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Nanocarriers for methotrexate delivery/codelivery in the frame of cancer diagnostics and treatment: a review

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Cancer is one of the most life-threatening family of diseases that cause death worldwide. As a highly researched and successful therapeutic agent, methotrexate (MTX) treats many solid tumours, hematologic malignancies, and autoimmune illnesses. Despite many benefits, methotrexate induces drug resistance and limits plasma half-life due to its poor pharmacokinetics. The variable biological availability have prompted researchers to investigate innovative delivery strategies for enhancing its therapeutic qualities. To develop more suitable methotrexate formulations, nanoparticles (NPs) have recently gained a significant interest. A wide range of nanoparticles, including polymer-based nanoparticles, carbon-based nanoparticles, lipid-based nanoparticles, as well as inorganic nanoparticles, can be deliver cancer chemotherapeutics such as methotrexate. Loading methotrexate into NPs can provide a delivery system that has shown great promise to carcinoma therapy. In this review, we will describe the feasibility of NP-based strategies to deliver methotrexate in cancer therapy, outlining the current state of the art and the challenges/promises for the future.

KEYWORDS

cancer therapy, methotrexate, nanoparticles, immune system, drug delivery

1 Introduction

Cancer is among the most important reasons for decrement of life expectancy. In 2019, according to a study by the World Health Organization (WHO) in 112 countries, cancer has been estimated to be the main reason for death before the age of 70. In many countries, the death rate resulting from cancer has increased compared to the death rate from stroke and heart disease. The most frequently diagnosed cancers include female breast carcinoma (11.7% of all occurrences), lung carcinoma (11.4%), colorectal carcinoma (10%), prostate

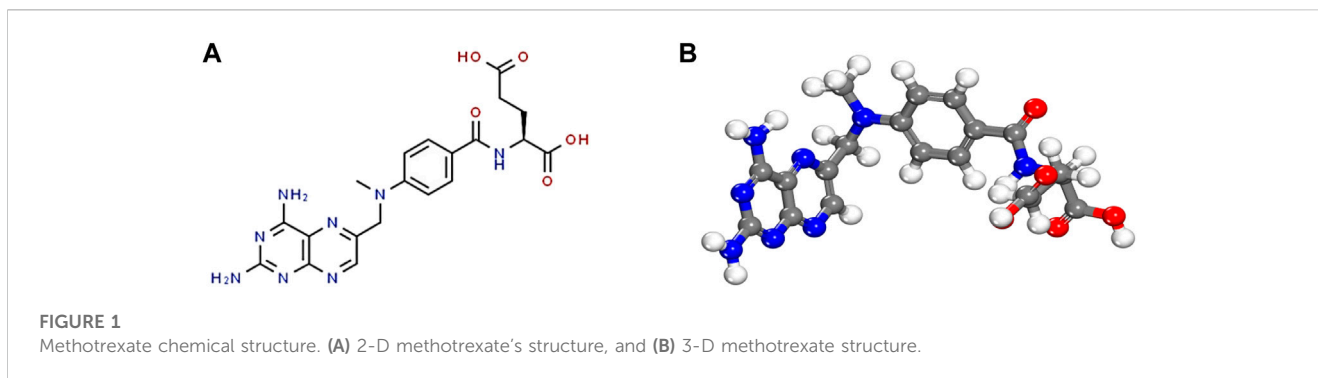
carcinoma (7.3%), and stoma carcinoma (5.6%). The most frequent deaths from cancers are due lung carcinoma (18% of all cancer deaths), followed by colorectal carcinoma (9.4%), liver carcinoma (8.3%), stomatal carcinoma (7.7%), and breast carcinoma (6.9%). Colorectal and prostate cancers are the most common cancers occurring and causing death among men population. In the women population, the most frequently leading reason of cancer death is breast cancer, followed by colorectal and lung cancers. Based on current clinical data, GLOBOCAN 2020 estimated the population with new cancer cases to be 19.3 millions per year, and reported around 10 million deaths from cancer globally (Fatima et al., 2021; Sung et al., 2021). Several immune-based strategies have been approved to treat cancer in recent years. Radiotherapy (RT) can affect the tumor immune state in a variety of ways, such as promoting the release of tumor-specific antigens by tumor cells and activating T-cells against tumors to overcome tumor immune tolerance mechanisms (Yang et al., 2022). RT treats patients by damaging DNA of tumor cells and generating cytotoxic reactive oxygen species (ROS) to control tumors. Meanwhile, the presence of hypoxic cells in tumors and toxic side effects in peripheral tissue lead to resistance to RT, which remains a challenge in RT research. At the same time, a minority of patients exhibit long-term response to immunotherapy due to the complexity of the mechanisms of tumor immune escape. As a result, single immunotherapy is restricted to a few tumors and can only benefit a fraction of patients. Due to the immunomodulatory effects of RT and reduced side effects in tumor immunotherapy, RT combined with immunotherapy has significantly improved the effective cure rate by eradicating tumor cells and amplifying the activity of immune cells in several clinical trials. Compared to immunotherapy, radioimmunotherapy has significantly shown better therapeutic effects with favorable efficacy to control the primary tumor and its metastasis by activating the immune response, indicating a synergistic effect of RT and immunotherapy (Pointer et al., 2022; Colciago et al., 2023). Radioimmunotherapy leads to immunogenic cell death (ICD) by promoting the release of tumor-associated antigens (TAAs), changing the tumor microenvironment (TME), and activating the immune system to exert an anti-tumor immune response. The synergy between immunotherapy and RT has become a hot field in oncology therapy to boost immune response (Yu et al., 2022; Colciago et al., 2023). In oncotherapy, nanoparticles (NPs) are able to overcome biological barriers, thus reducing the dose of chemotherapeutics needed and the severity of chemotherapy-associated side effects (Hassanisaadi et al., 2021; Ajalli et al., 2022). Nanotechnology can target neoplastic cells through the enhanced permeability and retention (EPR) effects or active targeting. As EPR depends on the nature of carcinoma cell leakage, only 20% of the medication can be delivered to the cell while the body excretes the rest. Cancer treatment usually uses cytotoxic mediators, including DNA-damaging agents and microtubule inhibitors (Pourmadadi et al., 2022). Chemotherapy is provided at the maximum likely dose that is safe and agreeable to the highest tolerable concentration. It is established that conservative chemotherapy has side effects on health, such as hair loss, cardiac and neurotoxicity, liver cell harm, kidney damage, sickness, vomiting, immunosuppressive effects, etc.

A nanocarrier (Nc) is used to locally deliver cytotoxic compounds to reduce side effects and advance medical results.

Nc is preferably deposited at the tumour location because of its size, high absorbency and EPR effect. The accumulation of NPs at the target sites yields incomplete neovascularization of tumor tissue and allows decreasing non-specific tissue damages and overcoming the barriers to chemotherapy. A mechanism called endocytosis can be more useful than conventional therapy. Targeted delivery of cytotoxic agents is due to ligands binding straight to the receptor, causing cell internalization (Sheikh et al., 2022). Numerous medical procedures have been performed with several projected anticancer therapies. When there is a real deal, the side effects and the transferring to the desired location are important worries. Also, another problem is drug resistance and toxicity of new anti-cancer treatments. Nanotechnology is a very versatile approach with great promises in medical treatments. A key problem to tackle when dealing with cancer concerns the translation of outcomes from *in vitro/in vivo* to clinical applications with focus on some parameters, including therapeutic delivery, toxicity, targeting, resistance, and stability. Accordingly, many NP-based approaches for anti-tumour therapies are practically unfeasible or ineffective. Two important mechanisms for drug delivery are targeting and active targeting pathways in nanomaterials. Different types of NPs as targeted delivery systems have been used for a variety of cancer therapies. The delivery systems based on NPs are subdivided in four forms to treat cancer, including 1) Viral NPs, 2) Organic NPs (dendrimer, polymeric, and liposomal), 3) Inorganic NPs (carbon, magnetic, gold, silica, and some metals), and 4) composite NPs (lipid polymer). All NPs are often coated by other materials due to the side effects of chemotherapy. Chemotherapy is a non-specific treatment that can be spread to numerous tissues and organs by ineffective pharmacokinetics. The main structure of nano-liposomes is based on biomembrane phospholipid for encapsulating particles to enhance the solubility in aqueous systems. Polymer NPs as a system of drug delivery have been applied to form multifunctional organic NPs. Poly(lactic-co-hydroxyacetic acid) is used as a polymer for drug delivery due to its good biological compatibility and biodegradability. Inorganic NPs include Au NPs, Fe₃O₄ NPs and Ag NPs, which can be fabricated according to relatively simple processes, have good stability but lower biocompatibility compared to polymers. Silicon NPs are another example of more biocompatible inorganic NPs. In general, any type of NP, both inorganic and organic, has advantages and disadvantages to treat cancer with a different range of results (Al-Zoubi and Al-Zoubi, 2022). Methotrexate (MTX) can be loaded into and released from many of these nano-systems as an anticancer drug for targeted therapy.

2 Methotrexate

Methotrexate (MTX) has roles such as antimetabolic, anti-proliferative and anti-inflammatory, employing folate antagonist. MTX could decrease the level of inflammation by the immune system and some inflammatory cells, for example, monocytes, mast cells, neutrophils, helper T and lymphocytes B. MTX prevents purines and pyrimidines synthesis by inhibiting dihydrofolate reductase (DHFR) and also thymidylate synthase (TYMS) which has a role in the construction of thymine residues, then its suppressing protein, deoxyribonucleic acid (DNA) and

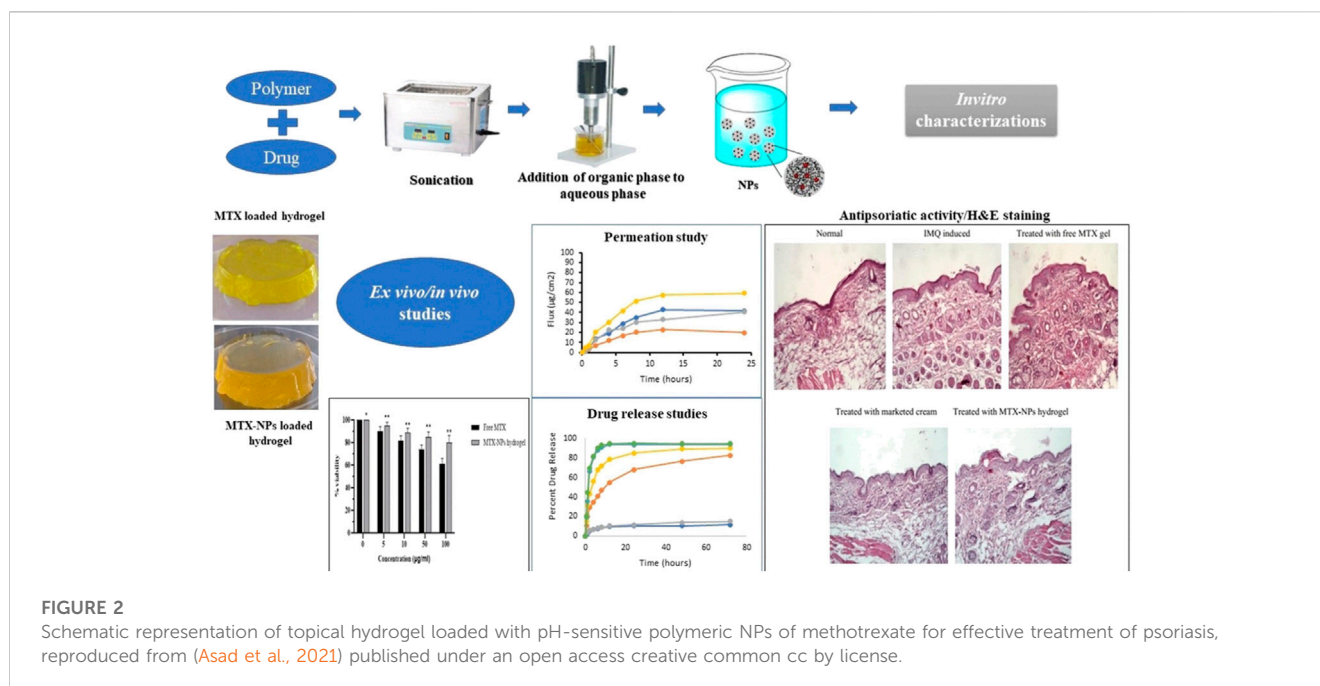


ribonucleic acid (RNA), so it leads to accumulation of a plethora of inflammatory cells (Zhao et al., 2022). Some chemotherapeutic drugs are likely to produce an antimicrobial resistance in gut microbiota by inducing dysbiosis. Methotrexate works by blocking the enzyme dihydrofolate reductase in bacterial and eukaryotic cells in DNA synthesis and is used to treat skin, breast, head, and neck cancers and inflammatory diseases such as rheumatoid arthritis (RA) (Guðmundsdóttir et al., 2021). Kidney excretes MTX, and delayed clearance may cause toxicity. Poisoning depends on MTX serum concentration and the duration of complete excretion from the body. However, impaired MTX clearance causes kidney problems and the use of hemodialysis clears effectively, but data are not available in these patients (Solignac et al., 2021). In cancer treatment with chemotherapy, some cancer cells are resistant to drugs and do not undergo apoptosis. For the treatment to be targeted quickly and accurately and for patients to experience less unpleasant side effects of chemotherapy, the bioavailability and stability of the drugs used in chemotherapy must be considered. Based on this, intelligent drug delivery systems and new stimuli-responsive materials have been developed. Clinical trials have shown that intelligent drug delivery systems have increased the efficiency of cancer therapy compared to free drugs without nano-carriers. Limitations of anticancer drugs include short half-lives, nonspecificity, widespread distribution, and toxicity. Nanocarriers can minimize these limitations through controlled delivery and release, which is desired in cancer treatment. Nanocarriers increase drug uptake and bioavailability, thus reducing side effects and increasing cytotoxicity to cancer cells (Alqosaibi, 2022). Methotrexate is used to treat tumours and lymphomas. High intravenous doses increase serum enzymes and, in the long term, increase liver enzymes, cirrhosis, and fibrosis. Methotrexate is a yellowish-orange brown and crystalline, odourless powder that is used in chemotherapy to prevent the RNA and DNA synthesis. The molecular formula is $C_{20}H_{22}N_8O_5$. Oral methotrexate is indicated to treat acute lymphoblastic leukaemia in children (Bethesda MD: National Library of Medicine US, 2022). Figures 1A, B are depicted 2 and 3-dimensional.

2.1 Methotrexate as an anticancer drug and resistance mechanisms

To decrease the unpleasant side effects on healthy tissues and cells, anticancer drugs require organic solvents and detergents for

their complete dissolution and stability. Nano-formulations transport anticancer drugs to the target site through a nano-carrier, improving efficacy, pharmacokinetics and bioavailability (Connie and Ariya, 2015). In clinical observations, despite the advantages of D- α -tocopheryl polyethylene glycol 1,000 succinate (TPGS) and poly(lactic-co-glycolic) acid (PLGA), no MTX delivery carrier was publicly claimed to use them (Almawash et al., 2022a). The remaining cells surviving after chemotherapy may develop drug resistance, possibly due to the presence of stem cells in heterogeneous tumours that cause resistant clones to form. Drug resistance occurs through the expression of genes that encode proteins resulting from the ATP-binding cassette (ABC) family. Multidrug flow pumps belong to this family, and drug withdrawal and overexpression pumps are among the main causes of chemotherapy failure. The capability of the drug to precisely target the cancer cell actively increases its concentration inside the cell and thus causes a localized cytotoxic effect (Xavier et al., 2022). In a clinical trial, it was shown that both MTX and folic acid, with a supramolecular approach, ligands are capable of interacting with folate receptor at the surface of cell, significantly reducing the side effects of the drug and the target cancer cells (Massaro et al., 2022). MTX is slightly soluble in water and has an oral bioavailability of less than 40%, which impairs its therapeutic effect. The oral uptake of MTX in the proton-dependent GIT depends on active transmission and dose. The oral bioavailability of MTX is decreased with increase in the dose above 25 mg; in fact, MTX is poorly bioavailability (~18% for doses >40 mg/m²) and ~80% is excreted unchanged into urine (Giri et al., 2022). Therefore, doses above 25% are more effective, although they may have side effects such as diarrhoea, anaemia, and intestinal toxicity. Liquid ion-based food (IL) systems are beneficial to improve pharmacokinetic properties and bioavailability, promote high intestinal absorption, better distribution, and limit drug metabolism. Preliminary results showed that MTX in the form of ionic fluids (IL) and body simulation fluids was 5,000 times more soluble and had a more anti-cancer effect than free MTX. Previous studies have shown that side effects in the slow distribution of MTX-IL are less than in the distribution of sodium MTX in organs such as the lungs, kidneys, heart and spleen without acute GIT accumulation (Moshikur et al., 2021). Therapeutic drug monitoring (TDM) is used as a valuable instrument for preventing the side effects of overdose and ineffective treatments that are unique to each patient and for assessing the effectiveness and safety of the treatment regimen. TDM is based on the level of active metabolite, which is usually plasma,



and is adjusted by adjusting the dose of the drug for each individual, which aims to increase effectiveness and decrease toxicity and bioavailability (Zhou et al., 2022).

3 Methotrexate drug delivery systems

3.1 Polymeric nanoparticles

3.1.1 Non-responsive polymeric nanoparticles

The potential of MTX-loaded polymeric NPs has been exploited for various outcomes. MTX was loaded into methoxy poly(ethylene glycol) (MPEG)-grafted chitosan (chitoPEG) copolymer (Seo et al., 2009). The mean size of the NPs was 50–300 nm with spherical shape. ¹H nuclear magnetic resonance (NMR) study demonstrated that MTX interacted with chitosan being embedded in the inner core, and the outer shell of the NPs was composed of MPEG (Figure 2). The loading efficiency of MTX was demonstrated to be between 65.2% and 94%. The cytotoxicity of the developed MTX-loaded NPs was evaluated on the B16F10 melanoma cells, and the outcomes revealed that MTX-loaded NPs presented less cytotoxicity compared to free MTX. In another study, MTX was enclosed into poly(lactide-co-glycolide) (PLGA) NPs by the process of solvent evaporation from a double emulsion (Jang et al., 2019).

The average size of the NPs was demonstrated to be 163.7 nm with an encapsulation efficiency of 93.3%, and the drug was released according to sustained kinetics (Figure 2). The cytotoxicity studies showed that the NPs presented higher antiproliferative activity against the cancerous cells, MFC-7 and CWR22Rv, in comparison with the free MTX. Moreover, the NPs were evaluated for properties of the pharmacokinetics and consequences of lymphatic delivery. The *in vivo* studies revealed high lymphatic efficiency and low accumulation of MTX in the kidney and liver. Furthermore, the fabricated NPs can be

investigated by applying different administration routes, and MTX was still detectable in the blood for a long time in comparison with free MTX. To evaluate the anti-breast cancer activity, polymeric NPs comprised of PLGA and PEG were developed by the double emulsion solvent-evaporation method. The PLGA-PEG NPs were co-loaded with MTX and trapoxin A (TPX) (Akbari et al., 2021a). The developed PLGA-PEG NPs exhibited smooth morphology and nanosize particle distribution. Moreover, the NPs presented promising results against MCF-7 breast cancerous cells. The co-loaded NPs elicited an effective inhibition of MCF-7 cells. Also, the flow cytometry analysis demonstrated cell growth inhibition by induction of apoptosis. Furthermore, the cell cycle analysis was also done, which revealed that there was a stopped cell cycle at G1. The dual drug-loaded PLGA-PEG NPs also exhibited the mitochondrial apoptosis. Altogether, the developed NPs proved to be promising in breast cancer therapy. To improve the effective concentration of MTX at the target site for cancer therapy, a novel system was developed that combined physical targeting along with chemical targeting (Lin et al., 2015). Poly(lactic acid) (PLA) and MPEG were used to encapsulate MTX in the nanobacilli-shaped carriers, and the dye Cy5.5 was conjugated with them. The nanobacilli had improved intratumoral uptake in the H22 xenograft mouse model. Moreover, the MPEG-PLA-MTX-Cy5.5 nanospheres released the drug into the tumour cells in a controlled manner to inhibit tumour growth and improve therapeutic efficiency. Similarly, another MTX loaded PLGA-beta cyclodextrin nanosystem was developed using double emulsion method to improve drug stability and solubility (Gorjikhah et al., 2017). The mean particle size ranged from 70 to 200 nm with an entrapment of 80%. The PLGA-β cyclodextrin NPs increased the cytotoxicity after releasing the drug in T47D breast cancer cell lines. In another study, PLA-PEG-PLA triblock polymeric NPs were synthesized to deliver MTX in a controlled way to the cancer cells. The double

emulsion method was adopted, and the prepared polymeric NPs were then covered with bovine serum albumin to enhance their biocompatibility. The average size of the NPs was reported to be in the range of 100–157 nm with an entrapping efficiency of 80.4% (Massadeh et al., 2016). In another study, biodegradable MPEG-PCL copolymer-based NPs were designed for their activity on breast cancer cells. MTX and curcumin were co-loaded in the NPs to improve the therapeutic activity against cancer cell lines. The nanoprecipitation method was adopted to develop self-assembled micelles. The average micelle size was found to be 88 nm. The cancer cell growth was inhibited with an increase in the concentration of the micelles in the treatment groups. The micelles improved the cytotoxicity against MCF-7 cancer cell lines (Saraei et al., 2019). Anti-inflammatory effects of MTX can also be relevant to the field of cancer treatment as some correlations were suggested between cancer development and persistent inflammation states. In this regard, MTX-loaded spherical chitosan and Pluronic F-127 NPs were developed using the ionic gelation technique and self-micellization for treating rheumatoid arthritis. The average size of the NPs was 181–417 nm, with a loading efficiency of 80.14%. The NPs prolonged the release of MTX for 48 h and improved the pharmacokinetic profile. There was an improved therapeutic activity with 3.48 times higher residence time in the inflamed tissues. The haematological study revealed that the NPs showed no adverse effects on the therapeutic activity. Chitosan was also employed to develop NPs and fucoidan to treat skin-related inflammatory issues. MTX was enclosed in the NPs with an entrapment efficiency of 80%–96% with a mean size of 300–500 nm. The NPs exhibited low cytotoxicity to the human keratinocytes and fibroblasts. *In vivo* studies showed that there was a significant reduction in the pro-inflammatory cytokines. Moreover, the NPs improved the permeation of MTX through the pig ear skin barrier as compared to free MTX. Altogether, this MTX topical delivery using this approach demonstrated promising anti-inflammatory activity (Barbosa et al., 2019). Polycaprolactone and polyethylene glycol (PCL-PEG) were used to develop NPs co-loaded with MTX and cisplatin for an effective treatment for non-small lung cancer (Zhang et al., 2017). The NPs were of spherical morphology and induced apoptosis in the tumour tissues as revealed by DAPI analysis. The PCL-PEG NPs improved the cytotoxicity against A549 cells by reducing bcl2 and cyclin D1 gene expression.

Iron oxide magnetic NPs (110–130 nm) were developed based on lignin as a natural polymeric-magnetic-nano-system via anti-solvent precipitation and ultrasonication for the targeted delivery of MTX. The drug loading and encapsulation efficiencies were calculated to be about 66.06% and 64.88%, respectively. The NPs, as a non-hemolytic material, exhibited a concentration-dependent release of MTX for the initial 24 h. MTT assay for NPs revealed higher cytotoxicity against breast cancer and macrophage cell lines in comparison to the free form of the drug by increasing caspase-3 activity and decreasing glutathione levels (Pathania et al., 2023).

Magnetic alginate beads coated with glutaraldehyde cross-linked chitosan were prepared to decrease the side effects and improve stability due to biodegradable and biocompatible nature of the NPs, resulting in an encapsulation efficiency of 75% and showing acceptable controlled release behavior. Cytotoxicity assay based on MTT revealed higher anticancer effect compared to the free MTX by increasing expression of Bax and decreasing the expressions of Bcl-2

and TNF- α in MCF-7 cells (Taran et al., 2023). β -CD/MTX complex was fabricated to enhance the solubility and stability of MTX in aqueous solvents. β -CD as a nanocarrier had a good capacity for loading and releasing MTX. MTT assay exhibited improvement of the MTX performance (Hadi et al., 2023). Arrow et al. (2023) prepared poly(lactide-co-glycolide) complexes to load MTX, which demonstrated inhibitory effects on human PVR-cells. Samani et al. (2023) synthesized folic acid-functionalized MTX-loaded perfluorohexane nanodroplets (NDs) with an alginate shell (FA-MTX/PFH@alginate NDs) as a tool for smart ultrasound-guided chemoradiotherapy with controlled release. This composite could enhance the anticancer activity of chemotherapeutics and reduce side effects to normal tissue during breast cancer treatment *in vitro* [cancer cell (4T1)] and *in vivo*. In addition, MTX-loaded nanostructures with 26.7 ± 1.5 nm showed high inhibitory effects on tumor growth. Furthermore this nanostructure had no significant hemolytic activity and organ toxicity in combination with ultrasound exposure and X-ray irradiation. Lastly, FA-functionalized MTX/PFH@alginate NDs were accumulated selectively in the tumor region (Samani et al., 2023).

3.1.2 Stimuli-responsive polymeric nanoparticles

Various polymeric NPs have been developed to enhance the effective therapeutic concentration of the drug at the target site (Lodhi et al., 2022; Shahrousvand et al., 2022; Yoosefi, 2022). Delivery approaches such as stimuli-responsive NPs are being exploited. Such stimuli include chemical and physical changes, and the chemical stimuli are divided into pH, enzyme, and redox activity. In contrast, the physical stimuli can include light, magnetic field or temperature. Hence, using stimuli-responsive approaches can be advantageous in the targeted delivery of tumour cells and other disorders (Li et al., 2013a).

Asad et al. (2021) demonstrated the effectiveness of pH-responsive MTX NPs loaded into chitosan hydrogel to treat psoriasis. Eudragit-100 (E100) was used to make the hydrogel stimuli-responsive (pH-sensitive). The adopted method was the o/w emulsion solvent evaporation method (Figure 2). The average size of the NPs was 256 nm, with a loading efficiency of 86%. The *ex-vivo* permeation study showed that 81% of MTX was retained in the epidermis following the hydrogel application. No significant symptoms of hyperkeratosis and parakeratosis were observed. Moreover, the studies on the imiquimod-induced psoriasis model exhibited promising therapeutic activity. Histopathological studies showed reduction of inflammation and redness in the MTX-treated groups following topical administration.

In another research, chitosan was employed with fucoidan to assemble MTX-loaded NPs to study their effectiveness in lung carcinoma. It was shown that NPs with an average size of 300 nm were stable in the stomach acidic environment and improved the oral bioavailability in lung cancer. The NPs induced apoptosis of the lung cancer cells and were safe toward fibroblasts. The NPs exhibited biological activity and could suppress the growth and proliferation of lung carcinoma cells about 7-fold more as compared to free drug. Altogether, the nanosystem was found to be mucoadhesive and pH-responsive for the MTX oral delivery (Coutinho et al., 2020). In a novel study, Ganoderma lucidum polysaccharide (GLP) was utilized because of its

antioxidant and antibacterial activity to develop a pH-sensitive nanosystem for the delivery of hydroxycamptothecin (HCPT) and MTX for cancer therapy. The developed NPs had a size of 190 nm and exhibited good loading capacity, i.e., 21.5% for MTX and 22.6% for HCPT. GLP NPs released the drugs under the acidic conditions of the tumour cells; however, the NPs remained stable at the other physiological pH. Moreover, tumour suppression was observed following the *in vivo* administration (Nezhad-Mokhtari et al., 2019). Similarly, an ultraviolet (UV) light and pH dual-responsive polymeric nanosystem was developed to deliver MTX in tumour therapy. Supramolecular polymeric NPs were fabricated from acetal-modified β -cyclodextrin-azobenzene and poly(α -cyclodextrin) (Dai and Zhang, 2018).

Supramolecular polymeric NPs were discovered to have an average size of 85 nm, with an encapsulation efficiency of 21.3%. pH responsiveness was due to acetal group, and UV responsiveness was due to azobenzene, which was attributed to the controlled release of MTX upon UV irradiation. Based on the function of pH, over 65% of MTX was released in 12 h according to fast delivery kinetics. Upon UV irradiation, the drug was released in a slow manner after the initial burst release at the tumor site. A derivative of chitosan, thiolated carboxymethyl chitosan, was used to develop another kind of NPs. The polymer self-assembled via disulfide linkage in deionized water to synthesize NPs. NPs were discovered to have an average size of 160 nm, with an encapsulation efficiency of 43.4%. The slow and sustained release of MTX from the polymeric NPs was beneficial for tumour suppression. The inhibition rate of the MTX-loaded NPs was estimated to be 90% against HeLa cells. Owing to the existence of the carboxyl group on the polymer, the NPs were swelled and released the drug in the acidic environment based on the function of the surface area of NPs in contact with water and pH (Gao et al., 2014). A pH-responsive nanohydrogel was also established for MTX delivery in cancer therapy. In this regard, poly(hydroxyethyl methacrylate) (PHEMA) was crosslinked with poly(ethyleneglycol) diacrylate (PEG-DA) via chain transfer polymerization technique. The mean size of MTX-loaded nanohydrogel was 194 nm. MTX was coupled with polymeric structure by a pH-responsive ester bond, facilitating the drug release in acidic pH. PHEMA and PEG-DA conjugated nanohydrogel induced cytotoxicity in the MCF-7 cancer cells. pH-responsive conjugate enhanced the toxicity and cellular uptake in a cancer animal model (Farzanfar et al., 2021).

To successfully deliver MTX and curcumin for treating colorectal cancer, three-block copolymers polycaprolactone-poly(ethylene glycol)-poly(caprolactone) (PCL-PEG-PCL) were proposed (Zhang et al., 2021). PCL-PEG-PCL NPs presented good biocompatibility, low toxicity, good stability, and amphiphilicity. These NPs exhibited an encapsulation efficiency of 77%–95%, with an average particle size ranging from 95 to 135 nm. PCL-PEG-PCL NPs demonstrated dose-dependent toxicity to CL40 and SW1417 cells. The hybrid NPs showed improved cellular absorbance in the cancer cells and induced apoptosis, as revealed by the fluorescence staining assay.

In a novel hybrid approach, chitosan NPs loaded with MTX were functionalized with photocatalytic TiO₂ NPs. The photocatalytic controlled release of MTX was facilitated through the developed nanosystem based on the function of TiO₂. Upon

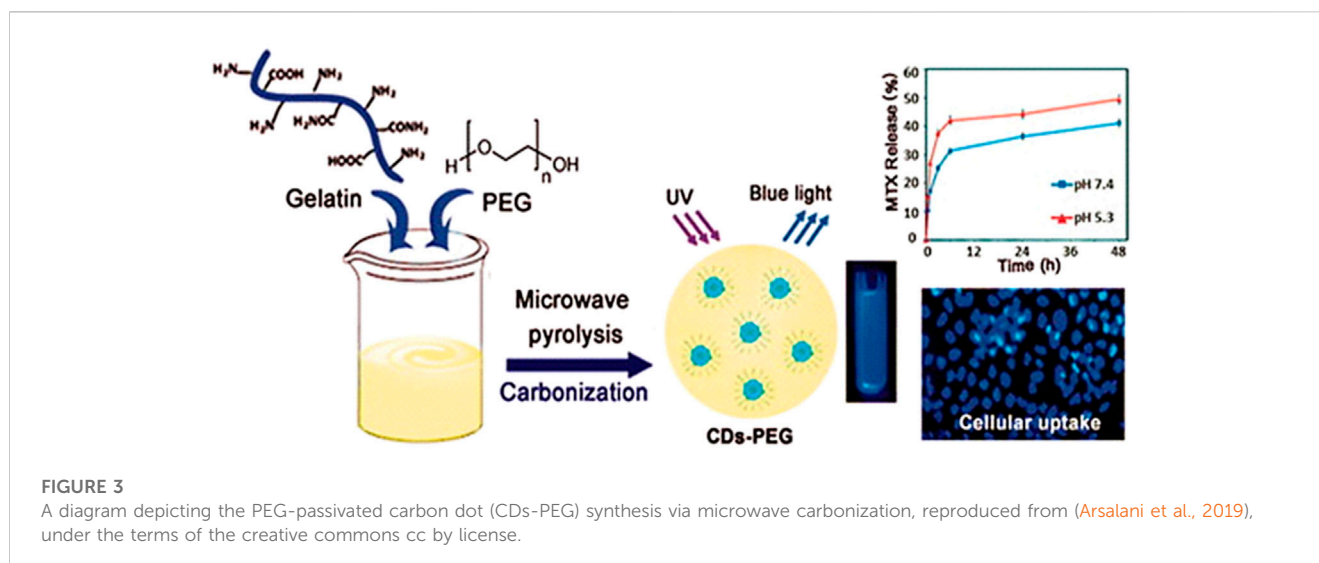
irradiation with UV, the fabricated drug delivery system released MTX as TiO₂ triggered the polymeric bond rupturing in the presence of light. The entrapment efficiency for MTX was around 75%, with a particle average size of 240 nm. CS NPs without TiO₂ NPs coating did not release a significant percentage of a drug upon UV irradiation, whereas TiO₂ NPs improved the release of MTX up to 40% in the presence of UV light. The cytotoxicity data confirmed that the developed nanosystem was cytotoxic against MCF-7 cells, hence showing promise in cancer therapy (Al-Nemrawi et al., 2022). In order to pass through the blood–brain barrier (BBB), which is as physical/biochemical barrier, the transactivator of transcription (TAT) peptide was used to produce mesoporous silica nanoparticles-linked TAT (MSN-NH-TAT) with the aim of improving MTX (MTX-loaded MSN-NH-TAT) penetration into the brain. As a result, brain-to-plasma concentration ratio and the drug blood terminal half-life was improved for delivery of MTX into the brain as compared with the use of free MTX (Shadmani et al., 2023). Poly(3-hydroxybutyrate)/chitosan-graft poly(acrylic acid)-conjugate hyaluronate was fabricated and used for targeted delivery of MTX to colon cancer cell lines (Caco-2). The overall copolymeric system showed the maximum drug loading efficiency about 69.7% \pm 2.7% (4.65 mg/g) and the release about 98.6% \pm 1.12%, at pH 7.4. The results of cytotoxicity exhibited IC₅₀ of 11.7 μ g/mL and enhanced level of DNA breakage as well as high reactive oxygen species (Hanna et al., 2023).

Biotin-PEG-conjugated nanogels of carboxymethyl polyethyleneimine (Biotin-PEG-CMPEI) were synthesized with a size of 100 nm and ζ -potential of +15 mV (pH = 7.4) for active targeted delivery of MTX in triple negative breast cancer (TNBC). This nano-system exhibited zero-order release kinetics of MTX at pH = 7.5 and a swelling-controlled release at pH = 5.5. The IC₅₀ of the MTX-loaded Biotin-PEG-CMPEI was about 10 folds lower than the free drug, while the unloaded nanogels had no significant toxicity. In a mouse model, the volume of tumor, animal mortality rate, lung metastasis and glomerular damage were lower after treatment with the MTX-loaded Biotin-PEG-CMPEI as compared to the group receiving the free drug (Abolmaali et al., 2022).

ROS-responsive hyaluronic acid–MTX was prepared combining chemotherapy with photothermal therapy (PTT) for cancer treatment. The synthesized thioketal moiety linker (TK) of MTX and hyaluronic acid (HA) (HA-TK-MTX (HTM)) in the presence of the photothermal agent IR780 (IHTM NPs) allowed reaching a tumor inhibition rate of 70.95% for 4T1 tumor-bearing mice due to ROS-responsive NPs (Yun et al., 2022). Amphiphilic cross-linked poly(vinyl alcohol-methyl methacrylate) (PVA-PMMA) copolymer NPs (NPs) as pH-responsive system were fabricated to load MTX. It was shown that 47.24% and 65.39% of the drug were released at pH = 7.4 and pH = 5.8, respectively, after 96 h. MTT assay confirmed the effectiveness of NPs against MCF-7 cells (Ziyae et al., 2023).

3.2 Carbon-based nanostructures

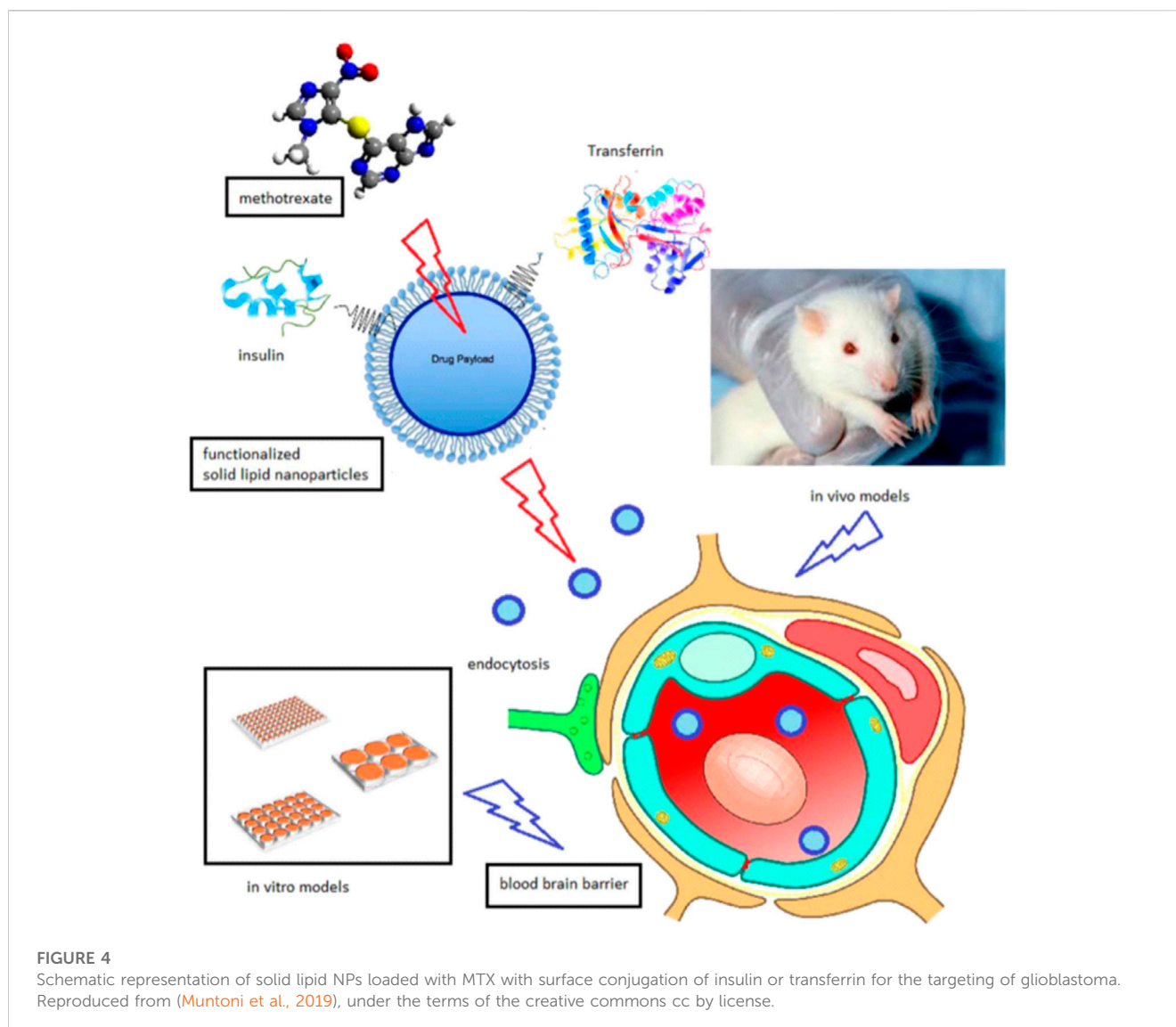
Recently, nanostructures based on carbon are gaining focus as medication delivery approaches to treat cancer effectively, inflammation, arthritis, and tumours. Carbon-based NPs have



improved water solubility, biocompatibility, high stability, and tunable surface modification properties (Li et al., 2010). In one research, carbon NPs were synthesized using sucrose and glucose using ultrasounds. Later, these carbon NPs were anchored with ethylene diamine and MTX to be delivered to cancer. These developed carbon NPs were used as fluorescent nanocarriers for MTX delivery in human lung cancerous cells (H157) (Ajmal et al., 2015). The NPs were studied through cell adhesion assay, LDH assay, and sulforhodamine B bioassay in H157 cells and presented cytotoxicity and high affinity to the cancer cells. Similarly, other graphene-based NPs, for example, graphene oxide (GO), were applied to deliver drugs. Graphene-based nanomaterials have high loading ability, interesting release profile and can simultaneously incorporate both hydrophilic and hydrophobic drugs. A GO-based nano-delivery system was prepared using Hummer's method followed by ultrasonication and loaded with MTX. The developed GO loaded with MTX presented cytotoxicity against different cell lines such as porcine skin fibroblasts (PEF), hepatocellular cancerous cells (HepG2 cells), and embryonic kidney cells derived from human (HEK293A cells) (Abdelhamid and Hussein, 2021). Various carbon-based drug delivery approaches are being exploited to deliver the drug to cancer cells. In a hybrid approach, multi-walled carbon nanotubes (MWCNTs) and C₆₀-fullerenes were conjugated by 1,3-dipolar cycloaddition using glycine and paraformaldehyde together to deliver MTX in breast cancer. The nanosystem was studied regarding cytotoxicity, cellular uptake, hemocompatibility, and pharmacokinetics. C₆₀-fullerenes/MWCNTs improved cellular uptake and retention along with improved cytotoxicity. C₆₀-fullerenes/MWCNTs allowed achieving improved pharmacokinetic profile with enhanced AUC, improved bioavailability, decreased clearance, and reduced IC₅₀ values (Joshi et al., 2017). Another hybrid approach was composed of fluorescent carbon dots (CDs-PEG) and PEG. The microwave pyrolysis approach used gelatin and PEG as starting materials to prepare CDs-PEG (Figure 3) (Arsalani et al., 2019). MTX-loaded CDs-PEG presented biocompatibility and anticancer activity. The average size of these nano-complexes was

6 nm with regular spherical morphology. CDs-PEG emitted blue photoluminescence with a highest quantum yield of 34%. The tumour growth was inhibited with improved targeted cancer therapy with good formulation stability.

In another study, oxidized MWCNTs together with chitosan were used in conjugation to deliver MTX to lung cancer. The developed system exhibited pH-responsive with fast and higher release in the acidic environment. A biocompatibility test was conducted using NSCLC H1299 and MRC-5 cells, showing the anticancer properties of the developed nano-system (Cirillo et al., 2019). Currently, proteins are being used as coating agents on the surface of carbon biomaterials using amide groups to improve cell adhesion and attachment. Moreover, the protein coating imparts better stability and compatibility in the biological environment. The used bovine serum albumin coating on carbon NPs was loaded with MTX. The NPs exhibited spherical morphology with improved stability. The developed nanosystem presented cytotoxicity in A549 lung cancer cells (83% cell death) with high apoptosis rate. Around 79% of MTX was released in 48 h and no hemocompatibility issues were reported (Muthukumar et al., 2014). Another carbon-based system, fullerenols (derivatives of fullerenes), was used as a nanocarrier for its improved cellular permeation. Fullerenols were conjugated with MTX via a hydrolysable ester bond. The developed MTX conjugated fullerenols improved the bioavailability, cytotoxicity, and pharmacokinetics of the drug (Bahuguna et al., 2018). The average fullerenols size was 94.60 nm with limited size dispersion. Compared to the free MTX, the protein binding was shown to be lower. The MTX release was pH-dependent, with an improved release (85%) in cancer cells. There was a significant reduction in IC₅₀ value for the MTX conjugated fullerenols. The fluorescent dye-tagged MTX conjugated fullerenols presented higher accumulation inside the cancer cells. Moreover, *in vivo* studies demonstrated the improved AUC by 6.15 times, and plasma half-life was improved by 2.45-fold as compared to the parenteral administration of a single dose. Also, conjugated fullerenols with MTX were compatible with erythrocytes while exhibiting higher toxicity to cancer cells.



3.3 Lipid-based nanoparticles

3.3.1 Non-responsive lipid-based nanoparticles

Lipid-based NPs are the most versatile drug delivery vehicles being exploited for their potential to treat various disorders, including cancer. Owing to the nature of lipids, these carriers can overcome the biological barriers. In one study, solid lipid NPs (SLNs) were developed using the fatty acid coacervation technique. Transferrin or insulin served as anchors, and they were stuffed with an MTX active lipophilic agent (Figure 4). Transferrin or insulin was selected because their proteins are overabundantly expressed on the blood-brain barriers. For the functionalization of proteins on SLNs, a PEG spacer was used. These developed SLNs presented higher cytotoxicity against glioblastoma cells and improved bioavailability in Wistar rats. Significant levels of SLNs were found in the tumour cells and suppressed the tumour growth in glioma models (Muntoni et al., 2019).

For the treatment of psoriasis systemic therapy, targeted therapy was developed using lipid NPs. MTX and etanercept were co-loaded into lipid NPs and mediated by carbopol hydrogel to deliver a drug into the skin with minimal transdermal permeation. The SNLs did not elicit cytotoxicity to the human keratinocytes and fibroblasts. Therapeutic amounts of drugs were deposited across the pig ear with reduced transdermal permeation. Cetyl palmitate was used as a lipid component, and the drugs were conjugated to the developed SLNs. The SLN size varied from 292 to 356 nm. The appearance of the SLN-loaded hydrogel was transparent, uniform, and glossy. The mucoadhesive characteristic of the hydrogel improved the MTX retention and reduced the systemic circulation in psoriasis (Ferreira et al., 2017). In a similar approach also addressed to MTX delivery, a pegylated lipid core containing stearic acid was created in order to overcome the limitation of low bioavailability of the drug. The pegylated SLN had an average size of 130 nm with limited size dispersion and spherical morphology. The developed SLNs were compatible with the blood cells and improved the pharmacokinetics

profile in tumour models. SLNs improved the blood circulation profile with efficient tumour cell uptake, as evident through SPECT imaging. Because of the presence of stearic acid, the bioavailability of the SLNs was effectively improved in BALB/c mice (Kakkar et al., 2015). Another topical treatment approach for psoriasis was developed using MTX-loaded nanostructured lipid carriers incorporated into the gel. The solvent diffusion technique was utilized to synthesize the nanostructured lipid carriers, which were later loaded into the carbopol gel following optimization. The deposition studies of the nanostructured lipid carriers were carried out on human cadaver skin which exhibited significant deposition across the biological barrier (71%) and resulted in the reduction of the psoriasis symptoms. The amount of pro-inflammatory cytokines were reduced following the gel application in the BALB/c mic, as compared to the marketed MTX gel (Agrawal et al., 2020). Lipid NPs have also shown promising results in osteosarcoma, where the traditional drug delivery systems could not slow the progression of the metastasis. MTX and edelfosine were co-loaded into the lipid NPs to synergistically improve the activity against osteosarcoma. Edelfosine suppressed the metastasis in the cancer cells, and MTX reduced the growth of osteosarcoma. Lipid NPs were found to be internalized in high concentration inside the cells irrespective of the expression of dihydrofolate reductase (González-Fernández et al., 2015). In another dual drug delivery approach, SLNs were fabricated to deliver MTX and calcipotriol for the topical therapy of psoriasis. Precirol ATO 5 and squalene were used in different ratios to synthesize the SLNs. The average particle size was in the range of 270–320 nm; however, the particle size increased with increasing concentration of Precirol. The developed SLNs improved the permeation of the loaded drugs across the skin (2.4–4.4 times higher in comparison with control). Confocal laser scanning microscopy (CLSM) exhibited improved permeation following the internalization of SLNs across the biological membrane. The promising outcome of the study was that drugs with different polarities were successfully embedded into one system without using any complex methodology. SLNs did not reveal any irritation to the skin and served as a potential vehicle for the efficient treatment of psoriasis (Lin et al., 2010).

SLNs have also been developed in a hybrid approach by anchorage with moieties such as fucose to target breast cancer. Gelucire 50/30 and stearic acid served as core, whereas stearyl amine and phospholipid 90 served as a coating. Fucose was anchored to the SLNs to target the breast cancer cells precisely while the off-site drug loading was minimized. SLNs demonstrated enhanced cellular absorbance in the breast cancerous cell with high drug release inside the cells and improved bioavailability. SLNs presented higher cytotoxicity at a lower IC_{50} value and facilitated programmed cell death, i.e., apoptosis. Moreover, the permeation profile was very promising, with improved anti-cancer activity due to the higher biodistribution of SLNS at the target site (Garg et al., 2016).

In order to prepare stable nanostructured lipid carriers (NLCs) for the encapsulation of MTX, glyceryl monostearate (GMS as solid lipids) and Miglyol 812 (M11 as liquid lipids) were used to reduce adverse side effects and increase the low solubility and non-specific targeting of MTX. The nanoystems exhibited long-term stability and spherical morphology along with an internal cubic crystalline

structure, as revealed by small-angle X-ray scattering (SAXS) analysis. MTX was encapsulated at $85 \pm 0.9\%$ and *in vitro* release of MTX was $52\% \pm 3.0$ in 24 h. Furthermore, the MTT assay showed that MTX-loaded NLCs exhibited more toxicity against HeLa and MCF-7 cell lines as compared to free drug. In addition, hemolysis was assessed to be $<10\%$ (Li et al., 2019).

Dendritic nanocarriers such as lysine dendrimers were used for drug delivery owing to their high drug-loading capacity and high resistance to proteolytic degradation. The poly-lysine dendritic nanocarrier (P₄LDN) for targeted chemotherapy was prepared and loaded with an epidermal growth factor receptor (EGFR)-specific short peptide E2 (ARSHVGYTGAR) and MTX. MTX was linked to the nanocarrier by using cathepsin B cleavable spacer (glycine–phenylalanine–leucine–glycine (GFLG)). The results showed that MTX released from the nanosystem decreased the percentage of cells in the G2/M phase and enhanced the percentage of cells in the S and G0 phase in both MDA-MB-231 (EGFR overexpressed) and SiHa (EGFR negative) cells (Narayanan et al., 2022).

3.3.2 Stimuli-responsive lipid-based nanoparticles

Currently, polymer and lipid-based hybrid NPs are being explored for their potential to deliver MTX in various disorders. Such hybrid NPs have high (Kumar et al., 2022) biocompatibility with improved blood circulation time (Surve et al., 2022). Also, these systems can further be modified in response to specific stimuli, including redox potential, pH, and temperature (Teixeira et al., 2017; Kumar et al., 2022; Chang et al., 2023).

Kumar et al. synthesized MTX-loaded nano-lipid polymeric system of poly(lactic-co-glycolic acid) (PLGA) in phosphatidylcholine (MTX-NLPHS) to release MTX through endocytosis against MCF-7 cell lines (encapsulation efficiency about $86.48\% \pm 0.31\%$). This system was not agglomerated due to highly negative zeta potential. *In vitro* release of MTX reached 100% in the long time. In a MTT study, the cell toxicity of MTX-NLPHS was lower with decreasing concentration of MTX. Meanwhile, at the higher concentration of MTX, toxicity was enhanced as compared to free MTX. ROS was also increased in the cells exposed to MTX-NLPHS, resulting from the controlled transport of drugs into the cells by PLGA (Kumar et al., 2023). In another study, NPs based on hyaluronan (HA)-coated lipid with low molecular weight (<10 kDa) and high molecular weight (700 kDa) were fabricated and tested for tumor targeting towards the HA receptor (CD44). *In vitro* results showed that NPs with high molecular weight influenced significantly the affinity towards CD44 on B16F10 murine melanoma cells. In addition, low-molecular-weight NPs had weak binding on B16F10 murine melanoma cell lines. Lastly, MTX as a chemotherapy drug was loaded into high-molecular-weight HA–tsNPs, resulting in slow drug diffusion with a half-life of 13.75 days (Mizrahy et al., 2014). Lipid polymer hybrid NPs were also tested to co-deliver two therapeutic drugs (Teixeira et al., 2017). Fucose-anchored lipid-polymer hybrid nanoparticles (Fu-LPHNPs) were fabricated to co-encapsulate MTX and aceclofenac (ACL) for targeted/controlled delivery purposes against MCF-7 and MDA-MB-231 cells. *In vitro* drug release studies showed that the release of ACL and MTX was rapid in the early 12 h (over 25%), then slower and at last the release was sustained over 168 h. The drug bioavailability when LPHNP was

used as carrier was greater than free MTX and ACL. It was reported that the therapeutic activity of MTX was improved by ACL and the inhibitory effect of MTX/ACL-loaded LPHNPs on cancer growth in a breast cancer mouse model was greater as compared to the simple combination of free MTX and ACL or MTX (Teixeira et al., 2017; Rui et al., 2022).

3.4 Inorganic nanostructures

At present, there has been a significant advancement in the use of inorganic compounds for developing drug delivery systems (Borse et al., 2023). Some selected inorganic NPs have low toxicity, tunable surface properties, improved intracellular drug delivery, and comprehensive functionality (Baqeri et al., 2022; Ekinci et al., 2023). Inorganic compounds such as calcium carbonate, iron oxide, silica, gold, and calcium phosphate are the most popular among the widely explored inorganic nanomaterials (Xu et al., 2006; Ram Prasad et al., 2019; Wu et al., 2022a). Currently, inorganic substances are being used to deliver MTX to different disorders.

Iron oxide and, specifically, Fe₃O₄ NPs have been studied to deliver MTX for effective anticancer treatment. Fe₃O₄ NPs (average size between 10 and 20 nm) were covered with a molecularly imprinted polymer (MIP) imparting thermal-responsive properties. These magnetic seeds could produce heat under the effect of an external magnetic field. Hence, the MIP-coated magnetic seeds exhibited superparamagnetic nature with an 80% MTX adsorption ratio. Under a magnetic field, these nanocarriers released MTX by cleavage of hydrogen bonds and demonstrated therapeutic activity in cancer cells. Therefore, this concept yielded a unique thermosensitive strategy for targeted drug delivery to cancerous tissue based on outer stimuli (Kubo et al., 2018). Although polymeric nanostructures are already being studied because of their biocompatibility, inorganic substances are often being used in conjunction with polymeric substances to increase their compatibility with healthy cells and tissues. The inclusion of gold NPs (Au NPs) into a polymeric shell facilitates imaging following the near-infrared photothermal stimulus. Following this approach, MTX and AuNPs were combined using the emulsion-diffusion-evaporation method and embedded into pegylated-poly(DL-lactic-co-glycolic acid) nanospheres (Lima and Reis, 2015). These developed hybrid nanostructures released MTX based on pH and temperature changes. Moreover, these Au-conjugated nanostructures demonstrated lower cytotoxicity to monocytes and macrophages as compared to Au NPs alone. The uptake of these nanostructures was higher in the cells based on clathrin and caveolae-dependent endocytosis. The presence of Au in the nanostructures dramatically decreased levels of pro-inflammatory cytokines that were produced by monocytes and macrophages. Moreover, based on the theranostic properties of Au, these nanostructures are novel vehicles for therapeutic as well as diagnostic function, showing highly effective for chemophotothermal therapy.

Calcium phosphate is another inorganic compound that can be utilized for developing drug delivery systems. In one study, calcium phosphate NPs (CaP-NPs) were synthesized using the reverse micelles technique and loaded with MTX. The average

size of the CaP-NPs was estimated to be 262 nm, with an entrapment performance of 58%. A minimum fraction of MTX was released at physiological pH, whereas over than 90% of the medication was released after three to 4 h at endosomal pH. The confocal imaging demonstrated the localization of CaP-NPs in CHO cell lines with promising intracellular drug delivery (Mukesh et al., 2009).

In another study, pH-sensitive magnetic hydrogels were developed via embedding of chemically precipitated Fe₃O₄ NPs into chitosan, carboxymethyl cellulose (CMC), and β-cyclodextrin (β-CD) hydrogel for the delivery of MTX (Naderi et al., 2020). The swelling capacity was observed for the hydrogels with magnetic NPs, and the maximum drug release (92%) was detected for the CMC-based hydrogels. The developed magnetic NPs were responsive to pH and magnetic field. A higher swelling index was observed for the magnetic hydrogel at high pH.

Silica is also used for fabricating nanocomposites for the delivery of MTX. Polypyrrole and mesoporous silica dioxide (PPy/mSiO₂) were used to synthesize nanocarriers to deliver MTX for cancer therapy. Moreover, graphene quantum dots (GQDs) were coated onto the surface of these nanocarriers. mSiO₂ improved the encapsulation efficiency of the nanocarrier, and PPy facilitated the heat development under exposure to outer near-infrared light. Upon irradiation with near-infrared light, the hydrogen bond between mSiO₂ and GQDs was broken, and then MTX was released in a controlled manner. PPy/mSiO₂ NPs coated with GQDs provided a better drug delivery platform based on the theranostic function of light (Zhao et al., 2017). Bovine serum albumin (BSA) was capped onto the AuNPs and loaded with MTX. The NPs demonstrated inhibition of breast carcinoma cells MCF-7. The NPs also induced the apoptosis based on the DNA ladder gel electrophoresis and flow cytometry analysis. These BSA-MTX-AuNPs improved uptake in MCF-7 cells as well as the stability and solubility of MTX, therefore demonstrating higher anti-cancer activity as compared to Au NPs alone. (Murawala et al., 2014). Some MTX-loaded nano-drug delivery systems are listed below with their composition and outcomes (Table 1).

The use of different phases including an inorganic/organic shell modified with polymer have been applied as multifunctional nanomaterials with the aim of protecting the inner core against oxidation, pH changes, high temperature, radiation, and aggressive agents. CoFe₂O₄/mSiO₂-NH₂/poly(methacrylic acid (MAA)-co-itaconic acid (IA))/MF₃ nanocomposite as a smart core/double shells (CDS) carrier was synthesized for the simultaneous release of MTX and doxorubicin (DOX) against MCF-7 human breast cancer cells. This nano-composite system was sensitive to pH for a controlled drug release. In addition, the *in vitro* cytotoxic studies revealed a noticeable tumor inhibition as compared with the free forms of drugs (Ghazimoradi et al., 2023).

A novel triple-responsive nanocarrier including magnetic (for directing drug to cancerous cells), pH (due to lower pH values of the cancerous cells) and temperature responsiveness (due to higher temperature of the cancerous cells) has been developed for the targeted delivery of anticancer drug. For this aim, superparamagnetic Fe₃O₄ NPs coated with poly(N-isopropylacrylamide) grafted with chitosan (size of

TABLE 1 Some MTX-loaded nano-drug delivery systems.

Nanostructures	Composition	Encapsulation efficiency and average size	Reported outcomes	Ref
Polymeric nanoparticles	PLGA	71% (MTX)	Improved cytotoxicity, inhibition of progression of breast cancer by controlled delivery of drugs	Hou et al. (2018)
		85% (Curcumin)		
		142 nm		
Polymeric nanoparticles	Chitosan	87% (MTX)	Enhanced tumour inhibition, increased bioavailability in breast cancer model	Naveen et al. (2020)
		201 nm		
Polymeric nanoparticles	PLGA, PEG	94% (MTX)	Dose-dependent cytotoxicity on SW1417 and CL40 cell lines on, elevated apoptosis in colorectal cancer	Meng et al. (2021)
		90% (Curcumin)		
		100 nm		
Polymeric hollow paramagnetic nanoparticles	PEG, silica, iron oxide	48% (Doxorubicin and MTX)	Improved the deposition profile of NPs and cytotoxicity in the lung cancer	Nozohouri et al. (2019)
		50 nm		
Wormlike polymeric nanoparticles	Silica, MPEG, PCL	6% (MTX)	Dose-dependent inhibition of viability in MCF-7 breast carcinoma cells	Gharebaghi et al. (2017)
		150 nm		
pH-responsive polymeric nanoparticles	PCL (Conjugation of amino acid-based amphiphile)	20.52% (MTX)	Membrane lytic behaviour in the cancer cells, higher antiproliferative activity in MCF-7 and HepG-2 cells	Macedo et al. (2019)
		178.5 nm		
Self-assembled nanoparticles	O-carboxymethyl chitosan	10%–19% (MTX)	Improved therapeutic index	Wang et al. (2011)
		187 nm–363 nm		
Inorganic nanoparticles	PEG, silver	39.6% (MTX)	Improved anticancer activity against MCF-7	Muhammad et al. (2016)
		12 nm		
Inorganic organic conjugate nanoparticles	Polyglycerol, Fe ₃ O ₄	2.2%–19.6% (MTX)	Improved uptake of NPs by human head and neck cancer cells and improved anti-cancer activity	Li et al. (2013b)
		12–32 nm		
Inorganic organic conjugate nanoparticles	Magnesium nitrate, aluminium nitrate layered double hydroxide	9%–42% (MTX)	Controlled release of MTX	Chakraborty et al. (2011)
		200–458 nm		
Solid lipid nanoparticles	Stearic acid, soya lecithin, sodium taurodeoxycholate	51.3% (MTX)	Sustained release of drug for improved anticancer activity	Ruckmani et al. (2006)
		270 nm		
Solid lipid nanoparticles loaded carbomer gel	Glyceryl monostearate, oleic acid	62% (MTX)	Localization in deeper skin tissues for effective treatment of psoriasis	Tripathi et al. (2018)
		212 nm		
Lipid-polymer hybrid nanoparticles	Tween-80, PLGA, glyceryl tripalmitate, phosphatidylcholine	70%–86% (MTX)	Improved cellular internalization in glioblastoma cells (U-87 MG)	Bhattacharya (2021)
		173–233 nm		
pH-responsive magnetic nanoparticles	Cyclodextrin, ferric chloride	89% (MTX)	Higher retention of the NPs in the osteosarcoma Saos-2 cells	Ahmadi et al. (2020)
		20–80 nm		
Polymeric nanoparticles	Chondroitin sulfate	75% (MTX)	Higher uptake of NPs in the joints with the reduction in inflammation	Shilpi et al. (2019)
		68% (Aceclofenac)		
		131 nm		
Polymer nanoparticles	Linseed mucilage and chitosan	49.97% ± 0.91% to 72.25% ± 0.66% (MTX)	MTX release from NPs was pH-dependent and had strong antitumor effects; non-vitocytotoxicity on healthy cells	Ghumman et al. (2023)
		192.1–246 nm		
Polymeric nanoparticles	Pullulan	12.8% for MTX and 6.7% for HCPT	Cytotoxicity of MTX/HCPT-loaded NPs on cancer cells was higher than that of individual drug loaded NPs	Vakilnezhad et al. (2019)
		185.7 ± 16.7 nm		

(Continued on following page)

TABLE 1 (Continued) Some MTX-loaded nano-drug delivery systems.

Nanostructures	Composition	Encapsulation efficiency and average size	Reported outcomes	Ref
Magnetic nanoparticles	MNP-MTX-FR Ab (anti-folate receptor antibody)	50–150 nm	Higher uptake by FRs on the tumor cells	Lodhi et al. (2022)
Complex polymeric nanoparticles	Bovine serum albumin (FO-MTX-BSA-NPs)	—	Targeted cytotoxic impacts on MCF-7, HepG2, and PC3 and high antioxidant activity	Al-Rahim et al. (2022)
Nanoparticles	I ₂ -IR783/MTX@NPs	240.6 ± 2.5 nm	Used against breast cancer by PDT-chemotherapy	Muangsoa et al. (2023)
Magnetic nanoparticles	MTX-Transferrin-functionalized Fe(Salen)-polypyrrole nanocomposites	350–400 nm	Targeted photo-/magneto-thermal cancer treatments	Kim et al. (2022)

85 nm) was fabricated and proposed for MTX delivery. This nanocarrier had the highest release percentage at pH = 5.5°C and 40°C, conditions which were similar to cancerous cell. The experiments of DAPI staining and MTT assay of MTX-loaded nanocarrier confirmed the effective anticancer performance by degrading the cell nucleus DNAs against the A549 lung cancer cell line (Moradi et al., 2022). In order to decrease severe adverse effects, combination of chemotherapy with photothermal therapy (PTT) or photodynamic therapy has been proposed (Muangsopa et al., 2023). Polydopamine (pDA)-modified ZIF-8 NPs loaded with MTX (pDA/MTX@ZIF-8 NPs) were fabricated for chemo-photothermal therapy: the nano-system displayed excellent photothermal effects under NIR irradiation, which yielded antitumor effect and allowed decreasing the dose of MTX. These pH-responsive NPs induced apoptosis through reducing mitochondrial membrane potentials (MMPs) in MG63 cells (Yin et al., 2022).

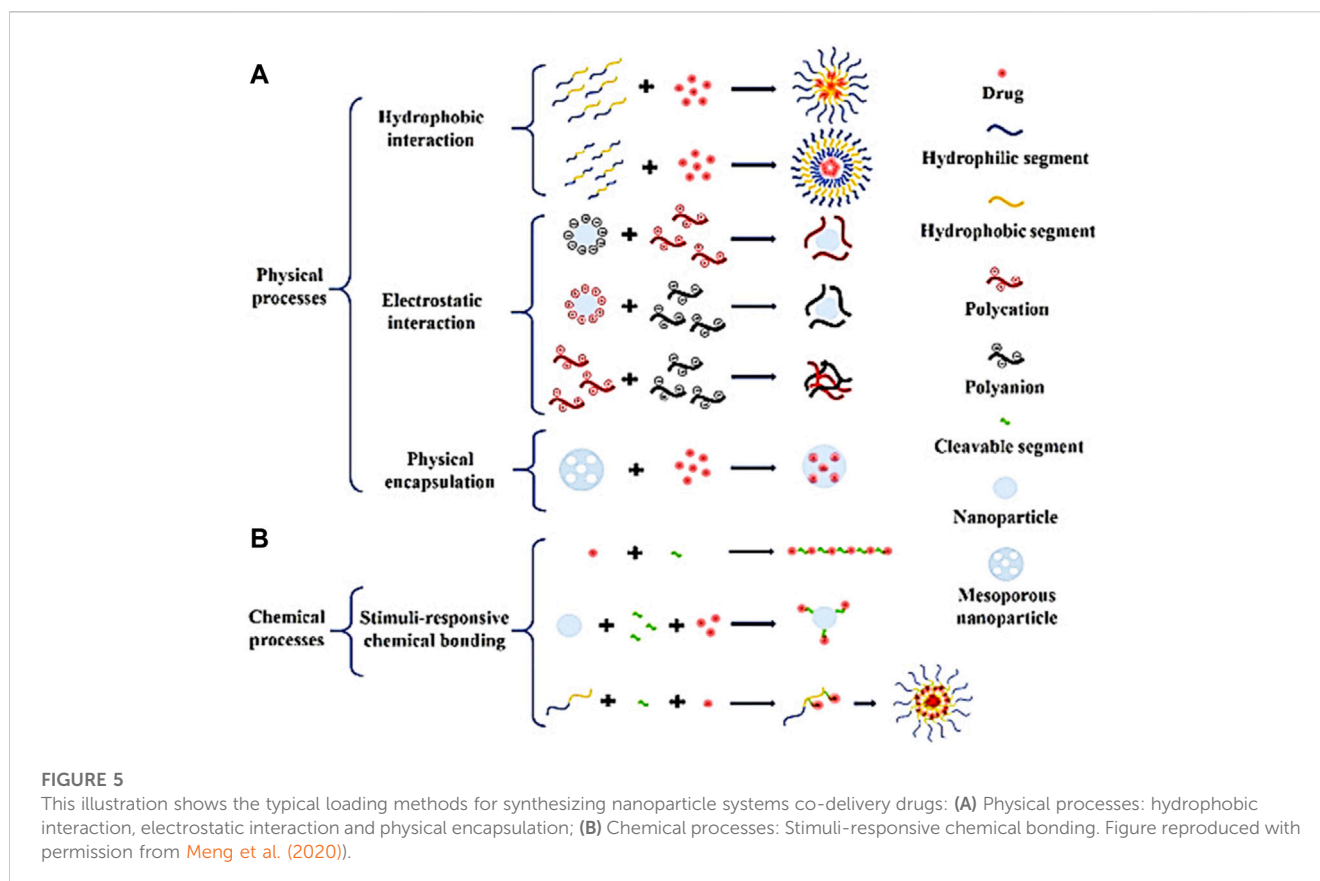
MoS₂ nanoflower (MoS₂ NF)-doped chitosan (CS)/oxidized dextran (OD) hydrogels were developed to treat colon cancer. 5-Fluorouracil (5-FU) was combined with polyethylenimine (PEI) and covered on MoS₂ nanoflower (MoS₂ NF)-doped CS/OD hydrogels to form 5-FU/PEI/MoS₂. In the next stage, 5-FU/PEI/MoS₂ was encapsulated by 1-tetradecanol (TD) to obtain TD/5-FU/PEI/MoS₂ (TFPM), which was in turn co-encapsulated with MTX in the CS/OD hydrogels. Because the electrostatic attraction between CS and OD is pH-sensitive, MTX and TD/5-FU/PEI/MoS₂ were released from the hydrogels at pH 7.4 (Liang et al., 2023).

In another study, Au and Fe NP conjugates with MTX were obtained by metal–vapor synthesis (MVS) and exhibited an average size of 8.3 and 1.8 nm, respectively. Anticancer activity based on MTT and NR assays was assessed. The results of Fe conjugates with MTX exhibited the highest toxicity as compared to MTX-loaded Au NPs against the lung adenocarcinoma cell line (Vasil'kov et al., 2023).

MnO₂ NPs coated with biocompatible poly(dimethyl-aminoethyl methacrylate-Co-itaconic acid) (DMAEMA-Co-IA)-conjugated MTX were prepared based on pH-sensitive targeted magnetic resonance imaging (MRI) contrast agent and innovative radio-sensitizing system. MTT assay for MnO₂@Poly(DMAEMA-Co-IA)-MTX NPs showed higher inhibitory effect on MCF-7 cell viability as compared to free MTX after 24 and 48 h with insignificant hemolytic activity (Ziyae et al., 2023).

4 Delivery and co-delivery systems of methotrexate with other drugs

The effectiveness of current cancer treatments is limited; therefore, researchers are studying the therapeutic and adverse effects of combining various drugs or treatment approaches (i.e., involving co-delivery drug systems) to achieve better outcomes (Mujokoro et al., 2020). Cancer treatments involving a single drug are often insufficient to treat cancer repeatedly due to potential drug resistance that might develop over time (Komarova and Wodarz, 2005; Zhang et al., 2017). Instead, a co-delivery drug system is required in combination therapies in the effort to avoid the development of drug resistance, just like in most clinical therapies where two or more therapeutic agents are used (Guo et al., 2014). The advantages offered by the co-delivery of drugs include the following: 1) extended half-lives of drugs, 2) enhanced drug targeting, 3) improved bioavailability, and 4) reduced adverse reactions to chemotherapy drugs (Torchilin, 2001; Qi et al., 2017). Co-delivery systems must be fabricated and designed according to the cargo (i.e., co-delivered drugs) and the vectors (i.e., the nanocarrier or nanoparticle) to finely tune the structural and physical properties. Therefore, effective co-delivery techniques should consider the interaction among the components, the balance of contradictory factors, and a synergistic treatment strategy (Yang et al., 2018; Meng et al., 2020). An example of co-delivered drugs currently in practice is paclitaxel (PTX) and cisplatin (DOP) which are delivered simultaneously for lung cancer therapy. Furthermore, choosing the appropriate vector type and drug loading strategy to successfully deliver cancer drugs is crucial. For example, when intravenously administering nanomedicine, factors to consider are 1) internalization, 2) circulation, 3) drug release, 4) accumulation, and 5) penetration to ensure a high level of therapeutic effectiveness (Blanco et al., 2015; Khan et al., 2015). Therefore, a weakness in one aspect of the co-delivery system will decrease its performance. In addition, designing co-delivery systems requires that vectors (i.e., micelles, organic/inorganic NPs, and microgels) and cargoes interact with each other and that the loaded reagents act synergistically (He et al., 2015; Xie et al., 2015; Xu et al., 2018). Moreover, it should be noted that although vectors (i.e., nanocarriers) pose some benefits (i.e., permeability and retention) as a result of their nanoscale, further modifications are still essential to enhance their



functionality, for example, their loading methods (as indicated in Figure 5). Furthermore, loading methods involve physical interactions (i.e., hydrophobic and electrostatic interactions), and they can be classified as either physical (i.e., simple agitation of reagents over time, e.g., polyanions) or chemical (i.e., several sequential chemical reactions, e.g., mesoporous NPs) process. These loading methods are widely studied and are found in the experimental data obtained from forty (40) different nanoparticle systems for the co-delivery of methotrexate (MTX) combined with other cancer-inhibiting drugs, reported in the literature from 2018 to 2022. Table 2 reports an overview of these studies with their advantages and the indications from *in vitro* and *in vivo* experiments for various cancers.

Recently, Shakeran et al. (2022) were the first to report on a new nanoparticle-based co-delivery system involving signal transducer and activator of transcription 3 (STAT3) siRNA (STAT3 is a key player in cell proliferation, survival, and cell division) (Ezra Manicum et al., 2022) and MTX in breast carcinoma cells. In this research, the authors functionalized mesoporous silica NPs with the polysaccharide chitosan, grafted covalently by aminopropyl triethoxysilane and glutaraldehyde, as illustrated in Figure 6. In addition, the co-delivery system (i.e., MTX and STAT3 siRNA) was studied using analytical methods (i.e., confocal laser scanning fluorescence microscopy and flow cytometric analysis) to treat breast carcinoma (i.e., MCF-7 cells). This co-delivery system was useful to considerably decrease the viability of the breast cancer cells. In another study, biodegradable mPEG-PCL copolymer NPs were fabricated by polymerization of ϵ -caprolactone dehydrated monomer in the presence of the initiator

(dry methoxy polyethylene glycol) and catalyst ($\text{Sn}(\text{Oct})_2$). These NPs were used as carriers for the delivery of MTX (average size of 51.89 ± 0.46 nm) or co-delivery of MTX and curcumin (average size of 88.21 ± 0.31 nm) after being conjugated with the drug(s). The amount of drug released from NPs at pH = 5.5 was higher than that released at pH = 7.4. MTT results showed lower cell survival with increasing drug concentration inside the NPs. The NPs conjugated with MTX and curcumin were associated to the highest cancer cell mortality due to pH sensitivity and synergistic effect/accumulation of drugs at tumor sites (Danafar et al., 2022).

In 2021, Shakeran et al. (2021) evaluated the cytotoxicity of the delivery system involving MTX-loaded, functionalized mesoporous silica NPs (MSNs) against breast cancer cells using confocal microscopy. Their findings were that the co-delivery system was effective against MCF-7 cells at a low dose of $0.5 \mu\text{M}$ MTX.

In addition, Rathee et al. (2022) demonstrated the promising carrier ability of nanostructured lipid carriers (NLCs) for MTX in the cytotoxic investigation (i.e., MTT assay) against MCF-7 and HeLa cell lines. This investigation revealed that the suggested drug delivery system exhibited significant cytotoxicity towards the specified cell lines, killing 55% of the HeLa cells and 40% of the MCF-7 cells.

Rui et al. (2022) recently synthesized a straightforward drug delivery system involving aminated mesoporous silica NPs (AMSNs) to load MTX, which was then encapsulated by electrostatic interaction with hyaluronic acid (HA). Spectroscopic methods (i.e., electron microscopic techniques, fourier transform infrared technique, and X-ray diffraction analysis) were used to confirm the successful synthesis of the drug delivery system. Flow

TABLE 2 MTX nanoparticle-based delivery and co-delivery systems to treat cancer.

Nanoparticle/Nanocarrier	Co-delivered drug	Advantages	Indication	References
Chitosan modified MSNs	MTX and STAT3	Decreased viability of breast cancer cells	Breast carcinoma	Shakeran et al. (2022)
Poly(DMAEMA-block-HEMA-block-AAc)	MTX and DOX	Small size micelles target poorly vascularized tumors		Saracai et al. (2019)
SPION	MTX	SPIONS can guide drugs to the desired locations in the body	Breast carcinoma	Ong et al. (2020)
PEG	MTX and DOX	Opsonization by alveolar macrophages	Lung carcinoma	Nozohouri et al. (2019)
chitosan-modified mesoporous silica	MTX	Controlled release and complete drug elution	Breast carcinoma	Shakeran et al. (2021)
Chitosan-based nanomicelle	MTX	The effective delivery system for cancer therapy		Mahmoodzadeh et al. (2019)
PEG	MTX and Cisplatin	Synergistic properties of cancer therapy	Lung carcinoma	Akbari et al. (2021b)
Poly(D,L-lactide-coglycolide)	MTX and Curcumin	Overcome sophisticated cellular pathway	Glioma	Mujokoro et al. (2020)
Liposomes	MTX	Increased residence time and drug release	Oral carcinoma	Jin et al. (2018)
Albumin lipid-polymer	MTX and CON	Increased cellular uptake of CON and MTX	Breast carcinoma and leukemia	Khalili et al. (2021)
Chitosan	MTX and PMX	Increased tumour growth and duration of blood circulation	Lung carcinoma	Chen et al. (2018)
Lipids	MTX	Improve the stability of bioactive agents and their bioavailability	Breast carcinoma	Rathee et al. (2022)
Poly(lactic-co-glycolic acid) PEG polymer	MTX and Trapoxin A	High antitumor activity	Breast carcinoma	Akbari et al. (2021a)
mPEG-bpoly(TAC-co-ATMC-g-S(CH ₂) ₁₀ COOH)	MTX and Doxorubicin (DOX)	Enhanced synergistic antitumor efficacy	Breast carcinoma	Li et al. (2019)
Gelatin	MTX	Four fold significant reduction in methotrexate IC ₅₀	Lung carcinoma	Abdelrady et al. (2019)
PEG	MTX and Curcumin	The local accumulation of the medication in the tumour's acidic tissue	Breast carcinoma	Curcio et al. (2018)
Lipid-Polymer	MTX and Aceclofenac	Synergistic anti-cancer effect	Breast carcinoma	Garg et al. (2017)
Aminated mesoporous silica nanoparticles	MTX	Good biocompatibility	Hepatoma	Rui et al. (2022)
Hydroxyapatite	MTX and Gemcitabine	Non-cytotoxic and dose-dependent cytotoxicity	Bone carcinoma	Ram Prasad et al. (2019)
Magnetite incorporated into chitosan	MTX	Higher cytotoxicity towards melanoma cells	Skin carcinoma	Bruckmann et al. (2022)
Niosomes	MTX and Curcumin	Effective codelivery and increased cytotoxicity	Colon carcinoma	Mousazadeh et al. (2022)
Pullulan	MTX and HCPT	Excellent therapeutic effects against drug-resistant tumors	Cervical carcinoma	Wu et al. (2022b)
PLGA	MTX and Curcumin	Significantly higher cytotoxicity	Breast carcinoma	Vakilinezhad et al. (2019)
Carrier-free	MTX, DOX, and Camptothecin	Synergistic therapeutic effect		Hou et al. (2018)
Liquid crystalline nanoparticles	MTX and Lactoferrin	Higher cellular uptake	Breast carcinoma	Mokhtar et al. (2022)
PEI and a p53 encoding plasmid DNA	MTX	Significant reduction of viability		Faria et al. (2019)
Conjugated albumin	MTX and Curcumin	Effective co-delivery		Nosrati et al. (2019)
Lipids	MTX, DOX and DTX	Synergistic cytotoxicity and reactive oxygen species	Breast carcinoma	Mahoutforoush et al. (2021)
PLGA	MTX and Paclitaxel	Promising the treatment of glioblastoma	Glioblastoma	Madani et al. (2020)
β-Cyclodextrin	MTX and 5-Fluorouracil	Localized sustained release	Breast carcinoma	Almawash et al. (2022b)

(Continued on following page)

TABLE 2 (Continued) MTX nanoparticle-based delivery and co-delivery systems to treat cancer.

Nanoparticle/Nanocarrier	Co-delivered drug	Advantages	Indication	References
Magnetic nanoparticles	MTX and DOX	Notable cytotoxicity	Breast carcinoma	Javanbakht et al. (2020)
Halloysite nanotubes	MTX and Biotin	Increase the cellular absorption and therapeutic effects	Leukemia	Massaro et al. (2022)
<i>Ganoderma lucidum</i> polysaccharides	MTX and HCPT	improved tumour-suppressive activity and reduced adverse effects <i>in vivo</i>	Breast carcinoma	Zheng et al. (2021)
PEGylated-nano gel	MTX and 6-mercaptopurine	Improved efficiency	Breast carcinoma	Nezhad-Mokhtari et al. (2019)
Polymer	MTX and Chrysin	Enhanced the efficacy	Breast carcinoma	Davaran et al. (2018)
Chitosan-coated-MOFs	MTX	Remarkable potency	Colon carcinoma	Khatibi et al. (2022)
Micelles	MTX and siRNA	Elevated tumor uptake and inhibited tumor growth	Cervical carcinoma	Hao et al. (2019)
Micelles	MTX and Mitomycin C	Improved the tumor accumulation and penetration		Li et al. (2015)
Dendritic chitosan	MTX and DOX	Apoptotic effects are higher	Breast carcinoma	Rahimi et al. (2017)
Fe ₃ O ₄ and PLGA-PEG	MTX	Higher cell viability	Breast carcinoma	Basu et al. (2018)
HBPLC-PEG-FA	MTX and DOX	pH-responsive behavior (DOX: 92.7% and MTX: 97.02% at pH 5, Biocompatible and biodegradable	Breast carcinoma	Aslani and Namazi (2022)
MgAl-LDH/Fe-MOF	MTX and DOX	Bioimaging potential, Controlled drugs release	Breast carcinoma	Pooresmaeil and Namazi (2022)
Niosomes nanocarrier	MTX and Cur	More toxicity	Colon carcinoma	Mousazadeh et al. (2022)
Cubosomes	MTX and Lactoferrin	Elevated tumor uptake and inhibited tumor growth	Breast carcinoma	Mokhtar et al. (2022)
MNPs-HA	MTX	Better time- and dose-dependent cytotoxic effect along with increase Bak1 protein levels	A549 lung cancer cell line	Dou et al. (2022)
Gli(gliadin)/CAS (sodium caseinate) nanogels	MTX	Enhancement of cancer cell apoptosis, good biocompatibility and pH-dependent drug release	HepG2 cells	Xu et al. (2022)
F-127 pluronic nanomicelles (PIP-NMs)	MTX	Enhancement of cancer cell apoptosis	MCF-7 cells	Mansour et al. (2022)

Methotrexate (MTX), Signal transducer and activator of transcription 3 (STAT3); Doxorubicin (DOX); Superparamagnetic iron oxide nanoparticles (SPION); Polyethylene glycol (PEG); Poly(lactic-co-glycolic acid) (PLGA); metal-organic frameworks (MOFs); 10-hydroxycamptothecin (HCPT); docetaxel (DTX); Conferone (CON); Pemetrexed (PMX); piperine (PIP); gliadin (Gli).

cytometry experiments further showed increased inhibitory effects of the co-delivery system on hepatoma (SMMC-7721) cells, while the medication-free carrier was highly biocompatible.

In another study, Bruckmann et al. (2022) showed the usefulness of MTX and magnetic NPs in an *in vitro* study against melanoma cells. In addition, the investigation reported the high cytotoxicity displayed by the drug delivery drug for use in skin cancer treatment.

Mousazadeh et al. (2022) described the of niosomes comprising MTX and curcumin (CUR) for the co-delivery impact on human colorectal carcinoma cell lines (HCT-116). These niosomes were made utilising the thin-film hydration approach, and the codelivery system was effective against the indicated cancerous cells.

MTX and 10-hydroxycamptothecin (10-HCPT)-loaded pullulan NPs were assessed by Wu et al. (2022b) as a synergistic medication delivery mechanism for cancer therapy. Furthermore, MTX served as a targeting compound and a cytotoxic drug in the synthesized co-delivery system. According to the authors, MHNPs

induced more cytotoxicity in HeLa cells when compared to individual drug-loaded NPs, suggesting that the co-delivery system make drugs to act synergistically and may be a practical antitumor agent.

In 2022, Mokhtar et al. (2022) described a hybrid nanocarrier co-delivery system for the concurrent distribution of MTX and aromatase inhibitor exemestane (EXE) for breast cancer treatment. First, EXE was enclosed physically in liquid crystalline NPs (LCNPs) using a carbodiimide reaction. Next, MTX was bonded chemically to lactoferrin (Lf), followed by the coating of the EXE-loaded LCNPs with MTX-Lf. The EXE and MTX exhibited a considerable synergistic effect on breast cancer cells after being embedded in the LCNPs. According to the study, dual drug-loaded LCNPs offer as a combined application of hormone treatment and chemotherapy agent for breast cancer.

Almawash et al. (2022b) synthesized a 5-Fluorouracil and MTX co-delivery system to be used as an anticancer agent in an *in vivo*

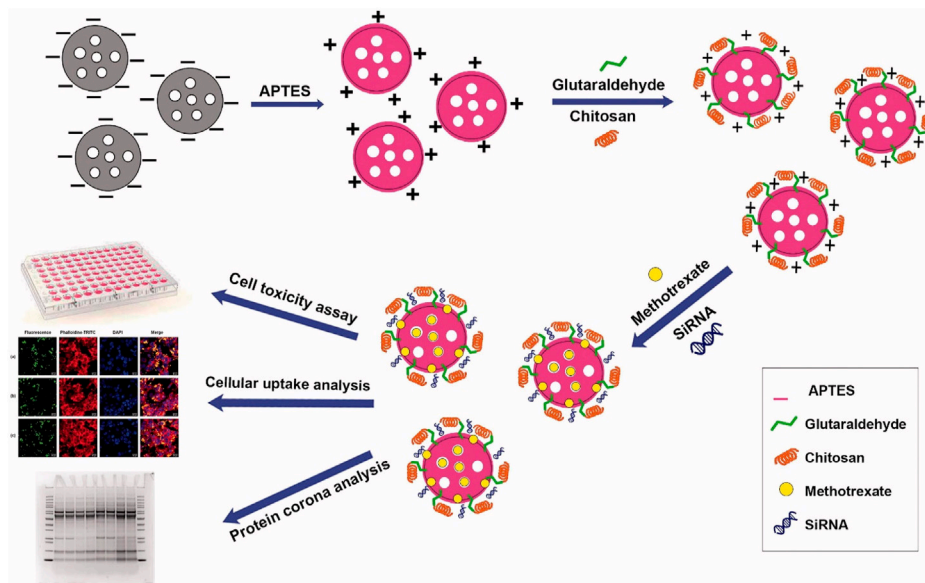


FIGURE 6
This illustration shows the delivery system involving signal transducer and activator of transcription 3 (STAT3) siRNA and MTX. Figure inserted with permission from (Shakeran et al., 2022).

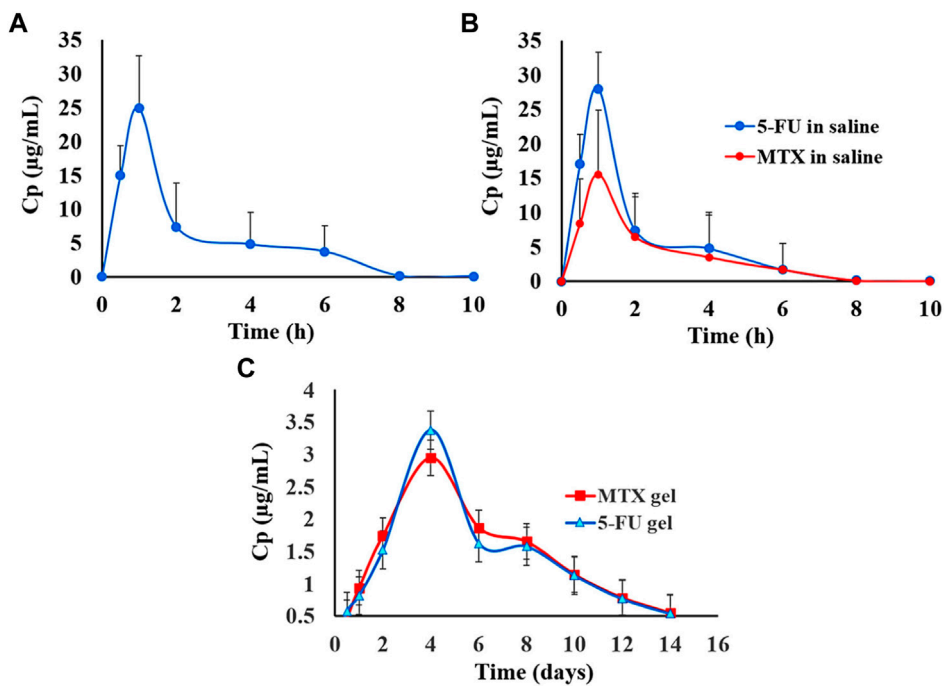


FIGURE 7
Graphical illustration comparing the *in vivo* release of 5-FU administered alone (A); 5-FU administered in combination with MTX (B); 5-FU administered alone and combined with MTX (C). Figure reproduced with permission from (Almawash et al., 2022b).

study on female rats against breast carcinoma. The study outcomes showed that this nano-system could load and release medication sustainably over 14 days, as illustrated in Figure 7.

An additional study by Massaro et al. (2022) reported on a “smart” co-delivery system involving MTX and biotin loaded on hallosite nanotubes (HNTs) for anticancer applications with a

specific focus on leukaemia. Thermographic and spectroscopic methods were used to characterize the nano-based drug delivery system. In the biological assays, both the biotinylated and MTX prodrugs played critical roles in the biological action of the cancerous treatment agents and showed promise.

Khatibi et al. (2022) created a delivery structure comprising a novel nanometal-organic framework (NMOF) covered with folic acid (FA) and chitosan (CS), referred to as MOF-CS-FA-MTX, for the delivery of MTX to colon carcinoma cells. The biological assays showed that the MOF-CS-FA-MTX co-delivery system has colon cancer inhibitory potential and is a viable system for cancer treatment.

Akbari et al. studied the efficient entrapment of Cisplatin (CDDP) and MTX into the bio-degradable poly(ϵ -caprolactone) (PCL)-poly(ethylene glycol) (PEG) copolymer, MTX@CDDP-loaded PCL-PEG, against lung cancer cell lines (Akbari et al., 2021b). Microscopic (i.e., scanning electron microscopy) characterization of the morphology and topology of the NPs, was carried out and it was established that the NPs had a spherical shape. The assay on lung cancer cells (A549) also showed that the formulation MTX@CDDP effectively killed lung cancer cells. Furthermore, the nanoparticle formulation MTX@CDDP-loaded PCL-PEG was promising in treating non-small-cell lung cancerous.

Khalili et al. (2021) synthesized a new co-delivery system consisting of MTX, conferone (CON), and a lipid/polymer hybrid NPs (i.e., MTX@CON-TLPN) for the concurrent delivery of multiple anticancer drugs. The synthesized material was characterized using spectroscopic methods (i.e., FTIR, ^1H NMR, dynamic light scattering (DLS), transmission electron microscopy (TEM), and SEM). This nano-system was adequate to deliver medications to cancer cells according to cell cycle modulation, cell death evaluation, and cellular internalization assessments.

In another research by Akbari et al. (2021a), they demonstrated the synergistic anticancer impact of a co-delivery system consisting of Trapoxin A (TPX) and MTX that were loaded on poly(lactic-co-glycolic acid)-polyethylene glycol NPs (PLGA-PEG) for successfully treating breast cancer (i.e., MCF-7 cells).

Mahoutforoush et al. (2021) used nanostructured lipid carriers (NLCs) to prevent carcinoma cell proliferation with MTX as a targeting moiety and chemotherapy agent in conjugation with DTX and DOX (NLCs/DTX/DOX/CS-MTX). Spectroscopic methods were used to evaluate the physicochemical characteristics of the synthesized nano-complexes, and the compounds were tested against human breast carcinoma cells (i.e., MFC-7) and lung carcinoma cells (i.e., A549). Among other factors, the NLCs/DTX/DOX/CS-MTX co-delivery system demonstrated synergistic cytotoxicity against cancer cells, with good potential for successful treatment.

Zheng et al. (2021) produced a nanoparticle-based drug co-delivery system from Ganoderma lucidum polysaccharides, MTX, and 10-hydroxycamptothecin (HCPT) (i.e., GLP-APBA-MTX/HCPT). According to this study, GLP-APBA-MTX/HCPT NPs were more effective and had fewer *in vivo* side effects as compared to using the free drugs, indicating that they could be an effective tumor-suppressing treatment.

In order to reduce side effects, colon-targeted NPs based on biocompatible poly(methacrylic acid) (PMAA) nanohydrogels were investigated for possible use in combined therapy by simultaneous

loading of MTX and chloroquine (CQ) (PMAA-MTX-CQ). Drug loading efficiency of this NP system was 4.99% for MTX and 25.01% for CQ1; furthermore, pH/enzyme-triggered drug release behavior was observed. Higher CQ release rate (76%) was detected under simulated acidic microenvironment of tumor tissue whereas 39% of CQ was released under normal physiological conditions. MTX release was facilitated by the presence of proteinase K enzyme. The reported findings suggested that dual release exerted a significantly higher killing effect against SW480 colon cells as compared with single-drug therapy. Furthermore, nanohydrogels did not elicit hemolysis (Rashidzadeh et al., 2023).

5 Conclusion, outlook, and perspectives

MTX is a drug with significant therapeutic potential for various ailments, including cancer and immunological disorders. However, it is well proven that the residual tumour cells can resist a great deal of stress after the chemotherapy process, leading to a tumour with a more complex composition. Because of MTX's poor pharmacokinetics, drug resistance, low bioavailability, and dose-dependent adverse effects, conventional drug delivery methods are constrained in their therapeutic potential. In this regard, the use of alternative delivery technologies has offered a significant chance to overcome these limitations of MTX. Drug delivery proposed platforms based on NPs, such as polymer and lipid-based NPs, as well as inorganic nanostructures, are extremely significant for improving the biological availability of MTX at the place of activity, as shown by *in vitro* studies and *in vivo* animal models. Consequently, the side effects of the drug on healthy tissues may be mitigated by synthesizing targeted NPs using a straightforward and effective procedure. However, a few main factors, such as nanomaterial-based MTX formulations, need to be considered to advance the use of NPs in delivery systems. In principle, the unique features of NPs allow for the controlled release of MTX. In this system, matrix type and drug loading technique are two important factors to consider for the delivery of anti-cancer medications. In another strategy, the co-delivery of small drug molecules like MTX and other pharmaceuticals may be regarded as an innovative technique to counteract some of the adverse effects of using MTX alone for an extended period. Co-delivery can reduce drug resistance, especially in long-term and overuse application. This strategy decreases undesirable effects without raising the initial dose, enabling clinical adoption for new objectives.

Due to uncontrolled drug release, lack of specificity in both normal and cancer cells, and drug resistance, novel drug delivery systems (DDSs) are needed not only to overcome adverse effects of MTX but also to increase therapeutic efficacies by improving bioavailability and targeting functions with the aid of delivery vehicles. Therefore, in the future, considerable attention should be paid to improve carriers including MOF- or COF-based stimuli-responsive DDSs such as pH-, temperature-, magnetic-, ion-, pressure-, H_2S -, redox-, and photo-responsive nano-platform. In addition, other (nano)systems relying on photodynamic and photothermal effects as well as their combination with other stimuli-driven responses (e.g., stimuli-responsiveness to H_2O_2) and/or other chemotherapeutics deserve to be considered to overcome drug resistance and side effects in normal cells.

Nevertheless, it cannot be ignored that the clinical implementation of these multifaceted approaches in the routine clinical practice has to face several obstacles from the viewpoints of both functional validation (e.g., quantitative assessment of the synergy between drugs and other co-strategies) and regulatory approval. Therefore, many of these carriers and “intelligent” biomaterials still require additional investigation to ensure the highest level of safety and effectiveness for human use.

Author contributions

Conceptualization, AR; writing-original draft preparation, MM, A-LE, MS, RB, and RE; writing-review and editing, SG, FB, and AR; supervision, AR, FB, and SG. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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