CHAPTER 1

Introduction: Cancer & Therapeutics

1.1 Cancer

Cancer is a collective term encompassing a wide range of diseases wherein abnormal cells undergo uncontrolled division and possess the ability to infiltrate and damage healthy body tissues. Neoplasm and malignant tumor are alternative terms used to describe cancer. Its capacity to spread throughout the body makes cancer a significant health concern [1]. Cancer continues to have a significant global impact as the second biggest cause of mortality.

1.1a Types of cancer

Tumors can be classified based on their location of occurrence, such as breast, brain, prostate, lung, ovary, and more. But within these tumor categories, other subtypes can be distinguished by variations in the molecular profile, morphology, or expression of specific markers. This phenomenon is referred to as intertumoral heterogeneity.

1.1b Epidemiology

According to International Agency for Research on Cancer (IARC) GLOBOCAN 2018 data from 185 countries exposed 2.3 million new cases of breast cancer (11.7%) and a 6.9 % death rate [2]. Cancer is a leading global cause of death, responsible for nearly 10 million deaths in 2020 [3]. The WHO (World Health Organization) estimates that cancer accounts for 13% of all deaths worldwide, with 8.2 million occurring annually [4]. Based on the 2020 Globocan data, breast cancer accounted for 10.6% of all cancer-related deaths

and 13.5% of all cancer cases in India. In India, breast cancer patients face lower survival rates than in Western nations due to early onset at a younger age, advanced stage at diagnosis, delays in initiating effective treatment, and inadequate healthcare services. Metastatic breast cancer exhibits a five-year survival rate of less than 30%, representing approximately 2.3 million new cases, accounting for 11.7% of all cancer cases. Breast cancer has now exceeded the most common type of cancer globally in 2021 [5]. The prevalence of cancer varies across developed and developing countries, as well as between genders. However, the incidence of five cancers, including lung, breast, colorectal, prostate, and stomach cancer, is the highest globally. (Figure 1.1) [6].

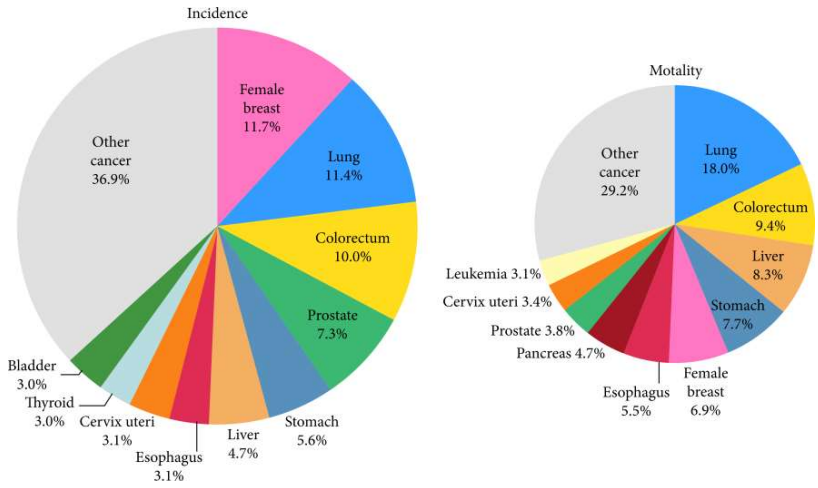

Figure 1.1: *Worldwide cancer incidence and mortality rate [6].*

1.2 Breast cancer

Breast cancer (BC) is a significant health concern among women due to its substantial impact on mortality and morbidity rates. Moreover, the effective management of breast cancer poses a significant challenge in healthcare [7]. Due to this, more than 14 million new cancer cases are reported yearly, with more than 600,000 deaths yearly [8]. Among

women in the US, approximately 12% are identified with BC, whereas 20-30% of cases are diagnosed as Triple-Negative Breast Cancer (TNBC) [9]. Notably, women with TNBC experience lower five-year survival rates (77%) compared to other types of breast cancers (93%). TNBC is more aggressive among all subtypes, spreading quickly to other organs and exhibiting recurrence despite intensive treatments (Figure 1.2). The name "triple-negative" is called due to the absence of growth hormone receptors, i.e., Human Epidermal Growth Factor Receptor-2 (HER-2), Estrogen (ER), and Progesterone (PR) receptors [10], typically found in breast cancer cells that play a crucial role in regulating cell growth ER is a protein present within cells of female reproductive tissue, other specific tissue, and cancer cells. Upon binding to the receptors within cells, the hormone estrogen has the potential to stimulate cell growth. Progesterone Receptor (PR) is another protein found in cells of female reproductive tissue, specific other tissues, and cancer cells. Progesterone binds to these receptors within cells and can induce cell growth. HER2 is a protein in average cell growth. In some. cancer cells, such as breast, ovarian, bladder, stomach, and esophageal cancers, HER-2 may be produced in larger-than-normal quantities. This overexpression can accelerate cancer cell growth and increase the chance of spreading to other body parts. Assessing the level of HER-2 on specific cancer cells can assist in treatment planning. HER-2 is called c-erbB-2, HER-2/neu, human EGF receptor 2, and HER-2 [11]. Additionally, TNBC is notorious for its high metastatic potential and increased possibility of recurrence, even following rigorous treatment protocols. However, the absence of these receptors in TNBC makes it challenging to target delivery, rendering conventional chemotherapy approaches and targeted drug delivery problematic, ultimately leading to poor therapeutic efficacy. Other treatments, such as local breast surgeries and mastectomy, also become complex and have high chances of tumor and local regional recurrence of tumor results. Breast cancer can `be

classified into two broad classes based on histopathological and molecular subtypes, further organized into five subcategories [12] (Figure 1.3).

1.2.1 Histological subtype of cancer

1.2.1a Pre-invasive (in-situ) breast carcinoma

Pre-invasive carcinoma is localized cancer confined to the primary site without spreading. This type of cancer is further classified into ductal carcinoma (DCIS) and lobular carcinoma (LCIS). In this condition, breast cancer cells are confined to the inner side layer of the milk ducts and prevented from invading the surrounding breast tissue or other body parts.

1.2.1b Invasive breast carcinoma

Invasive carcinomas encompass diverse tumors classified into histological subtypes, including invasive ductal (IDC) and invasive lobular, papillary tubular, medullary, and mucinous carcinomas. Among these, IDC is the most common, approximately 70-80 %. IDC originates in the milk ducts and extends beyond the ducts into the surrounding fatty tissue of the breast.

1.2.2 Molecular subtypes

Breast-cancer categorization based on molecular components holds more excellent value than histology. This classification involves analyzing genetic information derived from cancerous tissue. It has identified mainly 5 subtypes: luminal A, luminal B, triple-negative, HER-2-enriched, and normal-like. These subtypes provide crucial insights for implementing tailored treatment strategies and advancing research in targeted therapies.

1.2.2a Luminal A

This kind of breast cancer is distinguished by positive ER, PR receptor, and HER-2 negative. Furthermore, it has a modest expression of Ki-67 protein. Slow development with a lower grade of the tumor.

1.2.2b Luminal B

This particular subtype of breast cancer is known for being hormone-receptor-positive. However, it can be either HER-2 positive or HER2 negative, and Ki-67 protein expression is higher. Tumors grow at a faster rate and have a low-grade tumor, and have a generally worse prognosis than luminal A tumors.

1.2.2c Triple-negative breast cancer (TNBC)

TNBC is distinguished by the absence of ER, PR, and HER-2 receptors. It is usually recognized as the most aggressive kind of breast cancer.

1.2.2d Human epidermal growth factor receptor-2 enriched

This specific type of breast cancer is characterized by ER and PR negative but HER-2-positive. Tumors within this type exhibit a fast growth rate compared to the luminal A and B subtypes.

1.2.2e Normal-like

This particular subtype of breast cancer is ER and PR positive, whereas HER-2 is negative and displays less Ki-67. Overall, the prognosis for this subtype is generally favorable, although slightly less favorable than the luminal A subtype.

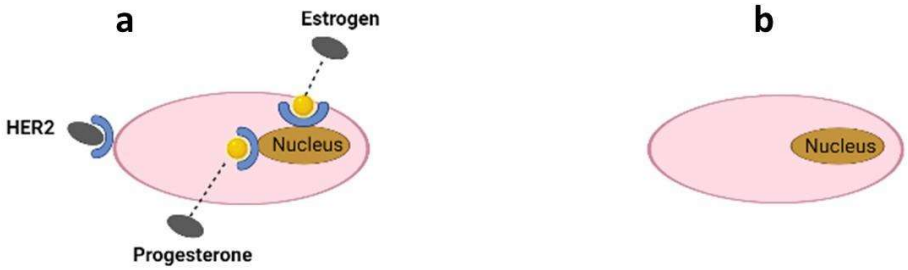

Figure 1.2: *a) Image represent the growth of hormonal receptor on the cell surface that express estrogen receptor (ER), progesterone receptor (PR), and, Human epidermal growth factor receptor 2 (HER-2), whereas b) Image indicates the absence of hormonal receptor on the cell surface (triple negative breast cancer).*

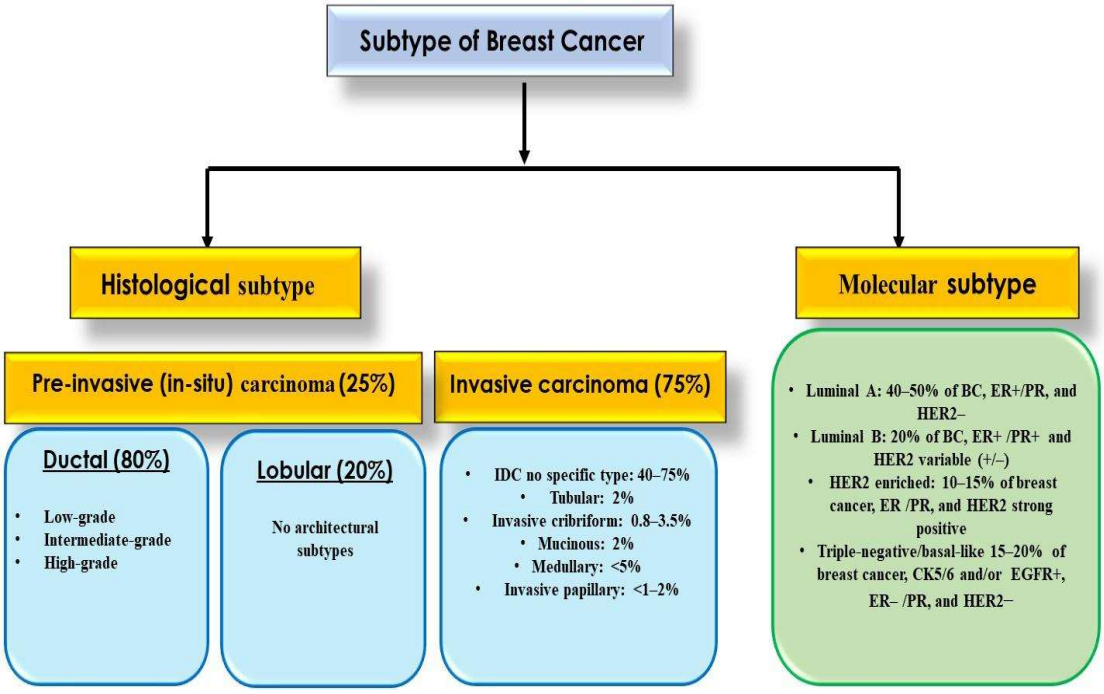

Figure 1.3: *Classification of Breast cancer. IDC: invasive ductal carcinoma.*

1.3 Common treatments for breast cancer

Most types of breast cancer are treated using surgery, radiotherapy, and chemotherapy. Breast cancer treatment is complex and constantly evolving, with many ongoing clinical trials on emerging therapies. Despite significant progress in the field, not all patients respond well to the treatment, so moving beyond these typical approaches is crucial. Next-generation nano-formulated platinum-based drug delivery has recently been clinically used; for example, Taxanes, anthracyclines (alone or in combination), and platinum-based chemotherapies are commonly used in breast cancer [13]. A few examples of chemotherapeutic drugs available in the market for cancer treatment include; Paclitaxel (Taxol), Doxorubicin, Cyclophosphamide, Capecitabine (Xeloda), Carboplatin, Fluorouracil (5-FU), Methotrexate, etc. However, these therapeutics have shown limited efficacy, leading to poor treatment responses and undesirable side effects [14,15], such as hair loss, fatigue, nausea, loss of appetite, vomiting, pain, weight gain, and diarrhea. So, new formulations other than those already commercially available must be developed.

1.4 Methotrexate (MTX)

Methotrexate belongs to a class IV molecule in Biopharmaceutical Classification System (BCS) [14]. The chemical structure of MTX consists of P-aminobenzoic acid, glutamic acid, and a pteridine ring (Figure 1.4). It is an antineoplastic and folic acid antagonist negatively charged drug with a molecular weight of 454.5 g/mol ($C_{20}H_{22}N_8O_5$) [15]. MTX has excellent potential to treat cancer, but its use is limited due to [16] less solubility (0.01 mg/mL), which gives rise to less permeable (Clog P = 0.53), rapid clearance (2-10 hours), and poor bioavailability. Dihydrofolate reductase is competitively

inhibited by methotrexate, which blocks the sites where folate binds to the enzyme [17]. MTX's capacity to block various enzymes involved in the pathways for synthesizing folate and de-novo nucleotides. Due to this, dihydrofolate (DHFA) conversion to tetrahydrofolate (THFA) production is inhibited, suppressing purine & pyrimidine synthesis. The synthesis of DNA and RNA, which are necessary for the proliferation of cancer cells, is prevented by this mechanism. [18]. Methotrexate has been shown to have remarkable anticancer activities in cells that overexpress folate receptors [19], indicating that it has significant anticancer potential in breast cancer [20] (Figure 1.5). This ability will influence MTX's anti-inflammatory [21] and antiproliferative actions, reflected in other pathways like methionine and adenosine [22]. Therefore, frequently used for the treatment of various malignancies, including leukemia, malignant lymphoma, breast cancer, neck & head cancer, osteosarcoma, and pediatric acute lymphocytic leukemia [23]. MTX also suffers from high non-specificity [24].

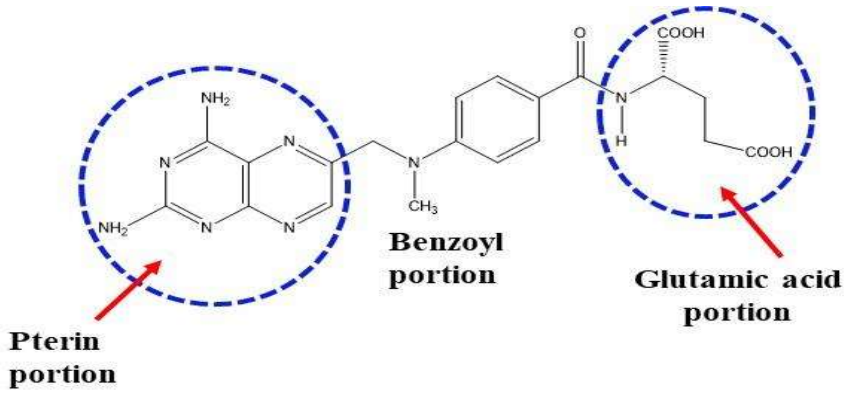

Figure 1.4: *Chemical Structure of Methotrexate*

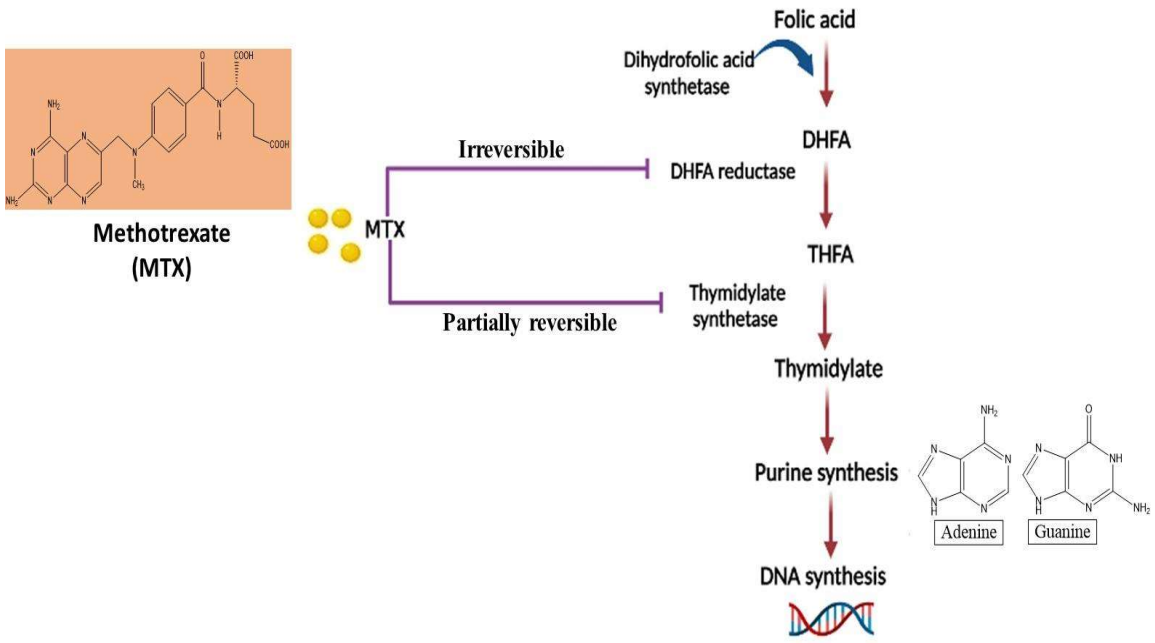

Figure: 1.5 *Mechanism of action of Methotrexate*

Despite its effectiveness in cancer treatment, methotrexate (MTX) is associated with harmful side effects, including neurotoxicity and toxicity to healthy cells, due to its non-specific drug delivery. Overcoming these adverse effects and enhancing therapeutic efficacy presents a considerable challenge [23].

1.5 Challenges in cancer treatment

Among the above treatments, chemotherapy is the most popular one. However, the drugs used are toxic and can have undesirable side effects. Due to the following factors,

- Numerous highly effective drugs have limited water solubility, restricting their clinical applications.

- Chemotherapeutic medications usually lack specificity, which decreases their ability to target cancer cells and raises the possibility of harming healthy cells.
- Following intravenous administration, most drugs exhibit inappropriate biodistribution, reducing therapeutic efficacy and side effects [25,26].

Challenges can be overcome by flowing strategies such as;

- Nanoparticles enhanced the drugs' solubility and stability [27].
- Targeted drug delivery system.
- Combination therapy (two or more drugs together) may overcome the problem of drug resistance.
- The polymeric nanocarrier has controlled drug release and reduced undesirable side effects.
- Stimulus-responsive nano-formulation (heat, pH, temperature, etc.) [28].

1.6 Nanoparticles

Nanoparticles as drug delivery systems have received much attention recently, especially for cancer treatment. It represents a new field of science with extraordinary potential to treat various human diseases. Nanoparticles have been synthesized using a top-down or bottom-up approach [29]. Due to their very small size (10-100 nm), they remain suspended in the solution, and such systems are known to exhibit colloidal behavior.

Figure 1.6: *Limitation of the chemotherapeutic drug.*

Due to their straightforward synthetic route, nanoparticle surfaces can be functionalized with various hydrophilic or phobic ligands. Besides, with slight variations in the synthesis protocol, several different drugs can be loaded and released with changes in pH, temperature or when in contact with a hydrophilic or hydrophobic atmosphere. Bottoms-up approaches have made significant advancements, making it possible to create nanoparticles with desired properties by following simple synthesis processes. Such nanoparticles can be modified to [30] improve the pharmacokinetics behavior of the drug. Small nanoparticle sizes enable the targeted delivery of hydrophobic anticancer drugs to specific locations within the body, reducing opsonization by the immune system.

This facilitates effective drug delivery while minimizing interference from the immune response [31]. The field of nanomedicine offers improvements in the stability, solubility, drug half-life, bioavailability, and area under the curve (AUC) of medications [26]. Moreover, nanomedicine enables targeted drug delivery, enhances drug accumulation at tumor sites through enhanced permeability and retention (EPR), and prolongs circulation time by mitigating rapid renal clearance and interaction with the reticuloendothelial system (RES). Several nanocarriers have been established and categorized into the following classes: hybrid, inorganic, and organic. The most common type used widely is organic nanocarriers. For example, polymeric nanoparticles, micelles, and liposomes have been popularly used. In contrast, inorganic nanocarriers such as; carbon nanobots, silica mesoporous, gold nanoparticles, quantum dots, and magnetic nanoparticles have been employed in several cases (Figure 1.7). Polymeric nanoparticles are extensively studied as therapeutic carriers due to their biocompatibility, biodegradability, and versatile design capabilities. Unlike liposomes, polymeric nanoparticles offer enhanced stability and a wide range of polymer options to suit specific drugs and applications. They are composed of biodegradable polymers such as aliphatic polyesters, polypeptides, PEG, HA, and dextran and have been approved for medical and pharmaceutical use due to their excellent safety profile.

Utilizing a nanoparticle-based drug delivery approach offers a potential solution to mitigate the harmful side effects of methotrexate (MTX). Leveraging the small size of nanoparticles, this approach enables the controlled release of the drug at a specific target site, promoting prolonged therapeutic activity while addressing the limitations of the drug. Chitosan has been more extensively used as a carrier among polymers due to its biocompatible nature.

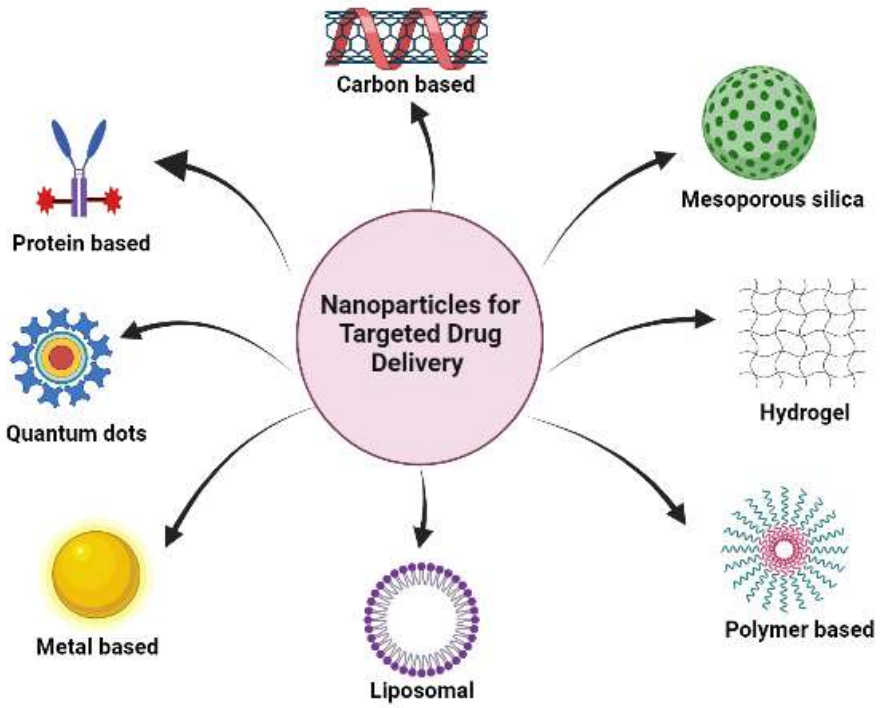

Figure 1.7: *Targeted drug delivery system used for breast cancer treatment.*

1.6.1 Polymer-based nanocarriers

Polymeric nanocarriers (PNCs) have been reliably used as drug delivery systems. Additionally, its surface is easily modified with the cancer-specific recognition components for drug delivery at the target site. Despite versatility, only a few polymers have been used as drug delivery systems. Among these proposed polymers, only a few of them are clinically approved. For example, Adagen, Genexol PM, Eligard, and Copaxone [32] have been widely employed and clinically approved.

1.7 Chitosan

Chitosan (CS) was first time discovered and discussed by Rouget in 1859 [33]. Chitosan is a mucopolysaccharide most plenty found naturally [34] with a high drug-loading ability [35]. It is produced by the deacetylation of chitin (exoskeleton of crab, shrimp, and fungal cell wall, etc.) [36–38]. Chitin is a linear homopolymer of poly-[→4)-N-Acetyl-D-glucosamine-(1→] [39][40] unit. Chitosan has units of N-acetyl-D-glucosamine and β-(1-4) linked D-glucosamine that are dispersed in the chain [41,42]. The degree of deacetylation affects the solubility [43], hydrophobicity, and capacity to interact electrostatically with polyanions. Reactive functional groups in the chitosan polymeric chain offer an extreme prospect for chemical alteration [44]. Chitosan shows Pka value in the amine group is about 6.5 [45]. Chitosan contains abundant amino (–NH2) groups, making it a promising candidate for surface modification. One of the remarkable characteristics of CS is its pH sensitivity. The protonation of the –NH2 groups causes CS to dissolve at pH values below 6.5 (the dissociation constant, pKa, of CS) [46]. At pH < 6 chitosan amine is protonated and pH > 6.5 chitosan amine are deprotonated and reactive [47]. Chitosan links interpolymeric, leading to the formation of fiber and network. Additionally, its -NH2 groups are responsible for its cationic nature [48], targeting activity [49], water solubility, and improved permeation [50,51]. The drug is characteristically liked with CS through ionic interaction, hydrogen bonding (H.B), and hydrophobic interaction. This feature makes CS an ideal material for pH-responsive drug carriers, particularly advantageous for controlled drug delivery. The non-toxic and biocompatibility of CS make it an excellent candidate for use as a drug delivery vehicle. [52,53]. Chitosan nanoparticles with high drug encapsulation efficiency and more circulating half-life [54]. Moreover, chitosan-based nanoparticles

exhibit enhanced permeation and retention (EPR), facilitating their rapid internalization into cancerous cells. Results in improved bioavailability of the drug within the tumor cells, enhancing the effectiveness [55]. Chitosan nanoparticles are extensively used for various applications, particularly in pharmaceutics and medicine [56].

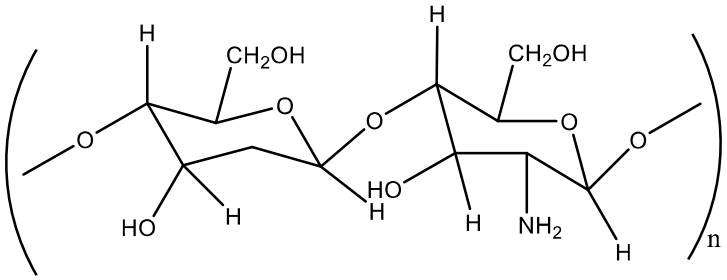

Figure 1.8: *Chemical Structure of Chitosan*

1.8 Chitosan nanoparticles synthesis methods

There are many methods available for synthesizing chitosan nanoparticles. Some are present here. Among all synthetic ionic gelation methods are the most commonly used (Figure 1.9).

1.8.1 Ionic gelation method

The ionic gelation method for chitosan nanoparticle synthesis was initially introduced by Calvo et al. In this method, chitosan nanoparticles are formed through the ionic interaction between the negatively charged sodium tripolyphosphate (STPP) as the cross-linking agent and the positively charged amino (NH2) groups of chitosan. Using a magnetic stirrer, the solution containing chitosan and STPP is continuously stirred at

room temperature [46]. By adjusting the ratio of chitosan to the cross-linker (STPP), the size and surface charge of the nanoparticles can be controlled [57].

1.8.2 Microemulsion Method

Maitra et al. initially developed the microemulsion method for chitosan nanoparticle preparation. In this method, a surfactant is dissolved in n-hexane along with the chitosan acidic solution. Subsequently, glutaraldehyde is added to the surfactant/hexane mixture under continuous stirring at room temperature, forming nanoparticles. Glutaraldehyde serves as a cross-linker in this process. The system is stirred overnight to allow for the completion of the crosslinking process. It should be noted that using glutaraldehyde as a cross-linking agent has a significant drawback as it is an antigenic agent. Additionally, this method is unsuitable for incorporating proteins or peptides into the nanoparticles as the covalent crosslinking can damage them. Furthermore, the size of the particles can be adjusted by varying the amount of glutaraldehyde used [58].

1.8.3 Self-Assembly

Chitosan nanoparticles undergo self-assembly in acidic water, with a hydrophobic core surrounded by a hydrophilic shell. This unique structure allows these nanoparticles to carry hydrophobic and hydrophilic drugs effectively. As a result, these amphiphilic nanoparticles have garnered significant attention in the field due to their versatile drug-delivery capabilities [59].

1.8.4 Polymerization

The concept of radical polymerization was initially proposed by [60–62]. Subsequently, Bravo-Osuna et al. utilized this method to prepare chitosan and thiolate chitosan-poly (isobutyl cyanoacrylate) nanoparticles. In their study, chitosan was continuously stirred

in diluted nitric acid along with a mixture of isobutyl cyanoacrylate, nitric acid, and ceric ammonium nitrate, all within an inert gas environment at 40°C for a duration of 40 minutes. After cooling to room temperature, sodium hydroxide was added to adjust the pH to 4.5, forming a nanoparticle dispersion [63].

1.8.5 Covalent cross-linking

Covalent cross-linking is crucial in synthesizing chitosan nanoparticles and their derivatives as drug carriers [64]. Glutaraldehyde, polyethylene glycol (PEG), and dicarboxylic acid are commonly used cross-linking agents. Fe_3O_4-chitosan nanoparticles have been prepared by covalent cross-linking chitosan with oleic acid-coated Fe3O4 nanoparticles using glutaraldehyde [65]. These nanoparticles exhibit superparamagnetic properties and are suitable for hyperthermia. Chitosan methotrexate nanoparticles have also been synthesized by covalently conjugating methotrexate to chitosan using glutaraldehyde. These conjugates demonstrate effective anticancer activity in MCF-7 cancer cells [66].

1.8.6 Precipitation

Two approaches for nanoparticle synthesis using the precipitation method, i.e., desolvation and diffusion. Desolvation, first applied by A. Berthold and K. Cremer [67] for chitosan microspheres, was later improved by Tian and Groves [58] to prepare 600-800nm chitosan nanoparticles [68]. Sodium sulfate is used in this method to precipitate the nanoparticles through hydrogen bonding. In diffusion methods, nanoparticles are obtained through the interaction of chitosan in the water phase with an organic solvent. However, this method often yields large nanoparticles, limiting their applications.

1.8.7 Spray Drying

This method offers a convenient one-step approach for the preparation of nanoparticle powder [69]. Mannitol microspheres containing chitosan nanoparticles loaded with protein were successfully prepared using this method. Additionally, chitosan iron oxide nanoparticles with different chitosan: iron oxide ratios were synthesized through spray-drying. The results revealed the crystallization of Fe_3O_4 within the chitosan matrix, and the nanoparticles exhibited stability in water along with superparamagnetic solid properties.

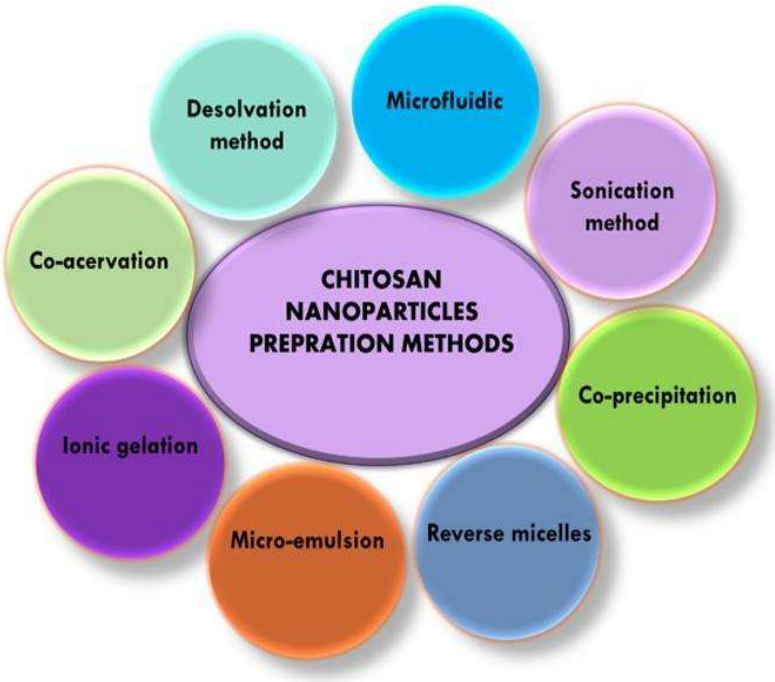

Figure 1.9: *Various methods for synthetic of chitosan nanoparticles*

1.9 Advantages of ionic gelation over other synthetic methods

1.9.1 Simplicity and cost-effectiveness

The ionic gelation method is relatively simple. It does not require complex synthesis procedures or expensive reagents, making it a cost-effective approach for chitosan nanoparticle production.

1.9.2 Mild reaction conditions

The synthesis process is performed under mild conditions, typically at room temperature, avoiding the need for harsh reaction conditions or high-energy inputs. This aspect is beneficial for preserving the biological activity of encapsulated or loaded materials.

1.9.3 Biocompatibility and Safety

Regulatory authorities generally recognize Chitosan and polyanions (STPP) used in the ionic gelation method as safe (GRAS) and have good biocompatibility. This makes chitosan nanoparticles suitable for biomedical and pharmaceutical applications, including drug delivery, tissue engineering, and wound healing.

1.9.4 Versatility and Tunability

The ionic gelation method allows for controlling the particle size and surface charge by adjusting the concentrations of chitosan and polyanion and the reaction parameters. This tunability enables the synthesis of chitosan nanoparticles with desired properties for specific applications.

1.9.5 High encapsulation

The gelation process helps retain the encapsulated materials within the chitosan matrix, preventing premature release and degradation.

1.9.6 Sustained release profile

Chitosan nanoparticles prepared using the ionic gelation method have shown the ability to provide sustained release of encapsulated substances. The porous structure of the nanoparticles allows for controlled release kinetics, leading to improved therapeutic efficacy and reduced dosing frequency.

Overall, the ionic gelation method offers a simple, cost-effective, and versatile approach for synthesizing chitosan nanoparticles with desirable characteristics for various biomedical and pharmaceutical applications.