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Drug delivery systems for differential release in combination therapy

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Introduction: Combination therapy with multiple therapeutic agents has wide applicability in medical and surgical treatment, especially in the treatment of cancer. Thus, new drug delivery systems that can differentially release two or more drugs are desired. Utilizing new techniques to engineer the established drug delivery systems and synthesizing new materials and designing carriers with new structures are feasible ways to fabricate proper multi-agent delivery systems, which are critical to meet requirements in the clinic and improve therapeutic efficacy.

Areas covered: This paper aims to give an overview about the multi-agent delivery systems developed in the last decade for differential release in combination therapy. Multi-agent delivery systems from nanoscale to bulk scale, such as liposomes, micelles, polymer conjugates, nano/micorparticles and hydrogels, developed over the last 10 years, have been collected and summarized. The characteristics of different delivery systems are described and discussed, including the structure of drug carriers, drug-loading techniques, release behaviors and consequent evaluation in biological assays.

Expert opinion: The chemical structure of drug delivery systems is the key to controlling the release of therapeutic agents in combination therapy, and the differential release of multiple drugs could be realized by the successful design of a proper delivery system. Besides biological evaluation *in vitro* and *in vivo*, it is important to speed up practical application of the resulting delivery systems.

Keywords: combination therapy, delivery system, differential release, drug carrier, therapeutic agent

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1. Introduction

With rapid developments in materials science, pharmaceutics and biotechnology, drug delivery system (DDS) has graduated from the concept of a pill to sophisticated programmable delivery system for sustained/controlled drug release from systemic delivery to organ and cellular targeting. Nowadays, several established DDSs, such as liposomes [1], micro/nanoparticles [2,3], micelles [4] and hydrogel systems [5], have presented numerous advantages in the clinic treatment of many diseases, especially in cancer treatment and tissue engineering. The use of DDSs can not only improve the therapeutic index of conventional drugs and reduce the side effects or symptoms during therapy, but also significantly facilitate the patient's compliance and comfort [6].

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Despite the widespread use of DDSs in medicine, most of the current DDSs are used to load and deliver a single drug, and only a few of them are applied to the delivery of drug cocktails. As combination therapies with multiple drugs have been routinely used in many cases of clinical treatment to improve outcomes [7], significant interest has been shown in the investigation of new approaches and systems for differential release of multiple drugs from a single delivery system.

Here, the research work on DDSs developed in the past decade for differential delivery of therapeutic agents in combination therapy is reviewed.

2. Combination therapy and multi-agent delivery systems

Generally speaking, the term combination therapy refers to the simultaneous administration of two or more pharmacologically active agents to treat a single disease. In addition, the combination of different types of therapy, such as surgery, photodynamic therapy, radiation therapy and/or chemotherapy, is also defined as combination therapy. In this review, the part of multi-agent chemotherapy is highlighted, but the latter condition is not addressed.

Owing to the complexity of body's defense mechanisms and multiple pathogenic factors of a disease, monotherapy with a single drug or drugs only hitting a single target may be inadequate during the treatment of diseases such as malignant tumors, hypertension, infection, cardiovascular maladies and immune disorders. So combined therapy with multiple therapeutic drugs that have non-overlapping toxicities but different mechanisms of action can give a better performance in disease treatment. Also, the synergy or an additive effect between chemotherapeutic agents may enhance further the therapeutic efficacy at a lower dose, and concurrently suppress drug resistance and intolerable side effects. Accordingly, combination therapy of multiple medications as a competitive and effective strategy for disease treatment has now commonly been used in the clinic.

Although DDSs with many advantages such as prolonged circulation time and improved biodistribution and availability have been established, most of them are designed for single drug delivery, which cannot fulfil the demands of combination therapy. So it is of great interest for many researchers to develop multi-agent delivery system (MADSs) that can control the release sequence, timing, dose and duration of each drug to achieve an effective combination therapy. On the other hand, compared with the discovery, synthesis and screening of new drugs, it is relatively timesaving and economical to exploit new MADSs that can maximize the therapeutic effect of existing drugs. To achieve this goal, some researchers use new techniques to engineer the established DDSs and others apply themselves to synthesizing new materials and/or designing carriers with new structure. In the last 10 years, a few MADSs from nanoscale to bulk scale and from simplex to composite have been fabricated.

In the following text, the characteristics of various MADSs, including the structure of drug carrier, drug-loading method and release behaviors, are described and discussed. First, the small-scale carriers (nano/micocarriers), which can realize passive or active targeting, and intracellular drug delivery by intravenous or oral administration are systematically introduced and analyzed (Section 3). Then, in Section 4, the delivery systems based on bulk materials used to release multiple drugs locally and sustainedly are presented and discussed.

3. The multi-agent delivery systems based on nano/microcarriers for combination therapy

Drug carriers in nano/microscale are usually designed and used to improve the biodistribution of therapeutic agents owing to their ability for passive targeting and even active targeting when ligands and antibodies are used as targeting factors. Taking the advantages of targeting delivery, established nanocarriers such as liposomes, micelles, nanoparticles, polymer-drug conjugates and new types such as dendrimers and nanocells (**Table 1**), as well as microparticles (**Table 2**), are engineered and fabricated for co-delivery of multiple agents in combination therapy.

3.1 Liposomes

Liposomes are closed spherical vesicles consisting of a membranous lipid bilayer that encapsulates a considerable volume of water (**Figure 1A**). The liposome bilayer is mainly based on natural and synthetic phospholipids and cholesterol, which affects the predominant physical and chemical properties of the resulting liposomes, including permeability, charge density and steric hindrance. As a unique structure, liposomes have the ability to be the carriers for both hydrophilic and hydrophobic compounds, encapsulating them either in the internal aqueous core or in the lipid bilayer, respectively. Liposomes as a single therapeutic agent carrier have mostly been developed and used in the clinic for cancer therapy. For example, liposomal formulations of the anthracyclines (Doxil) [8] and daunorubicin (DaunoXome) [9] are used for the treatment of a variety of cancers.

Nowadays, a series of studies is focusing on developing single liposome formulations that are able to co-encapsulate stably two or more therapeutic agents and coordinate the release behaviors of drugs after intravenous injection. Some successful examples are described as follows.

To overcome P-glycoprotein (Pgp)-mediated multi-drug resistance (MDR) phenotype in tumor cells, the combined use of cytotoxic drugs with a Pgp inhibitor (verapamil [VER]) is designed. As doxorubicin (DOX) and VER are weakly basic drugs, Wang *et al.* co-encapsulated them into

Table 1. Types of nanocarrier in multi-agent delivery systems.

Systems	Characteristics	Examples of drug loading	Ref.
Liposomes (50 – 600 nm)	Closed structures composed of outer lipid bilayer and inner aqueous phase	DOX and VER loaded in inner aqueous phase	[10]
		Isoniazid loaded in inner aqueous phase, rifampicin in lipid bilayer	[15]
Micelles (10 – 600 nm)	Colloidal core-corona structure with a hydrophobic core and hydrophilic shell assembled from amphiphilic block copolymers	PTX loaded in the core of micelle, plasmid DNA or antibody herceptin attached to the surface of shell	[21,26]
		PTX and CA4 loaded in the core of micelle	[29]
Polymer–drug conjugates (5 – 100 nm)	Complex of drugs and polymer connected with biodegradable linker	DOX and AGM attached to HPMA copolymer via a tetrapeptide linker (enzyme cleavable)	[34]
		PTX and ALN attached to HPMA copolymer respectively via Phe-Lys-*p*-aminobenzyl carbonate spacer and GFLG-*p*-nitrophenol group (both linkers are enzyme cleavable)	[37]
Polymer nanoparticles (50 – 400 nm)	Colloidal solid particles generally made by oil-water emulsion solvent evaporation method	Hydrophilic drugs VCR and VRP loaded in PLGA NPs	[53]
		Hydrophilic VCR and hydrophobic QC loaded in PLGA NPs	[55]
		Water-insoluble PTX and water-soluble GEM first linked together to form a conjugate, then loaded in a lipid-coated PLGA NP	[56]
Nanospheres (200 – 300 nm)	Polymeric particles generally made by water-oil-water double emulsion solvent evaporation method	PTX and hydrosoluble EN loaded in PLA nanospheres	[62]
		Lipophilic RA and water-soluble DNA loaded in nanospheres	[63]
Lipid (150 – 300 nm)	Hydrophobic particles with a monolayer of phospholipid coating	DOX and MMC loaded in polymer-lipid hybrid nanoparticles	[66]
Magneto-polymeric nanoparticles (several – 500 nm)	Polymeric particles with embedded magnetic nanocrystals or a magnetic core to achieve multifunction	DOX loaded in NPs and antibody herceptin attached to NPs with carboxyl group	[69]
		PTX and rapamycin loaded in the GMO outer lipid coating	[70]
Dendrimers (< 100 nm)	Globular branched macromolecule with a hydrophobic inner cavity and numerous surface functional groups	Phenylbutazone and mycophenolic acid respectively entrapped in the inner and bound on the surface of PAMAM dendrimer	[71]
		5FU and microRNA, respectively, loaded in the inner and bound on the surface of PAMAM dendrimer	[72]
Polymersome (100 nm)	Polymer-based shell composed of an aqueous lumen and a thick hydrophobic membrane (10 – 14 nm)	DOX and PTX, respectively, loaded in the lumen and the membrane of polymersome	[73]
Mesoporous silica nanoparticle (200 nm)	Inorganic engineered silica NP with numerous pores (pore size 3 nm)	DOX loaded in pores first, then siRNA conjugated with the DOX-loaded NP using PAMAM dendrimer as linker	[74]
Nanocell (180 – 200 nm)	NP consisting of a polymeric core and an extranuclear PEGylated-lipid envelope	DOX loaded in the nuclear PLGA-based nanoparticle, CA4 entrapped within the outer lipid coating	[75]

GMO: Glyceryl monooleate; NP: Nanoparticle.

Table 2. Types of microcarrier in multi-agent delivery systems.

Systems	Characteristics	Examples of drug loading	Ref.
Microparticles (1 – 500 μm)	Colloidal solid particles at microscale usually made by the double emulsion method	5FU and AODN encapsulated in PLGA microparticle	[76]
		Three drugs (isoniazid, rifampin, pyrazinamide) encapsulated in PLGA microparticle	[77]
		CpG DNA covalently linked to the antigen OVA-loaded microparticle by an acid-degradable crosslinker	[83]
Microspheres (40 – 125 μm)	Polymeric spheres at microscale	VER and vinblastine penetrated in the matrix of microsphere	[86]
Microcapsules (1 – 4 μm)	Hollow capsule assembled from a polyelectrolyte and amphiphilic surfactant by a layer-by-layer approach	Hydrophobic drug loaded in the shell, while hydrophilic drug loaded either in the shell or in the capsule	[87]

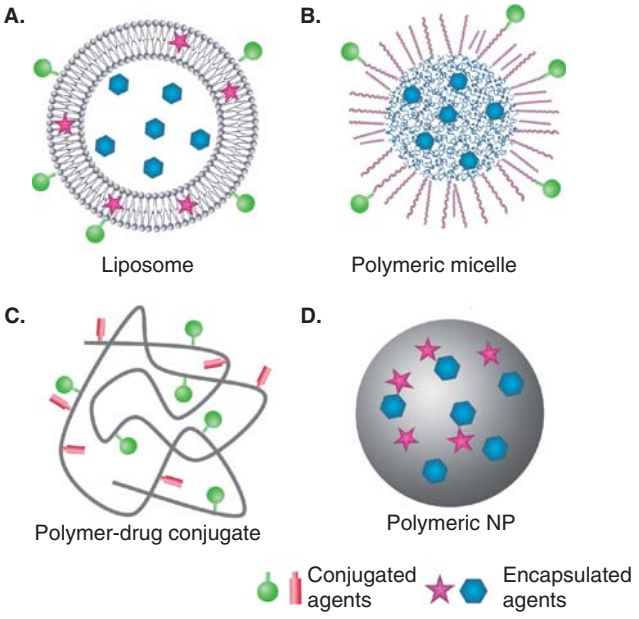

Figure 1. Common types of nanocarrier in multi-agent delivery systems for combination therapy. A. Liposome. **B.** Polymeric micelles. **C.** Polymer-drug conjugate. **D.** Polymeric NP.
NP: Nanoparticle.

the internal aqueous phase of Stealth liposomes (polyethylene glycol-coated, namely, PEGylated liposomes) by remote loading via a transmembrane pH gradient during the liposome preparation [10]. The *in vitro* release study presented the finding that DOX and VER had a similar release rate, < 10% after 12 h incubation in cell medium; and this liposome formulation had been demonstrated to be an effective reversal of MDR in DOX-resistant cell lines. Based on the work above, Wu *et al.*, conjugating transferrin (Tf) to the liposomes with DOX and VER, achieved selective targeting of tumor cells. The Tf-L-DOX/VER formulation showed high efficiency in overcoming drug resistance in K562 leukemia cells, with 5.2 and 2.8 times greater cytotoxicity than the non-targeted liposomes (L-DOX/VER) and the Tf-targeted liposomes containing DOX alone (Tf-L-DOX), respectively [11].

Sometimes, different drugs cannot be incorporated within a single liposomal formulation by one loading method, because of the different properties of drugs or the instability of the resulting formulation [12]. In this case, various loading technologies must be used to build a combination delivery system. Besides the established pH gradient loading, a new metal gradient method could be used to encapsulate drugs that can interact with transition metals. In the study of Abraham and co-workers, two distinct loading methods, pH gradient loading and transition metal gradient loading, were taken to co-encapsulate DOX and vincristine (VCR) within a single liposome [13]. DOX was first loaded into a manganese-sulfate containing liposomes relying on the formation of a manganese-DOX drug complex; VCR was subsequently loaded by means of an ionophore-generated transmembrane pH gradient formed by addition of the electroneutral ionophore A23187 (divalent cation/proton exchanger). In another work [14], the co-encapsulation of irinotecan and floxuridine within a single liposome was also realized using two loading methods. Floxuridine was passively entrapped in liposomes through pervasion as its concentration gradient during liposome production, whereas irinotecan was actively loaded using transition metal copper, as before. It is interesting that, by altering the ratio of cholesterol in the liposomal formulations, the release rates of both drugs could be coordinated.

In addition to encapsulating two different drugs in the internal aqueous core of liposomes, drugs with different solubilities (lipid soluble and water soluble) can be respectively incorporated in the lipid bilayer and aqueous phase of liposomes [15,16]. For example, isoniazid (INH, water soluble) and rifampicin (RIF, lipid soluble), which are used together to treat tuberculosis effectively, were successfully co-encapsulated in the same liposome formulation, respectively, in the aqueous phase and the lipid layer. The release periods of both drugs from the co-encapsulation form were extended compared with those of liposomes containing only one drug.

Bioactive macromolecules such as proteins, oligonucleotides (AODNs) and DNA are usually used in combination with small molecular drugs for the treatment of cancer.

Therefore, some liposome formulations co-encapsulating both bioactive agents and small molecular drugs have also been investigated [17-19]. Cationic liposomes that had been modified with truncated human basic fibroblast growth factor (tbFGF) peptide first encapsulated DOX, and then Msurvivin T34A plasmids (plasmids encoding the phosphorylation-defective mouse survivin threonine 34 → alanine mutant) were bound to the surface of liposome to form a co-delivery system [17]. This liposome formulation could simultaneously deliver these two agents (drugs and DNA) to the same cell and present high transfection efficiency, and consequently good synergistic/combined curative effect.

3.2 Micelles

Polymeric micelles are colloidal core-corona structures, spontaneously assembling from amphiphilic block or graft copolymer when its concentration is above critical micelle concentration (CMC) (**Figure 1B**). The hydrophobic core of micelles serves as a depot for poorly water-soluble drugs, whereas the outer hydrophilic shell can protect encapsulated drugs and prolong their blood circulation time, which makes it an appropriate carrier for drug delivery. Micelles carrying single poorly water-soluble drug with improved pharmacokinetics have been reported in the past few years. To reduce the additive or synergistic toxicity from more than one kind of excipient in the drug combination therapy, and to make the operation convenient in the clinic, micelles encapsulating and delivering multiple poorly water-soluble drugs have been investigated. In the report by Shin *et al.* [20], different combinations of several hydrophobic anticancer drugs, such as paclitaxel (PTX)/17-allylamino-17-demethyoxygeldanamycin (17-AAG), etoposide (ETO)/17-AAG, docetaxel (DCTX)/17-AAG and PTX/ETO/17-AAG, have been separately solubilized in the solution of poly(ethylene glycol)-*block*-poly(DL-lactic acid) (PEG-*b*-PLA) to form multi-drug micelles (MDMs). Compared with single-drug micelles (SDMs), the presence of 17-AAG in the MDMs not only makes the formulation stable for a longer time, but also stabilizes the encapsulated agents, avoiding early precipitation. Drugs in SDMs and MDMs have similar *in vitro* release kinetics, which suggested the drug release behavior from PEG-*b*-PLA micelles is controlled by a diffusion mechanism, and is not a result of the breakdown of micelles [20].

Another way to load therapeutics to micelles, besides encapsulating drugs in the core phase, is to form micelle-therapeutics conjugates by complexation or electrostatic interaction. In this way, bioactive agents can be incorporated with drug-loaded micelles and co-delivered to the target cells to achieve synergistic/combined therapeutic efficacy. A few systems that utilize drug-loaded micelles to form complexes with anionic gene [21-25], protein [26,27] or other types of drug [28] for multi-agent delivery have been developed in recent years. The amphiphilic copolymers poly{(*N*-methyldietheneamine sebacate)-*co*-[(cholesteryl oxocarbonylamido ethyl) methyl bis(ethylene) ammonium bromide] sebacate} (P(MDS-*co*-CES)) synthesized in Yang's lab [21] were used to prepare micelles and co-deliver anticancer drug PTX and interleukin-12-encoded plasmid to human breast cancer cells. Enhanced gene transfection of this system has been demonstrated by both *in vitro* and *in vivo* studies. Moreover, a good effect has been observed by replacing plasmids with synthetic siRNA molecules as well [21]. On the other hand, a system of a humanized monoclonal antibody Herceptin complexed onto the cationic surface of PTX-loaded P(MDS-*co*-CES) micelles was fabricated for targeting delivery of PTX to human breast cancer cells with overexpressed epidermal growth factor receptor (HER2) [26]. Compared with respective administration of pure Herceptin and BioPorter (a commercially available lipid-based protein carrier), the micelle co-delivery system showed greater efficiency for delivering Herceptin, leading to more accumulation of PTX molecules in the breast cancer cells, and therefore greater cytotoxicity and anticancer effect. Furthermore, the higher the HER2 expression level of cancer cells, the higher the cytotoxicity of PTX [26]. In the work of Bae *et al.* [28], amphiphilic block copolymer poly(ethylene glycol)-poly(β-benzyl L-aspartate) (PEG-PBLA) was first modified with hydrazide to form PEG-poly(aspartate hydrazide), followed by conjugating DOX and wortmannin (WOR, a phosphatidylinositol-3 kinase inhibitor) onto it through a hydrazine bond. Then the polymer-drug conjugates assembled into micelles with particle size < 100 nm. The *in vitro* cytotoxicity assay against human breast cancer cell line MCF-7 indicated that enhanced cytotoxic activities could be obtained with a low dose of DOX by concurrently delivering DOX and WOR with polymeric micelles. In the report from Wang and Ho, carboxyl acid functionalized amphiphilic copolymer $mPEG_{2000}$-PLA_{2000} was first conjugated with PTX, and then encapsulated combretastatin A4 (CA4, a neovasculature disruption agent) in the core phase during the self-assembly of micelles [29]. For this co-delivery system, a slower release of conjugated PTX than encapsulated CA4 was observed from the *in vitro* drug release study. The distinctive release profile between PTX and CA4 was ascribed to the slow hydrolysis of ester bond, which linked PTX and the $mPEG_{2000}$-PLA_{2000} copolymer. Significant neovasculature inhibition (> 90%) of this dual drug-loaded micelle was presented in the Matrigel plug assay *in vivo*. Furthermore, in Lewis lung carcinoma (LLC) tumor-bearing mice, this dual drug-loaded micelle also showed the greatest tumor growth inhibition effect of 76.6% after a 10-day treatment, whereas it was just 63.5 and 23.2%, respectively, for the single CA4-loaded micelle and the single PTX-conjugated one.

Lin and co-workers recently reported a multi-drug micelle to deliver concurrently anticancer drugs cytarabine (Ara-C) and 5-fluorouracil (5FU) [30]. Interestingly, this micelle was self-assembled from the random copolymers that were obtained by radical polymerization of these two drug derivatives containing the vinyl esters; and the drug release from the resulting multi-drug micelle was correlated to pH values, fast in pH 7.4 (simulated extracellular environment) and slow in pH 1.2 (simulated gastric juice).

3.3 Polymer-drug conjugates

In the last few decades, increasing interest has focused on polymer-drug conjugates as new 'nanomedicines' for disease treatment, especially for cancer treatment. Owing to rapid clearance from circulation, poor accumulation at the pathological sites and undesired toxicity to the normal cell/tissue when using conventional low-molecular-mass drugs, the concept of polymer-drug conjugates (**Figure 1C**) as a 'polymeric prodrug' was born from the desire to overcome these problems and improve the pharmacokinetics of parent free drugs. To form a polymer-drug conjugate, a small molecular drug is usually covalently bound to the side chain of a linear polymer normally by means of a linker, which is able to be cleaved and release drug on arrival at the target site but is stable during conjugate transport. Owing to advantages such as tumor selectivity by the enhanced permeability and retention (EPR) effect, decreased toxicity, increased drug solubility and bypassing of some mechanisms of drug resistance compared with the free drugs, many polymer-drug conjugates with established anticancer agents have undergone clinical evaluation, and some of them are expected to enter the market in the near future [31].

N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer is one of the matrices commonly used for polymer-drug conjugates, because of its known non-immunogenicity, non-toxicity and long circulation time. Many HPMA-based conjugates with a single drug for cancer treatment have been established, such as poly(HPMA)-GFLG-doxorubicin (GFLG is the tetrapeptide spacer) [32] and HPMA copolymer-paclitaxel (PNU166945) [33]. Recently, some research has focused on the fabrication of HPMA-based conjugates bearing a combination of two or more active agents with different mechanisms of action, to accomplish the simultaneous delivery of multiple drugs. Vicent and colleagues first used an HPMA copolymer to achieve the combination of endocrine therapy and chemotherapy by carrying the classical chemotherapeutic drug DOX as well as the aromatase-inhibiting antihormonal agent aminoglutethimide (AGM) [34]. The drugs were attached to the copolymers by means of a tetrapeptide linker that could be cleaved by lysosomal enzymes in the cancer cells. The two-drug-containing HPMA conjugate was stable in buffer alone, but released drugs in the presence of lysosomal enzymes, and different drug release profiles were observed compared with the single-drug-containing conjugates. In the initial release of ~ 30 min, a marked lag phase with little release appeared both for DOX and for AGM. After this time, however, the release rates of both drugs were enhanced relative to those formulations, respectively, with equal content of a single drug. The *in vitro* cytotoxicity tests against MCF-7 cells demonstrated that the conjugate with both drugs had a higher anticancer activity than that from the combination of two polymer conjugates carrying, respectively, a single DOX and a single AGM. Two subsequent studies suggested that some factors could improve the activity of two-drug-containing HPMA conjugate, including the complexity of cellar mechanisms, the different release profiles of drugs and the conditions of conjugate conformation [35,36].

Some other formulations of HPMA polymer conjugate for combination delivery of drugs have been reported lately [37-42]. After the achievement of targeting angiogenesis with a conjugate of HPMA copolymer and *O*-(chloracetyl-carbamoyl) fumagillol (an angiogenesis inhibitor, TNP-470) in mice [43], Satchi-Fainaro and colleagues fabricated HPMA conjugates with more than one therapeutic for the combination treatments of osteosarcomas and bone metastases. In one report from them, a HPMA copolymer was co-conjugated with anticancer drug PTX and bone-targeting agent alendronate (ALN) using different linkers, (Phe-Lys-*p*-aminobenzyl carbonate spacer for PTX and GFLG-*p*-nitrophenol groups for ALN), which could be cleaved by cathepsin B overexpressed in tumor endothelial and epithelial cells [37]. The agent ALN was used not only for its bone-targeting ability, but also for its potential pharmacological activity (such as antitumor and antiangiogenic activity). *In vitro* studies revealed that the anticancer and antiangiogenic activity of this conjugate against human umbilical vascular endothelial cells (HUVECs) was as effective as that of the combination of free drugs, which suggested its potential application as a bone-delivery system in cancer treatment [37]. In another work, both ALN and TNP-470 were attached to HPMA copolymer through a Gly-Gly-Pro-Nle linker, which can be cleaved by cathepsin K overexpressed in bone tissues [39]. This kind of polymer-drug conjugate simultaneously possessed the passive and active targeting ability, and presented synergistic antiangiogenic and antitumor potency both *in vitro* and *in vivo*. The growth inhibition against osteosarcoma could reach 96% with the HPMA copolymer-ALN-TNP-470 in mice, whereas it reached only 45% for combination of free drugs. Krakovičová *et al.* recently reported a controlled release of DOX and anti-inflammatory drug dexamethason (DEX) from a single HPMA copolymer for treatment of cancer [40]. In this system, DEX was first esterified with 4-(2-oxopropyl) benzoic acid (OPB) or 4-oxopentanoic acid (levulic acid [LEV]) to form its derivatives DEX-OPB and DEX-LEV. Then the respective derivate was covalently conjugated to the HPMA copolymers by means of pH-labile hydrazone bonds, followed by the attachment of free DOX in the same way. This formulation released drugs faster at pH 5 than at pH 7.4 owing to the hydrolysis of hydrazine bonds. Free DEX was released by enzymatic hydrolysis of ester bond in DEX derivatives, either on the polymer carrier, or after release of DEX derivative in the medium. Therefore, a pH-controlled dual drug release may be obtained in this way. HPMA polymeric conjugates with DOX attached through amide and hydrazone bonds were synthesized for the evaluation of synergic effect against cancer, in comparison with the combined administration of monoconjugates (HPMA-DOX conjugate just with an amide or a hydrazine as a linker) and the application of just one monoconjugate. The experimental results indicated that the mixed forms of the polymeric derivatives of

DOX can increase the antitumor efficacy remarkably as a result of the synergizing effect [42].

Poly(ethylene glycol) (PEG) is another classical copolymer for fabrication of polymer-drug conjugates because of enhanced biocompatibility, permeability and retention effect. However, owing to the poor loading capability from its only one or two terminal functional groups, PEG is usually branched or modified to obtain increased carrying capacity in practice, especially in MADSs [44-47]. Modified PEG simultaneously carrying anticancer agents epirubicin (EPI) and nitric oxide (NO) was designed owing to the potential synergistic effect and suppressed cardiotoxicity [44,48]. *In vitro* and *in vivo* studies confirmed this conjugate could increase the bioavailability drugs and was less cardiotoxic [44,48,49]. Polyethyleneimine (PEI), aptamer (Apt, a nucleic acid ligand)-modified PEG was utilized to attach small hairpin RNA (shRNA) and DOX to form shRNA/PEI-PEG-Apt/DOX conjugate. This kind of conjugate with synergistic effect from genes and chemotherapeutic drugs possesses the ability to destroy cancer cells selectively as well as having a wide therapeutic window in cancer therapy [50]. In the work of Chandna *et al.*, three agents, such as a synthetic analogue of luteinizing hormone-releasing hormone (LHRH, a targeting moiety), camptothecin (CPT, an anticancer drug) and a synthetic analogue of BCL2 homology 3 domain (BH3, a suppressor of cellular antiapoptotic defense), were incorporated into a DDS using PEG polymer as a carrier [51]. This kind of complex showed a high antitumor activity *in vitro* and *in vivo* owing to the improved biodistribution of DDS in the tumor site and the synergic effect of CPT and BH3, which cannot be achieved by separate application of free drugs.

Besides the synthetic polymers as matrices for drug conjugation, the bioactive macromolecules such as plasmid could be conjugated with DOX by means of intercalation to form a combined chemoimmunotherapy vehicle for cancer treatment [52]. This complex presented a prolonged clearance of ~ 3 h (10 min for free DOX) and higher anticancer efficacy both in mice bearing NCI-H358 xenografts and in mice bearing 4T1 murine allografts at a lower dose of DOX compared with application of free DOX. Furthermore, the systemic toxicity or cardiotoxicity was too weak to be observed, suggesting the plasmid-DOX complex with combined chemoimmunotherapy was more effective and safer than cytotoxic chemotherapy alone.

3.4 Nanoparticles

Nanoparticles (NPs) (**Figure 1D**), a kind of colloidal solid particulate with size < 1 μm, are prepared mainly from natural or synthetic polymers, and in some cases from the combination of polymer and other types of material. Depending on the method of preparation, therapeutic agents can be entrapped, dissolved in or attached to the nanoparticle matrix. Nanoparticles as a carrier for drugs not only improve the therapeutic efficacy of loaded drug by changing their pharmacokinetics and biodistribution, but also allow for a further functionalization to realize a specific purpose, such as active targeting or stimulus-responsive controlled release of drugs. In the last decade, interest in nanoparticles as a MADS has surged.

Poly(lactide-*co*-glycolide) (PLGA) is an FDA-approved polymer owing to its excellent biocompatibility and biodegradability, and has been widely used in particle fabrication for drug delivery systems. Some studies about using PLGA nanoparticles to co-load multiple drugs have been reported recently. For example, two hydrophilic drugs, vincristine sulfate (VCR) and verapamil hydrochloride (VRP), were co-loaded within PLGA nanoparticles prepared by the oil-in-water (O/W) emulsion solvent evaporation and salting-out method during the preparation process [53]. After optimizing the preparation parameters, two-drug-loaded PLGA nanoparticles (VCR-VRP-PLGANPs) with enhanced drug entrapment efficiency (~ 55% for VCR and 69% for VRP) and small particle size ~ 110 nm were obtained [53]. In a follow-up study, an optimized VCR-VRP-PLGANP was used for the reversal of multi-drug resistant against MCF-7 cells with high expression of Pgp. From the results of the *in vitro* assay, the co-delivery system of VCR-VRP-PLGANPs with low toxicity for normal tissue and fewer drug–drug interactions presented the highest reversal efficacy compared with other formulations of co-administrating two drugs, including VCR + VRP-PLGANPs, VRP + VCR-PLGANPs, VCR-PLGANPs + VRP-PLGANPs and the combination of free VCR and VRP, and would be the formulation with most potential for *in vivo* treatment of drug-resistant cancer [54]. Besides co-encapsulating two hydrophilic drugs, simultaneous delivery of hydrophilic and hydrophobic drugs can also be accomplished in a single PLGA NP. Hou and co-workers modified the method of O/W single-emulsion solvent evaporation mentioned above, successfully realizing the co-encapsulation of VCR (hydrophilic) and quercetin (QC, hydrophobic) in PLGA NPs despite the obviously different properties of these two drugs [55]. In another example, researchers proposed a concept of drug-conjugate strategy, which successfully enabled the loading of multiple drugs onto a single carrier [56]. Briefly, two anticancer drugs, PTX (water insoluble) and gemcitabine hydrochloride (GEM, water soluble) were first linked together to form a drug conjugate (PTX-GEM) by means of a hydrolysable ester bond using glutaric anhydride as a linker. Then the PTX-GEM conjugates with a stoichiometric ratio of 1:1 were loaded into a lipid-coated PLGA NP during particle preparation to achieve the dual drug delivery. Enhanced cytotoxicity of the resulting drug-loaded PLGA NPs was also demonstrated *in vitro* against XPA3 human pancreatic carcinoma cell line. In addition, Zhang *et al.* , attaching a reversible physical conjugate of DOX and an aptamer to a DCTX-encapsulated PLGA-*b*-PEG-based NP, manufactured a targeted drug delivery system that could simultaneously deliver both hydrophilic and hydrophobic drugs to cancer cells [57]. Some other reports about delivery of a drug cocktail from PLGA NPs have been presented recently as well, such as co-delivery of PTX and tariquidar (a Pgp modulator) to overcome tumor drug resistance [58] and co-delivery of three antiretroviral drugs (ritonavir, lopinavir, efavirenz) for the treatment of human immunodeficiency

type-1 (HIV-1) [59]. Besides PLGA NPs, NPs made from polyalkylcyanoacrylate [60] and chitosan [61] have also been fabricated for combined delivery of multiple therapeutic agents.

Nanospheres, a kind of NP with some aqueous areas distributed in polymeric matrix, are usually made by the water-in-oil-in-water (W/O/W) double emulsion-solvent diffusion/evaporation method. Owing to the two-phase structure, nanospheres allow entrapment of both hydrophilic and hydrophobic therapeutic agents, respectively, in the aqueous phase and the polymer matrix. Some research on using nanospheres to co-load agents with different physicochemical features has been reported, such as co-loading two drugs [62] or co-loading a drug and a bioactive agent (e.g., DNA) [63,64]. For example, in the work from Hammady *et al.*, poly(DL)-lactide polymer-based nanospheres with grafted selectin-specific ligands were used to co-encapsulate two antiangiogenic agents, endostatin (EN, hydrosoluble peptide) and PTX (water insoluble) [62]. The cellular uptake of nanopheres by activated HUVECs *in vitro* was improved as a result of the receptor-mediated endocytosis, and a synergetic antiangiogenic effect of the delivery system with two drugs (at lower doses), compared with free drugs, was observed in rat aorta tissue culture *ex vivo* models. On the other hand, Hammady and colleagues also used a single nanosphere to co-encapsulate a lipophilic drug (all-*trans* retinoic acid [RA]) and a water-soluble bioactive agent, calf thymus DNA, which can be substituted by a peptide, a protein or a plasmid DNA [63]. Drug release behavior was mostly influenced by the nature of the polymeric matrix and the porosity of the resulting nanospheres. The lower the microporosity the nanosphere had, the slower the drug release behaviors shown. A more pronounced antiangiogenic effect of an RA-loaded system than that of free drug was observed in rat aorta tissue culture, and its inhibitory action was sustained in a 14-day study.

Lipid or lipid-based nanoparticle composed of a solid hydrophobic core and a monolayer of lipid or polymer-lipid hybrid is one type of nanocarrier for bioactive agent, such as therapeutic peptides, proteins and antigens. In Wu's lab, this kind of carrier has been used for a MADS as well as a single-agent delivery system [65-67]. For example, a solid lipid NP incorporating an anionic polymer (dextran sulfate [DS]) was prepared to load and co-deliver DOX and chemosensitizers (e.g., VRP) [65]. The presence of anionic DS could enhance the encapsulation efficiency of cationic drugs by forming complexes, reduce the burst release, prolong the release time and meanwhile improve the anticancer effects. Based on their initial work about lipid NPs, Shuhendler *et al.* demonstrated true anticancer synergy of DOX and mitomycin C (MMC) by delivering them concurrently to human breast cancer cells, using a solid polymer-lipid hybrid nanoparticle (PLN) as a drug carrier [66]. In another report from Xu *et al.*, the complex of survivin-suppressor (iSur-DNA) and DCTX was encapsulated by a lipid-based envelope that consisted of PEG-disulfide-DOPE (DOPE: distearoylphosphatidylethanolamine) and folic acid-PEG-DOPE, via the lipid film hydration technique [68]. The introduction of folic acid, PEG and DOPE allows the resulting system to have active targeting, extended circulation time and drug release into the cytoplasm. This co-delivery system combined with iSur-DNA and DCTX provided a safe and efficient strategy for local treatment of human hepatocellular carcinoma.

For optimum treatment of diseases such as cancer, functionalizing the already existing drug carriers to improve their properties is important. In drug delivery systems, multifunctional nanoparticles with compound effects, for example, targeting specificity, diagnostic/imaging capability, optimized pharmacokinetics and combined delivery of pharmaceutic agents, is desired. Nowadays, magneto-polymeric nanoparticles used as multi-agent carriers for combination therapy have emerged. One example is from the report of Yang and co-workers [69]. They fabricated a magneto-polymeric nanoparticle by simultaneously encapsulating hydrophobic magnetic nanocrystals and anticancer drug DOX in a polymeric nanoparticle. Then the resulting NPs were conjugated with therapeutic antibodies using a carboxyl group to form MADSs, which concurrently had active targeting and potential imaging capability. Another example is about multifunctional nanoparticles consisting of superparamagnetic iron oxide core and glyceryl monooleate (GMO) lipid coating in which two hydrophobic anticancer drugs (PTX and rapamycin) were loaded [70]. High drug-entrapment efficiency (~ 95%), sustained drug release (> 2 weeks *in vitro*) and good antiproliferative effect against MCF-7 cells made this NP a potential multi-agent carrier in the combination therapy of cancer.

3.5 Other nanocarriers

Besides the nanocarriers presented above, some other forms of vehicles in the nano range, such as dendrimers [71,72], polymersomes [73], mesoporous silica nanoparticles [74], 'nanocells' [75], and so on, were established for co-delivering multiple therapeutic agents in the combination therapy.

Owing to the unique structure and characteristics including the inner interspace and terminal functional groups, dendrimers allow drug molecules to be entrapped in their interior and/or attached on the surface by hydrophobic/hydrogen bond interactions or by electrostatic interactions. Based on these properties, Zhao *et al.* investigated the competitive binding of multiple drugs to a single dendrimer and then produced a ternary dendrimer-drug complex with two drugs (**Figure 2A**), phenylbutazone (PBZ) and sulfamethoxazole (SMZ), which respectively localized in the interior pocket and bound on the surface of the dendrimer carrier by electrostatic interactions [71]. This so-called host–guest chemistry of dendrimer-drug complex provides a new way of designing and optimizing MADSs. Besides, microRNA (antisense-miR-21 oligonucleotide) and 5FU were also co-delivered to human brain glioma cells U251, where poly(amidoamine) dendrimer was used as a carrier. The cytotoxicity of 5FU

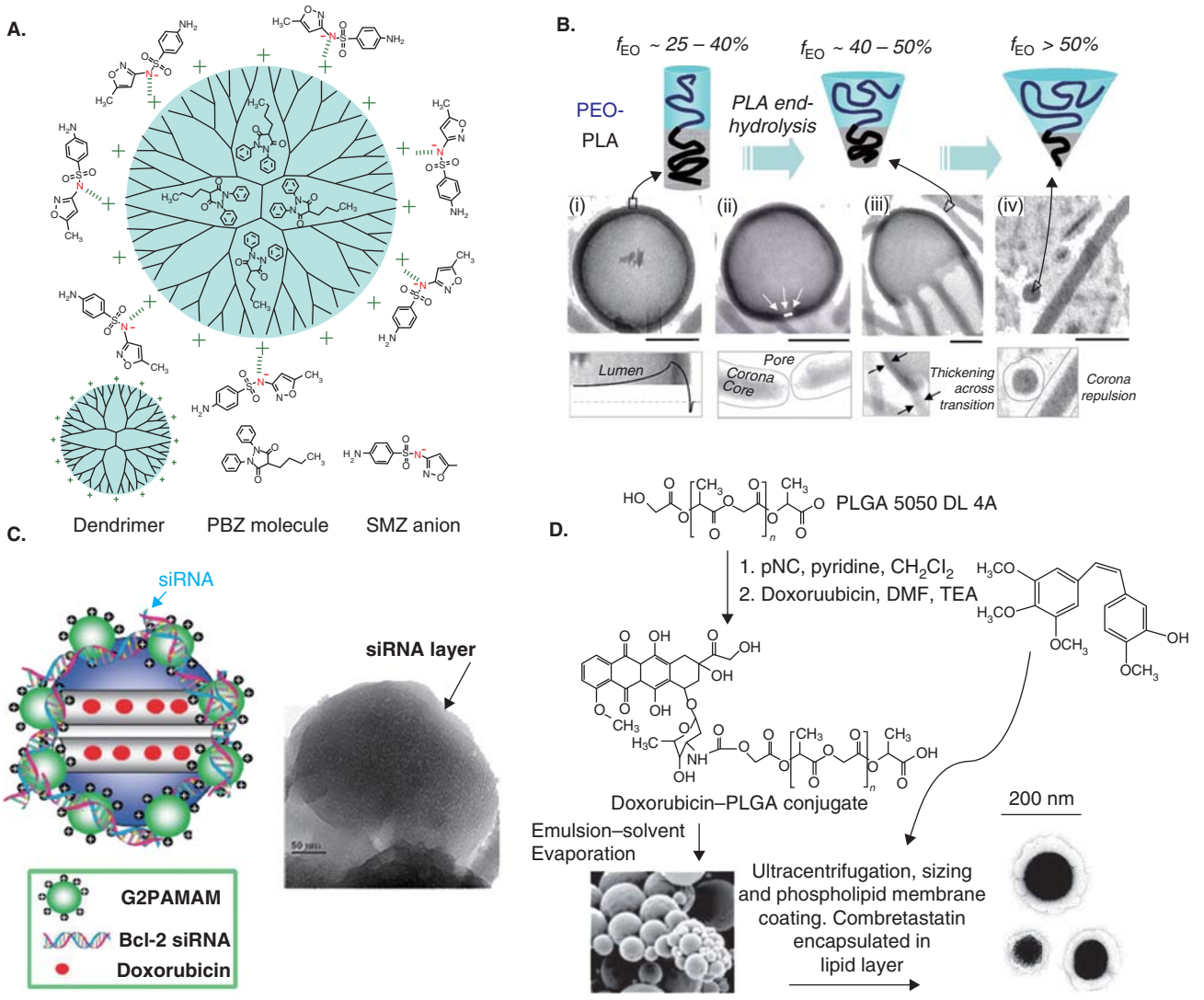

Figure 2. Types of nanocarrier with special structure in multi-agent delivery systems for combination therapy. A. Dendrimer carrier with phenylbutazone encapsulated in its interior pocket and sulfamethoxazole bound on its surface. **B.** TEM images of empty PEG-PLA-based polymersome and its topical key features such as the vesicular lumen and the corona-lined pore, and so on. **C.** Schematic diagram and TEM image of mesoporous silica nanoparticle-based MADS to co-load DOX and siRNA for combination therapy. **D.** Schematic diagram of synthesis process and SEM and TEM images of drug-containing nanocell.
Reproduced with permission from Ref. [71,73-75].
SEM: Scanning electron microscope; TEM: Transmission electron microscope.

was significantly improved by co-delivery of the miR-21 inhibitor, and consequently the apoptosis rate was increased and the migration of tumor cells was reduced [72].

Polymersome has a polymer-based shell structure, possessing an inner aqueous lumen and a thick hydrophobic periphery, so that a co-delivery system can be realized by concurrent loading of hydrophilic and hydrophobic drugs in this carrier as liposomes. A successful example is from Ahmed *et al.* [73]. In their work, PTX and DOX were co-loaded in a biodegradable polymersome fabricated from PEG-PLA and PEG-butadiene diblocks (**Figure 2B**). PTX was loaded in the core of polymersome membrane whereas DOX was encapsulated in the aqueous lumen. Prolonged circulation time and drug release of ~ 1 day from this polymersome system were obtained. Further *in vivo* study has demonstrated a more effective and sustained antitumor effect of this system than with the free drug cocktail [73].

Mesoporous silica nanoparticle (MSN) as a carrier for co-delivery of DOX and siRNA (**Figure 2C**) to overcome anticancer drug resistance was reported by Chen and colleagues [74]. DOX was first encapsulated inside the pores of MSN followed by modification of the DOX-loaded MSN

with amine-terminated polyamidoamine (PAMAM) dendrimers. Then the dendrimer-modified MSNs formed complexes with siRNAs to obtain a delivery system with chemical and genetic drugs. The synergy of these two agents against human ovarian cancer cells has also been observed in *in vitro* study. Also, the minimal premature release of DOX from the MSNs in the extracellular environment greatly limited the adverse side effects of DOX [74].

Sengupta and co-workers engineered a new drug carrier 'nanocell', which is a polymeric nanoparticle with an extranuclear PEGylated-lipid envelope (**Figure 2D**), for solid tumor treatment [75]. To fabricate the co-delivery system, an anticancer drug DOX was loaded in the nuclear PLGA-based nanoparticle after forming DOX-PLGA conjugates while an antiangiogenesis agent CA4 was entrapped within the outer lipid coating. This system allowed a first release of CA4 from the outer envelope to cause tumor vascular shutdown and then release of DOX to induce apoptosis, which could improve the therapeutic index and reduce side effect [75].

3.6 Microparticles

Microparticles served as a carrier of drugs are generally between 1 and 1000 μm. Compared with nanocarriers, microparticles with bigger volume can make it easier to encapsulate biomacromolecular drugs (e.g., protein, nucleic acid and antibody) or encapsulate more small molecular drugs. On the other hand, a long drug release period can be achieved with this system. Many methods have been developed to fabricate microparticles, including solidification of emulsion, coacervation, solvent evaporation and solvent extraction. Nowadays, many microparticle drug delivery formulations have undergone clinical trials, and some (**Table 2**) for combination delivery of therapeutic agents have been produced and evaluated in the laboratory.

On account of their excellent properties and a long safety record in the clinic, PLGA-based drug delivery systems and other injectable implants have already been successful commercially. Meanwhile, PLGA microparticles fabricated for delivery of two or more drugs have also been under study [76-79]. Hussain *et al.* simultaneously encapsulated antisense AODN and 5FU in a PLGA microparticle using a double emulsion method [76]. The resulting formulation released drugs significantly more slowly, with a long release period over 35 days, than the formulation containing a single drug. Khuller and co-workers co-loaded three drugs (isoniazid, rifampin and pyrazinamide) into a PLGA microsphere with diameter < 3 μm to treat murine tuberculosis by oral drug delivery [77]. Greater bioavailability of this co-delivery system was observed compared with that of free drugs.

Lamprecht and co-workers compared the performance between microparticle co-delivery systems made by different methods [80,81]. For example, they co-encapsulated nifedipine and propranolol HCl (lipophilic and hydrophilic drugs, respectively) in a single poly(ε-caprolactone) (PCL) microparticle prepared either by the oil-in-water or the water-in-oil-in-water solvent evaporation method [81]. The diameter of the resulting microparticles, encapsulation efficiency and state of two drugs as well as the drug release behaviors from the formulation prepared by each method were investigated. Based on the results of comparison, they concluded that the W/O/W technique is a promising method for co-encapsulation of both lipophilic and hydrophilic drugs.

As shown by some studies, a potent therapeutic antigen-specific immune response will be generated if antigens and cytosine-phosphorothioate-guanine oligodeoxynucleotides (CpG ODN) are co-delivered to the same antigen-presenting cell (APC) [82]. So developing a delivery vehicle to ensure the co-delivery of two agents is important. Some microparticle formulations were produced for this purpose [76,83-85]. For example, immunostimulatory acid-degradable microparticles were prepared as vehicles for combination delivery of CpG DNA and a protein antigen ovalbumin (OVA) [83]. Concretely, methacrylamide-modified CpG macromers were covalently linked to the polymer scaffold of acrylamide particles containing acid-degradable crosslinkers, and OVAs were simultaneously encapsulated during microparticle formation. The *in vivo* cytotoxicity assay demonstrated that the co-delivery of both antigen OVA and CpG from a single particle led to higher cytotoxic T-lymphocyte activity than the co-administration of OVA-loaded particles and free CpG with a corresponding amount. In addition, sustained protective immunity in the MO5 murine melanoma model could be induced by the systems until the loss of antigen expression in tumor.

In a report of Wu and co-workers, a chemosensitizer (VER) and an antineoplastic agent (vinblastine) were delivered concurrently by ionic polysaccharide microspheres with diameters ranging from 40 to 125 μm [86]. An effective release of each drug from the co-delivery system was shown in the *in vitro* test, and the release rate was controlled by hydration and swelling of the microsphere, and ion exchange as well. The uptake of VER from the co-delivery system by MDR cells was comparable to that of free VER, suggesting the bioactivity of chemosensitizer had not been weakened during the process. Manna and Patil encapsulated hydrophilic and hydrophobic drugs in the vacant core and the shell of a microcapsule during the process of microcapsule formation, by a layer-by-layer technique with a polyelectrolyte and amphiphilic surfactant [87]. In this system, hydrophilic drugs presented a relatively lower release rate than hydrophobic ones for the formation of tightly bound ionic complexes between the polyelectrolytes and the hydrophilic molecules. However, for hydrophobic drugs, there is no such kind of interaction; so differential release of drugs can be achieved in such a system.

4. The multi-agent delivery systems based on bulk materials for combination therapy

Different from systemic drug delivery by intravenous injection as nanocarriers, bulk materials including hydrogels, fibrous mats or films and composites of different carriers

(**Table 3**) are commonly used for local drug delivery. Furthermore, compared with drug accumulation in target sites by cellular uptake when using nanocarriers, bulk materials can be injected or implanted near the treated area, offering a depot from which drugs can be eluted slowly. Thus, a high local concentration of drug can be maintained in the pathological site over an extended period. On the other hand, the achievement of differential release of multiple agents in this kind of system is more likely owing to easier designing and construction of complicated structures in bulk scale.

4.1 Hydrogels

Hydrogels, swollen networks of crosslinked hydrophilic polymers, are appealing for biomedical applications owing to their tissue-like elasticity and good biocompatibility. Also, hydrogels possess high permeability for nutrients as well as water-soluble matter (e.g., hydrophilic drugs), which makes them a potential platform for drug release. To achieve the controlled drug-releasing application, properties of hydrogels such as permeability, stimuli-responsive ability, biodegradability and surface functionality have been engineered and optimized by researchers [88]. Nowadays, to design and fabricate new hydrogels as carriers for controlled release of multiple drugs is one of the hot topics in this area.

The releasing behavior of drugs from the hydrogel matrix depends not only on the physicochemical properties of drugs but also on how the drugs are being loaded. Based on this recognition, Mooney's group developed a new biodegradable hydrogel made from the oligomers of alginate and the crosslinker adipic dihydrazide, to load and release locally anticancer agents in a controllable way [89]. Three model drugs, methotrexate, DOX and mitoxantrone, were incorporated within the hydrogel in different ways. Methotrexate as a water-soluble drug was just dissolved in the pores of hydrogel networks. DOX was attached to the alginate backbone by covalently forming a hydrolysable hydrazone bond. Mitoxantrone was conjugated to the polymer by means of ionic interactions. So differential drug-release behaviors from this single formulation would be presented according to their loading methods and the controlled release could be achieved by different release mechanisms. In line with expectations, methotrexate with minimal hydrogel interaction, whose release is controlled by the diffusion, presented a rapid release profile. The release of DOX, lying on the hydrolysis extent of the hyrazone bond, was slower than that of methotrexate. The slowest release among the three drugs was observed for mitoxantrone, as it has a hydrogel degradation-relevant release mechanism. This successful example provided a feasible method to co-load and differentially deliver a variety of anticancer drug cocktails via the three different release mechanisms [89].

Recently, the authors reported a hyperbranched poly (amine-ester) (HPAE)-based hydrogel system for combination delivery of anticancer drugs. HPAE macromers with different modification degrees of terminal C = C were synthesized and used to fabricate an *in situ* gelling hydrogel with initiator ammonium persulfate (APS) [90]. This kind of system allows differential release of drugs with a prolonged release period through a two-step release procedure (step 1: diffusion out from HPAE molecules to hydrogel network; step 2: diffusion from hydrogel network to environmental release medium) (**Figure 3A**). Anticancer drugs, DOX and 5FU, were separately encapsulated in the different HPAE macromers, while leucovorin calcium (LC), as an adjuvant improving the anticancer effect of 5FU, was directly dispersed in the hydrogel matrix. Interestingly, it was found by the *in vitro* release study that the release behaviors of DOX and 5FU from the hydrogel with the same crosslinking density depended on the C = C modification degree of HPAE macromers where the drugs were encapsulated. Both DOX and 5FU were released slowly from macromers with high content of C = C but relatively fast from macromers with low C = C content, whereas LC was released the fastest among the three drugs for its one-step release (**Figure 3B** and **C**). Therefore, controlled/differential release of multiple drugs from a single delivery system can be conveniently achieved by loading different drugs into the HPAE macromers with different acrylation degrees [90].

Konishi *et al.* reported a dual release system of cisplatin and DOX based on a chemical crosslinked gelatin hydrogel, which presented a synergistic effect between these two drugs and significantly prolonged the survival rate of tumor-bearing mice [91]. Besides hydrogels from chemical crosslinking, a series of injectable physical hydrogels based on the blends of hyaluronan (HA) and methyl cellulose (MC) were fabricated for sustained combination therapy by co-delivery of different therapeutic agents [92]. To achieve a diversity of drug release profiles, the researchers dissolved drugs directly in the HAMC hydrogel matrix to get a fast diffusion-controlled release, and dispersed drug-loaded PLGA particles within the hydrogel to obtain a slow release behavior. The following *in vitro* release data indicated that, utilizing the combination of different drug release mechanisms, the release period of drugs can be adjusted from 1 to 28 days.

4.2 Nanofibrous mats

Nanofibrous mats, which possess the capability of encapsulation and controlled release for drugs, have been established as a type of drug delivery carrier or a tissue engineering scaffold. Nanofibrous mats are composed of core-shell polymer fibers commonly obtained by coaxial electrospinning, and recently by means of emulsion electrospinning. In emulsion electrospinning, the emulsion commonly used is a water-in-oil one, of which hydrophobic and hydrophilic drugs can be separately dissolved in the different phases (oily phase and aqueous phase), making the resulting fiber mat a potential carrier for MADSs in combination therapy.

Based on this characteristic, some MADSs constructed with fiber mats from biocompatible polymers have been

Table 3. Types of bulk material-based multi-agent delivery systems.

Bulk platforms	Characteristics	Examples of drug loading	Drug release	Ref.
Hydrogels	Swollen networks of crosslinked hydrophilic polymers	Methotrexate loaded in the pores of hydrogels, DOX and mitoxantrone attached to polymer matrix, respectively, by forming hydrazone bond and by ionic interactions	Release of methotrexate controlled by diffusion (fastest), DOX by covalent degradation and mitoxantrone by ionic dissociation (lowest)	[89]
		LC dissolved in hydrogel matrix, DOX and 5FU entrapped in the HPAE macromers with different contents of C = C	Release of LC controlled by diffusion, release of DOX and 5FU influenced by the C = C contents of HPAE macromers (the lower the C = C content, the rapider release, and vice versa)	[90]
Nanofibrous mats	A mat composed of core-shell polymer fibers commonly obtained by electrospinning	PTX loaded within the polymer matrix, DOX loaded in the inner aqueous phase of fibers	Release of DOX controlled by diffusion faster than that of PTX controlled by fiber degradation	[93]
		Tetra-layered nanofiber meshes: the first drug-loaded mesh, barrier mesh, the second drug-loaded mesh, and basement mesh (from top to bottom)	Time-programmed dual release achieved and controlled by morphological features of meshes	[95]
Composite materials				
Hydrogel/micelle composite	Hydrogel with embedded micelles	DOX encapsulated in pH- and themo-sensitive GPG micelles, Asp dispersed in the hydrogel	Asp: a short-term release, DOX: a long-term release and release rate accelerated by decreasing pH or increasing temperature	[96]
Polymer nanolayer/ micelle composite	Film with multiple layers and embedded micelles among the layers	Hydrophobic drugs (diclofenac or PTX) encapsulated in micelles, therapeutic polysaccharides (heparin or dextran sulfate) used to assemble the film with poly(β-amino ester)	Distinct drug release obtained, and governed by the intrinsic properties of the resulting polyelectrolyte multilayer including the drug component and other layering agents in the film	[97]
Mesoporous glass/micelle composite	Mesoporous bioactive glass with polypeptide-modified micelles attaching to its surface with hydrogen bonds	Water-soluble GS loaded in mesopores of glass and fat-soluble Nap in the core of micelles	A quick release for GS in acidic medium, whereas in basic medium for Nap	[98]
Hydrogel/ micro-particle composite	*In situ* crosslinking hydrogel with embedded microparticles	Chemokine MIP3α dispersed in hydrogel, pDNA and siRNA loaded in PEI-PLGA microparticles	Release of MIP3α attract dendritic cells to hydrogel first, then pDNA and siRNA co-delivered into the cells	[99]

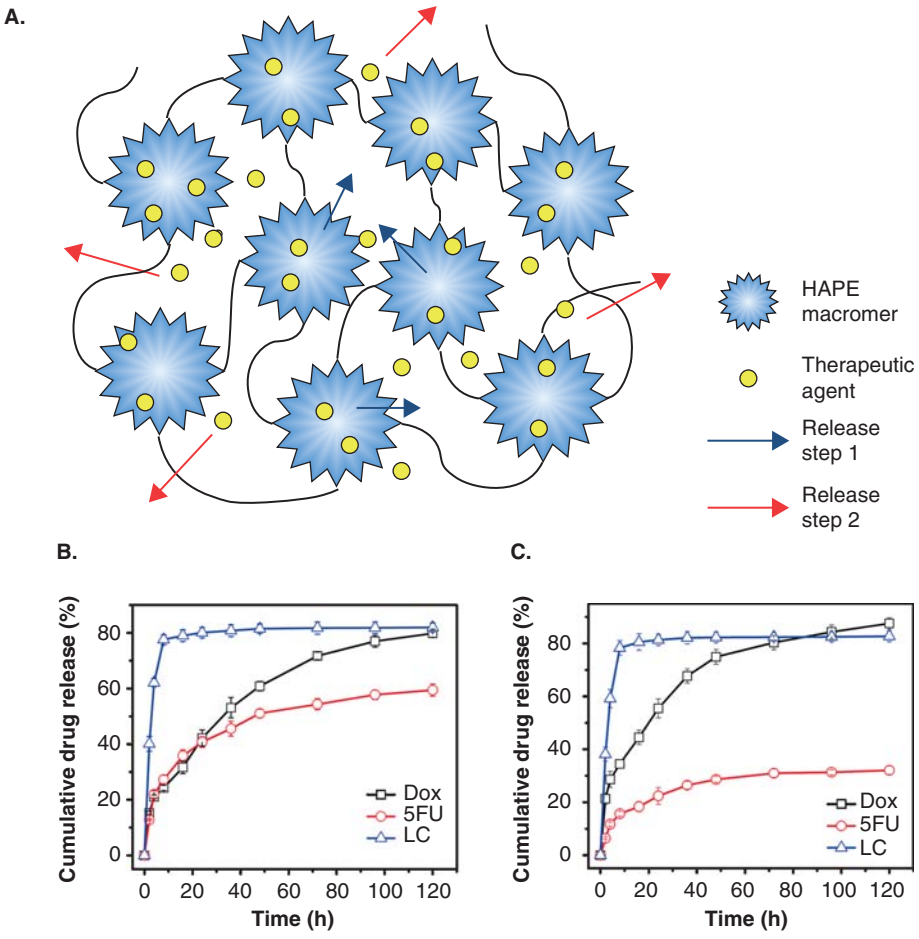

Figure 3. A. Schematic diagram of the two-step release procedure and combined release profiles of DOX, 5FU and LC from hydrogel B. H3-5FU/H5-DOX and C. H3-DOX/H5-5FU.

Reproduced with permission from Ref. [90].

H3: HPAE macromers with 18% terminal C = C groups; H5: HPAE macromers with 43% terminal C = C groups; Hn-drug: Drugs were encapsulated in macromer Hn.

developed. For example, a mat based on size-uniform PEG-*b*-PLA nanofibers was fabricated and concurrently loaded with anticancer agents PTX and DOX by Xu *et al.* [93]. Hydrophobic PTX was incorporated within the polymer matrix of nanofibers, with relatively hydrophilic DOX in the inner aqueous phase. *In vitro* release study revealed DOX release, controlled by a diffusion mechanism, was faster than PTX from both the single-agent formulation and the formulation co-loaded with two drugs. Besides, the release of PTX became faster from the dual drug-loaded fiber than from the single drug-loaded one, which might be because the degradation of fibers caused by the fast release of DOX accelerated the release of PTX. Cytotoxicity assay showed a more efficient inhibition against rat glioma C6 cell from the dual drug formulation compared with the single drug-loaded system, suggesting a promise of this fiber mat for multi-agent delivery in combination therapy. Also, rhodamine B and bovine serum albumin (BSA), as a small molecular drug and a macromolecular protein, were successfully incorporated into a poly(L-lactide-*co*-caprolactone) (PLCL) fiber mat by Yan *et al.* [94]. The drug release from this system could be controlled by adjusting the parameters of emulsion preparation.

In another work of Okuda *et al.* [95], a multilayered nanofiber mat was constructed from PLCL copolymers by sequential electrospinning with four layers, which were: i) the first drug-loaded mesh (top); ii) barrier mesh; iii) the second drug-loaded mesh; and iv) the basement mesh (bottom), respectively (**Figure 4**). Based on this structure, the release of the first drug could be controlled by the morphological features of drug-loaded mesh, such as fiber diameter and mesh thickness, whereas the second drug release could be adjusted by the features of barrier mesh as well as the drug-loaded mesh. Thus, a time-programmed dual drug release system was achieved.

4.3 Composite materials

Composite material composed of materials with different structures is one of the promising vehicles for multi-agent

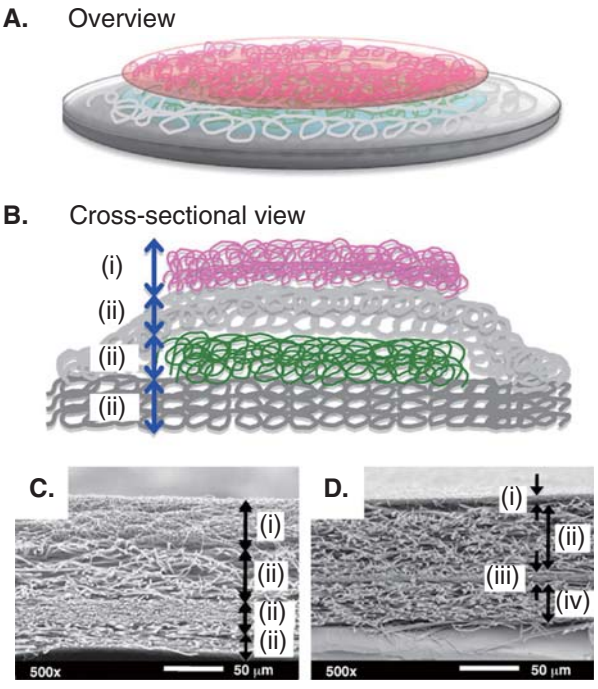

Figure 4. Schematic presentation of A. overview and B. cross-sectional view of tetra-layered nanofiber mat: i) the first drug-loaded mesh (top side); ii) barrier mesh; iii) the second drug-loaded mesh; and iv) the basement mesh (bottom side). C, D. Cross-sectional SEM images of tetra-layered nanofiber mat with different layer thicknesses.
Reproduced with permission from Ref. [95].
SEM: Scanning electron microscope.

delivery in combination therapy, as drug release behaviors can be different from carriers with different structures. A few composite systems have been designed and developed for combination delivery purpose, such as hydrogel/micelle composite [96], polymer nanolayer/micelle composite [97], mesoporous glass/micelle composite [98], hydrogel/microparticle composite [92,99] and alginate bead-embedded silk fibroin scaffold [100].

Take hydrogel/micelle composite as an example. Wei *et al.* [96] used pH- and thermo-sensitive micelles that were prepared from poly(L-glutamic acid)-*b*-poly(propylene oxide)-*b*-poly(L-glutamic acid) (PLGA′-PPO-PLGA′, abbreviated as GPG) to encapsulate drug DOX first. Then the DOX-loaded GPG micelles were mixed with poly(vinyl alcohol)/chitosan (PVA/CS) solution containing drug aspirin (Asp) to produce a hydrogel/micelle dual drug delivery system. As CS is a pH-sensitive material as well, the release behavior of Asp, which directly dissolved in the hydrogel, could be influenced by pH value of the release medium. However, the release of micelle-encapsulated DOX could be controlled by the environmental temperature as well as pH value. From the release experiments, Asp presented a short-term release owing to its good water solubility, whereas a sustained long-term release was observed for DOX. Moreover, the release rate of DOX was obviously accelerated by increasing temperature or decreasing pH value [96]. Similarly, siRNA-DNA-carrying microparticles were embedded in a quickly degradable hydrogel loaded with chemokines to build a composite system [99]. This system allowed sustained release of chemokines to attract immature dendritic cells and simultaneous delivery of siRNA and plasmid DNA to antigen-presenting cells.

A film with multiple layers and embedded micelles was a new composite fabricated by Kim *et al.* to co-deliver therapeutics with different physicochemical characteristics [97]. In this system, neutral hydrophobic drug-loaded micelles were incorporated within multiple nanolayers that were composed of hydrolytically degradable poly(β-amino ester) and anionic therapeutic polysaccharides (e.g., heparin and dextran sulfate) by the layer-by-layer (LbL) assembly technique (**Figure 5A**). After hydrolytic degradation of poly(β-amino ester), this multilayer platform could sustainedly release a variety of therapeutic agents, including therapeutic polysaccharides, hydrophobic drugs, and bioactive agents such as proteins, peptides and DNA. At the same time, differential release could be also realized with this multiple-layer system (**Figure 5B** and **C**).

Xia and colleagues reported a pH-controlled dual drug release system based on mesoporous bioactive glass (MBG) and polypeptide-modified micelles, in which the micelles were attached to the surface of mesoporous glass by means of hydrogen bonds (**Figure 5D**) [98]. Model drugs gentamicin sulfate (GS, water soluble) and naproxen (Nap, fat soluble) were separately loaded within the mesopores of MBG and the core of micelles. Owing to the interactions between GS and Si-OH groups of MBG, GS molecules were desorbed from the MBG rapidly in acidic medium, consequently giving a quick release. However, micelle-encapsulating Nap was released quickly when pH value increased, which resulted from the accelerated migration of ionized Nap molecules out of micelles under basic conditions (**Figure 5E** and **F**). Thus, multi-drug delivery can be realized in a controlled manner using this composite material.

5. Expert opinion

Combination therapy of multiple drugs is a promising strategy in the treatment of diseases, especially in treatment where adequate effects cannot be obtained with a single drug owing to the molecular complexity of diseases. Thereby, the realization of differential delivery of drugs with distinct therapeutic effect to the disease site from a single formulation is a new area in the development of DDS. However, because of the different properties of each drug such as solubility, biological degradability and the possible interaction between drugs, the stable encapsulation of multiple drugs in a single drug delivery vehicle presents a challenge to pharmaceutical processing. On the other hand, control of drug release behaviors (e.g., release order, timing and dose) is significant in practical applications. In the last 10 years, considerable efforts have been devoted to producing

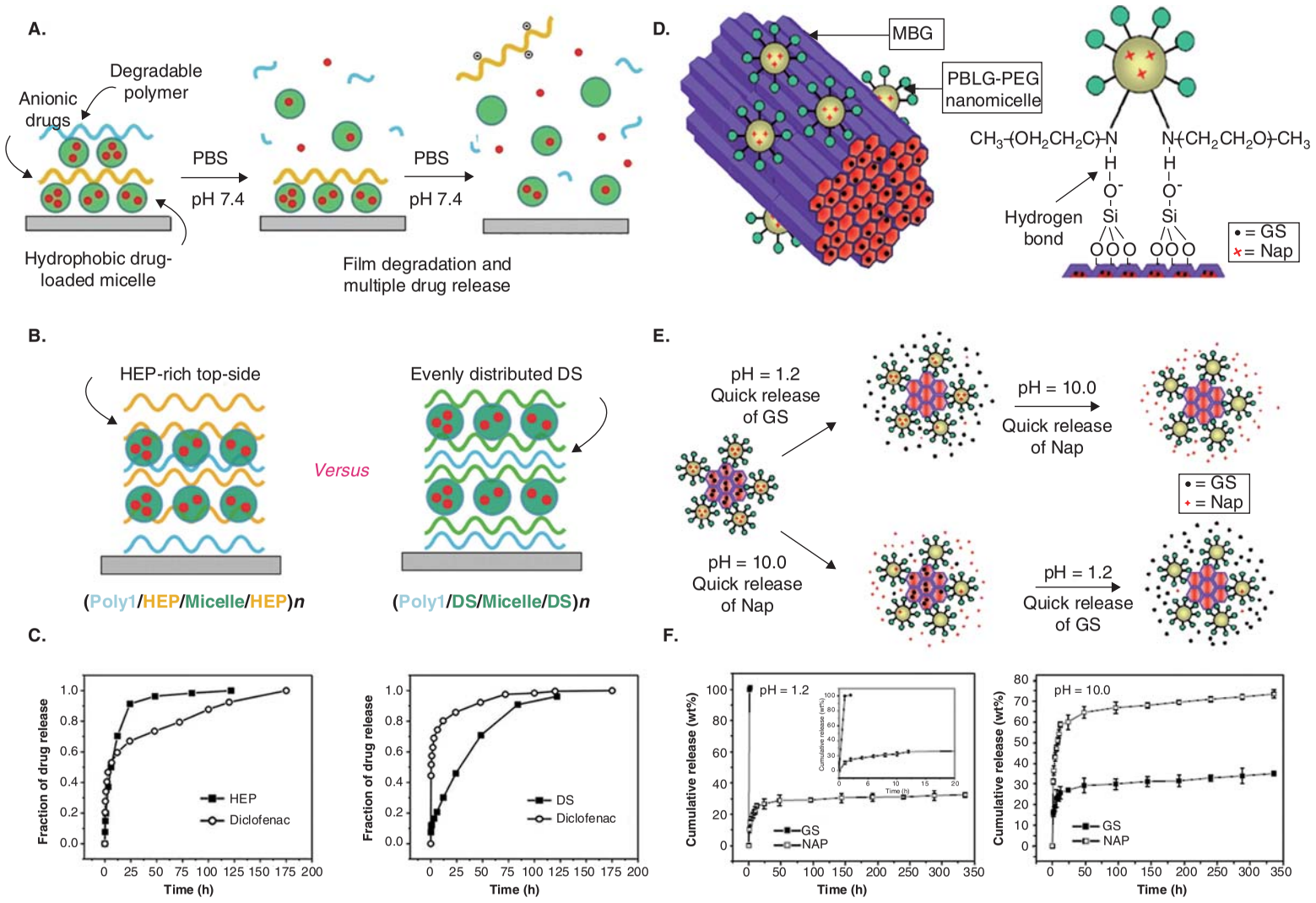

Figure 5. A. Schematic presentation of a composite film with multiple layers and embedded micelles, and release of multiple drugs from the film. B. Illustration of two different films and **C.** their corresponding different release profiles. **D.** Schematic diagram of mesoporous bioactive glass/PBLg-PEG nanomicelle composite-based dual-drug delivery system. **E.** Schematic illustration of pH-responsive drug release behaviors from the dual-drug delivery system. **F.** Real release profiles of drug GS and Nap from this system at different pH conditions.

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multi-agent delivery systems based on nano/microcarriers and bulk materials.

With the development of nanotechnology, many DDSs in nanoscale for co-delivery of multiple drugs by intravenous injection have been fabricated, such as liposomes, micelles, conjugates of drug and polymer, and nanoparticles. Utilizing various drug-loading techniques, including physical dispersion, dissolution, encapsulation and chemical covalent or electrovalent links, researchers incorporated multiple drugs and/or other bioactive agents within a single drug carrier. Generally, small molecular drugs are encapsulated into nanocarriers, whereas bioactive agents with large molecular mass are attached to the surface of carriers. Although enhanced therapeutic efficacy of nanocarrier-based MADSs has been demonstrated in numerous assays *in vitro*, there are some problems that needed to be improved for their future applications, such as system stability (e.g., susceptibility to aggregation) and drug leakage before reaching the disease site. Nowadays, some feasible ways, such as modification to nanocarriers with hydrophilic materials such as PEG, and targeting agents such as ligands and antibodies, or the design and fabrication of nanocarriers with a new structure, are used for optimization of the properties of nano-MADSs, including prolonged circulation time in the blood, elevated drug-encapsulating capability, active targeting and biocompatibility. The construction of multifunctional nano-MADSs with capabilities of signal-controlled drug release, imaging and/or diagnosis may be a possible development in the future.

Bulk material-based MADSs are usually used for localized and sustained release therapeutic agents by parenteral or topical injection. Hydrogel, especially injectable hydrogel that can form hydrogel-drug complex in the body by the sol-gel phase transition or by *in situ* chemical polymerization, is an attractive and typical bulk material for drug delivery. As hydrogel-based DDS can be formed without any organic solvent, it is suitable for delivery of labile agents such as proteins. Compared with targeted intracellular drug delivery of nano-MADSs, the release of drugs from bulk MADSs is relatively controllable by altering the material composition and properties (e.g., crosslinking density). However, as there are high water content and large pore sizes in most hydrogel matrix, the initial burst release for hydrophilic drugs and effective loading for hydrophobic drugs are still problems. Conjugation of drugs and gel matrix by chemical or physical interactions and surface-specific modification of hydrogel (e.g., coating a reduced permeability layer on the hydrogel surface with another material) are feasible methods to improve these problems, and consequently a differential release can also be achieved for each drug. On the other hand, fabrication of composite with different types of drug carrier is another way to get a MADS with excellent properties. For example, embedding nanocarriers such as micelles and particles in the hydrogel matrix may increase the biocompatibility of nanocarriers and prevent their migration away from the targeted site, and the drug release profile may also be improved by changes of the release process. Besides, smart hydrogels (namely, stimuli-responsive hydrogels) that can differentially release drugs by chemical or physical stimulation are of interest in the future development of MADS.

On the whole, although MADS is still in its infancy, progress on challenges to the fabrication of MADS with new structures and the achievement of a controlled release of multiple drugs would expand its great potential for combination therapy. In the coming years, drug delivery systems for differential release in combination therapy could be paid more and more attention, especially based on the smart nano/microcarriers and bulk materials in response to temperature, pH, light, magnetic/electric field, and so on. Meanwhile, it is important to give a risk assessment for the combination of drugs when designing or evaluating a MADS, as a drug combination can produce different effects, including synergism, additive effect and antagonism. One available method to determine this aspect is to perform the combination index-isobologram equation, which is widely used in pharmacology for both *in vitro* and *in vivo* bioassays. Finally, further studies on the biodegradation and toxicological effects of MADSs in humans are still called for to speed the practical applications of MADSs.

Declaration of interest

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