Co-encapsulation of flavonoids with anti–cancer drugs : A challenge ahead

Morgane Renault-Mahieux, Nathalie Mignet, Johanne Seguin, Khair Alhareth, Muriel Paul, Karine Andrieux

▶ **To cite this version:**

Morgane Renault-Mahieux, Nathalie Mignet, Johanne Seguin, Khair Alhareth, Muriel Paul, et al.. Co-encapsulation of flavonoids with anti–cancer drugs : A challenge ahead. International Journal of Pharmaceutics, 2022, 623, pp.121942. 10.1016/j.ijpharm.2022.121942 . hal-03882629

HAL Id: hal-03882629
https://hal.science/hal-03882629v1

Submitted on 22 Jul 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License

Version of Record: https://www.sciencedirect.com/science/article/pii/S0378517322004975
Manuscript_b0f2340cebbe004815cf57609a78d604

Co-encapsulation of flavonoids with anti-cancer drugs: a challenge ahead

Morgane Renault-Mahieux[1,2], Nathalie Mignet[1], Johanne Seguin[1], Khair Alhareth[1], Muriel Paul[2], Karine Andrieux[1]*

1. Université Paris Cité, CNRS, Inserm, UTCBS, F-75006 Paris, France ; nathalie.mignet@u-paris.fr; johanne.seguin@u-paris.fr; khairallah.alhareth@u-paris.fr; karine.andrieux@u-paris.fr

2. Pharmacy Department, AP-HP, Henri Mondor Hospital Group, F-94010, France ; morgane.renault@aphp.fr; muriel.paul@aphp.fr

*. Corresponding author

Abstract:

Flavonoids have been considered as promising molecules for cancer treatment due to their pleiotropic properties such as anti-carcinogenic, anti-angiogenic or efflux proteins inhibition. However, due to their lipophilic properties and their chemical instability, vectorization seems compulsory to administer flavonoids. Flavonoids have been co-encapsulated with other anti-cancer agents in a broad range of nanocarriers aiming to i) achieve a synergistic/additive effect at the tumor site, ii) delay drug resistance apparition by combining agents with different action mechanisms or iii) administer a lower dose of the anti-cancer drug, reducing its toxicity. However, co-encapsulation could lead to a change in the nanoparticles' diameter and drug-loading, as well as a decrease in their stability during storage. The preparation process should also take into accounts the physico-chemical properties of both the flavonoid and the anti-cancer agent. Moreover, the co-encapsulation could affect the release and activity of each drug. This review aims to study the formulation, preparation and characterization strategies of these co-loaded nanomedicines, as well as their stability. The *in vitro* assays to predict the nanomedicines' behavior in biological fluids, as well as their *in vivo* efficacy, are also discussed. A special focus concerns the evaluation of their synergistic effect on tumor treatment.

Keywords: nanomedicine, co-encapsulation, flavonoids, cancer, synergism

© 2022 published by Elsevier. This manuscript is made available under the CC BY NC user license
https://creativecommons.org/licenses/by-nc/4.0/

Abbreviations:

ASP: aspirin

BAI: baicalin

BCL: baicalein

CUR: curcumin

DCK: N^{α}-deoxycholyl-L-lysyl-methylester

DLS: dynamic light scattering

DOX: doxorubicin base

DOX-HCL: doxorubicin hydrochloride

DTX: docetaxel

EGCG: (-)-epigallocatechin gallate

EPR: enhanced permeability and retention

ETO: etoposide

GCA: glycyrrhizic acid

GEN: genistein

ICA: icaritin

PMX: pemetrexed

PLGA: poly(lactic-co-glycolic acid)

PTX: paclitaxel

QUE: quercetin

SEM: scanning electron microscopy

SGF: simulated gastric fluid

SIF: simulated intestinal fluid

SLB: silybin

TEM: transmission electron microscopy

TMX: tamoxifen

TMZ: temozolomide

TPT: topotecan

VIN: vincristine sulfate

1. Introduction

Flavonoids exerts interesting anti-cancer effects as well as reversal of anti-cancer drug resistance, or activity towards mechanisms involved in tumor invasion or development. It is common practice to administrate a combination of anti-cancer agents to overcome cross-resistance and achieve synergistically enhanced therapeutic effect, as well as to prevent toxic effects (Bayat Mokhtari et al., 2017). Combining an anti-cancer drug with flavonoids could maximize the treatment efficacy *via* an additional or a synergistic effect, thus allowing the administration of a lower dose of the anti-cancer drug and a decrease in its toxicity. It could also minimize the toxicity of anti-cancer drug treatments by scavenging free radicals, thus counteracting the ROS-mediated damages. Moreover, several flavonoids have been shown to inactivate several drug-resistance pathways, including the P-glycoprotein, an efflux protein transporting some anti-cancer drugs out of cells (Chen Chen et al., 2010; Cui et al., 2019; Kashyap et al., 2019).

The synergistic effect of two drugs is highly dependent on the dosage ratio of these two drugs. However, the difference in pharmacokinetic and biodistribution of the two drugs could prevent their synergistic effect. Nanocarriers encapsulating multiple drugs with different physicochemical and pharmaceutical properties could maintain the optimized synergistic drug ratio in a single carrier *in vivo* up to the point of intracellular uptake in the target cancer cell (Mayer et al., 2006; Tardi et al., 2007, 2009; Gurunathan et al., 2018). The use of a nanocarrier could translate the synergistic effect observed *in vitro* to a preclinical model by maintaining the fixed drug ratio (Harasym et al., 2007). A liposomal formulation (DSPC/DSPG/cholesterol (7:2:1 molar ratio)) encapsulating both cytarabin and daunorubicin in a 5:1 ratio (CPX-351) against acute myeloid leukemia in newly diagnosed patients was recently approved by the FDA and EMA as it showed an improved overall survival versus standard-of-care cytarabine plus daunorubicin chemotherapy (7+3 regimen) (Je et al., 2018; Alfayez et al., 2020). Preclinical *in vitro* testing determined the molar ratio 5:1 as the most effective as it displayed the greatest synergistic effect on a panel of tumor cell lines. The liposomal encapsulation maintained the synergistic drug ratio in plasma for 24 hours after injection and exhibited superior therapeutic activity compared to free drug cocktails, consistent with *in vivo* synergy (Tardi et al., 2009).

Co-encapsulation of a flavonoid with an anti-cancer drug has led to a synergistic or additive effect at the tumor site (Fang et al., 2018; Li et al., 2017; Pangeni et al., 2018b; Ramasamy et al., 2017; Ray et al., 2017; Wang et al., 2019, p. 20, 2015; Wong and Chiu, 2010, 2011; Zhu et al., 2017), the reversal of resistance (Hu et al., 2016; Meng et al., 2016) and delayed onset of drug resistance by combining agents with different mechanisms of action (Fatma et al., 2016; Jain et al., 2013; Li et al., 2017, 2018; Ramasamy et al., 2017; Sandhu et al., 2017; Wang et al., 2019; Zhu et al., 2017).

1.1 Anti-cancer properties of flavonoids

Flavonoids are natural polyphenols widely found in fruits, vegetables and tea. They consist of more than 6500 compounds and form one of the largest groups of plant metabolites (Panche et al., 2016).

Flavonoids are pleiotropic compounds with several properties such as antioxidant, antifungal, antibacterial, anti-carcinogenic, anti-angiogenic or anti-inflammatory activity (Dayoub et al., 2013; Nagaraju et al., 2013; Adan and Baran, 2015; Ahmad et al., 2016; Carvalho et al., 2017; Samie et al., 2018; Reyes-Farias and Carrasco-Pozo, 2019). They have demonstrated direct cytotoxicity on several tumor cell lines by inhibiting the cell cycle or inducing apoptosis. They also have demonstrated anti-angiogenic effects by targeting the VEGF or the bFGF signaling pathways, as well as by having an activity on matrix metalloprotease or directly on endothelial cells (Su et al., 2005; Touil et al., 2009, 2011; Mirossay et al., 2017). In addition, their anti-inflammatory properties may decrease the chronic inflammation that promotes tumor development, invasion and metastasis pathways (Gupta et al., 2018).

1.2 Flavonoids: a need for vectorization

Flavonoids are characterized by a common structure of two aromatic rings (A and B) which are linked through a three-carbon oxygenated heterocyclic ring (C) (Figure 1): this is the basic flavane skeleton. They are subdivided into flavonols, flavanols, flavones, flavanones, anthocyanidins and isoflavonoids according to the degree of unsaturation or oxidation of the C-rings, and depending on the carbon on which the B ring is attached to the C ring.

Flavonoids can exist as free aglycones and glycosidic conjugates (Bednarek et al., 2003; He, 2000). Aglycone flavonoids are hydrophobic molecules, but the glycosylation renders them more water-soluble (Plaza et al., 2014).

Depending on their structure, flavonoids are sensitive to light exposure, pH, temperature, solvent type (Jackman et al., 1987). For example, fisetin (FIS), quercetin (QUE), genistein (GEN) and myricetin are degraded in aqueous buffer at elevated temperature and at different rates depending on the pH (Yao et al., 2014; Wang and Zhao, 2016; Chaaban et al., 2017);

QUE and FIS are rapidly oxidized and degraded in water (Sokolová et al., 2012; Ramešová et al., 2015); flavonols are sensitive to light exposure and photodegradation is accelerated in the presence of aluminum ions (Smith et al., 2000).

Flavonoids can also be subject to rapid metabolism into the intestine by hydrolysis of the glucuroside, conjugation and deconjugation of the aglycone skeleton, in addition with elimination of conjugated metabolites by efflux transporters (Chen et al., 2003). There is also a hepatic first pass metabolism where they are conjugated by glucuronidation, sulfation, or methylation or metabolized into smaller phenolic compounds. The aglycon of flavones and flavanols is also degraded by a peroxidative mechanism and the resulting compounds can undergo further degradations by dioxygenase-catalyzed ring-fission reactions (Hinderer and Seitz, 1988).

The poor water solubility of most flavonoids is a major drawback for their administration. Combined with their poor absorption, susceptibility to degradation and rapid metabolism are the main factors for their low oral bioavailability (Hinderer and Seitz, 1988; Chen et al., 2003; Crozier et al., 2010).

To overcome these limitations, the use of nanocarriers could be a good alternative. Indeed, the encapsulation of flavonoids could increase their apparent solubility and allow their systemic administration. Oral bioavailability may also be enhanced by protection against flavonoid metabolism by the formulation. Therefore, the delivery of flavonoids using nanocarriers has been widely explored, and had led to an increase in their bioavailability and efficacy (Khushnud and Mousa, 2013; Mignet et al., 2013; Seguin et al., 2013; Aiello et al., 2019) (Figure 2).

1.3 Co-encapsulation of flavonoids with anti-cancer agents

Due to their multiple biological properties, flavonoids have been considered as one of the most promising candidates for combination therapy with anti-cancer drugs in order to increase tumor sensitivity and reduce toxicity (Kikuchi et al., 2019). Co-encapsulating flavonoids with anti-cancer agents could enable delivery of the optimized ratio to the target while protecting the flavonoids from degradation. The use of nanocarriers could also passively target the tumor site using the enhanced permeability and retention (EPR) effect, resulting in drug accumulation in the tumor site and decreased toxicity, as well as potential inhibition of intracellular endocytic uptake mediated drug resistance (Choi et al., 2016). As shown in Figure 3, research in the field of the co-encapsulation of flavonoids with other drugs has increased the last 10 years.

The aim of this review is to analyze how to co-encapsulate flavonoids with anti-cancer agents. The first part is dedicated to the formulation/preparation strategy and the challenge of this optimization. The second part reports the *in vitro* experiments to predict the *in vivo* behavior of these nanomedicines and finally their *in vivo* efficacy is discussed in the last chapter.

2. Formulation and preparation considerations

2.1 Choice of the nanocarrier for co-encapsulation

Co-encapsulating a flavonoid with another anti-cancer agent is a challenge. Indeed, the addition of a second drug in the system can create instability. The choice of the nanocarrier is important and depends on the co-encapsulated drugs and the target. As flavonoids are mostly hydrophobic and some anti-cancer drugs are hydrophilic, it is essential to choose a suitable nanocarrier capable of encapsulating both. The different nanocarriers developed to co-encapsulate flavonoids with anti-cancer drugs, as well as their compositions and preparation methods are reported in Table 1. Their characterization methods are reported in Table 2.

Polymeric micelles are spherical nanostructures formed by amphiphilic molecules capable of encapsulating lipophilic drugs in their hydrophobic internal core surrounded by the hydrophilic parts enabling their suspension in aqueous medium. They can release their load according to specific stimuli. As they are formed by the self-assembly of block copolymers held together by non-covalent interactions (Savić et al., 2006), the copolymer can be designed to respond to specific stimuli: pH (Gao et al., 2017), temperature (Lee et al., 2015), redox mechanism (Zhou et al., 2017). Several studies chose micelles to co-encapsulate flavonoids with anti-cancer agents, such as silibinin and docetaxel (DTX) (Dong et al., 2017) or QUE and DOX (Ramasamy et al., 2017) with high efficiency.

Solid-lipid nanoparticles are submicron-sized lipid emulsions where the liquid lipid (oil) has been replaced by a solid lipid. They can only encapsulate lipophilic drugs, such as baicalin (BAI) (Li et al., 2017). To overcome this limitation, hydrophilic drugs can be conjugated to or complexed with the lipids (Mehnert and Mäder, 2001).

Nanoemulsions present the same drawback: oil-in-water nanoemulsion have been proven very effective in delivering lipophilic drugs and increasing the *in vivo* efficacy of anti-cancer agents (Tagne et al., 2008) or improve the bioavailability of flavonoids (Wu et al., 2018), but they encapsulate only drugs with similar lipophilicity, such as baicalein (BCL) with paclitaxel (PTX) (Meng et al., 2016). To overcome this limitation, Pangeni *et al* designed water-in-oil-in-water nanoemulsions encapsulating both hydrophilic, amphiphilic and lipophilic

compounds (Pangeni et al., 2016, 2018a). Using this strategy, they successfully co-encapsulated QUE and pemetrexed (PMX) (Pangeni et al., 2018b).

Liposomes, lipid-polymer hybrid nanoparticles, polymeric nanoparticles and mesoporous silica nanoparticles are all capable of encapsulating both hydrophobic and hydrophilic drugs through different properties.

Liposomes are spherical vesicles made of phospholipids associated with other lipids such as cholesterol which stiffens the lipid bilayer and stabilizes it. Their amphiphilic properties allow them to self-assemble and form bilayers. Several studies have reported successful co-encapsulation of flavonoids and anti-cancer drugs in liposomes due to their ability to encapsulate i) hydrophobic drugs such as QUE in their lipid bilayer (Wong and Chiu, 2010), ii) hydrophilic drugs such as doxorubicin hydrochloride (DOX-HCL) or vincristine sulfate (VIN) in their aqueous core (Li et al., 2018; Wong and Chiu, 2010), and iii) amphiphilic drugs at the interface (Hu et al., 2010; Cosco et al., 2011). Moreover, their properties can be altered by modifying their formulation by adding functional groups to phospholipids, other lipids or polymers (Akbarzadeh et al., 2013).

Polymeric nanoparticles are formed by amphiphilic copolymers self-assembling in water into nanoparticles. They usually present better stability, sharper size distribution and more controlled drug-release profile than liposomes (Hu et al., 2010). Most of the reviewed studies chose this nanosystem to co-encapsulate flavonoids and anti-cancer drugs (Table 1). They typically encapsulate hydrophobic drugs, such as QUE and tamoxifen (TMX) (Jain et al., 2013), BCL and PTX (Wang et al., 2015) or DOX and icaritin (ICA) (Yu et al., 2020), but are able to encapsulate hydrophilic drugs by conjugation to the polymer or by attachment to the surface of the nanoparticles (Pan et al., 2019).

Lipid-polymer hybrid nanoparticles are multifunctional drug delivery platforms, which combines the mechanical advantages of polymeric core capable of encapsulating lipophilic drugs and the biomimetic advantages of the phospholipid shell in a single platform (Hadinoto et al., 2013). For example, they have been used to co-encapsulate GEN and PTX (Mendes et al., 2014).

Mesoporous silica nanoparticles are inorganic nanoparticles that can be functionalized. Their highly controlled porous structure and functionalization enable them to encapsulate both hydrophobic and hydrophilic drugs (Narayan et al., 2018). They were able to co-encapsulate flavonoids with lipophilic anti-cancer agents (Murugan et al., 2016; Fang et al., 2018).

226 Table 1. Types, formulation, preparation method and targets of nanocarriers co-encapsulating a flavonoid with an anti-cancer agent.

Flavonoid	Co-encapsulated drug	Flavonoids/drug encapsulation DL or EE (%)	Optimal wt ratio FLA/drug	Nanocarrier composition	Preparation method	Purification method	Formulation or process optimization	Administration route	Cancer	References
Micelles										
BAI	CUR	DL BAI : 3.50 ± 0.34 CUR : 7.46 ± 1.70	3.5:7.5*	quercetin dithiodipropionic acid oligomeric hyaluronic acid mannose ferulic acid polymer	Solvent-exchange	Centrifugation		Parenteral	NSCLC	(Wang et al., 2019)
QUE	DOX	Active loading capacity QUE: ~18 DOX: ~24	1:5	Poly(phenylalanine)-b-poly(L-histidine)-b-poly(ethylene glycol)	Solvent-exchange	Ultrafiltration		Parenteral	Squamous cell carcinomas	(Ramasamy et al., 2017)
SIL	DTX	DL SIL: 4.1 ± 0.2 DTX: 2.8 ± 0.3	6:4*	polyethylene glycol-blockpoly[(1,4-butanediol)-diacrylate-β-N,N-diisopropylethylenediamine]	Thin-film hydration	Dialysis		Parenteral	Metastatic breast cancer	(Dong et al., 2017)
Nanoemulsions										
BCL	PTX	EE BCL: 97.9 PTX: 97.1	1:1	Oil phase : Soybean oil and medium chain triglycerides Aqueous phase: Soybean lecithin, poloxamer 188, glycerol	High pressure homogenization	Ultracentrifugation	Preparation of complex: BCL-PL and PTX-cholesterol	Parenteral	Breast cancer	(Meng et al., 2016)
QUE	PMX	Non specified	2:1	Surfactant : Labrasol, Tween 80, Oil phase: Labrafil M 1944 CS Cremophor EL:PEG 400 Deionized water	Low-energy spontaneous emulsification		Complexation of PMX with DCK Screening of surfactants and co-surfactants Wt ratio of surfactants Concentration of drugs	Oral	Lung carcinoma	(Pangeni et al., 2018b)
NG	TMX	Non specified	Non specified	corn oil-lipid, labrasol and transcutol P	Mixing (SNEDDS)		Pseudo ternary diagram QbD based model	Oral	Breast cancer	(Sandhu et al., 2017)
Solid lipid nanoparticles										
BAI	DTX	DL BAI: 4.9 ± 0.6 DTX: 8.3 ± 0.6	37:63*	Soya lecithin, glyceryl monostearate Tf-PEG-hz-GMS, poloxamer 188	Emulsification	Centrifugation + filtration		Parenteral	Lung cancer	(Li et al., 2017)
Liposomes										
QUE	TMZ	DL QUE: 23.42 ± 2.17 TMZ: 15.87 ± 1.96	6:4*	Tween 80, poloxamer 188, DSPE-PEG2000 Glyceryl behenate, soy lecithin, cholesterol	Emulsification-evaporation and low temperature curing	Dialysis		Parenteral	Glioma	(Hu et al., 2016)
QUE	VIN	EE* QUE: 78.5 VIN: 78.3	1:2	Egg sphingomyelin, cholesterol,, ceramide-PEG2000	Thin-film hydration Loading of VIN with ionophore-mediated proton gradient	Size-exclusion chromatography	Optimization of the cholesterol ratio	Parenteral	Trastuzumab-insensitive breast tumor	(Wong and Chiu, 2010, 2011)
SIL	GCA	EE SIL: 24.37 GCA: 68.78		DPPC, cholesterol, PEG2000-DSPE	Thin-film hydration	Dialysis		Non specified	Hepatocellular carcinoma	(Ochi et al., 2016)
SLB	DOX-HCL	EE SLB: 93.07 ± 3.07 DOX-HCL: 95.74 ± 9.09	3:1	DSPE-PEG-cholic acid, cholesterol, phospholipids	Ethanolic injection Loading of DOX-HCL with ammonium sulfate gradient	Ultrafiltration		Oral	Hepatocellular carcinoma	(Li et al., 2018)
Lipid-polymer hybrid nanoparticles										

QUE	VIN	LE QUE: 5.1 ± 0.9 VIN: 12.3 ± 2.5	1:1	PLGA, cholesterol, stearic acid, DSPE-PEG2000	Nanoprecipitation	Ultrafiltration		Parenteral	Non-Hodgkin's lymphoma	(Zhu et al., 2017)
GEN	PTX	EE GEN: 97.99 ± 2.90 PTX: 97.80 ± 2.21	12:0.2	PLGA + Capric/caprylic triglyceride Pluronic F68 and F127 Phosphatidylcholine	Emulsification-evaporation Thin-film hydration	Filtration		Parenteral	Ehrlich Ascites Tumor	(Mendes et al., 2014)
Polymeric nanoparticles										
QUE QUE QUE	CUR ASP CUR + ASP	Similar entrapment QUE: 6.2 CUR: 1.5 ASP: 6.5	4.4:1:4.6 *	Chitosan- sodium hexametaphosphate	Nanoprecipitation	Dialysis			Colorectal cancer	(Ray et al., 2017)
QUE	TMX	EE QUE: 68.60±1.58 TMX: 67.16±1.24	2:1	PLGA	Emulsification-evaporation	Centrifugation	pH, type and concentration of PLGA, type and concentration of stabilizers, drugs loading ratio	Oral	Estrogen receptor positive breast cancer	(Jain et al., 2013)
QUE	DOX	DL QUE: 7.9 DOX: 3.6	6.9:3.1	Biotin-decorated poly(ethylene glycol)-b-poly(ε-caprolactone)	Thin-film hydration	Centrifugation		Parenteral	Breast cancer	(Lv et al., 2016)
BCL	PTX	EE BCL: 87.6 ± 3.1 PTX: 90.8 ± 2.9	5:1	Folate-valine-PTX Hyaluronic acid-lysine-BCL PLGA, poloxamer 188	Nanoprecipitation	Filtration		Parenteral	Lung cancer	(Wang et al., 2015)
QUE	ETO	EE QUE: non specified ETO: 62.46 ± 1.22	1:10	PLGA, poly-vinyl alcohol	Emulsification solvent evaporation	Centrifugation	O/W phase ratio polymers concentration Sonication time Drug:polymer ratio ETO:QUE ratio	Oral	Breast cancer	(Fatma et al., 2016)
ICA	DOX	DL ICA: 2.1 DOX: 10.8	3:1	PLGA-PEG, PLGA-PEG-aminoethyl anisamide	Nanoprecipitation	Ultrafiltration	ICA:DOX molar ratio	Parenteral	Hepatocellular carcinoma	(Yu et al., 2020)
EGCG	PTX	EE EGCG: 76.8 ± 9.1 PTX: 95.7 ± 7.3		PLGA, casein	Emulsion-precipitation	Centrifugation	Polymer/PTX ratio Casein/EGCG ratio	Parenteral	Breast tumor	(Narayanan et al., 2014)
Mesoporous silica nanoparticles										
QUE	DOX	Maximal DL QUE: 29.7 DOX: 32.6	1:1	Hyaluronic acid-silica	Nanoprecipitation	Centrifugation		Parenteral	Gastric carcinoma	(Fang et al., 2018)
QUE	TPT	DL QUE: 1,2 TPT: 1,8	4:6*	Silica, arginine-glycine-aspartic acid peptide, poly(acrylic) acid-chitosan	Nanoprecipitation	Centrifugation			Breast cancer (triple negative and MDR)	(Murugan et al., 2016)

**Calculated using the drug loading reported*

ASP = aspirin, BAI = baicalin, BCL = baicalein, CUR = curcumin, DCK = N$^{\alpha}$-deoxycholyl-L-lysyl-methylester, DL = Drug loading, DOX = doxorubicin base, DOX-HCL = doxorubicin hydrochloride, DSPE-PEG2000 = 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000], DTX = docetaxel, EE = encapsulation efficiency, EGCG = (-)-Epigallocatechin gallate, *ETO = etoposide, FLA = flavonoids, GCA = glycyrrhizic acid, GEN = genistein, ICA = icaritin, NG = naringenin, NSCLC = non small cell lung cancer, PEG = polyethylene glycol, PLGA = Poly (lactic-co-glycolic acid), PMX = pemetrexed, PTX = paclitaxel, QbD = quality by design, QUE = quercetin, SIL = silibinin, SLB = silybin,, SNEDDS = self-nano-emulsifying drug delivery systems, TMX = tamoxifen, Tf-PEG-hz-GMS = transferrin-polyethylene glycol-hydrazone-lyceryl monostearate, TMZ = temozolomide, TPT = topotecan, VIN = vincristine.*

233 Table 2. Characteristics and characterization methods of nanocarriers co-encapsulating a flavonoid with an anti-cancer agent.

Flavonoid / co-encapsulated drug	Minimal requirements					Additional characterization methods	References
	Size (nm)	PDI	Morphology	Zeta potential (mV)	Dosage of flavonoid (direct or indirect)		
Micelles							
BAI/CUR	121.0 ± 15	0.129	Spherical (TEM)	-20.33 ± 4.02	HPLC (UV detection) (Direct method)	Proton nuclear magnetic resonance	(Wang et al., 2019)
QUE/DOX	82.0 ± 3.4		Spherical (TEM, AFM)	-20	HPLC (UV detection) (Indirect method)	Proton nuclear magnetic resonance Gel permeation chromatography Critical micellar concentration X-ray diffractometry Fourier transform infrared spectroscopy	(Ramasamy et al., 2017)
SIL/DTX	85.3 ± 0.4	0.190 ± 0.1	Spherical (TEM)		HPLC (UV detection) (not specified)	Proton nuclear magnetic resonance u Matrix-assisted laser desorption-ionization – time of flight mass spectrometry Nile red assay	(Dong et al., 2017)
Nanoemulsions							
BCL/PTX	170.5 ± 6.5	0.113 ± 0.005	Spherical (TEM)	-42.4 ± 3.7	HPLC (indirect method)		(Meng et al., 2016)
QUE/PMX	13.2 ± 0.132)	0.095 ± 0.015	Spherical (TEM)	–3.99 ± 1.11	Non specified		(Pangeni et al., 2018b)
NG/TMX	52 – 73		Spherical (TEM)		Non specified	Self-emulsification time	(Sandhu et al., 2017)
Solid lipid nanoparticles							
BAI/DTX	135.5 ± 4.7	0.16 ± 0.05	Spherical (TEM)	-31.6 ± 3.5	HPLC (UV detection)	Proton nuclear magnetic resonance Colloidal stability	(Li et al., 2017)
Liposomes							
QUE/TMZ	196.5 ± 47.3	0.32 ± 0.09 (DLS)	Spherical (TEM)	30.5 ± 6.9	HPLC (UV detection) (direct method and indirect)	UV-Vis spectra Fourier transform infrared spectroscopy	(Hu et al., 2016)
QUE/VIN	135.9 ± 12.	0.161 ± 0.032 (DLS)			UV-vis (direct method)		(Wong and Chiu, 2011, 2010)
SIL/GCA	46.3 ± 0.4		Spherical (SEM, TEM)	-23.25 ± 0.83	HPLC (UV detection) (direct method)	Fourier transform infrared spectroscopy	(Ochi et al., 2016)
SLB/DOX-HCL	97.0 ± 2.2	0.239 ± 0.026 (DLS)	Spherical (TEM)		HPLC (UV detection) (direct method)	Stability in SGF and SIF (12 hours) Colloidal stability in plasma	(Li et al., 2018)
Lipid-polymer hybrid nanoparticles							
QUE/VIN	115.7 ± 5.5	0.22 ± 0.05	Spherical (TEM)	-36.4 ± 3.5	HPLC (VIN) UV-vis (QUE) (direct method)	Colloidal stability	(Zhu et al., 2017)
GEN/PTX	151.5 ± 5.6	0.17 ± 0.03	Spherical (TEM)		HPLC (UC detection) (direct method)	Nanoparticles tracking analysis Multiple light scattering	(Mendes et al., 2014)
Polymeric nanoparticles							
QUE/CUR	118 ± 5.6		Spherical (TEM, SEM)		UV-vis (direct method)		(Ray et al., 2017)
QUE/ASP	128 ± 0.9						
QUE/CUR/ASP	92 ± 0.7	0.12					
QUE/TMX	185.3 ± 1.20	0.184 ± 0.004			HPLC (UV detection) (direct method)	Stability in SGF and in SIF X-ray diffractometry Differential scanning calorimetry	(Jain et al., 2013)
QUE/DOX	105.8 ± 1.4	0.168 ± 0.023	Spherical (TEM)	-9.56 ± 1.80	HPLC (UV detection) (Direct method)		(Lv et al., 2016)

BCL/PTX	91.8 ± 2.3	0.1 ± 0.03	Spherical (TEM)	+3.3 ± 0.6	HPLC (UV detection) (not specified)	Proton nuclear magnetic resonance Fourier transform infrared spectroscopy	(Wang et al., 2015)
QUE/ETO	254.3	0.11	Spherical (TEM, SEM)		HPLC (UV detection) (direct method)		(Fatma et al., 2016)
ICA/DOX	~138 ~100 (TEM)		Spherical (TEM)	~3	HPLC (Not specified)		(Yu et al., 2020)
EGCG/PTX	230 ± 27 190 ± 12 (TEM)	0.18 (SEM)	Spherical (SEM, TEM)	-41 ± 3.4	HPLC (UV detection) (not specified)	Fourier transform infrared spectroscopy Raman in confocal microscopy BCA protein estimation assay Degradation of casein Colloidal stability	(Narayanan et al., 2014)
Mesoporous silica nanoparticles							
QUE/DOX	101.6 ± 3.21	0.123 ± 0.06	Spherical (TEM)	–28.56 ± 2.31	UV-vis (indirect method)	Fourier transform infrared spectroscopy Colloidal stability	(Fang et al., 2018)
QUE/TPT	72.9	0.03	Spherical (SEM,TEM)	+42.80	UV-vis (indirect method)	Thermogravimetric analysis Nitrogen adsorption/desorption isotherm Fourier transform infrared spectroscopy	(Murugan et al., 2016)

ASP = aspirin, BAI = baicalin, BCL = baicalein, CUR = curcumin, DOX = doxorubicin base, DOX-HCL = doxorubicin hydrochloride, DTX = docetaxel, EGCG = (-)-epigallocatechin gallate, ETO = etoposide, GCA = glycyrrhizic acid, GEN = genistein, HPLC = high performance liquid chromatography, ICA = icaritin, NG = naringenin, PMX = pemetrexed, PTX = paclitaxel, QUE = quercetin, SEM = scanning electron microscopy, SGF = simulated gastric fluid, SIF = simulated intestinal fluid, SIL = silibinin, SLB = silybin, TEM = transmission electron microscopy, TMX = tamoxifen, TMZ = temozolomide, TPT = topotecan, VIN = vincristine.

2.2 Optimization of the formulation and the process of preparation

2.2.1. Nanocarrier size

Once the drugs and carriers are chosen, several investigations must be conducted to optimize the formulation and process. Excipients and process are usually chosen with these critical parameters in mind: nanocarrier size and polydispersity (PDI), and drug loading. Zeta potential will not be discussed as it was mostly used to characterize the final nanocarriers or to assess the influence of nanocarrier surface modifications more than the influence of the co-encapsulation.

The size of the nanocarriers is of great importance as it influences their *in vivo* behavior. The pharmacokinetics of nanocarriers can be modified by a diameter under 5 nm, where they will be excreted by renal excretion (Choi et al., 2007), or by a diameter above 100 nm where they will be eliminated by the mononuclear phagocytic system more rapidly than with a diameter under 100 nm (Moghimi et al., 2001). Their internalization into cells (endocytosis) and their accumulation in the tumor *via* the EPR effect are also affected. The ideal diameter of a nanocarrier used for parenteral injection should be less than 200 nm (Peer et al., 2007). This size can be achieved directly as a function of the formulation and preparation process, or a size calibration may be required. There are many methods to measure the size of the nanocarriers: indirect methods such as light scattering (LS) or tunable resistive pulse sensing, and direct method such as electron microscopy (transmission electron microscopy (TEM) or scanning electron microscopy (SEM)), field flow fractionation and capillary electrophoresis. A combination of several methods, including a microscopic one to assess the morphology, is highly recommended (Gaumet et al., 2008). Most of the studies reviewed here assessed the size and polydispersity using dynamic light scattering (DLS) and TEM or SEM, and presented spherical particles with diameter under 200 nm.

Co-encapsulating two agents into one nanocarrier can sometimes increase its diameter. Ramasamy *et al* reported an increase of the co-loaded micelles' size compared to blank micelles identified by TEM (Ramasamy et al., 2017). Hu *et al* evidenced an increase in hydrodynamic diameter and PDI of liposomes co-encapsulating QUE and temozolomide (TMZ) (Hu et al., 2016). Moreover, Narayanan *et al* identified by DLS and TEM an increase in size with the casein shell formation around the poly(lactic-co-glycolic acid) (PLGA) nanoparticles as well as with drug loading, from 168 ± 18 nm to 230 ± 27 nm (Narayanan et al., 2014). Mendes *et al* encapsulated GEN by coating the PTX-PLGA-nanoparticles with a lipid bilayer incorporating GEN, which led to an increase in the size of the lipid-polymer hybrid nanoparticles (Mendes et al., 2014). On the contrary, other studies showed no impact of the co-encapsulation on the hydrodynamic diameter of nanocarriers compared to

unloaded nanocarriers (Li et al., 2017; Zhu et al., 2017; Pangeni et al., 2018b) or single-loaded nanocarriers (Wang et al., 2015; Lv et al., 2016; Li et al., 2017; Zhu et al., 2017). It should be noted that although some formulation optimization can lead to a better drug entrapment, it can also lead to an increase in nanocarrier size. For example, Jain *et al* showed that even if the use of PVA 1% as a surfactant increased the entrapment efficiency, the size was above than 200 nm and the PDI unacceptable, while the loading ratio of TMX and QUE and their total amount to be incorporated had no impact in term of size (Jain et al., 2013). It is difficult to discern a trend from these results: if the size never decreases after co-encapsulation, it can remain the same as after single-encapsulation or as empty nanocarrier. Co-encapsulation did not seem to have an impact in term of size compared to single-encapsulation, but few studies have compared both (Wang et al., 2015; Lv et al., 2016; Hu et al., 2016; Li et al., 2017; Zhu et al., 2017). The addition of a drug incorporating shell or bilayer around nanoparticles can lead to an increase in size (Mendes et al., 2014; Narayanan et al., 2014).

2.2.2. Drug loading

Drug loading is directly impacted by the chosen process and purification method. After preparation, a purification step is required to remove non-encapsulated drugs from the nanocarrier suspension. At this point, determination of the drug loading in the system is possible either by assaying the drug still encapsulated in the system after a step of disruption of this system (direct method), or by assaying the non-encapsulated drug and deducing the content of the still encapsulating drug (indirect method). This enable the determination of the encapsulation efficiency, which is the amount of entrapped drug divided by the total amount of drug added in the preparation, and the drug loading, which is the amount of total entrapped drug divided by the total nanoparticle weight, both expressed in percentage. Drug loading seems to be the better parameter to compare the encapsulation in different nanocarriers, but some publications presented only the encapsulation efficiency. Since flavonoids are mostly hydrophobic, the choice of the purification method depends on this. For example, in liposomes, hydrophobic flavonoids are located inside the lipid bilayer, near the water/lipids interface (Mohapatra and Mishra, 2011; Mignet et al., 2013) and can leak from the liposomes when aggressive methods are used such as ultrafiltration or centrifugation. In contrast, when flavonoids are covalently bound or within a nanoparticle, they are less prone to leakage with these methods. The different purification strategies used to prepare nanocarriers co-encapsulating flavonoids and anti-cancer agents were mainly centrifugation (Fang et al., 2018; Fatma et al., 2016; Jain et al., 2013; Li et al., 2017; Lv et al., 2016; Meng et al., 2016; Murugan et al., 2016; Narayanan et al., 2014; Wang et al., 2019), dialysis (Dong et al., 2017; Hu et al., 2016; Ochi et al., 2016; Ray et al., 2017), ultrafiltration (Li et al., 2018;

Ramasamy et al., 2017; Yu et al., 2020; Zhu et al., 2017) and size-exclusion chromatography (Wong and Chiu, 2011, 2010). We can discuss the efficiency of centrifugation and ultrafiltration as methods to separate non-encapsulated hydrophobic drugs from nanocarriers. Indeed, after centrifugation or ultrafiltration, the nanocarriers are usually found in the centrifugation pellet (except for micelles found in the supernatant) and resuspended after the supernatant elimination. However, the non-encapsulated hydrophobic drugs would also precipitate in the pellet and not be separated. Most studies report several washing steps of the nanoparticles that could help remove the likely precipitated flavonoid (Table 1). However, the washing medium was water (Fatma et al., 2016; Murugan et al., 2016; Zhu et al., 2017) or not defined (Jain et al., 2013; Narayanan et al., 2014; Li et al., 2018).

Drug loading is assessed after purification. As discussed in section 1.2, flavonoids are reactive and could be degraded during some preparation steps, depending on the pH or the temperature used (Gaber et al., 2017). An analytical method able to monitor the apparition of impurities, such as high performance liquid chromatography (HPLC) is highly recommended (Tsao, 2010; Corradini et al., 2011). Flavonoids can be detected by UV. Indeed, due to their polyphenolic structure, most flavonoids present two absorption bands in the UV spectrum: band I between 320 and 385 nm and band II between 250 and 285 nm which allow their UV detection. Their maximum absorption wavelengths differ according to the functional groups attached to the skeleton (Kumar and Pandey, 2013). As shown in Table 2, most studies used reverse phase HPLC coupled with a UV detector to assay the drugs. However, some used an indirect method to estimate drug loading. The direct method, consisting of the direct determination of flavonoids remaining in nanocarriers, should be preferred to the indirect method that could overestimate the loading if some degradation occurred during the process.

Co-encapsulating flavonoids with another drug may impact the loading of both drugs. Indeed, the two drugs might be in competition in the site of incorporation, or one may destabilize the structure needed for the other.

All type of nanocarriers described here have been used to successfully co-encapsulate lipophilic drugs, with some studies reporting formulation or process optimization. Jain *et al* compared the impact of the initial TMX:QUE ratio (1:1, 1:2 and 1:3) introduced for the preparation of co-loaded polymeric nanoparticles. Increasing the proportion of QUE introduced into the preparation did not impact the TMX loading, but QUE loading decreased significantly when the ratio TMX:QUE was 1:3 (from 4.10 ± 0.03 to 2.95 ± 0.23%), indicating that the best entrapment was achieved for an initial TMX:QUE ratio of 1:2 (Jain et al., 2013). Other teams co-encapsulating flavonoids with another lipophilic drug checked that the encapsulation of each drug was the same when they were either encapsulated alone or

co-encapsulated in the nanocarriers; however, the path to the optimized formulation was never reported (Mendes et al., 2014; Wang et al., 2015; Lv et al., 2016; Meng et al., 2016; Hu et al., 2016; Li et al., 2017). Yu *et al* observed that co-encapsulation of ICA and DOX increased the DOX loading (loading capacity from 8.3% encapsulated alone to 10.8% encapsulated with ICA) ; they hypothetised a π-π stacking interaction but did not investigated it (Yu et al., 2020). Pangeni *et al* optimized the concentrations of PMX complexed with N^{α}-deoxycholyl-L-lysyl-methylester (DCK) (PMX/DCK) and QUE introduced in the water-in-oil-in-water nanoemulsion by determining the maximum drug loading capacity with the minimum concentration of surfactants and co-surfactants introduced able to produce transparent nanoemulsion (Pangeni et al., 2018b). Fatma *et al* optimized the co-encapsulation of etoposide (ETO) and QUE in nanoparticles by evaluating the drug-polymer ratio and drug partitioning in the organic phase but the data were not reported (Fatma et al., 2016). These two studies report detailed results on the choice and proportions of the surfactant and cosurfactant to obtain the best nanoemulsion (Pangeni et al., 2018b) or on the influence of the excipients for a single-drug encapsulation (Fatma et al., 2016), but fail to report the loading of the co-encapsulated drugs.

The difficulty increases if the co-encapsulated drug and the flavonoid present different physicochemical characteristics. Nanocarrier formulations optimized for flavonoid incorporation and stabilization might not be optimal for the other drug. Formulation strategies including a compromise might be necessary and the co-encapsulation effect must be explored. For example, micelles can only encapsulate lipophilic drugs. Ramasamy *et al* had to convert DOX-HCL into doxorubicin base (DOX) in order to incorporate it into the micelles. In liposomes, flavonoids are inserted into the lipid bilayer, next to the polar head (Mignet et al., 2013), and can therefore destabilize the bilayer. It is important to study the interaction of flavonoids with the lipid bilayer and to characterize the influence of their incorporation. Wong *et al* optimized the best cholesterol ratio in the bilayer to co-encapsulate QUE and VIN by determining the encapsulation of each drug as a function of the cholesterol molar ratio (Wong and Chiu, 2010). To prevent destabilization, Murugan *et al* formulated pH-responsive mesoporous silica nanoparticles loaded with hydrophilic topotecan (TPT) via electrostatic interactions in the pores; to stabilize QUE, co-loaded with TPT, they conjugated it to the pH-responsive polymer coating the nanoparticles (Murugan et al., 2016). They confirmed the TPT encapsulation by thermogravimetric analysis and desorption curves, and the QUE conjugation by Fourier Transform InfraRed spectroscopy (FTIR). Narayanan *et al* designed PLGA–casein polymer–protein hybrid nanocarriers co-encapsulating (-)-epigallocatechin gallate (EGCG) and PTX. PTX was entrapped in the polymeric core while EGCG was retained by the protein shell. They optimized the PTX:PLGA:EGCG:casein ratio, and

confirmed that precipitating casein around the PTX-loaded PLGA nanoparticles did not affect PTX entrapment (Narayanan et al., 2014).

Other teams encapsulated both hydrophobic flavonoid and hydrophilic drug using the different compartments of the nanoparticles without describing detailed investigations. Li *et al* designed liposomes co-encapsulating the hydrophobic silybin (SLB) in the lipid bilayer and the hydrophilic DOX-HCL in the aqueous core at an optimized ratio 3:1 (Li et al., 2018). Zhu *et al* encapsulated both QUE and VIN in lipid-polymeric nanocarriers composed of PLGA, cholesterol, stearic acid and DSPE-PEG2000 (Zhu et al., 2017). Ray *et al* formulated spherical cross-linked chitosan-sodium hexametaphosphate nanoparticles encapsulating three drugs: QUE, curcumin (CUR) and aspirin (ASP). They also prepared two-drug loaded nanoparticles (Ray et al., 2017). For these three studies, no formulation optimization and/or preliminary testing was reported. Instability potentially caused by the co-encapsulation was not reported, nor during the process of preparation and neither during the storage.

Few studies reported the impact of co-encapsulation in term of drug loading or size, and gave only results on optimization of the nanocarriers taking into account the co-encapsulation. It is regretful that the influence and the validation of the purification step was never reported.

Co-encapsulating two drugs together might lead to physicochemical interactions between them, especially if both are loaded in the same compartment of the nanocarriers. Some studies specified that the drugs, due to their different hydrophobicity, are loaded in two different phases of the nanoemulsion (Pangeni et al., 2018b), or one into the lipidic bilayer and the other one into the aqueous core of the liposomes (Li et al., 2018). Similarly, core-shell nanoparticles can be loaded in their two compartments: one in the polymeric core and the other one in the phospholipidic bilayer coating (Mendes et al., 2014) or the casein shell (Narayanan et al., 2014). However, only Murugan *et al* experimentally demonstrated the localizations of one drug adsorbed on the pores of the mesoporous silica nanoparticles and the other on the outer layer (Murugan et al., 2016). No team studied the possible physicochemical interactions between the two drugs, even if they had similar lipophilic properties and consequently may be found in the same compartment of the nanocarrier.

From Table 1 and Table 2, we can observe that the minimal characterization of the nanocarriers are the same for co-encapsulation or single encapsulation: size distribution, surface charge, morphology and drug loading. Some characterizations might be interesting to apprehend the capacity of the system to co-encapsulate efficiently the two drugs. For instance, a lipid bilayer permeation study after incorporation of the flavonoid into the

liposome could be performed to assess whether this may lead to the leakage of the hydrophilic drug (Mingeot-Leclercq et al., 2001; Bensikaddour et al., 2008).

2.3 Shelf-stability of the co-encapsulating nanocarriers

Co-encapsulating a flavonoid with another anti-cancer agent can imply encapsulating two drugs with potential different physicochemical properties. Thus, a compromise in the formulation might have to be chosen, leading to an optimal formulation for both drugs, but not necessarily the best for the single-encapsulation of each drug. This can lead to a time-dependent instability of the formulation, such as leaking of the drugs outside the nanocarriers, aggregation of the nanocarriers, oxidation of the lipids, etc. Storage stability must be studied at least in term of preservation of size, polydispersity and drug encapsulation. Indeed, this information is crucial to determine the maximum delay between preparation and cell or *in vivo* experiments. It also provides data for the eventual mass-production and commercialization of the formulation, for which a consequent storage stability is mandatory.

Few studies reported the shelf-stability of their formulations. The results are summarized in Table 3. The conditions of these stability studies were heterogeneous in terms of duration, from 1 month to 6 months, temperature, relative humidity and storage form (in buffer, plasma or lyophilized) and did not refer to the standardized conditions described in the ICH Q1A(R2) guidelines. The optimized formulations showed good size stability, but even fewer publications studied the drug leakage.

Liquid nanoemulsions appear to be stable for several months at 4°C in term of size, PDI and drug retention; they are successful in retaining either lipophilic (QUE, BCL, PTX) or amphiphilic (PMX) drugs: less than 1% drug leakage in 6 months at 4°C for BCL/PTX nanoemulsions (Meng et al., 2016) and less than 3% of drug leakage after 3 months at 25°C for QUE/PMX nanoemulsions (Pangeni et al., 2018b). It is regrettable that too few studies have investigated the shelf stability for other types of nanocarriers. Suspensions of co-encapsulating liposomes showed leakage of either the lipophilic SIL or the hydrophilic GCA over time, with better retention of the lipophilic drug: 22% of SIL and 61% of GCA leaked from the liposomes after 3 months of storage at 4°C (Ochi et al., 2016). This result is not surprising as lipids can suffer degradation (oxidation, hydrolysis) leading to the leakage of encapsulated drugs. This is why some commercialized liposomal formulations, such as Ambisome® or Vyxeos®, are freeze-dried (Chengjun Chen et al., 2010). Freeze-drying was the method chosen by Jain *et al* to retain the lipophilic QUE and TMX inside nanoparticles, but there was no comparison with the suspension shelf stability (Jain et al., 2013). Because of the lack of shelf stability studies, it is impossible to determine a type of nanocarrier or a

process that guarantees the best retention of a flavonoid with an anti-cancer drug inside a nanocarrier.

Table 3. Shelf-stability in term of size and encapsulation of nanocarriers co-encapsulating a flavonoid with an anti-cancer agent.

Flavonoid / co-encapsulated drug	Type of nanocarrier	Storage stability			References
		Conditions	Size (nm) (PDI)	Drug leakage (%)	
QUE/DOX	Micelles	1 month, 4°C PBS pH 7.4 Serum media	 Stable Stable	 - -	(Ramasamy et al., 2017)
BCL/PTX	Nanoemulsion	6 months; 4°C	169.5 ± 6.0 (0.115 ± 0.001)	BCL < 1% PTX < 1%	(Meng et al., 2016)
QUE/PMX	Nanoemulsion	3 months 25 ± 2°C (60 ± 5% relative humidity)	Stable	<3% release	(Pangeni et al., 2018b)
QUE/VIN	Liposomes	180 days, 4°C	Stable	-	(Wong and Chiu, 2011, 2010)
SIL/GCA	Liposomes	3 months, 4°C	58.2 ± 3.8	Release SIL: 22%* GCA: 61%*	(Ochi et al., 2016)
QUE/TMX	Polymeric nanoparticles	25±2°C RH 55 ± 2% 3 months lyophilized	Stable	Stable	(Jain et al., 2013)

BCL = baicalein, DOX = doxorubicin base, GCA = glycyrrhizic acid, PMX = pemetrexed, PTX = paclitaxel, QUE = quercetin, SIL = silibinin, TMX = tamoxifen, VIN = vincristine.

**calculated using EE at day 0 and month 3*

3. *In vitro* prediction of the *in vivo* behavior of the co-encapsulating nanocarrier

To formulate the best nanomedicines, some *in vitro* experiments in biological medium can be performed to evaluate the behavior of the co-loaded nanocarriers: colloidal stability in cell culture medium, *in vitro* release, and calculation of the combination index evaluating the synergism during cell culture experiments. Those assays could permit to approach the *in vivo* behavior by combining the identification of the best ratio between the two drugs to obtain a synergistic or additive activity and verification of a similar release of both from the nanocarrier to always maintain this optimized ratio.

3.1 Colloidal stability in cell culture media

Colloidal stability of the nanocarriers co-encapsulating a flavonoid and an anti-cancer agent could be evaluated in plasma for a parenteral administration or in simulated intestinal fluid (SIF) and gastric fluid (SGF) in case of an oral route.

To predict its stability in plasma, only size was usually studied in 10% fetal bovine serum (FBS) in phosphate buffer saline (PBS) (Li et al., 2017; Zhu et al., 2017), in 20% serum in buffer (Fang et al., 2018) or in rat serum (Li et al., 2018) at 37°C. All these studies evidenced no size modification and no aggregation with duration of studies ranging from 12

hours (Li et al., 2018) to 24 hours (Zhu et al., 2017), 48 hours (Fang et al., 2018) or 72 hours (Li et al., 2017). Sometimes, the drug encapsulation were also monitored and showed good retention of the encapsulated drugs with no change in the encapsulation efficiency. This is surprising as there is an *in vitro* release of about 20% of the encapsulated drugs in aqueous buffer at 37°C: this might be explained by the fact that the dilutions for plasma stability assays are lower than those for *in vitro* release ones (1/10 versus 1/50 (Li et al., 2018) or unspecified versus 1/25 (Zhu et al., 2017)). Narayanan *et al* suspended EGCG and PTX co-loaded nanoparticles in PBS buffer (pH 7.4) with or without 20% serum, and evidenced an increase in particle size in the presence of serum, which could be due to the adsorption of serum proteins to the particle surface (Narayanan et al., 2014).

SGF and SIF are mimicking media containing enzymes and surfactants with a determined pH, 1.2 for SGF and 6.8 for SIF. In SGF and SIF, liposomes co-encapsulating SLB and DOX were able to retain at least 80% of the encapsulated drugs and did not change in size during 12 hours (Li et al., 2018). Freeze-dried PLGA-nanoparticles co-encapsulating TMX and QUE were stable in term of size, PDI and encapsulation 2 hours in SGF and 6 hours in SIF (Jain et al., 2013). These results suggest good stability in the digestive tract of these nanocarriers intended for oral administration.

3.2 *In vitro* release

The main goal of co-encapsulation is to deliver an optimized ratio of the two co-encapsulated drugs directly to the tumor site. After defining a drug ratio leading to a positive synergistic anticarcinogenic effect (positive synergistic drug ratio), similar release kinetic of the two drugs from the nanocarriers allows the conservation of this defined drug ratio in the tumor site.

To investigate whether the positive synergistic drug ratio was preserved during drug release from the nanocarriers, *in vitro* release experiments were mostly performed for formulations in different buffers with or without serum addition (Table 4). All *in vitro* release assays were performed using the dialysis method at 37°C, except for two that used the sample and separate method (Narayanan et al., 2014; Hu et al., 2016). Briefly, samples of the studied nanocarriers were transferred into dialysis bags (cut off 3000 Da (Wang et al., 2019), 3500 Da (Fang et al., 2018; Lv et al., 2016; Mendes et al., 2014; Ramasamy et al., 2017; Wong and Chiu, 2010), between 8000-14000 Da (Fatma et al., 2016; Jain et al., 2013; Li et al., 2017, 2018; Ochi et al., 2016; Yu et al., 2020) or unspecified (Meng et al., 2016; Zhu et al., 2017; Ray et al., 2017; Dong et al., 2017)) which were immersed in an adequate release medium. For pH-sensitive nanocarriers, the release medium was adapted to study the effect of pH using mainly a comparison between PBS pH 7.4 *versus* acetate buffer saline pH 5.5

(Ramasamy et al., 2017), phosphate-citric acidic buffered saline pH 5.5 (Dong et al., 2017) or an unspecified acidic environment (Murugan et al., 2016; Ray et al., 2017; Li et al., 2017; Fang et al., 2018; Li et al., 2018; Yu et al., 2020). For reduction-sensitive micelles, the effect of GHS concentration (reduction of a disulfide bond) on BAI and CUR release was studied by using a medium with GHS concentrations ranging from 0 to 10M in PBS (Wang et al., 2019).

Most release media contained surfactants (tween 80) to solubilize the lipophilic flavonoid and maintain sink conditions (Fang et al., 2018; Jain et al., 2013; Li et al., 2018; Lv et al., 2016; Mendes et al., 2014; Meng et al., 2016; Wang et al., 2015).

Table 4 reports the conditions of the *in vitro* release assays performed and their results. Drug release was presented as the percentage of released drug relative to the initial amount in the nanocarriers. Thus, the drug ratio could be considered as maintained when the release curves of each drug were superimposed.

Table 4. *In vitro* release profile of nanocarriers co-encapsulating a flavonoid with an anti-cancer agent.

In vitro release study in aqueous buffer				
Flavonoid / co-encapsulated drug	Conditions	Release of flavonoid (%)/ release of other drug (%)	Ratio maintained	References
Micelles				
BAI/CUR	37°C PBS pH 7.4	Biphasic		(Wang et al., 2019)
	100 rpm 72 hours	0M GHS : ~30/~30	Yes	
	0.5% Tween 80	1 M GHS: ~50/~60	Yes	
	From 0 to 10 M GSH	10 M GHS: ~80/~70	Yes	
QUE/DOX	37°C	Biphasic		(Ramasamy et al., 2017)
	PBS pH 7.4			
	24 hours	~7/~40	No	
	72 hours	~10/~40	No	
	ABS pH 5.5			
	24 hours	~20/~68	No	
	72 hours	~25/~90	No	
SIL/DTX	37°C 48 hours	Biphasic		(Dong et al., 2017)
	PBS 0.1 M pH 7.4	21.4/23.2	Yes	
	Phosphate-citric acid buffered saline pH 5.5	79.7/82.3	Yes	
Nanoemulsions				
BCL/PTX	37°C PBS pH 7.4	Biphasic		(Meng et al., 2016)
	2 hours	~40/~40	Yes	
	24 hours	83.8/81.2	Yes	
	0.5% (w/v) Tween-80			
Solid lipid nanoparticles				
BAI/DTX	37°C PBS 100 rpm			(Li et al., 2017)
	pH 7.4			
	24 hours	~70/~70	Yes	
	48 hours	~85/~85	Yes	
	pH 5.0			
	24 hours	~85/~85	Yes	
	48 hours	~85/~85	Yes	
Liposomes				
QUE/VIN	37°C 0.9% w/w sodium chloride 72 hours		Yes	(Wong and Chiu, 2011, 2010)
SIL/GCA	-37°C PBS pH 7.4 100 rpm			(Ochi et al., 2016)
	48 hours	~14/~88	No	
SLB/DOX-HCL	37°C 12 hours			(Li et al., 2018)
	3% v/v SDS			
	PBS pH 7.4	~50/~20	No	
	PBS pH 2.0	~20/~70	No	

Lipid-polymeric hybrid nanoparticles				
QUE/VIN	37°C PBS pH 7.4 100 rpm			(Zhu et al., 2017)
	6 days	~80-90/~80-90	Yes	
GEN/PTX	37°C 125 rpm			(Mendes et al.,
	2% SLS	~80/~10		2014)
	48 hours	~80/~70	No	
	60 days		No	
Polymeric nanoparticles				
QUE/CUR/ASP	37°C PBS 240h pH 7.4	Biphasic		(Ray et al., 2017)
	24 hours	~20/~50/~60	No	
	7 days	~92/~85/~99		
	pH 6.5 24 hours	~25/~59/~66	No	
QUE/TMX	37°C PBS pH 7.4 2.5 w/v Tween 80	Biphasic		(Jain et al., 2013)
	24 hours	44.23/24.92	No	
	20 days	94.23/85.88		
QUE/DOX	37°C PBS pH 7.4 120 rpm 0.5% Tween 80	Biphasic Similar release		(Lv et al., 2016)
	12 hours	~25/24.12	Yes	
	240 hours	~90/87.34	Yes	
QUE/ETO	37°C 100 rpm 0,1N HCl PBS			(Fatma et al., 2016)
	pH 6.8	65.8 ± 3.4/~58	No	
	24 hours	Not specified/65.6 ± 2.1	No	
	48 hours			
ICA/DOX	37°C PBS 1% BSA 24 hours			(Yu et al., 2020)
	pH 7.4	35/35	Yes	
	pH 5.5	>80/>80	Yes	
EGCG/PTX	37°C PBS pH 7.4 50 rpm	Sequential		(Narayanan et al.,
	72 hours	~49/~26	No	2014)
	10 days	~60/~28	No	
Mesoporous silica nanoparticles				
QUE/DOX	37°C PBS 100 rpm 1% Tween 80			(Fang et al., 2018)
	pH 7.4	~30/~30	Yes	
	pH 5.0	~80/~80	Yes	
QUE/TPT	37°C 48 hours			(Murugan et al.,
	pH 7.4	12.03/8.7	Yes	2016)
	pH 6.0	54.77/46.98	Yes	
	pH 5.0	80.72/76.24	Yes	
In vitro release study in buffer supplemented with serum				
Flavonoid / co-encapsulated drug	Conditions	Release of flavonoid (%)/ release of other drug (%)	Ratio maintained	References
Liposomes				
QUE/TMZ	37°C 10% human plasma (v/v) in PBS			(Hu et al., 2016)
	12 hours	~90/~75	No	
	24 hours	~90/~90		
Polymeric nanoparticles				
EGCG/PTX	37°C PBS pH 7.4 20% serum			(Narayanan et al., 2014)
	72 hours	~53/~25	No	

ABS = acetate buffer saline, ASP = aspirin, BAI = baicalin, BCL = baicalein, CUR = curcumin, DOX = doxorubicin base, DOX-HCL = doxorubicin hydrochloride, DTX = docetaxel, EGCG = (-)-Epigallocatechin gallate, ETO = etoposide, GCA = glycyrrhizic acid, GEN = genistein, GHS = gluthatione, HCl = hydrochloric acid, ICA = icaritin, NG = naringenin, PBS = phosphate buffer saline, PMX = pemetrexed, PTX = paclitaxel, QUE = quercetin, SLB = silybin, SIL = silibinin, TMX = tamoxifen, TMZ = temozolomide, TPT = topotecan, VIN = vincristine.

More than half of the formulations displayed a similar biphasic release kinetic of both drugs from the nanocarriers and thus could maintain the original drug ratio. This could allow the optimal ratio to be delivered to the tumor. However, very few teams studied the release in a complex medium supplemented with serum, and when they did, the ratio was not maintained. Of note, the release of EGCG and PTX from polymeric nanoparticles coated with casein was the same in aqueous buffer or complex medium (Narayanan et al., 2014).

Co-encapsulating TMX and QUE in polymeric nanoparticles did not retain the ratio of the co-encapsulated drugs during *in vitro* release or *in vivo* pharmacokinetics, suggesting that the formulation needs further optimization. Even though the effect of the ratio was not determined, this formulation had significantly better *in vivo* efficacy than free TMX or mixture of free TMX and free QUE (Jain et al., 2013). Similarly, there was no similar release kinetic of DOX and QUE from the micelles but *in vivo* experiments showed an improved efficacy of the antitumor effect of the co-loaded micelles compared with the mixture of the free drugs and with the DOX-loaded micelles (Ramasamy et al., 2017). In contrast, encapsulating SLB and DOX-HCL in liposomes did not permit the release of both drugs according to the most efficient ratio determined *in vitro* and, therefore, *in vivo* experiments did not evidence an impact of the co-encapsulated SLB on the tumor growth of hepatic tumor in compared to DOX-HCL encapsulated alone. However, the addition of SLB prevented cardiotoxicity. As the mixture of SLB-loaded liposomes and DOX-HCL-loaded liposomes was not studied, it is difficult to assess whether co-encapsulation was responsible for this effect (Li et al., 2018). The initial drug ratio of SIL and GCA co-encapsulated in liposomes could not be maintained during the release and no *in vivo* studies was conducted (Ochi et al., 2016).

In other cases, for specific applications, concomitant release was not desired but rather a sequential release. Indeed, Fatma *et al* required a sequential release for optimal combined efficacy, with a quicker release of QUE in order to inhibit the P-gp and enhance ETO effect (Fatma et al., 2016). Similarly, Narayanan *et al* wanted to release EGCG first to sensitize PTX-resistant breast tumor through glucose-regulated protein 78 inhibition (Narayanan et al., 2014, 2015). When GEN and PTX were co-encapsulated in lipid-polymeric nanoparticles, a temporal drug release was measured, with GEN being promptly released from the lipid bilayer due to the reversibility of the phospholipid-drug interaction and PTX being released when PLGA underwent degradation upon contact with water. This was of interest as GEN, an antiangiogenic agent, could initiate the inhibition of neoangiogenesis while the cytotoxic PTX sustainably released from the polymeric core into the tumoral tissue would promote its shrinkage. The best ratio of encapsulated GEN and PTX was evaluated *in vivo* on tumor growth and confirmed by of VEGF production assay and macroscopic evaluation of anti-angiogenic effect (Mendes et al., 2014).

As mentioned earlier, few teams characterized drug release in complex medium containing serum. Since flavonoids are known to bind to serum proteins (Bolli et al., 2010; Liu et al., 2014), it is questionable whether the drug release would be the same as in an aqueous buffer, and whether the optimized drug ratio would be maintained at the tumor site. Some teams preferred to directly study the pharmacokinetics *in vivo* but did not discuss the conservation of the optimized ratio, (Jain et al., 2013; Hu et al., 2016; Sandhu et al., 2017;

Pangeni et al., 2018a; Li et al., 2018), with the exception of Wong *et al* who showed that the ratio was conserved (Wong and Chiu, 2011). Narayanan *et al* evidenced *in vivo* the sequential release desired (Narayanan et al., 2014).

3.3 Combination index

The main goal of combination therapy is to achieve a positive synergistic effect in order to decrease the administered dose and drug-related toxicity. A combined effect that is better than each drug alone does not necessarily indicates synergism, as it can be the product of additivity or even a slight antagonist effect (Chou, 2010). An equation has been developed by Chou and Talalay to evaluate the synergism of two drugs by the combination index (CI) from cell culture experiments: $CI = (IC_{A/AB}/IC_A) + (IC_{B/AB}/IC_B)$, where IC_A and IC_B are the concentrations of A or B required to kill 50% of cells when A or B are used as single agents and $IC_{A/AB}$ and $IC_{B/AB}$ are the concentrations of A and B in the combination system required to kill 50% of cells. Synergism is defined by $CI < 0.9$, additivity by CI between 0.9 and 1.1 and antagonism by $CI > 1.1$.

CI was not evaluated for all nanocarriers. Studies that calculated CI are listed in Table 5. CI was determined using the free drugs (Meng et al., 2016; Wong and Chiu, 2010; Wang et al., 2015; Yu et al., 2020; Ramasamy et al., 2017) or the encapsulated drugs (Li et al., 2017; Wang et al., 2015; Fang et al., 2018; Wong and Chiu, 2011; Zhu et al., 2017; Ray et al., 2017). Evaluating the CI using free drugs can be very interesting to select a ratio before the formulation step, and to apprehend which loading of each drug can be achieved. Evaluating the CI using the drugs encapsulated separately or together is more accurate, because it will depend on the release of each drug from the nanocarriers.

CI was calculated using free and encapsulated drugs in only two studies. Wong *et al* reported that the best QUE:VIN ratio amongst 4 tested for synergistic effect was 1:2. They confirmed this CI using single or co-encapsulated VIN and QUE. This result was expected since the QUE:VIN ratio was maintained during *in vitro* release (Table 5, (Wong and Chiu, 2010)). Wang *et al* tested 7 BCL:PTX ratios as free or encapsulated drug. The best ratio was 5:1 using both conditions, and it was still the most synergistic on cells resistant to PTX (Wang et al., 2015).

Table 5. Combination index (CI) of the flavonoids and the other anti-cancer drugs evaluated for free drugs or encapsulated drugs.

Flavonoid / co-encapsulated drug	Synergistic studies Flavonoid:drug wt ratio with best CI (CI) study with free drugs	Flavonoid:drug wt ratio with best CI (CI) study with encapsulated drugs	References
Micelles			
QUE/DOX	4 ratios tested 1:5 (0.35, 0.58, and 0.55 in MCF-7,SCC-7, and MDA-MB-231 cancer cells respectively)		(Ramasamy et al., 2017)
Nanoemulsions			
BCL/PTX	MCF/tax cells 7 ratios tested 1:1 (0.382)		(Meng et al., 2016)
Solid lipid nanoparticles			
BAI/DTX		A549 cell line 1 ratio tested 37:63 (CI < 1)	(Li et al., 2017)
Liposomes			
QUE/VIN	4 ratios tested MDA-MB-231 cells: 1:2 (0.01) JIMT-1 cells: 1:2 (0.0000223)	1 ratio tested MDA-MB-231 cells: 1:2 (0.113) JIMT-1 cells: 1:2 (0.0900)	(Wong and Chiu, 2011, 2010)
Lipid-polymer hybrid nanoparticles			
QUE/VIN		Raji cells resistant to VIN 5 ratios tested 1:1 (0.25)	(Zhu et al., 2017)
Polymeric nanoparticles			
QUE/CUR/ASP		HCT-116 cell line Against two-drugs loaded 1 ratios but 3 dosages 2 µg/mL (0.19304) 5 µg/mL (0.33455) 10 µg/mL (0.55627)	(Ray et al., 2017)
BCL/PTX	7 ratios tested A549 cells: 5:1 (0.836) A549/PTX cells: 5:1 (0.798)	7 ratios tested A549 cells : 5:1 (0.707) A549/PTX cells :5:1 (0.513)	(Wang et al., 2015)
ICA/DOX	8 ratios tested Hepal-6 cells: 1:2 (0.24) Huh7 cells: 1:2		(Yu et al., 2020)
Mesoporous silica nanoparticles			
QUE/DOX	-	SGC7901/ADR cells 5 ratios tested 1:1 (0.19)	(Fang et al., 2018)

ASP = aspirin, BAI = baicalin, BCL = baicalein, CI = combination index, CUR = curcumin, DOX = doxorubicin base, DTX = docetaxel, ICA = icaritin, PTX = paclitaxel, QUE = quercetin, VIN = vincristine.

CI is a useful index for choosing the flavonoid:anti-cancer drug ratio to be co-encapsulated. However when CI was not determined, cytotoxicity assays were still performed to apprehend the formulation efficacy. Various methods were used: the comparison of single and co-encapsulation was evaluated (Wang et al., 2019), the co-loaded nanocarriers was compared to the free drugs alone (Jain et al., 2013; Narayanan et al., 2015; Lv et al., 2016; Hu et al., 2016; Murugan et al., 2016; Fatma et al., 2016; Dong et al., 2017; Li et al., 2018) or to the free mixture (Jain et al., 2013; Ochi et al., 2016; Lv et al., 2016; Murugan et al., 2016; Li et al., 2018). The co-encapsulating formulation could also be compared to the flavonoid single-loaded nanocarrier (Narayanan et al., 2015; Hu et al., 2016) or to the anti-cancer drug single loaded nanocarrier (Narayanan et al., 2015; Fatma et al., 2016; Lv et al., 2016; Dong et al., 2017; Li et al., 2018). The reversal index could also be evaluated by comparing the effect on cell lines resistant or not to the anti-cancer agent (Meng et al., 2016).

It should be noted that several other *in vitro* experiments were performed in an attempt to predict the *in vivo* behavior, such as cellular uptake of the nanocarrier into tumor cells (Fatma

et al., 2016; Hu et al., 2016; Lv et al., 2016; Meng et al., 2016; Murugan et al., 2016; Li et al., 2018; Wang et al., 2019), *in vitro* permeability assay using Caco-2 cells to assess the intestinal passage (Jain et al., 2013; Sandhu et al., 2017; Pangeni et al., 2018b), effect on the tumor cells protein expression (Yu et al., 2020; Fang et al., 2018; Ramasamy et al., 2017; Lv et al., 2016; Narayanan et al., 2015), effect on the ROS and glutathione generation by the tumor cells (Meng et al., 2016; Murugan et al., 2016; Ramasamy et al., 2017), or *in vitro* migration assay to evaluate the inhibitory effect of the drug on metastasis formation (Dong et al., 2017; Li et al., 2018; Pangeni et al., 2018b).

To conclude this section, an *in vitro* evaluation is always performed, most often to demonstrate an activity (cytotoxic or otherwise) of the association and/or the co-encapsulating formulation. However, there is no harmonization in the methodology used. For *in vitro* release experiments, condition variations concern duration, release medium, agitation and surfactant addition. It is to note the lacking of systematic MTT test and CI calculation, preventing a comparison between publications results. The main interest in the future would be to correlate the release ratio and the combination index to insure that a maintained release ratio is directly linked to a better *in vitro* efficacy.

4. *In vivo* efficacy

Most of the nanocarriers reviewed here were investigated *in vivo* using animal model experiments. It should be noted than none of these studies compared the co-loaded nanocarrier to the single-loaded nanocarriers mixture. We can emphasize that even if CI or *in vitro* release was not studied, or ratio not maintained, all formulations tested *in vivo* showed efficacy.

4.1 Breast cancer models

PTX/BCL co-loaded nanoemulsions evidenced a tumor inhibition rate of 77% on MCF-7/Tax tumor xenograft (Meng et al., 2016). Wong et al showed that the co-encapsulation of VIN and QUE in liposomes at a synergistic ratio led to low toxicity and good antitumor efficacy in estrogen negative, progesterone negative-and trastuzumab-insensitive xenograft models, with a dose of VIN given at two-thirds of its maximum tolerated dose (Wong and Chiu, 2011). Jain *et al* demonstrated that the combination of QUE and TMX in nanoparticles resulted in higher antitumor efficacy on female breast tumor-bearing rats in contrast to the free drug combination. In addition, co-encapsulation of an antioxidant could reduce the free radical induced oxidative stress generated during the course of chronic TMX therapy (Jain et al., 2013). QUE and DOX co-loaded nanoparticles significantly enhanced the *in vivo* anti-cancer efficacy of DOX in a drug-resistant MCF-7/ADR xenograft model (Lv et al., 2016). Micelles co-encapsulating DTX and SIL displayed a better anti-tumor effect on 4T1 tumor-bearing

mice compared to the free drug mixture (Dong et al., 2017). Self-emulsifying nano-emulsion co-encapsulating TMX and naringenin administered orally for 30 days resulted in a lower tumor burden in female breast cancer-bearing rats compared to the free drug mixture or the TMX encapsulated alone. Moreover, mortality was decreased, with complete animals survival for the co-encapsulating formulation (Sandhu et al., 2017). Mesoporous silica nanoparticles co-encapsulating TPT and QUE showed greater tumor rate inhibition on MDA-MB-231 breast tumor-bearing mice than free TPT or free QUE (Murugan et al., 2016).

4.2 Lung cancer models

Water-in-oil-in-water nanoemulsion co-encapsulating PMX and QUE for oral administration improved the oral bioavailability of both drugs *in vivo* with the result of a maximum tumor growth suppression in A549 cell-bearing mice (62.7%) in comparison with the control group (Pangeni et al., 2018b). The pH-sensitive micelles co-encapsulating BAI and CUR had a higher inhibition rate on A549 lung cancer-bearing mice than each free drugs and the single encapsulated CUR (Wang et al., 2019). PLGA-nanoparticles formed by prodrugs of BCL and PTX had a better effect than single-loaded nanoparticles *in vivo* on PTX-resistant A549 lung tumor-bearing mice (Wang et al., 2015). Co-encapsulated DTX and BAI in solid lipid nanoparticles could significantly increase the tumor inhibition rate in DTX-resistant A549 resistant tumor-bearing mice in comparison to single-loaded solid lipid nanoparticles, each free drug or free drug mixture (Li et al., 2017). DOX and QUE co-encapsulated in micelles demonstrated superior anti-cancer efficacy on SSC-7 tumor-bearing mice than free DOX, free QUE, mixture of free DOX and free QUE and DOX-encapsulating micelles (Ramasamy et al., 2017), even if the *in vitro* release of the drugs did not maintain the synergistic drug ratio.

4.3 Other cancer models

QUE and DOX co-loaded mesoporous silica nanoparticles had a better anti-tumor effect on SG7901/ADR tumor-bearing mice (gastric cancer model) than the free drugs mixture and the single-loaded nanoparticles (Fang et al., 2018).

Cholic-acid functionalized liposomes co-encapsulating DOX-HCL and SLB displayed enhanced liver accumulation and targeting, as well as more efficient inhibition of liver tumor growth in H22 tumor-bearing mice and HepG2 tumor-bearing nude mice (hepatocellular cancer models). In addition, co-loaded liposomes induced significantly less pathological damage to the cardiac tissue compared to free DOX-HCL administered with cholic-acid functionalized liposomes encapsulating only QUE (Li et al., 2018). Aminoethyl anisamide functionalized nanoparticles co-encapsulating DOX and ICA showed better liver accumulation, better tumor growth inhibition and better progression free survival in Hepa1-6

Luc tumor-bearing mice compared to DOX-nanoparticles or ICA-nanoparticles. They could also remodel the immune microenvironment to suppress hepatocellular carcinoma and induce better immunogenic cell death, leading to an antitumor vaccination effect (Yu et al., 2020).

VIN and QUE co-loaded in lipid-polymeric hybrid nanoparticles showed an enhanced antitumor efficacy on VIN resistant Raji tumor-bearing mice (Burkitt's lymphoma model) compared to free drugs alone or in combination and to the single-loaded nanocarrier alone (Zhu et al., 2017).

5. Concluding remarks

Flavonoids have been successfully co-encapsulated with anti-cancer agents in a broad range of nanocarriers. As the physicochemical properties of the drugs might differ, there is a need for formulation optimization depending on the drugs, and sometimes a compromise has to be made (Wong and Chiu, 2011), or prodrug can be synthetized (Wang et al., 2015). If several studies detailed some formulation optimization, so few reported an optimization of the formulation and of the process specific to the co-encapsulation. Moreover, as the storage stability of the co-encapsulating nanocarriers was rarely studied, one can wonder if those nanocarriers developed are really the most suitable for the aim of co-encapsulating flavonoids with an anti-cancer drug. Furthermore, the heterogeneity of the methodologies used for *in vitro* experiments between these publications prevents further comparisons in term of efficacy of theses drug combination strategies.

It is also difficult to conclude on the predictive value of the combination of *in vitro* release and CI. Surely, CI seems to be compulsory for selecting the best drug ratio, and *in vitro* release is an almost mandatory characterization before *in vivo* experiments. However, not assessing the CI or not maintaining the drug ratio during release did not prevent *in vivo* efficacy. We can hypothesize that maybe the efficacy of the combination would be better at an optimized drug ratio and with a similar release, but this will need confirmation (Wu et al., 2020). We can also wonder if there is a more efficient way to assess the efficacy of the drug combination. Recently, a study found a correlation between the Hill coefficient and the tumor response after treatment by DOX co-encapsulated with either irinotecan or gemcitabine in liposomes (Wu et al., 2020). Associating *in vitro* release with CI gives at least a solid clue for the *in vivo* efficacy.

Co-encapsulation has consistently led to a better *in vivo* anti-tumor effect compared to free drugs, a mixture of free drugs or one drug encapsulated alone into the nanocarriers. As co-encapsulation can be challenging, it is questionable whether co-encapsulation is worth the effort. For example, Mura *et al* evidenced that co-encapsulating sunitinib and

gemcitabine in squalene nanoparticles was as efficient *in vitro* as the mixture of the drug encapsulated alone in squalene nanoparticles, but did not confirm this result *in vivo* (Mura et al., 2016). On the other hand, liposomes co-encapsulating cytarabin and daunorubicin in a 5:1 ratio (CPX-351) against acute myeloid leukemia was approved by the FDA and EMA (Alfayez et al., 2020; Tardi et al., 2009). Moreover, Markovsky *et al* evidenced that PTX and DOX conjugated to PGA nanoparticles significantly increased the tumor inhibition rate on MDA-MB-231 tumor-bearing mice compared to a mixture of individually-conjugated drugs or free drug combination (Markovsky et al., 2014). This leads to the idea that co-encapsulation might be a promising approach. However, with regard to the co-encapsulation of flavonoids with an anti-cancer agent, this review highlights the absence of mixture of the individually encapsulated drugs as a control group *in vitro* or *in vivo* to be able to evidence a significant advantage of the co-encapsulation.

Due to the pleiotropic properties of flavonoids, some groups specifically studied the antioxidant (Jain et al., 2013; Li et al., 2018), antiangiogenic (Jain et al., 2013; Mendes et al., 2014), anti-metastasis (Dong et al., 2017) or macrophage reprogramming (Wang et al., 2019) properties of these molecules in order to evaluate their co-encapsulated formulation *in vitro* and/or *in vivo*. Some showed a decreased in toxicity on some vital organs (Jain et al., 2013; Li et al., 2018), or other an antiangiogenic effect within the tumor site (Jain et al., 2013; Mendes et al., 2014). This approach should be generalized for every design of nanocarriers co-encapsulating a flavonoid to highlight its additional effects.

7. Aknowledgments: na

8. Fundings: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. M.R.M. was partially supported by le Prix de la médaille (Assistance Publique – Hôpitaux de Paris) and l'Année Recherche.

9. References

Adan, A., Baran, Y., 2015. The pleiotropic effects of fisetin and hesperetin on human acute promyelocytic leukemia cells are mediated through apoptosis, cell cycle arrest, and alterations in signaling networks. Tumour Biol. 36, 8973–8984. https://doi.org/10.1007/s13277-015-3597-6

Ahmad, A., Ali, T., Park, H.Y., Badshah, H., Rehman, S.U., Kim, M.O., 2016. Neuroprotective Effect of Fisetin Against Amyloid-Beta-Induced Cognitive/Synaptic Dysfunction, Neuroinflammation, and Neurodegeneration in Adult Mice. Mol. Neurobiol. https://doi.org/10.1007/s12035-016-9795-4

Aiello, P., Consalvi, S., Poce, G., Raguzzini, A., Toti, E., Palmery, M., Biava, M., Bernardi, M., Kamal, M.A., Perry, G., Peluso, I., 2019. Dietary flavonoids: Nano delivery and nanoparticles for cancer therapy. Semin. Cancer Biol. https://doi.org/10.1016/j.semcancer.2019.08.029

Akbarzadeh, A., Rezaei-Sadabady, R., Davaran, S., Joo, S.W., Zarghami, N., Hanifehpour, Y., Samiei, M., Kouhi, M., Nejati-Koshki, K., 2013. Liposome: classification, preparation, and applications. Nanoscale Res Lett 8, 102. https://doi.org/10.1186/1556-276X-8-102

Alfayez, M., Kantarjian, H., Kadia, T., Ravandi-Kashani, F., Daver, N., 2020. CPX-351 (vyxeos) in AML. Leuk. Lymphoma 61, 288–297. https://doi.org/10.1080/10428194.2019.1660970

Bayat Mokhtari, R., Homayouni, T.S., Baluch, N., Morgatskaya, E., Kumar, S., Das, B., Yeger, H., 2017. Combination therapy in combating cancer. Oncotarget 8, 38022–38043. https://doi.org/10.18632/oncotarget.16723

Bednarek, P., Kerhoas, L., Einhorn, J., Frański, R., Wojtaszek, P., Rybus-Zając, M., Stobiecki, M., 2003. Profiling of Flavonoid Conjugates in Lupinus albus and Lupinus angustifolius Responding to Biotic and Abiotic Stimuli. J Chem Ecol 29, 1127–1142. https://doi.org/10.1023/A:1023877422403

Bensikaddour, H., Fa, N., Burton, I., Deleu, M., Lins, L., Schanck, A., Brasseur, R., Dufrêne, Y.F., Goormaghtigh, E., Mingeot-Leclercq, M.-P., 2008. Characterization of the Interactions between Fluoroquinolone Antibiotics and Lipids: a Multitechnique Approach. Biophys J 94, 3035–3046. https://doi.org/10.1529/biophysj.107.114843

Bolli, A., Marino, M., Rimbach, G., Fanali, G., Fasano, M., Ascenzi, P., 2010. Flavonoid binding to human serum albumin. Biochemical and Biophysical Research Communications 398, 444–449. https://doi.org/10.1016/j.bbrc.2010.06.096

Carvalho, D., Paulino, M., Polticelli, F., Arredondo, F., Williams, R.J., Abin-Carriquiry, J.A., 2017. Structural evidence of quercetin multi-target bioactivity: A reverse virtual screening strategy. Eur J Pharm Sci 106, 393–403. https://doi.org/10.1016/j.ejps.2017.06.028

Chaaban, H., Ioannou, I., Chebil, L., Slimane, M., Gérardin, C., Paris, C., Charbonnel, C., Chekir, L., Ghoul, M., 2017. Effect of heat processing on thermal stability and antioxidant activity of six flavonoids. Journal of Food Processing and Preservation 41, e13203. https://doi.org/10.1111/jfpp.13203

Chen, Chengjun, Han, D., Cai, C., Tang, X., 2010. An overview of liposome lyophilization and its future potential. Journal of Controlled Release 142, 299–311. https://doi.org/10.1016/j.jconrel.2009.10.024

Chen, Chen, Zhou, J., Ji, C., 2010. Quercetin: a potential drug to reverse multidrug resistance. Life Sci. 87, 333–338. https://doi.org/10.1016/j.lfs.2010.07.004

Chen, J., Lin, H., Hu, M., 2003. Metabolism of Flavonoids via Enteric Recycling: Role of Intestinal Disposition. J Pharmacol Exp Ther 304, 1228–1235. https://doi.org/10.1124/jpet.102.046409

Choi, H.S., Liu, W., Misra, P., Tanaka, E., Zimmer, J.P., Ipe, B.I., Bawendi, M.G., Frangioni, J.V., 2007. Renal Clearance of Nanoparticles. Nat Biotechnol 25, 1165–1170. https://doi.org/10.1038/nbt1340

Choi, J.Y., Thapa, R.K., Yong, C.S., Kim, J.O., 2016. Nanoparticle-based combination drug delivery systems for synergistic cancer treatment. Journal of Pharmaceutical Investigation 46, 325–339. https://doi.org/10.1007/s40005-016-0252-1

Chou, T.-C., 2010. Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res. 70, 440–446. https://doi.org/10.1158/0008-5472.CAN-09-1947

Corradini, E., Foglia, P., Giansanti, P., Gubbiotti, R., Samperi, R., Lagana, A., 2011. Flavonoids: chemical properties and analytical methodologies of identification and quantitation in foods and plants. Nat. Prod. Res. 25, 469–495. https://doi.org/10.1080/14786419.2010.482054

Cosco, D., Paolino, D., Maiuolo, J., Russo, D., Fresta, M., 2011. Liposomes as multicompartmental carriers for multidrug delivery in anticancer chemotherapy. Drug Deliv. and Transl. Res. 1, 66–75. https://doi.org/10.1007/s13346-010-0007-x

Crozier, A., Del Rio, D., Clifford, M.N., 2010. Bioavailability of dietary flavonoids and phenolic compounds. Mol. Aspects Med. 31, 446–467. https://doi.org/10.1016/j.mam.2010.09.007

Cui, J., Liu, X., Chow, L.M.C., 2019. Flavonoids as P-gp Inhibitors: A Systematic Review of SARs. Curr. Med. Chem. 26, 4799–4831. https://doi.org/10.2174/0929867325666181001115225

Dayoub, O., Andriantsitohaina, R., Clere, N., 2013. Pleiotropic beneficial effects of epigallocatechin gallate, quercetin and delphinidin on cardiovascular diseases associated with endothelial dysfunction. Cardiovasc Hematol Agents Med Chem 11, 249–264. https://doi.org/10.2174/18715257126661403092330 48

Dong, X.-Y., Lang, T.-Q., Yin, Q., Zhang, P.-C., Li, Y.-P., 2017. Co-delivery of docetaxel and silibinin using pH-sensitive micelles improves therapy of metastatic breast cancer. Acta Pharmacol. Sin. 38, 1655–1662. https://doi.org/10.1038/aps.2017.74

Fang, J., Zhang, S., Xue, X., Zhu, X., Song, S., Wang, B., Jiang, L., Qin, M., Liang, H., Gao, L., 2018. Quercetin and doxorubicin co-delivery using mesoporous silica nanoparticles enhance the efficacy of gastric carcinoma chemotherapy. Int J Nanomedicine 13, 5113–5126. https://doi.org/10.2147/IJN.S170862

Fatma, S., Talegaonkar, S., Iqbal, Z., Panda, A.K., Negi, L.M., Goswami, D.G., Tariq, M., 2016. Novel flavonoid-based biodegradable nanoparticles for effective oral delivery of etoposide by P-glycoprotein modulation: an in vitro, ex vivo and in vivo investigations. Drug Deliv 23, 500–511. https://doi.org/10.3109/10717544.2014.923956

Gaber, D.M., Nafee, N., Abdallah, O.Y., 2017. Myricetin solid lipid nanoparticles: Stability assurance from system preparation to site of action. European Journal of Pharmaceutical Sciences 109, 569–580. https://doi.org/10.1016/j.ejps.2017.08.007

Gao, W., Ye, G., Duan, X., Yang, X., Yang, V.C., 2017. Transferrin receptor-targeted pH-sensitive micellar system for diminution of drug resistance and targetable delivery in multidrug-resistant breast cancer. Int J Nanomedicine 12, 1047–1064. https://doi.org/10.2147/IJN.S115215

Gaumet, M., Vargas, A., Gurny, R., Delie, F., 2008. Nanoparticles for drug delivery: The need for precision in reporting particle size parameters. European Journal of Pharmaceutics and Biopharmaceutics 69, 1–9. https://doi.org/10.1016/j.ejpb.2007.08.001

Gupta, S.C., Kunnumakkara, A.B., Aggarwal, S., Aggarwal, B.B., 2018. Inflammation, a Double-Edge Sword for Cancer and Other Age-Related Diseases. Front Immunol 9, 2160. https://doi.org/10.3389/fimmu.2018.02160

Gurunathan, S., Kang, M.-H., Qasim, M., Kim, J.-H., 2018. Nanoparticle-Mediated Combination Therapy: Two-in-One Approach for Cancer. Int J Mol Sci 19. https://doi.org/10.3390/ijms19103264

Hadinoto, K., Sundaresan, A., Cheow, W.S., 2013. Lipid–polymer hybrid nanoparticles as a new generation therapeutic delivery platform: A review. European Journal of Pharmaceutics and Biopharmaceutics 85, 427–443. https://doi.org/10.1016/j.ejpb.2013.07.002

Harasym, T.O., Tardi, P.G., Harasym, N.L., Harvie, P., Johnstone, S.A., Mayer, L.D., 2007. Increased preclinical efficacy of irinotecan and floxuridine coencapsulated inside liposomes is associated with tumor delivery of synergistic drug ratios. Oncol. Res. 16, 361–374. https://doi.org/10.3727/000000006783980937

He, X.-G., 2000. On-line identification of phytochemical constituents in botanical extracts by combined high-performance liquid chromatographic–diode array detection–mass spectrometric techniques. Journal of Chromatography A 880, 203–232. https://doi.org/10.1016/S0021-9673(00)00059-5

Hinderer, W., Seitz, H.U., 1988. CHAPTER 2 - Flavonoids, in: Constabel, F., Vasil, I.K. (Eds.), Phytochemicals in Plant Cell Cultures. Academic Press, pp. 23–48. https://doi.org/10.1016/B978-0-12-715005-5.50009-1

Hu, C.-M.J., Aryal, S., Zhang, L., 2010. Nanoparticle-assisted combination therapies for effective cancer treatment. Ther Deliv 1, 323–334. https://doi.org/10.4155/tde.10.13

Hu, J., Wang, J., Wang, G., Yao, Z., Dang, X., 2016. Pharmacokinetics and antitumor efficacy of DSPE-PEG2000 polymeric liposomes loaded with quercetin and temozolomide: Analysis of their effectiveness in enhancing the chemosensitization of drug-resistant glioma cells. Int J Mol Med 37, 690–702. https://doi.org/10.3892/ijmm.2016.2458

Jackman, R.L., Yada, R.Y., Tung, M.A., Speers, R.A., 1987. Anthocyanins as Food Colorants —a Review. Journal of Food Biochemistry 11, 201–247. https://doi.org/10.1111/j.1745-4514.1987.tb00123.x

Jain, A.K., Thanki, K., Jain, S., 2013. Co-encapsulation of Tamoxifen and Quercetin in Polymeric Nanoparticles: Implications on Oral Bioavailability, Antitumor Efficacy, and Drug-Induced Toxicity. Mol. Pharmaceutics 10, 3459–3474. https://doi.org/10.1021/mp400311j

Je, L., Gl, U., Je, C., Lf, N., Tl, L., Ek, R., Rk, S., Sa, S., D, H., Sr, S., Rm, S., Dl, B., Je, K., Gj, S., Mj, W., Dh, R., A, H., K, B., M, C., Ac, L., Bc, M., 2018. CPX-351 (cytarabine and daunorubicin) Liposome for Injection Versus Conventional Cytarabine Plus Daunorubicin in Older Patients With Newly Diagnosed Secondary Acute Myeloid Leukemia. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 36. https://doi.org/10.1200/JCO.2017.77.6112

Kashyap, D., Garg, V.K., Tuli, H.S., Yerer, M.B., Sak, K., Sharma, A.K., Kumar, M., Aggarwal, V., Sandhu, S.S., 2019. Fisetin and Quercetin: Promising Flavonoids with Chemopreventive Potential. Biomolecules 9, 174. https://doi.org/10.3390/biom9050174

Khushnud, T., Mousa, S.A., 2013. Potential Role of Naturally Derived Polyphenols and Their Nanotechnology Delivery in Cancer. Mol Biotechnol 55, 78–86. https://doi.org/10.1007/s12033-012-9623-7

Kikuchi, H., Yuan, B., Hu, X., Okazaki, M., 2019. Chemopreventive and anticancer activity of flavonoids and its possibility for clinical use by combining with conventional chemotherapeutic agents. Am J Cancer Res 9, 1517–1535.

Kumar, S., Pandey, A.K., 2013. Chemistry and biological activities of flavonoids: an overview. ScientificWorldJournal 2013, 162750. https://doi.org/10.1155/2013/162750

Lee, R.-S., Lin, C.-H., Aljuffali, I.A., Hu, K.-Y., Fang, J.-Y., 2015. Passive targeting of thermosensitive diblock copolymer micelles to the lungs: synthesis and characterization of poly(N-isopropylacrylamide)-block-poly(ε-caprolactone). J Nanobiotechnology 13. https://doi.org/10.1186/s12951-015-0103-7

Li, S., Wang, L., Li, N., Liu, Y., Su, H., 2017. Combination lung cancer chemotherapy: Design of a pH-sensitive transferrin-PEG-Hz-lipid conjugate for the co-delivery of docetaxel and baicalin. Biomedicine & Pharmacotherapy 95, 548–555. https://doi.org/10.1016/j.biopha.2017.08.090

Li, Y., Yang, D., Wang, Y., Li, Z., Zhu, C., 2018. Co-delivery doxorubicin and silybin for anti-hepatoma via enhanced oral hepatic-targeted efficiency. Int J Nanomedicine 14, 301–315. https://doi.org/10.2147/IJN.S187888

Liu, S., Guo, C., Guo, Y., Yu, H., Greenaway, F., Sun, M.-Z., 2014. Comparative Binding Affinities of Flavonoid Phytochemicals with Bovine Serum Albumin. Iran J Pharm Res 13, 1019–1028.

Lv, L., Liu, C., Chen, C., Yu, X., Chen, G., Shi, Y., Qin, F., Ou, J., Qiu, K., Li, G., 2016. Quercetin and doxorubicin co-encapsulated biotin receptor-targeting nanoparticles for minimizing drug resistance in breast cancer. Oncotarget 7, 32184–32199. https://doi.org/10.18632/oncotarget.8607

Markovsky, E., Baabur-Cohen, H., Satchi-Fainaro, R., 2014. Anticancer polymeric nanomedicine bearing synergistic drug combination is superior to a mixture of individually-conjugated drugs. Journal of Controlled Release 187, 145–157. https://doi.org/10.1016/j.jconrel.2014.05.025

Mayer, L.D., Harasym, T.O., Tardi, P.G., Harasym, N.L., Shew, C.R., Johnstone, S.A., Ramsay, E.C., Bally, M.B., Janoff, A.S., 2006. Ratiometric dosing of anticancer drug combinations: Controlling drug ratios after systemic administration regulates therapeutic activity in tumor-bearing mice. Mol Cancer Ther 5, 1854–1863. https://doi.org/10.1158/1535-7163.MCT-06-0118

Mehnert, W., Mäder, K., 2001. Solid lipid nanoparticles: Production, characterization and applications. Advanced Drug Delivery Reviews, Lipid Assemblies for Drug Delivery 47, 165–196. https://doi.org/10.1016/S0169-409X(01)00105-3

Mendes, L.P., Gaeti, M.P.N., Ávila, P.H.M. de, Vieira, M. de S., Rodrigues, B. dos S., Marcelino, R.I. de Á., Santos, L.C.R. dos, Valadares, M.C., Lima, E.M., 2014. Multicompartimental Nanoparticles

for Co-Encapsulation and Multimodal Drug Delivery to Tumor Cells and Neovasculature. Pharm Res 31, 1106–1119. https://doi.org/10.1007/s11095-013-1234-x
Meng, L., Xia, X., Yang, Y., Ye, J., Dong, W., Ma, P., Jin, Y., Liu, Y., 2016. Co-encapsulation of paclitaxel and baicalein in nanoemulsions to overcome multidrug resistance via oxidative stress augmentation and P-glycoprotein inhibition. International Journal of Pharmaceutics 513, 8–16. https://doi.org/10.1016/j.ijpharm.2016.09.001
Mignet, N., Seguin, J., Chabot, G.G., 2013. Bioavailability of Polyphenol Liposomes: A Challenge Ahead. Pharmaceutics 5, 457–471. https://doi.org/10.3390/pharmaceutics5030457
Mingeot-Leclercq, M.-P., Gallet, X., Flore, C., Van Bambeke, F., Peuvot, J., Brasseur, R., 2001. Experimental and Conformational Analyses of Interactions between Butenafine and Lipids. Antimicrob Agents Chemother 45, 3347–3354. https://doi.org/10.1128/AAC.45.12.3347-3354.2001
Mirossay, L., Varinská, L., Mojžiš, J., 2017. Antiangiogenic Effect of Flavonoids and Chalcones: An Update. Int J Mol Sci 19. https://doi.org/10.3390/ijms19010027
Moghimi, S.M., Hunter, A.C., Murray, J.C., 2001. Long-circulating and target-specific nanoparticles: theory to practice. Pharmacological reviews 53, 283–318.
Mohapatra, M., Mishra, A.K., 2011. Photophysical Behavior of Fisetin in Dimyristoylphosphatidylcholine Liposome Membrane. The Journal of Physical Chemistry B 115, 9962–9970. https://doi.org/10.1021/jp1123212
Mura, S., Buchy, E., Askin, G., Cayre, F., Mougin, J., Gouazou, S., Sobot, D., Valetti, S., Stella, B., Desmaele, D., Couvreur, P., 2016. In vitro investigation of multidrug nanoparticles for combined therapy with gemcitabine and a tyrosine kinase inhibitor: Together is not better. Biochimie, Lipidomics and Functional Lipid Biology 130, 4–13. https://doi.org/10.1016/j.biochi.2016.08.003
Murugan, C., Rayappan, K., Thangam, R., Bhanumathi, R., Shanthi, K., Vivek, R., Thirumurugan, R., Bhattacharyya, A., Sivasubramanian, S., Gunasekaran, P., Kannan, S., 2016. Combinatorial nanocarrier based drug delivery approach for amalgamation of anti-tumor agents in bresat cancer cells: an improved nanomedicine strategies. Sci Rep 6. https://doi.org/10.1038/srep34053
Nagaraju, G.P., Zafar, S.F., El-Rayes, B.F., 2013. Pleiotropic effects of genistein in metabolic, inflammatory, and malignant diseases. Nutr. Rev. 71, 562–572. https://doi.org/10.1111/nure.12044
Narayan, R., Nayak, U.Y., Raichur, A.M., Garg, S., 2018. Mesoporous Silica Nanoparticles: A Comprehensive Review on Synthesis and Recent Advances. Pharmaceutics 10. https://doi.org/10.3390/pharmaceutics10030118
Narayanan, S., Mony, U., Vijaykumar, D.K., Koyakutty, M., Paul-Prasanth, B., Menon, D., 2015. Sequential release of epigallocatechin gallate and paclitaxel from PLGA-casein core/shell nanoparticles sensitizes drug-resistant breast cancer cells. Nanomedicine 11, 1399–1406. https://doi.org/10.1016/j.nano.2015.03.015
Narayanan, S., Pavithran, M., Viswanath, A., Narayanan, D., Mohan, C.C., Manzoor, K., Menon, D., 2014. Sequentially releasing dual-drug-loaded PLGA–casein core/shell nanomedicine: Design, synthesis, biocompatibility and pharmacokinetics. Acta Biomaterialia 10, 2112–2124. https://doi.org/10.1016/j.actbio.2013.12.041
Ochi, M.M., Amoabediny, G., Rezayat, S.M., Akbarzadeh, A., Ebrahimi, B., 2016. In Vitro Co-Delivery Evaluation of Novel Pegylated Nano-Liposomal Herbal Drugs of Silibinin and Glycyrrhizic Acid (Nano-Phytosome) to Hepatocellular Carcinoma Cells. Cell J 18, 135–148. https://doi.org/10.22074/cellj.2016.4308
Pan, J., Rostamizadeh, K., Filipczak, N., Torchilin, V.P., 2019. Polymeric Co-Delivery Systems in Cancer Treatment: An Overview on Component Drugs' Dosage Ratio Effect. Molecules 24. https://doi.org/10.3390/molecules24061035
Panche, A.N., Diwan, A.D., Chandra, S.R., 2016. Flavonoids: an overview. J Nutr Sci 5. https://doi.org/10.1017/jns.2016.41

962 Pangeni, R., Choi, J.U., Panthi, V.K., Byun, Y., Park, J.W., 2018a. Enhanced oral absorption of
963 pemetrexed by ion-pairing complex formation with deoxycholic acid derivative and multiple
964 nanoemulsion formulations: preparation, characterization, and in vivo oral bioavailability and
965 anticancer effect. Int J Nanomedicine 13, 3329–3351. https://doi.org/10.2147/IJN.S167958
966 Pangeni, R., Choi, S.W., Jeon, O.-C., Byun, Y., Park, J.W., 2016. Multiple nanoemulsion system for an
967 oral combinational delivery of oxaliplatin and 5-fluorouracil: preparation and in vivo
968 evaluation. Int J Nanomedicine 11, 6379–6399. https://doi.org/10.2147/IJN.S121114
969 Pangeni, R., Panthi, V.K., Yoon, I.-S., Park, J.W., 2018b. Preparation, Characterization, and In Vivo
970 Evaluation of an Oral Multiple Nanoemulsive System for Co-Delivery of Pemetrexed and
971 Quercetin. Pharmaceutics 10. https://doi.org/10.3390/pharmaceutics10030158
972 Peer, D., Karp, J.M., Hong, S., Farokhzad, O.C., Margalit, R., Langer, R., 2007. Nanocarriers as an
973 emerging platform for cancer therapy. Nature Nanotech 2, 751–760.
974 https://doi.org/10.1038/nnano.2007.387
975 Plaza, M., Pozzo, T., Liu, J., Gulshan Ara, K.Z., Turner, C., Nordberg Karlsson, E., 2014. Substituent
976 Effects on in Vitro Antioxidizing Properties, Stability, and Solubility in Flavonoids. J. Agric.
977 Food Chem. 62, 3321–3333. https://doi.org/10.1021/jf405570u
978 Ramasamy, T., Ruttala, H.B., Chitrapriya, N., Poudal, B.K., Choi, J.Y., Kim, S.T., Youn, Y.S., Ku, S.K.,
979 Choi, H.-G., Yong, C.S., Kim, J.O., 2017. Engineering of cell microenvironment-responsive
980 polypeptide nanovehicle co-encapsulating a synergistic combination of small molecules for
981 effective chemotherapy in solid tumors. Acta Biomaterialia 48, 131–143.
982 https://doi.org/10.1016/j.actbio.2016.10.034
983 Ramešová, Š., Sokolová, R., Degano, I., 2015. The study of the oxidation of the natural flavonol fisetin
984 confirmed quercetin oxidation mechanism. Electrochimica Acta 182, 544–549.
985 https://doi.org/10.1016/j.electacta.2015.09.144
986 Ray, L., Pal, M.K., Ray, R.S., 2017. Synergism of co-delivered nanosized antioxidants displayed
987 enhanced anticancer efficacy in human colon cancer cell lines. Bioactive Materials 2, 82–95.
988 https://doi.org/10.1016/j.bioactmat.2017.02.003
989 Reyes-Farias, M., Carrasco-Pozo, C., 2019. The Anti-Cancer Effect of Quercetin: Molecular
990 Implications in Cancer Metabolism. Int J Mol Sci 20. https://doi.org/10.3390/ijms20133177
991 Samie, A., Sedaghat, R., Baluchnejadmojarad, T., Roghani, M., 2018. Hesperetin, a citrus flavonoid,
992 attenuates testicular damage in diabetic rats via inhibition of oxidative stress, inflammation,
993 and apoptosis. Life Sci. 210, 132–139. https://doi.org/10.1016/j.lfs.2018.08.074
994 Sandhu, P.S., Kumar, R., Beg, S., Jain, S., Kushwah, V., Katare, O.P., Singh, B., 2017. Natural lipids
995 enriched self-nano-emulsifying systems for effective co-delivery of tamoxifen and naringenin:
996 Systematic approach for improved breast cancer therapeutics. Nanomedicine 13, 1703–
997 1713. https://doi.org/10.1016/j.nano.2017.03.003
998 Savić, R., Eisenberg, A., Maysinger, D., 2006. Block copolymer micelles as delivery vehicles of
999 hydrophobic drugs: Micelle–cell interactions. Journal of Drug Targeting 14, 343–355.
1000 https://doi.org/10.1080/10611860600874538
1001 Seguin, J., Brullé, L., Boyer, R., Lu, Y.M., Ramos Romano, M., Touil, Y.S., Scherman, D., Bessodes, M.,
1002 Mignet, N., Chabot, G.G., 2013. Liposomal encapsulation of the natural flavonoid fisetin
1003 improves bioavailability and antitumor efficacy. Int J Pharm 444, 146–154.
1004 https://doi.org/10.1016/j.ijpharm.2013.01.050
1005 Smith, G.J., Thomsen, S.J., Markham, K.R., Andary, C., Cardon, D., 2000. The photostabilities of
1006 naturally occurring 5-hydroxyflavones, flavonols, their glycosides and their aluminium
1007 complexes. Journal of Photochemistry and Photobiology A: Chemistry 136, 87–91.
1008 https://doi.org/10.1016/S1010-6030(00)00320-8
1009 Sokolová, R., Ramešová, Š., Degano, I., Hromadová, M., Gál, M., Žabka, J., 2012. The oxidation of
1010 natural flavonoid quercetin. Chem. Commun. 48, 3433–3435.
1011 https://doi.org/10.1039/C2CC18018A
1012 Su, S.-J., Yeh, T.-M., Chuang, W.-J., Ho, C.-L., Chang, K.-L., Cheng, Hsiao-Ling, Liu, H.-S., Cheng, Hong-
1013 Lin, Hsu, P.-Y., Chow, N.-H., 2005. The novel targets for anti-angiogenesis of genistein on

human cancer cells. Biochem. Pharmacol. 69, 307–318. https://doi.org/10.1016/j.bcp.2004.09.025
Tagne, J.-B., Kakumanu, S., Nicolosi, R.J., 2008. Nanoemulsion Preparations of the Anticancer Drug Dacarbazine Significantly Increase Its Efficacy in a Xenograft Mouse Melanoma Model. Mol. Pharmaceutics 5, 1055–1063. https://doi.org/10.1021/mp8000556
Tardi, P., Johnstone, S., Harasym, N., Xie, S., Harasym, T., Zisman, N., Harvie, P., Bermudes, D., Mayer, L., 2009. In vivo maintenance of synergistic cytarabine:daunorubicin ratios greatly enhances therapeutic efficacy. Leukemia Research 33, 129–139. https://doi.org/10.1016/j.leukres.2008.06.028
Tardi, P.G., Gallagher, R.C., Johnstone, S., Harasym, N., Webb, M., Bally, M.B., Mayer, L.D., 2007. Coencapsulation of irinotecan and floxuridine into low cholesterol-containing liposomes that coordinate drug release in vivo. Biochimica et Biophysica Acta (BBA) - Biomembranes 1768, 678–687. https://doi.org/10.1016/j.bbamem.2006.11.014
Touil, Y.S., Fellous, A., Scherman, D., Chabot, G.G., 2009. Flavonoid-Induced Morphological Modifications of Endothelial Cells Through Microtubule Stabilization. Nutrition and Cancer 61, 310–321. https://doi.org/10.1080/01635580802521346
Touil, Y.S., Seguin, J., Scherman, D., Chabot, G.G., 2011. Improved antiangiogenic and antitumour activity of the combination of the natural flavonoid fisetin and cyclophosphamide in Lewis lung carcinoma-bearing mice. Cancer Chemotherapy and Pharmacology 68, 445–455. https://doi.org/10.1007/s00280-010-1505-8
Tsao, R., 2010. Chemistry and Biochemistry of Dietary Polyphenols. Nutrients 2, 1231–1246. https://doi.org/10.3390/nu2121231
Wang, B., Zhang, W., Zhou, X., Liu, M., Hou, X., Cheng, Z., Chen, D., 2019. Development of dual-targeted nano-dandelion based on an oligomeric hyaluronic acid polymer targeting tumor-associated macrophages for combination therapy of non-small cell lung cancer. Drug Delivery 26, 1265–1279. https://doi.org/10.1080/10717544.2019.1693707
Wang, J., Zhao, X.-H., 2016. Degradation kinetics of fisetin and quercetin in solutions as effected by pH, temperature and coexisted proteins. Journal of the Serbian Chemical Society 81, 243–253. https://doi.org/10.2298/JSC150706092W
Wang, W., Xi, M., Duan, X., Wang, Y., Kong, F., 2015. Delivery of baicalein and paclitaxel using self-assembled nanoparticles: synergistic antitumor effect in vitro and in vivo. Int J Nanomedicine 10, 3737–3750. https://doi.org/10.2147/IJN.S80297
Wong, M.-Y., Chiu, G.N.C., 2011. Liposome formulation of co-encapsulated vincristine and quercetin enhanced antitumor activity in a trastuzumab-insensitive breast tumor xenograft model. Nanomedicine: Nanotechnology, Biology and Medicine 7, 834–840. https://doi.org/10.1016/j.nano.2011.02.001
Wong, M.-Y., Chiu, G.N.C., 2010. Simultaneous liposomal delivery of quercetin and vincristine for enhanced estrogen-receptor-negative breast cancer treatment. Anticancer Drugs 21, 401–410. https://doi.org/10.1097/CAD.0b013e328336e940
Wu, D., Pusuluri, A., Vogus, D., Krishnan, V., Shields, C.W., Kim, J., Razmi, A., Mitragotri, S., 2020. Design principles of drug combinations for chemotherapy. Journal of Controlled Release 323, 36–46. https://doi.org/10.1016/j.jconrel.2020.04.018
Wu, L., Bi, Y., Wu, H., 2018. Formulation optimization and the absorption mechanisms of nanoemulsion in improving baicalin oral exposure. Drug Development and Industrial Pharmacy 44, 266–275. https://doi.org/10.1080/03639045.2017.1391831
Yao, Y., Lin, G., Xie, Y., Ma, P., Li, G., Meng, Q., Wu, T., 2014. Preformulation studies of myricetin: a natural antioxidant flavonoid. Pharmazie 69, 19–26.
Yu, Z., Guo, J., Hu, M., Gao, Y., Huang, L., 2020. Icaritin Exacerbates Mitophagy and Synergizes with Doxorubicin to Induce Immunogenic Cell Death in Hepatocellular Carcinoma. ACS Nano 14, 4816–4828. https://doi.org/10.1021/acsnano.0c00708

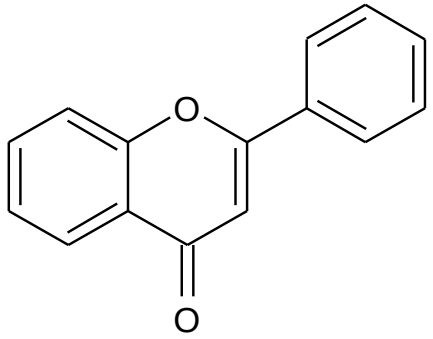
Zhou, Y., Wang, S., Ying, X., Wang, Y., Geng, P., Deng, A., Yu, Z., 2017. Doxorubicin-loaded redox-responsive micelles based on dextran and indomethacin for resistant breast cancer. Int J Nanomedicine 12, 6153–6168. https://doi.org/10.2147/IJN.S141229

Zhu, B., Yu, L., Yue, Q., 2017. Co-delivery of vincristine and quercetin by nanocarriers for lymphoma combination chemotherapy. Biomedicine & Pharmacotherapy 91, 287–294. https://doi.org/10.1016/j.biopha.2017.02.112

Figure 1. Chemical structure of flavonoid and their classes.

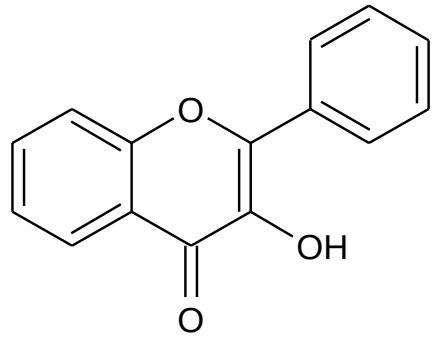
Figure 2. Total publications per year concerning flavonoids nanocarriers (web of science).

Figure 3. Total publications per year concerning flavonoids co-encapsulation (web of science).



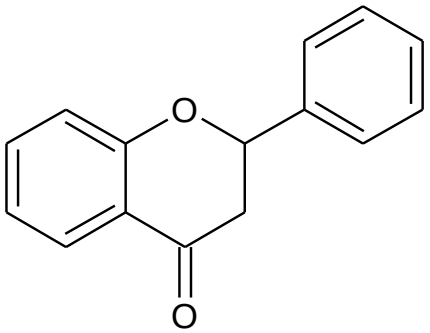
Flavone

e.g.: baicalein, baicalin (glucuroside), icaritin (phenylated)



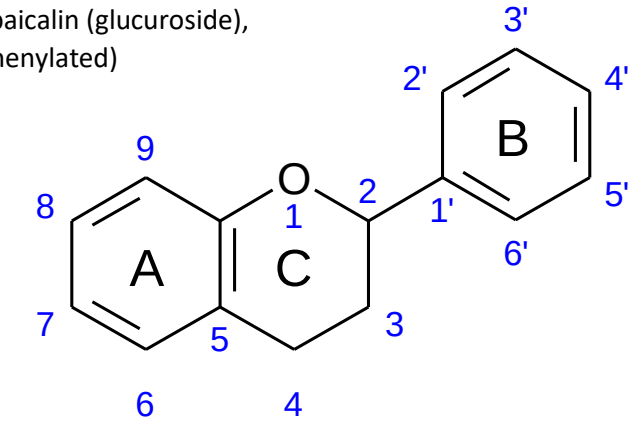
Flavonol

e.g.: quercetin

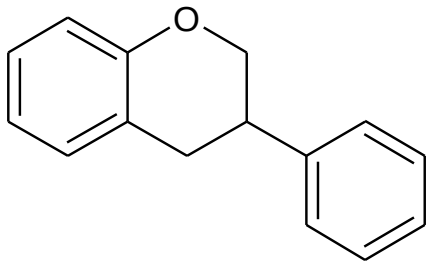


Flavanone

e.g.: naringenin, silibinin, silybin

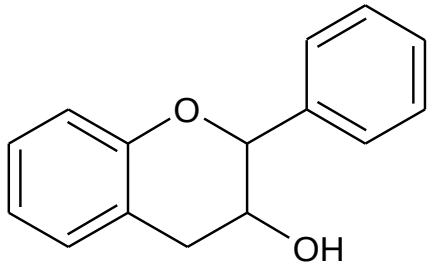


Flavonoid



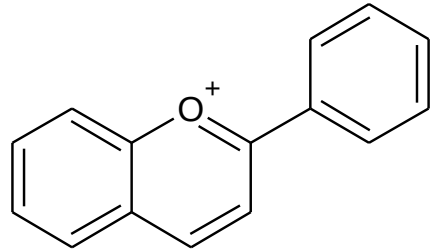
Isoflavonoid

e.g.: genistein (isoflavone)

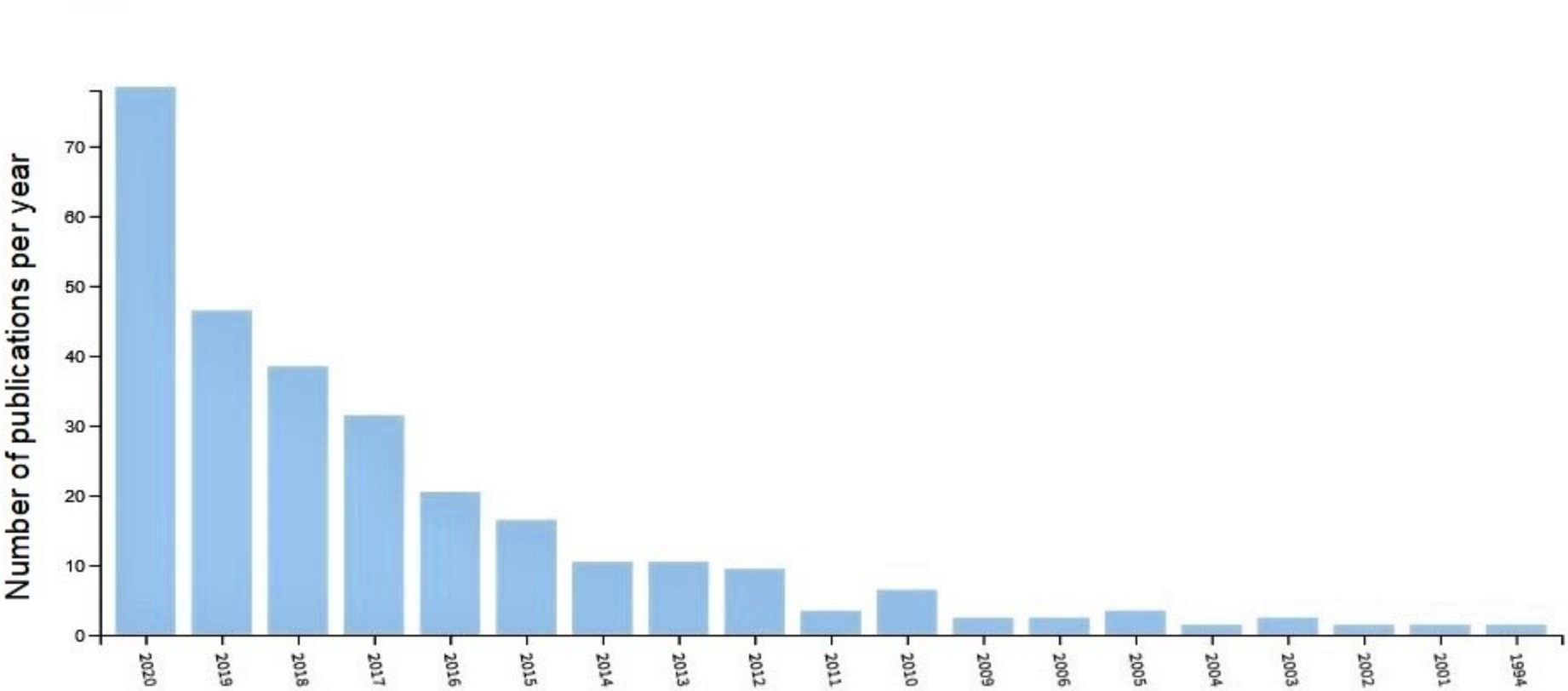


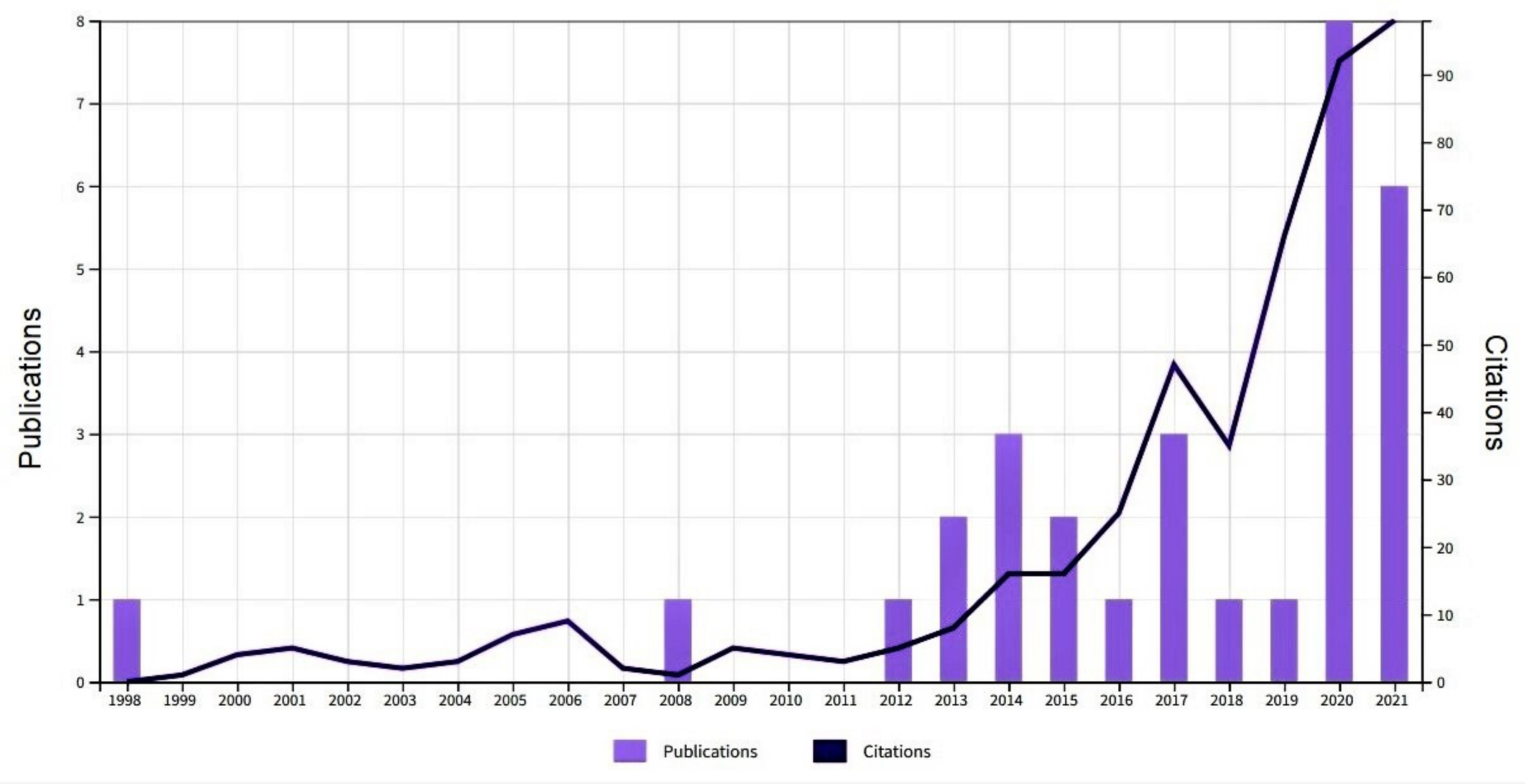
Flavanol

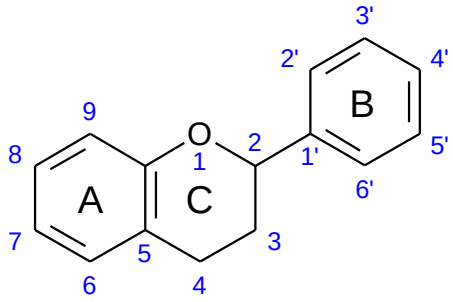
e.g.: epigallocatechin gallate



Anthocyanidin


Number of publications per year
0
10
20
30
40
50
60
70
2020
2019
2018
2017
2016
2015
2014
2013
2012
2011
2010
2009
2006
2005
2004
2003
2002
2001
1994

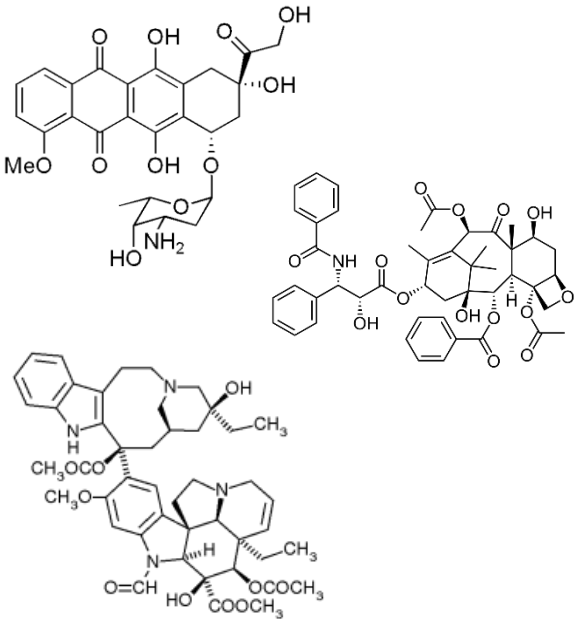

Publications
Citations
0
1
2
3
4
5
6
7
8
0
10
20
30
40
50
60
70
80
90
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019
2020
2021
Publications
Citations



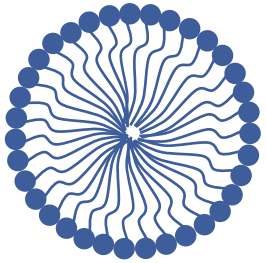
Flavonoid

+

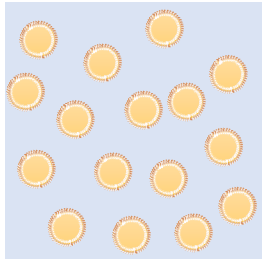
Anticancer drug



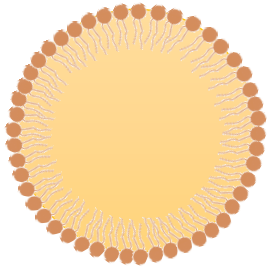
Co-encapsulation



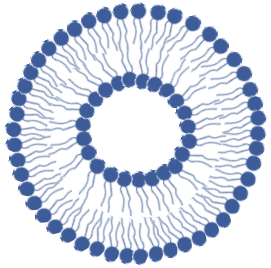
Micelle



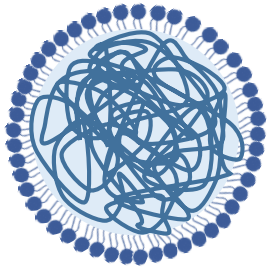
Nanoemulsion



Solid Lipid Nanoparticle

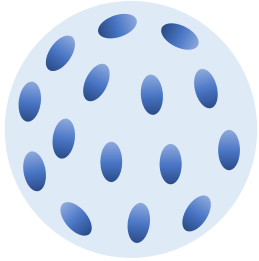


Liposome



Lipid-Polymer Hybrid Nanoparticle

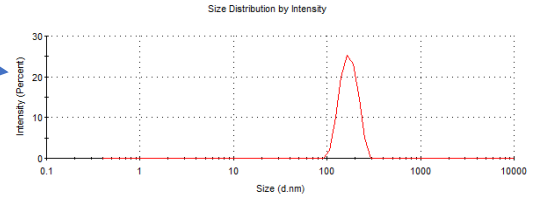
Polymeric Nanoparticle



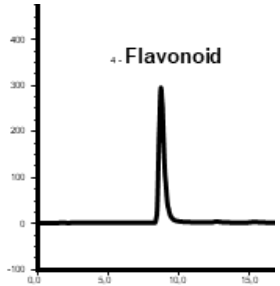
Mesoporous Silica Nanoparticle

Physico-chemical properties

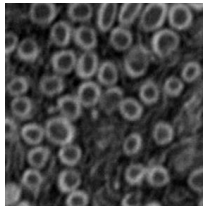
Diameter and PDI



Drug encapsulation

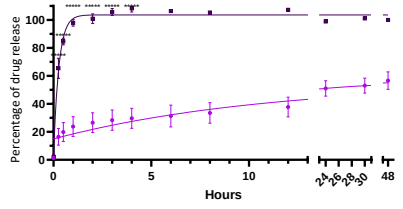


Morphology

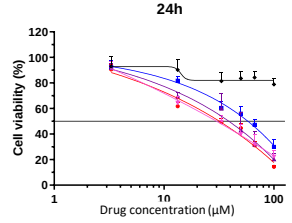


In vitro prediction of the in vivo behavior

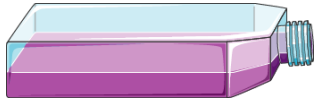
In vitro release



Cytotoxicity and Combination Index



Others



In vivo efficacy or mechanism assays