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Inulin-d-alfa-tocopherol succinate (INVITE) nanomicelles as a platform for effective intravenous administration of curcumin**

Giuseppe Tripodo[a], Gianfranco Pasut[b], Adriana Trapani[c], Anna Mero[b], Francesco Massimo Lasorsa[d], Theodora Chlapanidas[a], Giuseppe Trapani[c], Delia Mandracchia[c]*

[a]Department of Drug Science, University of Pavia, Viale Taramelli 12, 27100 Pavia, Italy.

[b]Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Via F. Marzolo 5, 35131 Padova, Italy

[c]Department of Pharmacy-Drug Sciences, University of Bari "Aldo Moro", Via Orabona 4, 70125 Bari, Italy.

[d]CNR Institute of Biomembranes and Bioenergetics, Via Amendola 165/a, 70126 Bari, Italy

*Corresponding Author: Department of Pharmacy-Drug Sciences, University of Bari "Aldo Moro", Via Orabona 4, 70125 Bari, Italy. Tel +39(0)805442046; Fax. +39(0)805442724. Email address: delia.mandracchia@uniba.it (D. Mandracchia)

** This work is dedicated to Prof. Andrea Latrofa in occasion of his retirement.

Abstract

The aim of this work was to evaluate the potential of INVITE based nanomicelles, an amphiphilic polymer constituted by Inulin (INU) and vitamin E (VITE), as a platform for improving the biopharmaceutical properties of hydrophobic drugs. For this purpose, curcumin was selected as a model and curcumin-INVITE nanomicelles were prepared.

This drug delivery system was characterized both *in vitro* for what concerns the physicochemical properties, blood compatibility and cellular uptake, and *in vivo* for the evaluation of the pharmacokinetic profile.

It was found that these nanomicelles release curcumin in a controlled manner and they are able to penetrate cellular membrane. Moreover, they showed an improved pharmacokinetic profile after intravenous administration.

In conclusion, INVITE micelles might constitute promising nanocarriers for improving the biopharmaceutical performance of hydrophobic drugs.

Keywords: Inulin, Vitamin E, micelle, curcumin, cell uptake, pharmacokinetic

1. Introduction

The biopharmaceutical performance of a drug is strictly depending on its physical-chemical properties. Among these, the aqueous solubility is a critical issue that often influence the bioavailability of oral administered drugs, as occurs for those belonging to class II of the Biopharmaceutic Classification System (BCS). Furthermore, the poor aqueous solubility represents an issue also for the parenteral administration. In fact, often, highly hydrophobic drugs, do not show favorable pharmacokinetic profiles following intravenous administration and present several formulation problems.

It has been estimated that about 40% of the drugs presently in clinical use and up to 75% of those currently under development could be considered poorly water-soluble drugs.[1] This issue requires the continuous development of advanced pharmaceutical formulations with the aim to improve the solubility profile of these therapeutic agents and desirably prolong their residence time in blood after a parenteral administration. One of the most common example of hydrophobic drug with significant formulation difficulties is paclitaxel, a well known anticancer drug. The intravenous administration of this drug is a challenge for pharmaceutical technologists because the so far adopted drug delivery strategies have shown serious drawbacks.[2] Curcumin is another common example of hydrophobic drug, which is characterized by low aqueous solubility, poor absorption and, moreover, is quickly metabolized and cleared by systemic circulation.

To date, several formulation strategies, have been proposed to overcome the above cited issues. In fact, many examples of drug delivery systems for hydrophobic drugs are reported in literature, including micro- and nano-particles,[3-5] hydrogels,[6-11] polymer-drug conjugates,[12-14] liposomes[15], cyclodextrins[16] and micelles.[17, 18] Among these, polymeric micelle systems, formed by an amphiphilic polymer with self-assembling properties in water, have attracted a lot of interest owing to their ability to deliver and eventually target non-water soluble drugs protected inside the hydrophobic core.[19]

In literature, there are numerous examples of polymeric micelles able to improve the bioavailability of curcumin. Abouzeid et al, proposed a micellar system based on polyethylene glycol-phosphatidylethanolamine (PEG-PE)/vitamin E for the co-delivery of paclitaxel and curcumin, designed to overcome multi-drug resistance in ovarian cancer. These micelles, having a mean particle diameter of 15.6 nm and a critical micellar concentration value of $1.66 \cdot 10^{-5}$ M, resulted effective in incorporating the two hydrophobic drugs.[20] In another study curcumin was encapsulated into monomethoxy poly(ethylene glycol)-poly(ε-caprolactone) (MPEG-PCL) micelles for colon cancer therapy. The micelles obtained in this study shown a mean particle size of 27.3 ± 1.3 nm a good re-solubility after freeze-drying and suitable encapsulation efficiency and drug loading.[21] Other studies, pointing on curcumin delivery by polymeric micelle, have been recently published and most of them use synthetic biocompatible polymers such as PEG, poly(ε-caprolactone) or Pluronic®.[22-26] From these studies it is also clear the need to optimize the curcumin delivery by controlling its release rate and to increase its pharmacokinetic profile, bioavailability and optimize its cellular uptake.

The aim of the present work was to evaluate the potential of polymeric micelles constituted by the natural molecules inulin (INU) and vitamin E (VITE), as a platform for improving the biopharmaceutical properties of hydrophobic drugs, such as curcumin, here selected as model.

Recently, we reported the synthesis and characterization of such new polymeric micelles, showing a great potential for drug delivery applications.[27] INU and VITE are two well-established natural molecules that exert beneficial effect on human health. While INU is a hydrophilic natural polysaccharide from fructose that in the last years has been used for different pharmaceutical applications,[28-30] VITE, that is a hydrophobic vitamin, is one of the most powerful anti-oxidant found in nature.[31, 32] By combining the physical-chemical properties of INU and VITE succinate it was possible to synthesize the polymeric amphiphile called INVITE, recently proposed for the therapy of urinary tract infections.[27] Here we are proposing the INVITE micelles as curcumin carrier for *i.v.* administration. The great advantage of INVITE micelles with respect to the most

common polymers used for nanocarriers is that INVITE can be considered as a "natural" polymer, being exclusively constitute by the two natural and cheap components INU and VITE. In this work, we demonstrate the effectiveness of INVITE micelles as carrier of curcumin whose biopharmaceutical performances have been significantly improved.

2. EXPERIMENTAL PART

2.1. Materials

All reagents were of analytical grade, unless otherwise stated. N,N-dimethylformamide (DMF), triethylamine (TEA), diciclohexylcarbodiimide (DCC), curcumin, pyrene, D-α-tocopherol succinate, poly(acrylic acid), NaCl, KCl, Na_2HPO_4, KH_2PO_4, DMSO-d_6, methanol, dichloromethane (DCM), Triton X-100, were purchased from Sigma-Aldrich (Milano, Italy). Inulin from dahlia tubers (INU, approx. 5000 Da), *N*-Hydroxysulfosuccinimide sodium salt (NHSS), Polisorbate 80 were purchased from Fluka (Milano, Italy). DMSO was purchased from Carlo Erba Reagents (Milano, Italy). Dialysis tubes with a MWCO 3.500 Da (Spectra/Por® 6) were purchased from Spectrum Labs.

2.2. Apparatus

FT-IR spectra (KBr pellets) were recorded in the range 4000–400 cm^{-1} using a Perkin–Elmer 1600 IR Fourier Transform Spectrophotometer (Monza, Italy). The resolution was 1 cm^{-1}.

UV-Vis analyses were performed using a Perkin–Elmer Spectrometer Lambda 25, Perkin-Elmer, (Monza, Italy).

^{1}H-NMR and ^{13}C-NMR spectra were recorded using a Varian Mercury 300 MHz instrument.

Centrifugations were performed with a Beckman Avanti 30 (Milano, Italy) equipped with a temperature control.

Ultraturrax model T25 apparatus (Janke and Kunkel, Germany) was used to prepare emulsion.

The mean size and polydispersity index (PDI) and the zeta potenzial of the INVITE M or INVITE MC micelles (empty or curcumin loaded micelles, respectively) were measured using a ZetasizerNano ZS (Malvern Instruments Ltd., Worcestershire, UK).

The morphological characteristics of INVITE M and INVITE MC micelles were examined by transmission electron microscope (TEM, Jeol-Jem-1200EXII, Japan).

The morphological changes of RBCs were analyzed by a Light Stereomicroscope (Leica Galen III) equipped with a Panasonic Camera (WV CP 230) and Leica Qwin v. 2.4 software

2.3. Synthesis and characterization of INVITE conjugates

Three Inulin-α-tocopherol succinate conjugates, called INVITE and numbered from 1 to 3 were synthesized according to a published procedure.[27]

Briefly, to a solution of VITE succinate (500 mg, 1 eq) in anhydrous DMF (4 mL) under nitrogen, DCC (2 eq) and NHSS (2 eq) were added. The activation reaction was carried out under stirring and nitrogen for 3 h at room temperature. Then, a solution of INU in anhydrous DMF and TEA (according to the following molar ratio: Z = 0.10 where Z indicates the molar ratio TEA/INU-repeating-units) was added. Then, the INU solution with TEA was added drop-wise to the VITE-NHSS ester solution and the reaction was carried out under nitrogen at 25°C for 12 h.

The amount of VITE was varied in order to obtain INVITE conjugates with different degree of derivatization, according to the following molar ratio Y=0.1 (INVITE 1) or Y=0.2 (INVITE 2) or Y=0.4 (INVITE 3) where Y indicates the molar ratio VITE/INU-repeating-units.

The three INVITE derivatives with different degrees of derivatization in VITE (named as INVITE 1-3 at 10, 18, 33% mol/mol in VITE groups, respectively) were obtained and characterized by FT-IR, ^{1}H-NMR and ^{13}C-NMR spectroscopy.

2.3.1. Determination of critical aggregation concentration (CAC)

CAC values for INVITE amphiphiles were calculated by fluorescence spectroscopy using the pyrene method. In this work, the method proposed by Kalyanasundaram *et al*. has been adopted.[33]

Excitation was done at 334 nm and emission were recorded in the range 350-450 nm wavelength. The slit for both excitation and emission were fixed at 5 nm. Pyrene was diluted in the INVITE samples solutions to yield a concentration of $2\cdot10^{-6}$ M.

In particular, 83.4 µl of a pyrene stock solution in acetone ($6\cdot10^{-3}$M) was diluted with water, to obtain a final concentration of pyrene $2\cdot10^{-5}$ M, and maintained under vigorous stirring and under nitrogen flow in order to totally remove the acetone. One hundred µl of the pyrene water solution were added to 0.9 mL of INVITE water solutions at various concentrations in the range of 10^{-7}– 2.5 mg/mL (final concentration of pyrene $2\cdot10^{-6}$ M) and was allowed to stand overnight to equilibrate. Fluorescence intensities of the pyrene entrapped in the micelle core were determined at room temperature and the ratio of the intensities I_1 and I_3 against INVITE concentration was plotted.

2.4. Preparation and characterization of empty or drug-loaded INVITE micelles

Micelles from INVITE 1, INVITE 2 and INVITE 3 conjugates were prepared by applying two different methods in order to evaluate which one was more suitable for curcumin drug loading.

2.4.1. Direct Dialysis from dimethylsulfoxide (DMSO)

100 mg of INVITE 1, INVITE 2 and INVITE 3 conjugates were dissolved in 10 mL of dimethylsulfoxide with or without 5 mg of Curcumin.[34] These polymer or polymer/drug dispersions were poured into a dialysis tubes (Spectra/Por® 6) with a MWCO 3.500 Da and sealed. Dialyses were carried out for 3 days against distilled water which was exchanged every 3 h.

The colloidal suspensions inside the dialysis tubes appeared transparent for the whole dialysis time and the curcumin loaded micelle suspensions appeared yellow/orange-colored.

At the end of the dialysis process, the drug loaded or not colloidal suspensions have been lyophilized and recovered with a 92-98 % w/w yields with respect to the starting polymer.

2.4.2. O/W Emulsion Technique

INVITE polymers (50 mg) were dispersed in double distilled water (5 mL) and left overnight under stirring to equilibrate. For drug loading, 2.5 mg of curcumin were dissolved in 1 mL of dichloromethane (DCM). This solution was added under constant stirring for 2 min at 11.000 rpm by Ultraturrax to the aqueous INVITE micellar dispersion. Then, the obtained emulsion was left under stirring overnight to permit the evaporation of the organic solvent and the formation of curcumin loaded micelles. After stirring the resulting dispersion was filtered through 0.45 µm filter membrane to remove drug precipitate and freeze dried. The samples were recovered with a yield of 95-97 % (w/w).

2.4.3 Drug loading evaluation by UV-VIS studies on INVITE micelles

For the evaluation of INVITE micelles drug loading, 2 mg of lyophilized micelles prepared by direct dialysis method or O/W emulsion method have been dissolved in 10 mL of DMSO.[34] The amount of drug loaded curcumin was determined by a UV-VIS Spectrometer Lambda 25, Perkin-Elmer, (Monza, Italy) by reading at 425 nm. Each measurement has been performed in triplicate. The loaded amount of curcumin was calculated by a calibration curve of curcumin in DMSO in the concentration range $0.5 \cdot 10^{-3}$-0.01 mg/mL (correlation coefficient $r^2 > 0.999$).

Curcumin drug loading (DL w/w %) in INVITE micelles was calculated based on the following formula: DL (w/w %) = (UV calculated weight of curcumin in micelles / weight of the feeding micelles plus curcumin) × 100

2.4.4. Size distribution and morphology of INVITE M and INVITE MC samples

The size distribution and the polydispersity index of empty INVITE micelles (INVITE M) or curcumin loaded INVITE micelles (INVITE MC) , were measured by using the ZetasizerNano ZS instrument.

In particular, INVITE M and INVITE MC aqueous solutions at concentration of 1 mg/mL were prepared and left to equilibrate overnight at 25°C under gently stirring. Then, the micellar dispersions were filtered on a 0.45 µm filter and the filtered mixture analyzed by ZetasizerNano ZS instrument. The measurement were performed in triplicate for each INVITE conjugate.

For transmission electron microscopy (TEM) studies, the INVITE samples were prepared as a 1 mg/mL solution and one drop was deposited on a copper grid. After drying at atmospheric pressure at 25 °C in a desiccator, the samples were negatively stained with uranyl acetate.

2.4.5. Drug release studies from INVITE micelles

10 mg of curcumin loaded INVITE micelles, prepared by direct dialysis as above described, have been solubilized in 1 mL of double distilled water and the solution poured in a 3500 Da MWCO dialysis membrane. The dialysis tubes were incubated in 10 mL of PBS pH 7.4 containing Tween 80 (0.5% wt) at 37 °C or in 10 mL of PBS pH 5.5 containing Tween 80 (0.5% wt) at 37 °C.

At scheduled times, all the release medium was removed and replaced by pre-warmed fresh release media. The amount of released curcumin has been valued by UV-VIS measurements and was calculated by a calibration curve of curcumin in PBS pH 7.4 containing Tween 80 (0.5% wt) in the concentration range $0.3 \cdot 10^{-3}$-0.01 mg/mL (correlation coefficient $r^2 > 0.999$) or in PBS pH 5.5 containing Tween 80 (0.5% wt) in the concentration range $0.3 \cdot 10^{-3}$ – 0.01 mg/mL (correlation coefficient $r^2 > 0.999$).

2.5 Blood compatibility by hemolysis assay

Freeze-dried INVITE 1MC, INVITE 2MC or INVITE 3MC were solubilized at a concentration of 5 mg/mL in a 0.9% NaCl solution in water and left to equilibrate under constant stirring for 24 h. Rat whole blood (0.2 mL), used immediately after isolation, was added to established amount of each sample to reach a final concentration (INVITE solution plus blood) of 1 mg/mL in INVITE and kept at 37°C for 30 min. Positive and negative controls were obtained by adding 0.2 mL of rat whole blood to 2.8 mL of 1% v/v Triton X-100 saline solution and to 2.8 mL of saline, respectively. All samples were incubated at 37 °C for 1h, 2h or 3 h. After incubation the samples were centrifuged at 2500 rpm, 25°C for 10 min and the absorbance of the supernatant was valued for release of hemoglobin at 540 nm. All the hemolysis experiments were performed in triplicate.

The percent of hemolysis of RBCs was calculated by the following formula: Hemolysis %= [(sample absorbance – negative control absorbance) / (positive control absorbance – negative control absorbance)] × 100

2.5.1 Morphological changes of RBCs

In order to observe morphological changes of treated RBCs at the early stages of hemolysis, the RBCs pellet derived from hemolysis assay was washed with 5 mL of 0.9 % NaCl solution in water followed by centrifugation at 25°C and 2500 rpm for 10 min, the supernatant has been discharged. Washing was continued until the supernatant was clear. Then, the RBCs pellet was resuspended in 3 mL of 0.9% saline solution and mounted on clean glass slides covered with cover slips and observed under a Light Stereomicroscope (Leica Galen III) equipped with a Panasonic Camera (WV CP 230) and Leica Qwin v. 2.4 software.

2.6. Cellular uptake studies

Uptake of INVITE 2MC into the cells was analyzed through an inverted Zeiss Axiovert 200 microscope (Zeiss, Milano, Italy) equipped with a 63×/1.4 oil objective. In particular, uptake of 0.1 and 0.01 mg mL^{-1} INVITE 2MC solution into human embryonic kidney (HEK 293) cells was imaged after 30, 60 and 120 min incubation at 37 °C in a 5% CO_2 atmosphere. Excitation (375 nm) and emission (525 nm) wavelengths of fluorescent probe were selected with appropriate filters mounted in Lambda 10-2 filter wheel controllers (Sutter Instruments, Novato, CA, USA) and images were captured by a CoolSNAP HQ CCD camera (Roper Scientific, Trenton, NJ) using the Metamorph/Metafluor software (Universal Imaging Corporation).

2.7. Pharmacokinetic studies

Six to eight weeks old female BALB/c inbred mice (20 ± 1 g), housed in a Specific Pathogen Free (SPF) animal facility, were used for this study. Procedures involving animals and their care were in conformity with institutional guidelines (D.L. 116/92 and subsequent implementing circulars), and experimental protocols (project ID: 24/2013) were approved by the local Ethical Committee of Padova University (CEASA). During *in vivo* experiments, animals in all experimental groups were examined continuously for a decrease in physical activity and other signs of disease or drug toxicity.

Ten mice were injected with INVITE micelles solution in PBS (dose: 50 µg per animal in curcumin equiv.) *via* tail vein after anesthesia with 5% isoflurane gas (mixed with O_2 in enclosed cages). At predetermined times, blood samples (100 µL) were withdrawn from the submandibular vein and collected in heparinized test tubes before centrifugation at 1,500 *g* for 15 min. To 50 µL of plasma, 450 µL of PBS was added and curcumin concentration was determined by fluorometer measurements (λ_{ex} = 375 nm; λ_{em} = 525 nm) on the basis of a calibration curve of curcumin in plasma/PBS (1 : 9) solution. Data were analyzed applying a two compartments model with PKSolver program.

3. RESULTS AND DISCUSSION

The enhancement of hydrophobic drugs bioavailability is a challenge for pharmaceutical technologists. In literature, a great number of papers are dealing with this issue. Among the used strategies, the incorporation of hydrophobic drugs into micellar systems is one of the most suitable method. In this work, we are going to show that inulin-d-α-tocopherol succinate (INVITE) micelles are valuable drug delivery systems for curcumin intravenous administration. Curcumin is not only an appropriate hydrophobic drug model, but also a molecule of growing scientific interest due to its countless therapeutic applications.

3.1. Synthesis of inulin-d-α-tocopherol succinate (INVITE) amphiphilic polymers

The synthesis of INVITE polymers has been performed by a published method.[27] What is particularly worth of mention at this stage is that the INVITE polymers have been obtained starting from two natural substances, INU and VITE, well known for their beneficial effects on human health. Furthermore, both INU and VITE could be considered as produced from renewable sources. This is an aspect that, nowadays, could not be ignored. In Table 1 the main physico-chemical properties of the obtained polymers are shown.

Table1. Physical-chemical characterization of INVITE 1-3 polymers

Sample	Mw ^{1}H-NMR [27] Da*	DD% mol/mol	CAC (mM)
INVITE 1	6687	10.4 ± 0.7	$22.3 \cdot 10^{-3}$
INVITE 2	7622	18.2 ± 0.6	$9.1 \cdot 10^{-3}$
INVITE 3	10568	33.1 ± 1.4	$2.4 \cdot 10^{-3}$

*INU Mw 5000 Da

As from Table 1, what is differentiating the three obtained INVITE polymers is the degree of derivatization (DD%) in VITE. Depending on the DD % the three polymers have been named as INVITE 1, INVITE 2 or INVITE 3 that, respectively, showed a DD % of 10, 18 and 33 mol/mol %, according to ^{1}H-NMR evaluations.

3.2. Critical aggregation concentration (CAC) evaluation

The CAC values for the three polymers, as from the pyrene method, resulted $22.3 \cdot 10^{-3}$, $9.1 \cdot 10^{-3}$ and $2.4 \cdot 10^{-3}$ mM, for INVITE 1, INVITE 2 and INVITE 3, respectively, and, as expected, at higher DD %, correspond lower CAC values (Table 1). These values of CAC are in accordance, or slightly lower, with respect to micellar systems based on PEG polyethylene glycol-phosphatidylethanolamine (PEG-PE)/VITE.[20,35] Furthermore, according to Duhem *et al*., VITE-glycolchitosan amphiphilic polymers shown a CAC of 2 ± 2 μg mL^{-1}.[36] In literature there are only few examples of INU-based amphiphilic polymers whose CAC has been valued. Thus, Srinarong *et al.* found a value of 0.009 % w/v for hydrophobized INU,[37] while Licciardi *et al.* found for their INU-based amphiphilic derivatives a CAC ranging $6 \cdot 10^{-2} - 5 \cdot 10^{-2}$ $mg \cdot mL^{-1}$ depending on the synthesized derivative.[38]

From the CAC values found for INVITE amphiphilic polymers it could be expected a good physical stability of the micelles as also confirmed by previously reported size stability studies.[27, 39]

3.3. Preparation and characterization of empty or curcumin loaded INVITE micelles

The preparation of INVITE micelles and their drug loading has been performed by two well established methods, by dialysis or by O/W emulsion.

Table 2 shows the results in terms of drug loading (DL), drug loading efficiency (DLe) and yield of curcumin loaded INVITE MC micelles obtained by direct dialysis or O/W emulsion method.

Table 2. Dependence of curcumin drug loading (DL), drug loading efficiency (DLe) and yield from the applied drug loading technique. All the experiments were performed with a drug/polymer ratio 1:20 and in triplicate (mean ± SD).

Sample	Drug loading technique	Solvent	DL w/w %	DLe* w/w %	Yield w/w (%)
INVITE 1MC	Direct dialysis	DMSO	2.4 ± 0.3	48 ± 1	97 ± 2
	O/W emulsion	DCM	1.1 ± 0.1	22 ± 2	95 ± 1
INVITE 2MC	Direct dialysis	DMSO	**3.5 ± 0.8**	**70 ± 1**	92 ± 3
	O/W emulsion	DCM	1.7± 0.2	34 ± 1	97 ± 2
INVITE 3MC	Direct dialysis	DMSO	2.2 ± 0.1	44 ± 1	98 ± 1
	O/W emulsion	DCM	0.7± 0.1	14 ± 2	97 ± 2

*Calculated as: theoretical weight of curcumin/actual weight of curcumin in the sample

As can be seen, between these two methods employed, the direct dialysis technique was superior in terms of drug loading with respect to the O/W emulsion technique. In particular, a reduction in drug loading > 47 % for all INVITE micelles when prepared by O/W emulsion with respect to the direct dialysis method has been observed. The reason of this outcome could be attributed to a better dispersion of the drug during the micelle formation process by dialysis. In particular, both INVITE and curcumin have been solubilized in DMSO and dialyzed against water (see experimental). The slow displacement of DMSO by water determine the formation of the INVITE polymeric micelles and the incorporation of curcumin within the micelle hydrophobic core before its precipitation in water was reached. Finally, part of the curcumin has been lost during the early phase of the dialysis process as also evidenced by the slightly yellow color of the washing media.

As for the O/W emulsion technique, the organic phase containing the dissolved drug was added under stirring into the micellar aqueous solution. So, it could be assumed that the fast dispersion and evaporation of the organic phase in water could not permit an efficient incorporation into the micelles, leading to drug precipitation resulting in a low drug loading.

Following the previous data, the chosen method for the preparation of empty INVITE micelles and for the curcumin loading was the dialysis one, due to more reproducible results and higher DL % values. It should be noted that the colloidal dispersions obtained after reconstitution in water of

the lyophilized micelles, resulted fully transparent and, in the case of the curcumin loaded micelles, orange-colored.

3.4. Size distribution and morphology of INVITE M and INVITE MC samples

The number-weighted size distribution for empty INVITE and loaded micelles has been measured by dynamic light scattering (DLS) and the results are reported in Table 3. It is noteworthy that the zeta potential values of INVITE M samples (Table 3) are in accordance with previous observations.[27, 39]

Table 3. Size distribution and zeta potential of empty (INVITE M) or curcumin loaded (INVITE MC) micelles.

Sample	Size (nm) ± sd	Zeta Potential* (mV)
INVITE 1M	7.1 ± 1.2	-28.8 ± 5.6
INVITE 2M	7.4 ± 1.9	-22.3 ± 4.2
INVITE 3M	13.1 ± 2.9	-34.0 ± 7.4
INVITE 1MC	7.3 ± 1.4	-23.4 ± 4.3
INVITE 2MC	7.9 ± 1.5	-28.9 ± 5.4
INVITE 3MC	19.3 ± 5.1	-32.7 ± 6.2

*Zeta potential of inulin = -9.14 ± 4.0

The observed size for INVITE micelles could be considered a clue on their cell-membrane penetration ability. Indeed, it has been demonstrated by a number of studies that nanosystems below 20 nm are able to penetrate the cell-membrane by passive diffusion. It is true for both normal and cancer cells.[40-42] For example, it was reported that nanosystems with dimensions ranging from 10 to 100 nm and whose surface charge is slightly positive or negative, should be able to access within disseminate tumour after parenteral administration.[40] For these reasons it could be supposed that the INVITE micelles could promptly penetrate the cell-membrane reaching high concentrations .

The zeta potential of INVITE M and INVITE MC micelles showed comparable negative values indicating that curcumin, as previously demonstrated, can be found in the inner core of the micelles,[39] so not contributing to the overall charge of the micelles. As previously stated,[27] the

observed negative charge can be attributed to the presence of inulin hydroxyl groups on the micelles outer shell.

The empty or drug loaded micelles have been further characterized by TEM to verify their morphology (Figure 1).

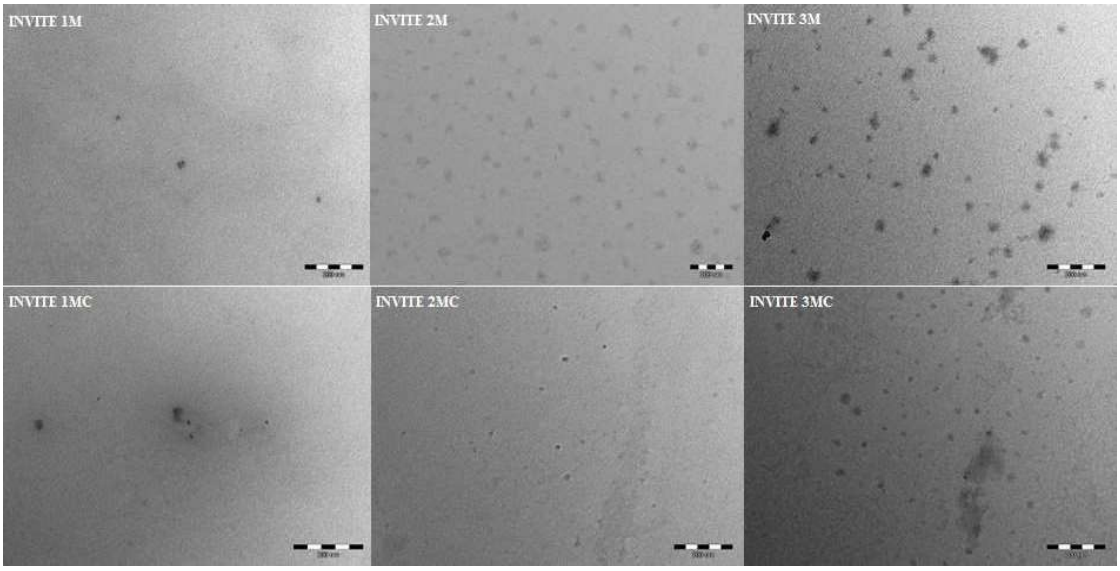

Figure 1. TEM photography of INVITE M (up) and INVITE MC (down) samples. The scale bar is 200 nm.

All the samples for TEM analysis have been prepared at the same concentration and dried in similar condition before staining with uranyl acetate. However, for samples INVITE 2M, INVITE 3M and INVITE 3MC the retrieved spots from the micelles look more as aggregates than single micelles. For this reason the actual size for these samples resulted higher than those found by DLS as also reported in other works.[43, 44] This behavior could be due to aggregation phenomena during the drying phase. Morros *et al.* reported that hydrophobically modified INU (HMI) are able to aggregate hierarchically at three levels, namely aggregates (<20 nm), flocks (<100 nm) and precipitates (>500 nm).[45] The smaller aggregates, which in the case of inulins can be classified as monomers and in the case of HMI as micelles, have dimensions below 15 nm.[45] In our study we did not found different populations from DLS studies but it seems reasonable that during the sample

preparation for TEM analysis a dehydration process occurs leading to aggregation phenomena. That is, in the dryer procedure, higher concentrations of the micelles are produced in a particular area which could favor the micelle aggregation. In addition, it should be considered that TEM analysis of micellar systems often is problematic due to the low contrast of the micelle itself.[46-48] These considerations could not be applied to INVITE 1M, INVITE 1MC and INVITE 2MC that shown discrete and round particles, not visibly aggregated, and with sizes that could be valued similar to those found by DLS studies. Furthermore, drug loaded or empty micelles did not show significant differences in size, probably due to strong interactions occurring between the micelle core and curcumin that avoid the increment in size distribution upon drug loading. Only INVITE 3MC shown a slight increase in size, probably due to the fact that the higher DD in VITE allowed the formation, inside the micelle core, of wider bridge-like structures with curcumin.

3.5. In-vitro curcumin release studies

Following the hypothesis by which the INVITE micelle core is formed by the formation of π-π interactions of the aromatic portion of VITE instead of more general hydrophobic interactions between the aliphatic chains,[34, 39] the drug loading of a hydrophobic drug bearing aromatic groups in the INVITE micelles, such as curcumin, should form a not-permanently-stable system. The release of the entrapped drug should occur in a controlled manner without any evident burst effect.

In order to evaluate the release behaviors of the INVITE in physiologic fluids or in conditions simulating the endosome or the tumour environments, release studies at pH 7.4 or 5.5 have been performed. Figure 2 shows the drug-release profiles, expressed as cumulative release % as a function of time in PBS solution at pH 7.4 or at pH 5.5 both in the presence of 0.5% Tween 80 up to 48 h.

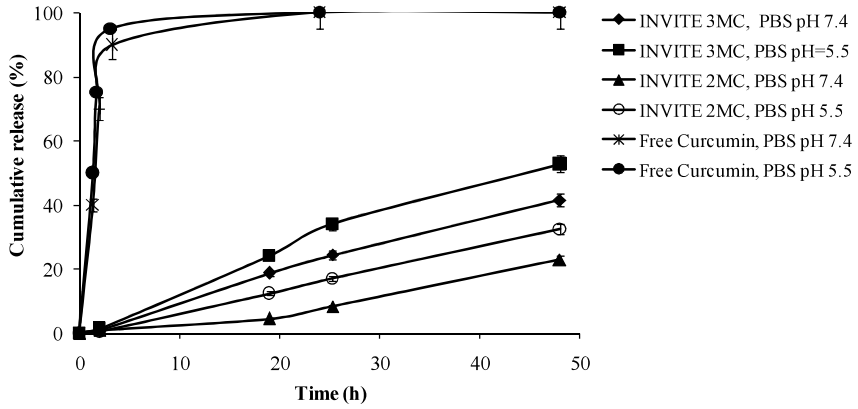

Figure 2. Cumulative release of curcumin from INVITE 2MC and INVITE 3MC at 37°C in PBS at pH 7.4 or in PBS at pH 5.5, INVITE 1MC sample is not included in the graph but is discussed.

As from Figure 2, the release curves from micelle systems clearly indicate a controlled release of curcumin in both media with no evident burst effect, while free curcumin is promptly released under the same conditions.

In particular, 42 % of curcumin from INVITE 3MC and 23% from INVITE 2MC was released after 48h in PBS pH 7.4. At pH 5.5 the curcumin release for both INVITE 3MC and INVITE 2MC was ≈ 10 % higher than that at pH 7.4 (53% for INVITE 3MC and 33% for INVITE 2MC as absolute values). The same release trend was obtained for INVITE 1MC where 11% of curcumin was released in PBS pH 7.4 and 23% at pH 5.5.

The release studies showed a clear trend in the amount of released curcumin depending on the different samples DD % in the order INVITE 3M > INVITE 2M > INVITE 1M. As previously stated, it is possible that the higher amount in VITE residues in INVITE 3M samples determines a "bridge-like" structure instead of a "centered-core" in the hydrophobic portion of the micelle so forming larger particles with respect to INVITE 1M and INVITE 2M systems determining faster release profiles. The observed release rates, different for the three INVITE systems, could not be attributed to different stability behaviors of the micelles since, from previously performed size

stability studies, no differences could be evidenced for the different samples up to several days.[27, 39] Furthermore, it should be noted that the release rate resulted almost constant with no time-related fluctuations so confirming the high physical stability of the proposed drug delivery system. Similar results in terms of curcumin release magnitude but with detectable burst effects, have been reported by Abouzeid et al. and by Sun et al.[20, 49]

The differential release rate at pH 7.4 or 5.5 may be useful for ocular or tumor delivery occurring in acidic environments.

3.6. Blood compatibility by hemolysis assay and changes in morphology of blood red cells (RBCs)

Drug delivery systems for intravenous administration should not produce any toxic effect to the blood cells. A well-established method to evaluate blood compatibility of micellar formulations intended for intravenous administration, is the hemolysis assay performed on whole blood.[22, 50, 51] This test evaluates the release of hemoglobin from the red blood cells (RBCs) in case of cellular rupture upon contact with a foreign material. The hemolysis associated with an adverse effect caused by a biomaterial determines the release of hemoglobin. The hemoglobin release could be effectively detected by spectroscopic evaluations. A value lower than 5% in hemolysis is considered a non-toxic effect. In the test shown in this study, Triton X-100 has been used as positive control (100 % hemolysis), while saline solution has been used as negative control (no hemolysis). Furthermore, the test has been performed on the three INVITE M samples (empty micelles) at a concentration of 1 mg/mL for 1, 2 or 3 h. All the tested samples do not induce RBCs hemolysis, even at the highest employed concentration, so confirming that the INVITE systems could be considered blood-compatible. Cytotoxic studies previously performed on fibroblasts showed that in a wide range of concentrations (below and above CAC) the INVITE systems could be considered as highly cell-compatible.[34] The visualization of the processed RBCs by optical microscopy confirmed the data from the hemolysis assay. In particular, taking in mind the complete membrane rupture by

Triton X-100, no visible damage nor shape modifications have been evidenced for all the tested INVITE M micelles whose morphology was in agreement with that of the negative control (saline solution), (see supporting information). This result strongly support the safe use of the INVITE micelles for intravenous administration.

Taking into account the low release rate of INVITE 1MC and INVITE 3MC which gave some problem in terms of dispersibility in water at high concentrations, in the following only the INVITE 2MC micelles have been used for further characterizations.

3.7. Cellular uptake

Due to the reduced size of the obtained micelles, it has been supposed that INVITE micelles could penetrate the cell-membrane easily and quickly. To verify this theory, cellular uptake studies have been performed on HEK 293 cells by valuing the fluorescence of curcumin inside the cells upon exposition to curcumin loaded INVITE micelles or curcumin alone at different times. As shown in Figure 3, cells treated INVITE 2MC, revealed a fast cellular uptake (within 30 min) concentrated in the cytosol of the cells. By contrast, no fluorescence was imaged when cells were incubated in the presence of control micelles (INVITE M), thus demonstrating the ability of INVITE 2MC to quickly and effectively cross the cellular membrane. As known also curcumin alone is able to cross the cellular membrane by passive diffusion,[49] but the INVITE 2MC system did not show any curcumin release in the incubation medium within 30 min so it can be concluded that the intracellular fluorescence was a result of the micelle internalization.

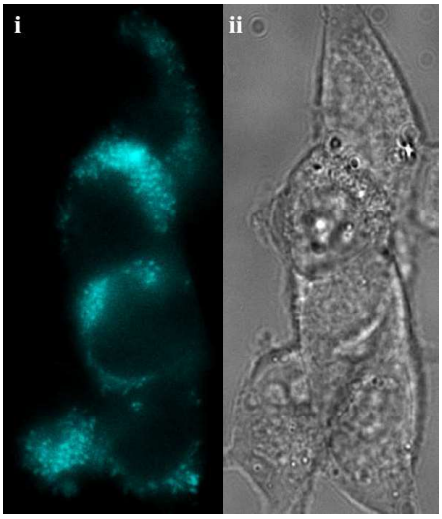

Figure 3. Uptake studies of INVITE 2MC on human embryonic kidney 293 cells. Cells were seeded onto 24 mm coverslips and treated at 37 °C in a 5% CO_2 atmosphere with 0.01 mg mL^{-1} of INVITE 2MC and internalized fluorescence (i) was acquired after 30 min. The integrity of the cells was confirmed in bright field images (ii). Images are representative of 3 independent experiments.

3.8. Pharmacokinetic studies

Pharmacokinetic studies were aimed to ultimately confirm that the INVITE systems are effective in improving the biopharmaceutical features of curcumin upon intravenous administration. The curcumin loaded INVTE 2MC micelles in PBS were administered to Balb/C mice e *via* intravenous tail vein injection at the dose of 50 µg per animal. The pharmacokinetic profile of INVITE micelles is reported in Figure 4 and the values of the main pharmacokinetic parameters are reported in Table 4. The data clearly show a great prolongation of curcumin half-life because in literature it has been widely demonstrated that not delivered curcumin ("naked" curcumin) is quickly eliminated from blood and could not be found in plasma just after few min.[49, 52-55]

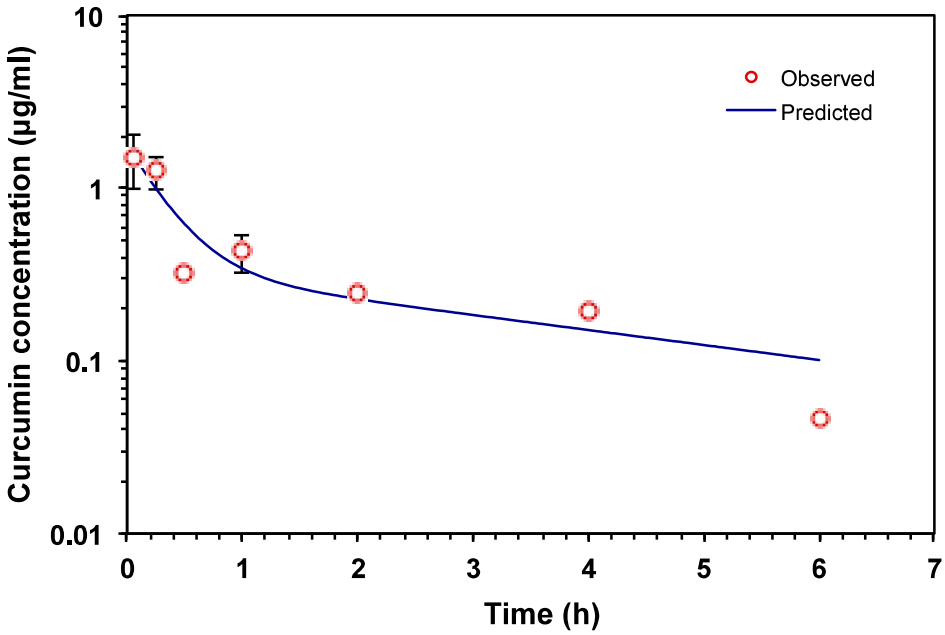

Figure 4. Pharmacokinetic profile of INVITE 2MC injected in Balb/C mice via tail vein at the dose of 50 µg per animal (curcumin equiv.)

Table 4. Pharmacokinetic parameters of INVITE 2MC

PK Parameter	Value
$t_{1/2}$ Alpha (h)	0.23
$t_{1/2}$ Beta (h)	3.47
V_d (mL)	2.93
Clearance (mL/h)	2.44
AUC 0-t (µg/mL * h)	1.65
AUC 0-inf (µg/mL * h)	2.15
MRT (h)	3.97

Conclusions

The effectiveness of INVITE systems for the delivery of curcumin chosen as model hydrophobic drug has been clearly demonstrated through different experimental evidences. It has been shown that curcumin loaded INVITE micelles are capable to provide a sustained release of the entrapped drug at different pH conditions and that their intravenous administration should not cause any adverse effect due to a proven blood compatibility. Furthermore, due to the nanometric dimensions of the INVITE systems, it has been demonstrated a fast cellular uptake (within 30 min) by human embryonic kidney 293 cells. Finally, pharmacokinetic studies on Balb/C mice showed the presence of curcumin in the blood up to 6 h, differently from "naked" curcumin that is quickly cleared from the blood stream. These results are strongly promising for the use of the INVITE systems in drug delivery of hydrophobic drugs.

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