



# Thymoquinone-based nanotechnology for cancer therapy: promises and challenges

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**Thymoquinone (TQ), the active ingredient of black seed, is a promising anticancer molecule that inhibits cancer cell growth and progression *in vitro* and *in vivo*. Despite the promising anticancer activities of TQ, its translation to the clinic is limited by its poor bioavailability and hydrophobicity. As such, we and others encapsulated TQ in nanoparticles to improve its delivery and limit undesirable cytotoxicity. These TQ-nanoparticle formulations showed improved anticancer and anti-inflammatory activities when compared with free TQ. Here, we provide an overview of the various TQ-nanoparticle formulations, highlight their superior efficacy and discuss up-to-date solutions to further enhance TQ bioavailability and anticancer activity, thus improving potential for clinical translation.**

## Introduction

Thymoquinone (TQ: 2-isopropyl-5-methylbenzo-1,4-quinone), the main active molecule in *Nigella sativa* essential oil, is a promising candidate for the treatment of various diseases including cancer. A systematic literature review emphasized TQ as a prominent molecule having promising antineoplastic effects against a wide range of solid and liquid tumors in *in vitro* and *in vivo* models [1]. What makes TQ interesting is its efficacy and selectivity against cancer cells and lack of toxicity in normal tissues [1]. For instance, TQ has shown anticancer activities against breast cancer cell lines by targeting peroxisome proliferator-activated receptors (PPARs) [2] and nuclear factor (NF)-κB [3]. It has shown chemo-preventive activities through inhibition of cell growth, induction of apoptosis and modulation of transcription factor NF-κB, as well as chemosensitizing efficacy to gemcitabine and oxaliplatin in pancreatic cancer [4]. TQ has also been reported to inhibit colon cancer growth and invasion, and induce cell cycle arrest and apoptosis in colon cancer cell culture and animal models [1]. Interestingly, TQ modulates Wnt signaling through glycogen synthase kinase (GSK)-3β activation, β-catenin translocation and reduction of

nuclear c-myc. TQ has been shown to mediate reactive oxygen species (ROS) damage in colon cancer, adult T cell leukemia and prostate cancer. In other systems, TQ exhibits strong antioxidant activity by upregulating superoxide dismutase (SOD), catalase (CAT) and glutathione (GPX) (reviewed in Ref. [5]).

Many studies have documented the adjuvant ability of TQ to improve the potency of several chemotherapeutic agents by enhancing their anticancer activity and/or alleviating their toxicity. Owing to these pleiotropic properties, TQ has been shown to alleviate toxicities related to chemotherapeutic agents such as cisplatin nephropathy, doxorubicin cardiotoxicity and acetaminophen hepatotoxicity, among others (reviewed in Ref. [1]). The oral administration of TQ was found to be safe in several animal models [6]. *In vivo*, TQ was not toxic at concentrations higher than its biologically active dose. The LD<sub>50</sub> of TQ in mice and rats ranged between 57 and 104 mg/kg when injected intraperitoneally, and 794 and 870 mg/kg when administered orally – concentrations that are much higher than the dose at which TQ exerted its anticancer activity (<10 mg/kg) (reviewed in Ref. [5]). Unfortunately, very few studies investigated the pharmacokinetic and pharmacodynamic characteristics of TQ. One study showed that TQ is reduced into hydroquinone by catalyzing liver enzymes [7] and was detected in the plasma of rats for up to 12 h post oral

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administration [8]. In rabbits, the absolute bioavailability of TQ upon oral administration was 58% with a lag time of 23 min, and 99% of TQ was bound to plasma proteins [9]. Identifying TQ binding targets *in vivo* and determining their distribution profile can greatly help in better understanding its pharmacological properties and the molecular mechanisms of action of this interesting compound.

Despite the promising anticancer activities of TQ, the main limitation for its clinical translation lies in its hydrophobicity, poor bioavailability, sparing solubility, light- and pH-sensitive nature [10], and capacity to bind to plasma proteins, which prevents it from reaching its targeted tumor sites. Owing to all these limitations, there are no reported studies on the anticancer therapeutic potential of TQ in humans. Studies on the safety profiles of TQ in humans are very limited. TQ was found to be well tolerated in patients up to 10 mg/kg/day in a Phase I clinical trial but showed no significant anticancer activity at this dose [11]. A recent Phase II clinical study evaluating the effect of 100 mg and 200 mg TQ on oral potentially malignant lesions is currently registered; however, it is still not open for participant recruitment (<https://clinicaltrials.gov/ct2/show/NCT03208790?term=thymoquinone&rank=1>).

Nevertheless, several clinical trials testing the effect of *Nigella sativa* on various diseases including beta thalassemia major in children, dyslipidemia and arsenical keratosis (<https://clinicaltrials.gov/ct2/results?cond=&term=nigella+sativa&cntry1=&state1=&recrs=#tableTop>) have shown that it is not toxic in patients at doses up to 1000 mg/day.

TQ nanoparticle research could serve as a new platform for overcoming these limiting factors to promote clinical testing of TQ. Nanoparticle encapsulation of TQ could improve its bioavailability, delivery and targeting capacity as well as protect it from unspecific binding. Thus, this review summarizes the most promising TQ nanoparticle formulations, their characteristics and applications, and highlights the approaches used for the formulation of clinically used drugs that are related to TQ. Nanoparticles of TQ are becoming more clinically attractive than free TQ because of their enhanced activity in modulating cancer hallmark targets *in vitro* and increased bioavailability and distribution *in vivo*. So far, several TQ nanoparticle formulations have been tested against colon, prostate, cervical and breast cancer, as well as leukemia and multiple myeloma [12–15]. These include polymeric, liposomal, niosomal, solid lipid nanocarriers (SLNs) and nanostructured lipid carriers (NLCs), among others (summarized in Table 1). In addition to reported anticancer activities, TQ nanoparticles were shown to exhibit antibacterial properties against *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhi* strains [16], antifungal potential against *Candida albicans* yeasts and *Candida* biofilms [17], and neuroprotective effects when administered orally in rats [18].

## Thymoquinone nanoparticles

### Polymeric TQ nanoparticles

Polymeric nanoparticles have many advantages that make them promising for drug delivery. This includes enhanced biodegradability, controlled or sustained release, small size and biocompatibility with tissues and cells. They are relatively nontoxic and are stable in blood and lack immunogenicity and thrombogenicity [19]. Polymeric nanoparticles can be synthesized by several meth-

ods depending on the drug to be encapsulated. As such, various polymeric nanoparticle formulations have been used for drug encapsulation and many have been FDA approved (Table 2). Below we discuss the polymeric formulations used for the encapsulation of thymoquinone.

### Poly-lactide-co-glycolide TQ nanoparticles

PLGA (poly-D,L-lactide-co-glycolide) is one of the most used polymers for the development of nanomedicine that has been FDA approved. It is associated with minimal systemic toxicity because it is hydrolyzed in the body to biodegradable lactic acid and glycolic acid, which are metabolized in the body via the Krebs cycle and removed as carbon dioxide and water [10,20]. Initially, the negatively charged PLGA nanoparticles and their poor transport across mucosal barriers limited their potential in drug delivery. However, the problem was soon overcome by blending PLGA nanoparticle formulations with alginate, chitosan, pectin, poly(propylene fumarate), polyvinyl alcohol (PVA) and poly(orthoester). Interestingly, PLGA nanoparticles were effective against *E. coli*, *S. aureus* and *S. typhi* strains, suggesting a wide range of therapeutic application in cancer therapy and food samples. PLGA was also used for encapsulation of diabetic, psychotic, hormonal and tetanus drugs.

TQ-PLGA nanoparticles are prepared by two methods. The nanoprecipitation method includes acetone-dissolved polymer, with or without emulsifier or stabilizer, that is added dropwise into the continuously rotating aqueous phase followed by a vacuum to evaporate the organic solvent. By contrast, the emulsification solvent evaporation method includes polymers that are dissolved in volatile organic solvent then added into the continuously rotating aqueous phase with or without emulsifier and sonicated [19]. Both methods follow a relatively simple procedure and are appropriate for the encapsulation of lipophilic drugs [21,22]. The main differences between the two methods lie in the scale-up and entrapment efficiency [21,22]. In the emulsification solvent-evaporation method, the possibility of scaling up is low because of the high-energy requirements in homogenization and the entrapment efficiency is moderate. The nanoprecipitation method, by contrast, is easily scaled up and leads to high entrapment efficiency, which makes it the most commonly used method for the preparation of PLGA nanoparticles [19].

TQ-loaded PLGA nanoparticles have been synthesized by emulsion solvent evaporation using anionic molecular micelles as emulsifiers [23]. These nanoparticles were more effective than free TQ in inhibiting the growth of MDA-MB-231 breast epithelial cancer cells [23]. The emulsion solvent evaporation method was also used by Nallamuthu *et al.* [16] to synthesize TQ-encapsulated PLGA nanoparticles and to evaluate its antioxidant and antibacterial activities. Notably, Soni *et al.* [24] employed emulsion solvent-evaporation and a stabilizer: PVA, to prepare dual drug-loaded TQ and paclitaxel nanoparticles. Paclitaxel-loaded PLGA nanoparticles showed higher efficacy in breast cancer cells at lower drug doses [24]. This suggests the feasibility of using TQ-loaded PLGA nanoparticles in clinical trials. In addition to paclitaxel, various anticancer drugs have been encapsulated by PLGA nanoparticles including vincristine sulfate, cisplatin, etoposide, doxorubicin, dexamethasone, rapamycin, among others [25]. Despite all the advantages, TQ-loaded PLGA nanoparticles have failed to reach the clinic so far. This could be attributed to low drug-loading efficiency, high-cost of production or difficulty of the scale-up.

TABLE 1

## Overview of TQ-nanoparticle formulations, their characteristics and applications

Particle class	Materials	Method	Size (nm)	Encapsulation efficiency (% EE)	Zeta potential (mV)	Biological effects	IC <sub>50</sub> of TQ versus TQ-NP formulation	Refs
Polymer carriers	PLGA	Emulsification solvent evaporation	100–200	10–35	–60 to –62	-Not toxic on normal breast cells -Inhibit proliferation of MDA-MB-231 breast cancer cells	TQ (0.132 ± 0.003 mg/ml) TQ-NP (0.030 ± 0.002 mg/ml)	[23]
			<200	62	–24.8	-Antioxidant and antibacterial property against <i>Escherichia coli</i> , <i>Staphylococcus aureus</i> and <i>Salmonella typhi</i> strains	N/A	[16]
	PHA-mPEG	Emulsification solvent evaporation	112–162	30–60	–18 to –27	-Not toxic to prenatal rat neuronal hippocampal and fibroblast cells	N/A	[15]
	PHV-mPEG	Modified emulsification solvent evaporation	200	25	–14	-Safe carriers for the controlled release of variety of hydrophobic drugs		[14]
	β-cyclodextrin	Self-assembly	445	N/A	21.8	-Not toxic to human periodontal fibroblasts -Enhance TQ's anticancer potential on MCF-7 breast cancer cells	TQ (24.09 ± 2.35 μM) TQ-NP (4.70 ± 0.6 μM)	[32]
	PEGylated	Nanoprecipitation	<50	80–97	–2.5 to –15	-Not toxic to normal peripheral blood mononuclear cells (PBMC) -Decrease the rate of migration of MCF-7 and HBL-100 cells -Hinder cytoskeletal actin polymerization through upregulation of miR-34a in a p53-dependent manner	N/A	[13]
	Ethyl cellulose (EC)	Emulsification solvent evaporation	235	91	–37	-Reduce tumor growth rate in colorectal cancer mouse xenograft models	N/A	[29]
	Polyaprolactone (PCL)		406	88	–13			
	PLGA		287	76	–19			
	PLGA (TQ-PTX dual-loaded drug)	Emulsification solvent evaporation	227	PTX (82.4) TQ (65.8)	PTX (–6.92) TQ (–10.43)	-Enhance anticancer activity as compared to the free drugs in MCF-7 breast cancer cells -Synergistic interaction between TQ and PXT	TQ (8.33 ± 0.98 μM) PXT (625.26 ± 23.6 nM) PXT-NP (219.69 ± 19.72 nM) TQ-NP (6.24 ± 0.98 μM) TQ-PTX dual-loaded drug (161.68 ± 17.4 nM)	[24]
	Amphiphilic deblock copolymers of PS-PEO-	Flash nanoprecipitation using inlet vortex mixer	79.2	80	N/A	-Not toxic to MCF-10-A normal breast cells -Enhance anticancer activity in MCF-7 breast cancer cells	TQ (>100 μM) TQ-NP (30 μM)	[33]

TABLE 1 (Continued)

Particle class	Materials	Method	Size (nm)	Encapsulation efficiency (% EE)	Zeta potential (mV)	Biological effects	IC <sub>50</sub> of TQ versus TQ-NP formulation	Refs
Lipid based	Nano-structured lipid carriers (NLC)	High pressure homogenization	75	N/A	-31	-Not toxic to rats and in normal human liver cells -Inhibit the formation of ethanol-induced ulcers -Modulate heat shock protein 70 (Hsp70)	TQ (10.5 ± 0.37 µg/ml) TQ-NP (20.1 ± 0.9 µg/ml)	[48]
		Hot pre-emulsion and crystallization	<50	95–97	>-30	-Not toxic toward normal cell lines (3T3-L1 and Vero) -Dose-dependent antiproliferative activity against breast (MCF-7 and MDA-MB-231) and cervical (Hela and SiHa) cancer cells -Induce apoptosis and cell cycle arrest	TQ (N/A) TQ/NP (4.47 ± 0.06 µM)	[49]
		High-pressure homogenization	33.39	98.96	-10.43	-Less toxic than pure TQ when administered orally to mice	N/A	[50]
		High-speed homogenization followed by ultrasonication	N/A	84.6–96.2	N/A	-Two- to three-fold increase in the bioavailability of TQ -Loaded-TQ show hepatoprotective and antioxidant efficacy	N/A	[51]
Liposomes		Thin film hydration	100	50–90	22	-Not toxic on normal periodontal ligament fibroblast -Inhibit the proliferation of breast cancer cell lines (MCF-7 and T47D); however, less potent than free TQ	TQ (15 µM and 40 µM in T47D and MCF-7, respectively) TQ-NP (75 µM and 200 µM in T47D and MCF-7, respectively)	[38]
Gold niosomes (Au-Nio)		Click chemistry	150	82	1.14	-Inhibit the viability of tamoxifen-resistant and AKT over expressing breast cancer cells -Inhibit MDM-2 and induce p53-mediated apoptosis	TQ (69.69 µl/ml in tamoxifen-resistant cells and 65.29 µl/ml in AKT overexpressing cells) TQ-NP (39.05 µl/ml in tamoxifen-resistant cells and 35.46 µl/ml in AKT overexpressing cells)	[39]
Solid-lipid nanoparticle (SLN)		Ultrasonication	N/A	60	N/A	-Improve drug delivery, release, uptake and distribution	N/A	[8]
		Solvent injection	165	70	-11.34	-Enhance bioavailability, distribution and delivery of drug to the brain	N/A	[45]
		Hot homogenization followed by micro emulsion	164–180	80–90	-45.40		N/A	[46,56]
Chitosan based	Chitosan (CS-NP)	Ionic gelation	150–200	60	30.3	-High drug targeting to the brain	N/A	[12]
	Myristic acid-chitosan	Self-assembly	150–200	N/A	N/A	-Decrease proliferation of MCF-7 breast cancer cells	TQ (186.89 µg/ml) TQ-NP (68.24 µg/ml)	[53]

TABLE 2

## Overview of most-relevant clinically approved nanoformulations related to TQ

Platform	Formulation (trade name)	Indication	Administration	Status	Nanoparticle mechanism of action and advantage over free drug	Refs
Liposomal	Pegylated Liposome Doxorubicin (Doxil <sup>®</sup> /Caelyx <sup>®</sup> )	HIV-related Kaposi's sarcoma, metastatic breast cancer, metastatic ovarian cancer	i.m.	FDA-approved	-Diminishes clearance rate and prolongs $\beta$ half-life of conventional Dox - Decreases the risks of irreversible cardiomyopathy - Increases concentration of Dox up to 16 times in targeted sites	[61]
	Liposomal annamycin (L-Annamycin)	Acute lymphocytic leukemia, acute myeloid leukemia	i.v.	Phase I/II	- Inhibits DNA replication and protein synthesis through inhibiting topoisomerase type II (TOP2) - Circumvents multidrug-resistant transporters such as p-glycoprotein - Ensures higher delivery proportion of annamycin and lowers associated cardiotoxicity	[62]
	Non-pegylated liposomal doxorubicin (Sarcodoxome)	Soft tissue sarcoma	i.v.	Phase I/II	- Intercalates with DNA and inhibits TOP2 - Shows acceptable hematologic, liver, renal and cardiac functions	[63]
	Liposomal lurtotecan (OSI-211, NX 2111)	Ovarian cancer Head and neck cancer	i.v.	Phase II	- Inhibits topoisomerase I - Increases lurtotecan accumulation in targeted sites up to 67%	[64]
	Lyso-thermosensitive liposomal Dox (ThermoDox)	Primary liver cancer	i.v.	FDA-approved	- Allows 25-time increase of Dox circulatory concentrations in targeted tumor sites	[65]
	Irinotecan HCl:Floxuridine Liposome (CPX-1)	Colorectal cancer	i.v.	Phase II	- Exhibits superior antitumor efficacy when compared with the conventional combination of irinotecan and floxuridine - Increases plasma levels and AUC for total irinotecan and floxuridine	[66]
Polymeric	Methoxy-PEG-poly(D,L-lactide) taxol (Genexol <sup>®</sup> -PM)	Metastatic breast cancer Non-small-cell lung cancer	i.v.	FDA-approved	- Reverses p-glycoprotein-mediated drug resistance <i>in vitro</i> - Increases tolerability of paclitaxel at high concentrations	[67]
	Doxorubicin loaded micellar Pluronics L61/F127 (SP1049C)	Adenocarcinoma of the esophagus and gastroesophageal junction	i.v.	Phase III	- Prevents overexpression of BCRP and activation of Wnt- $\beta$ -catenin signaling pathways as observed with Dox alone - Alters DNA-methylation profiles of cells epigenetically - Depletes subpopulation of cells with cancer stem cell properties/biomarkers	[68]
	Pegylated Camptothecin (Prothecan)	Various cancers	i.v.	Phase I/II	- Stabilizes the active lactone configuration of camptothecin - Increases circulation levels, solubility and bioavailability of camptothecin	[69]
	Poly-L-glutamate paclitaxel (Xyotax <sup>®</sup> )	Non-small-cell lung cancer, ovarian cancer	i.v.	Phase III	- Improves patients' survival rates	[70]
	Poly(iso-hexyl-cyanoacrylate) doxorubicin (Transdrug TD)	Hepatocellular carcinoma	i.a.	Phase I/II	- Increases survival rates after 18 months of treatment - Reduces pulmonary adverse effects of Dox	[71]
	Docetaxel PEG-PLGA nanoparticle (BIND-014)	Metastatic castration-resistant prostate cancer	i.v.	Phase II	- Targets prostate-specific membrane antigens (PSMA) - Improves intratumoral concentrations and exposure duration of docetaxel - Sustains plasma levels of docetaxel for 48 h at high doses ensuring lower clearance rates	[72]
	Camptothecin Cyclodextrin-PEG nanoparticle (nano-particle drug conjugate NDC) (CRLX101)	Platinum-resistant ovarian carcinoma, primary peritoneal cancer, metastatic renal cell carcinoma	i.v.	Phase II and FDA approved Fast Track approval in combination with paclitaxel and with Avastin <sup>®</sup>	- Inhibits HIF-1 $\alpha$ expression - Enhances water solubility, plasma concentrations, target tissue localization and retention of camptothecin - Sustains prolonged supply of active camptothecin with safe systematic administration and minimal immunogenicity (helps evading phagocytes and renal clearance)	[73]

Abbreviations: i.v., intravenous; i.m., intramuscular; i.a., intraarterial.

### **Polyethylene glycol and Polyvinyl pyrrolidone TQ nanoparticles**

Polyethylene glycol (PEG) is a water-soluble and FDA-approved polymer with nontoxic and nonimmunogenic properties, whereas polyvinyl pyrrolidone (PVP) is a water- and ethanol-soluble polymer that prolongs the circulating half-life of drugs and restricts their passage through the blood–brain barrier, therefore limiting associated neurotoxicity [13]. PEG has been used for the encapsulation of many drugs such as doxorubicin [26] and paclitaxel [27] (Table 2), all of which are used in the clinic for cancer treatment. The fact that most of the polymeric nanoparticles prepared with PEG polymers have reached clinical trials makes them a strong candidate for the encapsulation of TQ. TQ has been encapsulated in polymeric nanoparticles of PEG and PVP polymers [13]. PEG-TQ nanoparticles, prepared by the nanoprecipitation method, exhibited strong specificity to breast cancer cell migration by hindering cytoskeletal actin polymerization through upregulation of miR-34a in a p53-dependent manner [13]. Shah *et al.* [14] synthesized two amphiphilic TQ nanoparticle formulations by emulsion solvent-evaporation using the polyhydroxyalkanoates (PHA)-monomethoxy PEG (mPEG) and the poly(1-3-hydroxyvalerate)-block-mPEG (PHV-block-mPEG) diblock copolymers, respectively [14,15]. These polymers self-assembled into amphiphilic nanoparticles with a hydrophobic core and hydrophilic shell. The formulations were shown to be safe carriers for hydrophobic drugs like TQ and were nontoxic to prenatal rat neuronal hippocampal cells. Given their unique properties, PEG and PVP are preferred over other polymers for the nanoencapsulation of TQ and, thus, could be effective for the clinical translation of TQ.

### **Poly- $\epsilon$ -caprolactone TQ nanoparticles**

Poly- $\epsilon$ -caprolactone (PCL) is a biodegradable polyester that has received great attention owing to the hydrolytic degradation of its ester linkages under physiological conditions [19]. PCL is FDA-approved and has been used for tissue engineering and drug delivery [19]. It has been widely used for the encapsulation of various anticancer drugs such as Taxol<sup>®</sup> and tamoxifen [19]. Some of the desirable features of PCL include its good stability under ambient conditions, ease of processing, biodegradability, biocompatibility and high permeability [28]. Yet, PCL has not been widely used in the clinic owing to its low encapsulation efficiency and burst release, as well as its low tissue bioactivity, which is a consequence of its hydrophobic properties [28]. The combination of PCL with hydrophilic polymers could enhance its mechanical properties and degradation kinetics [28]. The encapsulation of TQ with PCL resulted in higher encapsulation rates and higher entrapment efficiency as compared with PLGA [29]. PCL-TQ-loaded nanoparticles were tested *in vivo* for the treatment of colorectal tumors in murine models and showed significantly higher therapeutic activity depicted in higher survival rates and reduced tumor volumes as compared with free TQ [29].

### **Cyclodextrin TQ nanoparticles**

Cyclodextrin (CD), a cyclic oligosaccharide with a hydrophilic external surface and a hydrophobic internal moiety, is another polymer used for drug encapsulation. Several features make CD a good drug carrier such as low toxicity, ability to protect the conjugated drug from biodegradation, availability of various cavity sizes and the possibility for chemical modification or

conjugation at several sites. CDs are of three types:  $\alpha$ -cyclodextrin,  $\beta$ -cyclodextrin and  $\gamma$ -cyclodextrin, and vary in their physiochemical properties.  $\beta$ -CD is the most useful and lowest priced. It is not metabolized in the upper intestinal tract; however, it is degraded by bacteria in the cecum and colon [30]. Initially, the use of  $\beta$ -CD in drug delivery was limited by its low solubility in aqueous solutions; however, various derivatives of  $\beta$ -CD have been developed to improve solubility. These include the hydrophilic  $\beta$ -CD derivatives: hydroxypropyl- $\beta$ -CD and carbonyl- $\beta$ -CD [31]. Interestingly, Abu-Dahab *et al.* [32] conjugated TQ with  $\beta$ -CD using a self-assembly method in which TQ was mixed with  $\beta$ -CD in a molar ratio of 1:0.25 (TQ:CD). These nanoparticles enhanced the antiproliferative activity of TQ against MCF-7 breast cancer cells in a time-dependent manner. The IC<sub>50</sub> of encapsulated TQ decreased from 25  $\mu$ M for free TQ to 5  $\mu$ M for TQ-CD nanoparticles [32]. Despite these significant results, TQ-loaded  $\beta$ -CD nanoparticles have not reached the clinic yet. Further studies are required to characterize the *in vivo* mechanism of action and safety of these systems.

### **Polystyrene-polyethylene-oxide TQ nanoparticles**

The uptake mechanism, uptake dynamics and the fate of TQ nanoparticles after cellular trafficking have rarely been investigated. Recently, we designed poly(styrene-*b*-ethylene oxide) (PS-PEO) TQ nanoparticles using flash nanoprecipitation and described their cellular uptake and trafficking [33]. PS-PEO is a nontoxic, amphiphilic diblock copolymer that offers enhanced stability and longer protection of the formulation from opsonization *in vivo*. Flash nanoprecipitation involves fast mixing of a stream (containing a molecularly dissolved solute) and stabilizing molecule (with an opposing stream containing a miscible solvent), which allows stabilization of the particles, thus enhancing drug encapsulation efficiencies and loading contents [34]. We provided evidence that the uptake of TQ nanoparticles is mediated by caveolin-dependent endocytosis in MCF-7 and MDA-MB-231 cells and by clathrin-dependent endocytosis in MDA-MB-231 cells. PS-PEO TQ nanoparticles showed equal or enhanced activity against MDA-MB-231 and MCF-7 breast cancer cell lines in comparison with free TQ [33].

### **Lipid-based TQ nanoparticles**

Lipid-based nanoparticles, the first nanodrug delivery system ever described, are isotropic nonstructural stable vesicles formulated by merging surfactant(s), oil and water [35]. This nanotechnology offers versatile chances to improve the bioavailability and stability of sensitive materials amid numerous advantages in drug delivery and penetration capacity [36]. So far, several lipid-based TQ-NP formulations have been prepared which are summarized below.

### **Liposomal TQ nanoparticles**

Among the various lipid-based formulations, liposomes are considered as the classical constructions of nanoparticles. They comprise artificial vesicles formed from a concentric amphiphilic phospholipid bilayer (the biological membrane) that entraps an aqueous core. Hydrophilic drugs are usually incorporated in the aqueous layer, whereas hydrophobic drugs like TQ are loaded into the lipid membrane. Currently, a considerable number of liposomal drugs for combating cancer and infectious diseases are FDA-approved and some are in clinical trials (Table 2) [37]. A TQ-loaded liposome (TQ-LP) formulation using 1,2-dipalmitoyl-sn-glycero-3-phosphocholine phospholipids has been prepared through the

conventional thin-film hydration technique [38]. Interestingly, this TQ-LP formulation was cytotoxic to MCF-7 and T47D breast cancer cell lines and showed negligible effects on the normal fibroblast cells [38].

### **Niosomes TQ nanoparticles**

Niosomes are novel drug delivery systems that have also been used for the preparation of TQ nanoparticles [39]. Niosomes are a special class of liposomes mainly composed of hydrated nonionic surfactants in addition to cholesterol or its derivatives [40]. Niosomes are used as alternatives to liposomes owing to their enhanced stability, biocompatibility, biodegradability, noncytotoxic and nonimmunogenic nature, as well as rigidity and high dispersive nature which prevents blockage of blood vessels [41,42]. Rajput *et al.* [39] encapsulated TQ in innovative multilamellar gold niosomes containing TQ and Akt-siRNA (siRNA-Nio-Au-TQ) to secrete both components systematically into cells. The prepared nanoparticles were tested *in vitro* against tamoxifen-resistant (MCF-7/Tam and T-47D/TAM) and Akt-overexpressing (MCF-7/Akt) cells and *in vivo* in a BALB/c (nu+/nu+) mouse xenograft model of MCF-7/TAM. In both cancer cell lines, treatment with siRNA-Nio-Au-TQ showed enhanced effects when compared with either free TQ or Nio-Au-TQ. These results were consistent with a significant decrease in tumor volumes when mice were treated with siRNA-Nio-Au-TQ intravenously for a period of 14 days. The uptake of siRNA-Nio-Au-TQ exhibited a time-dependent pattern with a maximum uptake achieved after 120–180 min incubation, the time at which the niosomes escape the lysosomal compartments. In fact, fluorescence imaging of the animals 3 h post injection showed that these unique TQ nanoparticles accumulated within the tumor target site. The underlying mechanism of action involved effectively knocking out Akt, thereby sensitizing cells to TQ in addition to enhancing p53-mediated apoptosis via MDM-2 inhibition [39]. This innovative method holds promise for TQ-targeted delivery and maintenance of effective TQ concentrations for a sustained period.

### **TQ solid lipid nanostructures**

Although liposomes are the common lipid-based nanoparticles, the need to have better control over drug release, delivery and stability necessitated the development of non-phospholipid-based nanoparticles. SLNs have been developed recently from lipids, which are solid at room and body temperature [43]. Several advantages of SLNs have been reported so far. These include increased bioavailability, protection of sensitive molecules, facilitation in controlled release of drugs and feasibility for industrial production because the use of organic solvents can be avoided [44]. Thus, SLNs have the potential to be used as alternative drug delivery vehicles for many lipophilic molecules. SLNs encapsulating TQ have a biphasic release trend of TQ characterized by an initial rapid or burst release followed by a slower controlled one [8,45]. Pathan *et al.* [8] reported a twofold increase in the relative bioavailability of TQ-SLN in the plasma of rats when administered orally in comparison with the parent drug; the quantification was possible even after 12 h post-administration. Similarly, Singh *et al.* [45] reported a fivefold increase of TQ-SLN (2999 g/l/h) in the plasma of rats after oral administration when compared with suspension TQ (484 g/l/h). In fact, this TQ-SLN formulation showed *in vitro* controlled drug release properties with maximum release of 70% at 24 h; whereas, suspension TQ

exhibited a time-dependent release with maximum release of 47%. This could be attributed to the fact that nanoparticles tend to have greater surface areas than the suspension molecules. Pharmacodynamically, the amount of TQ in the liver, spleen, kidneys, heart, brain and lungs was higher upon SLN administration. More so, this formulation had hepatoprotective activities against liver cirrhosis by lowering serum levels of liver-damaging enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP). Moreover, TQ-SLN exerted lower toxicity on Vero epithelial normal cells compared with other nanoparticles [46].

### **TQ nanostructured lipid carriers**

Similar to SLNs, nanostructured lipid carriers (NCLs) are novel colloidal lipid-based formulations that constitute a hybrid blend of incompatible solid and liquid lipids. Formulations based on NCLs improved the pharmacokinetic properties of a variety of drugs including timolol, doxorubicin, paclitaxel and hydrocortisone [47]. The active compound is usually incorporated between the fatty acid chains or between the lipid layers. NCLs have been shown to enhance drug absorption and dissolution rates in the gastrointestinal tract owing to higher drug loading and entrapment potential. NCLs also allow modulation of drug-release patterns and long-term stability of the incorporated drug during storage. Interestingly, they can be easily stabilized with minimal concentrations of surfactants [43]. Abdelwahab *et al.* [48] formulated TQ-NCL through high-pressure homogenization using hydrogenated palm oil, olive oil and phosphatidylcholine for the lipid phase and sorbitol, polysorbate 80 (surfactant), thimerosal and double-distilled water for the liquid lipid phase. This TQ-NCL formulation inhibited the formation of ethanol-induced ulcers by modulating heat shock protein (Hsp)70 and did not exhibit hepatotoxic symptoms neither in rats nor in WRL-68 normal human liver cells. Moreover, in rabbits, extravascular administration of TQ-NCL resulted in improved pharmacokinetic properties such as increased bioavailability and sustained concentrations in blood. This reflected better gastroprotective characteristics of the TQ formulation and its suitability for extravascular oral administration. Similarly, Ng *et al.* [49] used hydrogenated palm oil, lecithin and olive oil to prepare the lipid matrix, and sorbitol, polysorbate 80 (nonionic surfactant) and thimerosal for the aqueous matrix and then applied the hot pre-emulsion and crystallization method at room temperature to formulate TQ-NLC. These TQ-NCLs exhibited dose-dependent growth inhibitory effects against epithelial breast (MCF-7 and MDA-MB-231) and cervical (Hela and SiHa) cancer cells with no cytotoxic effects in normal monkey kidney epithelial and mouse embryo fibroblasts cells, Vero and 3T3 L1 cells. In comparison to normal cell lines, TQ-NCL induced non-specific phase cell-cycle arrest in the treated MDA-MB-231 cells at different exposure times followed by apoptosis. A recent TQ-NCL formulation was prepared and tested *in vivo* for acute and subacute toxicity using BALB/c mice [50]. In acute toxicity, a dose of 10 mg/kg of loaded TQ nanostructures was found to be less toxic than pure TQ. However, in the subacute toxicity study, a dose of 100 mg/kg of either TQ-NCL or 100 mg/kg of pure TQ did not exhibit mortality in mice after 28 days of oral administration, yet both molecules resulted in liver toxicity (elevated levels of ALT) after prolonged consumption without altering the function of other organs [50]. In another study, TQ-NCLs prepared by

Elmowafy *et al.* [51] had better bioavailability, increased half-life and decreased elimination rates in rats. This TQ nanoformulation improved most liver biomarkers and restored lipid peroxidation enzymes to normal levels (elevation of reduced glutathione and reduction of malondialdehyde) indicating promises of antioxidant power.

#### Chitosan-based TQ nanoparticles

Chitosan (CS), a copolymer of  $\beta$ -(1,4)-2-acetamido-D-glucose and  $\beta$ -(1,4)-2-amino-D-glucose, is a natural renewable pharmaceutical adjuvant with good biocompatible properties. CS-drug nanoparticle complexes have lately been considered to efficiently target tumors owing to their small sizes, selective adsorption and neutralizing effects on the tumor cell surfaces. Drugs carried by CS nanoparticles usually have sustained or controlled drug release, prolonged blood circulatory time and reduced drug toxicity to normal tissues [52]. So far, two CS-based TQ nanoparticles have been formulated. These include TQ-CS and TQ-myristic-acid-CS (TQ-MA-CS). Using the regular ionic gelation method, TQ-CS nanoparticles were prepared by Alam *et al.* [12] and assessed for their *in vitro* release profile. Similar to TQ-SLN, TQ-CS primarily showed a rapid initial release followed by a slow release pattern promoting a sustained escape of TQ from the nanoparticle core. *In vivo*, TQ-CS showed higher penetrating ability and better targeting of the brain when administered intranasally or intravenously, suggesting promising rapid drug delivery with reduced systemic exposure.

Polysaccharide-based nanogels, submicron hydrogel nanoparticles, have been intensively explored lately to address concerns related to biocompatibility, immunogenicity and toxicity. CS myristic nanogels have 3D internally cross-linked nanostructures with high specific areas available for interaction with chemicals in the gel environment. Once loaded in MA-CS nanogels, TQ significantly showed improved inhibition of human adenocarcinoma MCF-7 cell proliferation in a time- and dose-dependent manner when compared with suspension TQ, possibly owing to enhanced solubility and uptake [53].

#### Thymoquinone nanomedicine: fact or fiction?

During the past decade, several studies have promoted TQ as a promising anticancer agent, yet its inherent poor pharmacokinetic properties have hampered its chances for FDA approval and emergence as a potent medicine clinically. Our poor understanding of its toxicity in humans and the lack of proper knowledge of its exact molecular targets urges the search for new investigatory tools. The answer could lie in nanoparticle drug delivery systems that have made significant progress with clinically used drugs (reviewed in Table 2) and thus will most probably pave the way for TQ clinical trials in humans. Nanoparticle encapsulation of TQ provides the possibility of fluorescent labeling of the compound, thus tracing its route of entry, trafficking mechanisms as well as intracellular distribution. Our recently formulated PS-PEO TQ nanoparticles [33] enriched with Nile red dye could be traced in the cytoplasm and nucleus of breast cancer cells with no loss of biological activity. Unlike TQ nanoparticles, free TQ loses its activity when labeled thus making it hard to further study its efficacy. The major challenge is to identify the type of preparatory method to obtain more-effective formulations for TQ with guaranteed reproducibility.

As reviewed above, several formulations have been used for the encapsulation of TQ; however, a comparison between the different formulations and clear conclusions on which is better for enhancing the delivery and activity of TQ is still lacking. Given the properties of the various polymers used for TQ encapsulation, PEG and PVP polymers seem to be preferred over other polymers because they prolong the circulating half-life of the drug and increase its bioavailability. Despite their desirable properties, PLGA and PCL might not serve as good polymers for TQ encapsulation owing to their low drug-loading efficiency and low encapsulation efficiency. When comparing the reported  $IC_{50}$  of the various TQ-NP polymeric formulations used against human breast cancer,  $\beta$ -CD encapsulation was superior to other formulations because it showed the lowest  $IC_{50}$  value in MCF-7 cancer cell lines (Table 1). A possible disadvantage of this encapsulation, however, is that TQ was conjugated with  $\beta$ -CD using a self-assembly method; thus, it had to be freshly prepared and used within 24 h to maintain stability [32]. TQ- $\beta$ -CD encapsulation using the emulsification method that provides long-term kinetic stability [54] might increase the chances of clinical testing of this formulation.

For lipid-based formulations, liposomal encapsulations have been the only ones to reach the clinic for cancer treatment. SLNs have been used as alternatives to liposomes to treat several human diseases [55]. SLNs have been shown to enhance TQ delivery, uptake and bioavailability. One drawback of SLNs is the low drug-loading capacity, which led to an encapsulation efficiency (% EE) of TQ between 60 and 90% [8,45,46,56]. NLCs have been found to increase the drug loading of highly lipophilic molecules and protect them from degradation. TQ-NCL formulations have been found to increase % EE of TQ (>95%) more than TQ-SLN. They increased TQ bioavailability, lowered its toxicity and enhanced its anticancer activity. In fact, these NLCs represent promising strategies for increasing the stability of TQ over SLNs because they are made of FDA-approved surfactants and the preparation procedure is easily scalable for large-batch production by high-pressure homogenization. The fact that nanoformulations of some naturally derived agents that share common features with TQ have succeeded to reach the clinic provides promise for the clinical translation of TQ (Table 2). Some of these agents include Abraxane<sup>®</sup> (PLGA-paclitaxel), Doxil<sup>®</sup> and Myocel (liposomal doxorubicin), Theracurmin<sup>™</sup> (nanoparticle-based curcumin) and many more (Table 2). Given the similarity in physiochemical characteristics between paclitaxel, curcumin and TQ, the use of paclitaxel-based and/or curcumin-based nanoparticle formulations for the encapsulation of TQ can be very promising clinically.

Paclitaxel, the most widely used and effective plant-derived antineoplastic agent, is a highly lipophilic compound with very poor aqueous solubility [57] and its delivery has been associated with substantial challenges. Unlike TQ, paclitaxel has been FDA-approved for the treatment of breast and ovarian cancer. Several nanoparticle formulations have been used for the enhancement of paclitaxel availability. Several of these formulations have reached clinical trials and some have been FDA-approved including paclitaxel albumin-bound nanoparticles (nab-paclitaxel, Abraxane<sup>®</sup>) for the treatment of metastatic breast cancer and non-small-cell lung cancer [58], Genexol<sup>®</sup>-PM for treatment of breast, lung and ovarian cancer and Xyotax<sup>®</sup> for the treatment of non-small-cell lung cancer in Phase III clinical trials.

Curcumin, a diphenolic molecule extracted from the turmeric *Curcuma longa* roots, has potent effects against a wide range of solid and liquid cancers [59]. Similar to TQ, the poor bioavailability and poor solubility of curcumin have been the principle reasons behind the lack of its clinical translation. Theracurmin™ CR-011L is one of the few curcumin nanoparticle formulations that succeeded to reach clinical trials and has been shown to prolong the survival rate of patients with gemcitabine-resistant pancreatic cancer [60]. Currently, it is in Phase I clinical trials in patients with advanced malignancies (<https://clinicaltrials.gov/ct2/show/study/NCT01201694?term=THERACURMIN&cond=Cancer&rank=1>). Given the success of such formulations with paclitaxel and curcumin, it might be worth investigating the potential of developing similar formulations for TQ.

### Concluding remarks and future perspectives

Despite their superior activity over free TQ, TQ nanoparticle formulations have not reached the clinic. Considering their

stability and traceability *in vivo*, TQ nanoparticle formulations are more likely to reach the clinic than free TQ. To promote their clinical translation, several criteria should be fulfilled such as ensuring that nanoparticle sizes range between 10 and 100 nm and induce minimal side effects, in addition to establishing a simple, efficient and sustainable process to produce TQ nanoparticles for large-scale production. Once such TQ nanoparticles are prepared, human trials should be conducted to establish the toxicological profiles of these formulations and determine their effectiveness over free TQ. It is important to assess the efficacy of such nanoparticles in combination with orthodox chemotherapy to achieve better-targeted modalities to treat malignancies, reduce associated toxicities and sensitize tumor tissues without affecting the surrounding organs.

### Conflicts of interest

The authors declare no conflicts of interest.

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