CHAPTER 2

Literature review

2.1 Overview

This chapter explores the therapeutic applications of various nanocarriers in combination with methotrexate (MTX) as an anticancer drug. Current studies investigate the use of advanced chemotherapeutic nanocarriers such as inorganic nanomaterials (metal, metal oxide, and carbon-based particles) and organic nanomaterials (liposomes, polymeric nanoparticles, polymeric micelles, solid lipids nanoparticles, dendrimers). These nanocarriers are employed to enhance the effectiveness of MTX and introduce additional functionalities, including increased solubility, precise release, and passive and active targeting, aiming to improve MTX's bioavailability. The effectiveness of MTX in treating breast cancer has been extensively examined using *in-vivo* models, which provide better predictions of drug efficacy and toxicity compared to traditional models like the xenograft model due to their improved clinical relevance. Moreover, the chapter also discusses the latest advancements and challenges in advanced drug delivery systems (DDSs) for treating breast cancer. Breast cancer is a common type of cancer and a leading cause of mortality worldwide [70]. Rather than solely seeking new therapies, the strategy against cancer should prioritize improving existing therapies and diagnostics through innovative, effective, and plausible approaches [71]. Traditional treatments, which lack specific targeting mechanisms, pose a significant drawback as they harm both cancerous and noncancerous cells. This indiscriminate effect perpetuates systemic toxicity, leading to adverse effects and significantly impacting the overall quality of life for patients [72]. Early detection, on the other hand, has evident benefits, including significantly higher 5-year survival rates, reduced overall costs for patients, and less aggressive treatment

regimens [73,74]. Nanotechnology is an advanced tool for breast cancer treatment. It offers a potential solution by enhancing current treatment with superior targeting capabilities, reducing systemic toxicity, refining imaging techniques, and optimizing radiation therapy [75,76].

2.2 Role of Nanotechnology in Cancer diagnosis and therapy

Nanotechnology is a field focused on manipulating materials at the nanoscale (1-100 nm) [77,78]. Its unique physical and chemical properties make nanoparticles a promising tool in cancer diagnosis and therapy [79]. The remarkable potential of nanoparticles lies in their capacity to specifically target cancer cells, enabling the precise delivery of therapeutic agents and minimizing harm to healthy tissues. [80]. Numerous studies have demonstrated the potential of nanoparticle-based drug delivery systems to improve the pharmacokinetics and biodistribution of various chemotherapy drugs, such as paclitaxel, doxorubicin, methotrexate, and cisplatin, employed in breast cancer models [81,82]. Additionally, nanoparticles have been utilized for imaging and diagnosing breast cancer. Nanoparticle-based radiosensitizers have shown promise in enhancing the effectiveness of radiation therapy in breast cancer models. Also, they have been used as immunomodulators to augment immunotherapy efficacy. Several nanoparticles developed for breast cancer treatment, such as; metal-based, carbon, and polymer-based nanoparticles, have unique physicochemical properties that make them attractive for breast cancer diagnosis, imaging, and therapy [83].

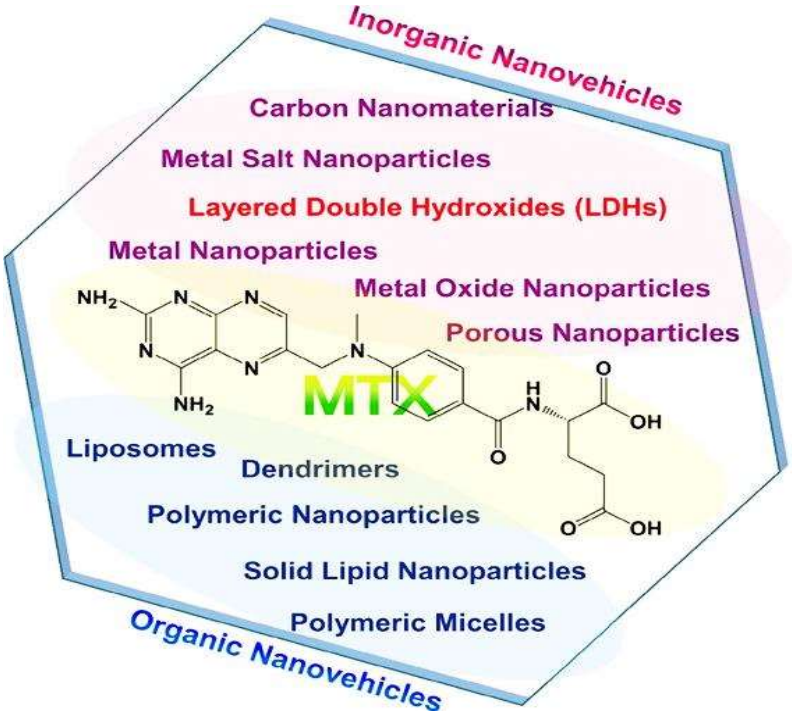

Figure 2.1: *Schematic diagram of methotrexate delivery nanocarrier.*

2.3 Metallic nanoparticles as drug delivery system

Metal nanoparticles have unique properties, including; small size, larger surface area, and tunable surface chemistry, making them suitable carriers for targeted drug delivery with excellent efficiency. Additionally, metallic nanoparticles possess unique optical properties that aid breast cancer imaging and diagnosis [84]. Several types of metallic nanoparticles, including gold, silver, and iron-based and metal oxide nanoparticles, have been intensively researched in the context of drug delivery for anticancer purposes. Nanoparticles of these materials have been utilized as carriers due to their biocompatibility, ability to bind with drug molecules, easy surface chemistry enabling targeted drug delivery, and high drug loading [85–87]. Methotrexate (MTX) is a

chemotherapy drug frequently used in cancer treatment. Only a few metal nanoparticles incorporated with methotrexate were reported, some presented here.

Dey et al. synthesized gold nanoparticles utilizing alginate and curcumin (Ccm) and loaded with MTX in their study. The resulting nanoparticles, MTX-Ccm-AuNPs, were evaluated for their effects on cell viability and cellular uptake in glioma (C6) and MCF-7 cell lines. The researchers observed that MTX-Ccm-AuNPs exhibited enhanced cellular uptake and demonstrated higher efficacy against cancer cells than free methotrexate. These findings highlight the potential of MTX-Ccm-AuNPs as a promising approach for improving drug delivery and enhancing the anticancer effectiveness of methotrexate [88].

Ghorbani et al. explored a pH-sensitive drug delivery with gold nanoparticles. They investigated the effectiveness of this system in delivering a combination of drugs, namely methotrexate (MTX), 6-mercaptopurine (MP), and doxorubicin (DOX). By utilizing gold nanoparticles, the researchers aimed to enhance the targeted delivery of these drugs. The researchers co-conjugated the drugs and gold nanoparticles (AuNPs) using a poly (ethylene glycol) block copolymer, forming MTX-MP-DOX-AuNPs. The loading amounts of MTX, MP, and DOX onto the AuNPs were determined as 49%, 12%, and 43%, respectively. To assess the efficacy of drug delivery, they employed HeLa, MCF-7, A549, and MDA-MB-231 cancer cell lines and successfully demonstrated the delivery of the triple combination of anticancer drugs using PEGylated AuNPs [89].

Numerous investigations have shown that Au-NPs could improve the effectiveness of treatment for breast cancer by boosting the dose supplied to cancer cells while minimizing harm to nearby healthy tissues [90,91]. Gold nanoparticles were also used in breast tumor photoacoustic and computed tomography (CT) imaging. [92,93]. Even though gold nanoparticles have been identified as a promising photo-synthesizer, their usage in

clinical settings has been constrained due to their low photothermal stability under repeated NIR irradiation. Gold nanoparticles' limited drug-loading capacity and regulated drug-release profile make them poor drug carriers [94,95].

Muhammad et al. used PEG-capped MTX-AgNPs for enhanced and efficient anticancer activity. Besides, nanoparticles were biocompatible to healthy cells and effective against cancer cells with 258.6 g/ml IC50 value, a significantly lower weight than free MTX concentration (512.7 g/ml). Further PEG-MTX-AgNPs displayed significantly reduced hemolytic activity compared to free MTX, indicating carrier compatibility with the blood [96]. In addition, silver nanoparticles have antitumor activity in breast cancer cell lines by inducing apoptosis and inhibiting cell proliferation [97].

Hamed Nosrati et al. developed iron-oxide NPs, coated with L-lysine and then encapsulated with MTX. This surface coating and conjugation process enabled the nanoparticles to be functionalized with MTX for targeted drug delivery. The author found that MCF-7 cell lines treated with F-Lys-MTX NPs have a tremendous anticancer impact [98].

According to Kohler et al., due to folate receptor targeting, MTX-conjugated iron oxide nanoparticles (MTX-IONPs) significantly increased their cellular absorption in MCF-7 and HeLa cells. Upon conjugation of methotrexate (MTX) with iron-oxide nanoparticles (IONPs), the MTX-functionalized NPs were efficiently internalized into tumor cells. These NP surfaces remained stable until they encountered specific enzymes that triggered the release of MTX, thereby minimizing the adverse effects on healthy cells [99].

Gao et al. prepared IONPs loaded with methotrexate (MTX). These MTX-conjugated IONPs were utilized for magnetic resonance imaging and exhibited remarkable therapeutic effects on MCF-7 cells. The combination of MTX chemotherapy and AC

magnetic field-induced hyperthermia resulted in excellent synchronous therapeutic outcomes. The cell viability analysis demonstrated a reduction in cell viability to 13% with MTX-IONPs due to a magnetic field and 64% without a magnetic field. These findings highlight the potential of MTX-IONPs for enhanced cancer therapy through a combined approach of chemotherapy and magnetic field-induced hyperthermia [100].

Lin et al. created the Cy-MTX-PEG-CS-IONPs, a drug and Cy5.5 dye-conjugated system. Compared to free MTX, Cy-MTX-PEG-CSIONPs significantly decreased the viability of HeLa. Concentration of MTX analysis in an animal model revealed that Cy-MTX-PEG-CS-IONPs had a substantially longer half-life (3.6 vs 0.4 hours), the higher area under the curve (18 vs 4 mg/h/L), high Mean residence time (MRT) (4.4 vs 0.4 hours), and less clearance (0.2 vs 0.9 L/hr.) than free MTX. After 15 days, Cy-MTX-PEG-CS-IONPs reduced tumor growth by around 1.6 times compared to free MTX [101]. Since these nanoparticles have magnetic qualities, they can precisely deliver drugs to cancer cells using magnetic fields [102]. Additionally, it has been demonstrated that iron oxide nanoparticles with imaging properties enabled early identification of breast cancer [103]. Inducing apoptosis and preventing cell proliferation, these nanoparticles have been proven to have anticancer activity in breast cancer cells [104]. Furthermore, titanium oxide nanoparticles and Zinc oxide nanoparticles have been shown to enhance the efficacy of chemotherapy drugs in breast cancer by increasing drug delivery to cancer cells [105]. These nanoparticles also offer antitumor activities in breast cancer cells [106].

2.4 Limitations of metal and metal oxide nanoparticles in cancer treatment

In summary, metal and metal oxide nanoparticles possess unique properties, making them promising contenders for drug delivery in anticancer therapy. However, it is critical to

recognize that additional study and development are required to fully explain their behavior, optimize their properties, and assure successful therapeutic application. Although these nanoparticles offer considerable potential, their utilization in breast cancer treatment is impeded by toxicity, which can adversely affect organs like the liver, kidneys, and lungs. The immune system may also identify metallic nanoparticles as foreign entities, resulting in inflammation and reduced treatment effectiveness. Moreover, the associated costs are limited, potentially impacting their accessibility as a viable treatment option. Furthermore, challenges concerning large-scale manufacturing, biological considerations, safety, government regulations, and overall cost-effectiveness hinder their commercialization prospects.

2.5 Metal salt nanoparticles

Metal salt nanoparticles, such as calcium carbonate (CC) and carbon-based-NPs, have been extensively explored for their potential in drug delivery applications. CC, known for its biodegradability and biocompatibility, has been studied as a drug carrier. [107]. However, its limitation in terms of micrometer size prompted the exploration of amorphous calcium carbonate nanoparticles (ACC) as an alternative carrier. [108]. Only a few research works were reported on metal salt nanoparticles loaded with methotrexate.

2.5.1 Calcium carbonate nanoparticles ($CaCO_3$-NPs)

In recent years, significant interest has been in utilizing calcium carbonate ($CaCO_3$) nanoparticles (NPs) for targeted drug delivery in cancer cells. These NPs offer several advantages, including their accessibility, affordability, safe, biocompatible, pH sensitivity, and slow biodegradability.

Die et al. [109] conducted a study on synthesizing methotrexate (MTX)-loaded amorphous $CaCO_3$-NPs using a gas diffusion technique. The authors observed that the collapse of MTX- $CaCO_3$-NPs occurred due to drug release and phase transformation combined. The research findings demonstrated that MTX- $CaCO_3$-NPs showed the maximum significant inhibitory effect on cancer cell viability, especially with prolonged exposure, indicating sustained release capabilities of the nanoparticles.

2.6 Carbon-based nanoparticles (C-NPs)

Due to their distinct physicochemical characteristics, carbon-based nanoparticles have become a promising contender for breast cancer treatment. Due to their excellent biocompatibility, they serve as the perfect delivery system for imaging and therapeutic compounds. Including drug administration, imaging, and photothermal therapy, C-NPs have been employed in several breast cancer treatment procedures. By delivering chemotherapy medications directly to the tumor site, carbon nanotubes have been demonstrated to enhance their effectiveness while minimizing side effects. As a drug carrier, graphene oxide has also been used to increase the bioavailability of therapeutic agents [110,111]. Nanoparticles, nanotubes, and graphene are carbon-based materials that have undergone extensive research for various uses, including biological labeling, bioimaging, medication administration, and electronic applications [112–114]. A few studies on carbon-based nanoparticles are presented here (Table 1.1).

2.6.1 Carbon nanotubes (CNT)

As drug delivery vehicles, carbon nanotubes (CNT) have several benefits, including a large surface area, surfaces that may be chemically modified with maximum drug-loading, and photoacoustic effects [115,116].

Das et al. developed multiwalled carbon nanotubes (CNTs) functionalized with fluorochromes (Alexa-fluor and AF488/647), radionuclides (99mTc), tumor-targeting ligands (FA), and anticancer drugs (MTX). Further, they observed that the cytotoxicity on A549 and MCF-7 cell lines found IC_{50} for MTX and MTX-CNT to be 2.13 & 7.36 mg/mL. Regarding tissue biodistribution, the study shows that the accumulation of drugs at the tumor site was ~19 times higher when compared to free MTX [117].

2.6.2 Graphene oxide (GO) nanoparticles as drug delivery system

Graphene oxide is frequently used in academic and industrial settings due to its favorable structural, electrical, thermal, and mechanical properties. However, the hydrophobicity of their surface acted as a barrier to their usage as drug delivery systems (DDSs) due to agglomeration [118].

Masoudipour et al. the researchers investigated the use of dopamine (DA)-coated graphene oxide (GO) as a carrier for the targeted delivery of methotrexate (MTX) to dopamine receptor sites. These nanoparticles were used in MCF-7 cells to evaluate the anticancer activities of this compound. Additionally, the study involved the utilization of overexpressed dopamine (DA) receptors and DA receptor-deficient HEK-293 (Human embryonic kidney) cell culture lines for conducting 96 experiments. The determination of IC_{50} values was carried out for free methotrexate (MTX), methotrexate-encapsulated graphene oxide (MTX-GO), and methotrexate-doxorubicin-coated graphene oxide

(MTX-DOX-GO) in MCF-7 cells, resulting in IC_{50} found 16, 18, and 15 μg/mL. Conversely, in HEK-293 cells, the corresponding IC_{50} for free MTX, MTX-GO, and MTX-DA-GO were found to be 73, 84, and 83 μg/mL [119].

2.7 Limitations of carbon-based nanoparticles in cancer treatment

Despite the possibility that carbon-based nanoparticles (C-NPs) could be helpful in the fight against breast cancer, there are certain drawbacks, such as; Biodistribution; C-NPs are absorbed by the liver or spleen, build-up in other tissues, and their treatment efficacy may be compromised. Furthermore, C-NPs may be challenging to get cleared from the body. They might be too large for the kidneys to filter out, which could lead to toxicity. Consequently, damaging living cells, especially when CNTs are not functionalized appropriately. At the same time, immune responses generated within the body against CNT may limit its effectiveness against cancer treatments. Manufacturing-related problems; It can take a long time and money to produce C-NPs. Table 2.2 presents the advantages and disadvantages of metallic and metal oxide nanomaterials for cancer therapies.

Table 2.1. *Summary of inorganic nanoparticles for methotrexate delivery*

Nano-carrier	Materials	EE (%)	LC (%)	Cell-line	Finding	Ref.
Metal-NPs	Gold Nanoparticle	–	25	–	Exhibit enhanced anticancer activity through improved controlled release of the drug.	[120]
	Alginate, curcumin-gold Nanoparticle	–	1.3	–	A dual drug molecule conjugated-nanocarrier can be achieved through a straightforward two-step process.	[88]
	Polyethylene glycol Au-NPs	36	11.5	–	A redox and pH dual-responsive nanocarrier	[89]
	Gelatin-gold Nanoparticles			MCF-7	In treating breast cancer, gelatin-coated gold nanoparticles serve as a highly effective pH-sensitive drug delivery method for methotrexate.	[121]
	BSA-capped Gold Nanoparticles	–	–	MCF-7	BSA-capped gold nanoparticles synthesized in-situ are an excellent carrier for delivering methotrexate to MCF-7 breast cancer cells.	[122]
	Platinum Nanoparticles	–	–	4T1	Chemoradiation therapy of 4T1 cancer cells using methotrexate-conjugated platinum nanoparticles can be performed utilizing X-ray irradiation.	[123]
	PEG and silver nanoparticles	39	–	MCF-7	The toxicity associated with hemolysis is reduced in comparison to free MTX.	[124]
	Graphene oxide silver nanoparticle	–	–	MCF-7 & HepG2	The cancer treatment utilizes a combination of graphene oxide-methotrexate and silver nanoparticles for precise targeting.	[125]
	PEG-Ag-NPs	–	–	–	PEG-capped silver nanoparticles loaded with methotrexate exhibit effective antitumor efficacy and biocompatibility.	[96]
Metal oxide NPs	Cy5.5-PEG-Chitosan-iron oxide	–	6.2	–	Using dual-modal imaging and self-targeted medication delivery.	[126]
	Chitosan-magnetite-iron Nanoparticles	74	39	MCF-7	The biosynthesis and characterization of chitosan-coated magnetite iron nanoparticles loaded with methotrexate and their effects on the MCF-7 cells up to 72 hours.	[127]
	Lipid base-Iron oxide nanoparticles	75	3	MDA-MB-231	Using a lipid-based homing system platform including a multifunctional superparamagnetic iron oxide nanoparticle to deliver methotrexate and mild heat for breast cancer therapy.	[128]
Metal Salt NPs	Calcium carbonate	–	30	–	The implant demonstrates a maximum drug loading and exhibits potent anti-cancer properties.	[129]

Carbon NPs	FA and 99Tc coated multiwalled CNT	86	33	A549 and MCF-7	The theragnostic application exhibits a high level of anti-cancer properties.	[117]
	Gelatin, graphene oxide	–	28	–	Biocompatible and high colloidal stability	[130]
	Dopamine, graphene oxide	81	19	MCF-7	Cancer cells can be targeted using high colloidal stability, sustained release, and DA dual chemotherapy synergy.	[119] [131]

Table 2.2. *Benefits and limitations of inorganic nanoparticles in cancer treatment.*

Nanoparticle Class	Benefits	Limitations
Gold NPs	Reliable biocompatibility established a platform for delivering a range of cancer medicines.	Toxicological problems can result from chemical pollutants from the synthesis with fewer immediate anticancer efficacy than other nanoparticles.
Silver NPs	Direct anti-cancer cell killing capacity, good biocompatibility.	The size of the particles must be adjusted for size-dependent cytotoxicity. With minimal delivery to the tumor, there could be off-target effects.
Iron Oxide NPs	The capacity for external magnetic stimulation enables control over the uptake, while the potential for functionalization with ligands offers an opportunity to enhance active targeting.	For active targeting to be clinically valuable, extensive research is required.
Zinc Oxide NPs	ROS, the release of cytokines and chemokines, and the death of cancer cells are all caused by innate action on molecular pathways. External stimulation, such as UV light, can cause cytotoxic consequences.	In vivo, treatment for off-target effects with minimal tumor formation is still necessary.
Titanium Dioxide NPs	Through the production of reactive oxygen species and induction of DNA damage, these nanoparticles exhibit similar direct cytotoxicity mechanisms to Zinc Oxide Nanoparticles in cancer cells.	Before tumor accumulation, NPs that typically collect in RES organs are removed by the renal system.

2.8 Polymer nanoparticles (P-NPs)

Polymeric nanoparticles are a promising class of nanomaterials for treating breast cancer. These are safe for in vitro and *in-vivo* usage since they comprise biodegradable and biocompatible monomers [132–134].

Due to this, they demonstrate the extraordinary bioavailability and effectiveness of chemotherapy by concentrating on the tumor location and minimizing side effects. Consequently, it facilitates the development of sustained drug delivery tailored to release medications under regulated conditions [135,136]. P-NPs have also been used as imaging agents for detecting and treating breast cancer. For instance, fluorescently labeled polymer nanoparticles have been demonstrated to successfully target breast cancer cells both *in-vitro* and *in-vivo* and show promising prospects for photodynamic therapy for breast cancer treatment. It can produce reactive oxygen species and cause cancer cells to die by inducing cell death by absorbing light, reducing tumors' size. Some research has also reported targeted methotrexate (MTX) delivery using P-NPs. Some examples are given below;

Jingou Ji et al. aimed to mitigate the toxicity of methotrexate (MTX) and enhanced nanoparticle targeting by developing folic acid (FA)-covalently bonded MTX-loaded chitosan (CS) nanoparticles cross-linked with sodium tripolyphosphate. The NPs exhibited average particles size ranging from 293.9 ± 24.2 to 401.5 ± 20.4 nm. *In-vitro* release studies conducted in PBS (pH 6.8) revealed that the MTX-NPs showed an initial burst release, followed by a controlled release. This sustained release profile can potentially reduce the adverse effects of the drug [137].

Taheri et al. developed a drug delivery system called MTX-HSA-LHRH, where luteinizing hormone-releasing hormone (LHRH) was functionalized on methotrexate

(MTX) conjugated to human serum albumin (HSA). The study demonstrated reduced breast cancer T47D cell line viability upon exposure to MTX-HSA-LHRH. The IC_{50} for free MTX, MTX-HSA with & without LHRH were determined to be 5.82 nM. The authors also observed that MTX-HSA-LHRH could specifically bind to the LHRH receptor, facilitating the internalization of MTX-HSA-LHRH particles into the cell via receptor-mediated endocytosis. A study revealed that in cancer cells, Folate receptors are commonly overexpressed; thus, conjugating folic acid (FA) to these receptors makes it a potent tumor-targeting drug [138].

Ji et al. investigated the employed FA-conjugated chitosan nanoparticles for targeted MTX (MTX/FA-CS) delivery. It was observed that the release profile of MTX varied as a function of the amount of MTX encapsulated in the chitosan on the nanoparticles. Specifically, at an MTX/chitosan ratio of 4/20, the release rate of MTX was 84%. Conversely, when the ratio was 1/20, the release rate of MTX decreased to 51% [137].

Chen et al. developed a PEG-chitosan NPs for the delivery of MTX (MTX-PEG-CS) to address numerous issues associated with drug delivery systems (DDSs), such as poor drug stability, short residence time during blood circulation, and high PEG-protein immunogenicity [71, 72]. The study revealed that NPs achieved an encapsulation efficiency of 87.7%. In HeLa cell culture lines, the viability of cells treated with free MTX and MTX-PEG-CS was compared. In contrast, free MTX exhibited a 34% suppression of cell growth after 24 hours. MTX-PEG-CS (at a concentration of 20 μM) demonstrated a 49% inhibition of cell growth. This finding suggests that PEG-CS nanoparticles may have functioned effectively as a delivery nanocarrier [24].

PASPs, poly (aspartic-acid) derivative-based polymer micelles, have several benefits as DDS nanocarriers, including low toxicity, biodegradability, biocompatibility, and inexpensive preparation costs [139].

Jiang et al. conducted a pharmacokinetic evaluation on PEG-coupled PASPs using a mouse model. The study determined the half-life ($t_{1/2}$), the area under the curve, and the total clearance of MTX-PEG-PASP to be 2.4, 6, and 19 mL/hr. Data obtained was further compared with the findings for non-PEGylated polymer micelles, which showed values of $t_{1/2}$ to be 1, 2.7, and 44 mL/hr. Suggesting PEGylated polymer micelles demonstrated an improved delay in MTX clearance from blood circulation [140].

Hanh Thuy Nguyen et al. developed multifunctional nanoparticles to target somatostatin receptors for combined chemo-photothermal therapy using polyaniline and methotrexate. The cytotoxicity study conducted on MCF-7 and MDA-MB-231 human breast adenocarcinoma cells and xenograft tumor-bearing BALB/c nude mice demonstrated enhanced suppression of tumor development compared to the control groups [141].

2.9 Biopolymer nanoparticles

Biopolymer nanoparticles can be used *in-vivo* safely as they are derived from natural or synthetic biodegradable and biocompatible polymers. For instance, pectin, chitosan, glucose, starch, dextran, amino dextran, proteins, gum, gelatine, collagen, alpha-amylase, casein, trypsin, glutamic acid, etc. Over other nanoparticle formulations, bio-polymer nanoparticles have several advantages, including good biocompatibility, adaptability, and targeted distribution. Among the all-biopolymers, chitosan has used most popularly as a drug carrier.

2.9.1 Chitosan

Chitosan is a possible nanomaterial for treating breast cancer since it is a naturally occurring cationic polymer generated from chitin. Chitosan has been demonstrated to have various distinctive qualities, including biocompatibility, biodegradability, low toxicity, and mucoadhesive, that make it an appealing choice for drug delivery and cancer therapy [36,142]. Chitosan has been studied in the context of breast cancer treatment due to its potential to carry chemotherapeutic drugs directly to tumor cells, augmenting their effectiveness while lowering off-target damage. In breast cancer cell lines and animal models, numerous studies have demonstrated that chitosan-based drug delivery systems can improve the therapeutic efficacy of several chemotherapeutic medicines such as doxorubicin, paclitaxel, and cisplatin [143–145]. It has been shown that chitosan nanoparticles can disrupt the tumor microenvironment, activates the immune system, modify cell signaling pathways to cause apoptosis (programmed cell death) and inhibit cell proliferation in breast cancer cells [146,147]. Furthermore, the possibility of using chitosan with other cancer treatments like radiation therapy and immunotherapy has also been researched. Chitosan-based nanoparticles have been exposed to stimulate the generation of reactive oxygen species after increasing radiation dosage within tumor tissue, hence improving the effectiveness of radiation treatment in the case of breast cancer. Additionally, it has been demonstrated that chitosan increases breast cancer cells' immunogenicity, making them more vulnerable to immune system-mediated eradication [36,148]. Further, chitosan's protonated amine groups may interact with the negatively charged plasma membrane to increase the permeability of cell membranes.

Their limitations constrain the clinical application of anticancer drugs. Mention those limitations, despite showcasing satisfactory effects in *in-vitro* studies [149]. Adding

chitosan has improved therapeutic characteristics, addressing low water solubility, light, thermal instability, and high toxicity [150]. It serves as an effective carrier for numerous anticancer drugs, including doxorubicin (DOX), curcumin (CUR), methotrexate, resveratrol, paclitaxel (PTX), doxazosin, ellagic acid, and 5-fluorouracil (5-FU) [49].

2.9.2 Chitosan nanoparticles (CS-NPs)

Chitosan nanoparticles (CS-NPs) have been extensively investigated for their applications in drug delivery, photodynamic therapy, and gene therapy due to their biocompatibility, biodegradability, and non-toxicity [151]. The systemic administration of methotrexate (MTX) can cause serious side effects due to its non-specific distribution in the body. To address this issue, chitosan nanoparticles (CS-NPs) were crosslinked with a crosslinker and employed as a drug delivery system for MTX in breast cancer therapy. [152]. In some studies, CS-NPs were crosslinked with glutaraldehyde and loaded with MTX, resulting in a sustained release of the drug over 72 hours and significant inhibition of breast cancer cell growth. In another study, CS-NPs were crosslinked with genipin and loaded with MTX, increasing loading capacity and sustaining drug release over 48 hours. Crosslinking CS-NPs with a crosslinker agent improved their stability and drug-loading capacity, making them more suitable for drug delivery systems for cancer therapy. Several studies have demonstrated the effectiveness of MTX-loaded CS-NPs in suppressing breast tumor growth both *in-vitro* and *in-vivo*. Breast cancer is a significant public health concern, and researchers have explored using chitosan-loaded methotrexate (MTX) nanoparticles as a potential alternative treatment.

Fathi et al. successfully developed a chitosan hydrogel with thermal and pH sensitivity. In a study conducted on MCF-7 cells, the researchers demonstrated the hydrogel's excellent biocompatibility [77]. In the study evaluating the release of the anticancer agent

doxorubicin (DOX), it was observed that the drug exhibited the highest release capacity (99%) under acidic conditions (pH 5.5) and at a temperature close to the body's normal temperature (37 °C) [153]. The research revealed that the chitosan-coated MTX-NPs had high biocompatibility *in-vitro* and *in-vivo*, suggesting their potential as a secure and reliable drug delivery agent for breast cancer treatment.

Another study evaluated the efficiency of chitosan-coated MTX nanoparticles in a breast cancer xenograft mice model. The study found that the chitosan-coated MTX nanoparticles significantly inhibited tumor growth compared to free MTX, and the nanoparticles showed reduced toxicity and increased bioavailability *in-vivo*. In conclusion, chitosan-coated MTX nanoparticles have great potential for breast cancer treatment and should be further investigated [154]. The use of chitosan-coated MTX nanoparticles in combination with ultrasound could be a promising strategy for breast cancer treatment [155].

Rajendiran M. et al. [156] used a straightforward chemical precipitation process to create and characterize chitosan-coated luminous rare earth doped terbium nanoparticles (LaF3:Tb3+/chi NPs) as a drug carrier for methotrexate (MTX). Entrapment efficiency was 83.82%, and particle size was reported to be 10–12 nm. According to research, free methotrexate does not have the same cytotoxic effect on MCF-7 breast cancer cell lines as MTX-coupled LaF3:Tb3+/chitosan nanoparticles have.

Amir Azadi et al. [157] prepared Nanogels loaded with methotrexate via ionic gelation to enhance pharmacokinetic parameters. The particle size ranged from 106-118 nm, while entrapment efficiency and drug loading capacity were 61% and 53%, respectively.

Meliha et al. [158] developed chitosan nanoparticles loaded with methotrexate (MTX) and labeled with radioactive metal (^{99m}Tc) for breast cancer imaging using a 4T1 breast

cancer cell line. Encapsulation efficiency was low (32 to 50 %), only showing considerable therapeutic efficacy but limited by internal toxicity.

Ehab M.M. et al. [127] reported MTX-loading magnetite iron nanoparticles chitosan coated and studied its effects on the MCF-7 cell line. Different formulations were used, and it was observed that the particles varied in size from 36-150 nm. Further, the methotrexate loading efficiency was 74.15% and 39.8%. MTX's release and anticancer activity improved when loaded with chitosan-coated Fe3O4-NPs for efficient cancer treatment.

Daniele et al. [159] reported the development of pH-responsive methotrexate-loaded chitosan nanoparticles used in *in-vitro* cytotoxic studies reported on the MCF-7 and HeLa cell lines. There were no *in-vivo* animal studies reported. The researchers described the development of chitosan nanoparticles encapsulated with MTX (MTX-CS-NPs). They incorporated an amino acid-based amphiphile called 77KS during the ionotropic method to achieve this. The addition of 77KS conferred pH-responsive characteristics to the nanoparticles, enabling the accelerated release of methotrexate as the pH decreased.

Furthermore, 77KS resulted in pH-dependent membrane-lytic activity, indicating its potential for disrupting cell membranes under specific pH conditions.

In their study, Rania et al. [20] developed chitosan nanoparticles loaded with methotrexate and subsequently coated them with hyaluronic acid (HA) and human serum albumin (HSA). The chitosan nanoparticles served as the core of the coating process. Different concentrations of HA and HSA solutions were utilized for coating. The researchers then evaluated the antitumor activity of these prepared NPs on MCF-7 cell lines.

Coating the NPs with HSA resulted in a slight size increase having a positive surface charge. The *in-vitro* cytotoxicity assessment revealed that HA and HSA-coated methotrexate-loaded chitosan nanoparticles exhibited enhanced anti-tumor properties compared to uncoated nanoparticles and free methotrexate. These findings indicate the potential of HA and HSA coating to improve the therapeutic efficacy of the nanoparticles against breast cancer cells. Nusaiba et al., 2022 also reported the formation of chitosan nanoparticles loaded with MTX and functionalized with photocatalytic TiO2-NPs. Entrapment efficiency for UV-sensitive drug release was 80%. Only *in-vitro* cytotoxicity was found on the MCF-7 cell line in this investigation [97]. In addition, Uv-sensitive medication release has been described with decreased therapeutic efficacy due to internal toxicity [160].

Zahra et al. [161] synthesized biodegradable carriers based on chitosan-modified mesoporous silica nanoparticles (MSN-APTES) to deliver methotrexate for breast cancer treatment. The author reports the particle size around 56 -73 nm with a loading capacity of 12 % and Zeta potential from +28 to +32 Mv, the potential for cytotoxicity in the MCF-7 cell line.

At a low dose (0.5 μM), the MTX-loaded MSN-APTES-chitosan had a favorable outcome. In this study, the authors described a novel approach to the synthesis of biodegradable MSNs with small and uniform sizes.,

Meliha et al. [152], in 2023, developed methotrexate-loaded chitosan nanoparticles labeled with Technetium-99m (99mTc) as an imaging probe for breast cancer diagnosis with an encapsulation efficiency of 64% and 70%.

The current research on chitosan-based nanoparticles for breast cancer treatment has shown promising results in increased efficacy and reduced toxicity compared to free

drugs. At the same time, using crosslinked CS-NPs loaded with MTX is a potential method for treating breast cancer since it provides better stability and drug loading capacity and ensures continuous drug release. However, more studies are required to explore the efficacy of this treatment approach and the optimal dosages and administration methods.

2.10 Advantages of chitosan nanoparticles over metal, metal oxide, and carbon-based nanoparticles in cancer treatment

Chitosan nanoparticles offer distinct advantages over metal, metal oxide, and carbon-based nanoparticles in cancer treatment. These advantages include:

2.10.1 Biocompatibility

Chitosan is a natural. It is biocompatible, biodegradable, and non-toxic, making it a safe option for biomedical applications. In contrast, metal, metal oxide, and carbon-based nanoparticles may exhibit varying cytotoxicity and biocompatibility concerns.

2.10.2 Controlled drug release

Chitosan nanoparticles can encapsulate and deliver therapeutic agents in a controlled manner. Their porous structure allows for the sustained and controlled release of drugs, ensuring a prolonged therapeutic effect. Metal nanoparticles and carbon-based nanoparticles may not possess the same degree of controlled drug-release capability.

2.10.3 Targeted drug delivery

Chitosan nanoparticles can be surface-functionalized with targeting ligands, such as antibodies or peptides, to recognize and bind to cancer cells specifically. This targeted approach enhances drug delivery to the tumor site, increasing therapeutic efficacy while

minimizing damage to healthy tissues. Metal nanoparticles and carbon-based nanoparticles may lack the specific targeting ability of chitosan nanoparticles.

2.10.4 Enhanced cellular uptake

Chitosan nanoparticles possess a positive surface charge, promoting interactions with negatively charged cell membranes. This electrostatic interaction facilitates the cellular uptake of nanoparticles and their cargo, improving intracellular drug delivery. Metal and carbon-based nanoparticles may not exhibit the same level of enhanced cellular uptake.

2.10.5 Immunomodulatory effects

Chitosan nanoparticles have inherent immunomodulatory properties, stimulating the immune system and enhancing the body's defense mechanisms against cancer. This immunomodulatory effect can potentially augment the efficacy of cancer treatment. Metal nanoparticles and carbon-based nanoparticles generally lack such immunomodulatory properties.

2.10.6 Ease of functionalization

Chitosan nanoparticles offer versatility in terms of surface modification and functionalization. They can be easily conjugated with various molecules, such as drugs, imaging agents, or targeting ligands, to enhance their therapeutic and diagnostic capabilities. Metal nanoparticles and carbon-based nanoparticles may have more limited options for surface functionalization.

2.10.7 Cost-effectiveness and availability

Chitosan, derived from renewable sources, is relatively abundant and cost-effective compared to metal, metal oxide, and carbon-based. This makes chitosan nanoparticles a more accessible option for cancer treatment.

In conclusion, metal, metal oxide, and carbon nanoparticles exhibit various adverse effects. Since these nanomaterials displayed toxicity and long-term safety issues, as demonstrated by *in-vivo* studies. Besides there, large-scale production is challenging require and regulatory approval [84]. Using chitosan-based nanoparticles is a promising avenue for developing safe and effective breast cancer treatments.

Table 2.3. *Summary of organic nanocarriers for methotrexate delivery*

Nanocarrier	Materials	EE (%)	LC (%)	Cell-line	Finding	Ref.
Liposome	DSPE-PEG2000, DPPG, DSPC and PEG4000	83	31	–	The concentration of MTX in the parietal lobe is 3.6 times greater than that of free MTX.	[162]
	Ara-C and DPPC	86	–	–	The release of MTX remains sustained even after a duration of 28 days.	[163]
Polymeric Nanoparticle	LHRH functionalized human serum albumin	–	–	T47D	The functionalization of LHRH enables internalization through LHRH receptor-mediated endocytosis.	[138]
	Folic acid conjugated chitosan	–	11	–	The release of MTX can be controlled by adjusting the ratio of MTX and chitosan.	[137]
	PEGylated chitosan	87	44	–	The inhibition of HeLa cells was 1.5-fold higher compared to free MTX.	[164]
	Chitosan-modified mesoporous silica nanoparticles	–	–	MCF-7	Chitosan-modified mesoporous silica nanoparticles function as biodegradable nanocarriers for methotrexate delivery, with potential applications in breast cancer treatment.	[161]
Solid Lipid Nanoparticle	Stearic acid, soya lecithin, and Sodium taurodeoxycholate	40	–	–	The formulation exhibits sustained release in mouse serum, resulting in a longer half-life and mean residence time (MRT).	[165]
	Hydrocarbonized porous silicon	12	–	–	The sustained release of the substance remains effective under different pH conditions.	[166]
	Imwitor, Neobee® Cremophor RH40 and Pluronic F127	70	-	A2780	There is a significant reduction in proinflammatory cytokines and cytokines driving T-cell activity.	[167]

	Stearic acid, Tween-80, soya lecithin and Triton X-100	22	7	–	The in-vivo biodistribution research with a radioisotope-labeled sample shows increased tumor uptake.	[168]
	Fucose, Pluronic F-68, Triton X-100 and Gelucire-50/13	84	15	–	The surface modification with fucose improves the targeting of tumors.	[169]
Polymeric micelle	mPEG–PLA	47	12	–	Achieving controlled release	[170]
	PEG-PLA	96	9	KBv	The chain length of PLA is utilized to achieve controlled and sustained release.	[171]
	PEG-PCL	–	~3	HeLa	The structural engineering of the diblock copolymer enables the development of a nanocarrier capable of loading multiple drugs.	[172]
Dendrimer	PAMAM and OEG	85	–	MCF-7	The nanocarrier exhibits excellent biocompatible and effectively inhibits tumor growth.	[173]

2.11 Problem Statement

This thesis aims to investigate the impact of encapsulated methotrexate on its pharmacokinetic properties, aiming to enhance its effectiveness in treating breast cancers while minimizing side effects. Additionally, the study proposes using fluorescent nanoparticles in conjunction with methotrexate to potentially improve treatment efficacy and enable precise bioimaging of tumors. This research can be described in three points below.

i) To enhance the pharmacokinetic properties of methotrexate (MTX), including its half-life, bioavailability, volume of distribution (Vd), and mean residence time (MRT), while simultaneously reducing its toxicity. This will be achieved by developing a polymeric nanocarrier as a controlled-release system that specifically targets the desired site of action.

ii) To assess the pharmacokinetic parameters and biodistribution of the developed nanoparticles in a rat model. This evaluation will investigate how the nanoparticles are absorbed, distributed, metabolized, and eliminated within the body and determine their concentration in various tissues and organs.

iii) To evaluate the nanoparticles as both a bioimaging agent and an effective antitumor treatment in a rat model of chemically-induced cancer using N-methyl-N-nitrosourea (NMU) and 7,12-dimethylbenz[a]anthracene (DMBA). The nanoparticles will be examined for their potential to serve as contrast agents for imaging purposes, allowing for non-invasive visualization of tumors. Additionally, their efficacy as an anti-tumor therapy will be assessed, focusing on their ability to inhibit tumor growth and improve overall survival in the rat model.