Nanostructured Polymeric Functional Micelles for Drug Delivery Applications

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Summary: Inulin (INU) is a polyfructose which have the unique features to not bind plasmatic proteins and to be totally filtered by the kidneys where it is neither secreted nor reabsorbed. These behaviors make it an ideal candidate to target and delivery drugs into the kidneys for the therapy of urinary tract affections. On the other side, Vitamin E (VITE) is a hydrophobic molecule with important antioxidant activities. Therefore, the chemical conjugation of INU and VITE, led to amphiphilic INVITE polymers able to self-assemble in nanostructured micelle.
The obtained micelle have been drug loaded with the model hydrophobic drug curcumin (solubility $\approx$ 11 ng/ml), the drug release and micelles stability studies have been performed *in-vitro* in simulated physiological fluids. Furthermore, biological studies on suitable cell lines have been carried out to assess the cytotoxicity of INVITE conjugates.

Keywords: inulin; micelles; self-assembly; urinary tract; vitamin E

Introduction

Inulin (INU) is a naturally occurring linear polyfructose, obtained by extraction from several plants like Dahlia tuber, Jerusalem artichoke and chicory. Moreover, being Inulin obtained from natural sources and being also used for the production, e.g., of bioethanol it could be considered as a renewable energy sources.[1] In the last years, INU found several applications as a valuable polymer for drug delivery purposes. It has been proposed for the formulation and production of different drug delivery systems such as hydrogels for colon targeted drug delivery, or for the therapy of iron deficiency anemia.[2–7]

INU, when intravenously administrated, does not bind to plasmatic proteins, is freely filtered by kidneys where it is neither secreted nor reabsorbed and it is not metabolized by the kidney. For this reasons INU is employed since almost 60 years for the evaluation of renal functionality.[8] In fact, INU, which has a mean molecular radius of 1.5 nm and a molecular weight of approximately 5.200 Da, is considered an ideal marker and the gold standard for measuring glomerular filtration rate (GFR).

Taking into consideration this behaviors it can be postulated that Inulin will reach the kidneys at high concentrations due to the lack in reabsorption and secretion, making it an ideal candidate for drug targeting and delivery to the urinary tract.

Vitamin E (VITE) shows several biological activities determining beneficial effect on human health. It is a required nutrient for humans, i.e., for the prevention of VITE deficiency symptoms, including peripheral neuropathy and hemolytic anemia. VITE is a potent lipid-soluble, chain-breaking antioxidant.[9] Furthermore, VITE is an antioxidant, anti-inflammatory and antithrombotic. It protects the cell membranes, especially in the lungs and red blood cells, against damage caused by various pollutants, peroxides, and free radicals formed during metabolic processes.[10,11] It has been shown that VITE works

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synergistically with other antioxidant nutrients, such as vitamin C, β-carotene, to quench free radicals, peroxides, and other potentially harmful substances.[11] In regard to its anti-inflammatory effects, VITE inhibits the enzyme lipoxygenase, which is responsible for the formation of leukotrienes that cause inflammation.[12,13] This can be useful in the treatment of asthma and other inflammatory conditions such as arthritis. At higher doses, vitamin E has been shown to exhibit antithrombotic activity by increasing the production of prostaglandins.[11,13] Due to its hydrophobicity, VITE has been employed to synthesize amphiphilic molecules able to increase solubility of hydrophobic molecules through their incorporation.[11] Various tocopherol esters are commercially available that include acetate, succinate, tartrate, nicotinate, and polyethylene glycol-1000 succinate. The esterification of tocopherols improve its stability, in fact tocopherol esters have been found to be more stable against oxidation than tocopherol itself.[14] The stability of these VITE esters depends from their chemical structure in fact, e.g. within the gastrointestinal tract, ester hydrolysis is considerably fast for vitamin E acetate and slower for vitamin E succinate and D-α-tocopherol polyethylene glycol succinate (TPGS).[11,15,16] Importantly, VITE esters showed to retain the biological properties of native VITE as well as specific behaviors such as anti-cancer activity.[15,16]

Both INU and VITE result approved for injectable and are well tolerated even at high concentrations.

With the aim to combine the unique properties of INU and VITE for pharmaceutical applications a new INVITE bioconjugate, able to produce amphiphilic systems self-assembling in nanostructured DDS even at low concentrations, has been synthesized and evaluated in this work. The obtained amphiphilc polymers are provided with hydrolyzable ester groups linking INU and VITE and the degree of functionalization, intended as the amount of INU repetitive unit functionalized with VITE, has been varied to tailor the final chemical-physical properties of the obtained bioconjugates. One derivative has been selected and its cytotoxicity, on a selected cell line, has been tested. Subsequently, the INVITE micelle system has been loaded with a hydrophobic model drug (curcumin) and the stability of the drug-loaded micelles has been valued in simulated physiological condition.

Experimental Part

Materials

All reagents were of analytical grade, unless otherwise stated. Dimethylsulfoxide (DMSO), curcumin, D-α-tocopherol succinate (Vitamin E succinate, VITE) were purchased from Sigma-Aldrich (Milano, Italy). Inulin from dahlia tubers (INU, approx. 5000 Da), was purchased from Fluka (Milano. Italy). Dulbecco modified Eagle's medium (DMEM) with 4.5 g/l glucose and glutamine was purchased from Lonza, Milan (Italy). Foetal Bovine Serum (FBS, Eu approved) was purchased from EuroClone, Milan (Italy). Human adult dermal fibroblasts as primary cells were purchased from International PBI, Milan (Italy).

INVITE Synthesis

Inulin-D-α-tocopherol succinate conjugate was synthesized by a known carbodiimide chemistry obtaining a final product with a derivatization degree in VITE of $26 \pm 1.2\%$ mole/mole. The obtained amphiphilic polymer showed a critical aggregation concentration of 0.4 mg/ml corresponding to $2.5 \cdot 10^{-3}$ mM as calculated by the pyrene method.

INVITE Micelle Preparation

In a standard procedure for INVITE micelles preparation 100 mg of INVITE polymer were solubilized in 10 ml of DMSO with or without 3 mg of Curcumin. After complete solubilization, the solution was poured in a dialysis tube (Spectra/Por® 6) with a MWCO 3.500 Da and dialyzed

against distilled water for 3 days by changing the dialysis water every 3 hours. The colloidal suspensions inside the tubes appeared transparent for the whole dialysis time, additionally, the drug loaded micelles suspension appeared yellow. At the end of the dialysis process, either the drug loaded or the placebo (INVITE micelles without drug) colloidal suspensions have been lyophilized and recovered with a 86% w/w yields with respect to the starting polymer.

Drug Loading Evaluation by UV Vis Analysis on INVITE Micelles

For the evaluation of INVITE micelles drug loading, 2 mg of lyophilized drug-loaded micelles were dissolved in 20 mL of DMSO. The amount of drug loaded curcumin was determined at 425 nm by a UV-VIS Spectrometer Lambda 25, Perkin-Elmer, (Monza, Italy). Each measurement was performed in triplicate. The loaded amount of curcumin was calculated with respect to a calibration curve of curcumin solutions in DMSO in the concentration range $0.5 \cdot 10^{-3}$–0.01 mg/ml (correlation coefficient $r^2 > 0.998$).

Cell Culture

Adult dermal fibroblast as primary cells were purchased from International PBI (Milan, Italy). The cells were cultured in DMEM containing 10% fetal bovine serum (FBS, Eu approved) and 1% antibiotic solution (100U/ml penicillin, 100 μg/ml streptomycin, Sigma-Aldrich). After expansion, the cells were detached for the cytotoxicity studies.

Cytotoxicity Studies

The effects of INVITE micelle on cell viability were assessed with the 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium (MTT) assay, using 96 Well Cell Culture Cluster with 10,000 fibroblasts plated in contact to 100 μl of INVITE samples at different concentrations, following a protocol previously set up by the research group.[17]

Briefly, fibroblasts were cultured in wells in DMEM supplemented with FBS for 24 h at 37 °C, then media were removed and fresh DMEM (without serum) and containing the INVITE samples at stated concentrations, was added. After 48 h, 25 μl of MTT solution (5 mg/mL in DMEM) were added into wells. Cells were incubated for 2 h at 37 °C to allow MTT reduction by mitochondrial dehydrogenase in viable cells. After 2 h, a suitable detergent was added to dissolve the resulting blue formazane crystals. Results were revealed by a multiwell scanning spectrophotometer (Microplate Reader Model 680, Bio-Rad Laboratories, USA). The optical density was measured at 570 nm with 655 nm as reference wavelength. Cell viability was calculated as the percentage of untreated cells (control).

Drug Release Studies from INVITE Micelles

10 mg of INVITE curcumin-loaded or placebo micelles have been solubilized in 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% w/w) at 37 °C or 25 °C, in static or dynamic conditions. 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 measurement.

Results and Discussion

Herein, Inulin-D-α-tocopherol succinate (INVITE) polymer/micelles have been valued for their cytocompatibility toward fibroblasts while INVITE micelles were valued for their ability to incorporate and release the hydrophobic model drug curcumin, Figure 1.

Curcumin is the product obtained by solvent extraction of turmeric i.e., the ground rhizomes of Curcuma longa L. (Curcuma domestica Valeton) and purification of the extract by crystallization.[18] Its water solubility was found to be 3–11 ng/ml depending from the source.[19] Because of its important pharmacological effects,

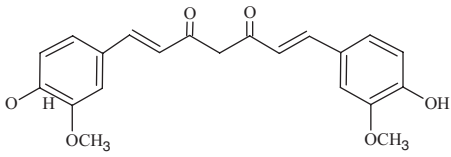

Figure 1.
Chemical structure of Curcumin.

efforts have been spent to increase curcumin water solubility by adopting different successful approaches such as complexation with cyclodextrins or inclusion in micelle systems.[19,20]

In the present work curcumin was used as a model hydrophobic drug. The drug loading of curcumin in INVITE micelles has been performed by a dialysis method. In particular, both INVITE and curcumin have been solubilized in DMSO and dialyzed against water for several days (see experimental part). In this way, the slow displacement of DMSO by water determined on one side the formation of the INVITE polymeric micelles and, on the other, the incorporation of curcumin within the micelles hydrophobic core. How curcumin is structurally positioned inside the micelle is under investigation, but preliminary data are indicating possible π-π stacking interactions as the base for curcumin loading and micelle stabilization instead of a "typical" physical interaction due, i.e., to the hydrophobic interaction between the VITE alkyl chains, Figure 2. These preliminary studies have been performed by ^{1}H-NMR. In particular, it has been seen that in D_2O the signals of the protons close to the aromatic ring of VITE disappear, while the signals of the alkyl-chain protons are still detectable. This result is probably indicating the formation of an "aromatic" hydrophobic inner core which excludes the deuterium oxide and does not allow any detection of the protons included into the "dry" space. This assumption would also be confirmed by the clear detection of the alkyl chain protons signals of VITE in the ^{1}H-NMR spectrum so indicating that these protons can be found outside the water-exclusion area, i.e., within the outer hydrophilic shell. On the other side, the ^{1}H-NMR signals of curcumin (D_2O), when incorporated in the INVITE micelle system, resulted not detectable. Also in this case, it probably indicates the complete incorporation of curcumin molecules into the micelle hydrophobic core. These data are supporting that the physical forces driving micelle stabilization and curcumin incorporation are probably π-π stacking interactions. Further studies will be aimed to confirm these assumptions.

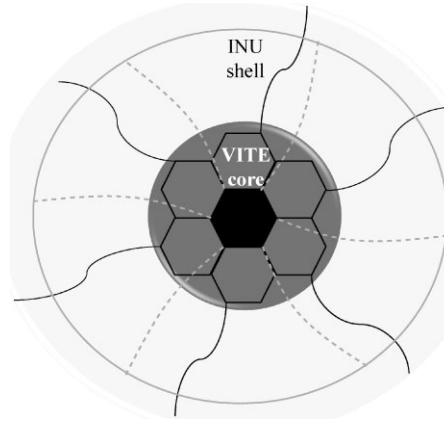

Figure 2.
Schematic representation of self-assembled INVITE nanostructures in aqueous environment; the black and dark-gray colors represent the VITE inner hydrophobic core while the light-gray colors represent the INU outer hydrophilic shell.

The lyophilized and rehydrated micelles resulted in a transparent colloidal-suspension. Additionally, the drug loaded micelles resulted in a homogeneous yellowish color colloidal-suspensions, suggesting that the incorporated curcumin is molecularly dispersed into the micelle (no precipitation in water or aggregation).

The nominal amount of loaded curcumin was 3% w/w, that resulted in a final drug loading of 1.75% w/w as calculated by UV-VIS analysis, meaning a drug loading efficiency of 58%. The drug loading calculated as mole/mole ratio resulted 21%. Considering that the final drug loaded micelle-suspension resulted clear and yellow (no precipitate), it is possible to hypothesize that some curcumin has been

washed out during the DMSO displacement by water. The freeze dried, drug loaded or placebo, micelles resulted in soft, coherent and not collapsed powders easily re-dispersible in water with formation of colloidal dispersions (Figure 3).

Placebo INVITE micelles have been subjected to cytotoxicity studies to confirm the expected biocompatibility of INVITE systems, Figure 4.

The tested polymer concentrations were between 6.6 and 0.00391 mg/ml. In particular, at concentrations between 5 and 0.00391 mg/ml, the fibroblasts viability was good, ranging from 80 to 115%, no evidence of interaction with cellular metabolism was highlighted. Higher concentration resulted in lower viability. It should be noted that critical aggregation concentration (CAC) for these systems (data not shown) was found to be 0.4 mg/ml ($2.5 \cdot 10^{-3}$ mM) meaning that both micelles (up to ≈5 mg/ml) and free polymer could be considered not cytotoxic. This is an encouraging result because indicates that even at high concentrations, i.e. at the site of administration, the INVITE system would not result in any cytotoxic effect.

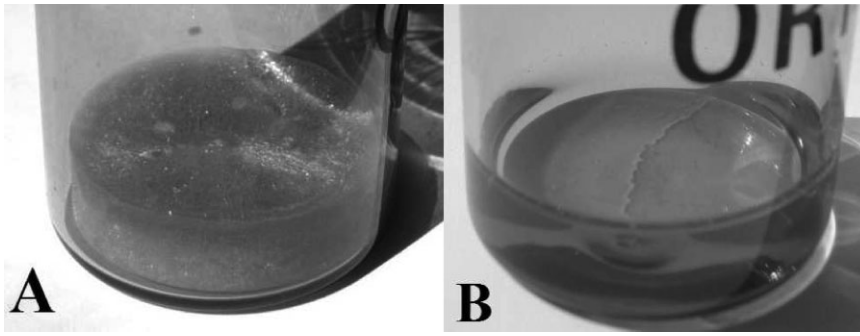

Figure 3.
Freeze-dried INVITE curcumin-loaded micelles before (A) and after (B) reconstitution with water.

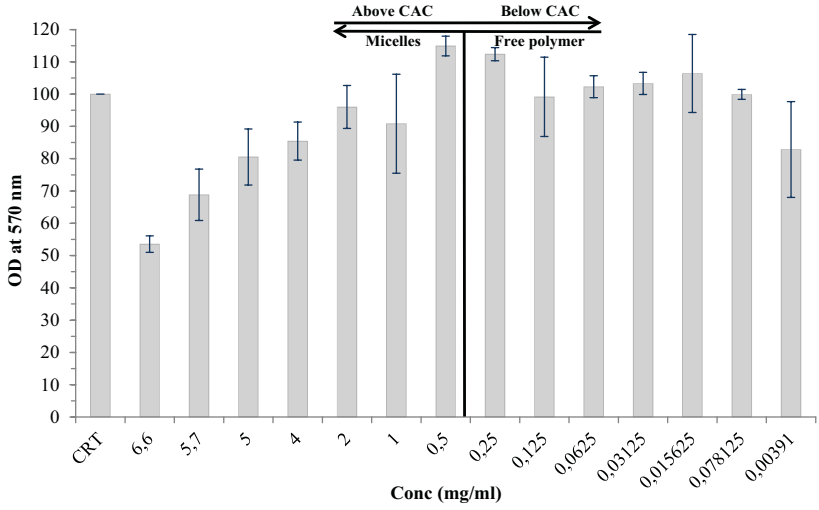

Figure 4.
Cytotoxicity studies on fibroblasts of INVITE at different concentrations above or below critical aggregation concentration (CAC).

Thinking to these systems for parenteral administration, release studies have been performed in simulated physiological conditions, Figure 5.

These studies were aimed to verify the stability of drug loaded micelles in physiological conditions, in fact, when injected in the blood stream the micelles should not release the incorporated drug until, e.g., the formulation reaches the urinary tract or the release is triggered by a specific stimulus.

As shown in Figure 5, as expected, free curcumin is promptly dissolved because of the adopted sink conditions. Furthermore, it demonstrates that the dialysis membrane does not influence the drug dissolution process. The complete release of curcumin from INVITE micelles is achieved after 250 h at 37 °C under stirring. The slow release rate of the drug in the absence of specific triggers indicates, on one side, that the micelle system results stable in the release medium, on the other, that the drug strongly interacts with the micelle core. In this contest, the release of the drug from the micelle is both influenced by the drug-polymer physical interaction and by the subsequent drug diffusion through the micelle outer shell. The drug-polymer interaction could be explained, as previously theorized, by the formation of π-π stacking interactions. These physical forces stabilize the drug into the micelle hydrophobic core reducing the release rate due to the higher affinity of the drug with the hydrophobic micelle environment rather than with the hydrophilic medium. On the other side, considering the hydrophilic layer of each micelle as a membrane between the donor (hydrophobic micelle core) and acceptor (release medium) compartments, the diffusion of the drug from the micelle to the release medium would be regulated by the first Fick's law. When the system is maintained without stirring (static) a significant reduction in the dissolution rate could be noted. Even if sink conditions are maintained (release volume 10 times the saturation volume of curcumin), the static conditions of the release medium would not assure to maintain the steady-state conditions as from first Fick's law with subsequent reduction of the dissolution rate. This assumption might explain the differences of the release profiles shown by the dynamic and static release experiments at 37 °C.

The release studies at 25 °C (with stirring) have been performed to verify the stability of the formulation after reconstitution at room temperature taking into consideration a multi-dose treatment or a long infusion time, e.g., during

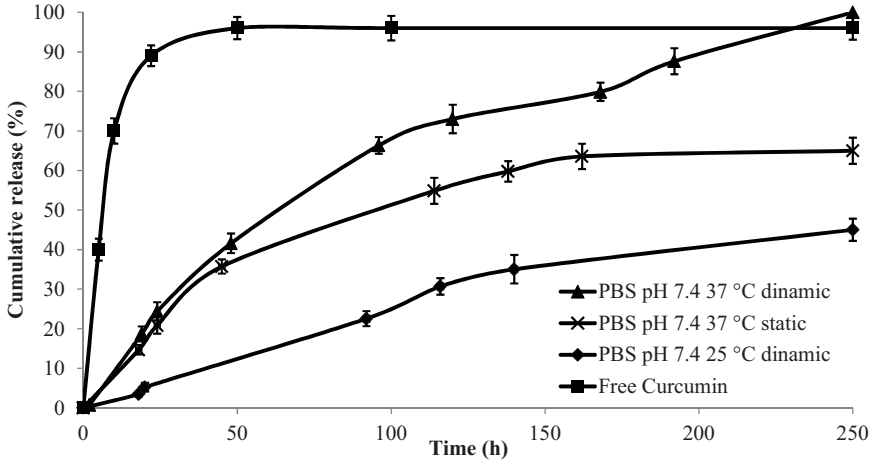

Figure 5.
Cumulative release of Curcumin from INVITE micelles in PBS at pH 7.4 at 37 °C with stirring and without stirring at 25 °C or at 37 °C.

phleboclysis administration. After 18 h the release of curcumin resulted $3.5 \pm 0.3\%$ indicating a high stability of the micelle system in these conditions. It would be supposed that such a formulation could be administered even at low concentrations (large volumes) and for a prolonged time maintaining its pharmaceutical stability. On the other side, when injected in the blood stream, the drug loaded micelles could result enough stable to reach unaltered the kidneys. Preliminary studies (data not shown) are indicating a remarkable drug release in simulated urine.

Conclusion

In this work a new Inulin-D-α-tocopherol succinate (INVITE) amphiphilic system self-assembling in nanostructured micelles has been tested for its cytotoxicity, for its ability to load hydrophobic drugs and for its drug release behaviors. This system has been thought for the drug targeting and delivery to the kidneys. The INVITE polymer resulted cytocompatible in a wide concentration range above and below the critical aggregation concentration. The release studies shown that INVITE micelles systems reconstituted in PBS pH 7.4 are stable at 25 °C for several hours so to allow a prolonged administration time as it happens during long time infusion. Furthermore, they resulted enough stable at both dilution and temperature to reach the kidneys before the entrapped drug is released. Further studies will be aimed to verify the drug release in conditions mimicking the urinary tract and in-vivo studies to verify the drug targeting.

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