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Amphiphilic inulin-D-α-tocopherol succinate (INVITE) bioconjugates for biomedical applications

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ABSTRACT

Herein is reported the synthesis and characterization of innovative inulin (INU)–vitamin E succinate (VITE) bioconjugates (INVITE). The obtained amphiphilic INU-based polymers, self-assembling in nanostructures, have been thought as new drug delivery systems (DDS) for the therapy of urinary tract infections (UTI).

The synthesis of INVITE bioconjugates was carried out in bulk, without isolation of intermediate products, to reduce the amount of solvents used in the purification steps and to prevent possible VITE oxidation during work up.

Six different INVITE conjugates (INVITE 1–6) have been synthesized by varying both the relative amount of VITE with respect to INU repetitive units and the reaction temperature.

Afterwards, the ability of the new conjugates to form micelle systems, by applying two different established methods for critical aggregation concentration (CAC) evaluation, has been verified. Both methods produced similar CAC values ranging from 2.5×10^{-3} mM to 2.4×10^{-2} mM in agreement with the different degrees of derivatization shown by the INVITE 1–6 conjugates.

The mean diameter of prepared INVITE micelles, resulted in the range 24–60 nm. The size of the obtained INVITE micelles did not change as measured at different time points up to 12 days, so confirming their stability upon storage.

1. Introduction

Polymers, whether naturals, semisynthetic or synthetic are fundamental materials for the modern pharmaceutical technology. Lots of examples of polymeric drug delivery systems (DDS) such as hydrogels (Giammona et al., 2006; Mandracchia, Pitarresi, Palumbo, Carlisi, & Giammona, 2004; Pitarresi, Saiano, Cavallaro, Mandracchia, & Palumbo, 2007b; Saiano, Pitarresi, Mandracchia, & Giammona, 2005; Tripodo, Pitarresi, Cavallaro, Palumbo, & Giammona, 2009), nanoparticles (Craparo, Cavallaro, Bondi, Mandracchia, & Giammona, 2006), macromolecular bioconjugates (Palumbo, Pitarresi, Mandracchia, Tripodo, & Giammona, 2006), tissue engineered materials (Innocente et al., 2009; Nottelet et al., 2007, 2009; Palumbo et al., 2006) artificial oxygen carriers (Mandracchia et al., 2007) are present in literature. Among biopolymers, inulin (INU) is a natural polymer obtained from several plant (Jerusalem artichoke, chicory, dahlias, and dandelions) and it belongs to a class of fibers known as non-digestible oligosaccharide (ODN) that exert beneficial effects on physiological functions (Pool-Zobel, van Loo, Rowland, & Roberfroid, 2002; Roberfroid, 2007; Taper & Roberfroid, 2005).

INU has some unique behaviors: when it is intravenously administrated, it 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 these reasons INU is employed since almost 60 years for the evaluation of renal functionality (Schwartz & Furth, 2007). In fact, INU, which has a mean molecular radius of 1.5 nm and a molecular weight of approximately 5200 Da, is considered an ideal marker and the "gold standard" for measuring glomerular filtration rate (GFR). Taking into account these properties, INU could be considered as a polymer able to reach high concentrations in the urinary tract after intravenous administration.

The term urinary tract infection (UTI) is used for any infection occurring in the urinary tract. UTI is most commonly bacterial, but fungal, viral and parasitic infections can occur. These infections affect a wide range of the world population, hospitalized or not, with high prevalence among women, elderly and children and is consequently associated with high healthcare and social costs. This is why there is a great interest to find appropriate pharmaceutical approaches to solve this broad class of diseases affecting the urinary

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tract. One of the most common UTI is the infection of the bladder causing cystitis, but severe infections could occur in other parts of the urinary tract causing urosepsis, pyelonephritis, urethritis and prostatitis.

Most of the UTI are treated with broad-spectrum antimicrobial agents that often lead to antimicrobial resistance and other important side effects. A modern strategy needs a very specific antimicrobial therapy, thus, numerous challenges in the diagnosis and treatments of UTI are still open (Beveridge, Davey, Phillips, & McMurdo, 2011; Johansen et al., 2011).

Introducing vitamin E (VITE) in the therapeutic protocols resulted in beneficial effects, i.e., by reducing the urinary tract oxidative stress. This pathological condition occurs when the production of oxidants exceeds the capacity of the enzymatic and non-enzymatic antioxidant systems. Oxidative stress condition has been demonstrated in chronic kidney diseases (CKD), in hemodialysis (HD) patients, in focal segmental glomerulosclerosis (FSGS), in IgA nephropathy and during infectious diseases (Fryer, 2000; Haugen & Nath, 1999; Johansen et al., 2011; Tucker & Townsend, 2005). Clinical studies demonstrated that VITE, particularly in the form of α-tocopherol, has beneficial effects in kidney diseases with high oxidative stress (Fryer, 2000; Tucker & Townsend, 2005). Furthermore, VITE exerts most of its beneficial effects by preserving the integrity of the biological membranes, stabilizing their permeability and fluidity and preventing apoptosis due to oxidative stress (Thabet & Chan, 2006).

By a pharmaceutical point of view, VITE is a low water soluble and highly lipophilic molecule. It shows a great safety profile and is approved for injection. VITE would be an optimal candidate to form amphiphilic systems for biomedical applications, i.e., by conjugation with a hydrophilic polymer.

In the last few years, several papers on INU based DDS have been published (Mandracchia et al., 2011; Pitarresi et al., 2008; Tripodo, Pitarresi, Palumbo, Craparo, & Giammona, 2005). Interestingly, modified INU has been also employed in the preparation of amphiphilic systems for surfactant applications (Exerowa, Platikanov, Levecke, & Tadros, 2009b).

Taking into account the above considerations, the main aim of this work is to chemically functionalize INU with VITE to form an amphiphilic polymer able to self-assemble in water in micelle systems.

As known, micelles are formed when amphiphiles are placed in water at specific concentrations. They consist of an inner core of assembled hydrophobic segments capable of solubilizing lipophilic substances and an outer hydrophilic corona serving as a stabilizing interface between the hydrophobic core and the external aqueous environment.

However, the disadvantage of surfactant micelles is their rapid break down upon dilution, which can result in premature leakage of the drug and its precipitation *in situ*. These limitations of surfactant micelles as drug delivery carriers aimed the researcher to form micelles with significantly enhanced stability and solubilizing activity. The use of polymer-based micelles has gained much attention because of the high variety of available polymers, their biocompatibility, biodegradability, and the multiplicity of functional groups available for the conjugation with different molecules. Polymeric micelles are widely studied as injectable drug delivery systems (DDS) for sparingly soluble drugs such as paclitaxel, indometacin, amphotericin B, doxorubicin, and dihydrosterone, resulting in interesting drug carriers (Craparo et al., 2008; Tao, Liu, Chen, Yang, & Liu, 2012).

In literature, so far, only few surfactants based on INU or micelles based on VITE are reported (Exerowa et al., 2009b; Folmer et al., 2009; Gotchev, Exerowa, Khristov, Levecke, & Tadros, 2010; Tadros, Vandamme, Levecke, Booten, & Stevens, 2004).

In this paper, the synthesis and characterization of INU–VITE bioconjugates self-assembling in nanostructured micelles to be used in the therapy of urinary tract infections (UTI) is reported. These micelle systems should be able to incorporate a hydrophobic drug and to release it in a controlled manner.

By a chemical point of view, VITE-succinate was linked to INU by an ester bond providing the obtained amphiphilic INU based polymers with hydrolysable groups. The possible hydrolysis of the ester bond would determine the partial release of VITE or VITE-succinate (used in the synthetic procedure) that, as explained, could exert beneficial effect on human health.

These new inulin–vitamin E polymers open a new gate in the wide field of polymeric surfactants because of its industrial potential and very broad range of application.

2. Materials and methods

2.1. Materials

All reagents were of analytical grade, unless otherwise stated. *N*,*N*-dimethylformamide anhydrous 99.8% (DMF), triethylamine ≥99% (TEA), *N*,*N*′-dicyclohexylcarbodiimide 99% (DCC), curcumin from Curcuma longa (Turmeric) ≥65%, pyrene for fluorescence ≥99.0%, D-α-tocopherol succinate semisynthetic 1210 IU/g, DMSO-d6 99.96 atom % D were purchased from Sigma-Aldrich (Milano, Italy). Inulin from dahlia tubers (INU, approx. 5500 Da), *N*-hydroxysulfosuccinimide sodium salt ≥98% (NHSS) were purchased from Fluka (Milano. Italy).

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).

Fluorescence measurements were taken by using 1 cm path length quartz cuvettes in a Luminescence Spectrometer LS 55, Perkin Elmer, (Monza, Italy).

^{1}H-NMR and ^{13}C-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.

The mean size and polydispersity index (PDI) of the INVITE micelles were measured using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) after suitable dilution of concentrated bulk suspensions in demineralized water. The zeta potential, i.e. the surface charge of INVITE micelles was determined by laser Doppler velocimetry with the same instrument after dilution with KCl 1 mM.

The thermal properties of prepared INVITE conjugates were examined with a DSC 822E Mettler-Toledo instrument (Milano, Italy) with a refrigerated cooling accessory.

2.3. Synthesis of inulin-D-α-tocopherol succinate conjugates

Six inulin-D-α-tocopherol succinate conjugates, named INVITE and numbered 1-6 were synthesized. Briefly, VITE-succinate (500 mg, 1 eq) was dissolved in 4 ml of anhydrous DMF under nitrogen and DCC (2 eq) and NHSS (2 eq) were added to form the VITE–NHSS reactive ester. The activation reaction was carried out under nitrogen for 3 h. The amount of VITE was varied in order to obtain INVITE conjugates with different degrees of derivatization, according to the following molar ratio $Y = 0.1$ (INVITE 1 and 4) or $Y = 0.2$ (INVITE 2 and 5) or $Y = 0.4$ (INVITE 3 and 6) where Y indicates the molar ratio VITE/INU-repeating-units. Then, INU was dissolved

in anhydrous DMF under nitrogen and TEA, as a catalyst, was added according to the following molar ratios: $Z = 0.10$ where Z indicates the molar ratio TEA/INU-repeating-units. Then, the INU solution with TEA was added drop by drop to the VITE–NHSS solution and the reaction was carried out under nitrogen at 25 °C (INVITE 1, 2 and 3) or 40 °C (INVITE 4, 5 and 6) for 12 h. After this time, the DMF solution of INVITE was filtered-off to remove the formed dicyclohexylurea and the resulting clear solution was precipitated in a tenfold excess of acetone with respect to the reaction DMF volume. In this solvent inulin is insoluble while DCC, NHSS and VITE are freely soluble. So, the precipitation solvent was used also for the purification of the final products. In particular, the suspension of INVITE in acetone was stirred for 20 min at room temperature and then centrifuged for 10 min at 11,000 rpm/4 °C. This procedure was repeated five times for all the samples, finally, the white solid has been collected, dried under vacuum and stored under nitrogen at 4 °C until use. In literature, similar purification procedures have been successfully applied for other INU derivative (Tripodo et al., 2005; Pitarresi et al., 2008).

The six INVITE derivatives with different degrees of derivatization in VITE (named as INVITE 1–6 at 9, 16, 34, 11, 20, 37 mol%/mol% in VITE groups, respectively) were obtained and characterized by FT-IR, ^{1}H-NMR and ^{13}C-NMR spectroscopy and DSC analysis.

2.4. Evaluation of VITE oxidation during the reaction work-up and quantitative determination of VITE in INVITE conjugates by UV studies

UV studies have been performed aimed to verify the possible oxidation of VITE during the reaction work-up. Stock solutions of representative INVITE samples were prepared in distilled water or DMSO while VITE samples were prepared in DMSO. In particular, the samples INVITE 1 and 3 as representative of reactions performed at 25 °C and two different degrees of derivatization (*DD*) in VITE and INVITE 6 representative of reactions performed at 40 °C have been selected. The standard solutions of INVITE 1, 3 and 6 in water or DMSO have been used to verify the linearity of the samples at $\lambda = 290$ nm in a concentration range 0.001–1.0 mg/ml in water or DMSO. The chosen concentration for subsequent INVITE stability studies was 0.5 mg/ml. In particular, freshly synthesized samples of INVITE 1, 3 and 6 have been solubilized in N_2 degassed anhydrous DMSO at a 0.5 mg/ml concentration and the spectrum immediately measured at 25 °C in the range 320–190 nm. In the same way, VITE was solubilized in N_2 degassed anhydrous DMSO at a 0.2 mg/ml concentration (this concentration has been chosen because is almost corresponding to the amount of VITE in a 0.5 mg sample of INVITE 3) and the spectrum acquired.

The obtained absorbance values at 290 nm for each INVITE samples were also used for *DD* evaluation. With this aim a six points calibration curve in DMSO from VITE was obtained with a $R^2 = 0.9949$ in the concentration range 0.0333–0.333 mg/ml. All measurements were performed in triplicate. The *DD* % of INVITE samples as from UV studies has been calculated applying the following equation:

$$(W_{vite}/MW_{vite})/\left(\frac{(W_{ts} - ((W_{vite}/MW_{vite}) \times MWR_{invite})}{MWRU_{inu}}\right) \times 100 \quad (1)$$

where W_{vite} is VITE weight in the sample as calculated from VITE calibration curve at 290 nm, MW_{vite} is the molecular weight of VITE (530.78 Da), W_{ts} is the total weight of the starting sample (0.5 mg), $MWRU_{invite}$ is the molecular weight of INVITE repeating unit (repeating unit of INU functionalized with VITE) (674.78 Da), $MWRU_{inu}$ is the molecular weight of INU not functionalized repeating unit (162 Da).

2.5. Differential scanning calorimetry

All measurements were made in triplicate using non hermetic aluminum pans. The sample amount was 5 mg exactly weighed.

Differential scanning calorimetry (DSC) curves were obtained over a temperature range of 25–200 °C for INU and vitamin E succinate with a heating rate of 5 °C/min while to determine the T_g of INVITE conjugates the samples was heated to 160 °C with rate of 30 °C/min, then cooled to -20 °C with a cooling rate of 30 °C/min and heated again to 160 °C with rate of 30 °C/min. During measurements the sample cell was purged with nitrogen at a flow rate of 50 ml/min. The midpoint of the deflection in the heat flow versus temperature curve, referred to the second heating cycle, was taken as the T_g.

2.6. Determination of the critical association concentration (CAC)

The critical association concentration (CAC) of selected INVITE 1, INVITE 2 and INVITE 3 conjugates in ultrapure water at 25 °C was determined using two different methods in order to compare the obtained results.

The first method was by fluorescence spectroscopy using pyrene, a hydrophobic fluorescence probe that preferentially partitions into the hydrophobic core of the micelle. We used the method proposed by (Kalyanasundaram & Thomas, 1977) by considering the change in the vibronic fine structure of the pyrene emission and by monitoring the changes in the ratio of the intensities I_1 and I_3 of the first and third vibronic peaks.

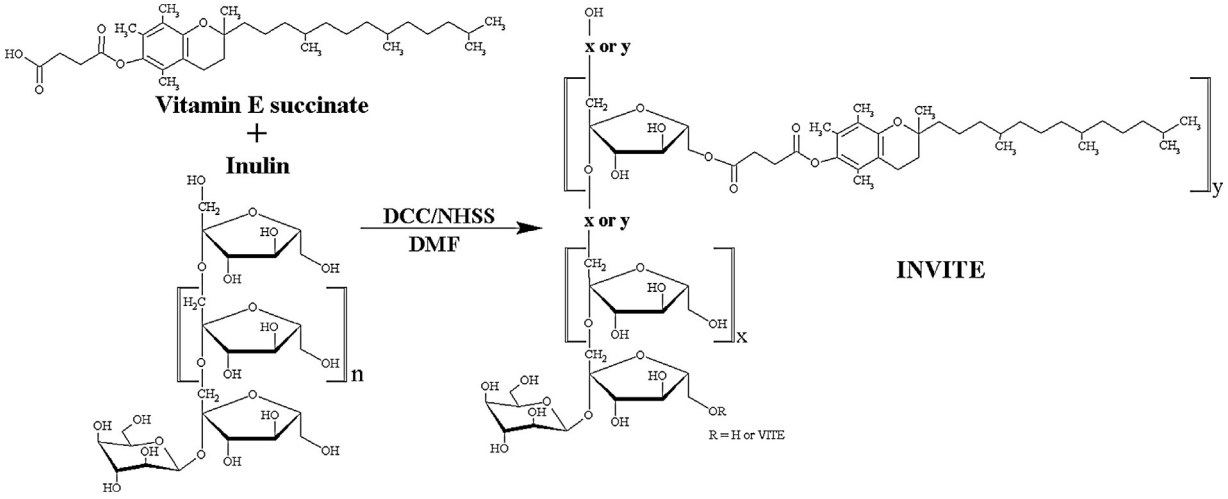
Excitation was done at 334 nm and emission were recorded in the 350–450 nm wavelength range. The slit for both excitation and emission were fixed at 5 nm. Pyrene was diluted in the INVITE samples solutions to yield a concentration of 2×10^{-6} M.

In particular, a stock solution of pyrene in acetone (6×10^{-3} M) was prepared, then, a small amount of this pyrene stock solution (83.4 μl) was diluted with water to obtain a final concentration of pyrene 2×10^{-5} M. This pyrene water solution was maintained under vigorous stirring and under nitrogen flow in order to totally remove the acetone. 100 μl of the pyrene water solution was 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 2×10^{-6} M) and was allowed to stand overnight at 37 °C 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.

The other method, concerning the use of curcumin as a probe, was recently published (Mondal & Ghosh, 2012). In particular, a stock solution of curcumin in methanol (1 mg/ml) was prepared. Then, a small amount of the curcumin stock solution (8.4 μl) was added to 2.5 ml of different INVITE water solutions at various concentrations in the range of 10^{-7}–2.5 mg/ml at 37 °C. Absorption spectra at 425 nm were recorded 25 °C for each INVITE/curcumin solutions and the absorption values were plotted as a function of different INVITE concentrations. To obtain the CAC value as mM, the found CAC values in mg/ml (by the curcumin or pyrene methods) have been divided for the molecular weights of INVITE bioconjugates that, in turn, have been calculated from DD following equation:

$$M_w = 30.86 \times \left(\frac{(MWRU_{invite} \times DD) + (MWRU_{inu} \times (100 - DD))}{100}\right) \quad (2)$$

where, 30.86 is the number of repeating unit in native INU having a M_w of 5000 Da, $MWRU_{invite}$ is the molecular weight of INVITE repeating unit (repeating unit of INU functionalized with VITE) (674.78 Da), $MWRU_{inu}$ is the molecular weight of INU not functionalized repeating unit (162 Da) and *DD* is the derivatization degree



Scheme 1. Scheme of reaction between Inulin and α-tocopherol succinate leading to INVITE conjugates.

as calculated by ^{1}H-NMR. The CAC values from the so calculated molecular weights were obtained as mmoles of macromolecules per ml (molar) and subsequently (by multiplying this value for 1000) as mM.

2.7. Physical–chemical analysis and stability studies on INVITE samples by size and zeta potential measurements

The hydrodynamic size of the INVITE 1, INVITE 2 and INVITE 3 micelles and the polydispersity index, were measured by using the Zetasizer Nano ZS instrument.

In particular, INVITE aqueous solutions at concentrations above CAC were prepared and left to equilibrate overnight at 25 °C under gently stirring. After this time, solutions were filtered by 0.45 μm filter and analyzed by Zetasizer Nano ZS instrument. The measurement were performed in triplicate for each INVITE conjugate. The solutions were further kept at 25 °C for pre-defined storage time up to 12 days to verify their physical stability, evaluated by measuring the size of the micelles after these periods of storage.

Aimed to verify the hydrolytic stability of VITE–INU ester bond in water the same samples used for the stability studies (at the same time points), after lyophilization, have been suspended in acetone, in which solvent VITE is soluble while INU is insoluble, and the amount of hydrolyzed VITE has been valued by UV studies performed on dried supernatant re-suspended in DMSO. Furthermore, supposing that the micelle system could improve the hydrolytic stability of INVITE conjugates ester bonds, the hydrolysis stability studies have been performed on INVITE samples in aqueous solution under the CAC values found for each samples.

Moreover, INVITE aqueous solutions were used to define the *Z*-potential of the micelles at 1 mg/ml supplemented with KCl 1 mM.

3. Results and discussion

3.1. Synthesis and characterization of the INVITE bioconjugates

The main idea of this paper points on the synthesis of inulin-D-α-tocopherol succinate based bioconjugates (INVITE) able to produce amphiphilic systems self-assembling in nanostructured DDS, even at low concentrations. These systems could be proposed for the drug delivery and targeting of specific drugs for the therapy of urinary tract infections. By a chemical point of view, VITE was linked to INU by exploiting the known and simple chemistry of carbodiimide by activating the carboxyl group of VITE-succinate as a reactive ester. Then, the activated molecule reacted with the hydroxyl groups of INU, most probably the primary one being the most acidic as also supported by ^{13}C-NMR studies (see later), forming the INVITE bioconjugate (Scheme 1). In view of a more ecological chemistry, no isolation of intermediate products was performed, in fact, the reactions were carried out in bulk to strongly reduce the use of solvent and energy also in consideration of possible scaling-up and industrial applications.

The obtained INVITE systems, due to their amphiphilic nature, should be able to solubilize and incorporate hydrophobic drugs while targeting them in the urinary tract.

Table 1 shows the reaction conditions for the synthesized INVITE samples, the yield and the derivatization degree. In particular, six INVITE conjugates were synthesized by maintaining constant the amount of DCC and NHSS and by varying the amount of VITE-succinate (*Y* ratio) and the temperature to evaluate the influence of these parameters on the degree of derivatization (*DD*) of the INVITE conjugates. The *DD* has been calculated by ^{1}H-NMR or by UV studies. The obtained INVITE conjugates, their nomenclature, degree of derivatization as mol%/mol% of VITE with respect to INU (*DD*) and

Table 1
Reaction conditions for the synthesis of INVITE conjugates, their nomenclature, *DD* and yield.

Sample	*Y*	*T* (°C)	Yield % (w/w)	*DD* ^{1}H-NMR (mol/mol%)	*DD* UV (mol/mol%)
INVITE 1	0.1	25	90 ± 3	9.1 ± 0.5	8.9 ± 0.3
INVITE 2	0.2	25	98 ± 5	16.4 ± 0.8	NC
INVITE 3	0.4	25	98 ± 2	34.3 ± 1.2	33.3 ± 0.7
INVITE 4	0.1	40	92 ± 6	11.4 ± 0.2	NC
INVITE 5	0.2	40	87 ± 5	20.7 ± 0.5	NC
INVITE 6	0.4	40	85 ± 2	37.2 ± 1.2	29.8 ± 1.5

NC = not calculated *Y* = moles of VITE succinate/moles of inulin repeating units *DD*% ^{1}H-NMR = degree of derivatization calculated by ^{1}H-NMR *DD*% UV = degree of derivatization calculated by UV.

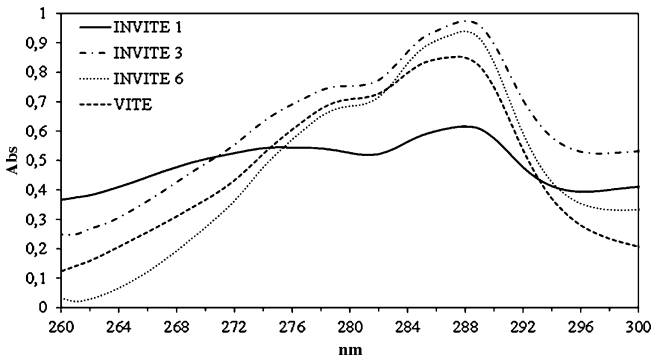


Fig. 1. Stability studies of VITE after reaction with INU by valuing the presence of the characteristic peak of VITE at 284 nm performed by UV studies.

yield (expressed as w/w % respect to starting INU) are summarized in Table 1.

As shown, all INVITE conjugates were obtained with high yield ranging from 85% to 98% w/w. The samples with the lower yield resulted the INVITE 5 and INVITE 6 corresponding to the samples synthesized at 40 °C. It could be postulated that a partial degradation of the INU backbone occurs at 40 °C leading to the formation of low molecular weight oligomers being removed during the purification steps. In this way, it should be considered that possible oligomers with a relatively high content in VITE would result more soluble in the purification solvent with respect to the polymeric INU. This assumption is also confirmed by the yield of INVITE 4 that show a yield similar to the sample obtained at 25 °C. Probably, the lower *DD* in VITE with respect to the samples INVITE 5 and 6, resulted in a low solubility in the precipitation solvent. On the other side, the *DD* of INVITE 1, 2 and 3 is very similar to the *DD* of INVITE 4, 5 and 6 and is in agreement with the stoichiometry of reaction; this result confirms that differences in terms of reaction temperature do not significantly influence the *DD* of the obtained conjugates if not in terms of final yield. Interestingly, the *DD* as calculated by UV studies is fitting with *DD* calculated by ^{1}H-NMR (Table 1).

3.2. Studies on VITE oxidation after reaction with INU

Being VITE subjected to oxidation phenomena, it is fundamental, before subsequent characterizations, to verify that VITE is not oxidized during the reaction work-up (Gruszka, Pawlak, & Kruk, 2008). With this aim, UV studies have been performed in DMSO by valuing the UV spectra of three different INVITE samples and a standard solution of VITE as shown in Fig. 1.

In literature, it is reported that the disappearance of the peak with the characteristic shoulder of VITE centered at around 284 nm is strongly indicative of the tocopherols oxidation (Gruszka et al., 2008).

As from Fig. 1, the characteristic peak of VITE resulted unaltered for all the tested samples that are well representative of all the applied reaction conditions. This result is fundamental to verify that the VITE linked to INU would maintain its main structure (antioxidant) after the reaction and purification procedures.

3.3. Thermal behaviors of INVITE conjugates

The melting point of VITE succinate resulted 76 °C. The INU thermogram shows a melting peak at 180 °C.

Table 2 shows the T_g and the ΔC_p values for the prepared INVITE conjugates. Each sample showed a single T_g so indicating that no blend but only bonded INU–VITE is present. The T_g of the different

Table 2
Thermal behaviors of INVITE samples as determined by DSC studies.

Sample	T_g (°C)	ΔC_p (J/g/K)
INVITE 1	68.5	0.57
INVITE 2	77.7	0.51
INVITE 3	82	0.52
INVITE 4	79.4	0.34
INVITE 5	95.3	0.35
INVITE 6	100.7	0.32

Inulin m.p. = 180 °C.
Vitamin E succinate m.p. = 76 °C.

conjugates (INVITE 1, 2, 3 and INVITE 4, 5, 6) increases by increasing the degree of derivatization in VITE.

Probably, the increasing amount of VITE content in the INVITE samples determines an increase of polymer chain stiffness leading to higher T_g values in the indicated order INVITE 3 > INVITE 2 > INVITE 1. Furthermore, a single thermal transition, as the one seen for INVITE bioconjugates, is indicative of a homogeneous system neither phase separating nor blended.

3.4. Spectroscopy studies

FT-IR spectra of the INVITE conjugates, Fig. 2, confirmed the formation of a new ester bond between INU and VITE. In particular, INVITE spectra showed the presence of bands referred to INU (1646 cm^{-1}) and a new band at 1742 cm^{-1} (C=O stretching of esters). Moreover, depending on the *DD* of the sample the intensity of the signal at 1742 cm^{-1} changed, i.e., higher is the *DD* higher is the absolute and relative intensity of the ester peak.

^{1}H-NMR spectra of INVITE conjugates, Fig. 3, showed a new peak at ~0.8 ppm belonging to VITE alkyl chain (12*H*, m, CH_3). Derivatization degree (*DD* mol%/mol%) was calculated as ratio between the integral of the peak at ~0.8 ppm (VITE, m, 12*H*) and the integral of the peak at 3.5–4.0 ppm (fructose ring, m, 7*H*). *DD* values for the INVITE 1–6 conjugates are shown in Table 1 and are in agreement with the expected amount of VITE (*Y* values of reaction condition). Furthermore, ^{1}H-NMR spectra of INVITE bioconjugates did not show any detectable amount of impurities from DCC or NHSS. So confirming that the applied purification condition resulted successful. Similar considerations on the purification method efficiency could be done from the ^{13}C-NMR spectra.

^{13}C-NMR spectrum of INVITE conjugates confirmed the good course of the reaction between INU and VITE. In Fig. 4 are evidenced the most two significant peaks; at 172.23 ppm (VITE–**C**OO–CH2CH2–COO–INU) and 168.94 ppm (VITE–OOC–CH2CH2–**C**OO–INU) referred to the two carbonyl succinic ester bonds, one of them relative to the new formed ester VITE–INU, that shift to higher fields with respect to the unbonded carboxyl group of VITE (VITE–OOC–CH2CH2–**C**OOH 173.99 ppm).

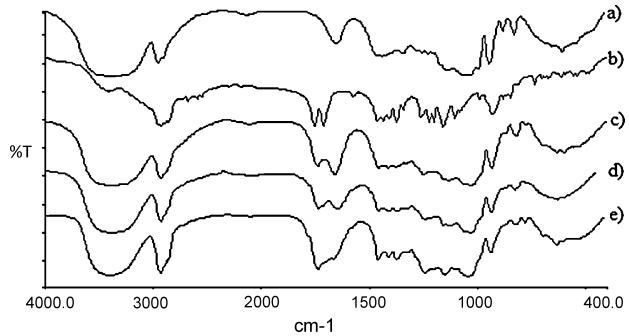


Fig. 2. FT-IR spectra of: (a) Inulin, (b) Vitamin E succinate, (c) INVITE 1, (d) INVITE 2 and (e) INVITE 3.

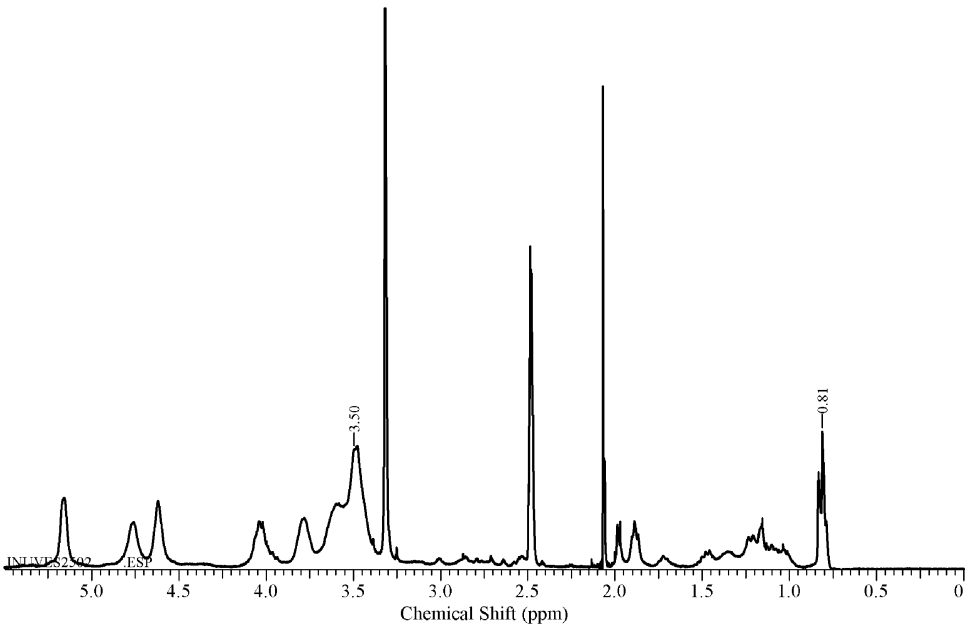


Fig. 3. Representative ^{1}H-NMR spectrum of INVITE conjugates in DMSO-d_6.

This also confirms the validity of the purification steps as demonstrated by the absence of not reacted carboxyl groups belonging to VITE.

Considering that the reactions performed at 40 °C did not give particularly benefit to the final products in terms of yields or *DD* and aimed to reduce energy-consuming procedures, only the INVITE 1, INVITE 2 and INVITE 3 conjugates, obtained at 25 °C, have been further characterized.

3.5. CAC determination

Micelle formation determines a discontinuity in the physical properties of the solution such as surface tension, viscosity, conductivity and light scattering (Inoue, Ebina, Dong, & Zheng, 2007; Kumaraguru & Santhakumar, 2006). According to Rosen (Mathias, Rosen, & Davenport, 2001) CMC can be estimated by a plot of some physical property versus the surfactant concentration.

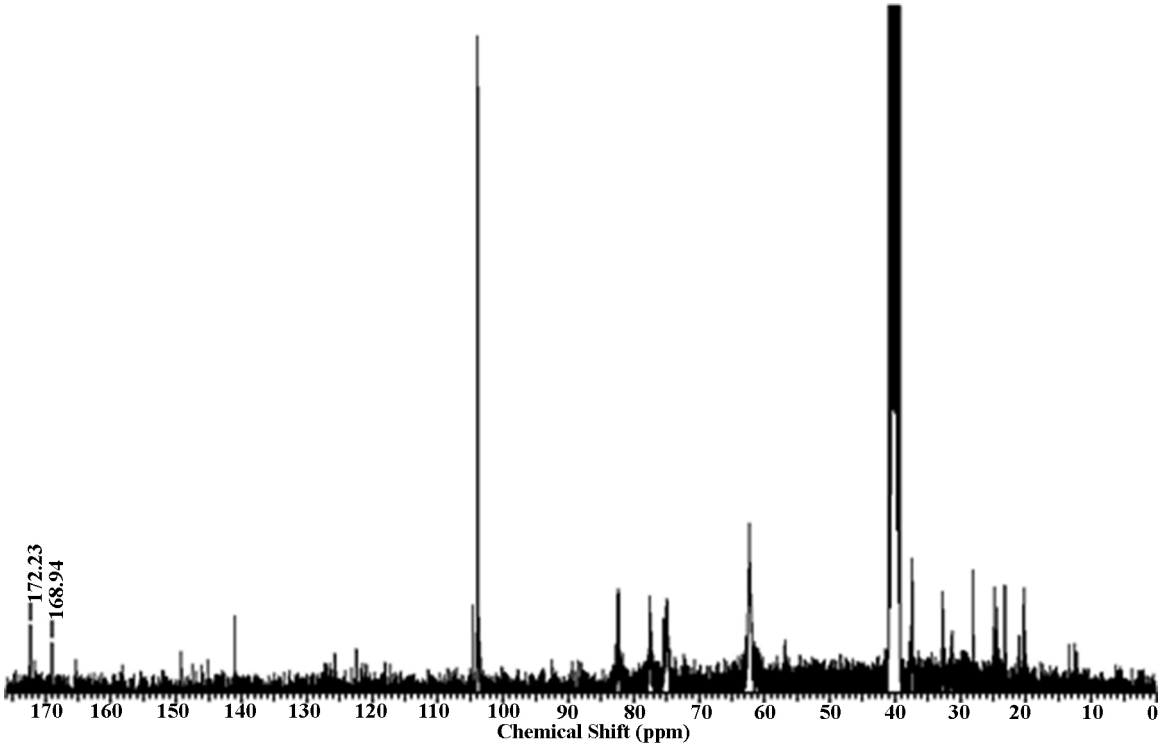


Fig. 4. Representative ^{13}C-NMR spectrum of INVITE conjugates in DMSO-d_6.

Table 3
Critical Association Concentration (CAC) values for INVITE 1, INVITE 2 and INVITE 3 samples as calculated by the curcumin or pyrene methods at 25 °C (see experimental part for details).

Sample	CAC (mM)	
	Curcumin	Pyrene
INVITE 1	7.5×10^{-2}	2.4×10^{-2}
INVITE 2	6.0×10^{-2}	1.6×10^{-2}
INVITE 3	3.8×10^{-2}	2.5×10^{-3}

The ability of polymeric micelles to act as an effective drug delivery system is controlled to a large extent, by their size and their CAC, defined as the copolymer concentration below which only single chains exist. In solutions of the copolymer at higher concentration than the CAC, micelles and single chains coexist. This definition is commonly used to describe polymeric micelles, in analogy with the CMC of surfactant micelles, although the mechanism of micellization may not be the same for amphiphilic polymers and surfactants (Francis, Cristea, & Winnik, 2004). In fact one of the difference between the two types of assemblies from the physicochemical point of view is that the concentration at which the association first take place, known as critical association concentration (CAC) is lower by several orders of magnitudes than typical surfactant CMC values, making polymeric micelles more stable toward dilution in biological fluids. The CAC is indicative of the tendency of these amphiphilic molecules to freely organize themselves in (nano)particulate systems probably leading to a micelle-like structures formed by one or more self-assembling polymer chains. Furthermore the obtained CAC values can represent a useful tool to define the system stability after dilution.

In this paper we determined the CAC of INVITE 1, INVITE 2 and INVITE 3 polymeric micelles by two different methods. The first one was the most commonly used method for the CMC determination, performed by fluorescence spectroscopy using pyrene, being a hydrophobic fluorescence probe that preferentially partitions into the hydrophobic core of the micelle. Pyrene undergoes changes in its photophysical properties as a result of the change on its micropolarity it experiences upon diffusion from bulk water (hydrophilic environment) into the micelle core (hydrophobic environment). So far, between two methods existing to estimate CAC of polymeric micelles with pyrene fluorescence, the original method proposed by Kalyanasundaram and Thomas (1977) by considering the change in the vibronic fine structure of the pyrene emission and by monitoring the changes in the ratio of the intensities I_1 and I_3 of the first and third vibronic peaks has been applied.

The spectra showed five emission peaks at 372 (I_1), 378 (I_2), 383 (I_3), 390 (I_4) and 393 (I_5) nm, in agreement with literature (Kalyanasundaram & Thomas, 1977; Mitsionis & Vaimakis, 2012). In polar solvent, like water, pyrene shows high fluorescence intensity for emission peak I_1 and I_3. Below the CAC, pyrene senses the polar environment of water molecules and, consequently, the ratio between emission intensities I_1 and I_3 is high. Above the CAC, when the micelles were formed, pyrene is solubilized into the core of the micelles that behaves like a non-polar solvent, so the environment sensed by pyrene is less polar, causing the decreases of I_1/I_3 ratio.

Plotting the ratio I_1/I_3 versus the INVITE bioconjugate concentration, a typical sigmoidal decrease at around the CAC is obtained and the resulting values are summarized in Table 3. The CAC values for INVITE 1, INVITE 2 and INVITE 3 were estimated from the interception of the rapidly varying part and the nearly horizontal part at high concentration of the pyrene I_1/I_3 ratio plot (Aguiar, Carpena, Molina-Bolivar, & Ruiz, 2003).

The other method applied for CAC determination was the curcumin method by using the curcumin as a hydrophobic probe. INVITE conjugates enhance the absorption intensity of curcumin, probably because of the hydrophobic interaction between curcumin and micelle system. The CAC values have been determined from the break point of premicellar and postmicellar regions from the plot of absorbance peak of curcumin against conjugates concentrations for INVITE 1, INVITE 2 and INVITE 3.

The obtained data from curcumin absorbance and pyrene fluorescence methods are reported in Table 3. As shown, the results obtained from the two different methods are consistent.

To compare the obtained CAC results for INVITE bioconjugates to those of commonly used molecular surfactants, the CAC, as found from pyrene method, has been re-calculated as mmole of hydrophobic groups per liter. To do that, it has been considered that one molecule (one polymeric chain) of INU or functionalized INVITE contains 30.86 repeating units of fructose (M_w native INU/M_w repeating unit, 5000 Da/162 Da). For example, for INVITE 1, from its *DD*, we know that the 9% of the 30.86 unit of fructose will be found functionalized with VITE, it means that 2.78 of 30.86 units will show a VITE moiety. In this way, 2.78 became a factor by which the found mM CAC value for the INVITE 1 should be multiplied to find the CAC as mmoles of hydrophobic groups per liter (CAC2) because 1 equivalent of INVITE will correspond to 2.78 equivalents of VITE. Appling this method to all the samples the CAC2 for INVITE 1, INVITE 2 or INVITE 3 were calculated respectively as 6.7×10^{-2} mM, 7.9×10^{-2} mM and 2.6×10^{-2} mM. By comparing these values with those from some commonly used surfactant molecules such as sodium dodecyl sulfate (SDS) (7.96 mM) dodecyltrimethylammonium bromide (DTAB) (13.88 mM) Tween-20 (0.053 mM), it is possible to note that INVITE micelles should result stable upon dilution and, above all, INVITE 3 stability in water could be higher than other commonly employed polymeric or not micelle systems.

Moreover, the CAC values, as calculated by both pyrene or curcumin methods, decrease by increasing the derivatization degree of INVITE conjugates, this behavior confirmed that CAC is regulated thermodynamically and its value is affected, above all, by the relative amount of the hydrophobic portions forming the core of the micelles (Chandrasekharan et al., 2011). These results suggest a better thermodynamic stability of the INVITE micelle systems, delaying their disorganization in single (macro)molecules after dilution in an aqueous environment such as physiological fluids. In fact, usually, after intravenous administration micelle system will be, inevitably, diluted and this dilution could cause a premature release of the incorporated drug due to breaking up of the micelles and, as a consequence, the loss of the therapeutic efficacy. The obtained low values in CAC for INVITE polymers are fundamental considering the final aim of INVITE conjugates as DDS targeted to the urinary tract and encourage the use of these systems for drug delivery applications also at high dilution volumes.

3.6. Characterization of INVITE micelles

The hydrodynamic size of the micelles and the polydispersity index, as measured by using the Zetasizer instrument at a concentration of 1 mg/ml (which correspond to 1.5×10^{-1} mM for INVITE 1, to 1.3×10^{-1} mM for INVITE 2 and to 9.6×10^{-2} mM for INVITE 1) and so above the CAC for all the samples, was 60 nm (PI: 0.25) for INVITE 1, 54 nm (PI: 0.3) for INVITE 2 and 24 nm (PI: 0.27) for INVITE 3 micelles (Table 4). As shown, the hydrodynamic size decreases with increasing the degree of derivatization (size INVITE 1 > INVITE 2 > INVITE 3) most probably due to stronger hydrophobic interactions within the VITE portions of more functionalized INVITE bioconjugates leading to a reduction of the micelle hydrodynamic size.

The size measurements were carried out on the micelle systems immediately after the preparation and also after pre-defined storage time at 25 °C to verify their physical stability.

Table 4
Dimensional and Zeta Potential values for INVITE micellar systems as from laser light scattering measurements.

Sample	Mean diameter (nm ± *SD*)	PI	Zeta potential* (mV)
INVITE 1	60.5 ± 10.9	0.25 ± 0.05	−24.8 ± 5.6
INVITE 2	54.8 ± 10.0	0.30 ± 0.03	−22.3 ± 4.2
INVITE 3	24.0 ± 5.9	0.27 ± 0.04	−34.0 ± 7.4

* Inulin zeta potential = −9.14 ± 4.0 mV.
PI = polidispersity index.

In particular, the stability study was performed by measuring the size of the micelles in ultrapure water at designated time points of 0 day, 6 days and 12 days. The size of the micelles measured by Zetasizer versus time is shown in Fig. 5. In aqueous environment after 6 or 12 days the INVITE micelles hydrodynamic diameter variation resulted not significant so indicating that the INVITE systems did not show any dimensional instability (no aggregates) with respect to the indicated storage time after reconstitution (Fig. 5). Furthermore, it has been verified that the ester bond between INU and VITE in INVITE conjugates is not hydrolyzed during the stability studies (see Section 2.7). By these studies, no free VITE has been detected, so confirming that INVITE micelles are also stable by a hydrolytic point of view. Supposing that the micelle system could improve the hydrolytic stability of INVITE conjugates ester bonds, the same hydrolysis stability studies have been performed on INVITE samples in aqueous solution but under the CAC values found for each samples. Also in this case, no presence of free VITE has been detected.

It is known that the presence of charges on the micelle surface could improve the stability of the micelle system due to electrostatic repulsion forces. Table 4 shows *Z*-potential for INVITE 1, 2 and 3 micelles. As expected, *Z*-potential is negative due to the presence of hydroxyl groups of INU on the external surface of micelles. In fact, It should be noted that fructose (that forms the repeating unit of INU) shows a p*K*a of 12.03 making it the most acidic carbohydrate after maltose and lactose. So, it would be reasonable that the found negative values of zeta potential are due to dissociation phenomena (at the equilibrium) of the INU hydroxyl groups that even if reduced in magnitude could contribute to the found potential. Another reason that would explain this assumption, or the reason of the phenomenon itself, is the use of triethylamine during the reaction between INU and VITE that lead to the deprotonation of the INU hydroxyl groups. Since all the synthetic and purification steps were performed in aprotic solvents, it is reasonable to think that the deprotonated hydroxyl groups from INU will be found in this form also when the INVITE samples, dried from acetone, are put in water. (Dziechciarek, van Soest, & Philipse, 2002; Ferreira, Coutinho, & Gama, 2011; Oosten, 1990; Yang, Wang, Ma, Li, & Huang, 2014). In literature, another explanation for negative zeta potential of nanoparticulates from neutral polysaccharides, is found in the preferential absorption of hydroxyl ions (from water) on the particles surface (Hornig, Bunjes, & Heinze, 2009; Park, Huang, Corti, & Franses, 2010).

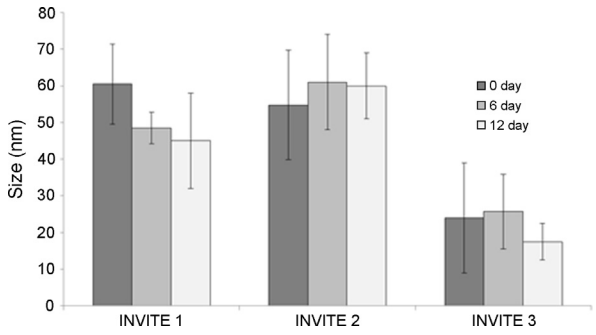


Fig. 5. Stability studies of INVITE 1, 2 and 3 micellar systems carried out for 12 days in ultrapure water valuing size changes by laser light scattering measurements at different time points.

Furthermore, the difference in the *Z*-potential values of INVITE conjugates resulted similar in magnitude, so supporting that the main contribution to the negative values in *Z*-potential is from INU hydroxyl groups mostly present on the outer micelle shell.

4. Conclusions

In this paper, six new amphiphilic bioconjugates based on inulin and D-α-tocopherol succinate, called INVITE 1–6 were synthesized and characterized by FT-IR, ^{1}H-NMR, ^{13}C-NMR spectroscopy, DSC and Zetasizer studies.

The obtained INVITE micelles exhibited low CAC values (range 2.5×10^{-3} mM to 7.5×10^{-2} mM), low size (24–60 nm), a narrow distribution ($0.25 < PI < 0.3$) and resulted stable in water up to 12 days. In particular INVITE 3 looks like a good candidate to be used for drug delivery applications thanks to its low CAC (2.5×10^{-3} mM) and size (24 nm).

According to the work basic concepts that address INU as an optimal polymer for passive renal targeting, the developed INVITE systems could be proposed as potential drug delivery systems for the delivery of anti-infective or anti-inflammatory drugs directly to the urinary tract reducing the side effects related to undesired systemic distribution of the drug. Furthermore, the in-vivo hydrolytic or enzymatic degradation of the ester link between INU and VITE should allow the release of VITE acting as an anti-oxidant in environments suffering of high oxidative-stress and aiding the regression of the pathology. Further studies will be addressed to test INVITE formulations for their ability to solubilize and incorporate hydrophobic drugs commonly used for the therapy of infective or inflammatory disease of the urinary tract as well as for their biocompatibility and drug targeting in-vivo.

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