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Abstract: Here, in vivo long-circulating behaviours of Inulin-based nanomicelles are demonstrated for the first time. We show the synthesis and evaluation of biotin-decorated polymeric INVITE micelles constituted of substances of natural origin as Inulin (INU) and Vitamin E (VITE) as long-circulating carriers for receptor-mediated targeted drug delivery. The resulting INVITE or INVITE-BIO micelles, nanometrically sized, did not reveal any cytotoxicity after 24 h of incubation with Caco-2 cells. Moreover, in vitro studies on Caco-2 cells monolayers indicated that the transport of INVITE-BIO micelles was faster than surface unmodified INVITE micelles. In vivo optical imaging studies evidenced that, upon intravenous administration, INVITE-BIO micelles were quantitatively present in the body up to 48 h. Instead, after oral administration, the micelles were not found in the systemic circulation but eliminated with the normal intestinal content. In conclusion, INVITE-BIO micelles may enhance drug accumulation in tumor-cells over-expressing the receptor for biotin through receptor mediated endocytosis.

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**Dear**
**Tatiana K. Bronich, PhD,**
**University of Nebraska Medical Center, Omaha, Nebraska, USA**
**Editor-in-Chief of NANOMEDICINE: NANOTECHNOLOGY, BIOLOGY AND MEDICINE.**

Please find enclosed our manuscript "**Design, synthesis and evaluation of Biotin decorated Inulin-based polymeric micelles as long-circulating nanocarriers for targeted drug delivery**" from Delia Mandracchia, Antonio Rosato, Adriana Trapani, Theodora Chlapanidas, Isabella Monia Montagner, Sara Perteghella, Cinzia Di Franco, Maria Luisa Torre, Giuseppe Trapani, Giuseppe Tripodo* for submission as a research paper to **NANOMEDICINE: NANOTECHNOLOGY, BIOLOGY AND MEDICINE**.

The chemical combination of Inulin, Vitamin E and Biotin, which are all natural substances, led to the formation of nanomicelles with long-circulating and targeting behaviors. *In-vivo* and *in-vitro* performances of inulin-vitamin E nanomicelles were evaluated before and after bioconjugation with biotin, chosen as a tumor targeting substances. It has been assessed that the obtained systems can be found in the systemic circulation up to 72 h after intravenous administration. On the other side, when orally administered, no systemic absorption was detected attributing this behavior to the activation of an efflux system as demonstrated by a transport study performed on Caco-2. A deep physicochemical characterization was further performed.
In our opinion these results, together with the interesting properties of the applied material are of wide interest for the reader of **NANOMEDICINE: NANOTECHNOLOGY, BIOLOGY AND MEDICINE**.
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Sincerely yours
**Dr. Giuseppe Tripodo**

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***Graphical Abstract: Text**

Graphical abstract text

Long circulating micelles from the natural substances Inulin, Vitamin E and Biotin were produced by simple chemical procedures. *In vivo* and *in vitro* experiments clearly addressed the prepared nanosystems as valuable drug delivery systems with targeting behaviours.

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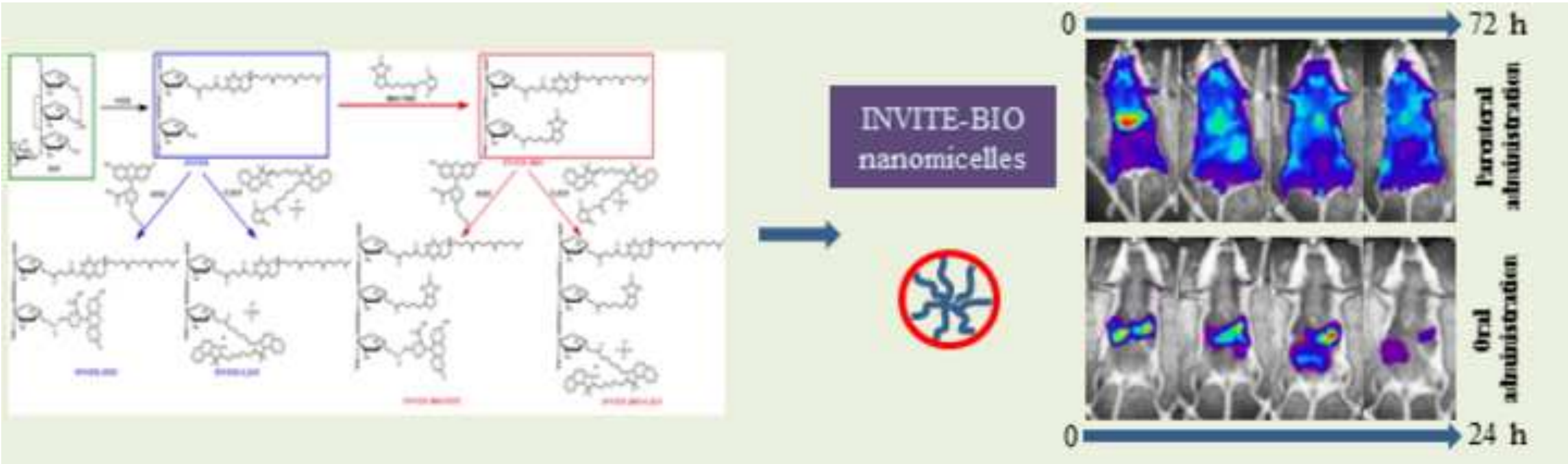

# Design, synthesis and evaluation of Biotin decorated Inulin-based polymeric micelles as long-circulating nanocarriers for targeted drug delivery

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## Abstract

Here, *in vivo* long-circulating behaviours of Inulin-based nanomicelles are demonstrated for the first time. We show the synthesis and evaluation of biotin-decorated polymeric INVITE micelles constituted of substances of natural origin as Inulin (INU) and Vitamin E (VITE) as long-circulating carriers for receptor-mediated targeted drug delivery. The resulting INVITE or INVITE-BIO micelles, nanometrically sized, did not reveal any cytotoxicity after 24 h of incubation with Caco-2 cells. Moreover, i*n vitro* studies on Caco-2 cells monolayers indicated that the transport of INVITE-BIO micelles was faster than surface unmodified INVITE micelles. *In vivo* optical imaging studies evidenced that, upon intravenous administration, INVITE-BIO micelles were quantitatively present in the body up to 48 h. Instead, after oral administration, the micelles were not found in the systemic circulation but eliminated with the normal intestinal content. In conclusion, INVITE-BIO micelles may enhance drug accumulation in tumor-cells over-expressing the receptor for biotin through receptor mediated endocytosis.

**Keywords: Inulin, Vitamin E, Biotin, Cancer, Optical Imaging, Micelle**

Inulin (PubChem CID: 16219508)

Vitamin E (PubChem CID: 14985)

Vitamin E Succinate (PubChem CID: 20353)

Biotin (PubChem CID: 171548)

Biotin-NHS (PubChem CID: 6710714)

Cy5.5 NHS ester (PubChem CID: 52918950)

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**Number of Figures: 7**
**Number of References: 57**
**Number of Tables: 2**

## 1. Background

Polymeric micelles are interesting nanostructured platforms characterized by a core-shell structure and obtained by self-assembly of amphiphilic polymers in aqueous solutions. The core is formed by the hydrophobic portion of the polymer, while the hydrophilic part constitutes the shell. Due to their small size (10-100 nm), low toxicity, capacity to solubilize lipophilic drugs in the core, and high drug loading these nanocarriers are most investigated drug delivery systems.[1] Among the hydrophobic polymers, poly(propylene glycol (PPG), poly(D,L-lactide) polycaprolactone are often employed, while the polyethylene glycol (PEG) is frequently used as hydrophilic moiety and these remarks, taken together, confirm that the majority of polymeric micelles described in literature are based on biodegradable and synthetic copolymers.

In the context of our research project aimed at evaluating biodegradable amphiphilic polymers of natural origin and from renewable resources, according to the sentence "learning from Nature, discovering through Nature", we designed nanomicelle systems based on Inulin (INU, a fructan-type oligosaccharide) and Vitamin E (VITE) denoted as INVITE. The INVITE nanomicelles, previously demonstrated effective biomedical and pharmaceutical properties, such as high biocompatibility, suitability for intravenous administration, free and quick cross of cellular membrane, solubilisation and delivery of highly hydrophobic drugs, and favourable pharmacokinetic *in vivo* after intravenous administration.[2-5]

Why Inulin? Because it is a natural polysaccharide extracted from many plants, hydrophilic, cheap, FDA-approved and routinely used by intravenous injection, i.e., it has an exceptionally-high safety profile. Furthermore, it has been used for pharmaceutical applications in different forms such as hydrogels [6-10], micelles [11-13], nanoparticles[14], iron-supplementing systems [15] and more. When INU is intravenously administrated, it does not bind to plasmatic proteins[16, 17] and it is freely filtered by the kidney where it is neither secreted nor reabsorbed and it is not metabolized by the kidney.[18] INU, shows a mean molecular radius of 1.5 nm and a molecular weight of approximately 5.000 Da .[19] Moreover, notable applications of inulin concern stabilization of proteins, modified drug delivery and targeting and adjuvanting vaccine formulations.[19]

Why vitamin E? It is a vitamin normally found in many foods, especially in olive oil and other fat-derived nutrients.[20] It is one of the most powerful anti-oxidant that Nature uses in its cycles, and in human body enters in several processes including cancer and oxidative stress, fighting them.[21, 22] Vitamin E is hydrophobic and its use for pharmaceutical applications is widely documented.[23, 24]

Concerning the targeting properties of polymeric micelles, their small size allows to achieve passive targeting by extravasation through the leaky tumor vessels *via* enhanced permeability and retention effect (EPR) effect. However, to increase the intracellular uptake of these drug delivery systems to the target site, the presence of an active targeting moiety on the surface of these nanocarriers would be most suitable in order to exploit the receptor mediated active targeting strategy.

The aim of the present work was to evaluate biotin surface modified INVITE nanomicelles as carriers for targeted drug delivery. Why Biotin? Among the cellular surface targets potentially useful for receptor mediated targeted drug delivery, biotin, a natural nutrient, is widely employed because of its over expression in several tumors and for its strong interaction with avidin.[25] Indeed, several aggressive cancer lines such as leukemia (L1210FR), ovarian (OV 2008, ID8), colon (Colo-26), mastocytoma (P815), lung (M109), renal (RENCA, RD0995), and breast (4T1, JC, MMT06056) cancer cell lines [26, 27] overexpress receptors for biotin.[28] It is important to be underlined that biotin cannot be synthesized by mammalian cells, thus, biotin must be obtained from exogenous sources via intestinal absorption.[29]

The so called sodium-dependent multivitamin transporter (SMVT) is an important membrane transporter for biotin which is found along the small and large intestines. Several essential nutrients such as biotin, are taken-up by this transporter which have been shown as the responsible for the antitumor activity of biotin functionalized camptothecin on multi-drug resistant human ovarian cancer cell line A2780.[30, 31] Interestingly, its SMVT overexpression was found superior with respect to folate receptor.[32] This is mostly due to the fact that biotin belongs to a particular category of exogenous micronutrients which are required for cellular functions and, particularly, for cell growth.[33] Consequently, the biotin demand in tumors, is higher than normal tissues.[34]

In 2006 Park Keun-Hong and coworkers were among the pioneers in preparing nanogels from pullulan and biotin (PU/Bio) as a valuable method to deliver anticancer drugs using specific receptor-mediated targeting between biotin and tumor cells.[35] In the last years, more and more evidences point on the effectiveness in using biotin as a drug targeting molecule.[26, 36, 37] Moreover, biotin does not bind to plasmatic protein unless in very small amount.[38] In this way, we would not substantially modify the plasmatic behaviors of INU, while modifying the hydrodynamic properties of the polymer.

These premises, led us to hypothesize that a drug delivery system composed by INU-based micelles would retain the main behaviors of the parent polymer, since the external shell of the micelle would be chemically composed by the polysaccharide and, eventually, by the non plasma-protein binder biotin. What would be modified should essentially be, the spatial conformation of the polymer especially when BIO is found on the surface of the micelle. Since glomerular filtration is strongly influenced by size and shape of the substances we thought that such a system, based on INU, would "acquire" long-circulation behaviors to be exploited for drug delivery purposes.

Thus, based on the mentioned rational design, herein we investigated the amphiphilic inulin-vitamin E (INVITE) bioconjugate, surface modified with biotin (INVITE-BIO), as specific carriers with long-circulating and targeting behaviors. In particular, the synthesis and characterization of INVITE-BIO nanomicelles are described in this paper. Moreover, the fate of the targeted micelles was monitored *in vitro* on Caco-2 cells as well as *in vivo* by optical imaging biodistribution studies.

## 2. Methods

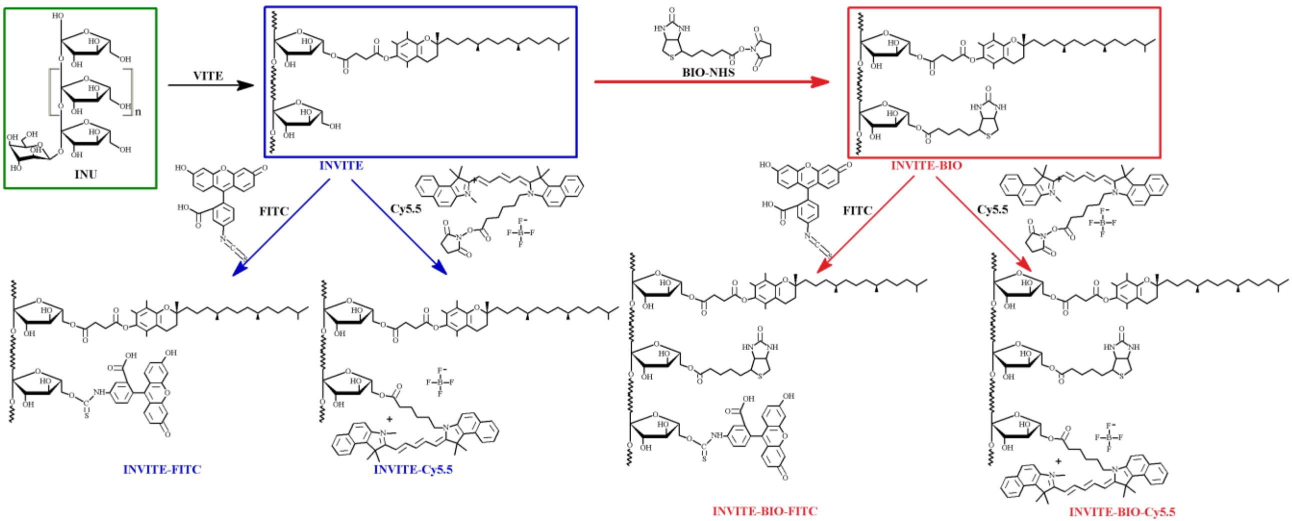
### 2.1 Materials and cell lines

All reagents were of analytical grade, unless otherwise stated. Anhydrous *N*,*N*-dimethyl formamide 99.8 % (DMF), triethylamine ≥99 % (TEA), N,N′-dicyclohexyl carbodiimide 99 % (DCC), pyrene, d-α-tocopherol succinate semisynthetic 1210 IU/g, inulin from dahlia tubers (INU, approx. 5500 Da), fluorescein 5-isothiocyanate (FITC) and *N*-Hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich (Milan, Italy). *N*-hydroxysulfosuccinimide sodium salt ≥98 % (NHSS), biotin and DMSO-d6 99.96 atom % D were purchased from TCI Europe, Zwijndrecht, Belgium. Cyanine5.5 NHS ester (Cy5.5) was from Lumiprobe, Hallandale Beach, USA. Caco-2 cells (Caco-2 Passage 43) were obtained from the European Collection of Authenticated Cell Cultures Cell Bank (ECACC, Salisbury, UK). All reagents used for cell cultures were purchased from Eurcolone (Milan, Italy). Fetal bovine serum were obtained from Hyclone (GE Healthcare, Milan, Italy).

## 3. Results

### 3.1 Synthesis and characterization of INVITE and INVITE-BIO and their derivatives

In this work we synthesized and characterized five new INVITE derivatives plus one intermediate, namely we synthesized: i) FITC derivative of INVITE to be used for *in vitro* transport studies on Caco-2 cell monolayer, named INVITE-FITC, ii) Cy5.5 derivative of INVITE to be used for *in vivo* biodistribution studies by NIR fluorescence imaging, named INVITE-Cy5.5, iii) *N*-hydroxysuccinimide ester of biotin for subsequent conjugation to INVITE, named BIO-NHS, iv) biotin derivative of INVITE to be used for cytotoxicity studies on Caco-2 cells, named INVITE-BIO v) FITC derivative of INVITE-BIO to be used for *in vitro* transport studies on Caco-2 cell monolayer, named INVITE-BIO-FITC, and vi) Cy5.5 derivative of INVITE-BIO to be used for *in vivo* biodistribution studies by NIR fluorescence imaging, named INVITE-BIO-Cy5.5. The synthetic pathways followed by us are summarized in Scheme 1. All the new molecules were characterized by $^{1}$H-NMR (performed for all the samples), $^{13}$C-NMR (INVITE, INVITE-BIO), CAC (INVITE, INVITE-BIO), SEM (INVITE-BIO), elemental analysis for sulphur detection by SEM (INVITE, INVITE-BIO). Purifications were performed by washing the reaction product in selected solvents and by further exhaustive dialysis. Furthermore, as shown below, due to the nanometric dimension of the obtained micelles, sterile filtration has been used throughout *in vitro*/*in vivo* studies.




**Scheme 1.** Schematic representation of the synthesis performed in the present work for the production of INVITE, INVITE-FITC, INVITE-Cy5.5, INVITE-BIO,INVITE-BIO-FITC and INVITE-BIO-Cy5.5.

The synthesis of INVITE-BIO proceeded through the isolation of the *N*-hydroxysuccinimide ester of biotin which was characterized by $^{1}$H-NMR and melting point confirming identity and purity of the sample (see SI1 for $^{1}$H-NMR of BIO-NHS). INVITE polymer, in the presence of TEA, freely and quantitatively reacted with the synthesized BIO-NHS. The NMR ($^{1}$H, $^{13}$C) spectra were acquired after exhaustive dialysis to assure high purity of the samples. $^{1}$H-NMR spectrum of the synthesized INVITE-BIO is shown in Figure 1.

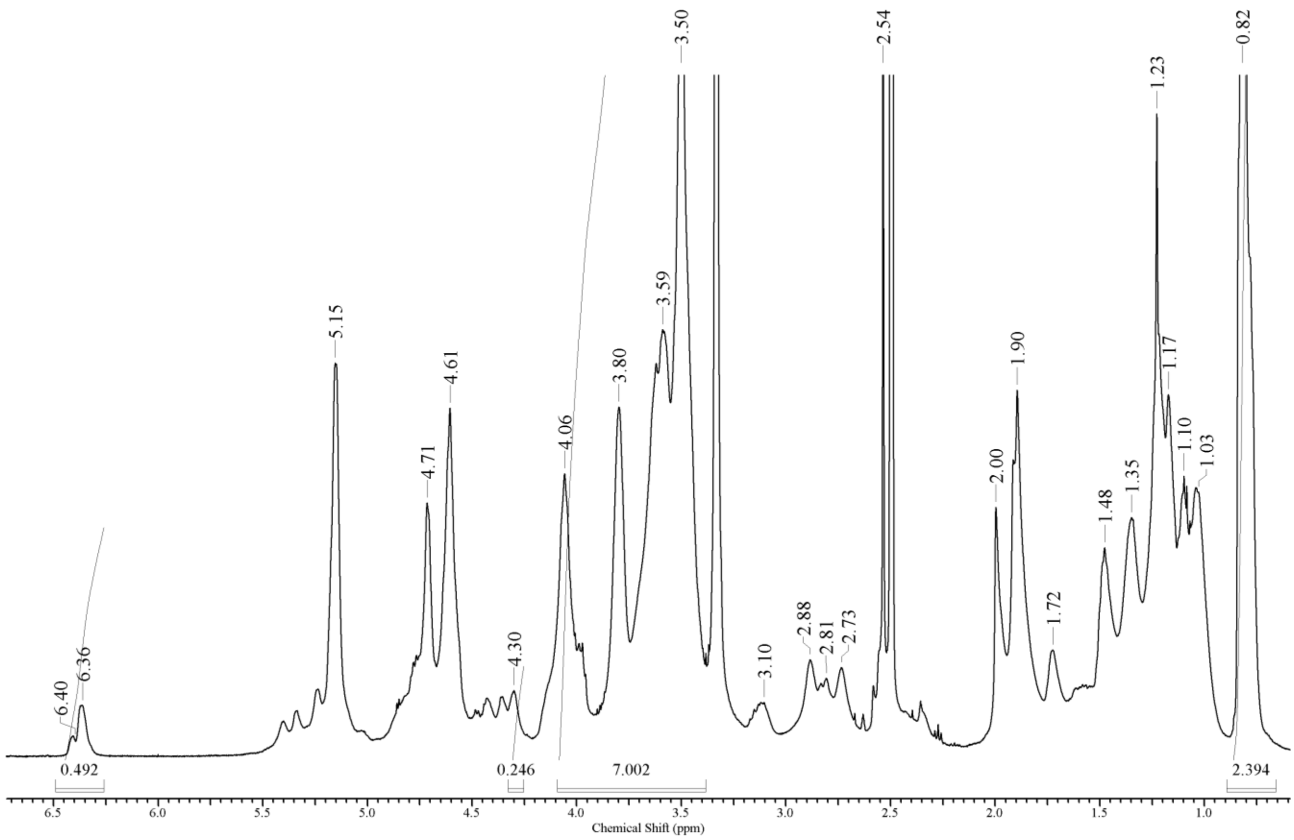



**Figure 1.** $^{1}$H-NMR spectrum, registered in DMSO-$d_6$, of the synthesized INVITE-BIO polymer: integrals values are shown for the peaks at ppm 6.40/6.36 (2N**H** BIO), 4.30 (1**H** BIO), 4.06-3.50 (7**H** INU) and 0.82 (12**H** VITE), please see experimental part for full peak attribution.

The peaks at ppm 6.40 (s, 1H, N**H**, BIO), 6.36 (s, 1H, N**H**, BIO) and 4.30 (s, 1H, -NH-**CH**-CH-, ring BIO) were used to identify BIO in the conjugate and to calculate the DD. In particular, the peak integrals at ppm 6.40 and 6.36 relative to the two N**H** protons in BIO ring were used to confirm the DD value obtained by comparing the integral of the proton of BIO at ppm 4.30 with the integral from the 7 protons belonging to Inulin ring. This additional measure was necessary due to the overlapping of the peak at ppm 4.30 with some from INU. A further confirmation of the chemical conjugation was from $^{13}$C-NMR study which shown a significant upfield shift of the carbonyl carbon at ppm 174.96 of free carboxyl group from BIO to ppm 173.98 for carbonyl carbon of the new-formed ester in INVITE-BIO (see SI2). A similar outcome arises from FT-IR studies (see SI3-4).

Critical aggregation concentration (CAC) was determined to evaluate the effect of the BIO pendant group on the aggregation ability of INVITE which in turn has demonstrated CAC values as low as a $10^{-3}$ magnitude in mM. The CAC value for INVITE-BIO resulted $1.2 \cdot 10^{-3}$ mM, predicting a good stability at dilution for the synthesized system in aqueous environment, Table 1. Moreover, it indicates that BIO does not influence the INVITE hydrophobic core, likewise because BIO could be found on the nanomicelle surface. This result is of notable importance since the recognition of the micelles by specific receptors for BIO could occur only if BIO is exposed to the environment. To confirm this assumption, an elemental analysis of the nanomicelles surface was performed by the SEM equipment provided with an energy dispersive X-Ray detector. The results, which should be considered of qualitative nature, showed that no sulphur occurs on INVITE micelles, while a detectable percentage was observed on the surface of INVITE-BIO micelle.

**Table1.** Physical-chemical characterization of INVITE and INVITE-BIO polymers

| Sample | Mw ($^1$H-NMR) Da* | Size (nm) ± sd | CAC (mM) |
|---|---|---|---|
| **INVITE** | 8148 | 8.7 ± 0.6 | $8.5 \cdot 10^{-3}$ |
| **INVITE-BIO** | 9874 | 11.2 ± 1.2 | $1.2 \cdot 10^{-3}$ |

*INU Mw 5000 Da

SEM studies allowed to establish both morphology and size range of the micelles, Figure 2. The number-weighted size distribution for empty INVITE and loaded micelles has been measured by dynamic light scattering (DLS) which shown a mean diameter size of 11.2 ± 1.2 nm and a polydispersity index of 0.38.

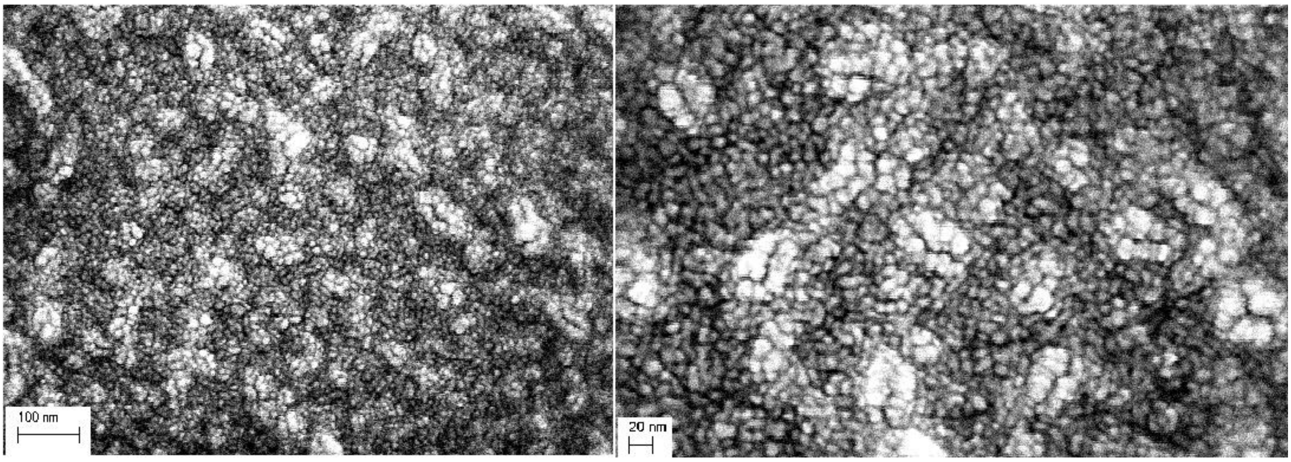



**Figure 2.** SEM microphotograph of INVITE-BIO micelles at the dry state: left 100Kx, right 200Kx.

From the SEM microphotograph it was evident a round shape of the micelles and it confirmed a particle size of about 10 nm. Obviously, being the measurement performed at the dry state, no discrete micelle could be seen but it is clear the presence of single structures. As summarized in Scheme 1, different INVITE and INVITE-BIO derivatives have been synthesized in order to broadly characterize the obtained product by *in vitro/in vivo* studies. The main $^1$H-NMR data for the identification and DD calculation relative to the obtained conjugates have been summarized in Table 2.

**Table 2**. Main diagnostic peaks from $^1$H-NMR analysis for each INVITE derivative, the peak integral used for degree of derivatization (with respect to INU fructose protons) and found DD vs theoretic one.

| Sample | Diagnostic peaks (ppm)$^{\Delta}$ | Integral for DD (peak ppm)$^{*}$ | Found DD % (Theoretic) |
|---|---|---|---|
| INVITE | 2.89, 2.71, 1.99, 1.88, 1.72, 1.48, 1.33, 1.21, 1.04, 0.81 | 0.81 | 19.9 ± 0.90 (20.0) |
| INVITE-FITC | 8.00, 7.68, 7.12-7.18, 6.84 | 8.00 | 0.97± 0.05 (1.00) |
| INVITE-Cy5.5 | 8.43, 8.23, 8.05, 7.71, 7.48, 1.95 | 8.05 | 1.95±0.10 (2.00) |
| INVITE-BIO | 6.40, 6.36, 4.30, 3.10, 2.81/2.58 | 4.30/6.40/6.36 | 24.6 ± 1.20 (25.0) |
| INVITE-BIO-FITC | 7.99, 7.66, 7.13-7.17, 6.85 | 7.99 | 0.99± 0.05 (1.00) |
| INVITE-BIO-Cy5.5 | 8.43, 8.23, 8.05, 7.71, 7.48, 1.95 | 8.05 | 2.01±0.10 (2.00) |

$^{\Delta}$ Diagnostic peaks are referred to the substituent, i.e., VITE, FITC, Cy5.5 or BIO.
$^{*}$ The ppm indicates the peak which integral has been related to the integral relative to the 7 protons of INU fructose backbone.

It is important to underline that the amount of Cy5.5 used for the reactions is calculated to administrate an established dose of NIR probe for each animal. In particular, it has been calculated that one molecule of Cy5.5 should be present for each micelle. This assumption arises from the fact that each micelle is composed of 2-3 INVITE chains [39] and every chain contains ≈ 30.86 of fructose repeating units (degree of polymerization). It means that a single micelle will contain ≈ 62-93 fructose repeating units so a DD at around 2 % (2 Cy5.5 every 100 RU of fructose) should assure, statistically, one molecule of Cy5.5 for each micelle.

### 3.2 Biological *in vitro* characterization of INVITE and INVITE-BIO

The *in vitro* cytotoxicity of INVITE-BIO micelles was evaluated by MTT test using Caco-2 cells up to 24 h of incubation. INVITE-BIO micelles did not show any appreciable cytotoxicity (see SI5). On the other side, these findings confirm our previous data on fibroblasts, mesenchymal stromal cells and erythrocytes with respect to INVITE micelles.[3-5]

To perform the transport experiments on Caco-2 cells, INVITE-BIO was functionalized with FITC. At the same time also INVITE micelles were functionalized with FITC to measure their crossing by fluorescence measurement in the different media and to compare two similar systems, one containing BIO and one in which such targeting moiety is missing.

Here, we performed transport experiments in both directions, i.e., apical to basolateral (AB) and basolateral to apical (BA), sampling from both sides, i.e., from donor or acceptor compartment. In all the performed experiments the sample concentration was maintained above CAC.

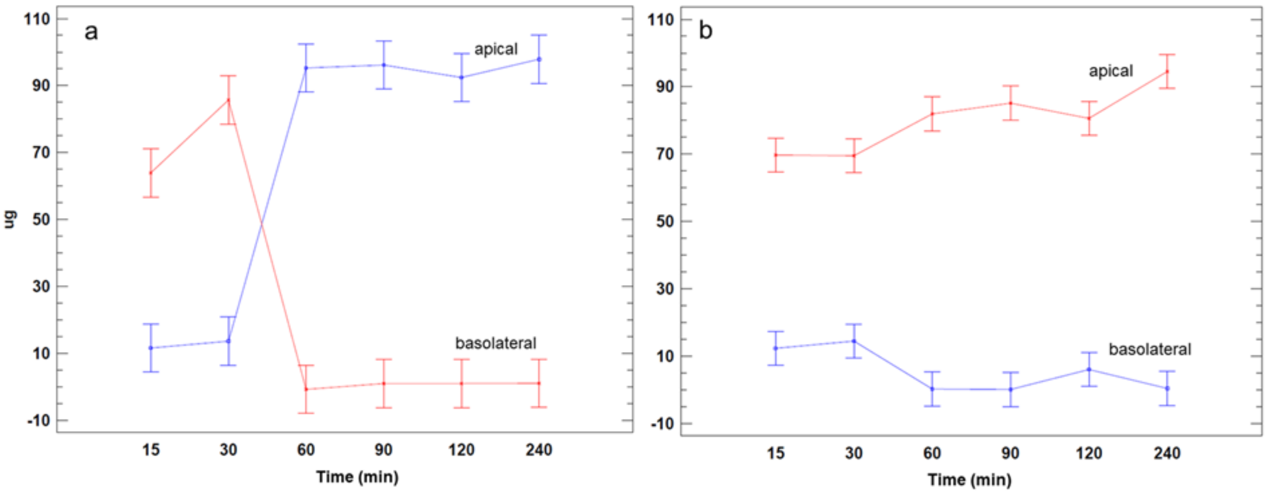



**Figure 3.** Transport study on Caco-2 cells of INVITE-BIO-FITC a) sample was loaded in the apical side and the sampling was performed in both apical (blue) and basolateral (red) side up to 240 min b) sample was loaded in the basolateral side and the sampling was performed in both apical (red) and basolateral (blue) side up to 240 min.

The results obtained from such transport study, clearly indicated that within 30 min the whole amount of fluorescein-labelled INVITE-BIO micelles were transported from the apical side (AP) to the basolateral one (BL) when the INVITE-BIO-FITC micelles were loaded in the AP compartment, *i.e.*, the micelles cross "freely" the cell-monolayer, Figure 3a and b. After 30 min, the same amount of micelles is re-transported in the opposite direction BL to AP, Figure 3a. When INVITE-BIO-FITC micelles were loaded in the BL compartment no transport was detected, Figure 3b.

To clearly attribute any effect to the presence of BIO on the surface of the micelles, INVITE-FITC micelles instead of INVITE-BIO-FITC, were used for the same transport study (Figure 4 a and b).

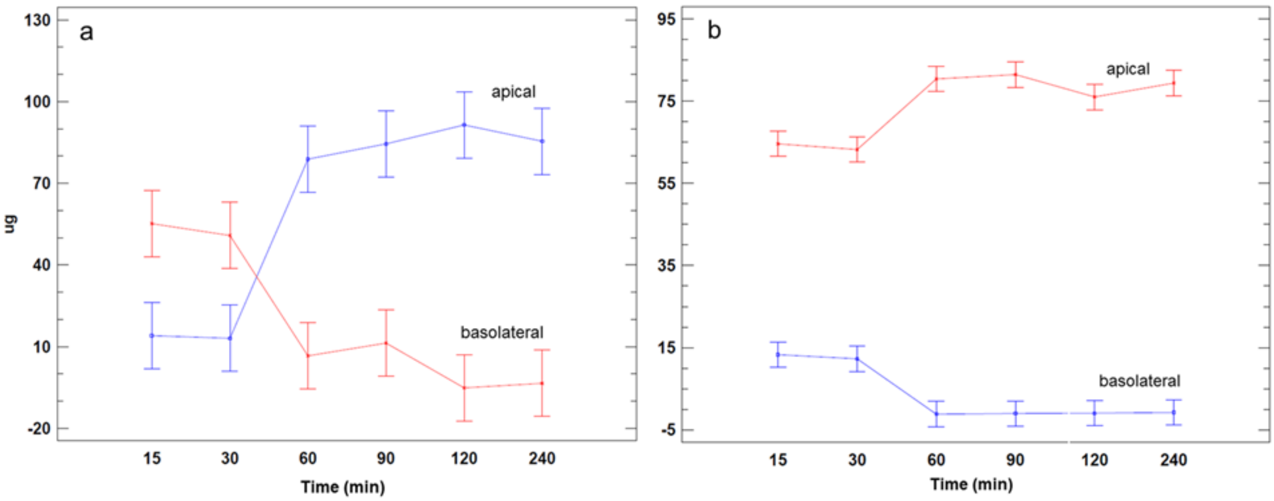



**Figure 4.** Transport study on Caco-2 cells of INVITE- FITC a) sample was loaded in the apical side and the sampling was performed in both apical (blue) and basolateral (red) side up to 240 min b) sample was loaded in the basolateral side and the sampling was performed in both apical (red) and basolateral (blue) side up to 240 min.

As seen for INVITE-BIO-FITC, even INVITE-FITC showed a fast crossing of Caco-2 monolayer from AP to BL with a complete efflux BL to AP after 30 min. On the other side, when INVITE-FITC was loaded in the BL compartment, no crossing was evidenced.

### 3.3 Biodistribution analysis of INVITE-BIO and INVITE in BALB/c mice by optical imaging studies.

INVITE polymers were chemically conjugated to an established amount of Cy5.5 probe to perform optical imaging studies (see SI6-7). The *in vivo* biodistributions of Cy5.5-labeled INVITE and INVITO-BIO micelles were assessed in BALB/c mice after oral or i.v. administration.

As from figure 5, when the INVITE or INVITE-BIO micelles were administered intravenously, their presence in the whole body was detected at least up to 48 h.

Furthermore, the quantitative determination of the residual fluorescence, indicated that almost the whole amount was still in the body throughout the period of analysis and, after an initial very rapid phase of hepatic accumulation, it appeared homogeneously distributed in all body compartments.

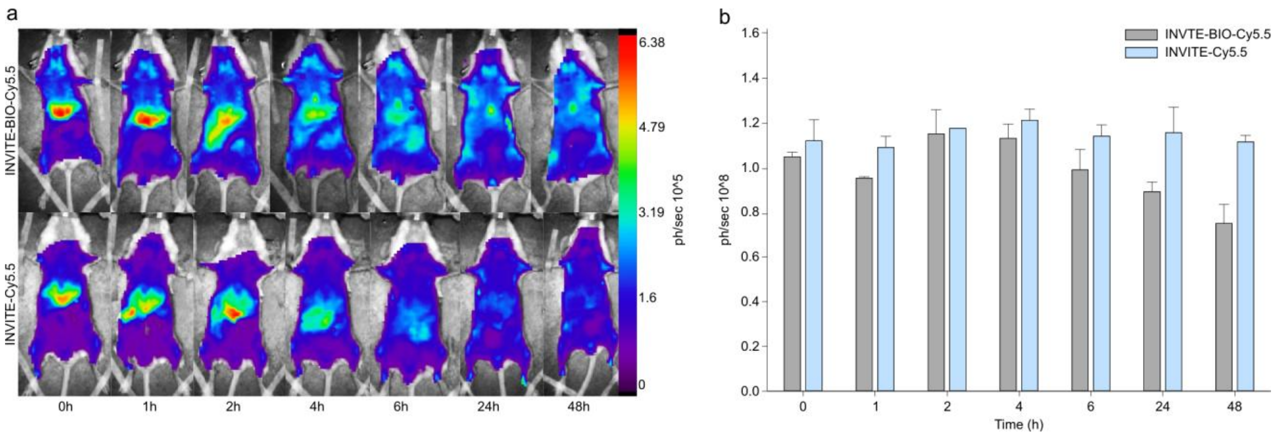



**Figure 5.** Biodistribution analysis of INVITE-BIO and INVITE in BALB/c mice. (a) Mice were injected i.v. with Cy5.5-labeled-INVITE-BIO (upper panels) or Cy5.5-labeled-INVITE (lower panels). Biodistribution kinetics of the compounds was assessed by fluorescence optical imaging as total body scanning with a 670 nm laser and a 693LP filter; spatial resolution/scan step was fixed at 1.5 mm, exposure time was 0.5 seconds, and laser power was automatically adjusted for each scan session. The figure shows one representative experiment of two that produced similar results. (b) The vertical histograms represent the total photons emitted from ROI of the total body. Data are expressed as photon flux and quantified as photon × second$^{-1}$. Two mice per group were analyzed and data are reported as mean ± S.D.

On the other hand, when the biodistribution of Cy5.5-labeled INVITE and INVITE-BIO was studied upon oral administration, the fluorescent signals were detected to progress along the intestinal tract of the animals, and were eliminated in 24 h, Figure 6.

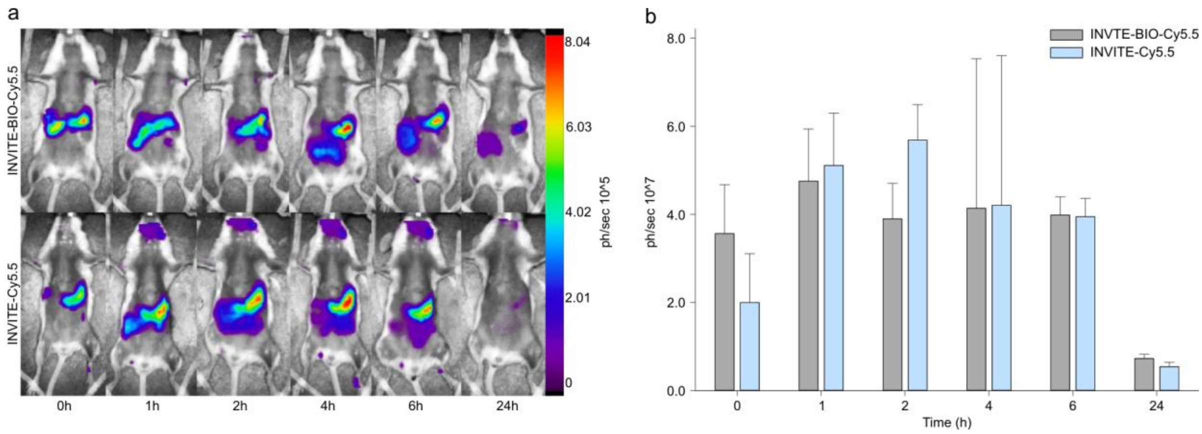



**Figure 6.** Biodistribution analysis of INVITE-BIO and INVITE in BALB/c mice. (a) Mice were administered by the oral route with Cy5.5-labeled-INVITE-BIO (upper panels) or Cy5.5-labeled-INVITE (lower panels). Biodistribution kinetics of the compounds was assessed by fluorescence optical imaging. The figure shows one representative experiment of two that produced similar results. (b) The vertical histograms refer to the total photons emission from mouse total body ROI.

Data are expressed as photon flux and quantified as photon × second$^{-1}$. Two mice per group were analyzed and data are expressed as means ± S.D.

## 4. Discussion

The preparation of long-circulating nanocarriers for drug delivery purposes is often challenging, mostly because a great number of colloidal delivery systems are rapidly cleared from the blood stream when intravenously injected.[40] Thus, upon intravenous injection, nanoparticulate systems could be rapidly cleared from the blood (even within few minutes) by the reticulum endothelial system (RES) and, particularly, by the hepatic Kupffer cells.[40, 41] Among the adopted strategies to overcome nanoparticles clearance, their surface functionalization with sterically stabilizing macromolecules such as PEG is one of the most applied. Despite the effectiveness of the approach, it requires at least an additional chemical step for the functionalization and involves a synthetic polymer within the structure of the nanosystem which could be or not composed by biodegradable substances. Without any doubt, building up a nanoparticulate system with “native” stealth behaviours is a desirable goal.

In 2001, Moghimi et al re-proposed, in a review article, a sentence from Aristotele: “If one way be better than another, you may be sure it is nature's way”, further stating that: “it seems appropriate to design a long-circulating carrier based on nature's principles”.[42] This is exactly what we are aiming to; the modification of natural substances to work as they are designed. Thus, we started from the experimental evidences which demonstrate that INU, as well as BIO, does not bind to plasmatic proteins.[16-18, 38] Furthermore, INU is freely filtered by the kidney and is neither reabsorbed nor secreted in the urinary tract. Supported by these data, we designed to use biotinylated (targeted), or not, INU-based nanomicelles to escape the immune system of the blood (i.e., RES) and to avoid plasma protein binding. At the same time, we thought that modifying size and shape of native INU by including it in a biotinylated micelle system would reduce, if not avoid, the glomerular filtration of the substance so working as a reservoir/long-circulating/targeted drug delivery systems. With these aims in mind, INU was functionalized with Vitamin-E (VITE) to gain the amphiphilic polymer INVITE which, in turn, was further functionalized with Biotin (BIO) to provide the system with tumor-targeting moieties and to evaluate the effect of BIO on the circulating behaviours of the carrier. To test the efficacy of INVITE and INVITE-BIO micelles were conjugated also with various molecules including different fluorescent probes.

Since Caco-2 cells are from human epithelial colorectal adenocarcinoma, in our study, they were used as a cellular model to simulate the oral absorption. In fact, Caco-2 cells, are often used to simulate the gut epithelial cells and are usually grown in monolayers in single-cell culture or in the so-called Transwell® system which allows to simulate the transport (or passage) of drugs or nanoparticulate across the epithelial layer. [43]

In our study, Caco-2 cells were chosen to evaluate the possibility of oral administration of INVITE-BIO micelles and because of the expression of the receptor for biotin on their surface.[44, 45] Several papers in literature report on strongly increased transport rate of biotinylated molecules in the gut.[30, 46] Thus, it has been supposed that the presence of biotin on the micelle surface should increase the transport rate of the micelles with respect to BIO un-decorated micelles when orally administered.[47]

In general, it is accepted that substances that are transported by passive diffusion, usually show a comparable permeability in both directions in the Caco-2 model.[48] Based on these findings, an active transport P-glycoprotein (Pgp)-mediated efflux seems as the most probable mechanism involved in the case examined. This hypothesis is supported by at least two reasons i) no passive (concentration) equilibrium is reached by the system in both directions and this would explain the active transport of the micelles, ii) the efflux is active (again no equilibrium) but induced (activated) by AP to BL passage, thus, it supports the Pgp activation, only in one direction, Figure 3.

To clearly attribute any effect to the presence of BIO on the surface of the micelles, INVITE-FITC micelles instead of INVITE-BIO-FITC, were used for the same transport study (Figure 4 a and b).

As seen in Figure 3 and 4, the amount of INVITE-BIO-FITC crossed at time 30 min resulted almost 50 % higher than INVITE-FITC. This result should be linked to the presence of BIO on the INVITE-BIO micelles surface.

Different studies in literature could help in understanding the main gained outcomes. Thus, for example, previously, it has been reported that Caco-2 cells internalized labelled lactoferrin only from the apical membrane side, but not from the basolateral side, so suggesting that Caco-2 cells expressed the lactoferrin receptor in the AP side, and that the cellular membrane of the cells bound lactoferrin specifically.[44] It should be noted that lactoferrin is a glycoprotein which oligosaccharide portion is often involved in their recognition. It is also known that different substances show opposite behaviors in terms of flow direction, i.e., BL to AP transport is strongly supported and this is indicative of secretion of the substances from the cells into the lumen.[48] Francis and co-worker found that dextran and hydroxypropylcellulose hydrophobically modified by polyoxyethylene cetyl ether, shown a Caco-2 cell permeability higher in the BL-AP direction, compared to the AP-BL permeability[49]; these results are similar to other findings obtained on hydrophilic dendrimers.[50] In our system it is clear that the efflux mechanism is not influencing the micelles uptake in the early stage of the experiment and it is known that if inadequate concentration is achievable in the donor compartment for the detection of transported solute into the receiver phase, the experiment may not be concluded, but this is not our circumstance.[51] Furthermore, in the case of micellar system, it is broadly demonstrated that amphiphilic molecules and macromolecules are often substrate for the efflux pump Pgp when in the monomer form, *i.e.* below their CMC/CAC, while do not show P-gp inhibition effect when in the "micellear" form. Of course Pgp, has to be considered as the main pump in the substances' efflux and its inhibition causes the block of the efflux itself.[51, 52]

In the absence of specific Pgp inhibitors, it could be supposed that the INVITE based micelles are taken-up by Caco-2 cells by an active mechanism which acts only in the apical side of the monolayer determining the efflux of the micelles in the BL-AP direction after 30 min incubation. The presence of BIO on the micelles surface may stress this mechanism in terms of an increased amount of micelles taken-up.

On the contrary, the micelles did not activate any active mechanism when loaded in the BL compartment nor activate the efflux pump. No passive diffusion could be evidenced in both directions. So, it could be postulated that the presence of BIO on the micelle surface: i) increases the amount of taken-up micelles, ii) the micelles are subjected to an active transport, iii) no passive diffusion is detected, iv) the micelles activate an efflux mechanism uniquely in the direction AP-BL and v) no transport nor passive diffusion could be appreciated in the BL-AP direction. As for the transport mechanism of polymeric micelles across Caco-2 cells monolayer only few investigations have been reported in the literature on this topic which deserves to be thoroughly studied in future studies.[53] Based on the reported conclusions, the transport of polymeric micelles occurs via endocytosis by transcellular pathway rather than the paracellular one.[53]

*In vivo* studies demonstrated that, after intravenous administration, the micelles were not eliminated from the body up to 48 h, so supporting the premises of our work, Figure 5. The micellar systems we used in this study are mainly constituted by INU which could be found in the outer shell of the micelles which are nanometrically sized. It could be postulated that after intravenous administration the micelles do not bind to plasmatic proteins and, since they are really small in dimension, could spread in the whole body including the capillary system. On the other side, the filtration by the kidney could be impaired due to the different hydrodynamic size of the polymer when constituting the micelle with respect to the INU in its "natural" linear form. It should be remembered that filtration is a "mechanical" process and plasmatic protein or antibody binding are "chemical" processes. Consequently, being the chemical properties of INU not altered even when BIO is used to decorate the micelles, it could retain its main behaviours, while the mechanical properties could be altered by the different conformation of the polymer. Considering the long permanence time, the possible payload of the prepared drug delivery systems could be released by following established and predictable kinetics. Notably, even 48h post-injection, animals did not show any appreciable sign of toxicity. An independent study was performed to verify the presence of the INVITE-Cy5.5 nanomicelles up to 72 h (see SI8). This study confirmed that the nanomicelles are still distributed in the body up to the tested time and that the intensity of the signal is comparable to that at 48 h.

In the literature some examples can be found of polysaccharide based micelles which have been studied for their biodistribution *in vivo.* For example, in a recent study from Zhang et al., pH-responsive dextran-g-poly(lactide-co-glycolide)-g-histidine copolymer micelles, were studied for the intracellular delivery of paclitaxel and intravenously administered in mice. The study was performed by NIR fluorescence and the animals were sacrificed after 24 h.[54] Another system based on chitosan micelles showed a remarkable tumor targeting with limited body diffusion.[55] Another polysaccharide of great pharmaceutical interest is hyaluronic acid (HA) [56] which has been also studied in the form of micelles for drug targeting and delivery. In a recent study, Thomas et al., described the use of HA micelles for targeting CD44 overexpression in cancer cells. They found that after intravenous administration in mice, the micelle system was distributed in the body up to 5 days.[57]

On the other side, no systemic absorption was detected upon oral administration. This last finding well fits the transport studies, suggesting that Pgp activation determines an important efflux of the micelles as seen in the Caco-2 cell line. On the other side, at 24 h small amount of fluorescence could be detected, thus, it could be proposed the use of the INVITE based micelles for the oral delivery of highly hydrophobic drugs also considering that INU is specifically degraded by the microflora found into the colon.

## 5. Conclusions

Rationally designed nanomicelles based on inulin and vitamin-E, surface modified with biotin were prepared and investigated in the present study. In particular, the synthetic procedures from INVITE to INVITE-BIO nanocarriers and their fluorescent derivatives resulted efficient and reproducible. A deep *in vitro*/*in vivo* characterization of the resulting micelles was performed to better understand the fate of the proposed system after their administration. *In vitro* studies on Caco-2 cells indicated that the biotin-surface-modified micelles transport was faster than INVITE micelles (without biotin), but both stimulated the efflux protein Pgp. MTT test did not reveal any cytotoxicity of both INVITE or INVITE-BIO micelles after 24 h of incubation with the cells. Finally, *in vivo* optical imaging studies evidenced that, when intravenously administered, the micelles where quantitatively present in the body up to 48 h. After oral administration, due to Pgp efflux pump activation, the micelles were not found in the systemic circulation but were likely eliminated with the normal intestinal content. Therefore, it can be concluded that intravenously administered biotin conjugated INVITE polymeric micelles as long-circulating drug delivery systems may increase drug accumulation in tumor cells over-expressing the receptor for biotin through receptor mediated endocytosis. In particular, INVITE-BIO micelles should be promising nanocarriers for targeted drug delivery of lipophilic antitumor drug (*e.g*. paclitaxel) in the treatment of tumors expressing the biotin receptor.

**Appendix A. Supplementary data**

Supplementary data to this article can be found online at…(see attached file at submission)

## References

[1] V.P. Torchilin, Structure and design of polymeric surfactant-based drug delivery systems, Journal of Controlled Release, 73 (2001) 137-172.

[2] D. Mandracchia, A. Trapani, T. Chlapanidas, G. Trapani, G. Tripodo, Enzyme controlled release of celecoxib from inulin based nanomicelles, Journal of Cellular Biotechnology, 1 (2015) 107-118.

[3] G. Tripodo, G. Pasut, A. Trapani, A. Mero, F.M. Lasorsa, T. Chlapanidas, G. Trapani, D. Mandracchia, Inulin-D-alpha-Tocopherol Succinate (INVITE) Nanomicelles as a Platform for Effective Intravenous Administration of Curcumin, Biomacromolecules, 16 (2015) 550-557.

[4] G. Tripodo, T. Chlapanidas, S. Perteghella, B. Vigani, D. Mandracchia, A. Trapani, M. Galuzzi, M.C. Tosca, B. Antonioli, P. Gaetani, M. Marazzi, M.L. Torre, Mesenchymal stromal cells loading curcumin-INVITE-micelles: A drug delivery system for neurodegenerative diseases, Colloids and Surfaces B-Biointerfaces, 125 (2015) 300-308.

[5] G. Tripodo, D. Mandracchia, R. Dorati, A. Latrofa, I. Genta, B. Conti, Nanostructured Polymeric Functional Micelles for Drug Delivery Applications, Macromolecular Symposia, 334 (2013) 17-23.

[6] G. Tripodo, G. Pitarresi, F.S. Palumbo, E.F. Craparo, G. Giammona, UV-photocrosslinking of inulin derivatives to produce hydrogels for drug delivery application, Macromolecular Bioscience, 5 (2005) 1074-1084.

[7] G. Pitarresi, G. Tripodo, R. Calabrese, E.F. Craparo, M. Licciardi, G. Giarnmona, Hydrogels for Potential Colon Drug Release by Thiol-ene Conjugate Addition of a New Inulin Derivative, Macromolecular Bioscience, 8 (2008) 891-902.

[8] G. Pitarresi, G. Tripodo, D. Triolo, C. Fiorica, G. Giammona, Inulin vinyl sulfone derivative cross-linked with bis-amino PEG: new materials for biomedical applications, Journal of Drug Delivery Science and Technology, 19 (2009) 419-423.

[9] G. Tripodo, G. Pitarresi, G. Cavallaro, F.S. Palumbo, G. Giammona, Controlled Release of IgG by Novel UV Induced Polysaccharide/Poly(amino acid) Hydrogels, Macromolecular Bioscience, 9 (2009) 393-401.

[10] D. Mandracchia, N. Denora, M. Franco, G. Pitarresi, G. Giammona, G. Trapani, New Biodegradable Hydrogels Based on Inulin and alpha,beta-Polyaspartylhydrazide Designed for Colonic Drug Delivery: In Vitro Release of Glutathione and Oxytocin, Journal of Biomaterials Science-Polymer Edition, 22 (2011) 313-328.

[11] M. Licciardi, C. Scialabba, C. Sardo, G. Cavallaro, G. Giammona, Amphiphilic inulin graft co-polymers as self-assembling micelles for doxorubicin delivery, Journal of Materials Chemistry B, 2 (2014) 4262-4271.

[12] D. Mandracchia, G. Tripodo, A. Latrofa, R. Dorati, Amphiphilic inulin-d-alpha-tocopherol succinate (INVITE) bioconjugates for biomedical applications, Carbohydrate polymers, 103 (2014) 46-54.

[13] P. Muley, S. Kumar, F. El Kourati, S.S. Kesharwani, H. Tummala, Hydrophobically modified inulin as an amphiphilic carbohydrate polymer for micellar delivery of paclitaxel for intravenous route, International Journal of Pharmaceutics, 500 (2016) 32-41.

[14] M. Licciardi, C. Scialabba, G. Cavallaro, C. Sangregorio, E. Fantechi, G. Giammona, Cell uptake enhancement of folate targeted polymer coated magnetic nanoparticles, Journal of Biomedical Nanotechnology, 9 (2013) 949-964.

[15] G. Pitarresi, G. Tripodo, G. Cavallaro, F.S. Palumbo, G. Giammona, Inulin-iron complexes: A potential treatment of iron deficiency anaemia, European Journal of Pharmaceutics and Biopharmaceutics, 68 (2008) 267-276.

[16] E. Lentjes, K.W. Florijn, P.C. Chang, W. Vandam, INULIN MEASUREMENT IN SERUM AND URINE WITH AN AUTOANALYZER, CORRECTED FOR GLUCOSE INTERFERENCE, European Journal of Clinical Chemistry and Clinical Biochemistry, 32 (1994) 625-628.

[17] M. Nishida, M. Uechi, S. Kono, K. Harada, M. Fujiwara, Estimating glomerular filtration rate in healthy dogs using inulin without urine collection, Research in Veterinary Science, 93 (2012) 398-403.

[18] M.E. Kerl, C.R. Cook, Glomerular filtration rate and renal scintigraphy, Clinical Techniques in Small Animal Practice, 20 (2005) 31-38.

[19] M.A. Mensink, H.W. Frijlink, K.v.d.V. Maarschalk, W.L.J. Hinrichs, Inulin, a flexible oligosaccharide. II: Review of its pharmaceutical applications, Carbohydrate Polymers, 134 (2015) 418-428.

[20] Y. Zhao, F.J. Monahan, B.A. McNulty, M.J. Gibney, E.R. Gibney, Effect of vitamin E intake from food and supplement sources on plasma alpha- and gamma-tocopherol concentrations in a healthy Irish adult population, British Journal of Nutrition, 112 (2014) 1575-1585.
[21] J.M. Tucker, D.M. Townsend, Alpha-tocopherol: roles in prevention and therapy of human disease, Biomedicine & Pharmacotherapy, 59 (2005) 380-387.
[22] M.A. Thabet, J.C.M. Chan, Vitamin E in renal therapeutic regimens, Pediatric Nephrology, 21 (2006) 1790-1801.
[23] P. Chandrasekharan, D. Maity, C.X. Yong, K.-H. Chuang, J. Ding, S.-S. Feng, Vitamin E (D-alpha-tocopheryl-co-poly(ethylene glycol) 1000 succinate) micelles-superparamagnetic iron oxide nanoparticles for enhanced thermotherapy and MRI, Biomaterials, 32 (2011) 5663-5672.
[24] N. Cao, S.-S. Feng, Doxorubicin conjugated to D-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS): Conjugation chemistry, characterization, in vitro and in vivo evaluation, Biomaterials, 29 (2008) 3856-3865.
[25] W.J. Yang, Y.Y. Cheng, T.W. Xu, X.Y. Wang, L.P. Wen, Targeting cancer cells with biotin-dendrimer conjugates, European Journal of Medicinal Chemistry, 44 (2009) 862-868.
[26] S.Y. Chen, X.R. Zhao, J.Y. Chen, J. Chen, L. Kuznetsova, S.S. Wong, I. Ojima, Mechanism-Based Tumor-Targeting Drug Delivery System. Validation of Efficient Vitamin Receptor-Mediated Endocytosis and Drug Release, Bioconjugate Chemistry, 21 (2010) 979-987.
[27] J.-F. Shi, P. Wu, Z.-H. Jiang, X.-Y. Wei, Synthesis and tumor cell growth inhibitory activity of biotinylated annonaceous acetogenins, European Journal of Medicinal Chemistry, 71 (2014) 219-228.
[28] G. Russell-Jones, K. McTavish, J. McEwan, J. Rice, D. Nowotnik, Vitamin-mediated targeting as a potential mechanism to increase drug uptake by tumours, Journal of Inorganic Biochemistry, 98 (2004) 1625-1633.
[29] H.M. Said, Cell and Molecular Aspects of Human Intestinal Biotin Absorption, Journal of Nutrition, 139 (2009) 158-162.
[30] S. Ramanathan, S. Pooyan, S. Stein, P.D. Prasad, J.H. Wang, M.J. Leibowitz, V. Ganapathy, P.J. Sinko, Targeting the sodium-dependent multivitamin transporter (SMVT) for improving the oral absorption properties of a retro-inverso Tat nonapeptide, Pharmaceutical Research, 18 (2001) 950-956.
[31] T. Minko, P.V. Paranjpe, B. Qiu, A. Lalloo, R. Won, S. Stein, P.J. Sinko, Enhancing the anticancer efficacy of camptothecin using biotinylated poly(ethyleneglycol) conjugates in sensitive and multidrug-resistant human ovarian carcinoma cells, Cancer Chemotherapy and Pharmacology, 50 (2002) 143-150.
[32] L. Bildstein, C. Dubernet, P. Couvreur, Prodrug-based intracellular delivery of anticancer agents, Advanced Drug Delivery Reviews, 63 (2011) 3-23.
[33] A.D. Vadlapudi, R.K. Vadlapatla, D. Pal, A.K. Mitra, Functional and Molecular Aspects of Biotin Uptake via SMVT in Human Corneal Epithelial (HCEC) and Retinal Pigment Epithelial (D407) Cells, Aaps Journal, 14 (2012) 832-842.
[34] G. Tripodo, D. Mandracchia, S. Collina, M. Rui, D. Rossi, New perspectives in cancer therapy: the biotin-antitumor molecule conjugates, Medicinal Chemistry, S1-004 (2014).
[35] K.H. Park, D. Kang, K. Na, Physicochemical characterization and carcinoma cell interaction of self-organized nanogels prepared from polysaccharide/biotin conjugates for development of anticancer drug carrier, Journal of Microbiology and Biotechnology, 16 (2006) 1369-1376.
[36] S. Maiti, N. Park, J.H. Han, H.M. Jeon, J.H. Lee, S. Bhuniya, C. Kang, J.S. Kim, Gemcitabine-Coumarin-Biotin Conjugates: A Target Specific Theranostic Anticancer Prodrug, Journal of the American Chemical Society, 135 (2013) 4567-4572.
[37] H. Yuan, K. Luo, Y.S. Lai, Y.J. Pu, B. He, G. Wang, Y. Wu, Z.W. Gu, A Novel Poly(L-glutamic acid) Dendrimer Based Drug Delivery System with Both pH-Sensitive and Targeting Functions, Molecular Pharmaceutics, 7 (2010) 953-962.
[38] D.M. Mock, M.I. Malik, Distribution of biotin in human plasma: most of the biotin is not bound to protein, The American Journal of Clinical Nutrition, 56 (1992) 427-432.
[39] L. Catenacci, D. Mandracchia, M. Sorrenti, L. Colombo, M. Serra, G. Tripodo, In-Solution Structural Considerations by H-1 NMR and Solid-State Thermal Properties of Inulin-D-alpha-Tocopherol Succinate

(INVITE) Micelles as Drug Delivery Systems for Hydrophobic Drugs, Macromolecular Chemistry and Physics, 215 (2014) 2084-2096.
[40] S.M. Moghimi, J. Szebeni, Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties, Progress in Lipid Research, 42 (2003) 463-478.
[41] E.F. Craparo, D. Triolo, G. Pitarresi, G. Giammona, G. Cavallaro, Galactosylated micelles for a ribavirin prodrug targeting to hepatocytes, Biomacromolecules, 14 (2013) 1838-1849.
[42] S.M. Moghimi, A.C. Hunter, J.C. Murray, Long-circulating and target-specific nanoparticles: Theory to practice, Pharmacological Reviews, 53 (2001) 283-318.
[43] J.M. Gamboa, K.W. Leong, In vitro and in vivo models for the study of oral delivery of nanoparticles, Advanced Drug Delivery Reviews, 65 (2013) 800-810.
[44] K. Ashida, H. Sasaki, Y.A. Suzuki, B. Lonnerdal, Cellular internalization of lactoferrin in intestinal epithelial cells, Biometals, 17 (2004) 311-315.
[45] H.M. Said, C. Kumar, Intestinal absorption of vitamins, Current Opinion in Gastroenterology, 15 (1999) 172-176.
[46] A.D. Vadlapudi, R.K. Vadlapatla, D. Kwatra, R. Earla, S.K. Samanta, D. Pal, A.K. Mitra, Targeted lipid based drug conjugates: A novel strategy for drug delivery, International Journal of Pharmaceutics, 434 (2012) 315-324.
[47] S.Y. Chae, C.-H. Jin, H.J. Shin, Y.S. Youn, S. Lee, K.C. Lee, Preparation, characterization, and application of biotinylated and biotin-PEGylated glucagon-like peptide-1 analogues for enhanced oral delivery, Bioconjugate Chemistry, 19 (2008) 334-341.
[48] N.G.M. Schipper, T. Osterberg, U. Wrange, C. Westberg, A. Sokolowski, R. Rai, W. Young, B. Sjostrom, In vitro intestinal permeability of factor Xa inhibitors: Influence of chemical structure on passive transport and susceptibility to efflux, Pharmaceutical Research, 18 (2001) 1735-1741.
[49] M.F. Francis, M. Cristea, Y.L. Yang, F.M. Winnik, Engineering polysaccharide-based polymeric micelles to enhance permeability of cyclosporin a across Caco-2 cells, Pharmaceutical Research, 22 (2005) 209-219.
[50] M. El-Sayed, M. Ginski, C. Rhodes, H. Ghandehari, Transepithelial transport of poly(amidoamine) dendrimers across Caco-2 cell monolayers, Journal of Controlled Release, 81 (2002) 355-365.
[51] N.F.H. Ho, J. Nielsen, M. Peterson, P.S. Burton, Quantitative and Mechanistic Assessment of Model Lipophilic Drugs in Micellar Solutions in the Transport Kinetics Across MDR1-MDCK Cell Monolayers, Journal of Pharmaceutical Sciences, 105 (2016) 904-914.
[52] M.M. Nerurkar, N.F.H. Ho, P.S. Burton, T.J. Vidmar, R.T. Borchardt, Mechanistic roles of neutral surfactants on concurrent polarized and passive membrane transport of a model peptide in Caco-2 cells, Journal of Pharmaceutical Sciences, 86 (1997) 813-821.
[53] N. Li, X.-R. Li, Y.-X. Zhou, W.-J. Li, Y. Zhao, S.-J. Ma, J.-W. Li, Y.-J. Gao, Y. Liu, X.-L. Wang, D.-D. Yin, The use of polyion complex micelles to enhance the oral delivery of salmon calcitonin and transport mechanism across the intestinal epithelial barrier, Biomaterials, 33 (2012) 8881-8892.
[54] J. Zhang, K. Chen, Y. Ding, X. Xin, W. Li, M. Zhang, H. Hu, M. Qiao, X. Zhao, D. Chen, Self-assembly of pH-responsive dextran-g-poly(lactide-co-glycolide)-g-histidine copolymer micelles for intracellular delivery of paclitaxel and its antitumor activity, Rsc Advances, 6 (2016) 23693-23701.
[55] H. Chen, Y. Chen, H. Yang, W. Xu, M. Zhang, Y. Ma, S. Achilefu, Y. Gu, A dual-targeting nanocarrier based on modified chitosan micelles for tumor imaging and therapy, Polymer Chemistry, 5 (2014) 4734-4746.
[56] G. Tripodo, A. Trapani, M.L. Torre, G. Giammona, G. Trapani, D. Mandracchia, Hyaluronic acid and its derivatives in drug delivery and imaging: Recent advances and challenges, European Journal of Pharmaceutics and Biopharmaceutics, 97 (2015) 400-416.
[57] R.G. Thomas, M. Moon, S. Lee, Y.Y. Jeong, Paclitaxel loaded hyaluronic acid nanoparticles for targeted cancer therapy: In vitro and in vivo analysis, International Journal of Biological Macromolecules, 72 (2015) 510-518.

Supplementary Material

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