-free material (Vainionpaa et al. 1987). PLA-PGA exhibits good biocompatibility with lesser toxicity, but it reduces the osteo-inductivity and causes inflammation at the site surgery (Naughton et al. 1995). PLGA-HA composite produces good compatibility without producing any

inflammation and infection (West and Hubbell 1986). The degradation rate of PCL is slow, so it supports higher angiogenesis in acidic medium (Hutmacher et al. 1996). While the degradation of chitosan can cause inflammation after the 12 weeks of implantation. So calcium-based bioceramics or composites are better options for the implant fabrication because they make compatibility with the body tissues without producing inflammation (Lu et al. 1999; Apelt et al. 2004).

20.6 Conclusion

All polymers, metals, bioceramics, and composites are having their specific applications in selected tissue engineering field. Generally metals are the first choice as an implant for the repair and replacement of the bone. But metals have a lot of disadvantages such as lack of biodegradability in the body. In some cases, due to toxic effects, metals are replaced by hybrid materials. It is normal that the coming era of biocompatible and degradable materials will show immeasurable changes in the implants and tissues interfacing in the light of learning picked up from the latest research. The further research makes the possibility to produce ideal implants in the future for tissue engineering.

References

Adams JE, Zobitz ME, Lewallen DG et al (2005) Canine carpal joint fusion: a model for four-corner arthrodesis using a porous tantalum implant. J Hand Surg Am 30(6):1128–1135

Ahmed TAE, Dare EV, Hincke X, Al AET (2008) Fibrin: a versatile scaffold for tissue engineering applications. Tissue Eng 14(2):199–215

Albanna MZ, Bou-akl TH, Blowytsky O et al (2013) Chitosan fibers with improved biological and mechanical properties for tissue engineering applications. J Mech Behav Biomed Mater 20:217–226

Al-khateeb KAS, Mustafa AA, Faris A, Sutjipto A (2012) Use of porous alumina bioceramic to increase implant osseointegration to surrounding bone. Adv Mater Res 445:554–559

Amanda BL, Kibret M (2014) Biodegradable polyphosphazene biomaterials for tissue engineering and delivery of therapeutics. Bio Med Res Int. https://doi.org/10.1155/2014/761373

Ambrosio AMA, Allcock HR, Katti DS, Laurencin CT (2002) Degradable polyphosphazene/poly (a -hydroxyester) blends: degradation studies. Biomaterials 23:1667–1672

Apelt D, Theiss F, EI-Warrak AO, Zlinszky K, Bettschart-Wolfisberger R et al (2004) In vivo behavior of three different injectable hydraulic calcium phosphate cements. Biomaterials 25:1439–1451

Armitage DA, Parker TL, Grant DM (2002) Biocompatibility and hemocompatibility of surface-modified NiTi alloys. J Biomed Mater Res A 66(1):129–137

Bobyn JD, Stackpool GJ, Hacking HA et al (1999) Characteristics of bone ingrowth and interface mechanics of a new porous. J Bone Joint Surg Br 81(5):907–914

Brien FJO (2011) Biomaterials & scaffolds for tissue engineering. Mater Today 14(3):88–95

Bueno EM, Glowacki J (2009) Cell-free and cell-based approaches for bone regenration. Nat Rev Rheumatol 5(12):685–697

Cama G, Barberis F, Botter R, Cirillo P, Capurro M et al (2009) Preparation and properties of macroporous brushite bone cements. Acta Biomater 5:2161–2168

Conconi MT, Lora S, Baiguera S, Boscolo E et al (2004) In vitro culture of rat neuromicrovascular endothelial cells on polymeric scaffolds. J Biomed Mater Res A 71(4):669–674

Davies JE (2007) Bone bonding at natural and biomaterial surfaces. Biomaterials 28(34):5058–5067

Dhandayuthapani B, Yoshida Y, Maekawa T, Kumar DS (2011) Polymeric scaffolds in tissue engineering application: a review. Int J Poly Sci:1–19

Engin NO, Tas AC (1999) Manufacture of macroporous calcium hydroxyapatite bioceramics. J Eur Ceram Soc 19(13–14):2569–2572

Erbel R, Di Mario C, Bartunek J, Bonnier J et al (2007) Temporary scaff olding of coronary arteries with bioabsorbable magnesium stents: a prospective, non-randomised multicentre trial. Lancet 369:1869–1875

Farooq I, Imran Z, Farooq U et al (2012) Bioactive glass: a material for the future. World J Dent 3(2):199–201

Gao JIN, Ph D, Crapo PM et al (2006) Macroporous elastomeric scaffolds with extensive micropores for soft tissue engineering. Tissue Eng 12(4):917–925

Giannoudis PV, Dinopoulos H, Tsiridis E (2005) Bone substitutes: an update. Injury 36(3):S20–S27

Gooptu B, Lomas DA (2008) Polymers and inflammation: disease mechanisms of the serpinopathies. J Exp Med 205:1529–1534

Greiner C, Oppenheimer SM, Dunand DC (2005) High strength, low stiffness, porous NiTi with superelastic properties. Acta Biomater 1:705–716

Guo B, Lei B, Peng L, Ma PX (2015) Functionalized scaffolds to enhance tissue regeneration. Regen Biomater 2(1):47–57

Hench LL (1993) Bioceramics: from concept to clinic. Am Ceram Soc Bull 72:93–98

Hentrich RL Jr, Graves GA Jr, Stein HG, Bajpai PK (1971) Evaluation of inert and resorbable ceramics for future clinical orthopedic applications. J Biomed Mater Res 5(1):25–51

Heublein B, Rohde, Kaese V et al (2003) Biocorrosion of magnesium alloys: a new principle in cardiovascular implant technology ? Heart 89(6):651–656

Hong Z, Reis RL, Mano JF (2008) Preparation and in vitro characterization of novel bioactive glass ceramic nanoparticles. J Biomed Mater Res A 88(2):304–313

Hutmacher D, Hurzeler MB, Schliephake H (1996) A review of material properties of biodegradable and bioresorbable polymers and devices for GTR and GBR applications. Int J Oral Maxillofac Implants 11:667–678

Kokubo T, Takadama H (2006) How useful is SBF in predicting in vivo bone bioactivity? Biomaterials 27(15):2907–2915

Laurencin CT, El-Amin SF, Ibim SE, Willoughby DA, Attawia M et al (1996) A highly porous 3-dimensional polyphosphazene polymer matrix for skeletal tissue regeneration. J Biomed Mater Res 30(2):133–138

Laurencin CT, Ambrosio AM, Sahota JS (2003) Novel polyphosphazene-hydroxyapatite composites as biomaterials. IEEE Eng Med Biol Mag 22:18–26

Lin F, Yan C, Fan W et al (2010) Preparation of mesoporous bioglass coated zirconia scaffold for bone tissue engineering. Adv Mater Res 365:209–215

Liu X, Ma MX (2004) Polymeric scaffolds for bone tissue engineering. Ann Biomed Eng 32:477–486

Liu C, Xia Z, Czernuszka JT (2007) Design and development of three-dimensional scaffolds for tissue engineering. Chem Eng Res Des 85(7):1051–1064

Lu JX, About I, Stephan G, van Landuyt P, Dejou J et al (1999) Histological and biomechanical studies of two bone colonizable cements in rabbits. Bone 25:41S–45S

Luz GM, Mano JF (2011) Preparation and characterization of bioactive glass nanoparticles prepared by sol–gel for biomedical applications. Nanotechnology 22(49):494014. https://doi.org/10.1088/0957-4484/22/49/494014

Ma PX (2004) Scaffolds for tissue fabrication. Mater Today 7:30–40

Madihally SV, Matthew HWT (1999) Porous chitosan scaffolds for tissue engineering. Biomaterials 20:1133–1142

Michiardi A, Aparicio C, Planell JA, Gil EJ (2006) New oxidation treatment of NiTi shape memory alloys to obtain Ni-free surfaces and to improve biocompatibility. J Biomed Mater Res B 77(2):249–256
Naughton GK, Tolbert WR, Grillot TM (1995) Emerging developments in tissue engineering and cell technology. Tissue Eng 1:211–219
Okazaki Y (2001) A new Ti – 15Zr – 4Nb – 4Ta alloy for medical applications. Curr Opinion Solid State Mater Sci 5:45–53
Park JB, Lakes RS (1992) Biomaterials – an introduction, 2nd edn. Plenum Press, New York
Payne RG, Mcgonigle JS, Yaszemski MJ, Yasko AW et al (2002) Development of an injectable, in situ crosslinkable, degradable polymeric carrier for osteogenic cell populations. Part 3. Proliferation and differentiation of encapsulated marrow stromal osteoblasts cultured on crosslinking poly(propylene fumarate). Biomaterials 23:4381–4387
Pilliar RM (2009) Metallic biomaterials. In: Narayan R (ed) Biomedical materials. Springer, New York, pp 41–81
Prymak O, Bogdanski D, Ko M, Esenwein SA et al (2005) Morphological characterization and in vitro biocompatibility of a porous nickel – titanium alloy. Biomaterials 26(29):5801–5807
Puoci F (2015) Advanced polymers in medicine. Springer, Berlin
Rai R, Tallawi M, Grigore A, Boccaccini AR (2012) Progress in polymer science synthesis, properties and biomedical applications of poly(glycerol sebacate) (PGS): a review. Prog Polym Sci 37(8):1051–1078
Rezwan K, Chen QZ, Blaker JJ, Boccaccini AR (2006) Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering. Biomaterials 27(18):3413–3431
Sachlos E, Czernuszka J (2003) Making tissue engineering scaffolds work. Review: the application of solid freeform fabrication technology to the production of tissue engineering scaffolds. Eur Cell Mater 5:39–40
Salgado J, Coutinho OP, Reis RL (2004) Bone tissue engineering: state of the art and future trends. Macromol Biosci 4(8):743–765
Sheikh Z, Sima C, Glogauer M (2015) Bone replacement materials and techniques used for achieving vertical alveolar bone augmentation. Materials 8:2953–2993
Siraparapu YD, Bassa S, Sanasi PD (2013) A review on recent applications of biomaterials. Intl J Sci Res 1:70–75
Staiger MP, Pietak AM, Huadmai J, Dias G (2006) Magnesium and its alloys as orthopedic biomaterials: a review. Biomaterials 27(9):1728–1734
Tarnita D, Tarnita DN, Bizdoaca et al (2009) Properties and medical applications of shape memory alloys. Romanian J Morphol Embryol 50(1):15–21
Temeno JS, Mikos AG (2000) Injectable biodegradable materials for orthopedic tissue engineering. Biomaterials 21:2405–2412
Thamaraiselvi TV, Rajeshwari S (2004) Biological evaluation of bioceramic materials – a review. Trends Biometer Artif Organs 18(1):9–17
Vainionpaa S, Kilpikari J, Laiho J, Helevirta P et al (1987) Strength and strength retention vitro, of absorbable, self-reinforced polyglycolide (PGA) rods for fracture fixation. Biomaterials 8:46–48
Vert M, Li SM, Guerin P et al (1992) Macromoleculaires, bioresorbability and biocompatibility of aliphatic polyesters. J Mater Sci Mater Med 3(6):432–446
Wang Y, Ameer GA, Sheppard BJ, Langer R (2002) A tough biodegradable elastomer. Nat Biotechnol 20(6):602–606
West J, Hubbell J (1986) Bioactive polymers, synthetic biodegradable polymer scaffolds. Chapter 5. In: Bioactive polymers. Springer, New York
Yang C, Hillas PJ, Julio AB et al (2004) The application of recombinant human collagen in tissue engineering. BioDrugs 18(2):103–119
Yaszemski MJ, Payne RG, Hayes WC et al (1995) The ingrowth of new bone tissue and initial mechanical properties of a degrading polymeric composite scaffold. Tissue Eng 1(1):41–52
Zohora FT, Yousuf A, Anwarul M (2014) Biomaterials as porous scaffolds for tissue engineering applications: a review. European Sci J 10(21):186–209

Oocyte Cryopreservation: Paradigm in Assisted Reproduction Technology

21

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21.1 Introduction

Cryobiology is about to know and develop scientific understanding of the changes induced by very low temperature on living things at cell and tissue level. Paradoxical effects of low temperature in nature have been known for centuries through examples such as hibernation and frostbite (Boyle 1683). In the far past, people were not aware about the practical applications of cryopreservation of animal cells, human cells, or whole organ such as the kidney, heart, liver, and cornea including other soft and delicate tissues. Vitrification made it possible to cryopreserve valuable and endangered plant species. A milestone was achieved in the area of cryobiology when Christopher Polge and his team observed the protective ability of glycerol for cryopreservation of cell and other delicate tissues. This accidental search helped Polge and his team to cryopreserve spermatozoa of cattle and chickens successfully (Polge et al. 1949).The birth of the first calf from frozen spermatozoa of bovine by artificial insemination in 1951 opened new doors for production of superior animals due to practical ability of cryobiology. Cryobiology is now being used for conservation and improvement of endangered and valuable species of wildlife and superior domestic animals (Pukazhenthi and Wildt 2003). Currently many more approaches are being developed for manipulation and assessment of genetic integrity of cell at cytological, phenotypical, and molecular level especially with relevance to genetic stability. Curiosity to know about the totipotency and pluripotency combined with high-level understanding of epigenetic opened new research areas to be exploited for cryobiologists. Cellular reprogramming reduced production of free radical, and avoiding apoptosis during cryopreservation along with other advances ensures that the future is bright for the next generation of cryobiologists.

Cryopreservation of oocytes will prove paradigm for making oocytes readily available for conducting experiments throughout the year. Oocyte cryopreservation

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is helpful to preserve endangered, extinct, and valuable species of animals and plants. An assisted reproduction technology (ART) for rare, vulnerable, and valuable animals is not possible without oocyte cryopreservation. The oocyte cryopreservation is the key of success for production of animals particularly which have low reproduction ability (Cecim et al. 1995).The use and storage of embryos in case of humans is illegal, unethical, and immoral which can be overcome by oocyte cryopreservation (Gosden and Nagano 2002).

21.2 Principles Used in Cryopreservation

The cells are kept in state of suspended animation for indefinate time from which these cells can be restored in viable condition for future use in assisted reproduction technologies (ART). It helps in maintaining their viability for a very long period of cryopreservation. Most of the cells including the mammalian cells are cryopreserved at −196 °C which freezes all biological activities. The critical point in the process of cryopreservation is cooling the sample to −196 °C followed by thawing to achieve desired normal temperature 37 °C. The rapid cooling of cells and tissues also solidifies water present in the cell. This can induce many damages in oocytes like ruptured zona pellucida, splitting of oocyte in two halves, leakage of contents, shrunken or fragmented cytoplasm, change in shape, and partial or complete denudation of cytoplasm due to formation of ice crystals. The best method of cryopreservation is to restrict the formation of ice crystals (Lovelock 1954). During solidification process, there are chances to achieve exceptionally high level of electrolytes and other available solutions in the cell. This increased level may be highly toxic for proteins present in the cell; therefore, change in the level of concentration of solution in the cell must be avoided for better cryopreservation process. The rewarming of sample releases free water due to melting of solid ice which results in the decreased osmolarity of rest of solution. During the thawing process, slow rewarming may increase the risk of recrystallization and release of free water; this may induce many types of damages in the cells. The Rapid rate of rewarming leads to sudden fall of extracellular osmotic pressure, this may be responsible for cell swelling and inducing much kind of cell damages (Mazur 1980). This is known as osmotic shock; keeping safe from osmotic shock to the cells is the third most important target of an ideal process of cryopreservation. Cryopreservation process involves the utilization of chemicals additionally to restrict the damage of cell and internal organs of cell. The chemicals used for this purpose are known as cryoprotectants. These cryoprotectants are classified in two groups.

A. Permeating Cryoprotectants
B. Non-Permeating Cryoprotectants

21.2.1 Permeating Cryoprotectants

Permeating group of cryoprotectants: This group includes chemicals having small structure. These small structured molecules restrict formation of crystals of ice due to formation of hydrogen bond with water. These chemicals have ability to permeate the cell membrane (Friedler et al. 1988). The often used members of permeating group of cryoprotectants are PROH (propylene), DMSO (dimethyl sulfoxide), GLY (glycerol), and EG (ethylene glycol).

21.2.2 Non-permeating Cryoprotectants

These chemicals are not able to permeate membrane. These help to increase the concentration of permeating group of cryoprotectants and also restrict the formation of crystals of ice. These are used in the process of thawing. The sucrose is normally used as a non-permeating cryoprotectant. The disaccharides and many other non-permeating chemicals complete this group.

21.2.3 Removal of Cryoprotectants

Finally in cryopreservation, the cryoprotectants are removed from the cryopreserved oocyte. Often 1–2 M concentration is used during cryopreservation of oocyte in slow freezing method, while in rapid freezing method, concentration of 4–8 M is preferred. Rapid rehydration is used when permeating cryoprotectants were used for freezing; however, this is a very slow process and needs a lot of expertise. Removal of cryoprotectants can be achieved quickly using non-permeating chemicals such as sucrose (Camus 2004; Massip 2001).

21.3 Types of Cryopreservation

Two strategies are being currently practiced for mammalian oocytes cryopreservation.

1. Slow freezing also called equilibrium freezing or programmable freezing
2. Vitrification also called nonequilibrium freezing

21.3.1 Slow Freezing

For cryopreservation of mammalian cells and oocytes using slow freezing method, solution of cryoprotectant(s) 1–2 mol/L is prepared, and cells are cooled at very low speed (0.3–0.5 °C/min) to concentrate the contents present inside the cell applying dehydration principal. The protocol for slow freezing was first standardized by

(Whittingham et al. 1972). In this technique, cells are subjected to −80 °C prior to plunging them in liquid nitrogen.

21.3.2 Vitrification

Vitrification is recent development in cryopreservation used for rapid cooling of cells. This approach restricts the formation of ice in the form of crystals in both sides of the cell, i.e., intercellular and extracellular (Rall and Fahy 1985). Since vitrification is rapid cooling approach, this high concentration of cryoprotectants (5–7 M) is used at ultrahigh speed of cooling (2000–25,000 °C/min). This approach completely inhibits the formation of intercellular ice (Porcu et al. 2004). Vitrification is cost-effective, rapid, and an easy method of cryopreservation. According to Purohit et al. 2012, the immature cumulus oocytes are more vulnerable to bear the stress induced during vitrification and achieve high fertilization rate.

21.4 Oocyte Cryopreservation

Oocyte is female gametocyte or germ cell involved in reproduction. The oocytes of animals have high water content and low tolerance capacity toward cooling. This along with large volume and sensitivity makes them difficult to cryopreserve (Leibo 1980). The first attempt of cryopreservation of mammalian oocyte was practiced in 1958 by reviving unfertilized form of oocyte using mouse as experimental organism (Sherman and Lin 1958). The birth of first live offspring with in vitro fertilization (IVF) techniques using cryopreserved oocytes of mouse was performed in 1977 (Whittingham 1977). Later on, many successful attempts were made using mouse and other species of animals like cow, rabbit, pig, cat, and hamster. Despite these notable success stories, the optimal and effective protocol for cryopreservation of oocytes is yet to be standardized. Undoubtedly developing optimized and standard protocol will be seen as advancement in the area of cryobiology which will prove panacea in reproductive biology and assisted reproductive technology for conservation of superior genetic traits in living beings. The science of cryobiology has competency to ensure the supply of oocytes instantly round the clock for advanced research experiments in area of assisted reproductive technology (ART) (Moawad et al. 2012). Furthermore, oocyte cryopreservation makes possibility of salvaging genetic material from prepubertal, infertile, pregnant, or even from dead animals. This makes possible to create oocyte bank and survival of endangered species in animals. Oocyte cryopreservation also made disease testing possible before birth of child (Clark and Swain 2013). As per reports, the number of children born from cryopreserved oocytes after fertilization has exceeded 1000 universally (Rodriguez-Wallberg and Oktay 2012). First successful births after using mammalian cryopreserved oocytes as given in Table 21.1 prove the potential and significance of cryopreservation.

Table 21.1 First births resulting from the successful cryopreservation of mammalian oocytes

Species	Method	Authors
Mouse	Slow cooling	Parkening et al. (1976)
	Vitrification	Kono et al. (1991)
Rabbit	Slow cooling	Al-Hasani et al. (1989)
	Vitrification	Vincent et al. (1989)
Cattle	Slow cooling	Fuku et al. (1992)
	Vitrification	Vajta et al. (1998)
Human	Slow cooling	Chen (1986)
	Vitrification	Kuleshova et al. (1999)
Horse	Vitrification, CryoLoop	Maclellan et al. (2002)

Cryopreservation of oocytes will have multiple applications for animals and human beings. Particularly in the context of farm animals, oocytes can be retrieved from live animals even if they are prepubertal, infertile, or pregnant. Oocytes can also be retrieved from the ovaries of dead animals. The oocytes collected from any source can be subjected to freezing for creating repositories in the form of oocyte banks. Thus, valuable genetic bloodlines from females can be preserved, and oocytes rather than embryos from a proven sire can be marketed. Frozen-thawed oocytes could be also later used for producing live offspring for faster multiplication of superior germplasm or for use in reproductive technologies like production of cloned or transgenic livestock or production of embryonic stem cells.

21.5 Morphological Damages and Their Controls in Cryopreservation

Cryopreservation induces many functional and morphological damages. The type and intensity of damages are influenced by size of cell, quality of oocyte, species of animals, and concentration of cryoprotectant used in cryopreservation. The types of damages induced in cryopreserved oocytes reduce the fertility rate of oocytes even render them unfit for use in assisted reproductive technologies. Furthermore, scientists have studied many variables that can affect the success of the cryopreservation. The major variables include temperature (during cryoprotectant addition, freezing, and thawing), types of cryoprotectant, additives, vessels for holding the oocytes, types of machinery, and the osmotic properties of solution.

21.6 Sheep: Important Domestic Animal

Sheep (*Ovis aries*) is one of the first species to be domesticated. It has been in close association with humans from ancient time. These species are human-supporting animals in the universe and gained special importance in the Arab world countries. It is one of economically important animals because these countries have very less cultivable land with low fertility. Sheep produces fiber, wool, manure, and meat

Fig. 21.1 French straw (0.25 ml) containing oocytes. *PVA* polyvinyl powder, *LS* loading solution, *VS* vitrification solution

also. Currently sheep is being used as model animal for conducting experiments for new findings in the scientific world. Sheep has resemblance in physiology, reproduction, and metabolic process with many mammals. This makes it suitable model animal for scientific research. Effective reproduction strategies may be helpful to multiply their number many times, which definitely will open doors for employment and livelihood for rural people, farmers especially in the developing countries. The multiplication of sheep is possible by assisted reproduction technologies. The oocyte cryopreservation is the first step in this direction (Mohammadpour 2007). Sheep has high resemblance with human in respect to body weight, size, and other major biological processes. Scientists have used sheep for studying serology, anesthesia, radio immunoassays, and catheterization techniques. They have also studied several other body defense mechanisms along with placental route of transfer of metabolites and drugs (Hau and Van Hoosier 2010). Our study focused on cryopreservation of cumulus oocyte complexes of sheep due to resemblance of many metabolic processes with human. Keeping in view the above discussed facts, we conducted our study on cryopreservation of cumulus oocyte complexes of sheep using DMSO 40%, glycerol 40%, and combination of DMSO 20% + 20% glycerol for the purpose of reduction of cost and applications in assisted reproduction technology. Sheep ovaries were collected from the slaughterhouse at New Delhi immediately after slaughter to avoid any ethical issue.

21.6.1 Vitrification Using Glycerol or DMSO

For each trial, the immature oocytes were divided into four groups, each containing at least 20–30 oocytes. The oocytes of group 1 (G40) and group 2 (D40) were exposed to the final concentration of the cryoprotectant in a two-step manner by equilibrating the oocytes serially to 20% and 40% of the cryoprotectant. The oocytes were placed into culture dishes containing 2 ml of medium with G20 or D20 for 1 min before exposure to G40 or D40 solution. Groups of around ten oocytes were then loaded into a 0.25 ml French straw, suspended in a 30 μl microdrop of G40 or D40 solution. The straw was then sealed with polyvinyl alcohol powder. The oocytes were then loaded into the straws as described below (Fig. 21.1).

Nearly half straw having the vitrifying solution plunged quickly in liquid nitrogen and the immersion of remaining part of straw achieved slowly to avoid rupture. This plunging in liquid nitrogen was done within 60 s of the final exposure of oocytes to G40 or D40 cryoprotectant solution (Gautam et al. 2008).

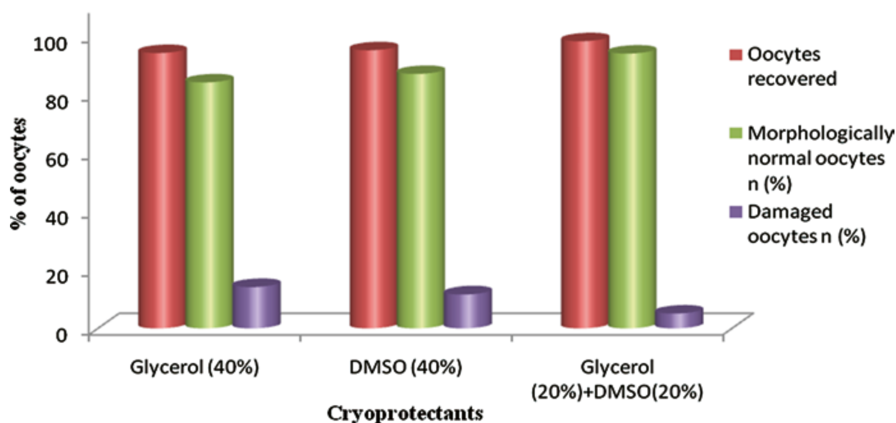

Fig. 21.2 Normal oocytes recovered using different cryoprotectants in sheep

21.6.2 Vitrification Using Combinations of Glycerol and DMSO

The oocytes of groups 3 (G20) and 4 (D20) then subjected to the final concentration of the cryoprotectants in a two-step manner. The oocytes were placed in culture dishes containing 2 ml of TF medium with G10 or D10 for 60 s and then subsequently suspended in G20 or D20 for 30 s. Groups of around ten oocytes were loaded into a 0.25 ml French straw, suspended in a 100 μl microdrop of G20 or D20 solution. The straw was then sealed with polyvinyl alcohol powder. The straws were then plunged in liquid nitrogen as mentioned above and stored for at least 60 days (Kumar 2015).

21.6.3 Thawing

For thawing, the straws were held in air for 5–10 s and then plunged in a water bath at 37 °C for 10–15 s. Contents of straw were decanted in a culture dish, and the oocytes were then quickly transferred to fresh TF medium containing 0.5 M sucrose. A three-step dilution was used for the oocytes treated with different cryoprotectants or their combinations (0.5, 0.33, and 0.17 M sucrose, with 1 min equilibration in each solution). After that, oocytes were transferred to fresh TF medium for proper washing with it.

21.6.4 Morphological Evaluation of Recovered Oocytes

Thawed oocytes were observed under inverted microscope at 400 ×. The percentage of morphologically normal oocytes was very good which is minimum 84.2 ± 2.3% for glycerol 40% and maximum 94.09 ± 1.1% for 20% glycerol +20% DMSO (Fig. 21.2) is a success of our study. The types of damages observed after

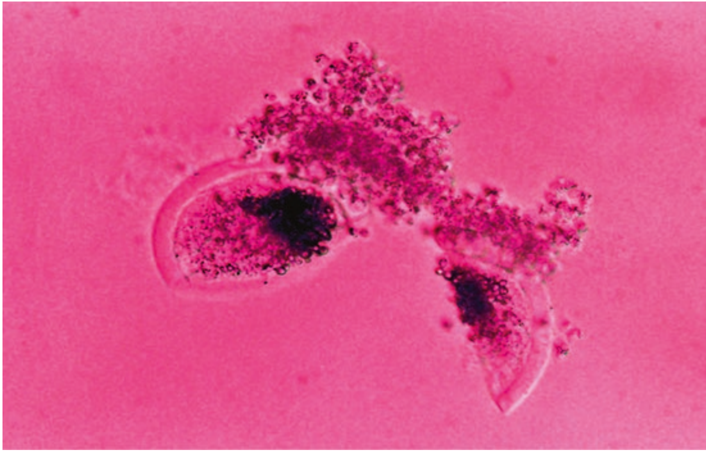

Fig. 21.3 Oocyte broken into two halves

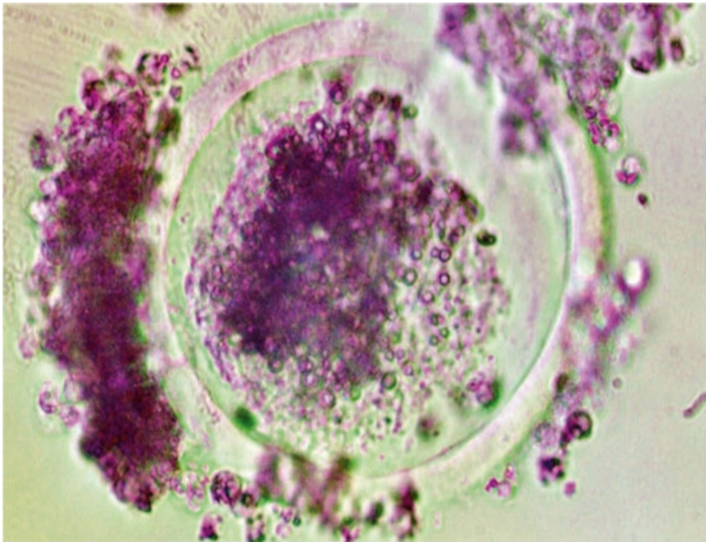

Fig. 21.4 Damaged zona

vitrification and thawing of immature oocytes using different combination of cryoprotectants and concentrations used in the present study included oocytes split in two halves (Fig. 21.3), damaged zona (Fig. 21.4), fully denuded oocytes (Fig. 21.5), shrunken cytoplasm, and change in shape (Fig. 21.6).

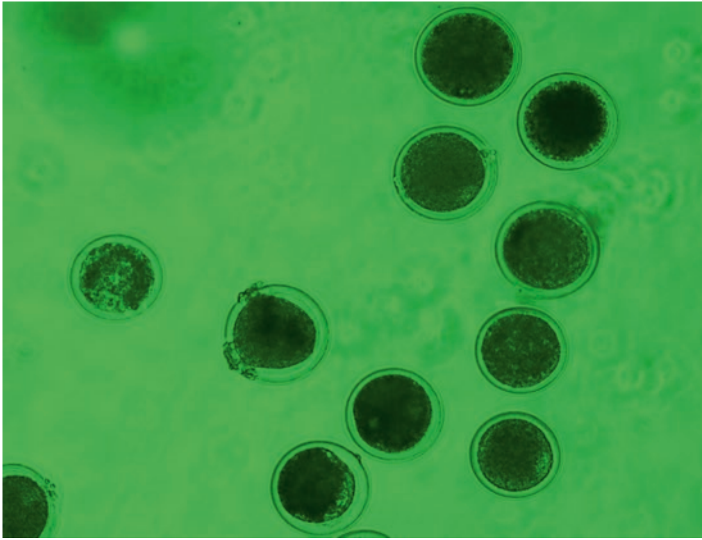

Fig. 21.5 Fully denuded oocytes

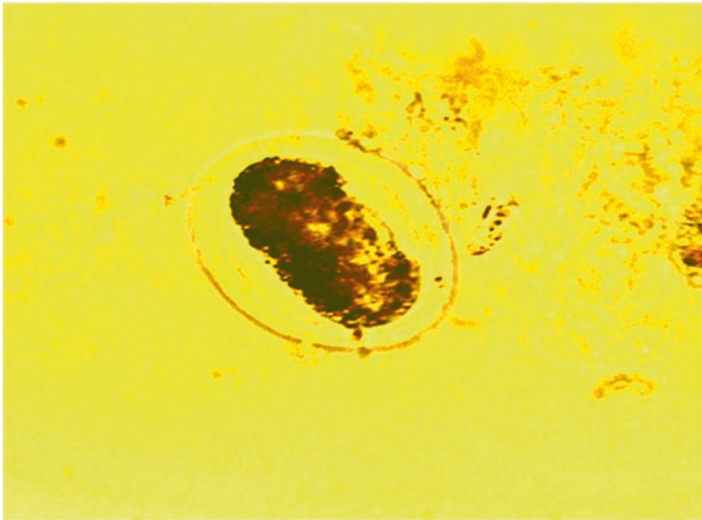

Fig. 21.6 Shrunken cytoplasm and change in shape of oocyte

21.7 Conclusion

Cryopreservation has great potential for germplasm conservation. Oocyte cryopreservation is emerging area having numerous developing applications for advanced research in biomedical and assisted reproductive technology fields. It has

been reported that cryopreservation did not affect the morphology of oocytes. Therefore, it is recommended that vitrification is a suitable technique to cryopreserve the oocytes for further use in assisted reproduction technology for production of superior animals in the future as proved in our study having high (84–94%) recovery rate of morphologically normal oocytes after cryopreservation. Further research is required to develop new protocols for cryopreservation and to check fertilization competency of cryopreserved oocytes.

References

Al-Hasani S, Kirsch J, Diedrich K, Blanke S, Van der Ven H, Krebs D (1989) Successful embryo transfer of cryopreserved and in vitro fertilized rabbit oocytes. Hum Reprod 4(1):77–79

Boyle R (1683) New experiments and observations touching cold. R Davis, London

Camus M (2004) Human embryo cryopreservation: review of clinical issues related to the success rate. In Proceedings of symposium on cryobiology and cryopreservation on human gametes and embryos, ESHRE Campus, 24–26

Cecim M, Kerr J, Bartke A (1995) Infertility in transgenic mice overexpressing the bovine growth hormone gene: luteal failure secondary to prolactin deficiency. Biol Reprod 52(5):1162–1166

Chen C (1986) Pregnancy after human oocyte cryopreservation. Lancet 327(8486):884–886

Clark NA, Swain JE (2013) Oocyte cryopreservation: searching for novel improvement strategies. J Assist Reprod Genet 30(7):865–875

Friedler S, Giudice LC, Lamb EJ (1988) Cryopreservation of embryos and ova. Fertil Steril 49(5):743–764

Fuku E, Kojima T, Shioya Y, Marcus GJ, Downey BR (1992) In vitro fertilization and development of frozen-thawed bovine oocytes. Cryobiology 29(4):485–492

Gautam SK, Verma V, Palta P, Chauhan MS, Manik RS (2008) Effect of type of cryoprotectant on morphology and developmental competence of in vitro-matured buffalo (*Bubalus bubalis*) oocytes subjected to slow freezing or vitrification. Reprod Fertil Dev 20(4):490–496

Gosden R, Nagano M (2002) Preservation of fertility in nature and ART. Reproduction 123(1):3–11

Hau J, Van Hoosier GL (2010) Handbook of laboratory animal science, 3rd edn. CRC Press, Boca Raton

Kono T, Kwon OY, Nakahara T (1991) Development of vitrified mouse oocytes after IVF. Cryobiology 28:50–54

Kuleshova L, Gianaroli L, Magli C, Ferraretti A, Trounson A (1999) Birth following vitrification of a small number of human oocytes: case report. Hum Reprod 14(12):3077–3079

Kumar S (2015) Cryobiological effects and apoptotic gene expression on cumulus oocyte complexes in sheep (Ovis areis). Unpublished doctoral thesis, Chaudhary Devi Lal University, Sirsa

Leibo SP (1980) Water permeability and its activation energy of fertilized and unfertilized mouse ova. J Membr Bio 53(3):179–188

Lovelock JE (1954) The protective action of neutral solutes against haemolysis by freezing and thawing. Biochem J 56(2):265

Maclellan LJ, Carnevale EM, Da Silva MC, Scoggin CF, Bruemmer JE, Squires EL (2002) Pregnancies from vitrified equine oocytes collected from super-stimulated and non-stimulated mares. Theriogenology 58(5):911–919

Massip A (2001) Cryopreservation of embryos of farm animals. Reprod Domes Anim 36(2):49–55

Mazur P (1980) Limits to life at low temperatures and at reduced water contents and water activities. In: Limits of life, Springer, The Netherlands, 1–23

Moawad AR, Fisher P, Zhu J, Choi I, Polgar Z, Dinnyes A, Campbell KH (2012) In vitro fertilization of ovine oocytes vitrified by solid surface vitrification at germinal vesicle stage. Cryobiology 65(2):139–144

Mohammadpour AA (2007) Comparative histomorphological study of ovary and ovarian follicles in Iranian Lori-Bakhtiari sheep and native goat. Pak J Biol Sci 10(4):673–675
Parkening TA, Tsunoda Y, Chang MC (1976) Effects of various low temperatures, cryoprotective agents and cooling rates on the survival, fertilizability and development of frozen-thawed mouse eggs. J Exp Zool 197(3):369–374
Polge C, Smith AU, Parkes AS (1949) Revival of spermatozoa after vitrification and dehydration at low temperatures. Nat 164(4172):666
Porcu E, Fabbri R, Damiano G, Fratto R, Giunchi S, Venturoli S (2004) Oocyte cryopreservation in oncological patients. Eur J Obstet Gynecol Reprod Biol 113:14–16
Pukazhenthi BS, Wildt DE (2003) Which reproductive technologies are most relevant to studying, managing and conserving wildlife? Reprod Fertil Dev 16(2):33–46
Purohit GN, Meena V, Solanki K (2012) Morphological survival and subsequent *in vitro* maturation of denuded and cumulus compact Bubaline oocytes cryopreserved by ultra-rapid cooling. J Buff Sci 1(1):78–83
Rall WF, Fahy GM (1985) Ice-free cryopreservation of mouse embryos at −196°C by vitrification. Nature 313:573–575
Rodriguez-Wallberg KA, Oktay K (2012) Recent advances in oocyte and ovarian tissue cryopreservation and transplantation. Best Pract Res Clin Obstet Gynaecol 26(3):391–405
Sherman JK, Lin TP (1958) Survival of unfertilized mouse eggs during freezing and thawing. Exp Bill Med 98(4):902–905
Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, Callesen H (1998) Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. Mol Reprod Dev 51(1):53–58
Vincent C, Garnier V, Heyman Y, Renard JP (1989) Solvent effects on cytoskeletal organization and in vivo survival after freezing of rabbit oocytes. J Reprod Fertil 87(2):809–820
Whittingham DG (1977) Fertilization *in vitro* and development to term of unfertilized mouse oocytes previously stored at −196 °C. J Reprod Fertil 49(1):89–94
Whittingham DG, Leibo SP, Mazur P (1972) Survival of mouse embryos frozen to −196°C and −269 °C. Sci 178(4059):411–414

Chemiluminescence 22

Heena Rekhi, Ripneel Kaur and Ashok Kumar Malik

22.1 Introduction

Luminescence is the production of light by organisms. Reactions occurring inside the bioluminescent organism release the energy and emit light. Bioluminescent organisms such as fireflies, glowworms, anglerfish, and those responsible for the phosphorescence of the sea are often familiar (Fig. 22.1). Still there are many marine organisms such as shrimp and squid which show more vivid effects. These chemical species are different from fluorescence.

Most bioluminescent reactions involve luciferin and luciferase. In the presence of oxygen enzyme, luciferase acts as an organic molecule luciferin to produce energy in terms of photon and oxyluciferin. Some bioluminescent organisms produce their own light, either making all of the ingredients themselves or making everything their own. Instead, some have a symbiotic relationship with bioluminescent bacteria that live inside their bodies which don't produce their own light. Various biochemical sequences are used to produce light. In the simplest case, luciferin is a small heterocyclic organic molecule whose enzyme-catalyzed oxidation leads to the formation of product, oxyluciferin. Luciferase involved uses common cofactors in the reaction shown in mechanism (Fig. 22.2). These cofactors are often central to light-emitting reaction and thus can be coupled to many reactions of biological significance, for example, bioluminescent reactions found in firefly (ATP as cofactor), *Renilla* (PAPS as cofactor), and luminous bacteria (NADH or NADPH as cofactor).

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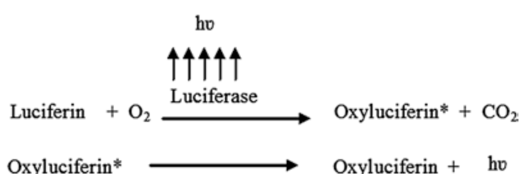
Fig. 22.1 Bioluminescent lucifer, mushrooms, and fluorescent corals. (Adapted from Google images)

Fig. 22.2 Bioluminescent reaction mechanism. (Adapted from Fraga et al. 2006)



22.2 Characteristics of the Light Emission

A large amount of energy is released when bioluminescence results from a chemical reaction which leads to the product molecule populated in excited electronic state instead of being dissipated in the form of heat as in a normal chemical reaction. Bands are found to be broad for the bioluminescence spectra with widths at half-height around 60–100 nm. Visible radiation appears in the wavelength range of 400–700 nm (Fig. 22.3). Terrestrial organisms have yellow-green bioluminescence color, whereas marine species show bioluminescence 450–510 nm. Maximum transmission luminescence has been achieved in ocean water for blue to green, whereas terrestrial group achieves the maximum transmission for yellow light.

22.3 Chemiluminescence

Light is emitted from the chemical reactions at ordinary room temperatures in chemiluminescence. The radiation produced may be in the UV, infrared, or visible region. The radiative process is similar to fluorescence when the excited state is a singlet and is similar to phosphorescence when the excited state is triplet. Several mechanisms have been described to explain the manner by which chemical energy is provided for chemiluminescent reactions. These include reactions involving peroxide decomposition, singlet oxygen, ion radicals, and chemically initiated electron exchange. This is a multistep process (Scheme 22.1); the final quantum yield is the product of the chemical and physical efficiencies of the processes involved.

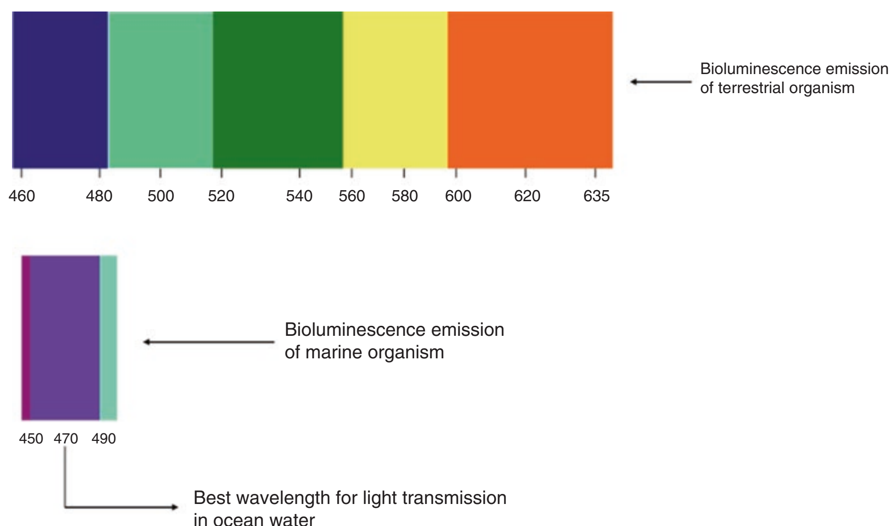
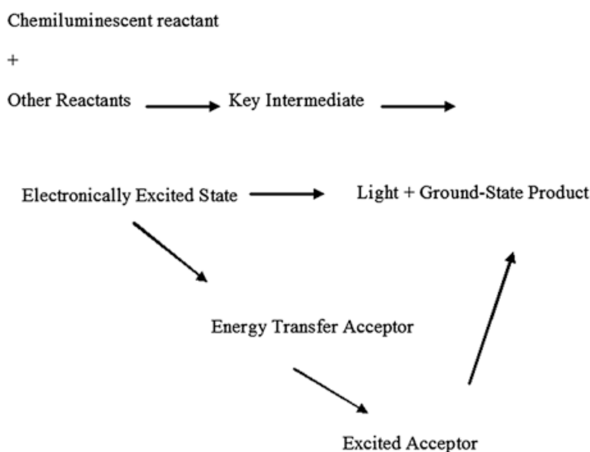


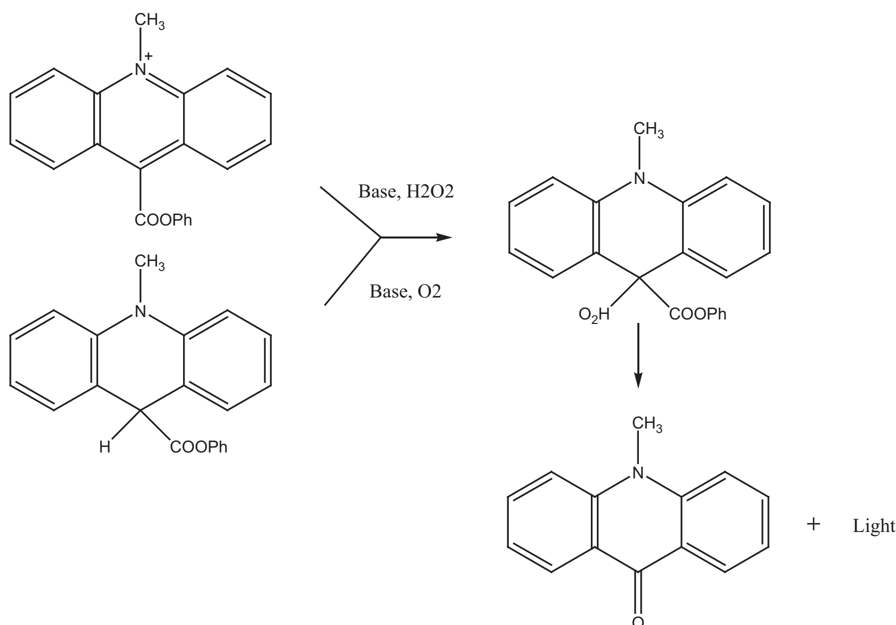
Fig. 22.3 Bioluminescence emission of various organisms

Scheme 22.1 Chemiluminescent reaction



22.3.1 Mechanism of Chemiluminescence

It is an interesting and important area; an understanding of this can be expected to improve the application of phenomenon. Compounds may react with oxygen, usually in the form of carbanion or electron-rich species to produce peroxide. A peroxide can also be produced if the structure of the compound invites attack by hydrogen peroxide, which is a powerful nucleophile in aqueous solution. The purpose is to produce an intermediate derived from luminol which will react with peroxide or its oxidation product superoxide ion. The fluorescent product is derived from the reactant.



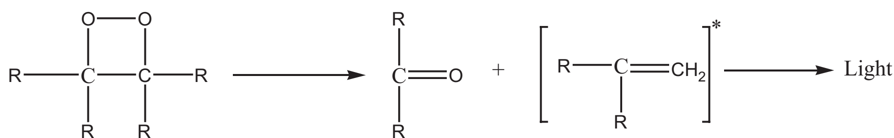
Chemiluminescence is classified on the basis of phase in which luminescence occurs, i.e., gas, liquid, or solid.

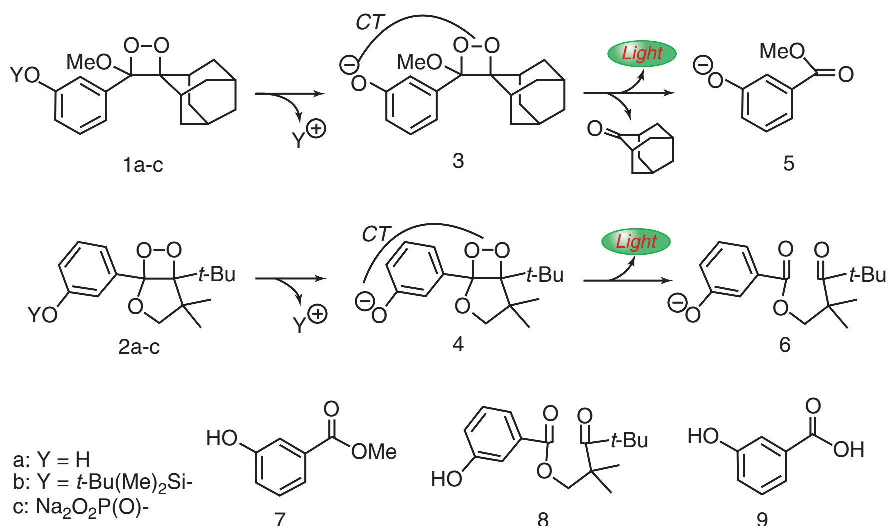
22.3.2 Liquid Phase Chemiluminescence

Liquid phase chemiluminescent reactions progress through the decomposition of intermediates with the instantaneous formation of two carbonyl groups. The concerted formation of these groups meets the energy requirements for the chemiluminescent reaction.

22.3.3 Peroxide Decomposition

Some dioxetanes are stable at room temperature but most decompose below 80 °C and require careful synthesis and handling. Simple dioxetanes decompose to produce carbonyl compounds in excited states, including singlet states, and thus the decomposition is accompanied by the emission of light. Both photochemical and chemiluminescent methods have been used to determine the relative yields of the excited states.

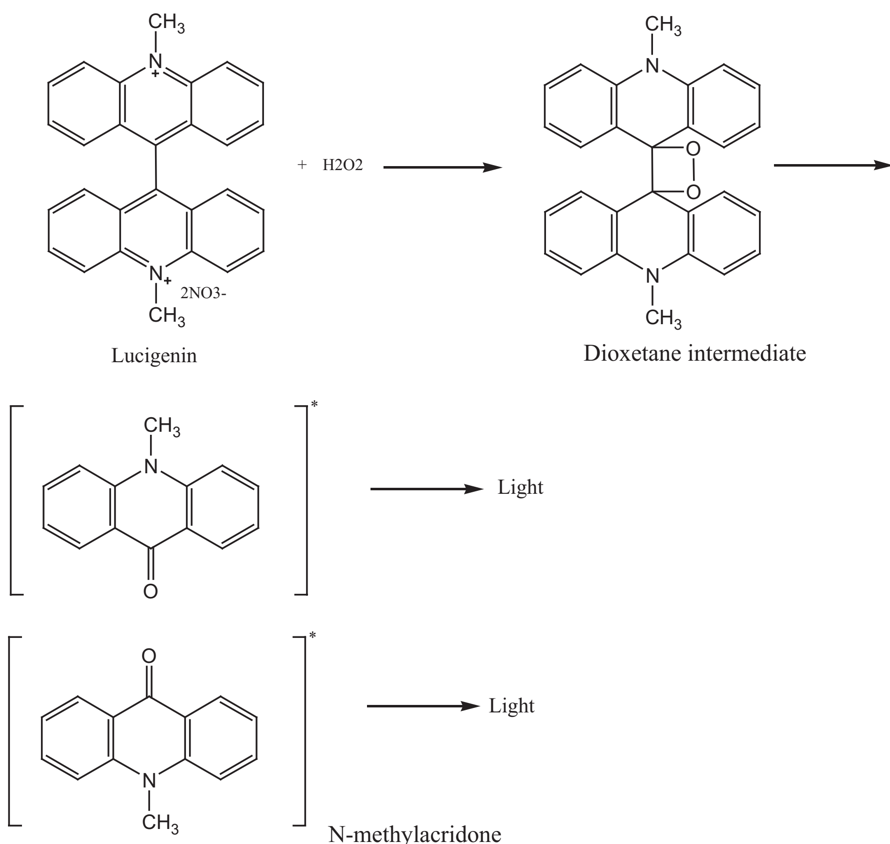




Scheme 22.2 Base-induced chemiluminescent decomposition of 3-oxyphenyl-substituted 1,2-dioxetanes. (Adapted from Watanabe et al. 2014)

The highest yield of excited product in dioxetane decomposition reaction is produced from the decay of tetramethyl-1,2-dioxetane, where 50% acetone produced is in the triplet form; the singlet state is formed only in the low yields. Watanabe et al. describe the chemiluminescent decomposition of 3-hydroxyphenyl-substituted dioxetanes in an aqueous system (Watanabe et al. 2014). Considerable attention has been received for the possible applications in modern biological and clinical analysis using chemiluminescence which includes the intramolecular charge-transfer-induced decomposition (CTID) of oxidophenyl-substituted dioxetanes. Typical examples are adamantylidene-substituted dioxetanes (1) and bicyclic dioxetanes (2), which undergo chemiluminescent CTID through unstable oxidophenyl-substituted dioxetane (3) or (4) produced by deprotonation (Scheme 22.2).

Gleu and Petsch reported the chemiluminescent substances acridine derivatives and lucigenin (Gleu and Petsch 1935). Its derivatives produce strong chemiluminescence (CL) properties with reducing agents like hydrogen peroxide. Veazey et al. have described the CL reaction oxidized by lucigenin in various reaction steps (Veazey et al. 1984). The reaction between lucigenin and hydrogen peroxide produces light which proceeds without a catalyst, although it is accelerated by metal ions leading to an intensification of chemiluminescence. Light emission occurs on the addition of numerous nucleophiles and reducing agents to solutions of lucigenin.



22.3.4 Electron Transfer Chemiluminescence

Electron transfer reactions are capable of producing excited products which may decay to the stable state with the release of energy, for example, resonance energy transfer. The oxidation of DBA which has been reported by Dong et al. (2016) arises via one-electron oxidation in nonaqueous solution. However, DBA oxidation is complicated at the GCE bare in an aqueous medium (Ahlberg et al. 1981). Dissolved oxygen can react with DBA-oxidized product (Luc^{*+}) to generate CL (Cui et al. 2007). Sun et al. (2001) have reported the transfer of energy during the chemiluminescence process. ECL emission was tested under the oxygen and inert atmosphere.

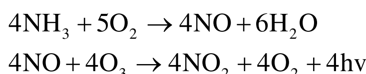
22.3.4.1 Gas Phase Chemiluminescence

Air pollution can be monitored by it and has been studied in simple gases, flames, metal vapors, chemical lasers, molecular beams, etc. Ozone and atomic oxygen can react with other gases such as oxides of nitrogen, sulfur dioxide, olefins, and

hydrogen sulfide to emit light. NO is a relatively unstable molecule which forms NO_2 (especially) on oxidation in the presence of O_3. Photomultiplier tube or solid-state devices have been used to measure the quantity of light produced by the reaction of each NO molecule. Proposed mechanism is as follows:

$$NO + O_3 \rightarrow NO_2 + O_2 + hv$$

Total oxides of nitrogen (NOx) can be measured using chemiluminescence technique by passing the sample over a hot catalyst to reduce all oxides of nitrogen to NO. Just prior to the reaction chamber, this is done within instrument. The resulting signals may be compared indirectly to measure NO_2 in some instruments that can perform the automatic switching of the catalyst in and out of the sample path. Measurements of ammonia (NH_3) are done by variations of chemiluminescence:



$$4NH_3 + 5O_2 \rightarrow 4NO + 6H_2O$$
$$4NO + 4O_3 \rightarrow 4NO_2 + 4O_2 + 4hv$$

The base of CL reactions is the production of light in a flame in which high temperature stimulates the chemical reaction forming the intermediates (Stiles et al. 1994). This methodology has been used to detect the different elements like nitrogen, phosphorous, sulfur, boron, arsenic, antimony, and halogens.

Dunlea et al. (2007) have estimated nitro chemiluminescence (NO_x) in a polluted urban environment. They observed interference peaks in the ambient ozone concentration and found an important issue with CL screening regarding their inability to specifically detect nitrogen dioxide molecule. The CL NO_x monitor interference shows a reasonable association with the measured concentration of ozone given in Fig. 22.4.

22.3.5 Solid Phase Chemiluminescence

Light emission from solids, produced by processes such as electro luminescence, has been widely investigated (Jeon et al. 2015) and has found numerous applications in cathode-ray tube and x-ray screens. However, the number of solids which undergo chemical oxidation reactions leading to chemiluminescence is small. Best example illustrated the solid phase chemiluminescence produced from the oxidation of siloxene. The basic formula is $(Si_6H_6O_3)_n$ and structure is in Fig. 22.5. Oxidation of siloxene with hydrogen peroxide, permanganate, ceric sulfate, chromic acid, nitric acid, or other strong oxidants results in red chemiluminescence (Rauhut 1979). An energy transfer process after oxidation to hydrosiloxane present in the structure is believed to be responsible for the emission. Electron transfer reactions have been reported to produce chemiluminescence in solid phase reactions. Examples include the luminescence from anthracene crystals subjected to an alternating current in complexes of Nd(III), Er(III), and Yb(III) (Lazarides et al. 2007).

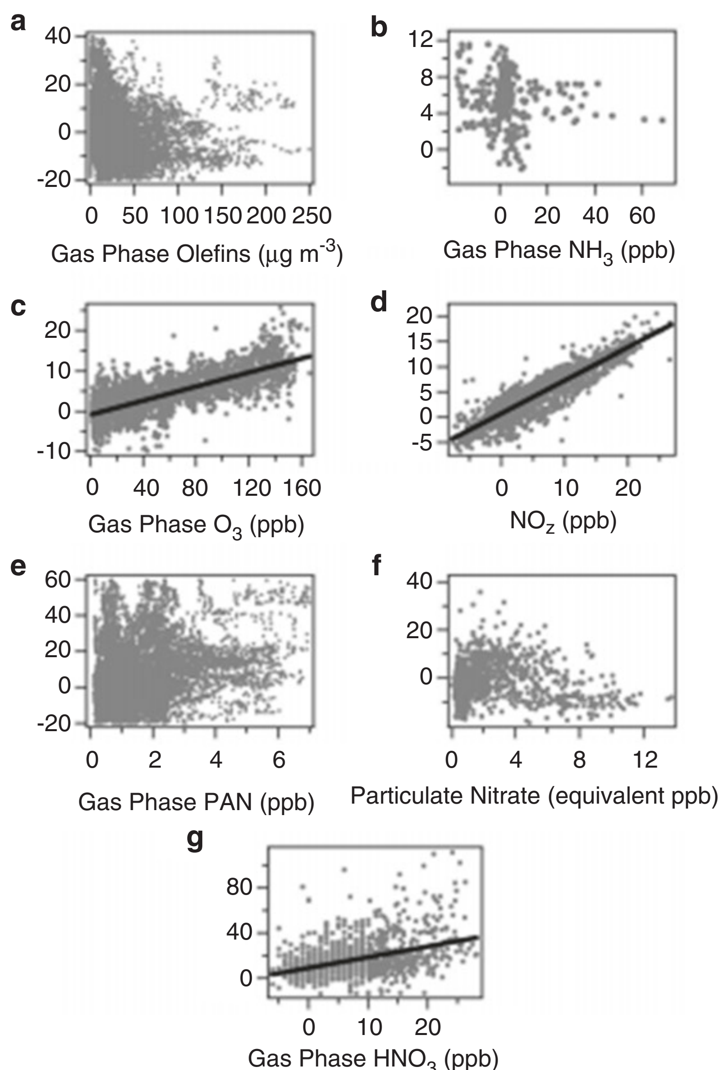


Fig. 22.4 CL NO_x monitor interference linear regression plots. (Adapted from Dunlea et al. 2007)

22.4 Analytical Applications of Chemiluminescence

The CL agents mainly utilized for the investigative purposes are luminol (Khan et al. 2014), hydrogen peroxide, and fluorescein. Luminol chemiluminescence (LCL) is commonly used for the qualitative and quantitative analysis of macromolecules. Biosensors for environmental monitoring and cellular localization in pharmaceutical industry and as biological tracers and several other immunoassays use luminol-based techniques. LCL has a lot of merits due to its selective nature, ease

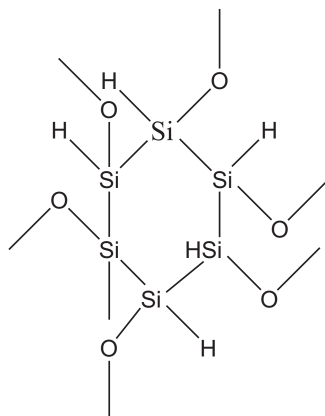


Fig. 22.5 Basic structure of siloxene

of use, being economical, and high sensitivity. Luminol is a crystalline, diprotic with pKa values of 6.74 and 15.1 soluble in polar solvents (Barni et al. 2007). An adverse analytical application of CL has been there including the detection of inorganic and trace metal analysis (Zhang et al. 2007). The toxicity has not been completely explored, although some part of it is described in the cataract, skin, breathing, and gastrointestinal tract (Rose and Waite 2001). Some luminol derivatives have been developed by researchers to maximize its intensity and increase the emission wavelength range in the visible region due to its great application part (Jiao et al. 2011). Many substances are known to stimulate or hinder the chemiluminescence of luminol, and good reviews of their use in analytical chemistry have been presented by Cui et al. (2004). There are a number of compounds which provide stronger inhibition for LCL, whereas benzoic acid and sulfosalicylic acid showed weak signals. Hydroxyl group proves to be an essential element for the CL inhibition, whereas the acidic group proves to be favorable for the CL enhancement shown in Figs. 22.6 and 22.7.

Since a number of metal ions may activate a particular chemiluminescent material under similar conditions, some form of prior treatment is essential to gain a degree of analyte selectivity. This lack of specificity has probably hindered attempts to apply chemiluminescent trace metal analysis in the past. Seitz et al. give the best example of trace metal analysis that applies to biological samples, in which chromium is determined by adding excess ethylenediaminetetraacetic acid (EDTA) to the sample (Seitz et al. 1972).

Over the past few years, chemist has been attracted by the analytical applications of carbon nanostructure chemiluminescence detection (CNS-CL). Recently CNSs revealed to be chemiluminescent (Amjadi et al. 2014; Lin et al. 2011; Bulgakov et al. 2009; Amjadi et al. 2014) can usefully address selectivity and sensitivity requirements. Furthermore CNSs have been used as reagents as well as catalysts which emit light upon direct oxidation, (Amjadi et al. 2014; Wang et al. 2013; Chen et al. 2011), quenchers (He et al. 2013), biomolecule (Wu et al. 2015; Bi et al. 2009), background reducer (Gao and Li 2013), catalyzer support (Zhang and Cui

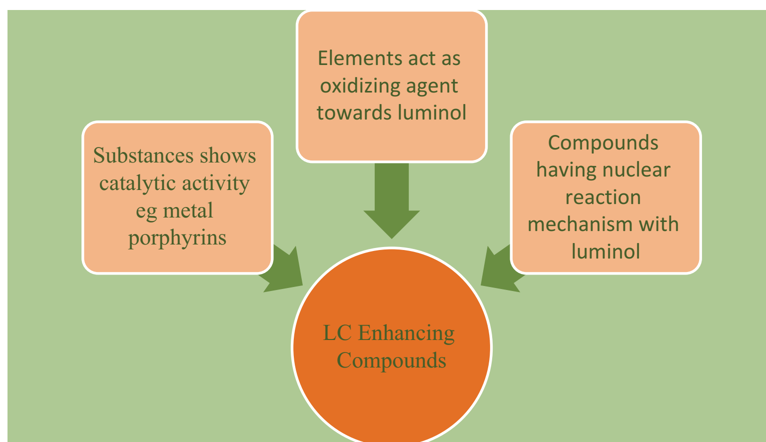


Fig. 22.6 LC enhancing compounds

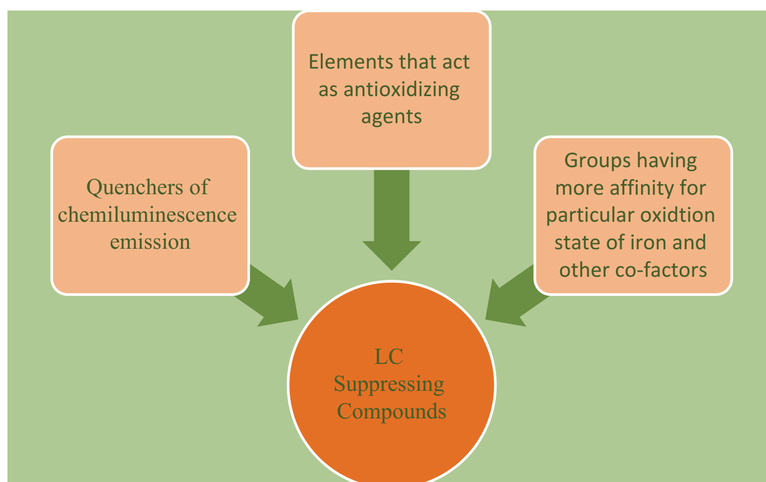


Fig. 22.7 LC suppressing compounds

2014; Safavi et al. 2009), chemiluminescence resonance energy transfer (CRET) (Lee et al. 2012; Gao et al. 2014), and even recognition elements (Huamin et al. 2013; Qiu et al. 2012a, b). Due to the great potential of CNS analytical applications, it has gained the attention of many researchers.

22.5 Graphene Molecularly Imprinted Polymer

The synthetic materials with selective cavities of different form and functional groups of the target analyte form the molecularly imprinted polymers. Magnetic molecularly imprinted polymers (MMIPs) are generally formed by encapsulating a

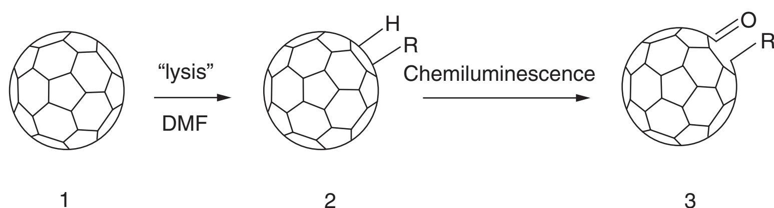


Fig. 22.8 Chemiluminescence of C_{60} fullerene in DMF. (Adapted from Papadopoulos et al. 2001)

magnetic particle with an organic polymer in order to facilitate the separation of MIPs from the reaction solution. These are readily used in crude samples containing a lot of suspended solid materials.

22.5.1 Graphene Oxide (GO)-Catalyzed CL Reactions

The synthesized graphene oxide-catalyzed CL reactions have possessed more catalytic activities with more binding sites than graphene, for example, luminol-H_2O_2 and luminol-O_2 (Hao et al. 2013; Wang et al. 2012; Song et al. 2013; He and Cui 2012; Yang et al. 2014a, b). Wang et al. (2012) have illustrated six times enhancement in CL intensity due to graphene oxide. It is concluded from the spectral analysis that the graphene oxides improved the electron transfer reaction provided with the more yield of oxygen on GO surface.

22.6 Chemiluminescent Reaction of Fullerenes

A radiation dosimetry method was developed using the CL emission of fullerene C_{60}. As a dosimeter, it has described well the relation between CL fullerene intensity and an irradiation dose. Papadopoulos et al. found the stable products with CL reaction of fullerenes with Fenton's reagent (Papadopoulos et al. 2001) (Fig. 22.8).

22.7 Chemiluminescent Reactions for Cancer Detection Therapy

Researchers are currently fascinated in oncology for perfect diagnosis with an efficient tool for timely detection by the methods provided by CL. For monitoring the treatment of cancer, patients have undergone chemotherapy and radiotherapy. CL techniques have been widely applied nowadays. Yao et al. recently reported the determination of anticancer drug mitoxantrone (MTX), having high efficiency in the treatment of breast cancer by the copper-based CL method (Yao et al. 2014). The complex has provided lower level of concentration in CL reaction as compared to schedule emission of CL luminol. Indeed, when 10^{-7} $molL^{-1}$ luminol reacted with

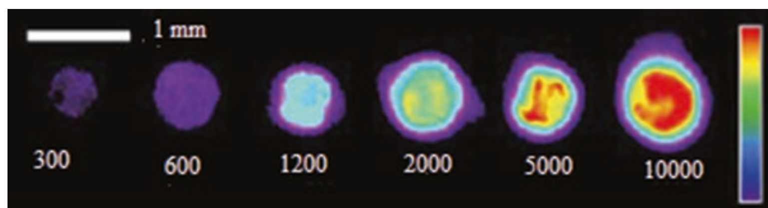


Fig. 22.9 Imaging CL microscopy (Creton and Jaffe 2001)

other oxidants, CL was hardly observed. Probably this fact is the main reason for the reduction in interference of other substances. In modern existence, considerable attention was received by the new oxidant reagents developed for the CL reaction, which are used to extend the application part.

22.7.1 Analytical Technique CL As Detection Technique

22.7.1.1 Imaging Microscopy

To accomplish chemiluminescence and bio imaging microscopy analysis, optical microscope can be easily connected to similar imaging devices for the process of execution. To target the maximum analytical detectability, the light gathering system should be modified as small-sized sample leads to a very weak emission of light (Christenson et al. 2002). Contact of external light can be avoided by enclosing the whole microscope or sample area in a dark box. Specific substrates or reagent solutions are used to accomplish its detection. The localization and quantification of biomolecules in single cell as well as tissue sections is best represented by ultrasensitive analytical figure (Fig. 22.9). Bio- and chemiluminescence can detect inorganic and organic molecules and enzymes by the use of appropriate reagents coupled with enzymatic reactions.

The study of metabolites in living cells and tissues has been done by using chemiluminescence imaging. The methods for the real-time image formation have been developed successfully by exploiting the specific advantages of CL detection such as, for example, high sensitivity and rapidity. The release of nitric oxide is followed by proper stimulation which can be measured in different cell cultures and tissues by the chemiluminescence imaging on the sample of a hydrogen peroxide/luminol solution which undergoes a chemiluminescent reaction (Wiklund et al. 1997).

22.7.1.2 Liquid Chromatography

The widespread applicability of CL reaction is its detective approach in liquid chromatography. As far as the trace analysis is concerned, fluorescence serves as the most efficient detection technique where the elimination of excitation source in CL mode reduces stray light, background emission, and unsteadiness of light source. Analysis of FICs with luminol-H_2O_2 system via post column method for the detection of CL has been developed by Ariga et al. for high-performance liquid

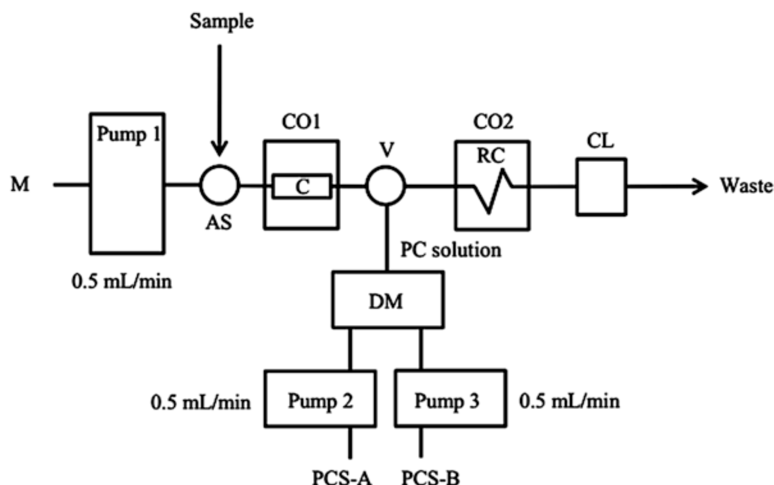


Fig. 22.10 Scheme of HPLC system. (Adapted from Ariga et al. 2016)

chromatography (HPLC). Delivery of the solutions has been done separately using two pump systems; thereafter thorough mixing of luminol with H_2O_2 was feasible in post column reaction (Ariga et al. 2016). Figure 22.10 shows the analytical approach of HPLC can be employed for selective detection of FIC due to its capacity to bind Fe(III), irrespective of its structural feature. The limits of detection were quite low.

Yong Xie et al. have developed an online analysis method by HPLC-DAD coupled with chemiluminescence (CL) for simultaneous detection and identification of antioxidants in three natural plants of traditional Chinese medicine “she gan” (Xie et al. 2014).

22.7.1.3 Capillary Electrophoresis

Chemiluminescence detection with capillary electrophoresis mode as a prior separation analytical technique has started is being explored. Research area in this field serves as a strong analytical device to resolve and quantify biomedical analytes. CL reactions are employed for postcapillary detection of various groups like luminol, firefly, acridinium esters, luciferase peroxyoxalates, acidic potassium permanganate, etc. and speed up the CL reaction after the separation of analytes in electrophoretic capillary. Application of different interfaces is used to set up the channel depending upon the type of analytes targeted with the combination of CL reagents in Fig. 22.11.

Su et al. have designed the microchip based on the principle of flow injection chemiluminescence system and capillary electrophoresis (Su et al. 2004). It has three main channels, five reservoirs, and a detection cell. By using permanganate chemiluminescent system, dopamine and catechol were separated and detected on the prepared microchip. Acridinium esters can also be successfully employed in CE as CL detection system. CE analysis of amino acids, peptides, and proteins can be achieved by their derivatization with acridinium esters. Cao et al. characterized a

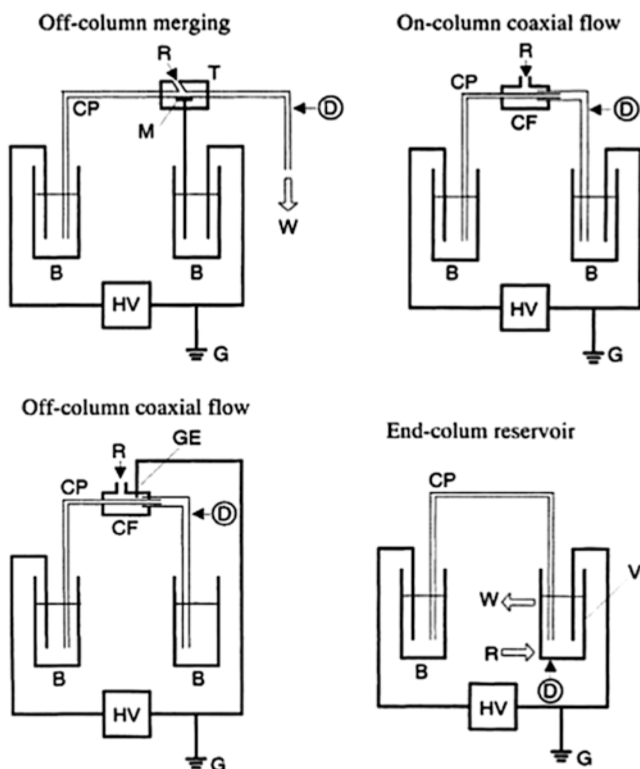


Fig. 22.11 Devices using capillary electrophoresis with chemiluminescence with different interfaces. (Adapted from Cao et al. 2002)

new end-column electro-chemiluminescence (ECL) detection technique coupling to capillary electrophoresis (Cao et al. 2002). Exclusive to the use of decoupler, a platinum electrode was used to directly connect to an inner diameter of capillary. During the optimization of various detection parameters, the distance between the capillary and an electrode was an important one for the determination of actual concentration of $Ru(bpy)_3^{2+}$ in the suitable detection region.

22.7.1.4 Gas Chromatography

A lot of chemiluminescence detectors have been developed along with the gas chromatography detection technique. The various detectors involve the flame photometric detector (FPD), sulfur chemiluminescence detector (SCD), thermal energy analysis (TEA) detector for reaction analysis of nitrosamines, and redox chemiluminescence detector (RCD) for the specific detection of compounds containing heteroatoms. FCLD for S, Se, Te, and P compounds are promisingly accepted (Ramírez et al. 2015). Figure 22.12 captured a conventional device for the detection of CL in gas chromatography.

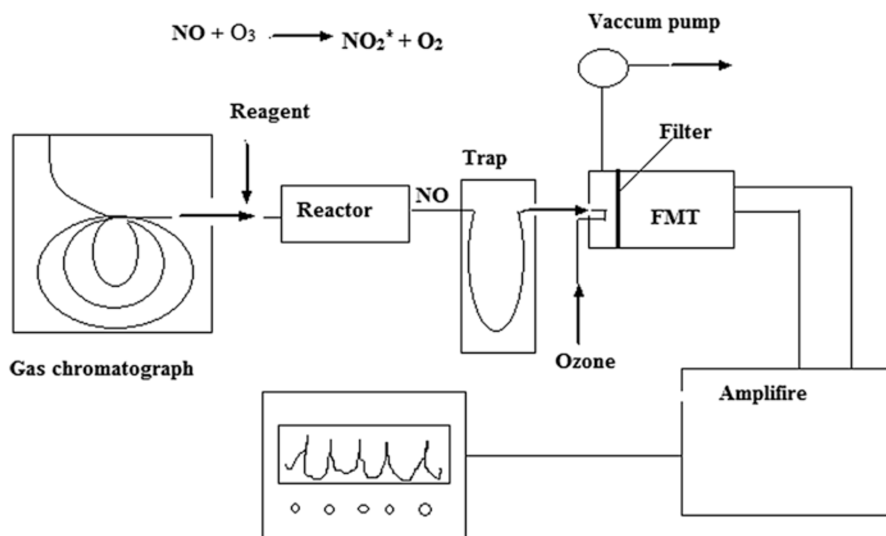


Fig. 22.12 Schematic diagram for CL detection in gas chromatography

The methods of flowing stream involve the mixing of chemiluminescent reagent along with analyte stream to carry out the analysis of CL emission after its incorporation. The examination of CL reactions in liquid phase seems to be simple for FIA because of its feasibility, robustness, precision, and quick response (García-Campaña and Baeyens 2000). The injection of a sample along with CL reagent into the flowing stream is in close proximity to detector emission that occurs in a cell placed in front of the detector. Various experimental variables like temperature, pH, flow rates, dimension of mixing, and detector coils needed to be optimized for increasing the sensitivity of detection. There are widespread applications in quality control in pharmaceutical analysis using flow injection method. The emergence of the immobilization technique has provided a preface of enzymatic reactors, which can be positioned before the CL reaction takes place. The substrate and one of the products of enzymatic reaction are actively involved in CL reaction. The substrates detected by this method are amino acids, glucose, choline, aldehydes, cholesterol, and lactate that generate peroxide during a movement through a selective column reactor with immobilized oxidase enzyme in the presence of oxidant, oxygen, present in the samples. For the post column determination of peroxide, luminol is used along with peroxide catalyst. The determination of glucose by CL emission which is produced when hydrogen peroxide is formed by the reaction of luminol with immobilized glucose oxidase in the presence of potassium hexacyanoferrate is shown in Fig. 22.13.

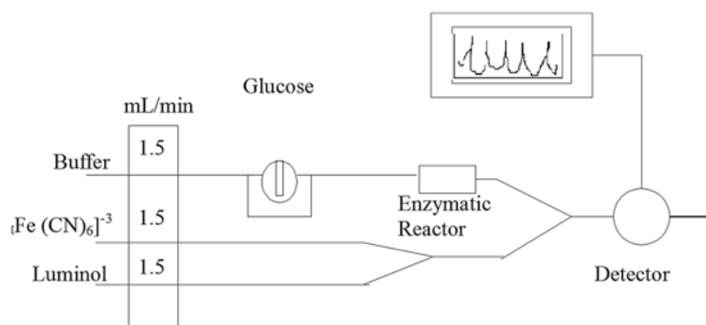


Fig. 22.13 Scheme of FIA. (Adapted from García-Campaña and Baeyens 2000)

22.8 Conclusion

Chemiluminescent reactions occur in solid, liquid, and gas phase and involve a diversity of organic and inorganic molecules. The analytical potential of most chemiluminescent reactions is unexplored, and only liquid phase reactions have found widespread application. In the last decade, recent advancement has been achieved with respect to the CL reagents as labels to derivatize and selectively find out the analytes. A variety of analytical applications have been found in CL reactions in various scientific fields such as pharmaceutical, environmental, and food analysis and in clinical laboratories for diagnosis of disease, prognosis, and monitoring of patient treatments. The widespread applications of CL techniques can be attributed to their sensitivity, simplicity, cost-effectiveness, and wide linear range.

References

Ahlberg E, Hammerich O, Parker VD (1981) Electro-transfer reactions accompanied by large structural changes 1. Lucigenin-10,10-dimethyl-9,9 –biacridylidene redox system. J Am Chem Soc 103:844–849

Amjadi M, Manzoori JL, Hallaj T (2014) Chemiluminescence of graphene quantum dots and its application to the determination of uric acid. J Lumin 153:73–78

Ariga T, Imura Y, Suzuki M, Yoshimura E (2016) Determination of ferric iron chelators by high-performance liquid chromatography using luminol chemiluminescence detection. J Chromatogr B 1014:75–82

Barni F, Lewis SW, Berti A, Miskelly GM, Lago G (2007) Forensic application of the luminol reaction as a presumptive test for latent blood detection. Talanta 72:896–913

Bi S, Zhou H, Zhang SS (2009) Multilayers enzyme-coated carbon nanotubes as biolabel for ultrasensitive chemiluminescence immunoassay of cancer biomarker. Biosens Bioelectron 24:2961–2966

Bulgakov R, Galimov D, Kinzyabaeva Z (2009) Chemiluminescence produced by oxidation of fullerene hydride $C_{60}H_{36}$ with oxygen in solution. Russ Chem Bull 58:857–858

Cao W, Liu J, Yang X, Wang E (2002) New technique for capillary electrophoresis directly coupled with end-column electrochemiluminescence detection. Electrophoresis 23:3683–3691

Chen H, Lin L, Lin Z, Lu C, Guo G, Lin JM (2011) Flow-injection analysis of hydrogen peroxide based on carbon nanospheres catalyzed hydrogen carbonate-hydrogen peroxide chemiluminescent reaction. Analyst 136:1957–1964

Christenson MA, Dyke KV, Dyke CV, Woodfork K (2002) Luminescence biotechnology: instruments and applications. CRC Press, Boca Raton, p 469

Creton R, Jaffe LF (2001) Chemiluminescence microscopy as a tool in biomedical research. BioTechniques 31:1098–1105

Cui H, Shi MJ, Meng R, Zhou J, Lai CZ, Lin XQ (2004) Effect of pH on inhibition and enhancement of luminol-H_2O_2-Co^{2+} chemiluminescence by phenolic compounds and amino acids. J Photochem Photobiol 79:233–241

Cui H, Zhang H, Shi MJ, Wang W, Dong YP, Guo JZ (2007) Electrogenerated chemiluminescence of lucigenin in ethanol solution at a polycrystalline gold electrode. Electroanalysis 19:1703–1710

Dong YP, Zhoua Y, Wang J, Zhu JJ (2016) Electrogenerated chemiluminescence resonance energy transfer between lucigenin and CdSe quantum dots in the presence of bromide and its sensing application. Sensors Actuators B Chem 226:444–449

Dunlea EJ, Herndon SC, Nelson DD, Volkamer RM, Martini FS, Sheehy PM, Zahniser MS, Shorter JH, Wormhoudt JC, Lamb BK, Allwine EJ, Gaffney JS, Marley NA, Grutter M, Marquez C, Blanco S, Cardenas B, Retama A, Ramos Villegas CR, Kolb CE, Molina LT, Molina MJ (2007) Evaluation of nitrogen dioxide chemiluminescence monitors in a polluted urban environment. Atmos Chem Phys 7:2691–2704

Fraga H, Fernandes D, Novotny J, Fontes R, Esteves da Silva JC (2006) Firefly luciferase produces hydrogen peroxide as a coproduct in dehydroluciferyl adenylate formation. Chem Biochem 7:929–935

Gao Y, Li B (2013) G-quadruplex DNAzyme-based chemiluminescence biosensing strategy for ultrasensitive DNA detection: combination of exonuclease III-assisted signal amplification and carbon nanotubes-assisted background reducing. Anal Chem 85:11494–11500

Gao H, Wang W, Wang Z, Han J, Fu Z (2014) Amorphous carbon nanoparticle used as novel resonance energy transfer acceptor for chemiluminescent immunoassay of transferrin. Anal Chim Acta 819:102–107

García-Campaña AM, Baeyens WRG (2000) Principles and recent analytical applications of chemiluminescence. Analusis 28:686–698

Gleu K, Petsch W (1935) Die Chemiluminescenz der Dimethyl-diacridyliumsalze. Angew Chem 48:57–59

Hao M, Liu N, Ma Z (2013) A new luminol chemiluminescence sensor for glucose based on pH-dependent graphene oxide. Analyst 138:4393–4397

He Y, Cui H (2012) Synthesis of highly chemiluminescent graphene oxide/silver nanoparticle nano-composites and their analytical applications. J Mater Chem 22:9086–9091

He Y, Huang G, Cui H (2013) Quenching the chemiluminescence of acridinium ester by graphene oxide for label-free and homogeneous DNA detection. ACS Appl Mater 5:11336–11340

Huamin Q, Lulu F, Li X, Li L, Min S, Chuannan L (2013) Determination sulfamethoxazole based chemiluminescence and chitosan/graphene oxidemolecularly imprinted polymers. Carbohydr Polym 92:394–399

Jeon YI, Bharat LK, Yu JS (2015) White-light emission of $Ca_2La_8(GeO_4)_6O_2$: Tb_3þ/Sm_3þ nanocrystalline phosphors for solid-state lighting applications. J Lumin 166:93–100

Jiao TF, Xing YY, Zhou JX (2011) Synthesis and characterization of functional cholesteryl substituted Luminol derivative. Mater Sci Forum 694:565–569

Khan P, Idrees D, Moxley MA, Corbett JA, Ahmad F, Figura GV, Sly WS, Waheed A, Hassan MI (2014) Luminol-based chemiluminescent signals: clinical and non-clinical application and future uses. Appl Biochem Biotechnol 173:333–355

Lazarides T, Alamiry MAH, Adams H, Pope SJA, Faulkner S, Weinstein JA, Ward MD (2007) Anthracene as a sensitiser for near-infrared luminescence in complexes of Nd(III), Er(III) and Yb(III): an unexpected sensitisation mechanism based on electron transfer. Dalton Trans 15:1484–1491

Lee JS, Joung HA, Kim MG, Park CB (2012) Graphene-based chemiluminescence resonance energy transfer for homogeneous immunoassay. ACS Nano 6:2978–2983

Lin Z, Xue W, Chen H, Lin JM (2011) Peroxynitrous-acid-induced chemiluminescence of fluorescent carbon dots for nitrite sensing. Anal Chem 83:8245–8251

Papadopoulos K, Triantis T, Boyatzis S, Dimotikali D, Nikokavouras J (2001) Photo- radio- and sono storage chemiluminescence of buckminsterfullerene C-60. J Photochem Photobiol A 143:93–97

Qiu H, Luo C, Sun M, Lu F, Fan L, Li X (2012a) A chemiluminescence array sensor based on graphene-magnetite-molecularly imprinted polymers for determination of benzenediol isomers. Anal Chim Acta 744:75–81

Qiu H, Luo C, Sun M, Lu F, Fan L, Li X (2012b) A chemiluminescence sensor for determination of epinephrine using graphene oxide-magnetite-molecularly imprinted polymers. Carbon 50:4052–4060

Ramírez N, Vallecillos L, Lewis AC, Borrull F, Marcéc RM, Hamiltona JF (2015) Comparative study of comprehensive gas chromatography-nitrogen chemiluminescence detection and gas chromatography-iontrap-tandem mass spectrometry for determining nicotine and carcinogen organic nitrogen compounds in thirdhand tobacco smoke. J Chromatogr A 1426:191–200

Rauhut MM (1979) Chemiluminescence In kirk-othmer encycl Chem Technol 5: 416–450

Rose AL, Waite TD (2001) Chemiluminescence of luminol in the presence of iron(II) and oxygen: oxidation mechanism and implications for its analytical use. Anal Chem 73:5909–5920

Safavi A, Maleki N, Doroodmand MM, Koleini MM (2009) Carbon nanostructures as catalytic support for chemiluminescence of sulfur compounds in a molecular emission cavity analysis system. Anal Chim Acta 644:61–67

Seitz WR, Suydam WW, Hercules DM (1972) Determination of trace amounts of chromium(III) using chemiluminescence analysis. Anal Chem 44:957–963

Song Y, Yang T, Zhang T, Jin N, Zhao Y, Fan A (2013) Ultrasensitive chemiluminescent immunoassay labeled with graphene oxide. Anal Methods 5:3646–3649

Stiles DA, Calokerinos AC, Townshend A (1994) Flame chemiluminescence analysis by molecular emission cavity detection. Wiley, New York

Su R, Lin JM, Qu F, Chen Z, Gao Y, Yamada M (2004) Capillary electrophoresis microchip coupled with on-line chemiluminescence detection. Anal Chim Acta 508:11–15

Sun YG, Cui H, Lin XQ (2001) Study of electrochemiluminescence of lucigenin at glassy carbon electrodes in NaOH solution. J Lumin 92:205–211

Veazey RL, Nekimken H, Nieman TA (1984) Chemiluminescent reaction of lucigenin with reducing sugars. Talanta 31:603–606

Wang DM, Zhang Y, Zheng LL, Yang XX, Wang Y, Huang CZ (2012) Singlet oxygen involved Luminol Chemiluminescence catalyzed by graphene oxide. J Phys Chem 116:21622–21628

Wang DM, Gao MX, Gao PF, Yang H, Huang CZ (2013) Carbon nanodots-catalyzed chemiluminescence of luminol: a singlet oxygen-induced mechanism. J Phys Chem C 117:19219–19225

Watanabe N, Oguri A, Horikoshi M, Takatsuka H, Ijuin HK, Matsumoto M (2014) Chemiexcitation efficiency for the charge-transfer-induced chemiluminescent decomposition of 3-hydroxyphenyl-substituted dioxetanes in an aqueous system. Tetrahedron Lett 55:1644–1647

Wiklund NP, Cellak S, Leone AM, Iversen HH, Gustafsson LE, Brundin L, Furst VW, Flock A, Moncada S (1997) Visualisation of nitric oxide released by nerve stimulation. J Neurosci Res 47:224–232

Wu Y, Nie F, Xia D (2015) Chemiluminescence assay for the glycoprotein tenascin-C based on aptamer-modified carboxylated magnetic carbon nanoparticles. Microchim Acta 182:227–232

Xie GY, Zhu Y, Shu P, Qin XY, Wu G, Wang Q, Qin MJ (2014) Phenolic metabolite profiles and antioxidants assay of three Iridaceae medicinal plants for traditional Chinese medicine "She-gan" by on-line HPLC–DAD coupled with chemiluminescence (CL) and ESI-Q-TOF-MS/MS. J Pharm Biomed Anal 98:40–51

Yang L, Zhang R, Liu B, Wang J, Wang S, Han MY, Zhang Z (2014a) p-Conjugated carbon radicals at graphene oxide to initiate Ultrastrong chemiluminescence. Angew Chem 53:10109–10113
Yang Z, Zhu J, Dai H, Li J, Shen J, Jiao X, Hu X, Ju H (2014b) Graphene oxide based ultrasensitive flow-through chemiluminescent immunoassay for sub-picogram level detection of chicken interferon-γ. Biosens Bioelectron 51:356–361
Yao H, Zhang M, Zeng W, Zeng X, Zhang Z (2014) A novel chemiluminescence assay of mitoxantrone based on diperiodatocuprate (III) oxidation. Spectrochim Acta A Mol Biomol Spectrosc 117:645–650
Zhang H, Cui H (2014) High-density assembly of chemiluminescence functionalized gold nanodots on multiwalled carbon nanotubes and their application as biosensing platforms. Nanoscale 6:2563–2566
Zhang C, Qi H, Zhang M (2007) Homogeneous electrogenerated chemiluminescence immunoassay for the determination of digoxin employing Ru(bpy)2(dcbpy)NHS and carrier protein. Luminescence 22:53–59