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PEGylation of coenzyme Q_{10} and *in vitro* release studies

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Methoxy polyethylene glycol conjugated with coenzyme Q_{10} (mPEG)-CoQ_{10} and analog adducts with amino acids as spacers were synthesized as a new drug delivery systems for CoQ_{10}. Alanine and branched chain amino acids (valine, leucine and isoleucine) were conjugated to mPEG by an amide linkage and to CoQ_{10} by an ester bond. Recently, branched chain amino acids (BCAAs), which are released along with $CoQ_{10,}$ have received increasing attention as 'anti-fatigue' elements. FT-IR and ^{1}H NMR spectroscopic analysis were useful to characterize the synthesized conjugates. Studies *in vitro*, in buffer solutions at different pH and in the presence of esterase were conducted. The hydrolysis studies showed a specific cleavage dependent on the pH of the medium and by the presence of proteolytic enzymes. The results showed the improvement of the pharmacokinetic properties of CoQ_{10}. The antioxidant activity of the synthesized conjugates was also evaluated by DPPH assay.

1. Introduction

Chemically, coenzyme Q (CoQ) is similar to vitamin K. In humans, it is synthesized in the body and is called coenzyme Q_{10} (CoQ_{10}) since the side chain presents ten isoprene units in its structure. Two are the redox states of CoQ_{10}: the oxidised ubiquinone and the reduced one ubiquinol. CoQ_{10} is a cofactor in the mitochondrial electron transport chain (respiratory chain) and it is therefore essential for the production of ATP acting as a mobile redox agent shuttling electrons and also protons (Ernster and Dallner 1995) (**Fig. 1**). Furthermore, CoQ_{10} in its reduced state (ubiquinol) is a potent lipophilic antioxidant and is able of recycling and regenerating other antioxidants such as tocopherol and ascorbate (Crane 2001).The central role of coenzyme Q_{10} in mitochondrial electron transport chain and its well known antioxidant properties constitute the basis for its clinical uses. Different studies show a possible benefical effect in different diseases, such as cardiovascular, neurodegenerative, inflammatory and tumoral diseases together with diabetes and hyperlipidemia. Besides, recently, CoQ_{10} has received increasing attention as dietary supplement. Since CoQ_{10} is principally present in the myocardium, a reduction of CoQ_{10} was reported as possible cause of heart failure. For these reasons, cardiovascular together with neurodegenerative diseases are the principal field of application (Overvad et al. 1999; Langsjoen and Langsjoen 1999). Besides, different studies correlate $CoQ_{10}$with physical exercise and have confirmed its effect in improving physical performance and in opposing exercise-related damage (Littarru and Tiano 2010).

However, CoQ_{10} exhibits poor solubility in water, its absorption is low and very limited from the gastrointestinal tract. Its low oral bioavailability is due to its low intestinal permeability and great molecular weight and furthermore, it shows instability in the presence of certain enzymes and oxidants (Bhagavan and Chopra 2006). To overcome this limitation, a number of research efforts have been made to improve its water dispersibility and bioavailability, including cyclodextrin (CD) inclusions, liposomes, polymeric micelles and self-nanoemulsifyng drug delivery systems but, in these forms, only intermolecular forces are employed (Kumar et al. 2016).

Polyethylene glycol (PEG), HO-$(CH_2CH_2O)_n$-H, is at the basis of all approved PEGylated products in its linear or branched form. The great flexibility, ascribed to the absence of bulky substituents along the chain and the high hydratation of the polymeric backbone are the two key properties of this polymer. PEG has been approved by FDA for human use (Pasut and Veronese 2012).

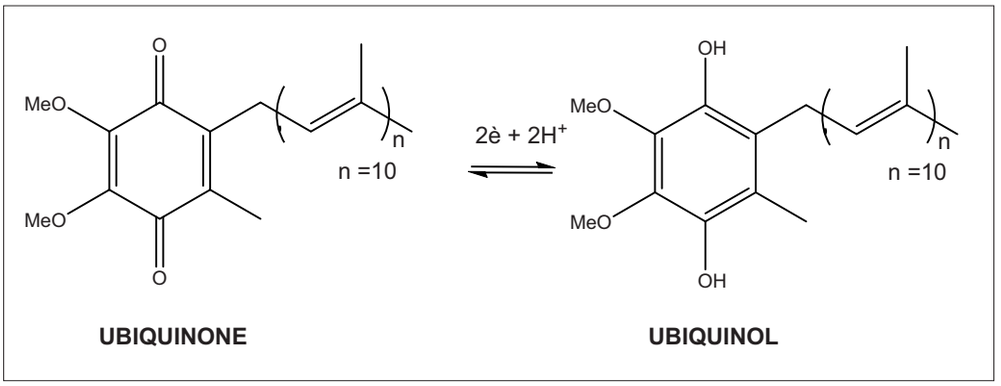

Fig. 1: Coenzyme Q_{10}: redox state reduced (ubiquinol) [chemical name: 2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-hydroquinone] and redox state oxidized (ubiquinone) [chemical name: 2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-benzoquinone].

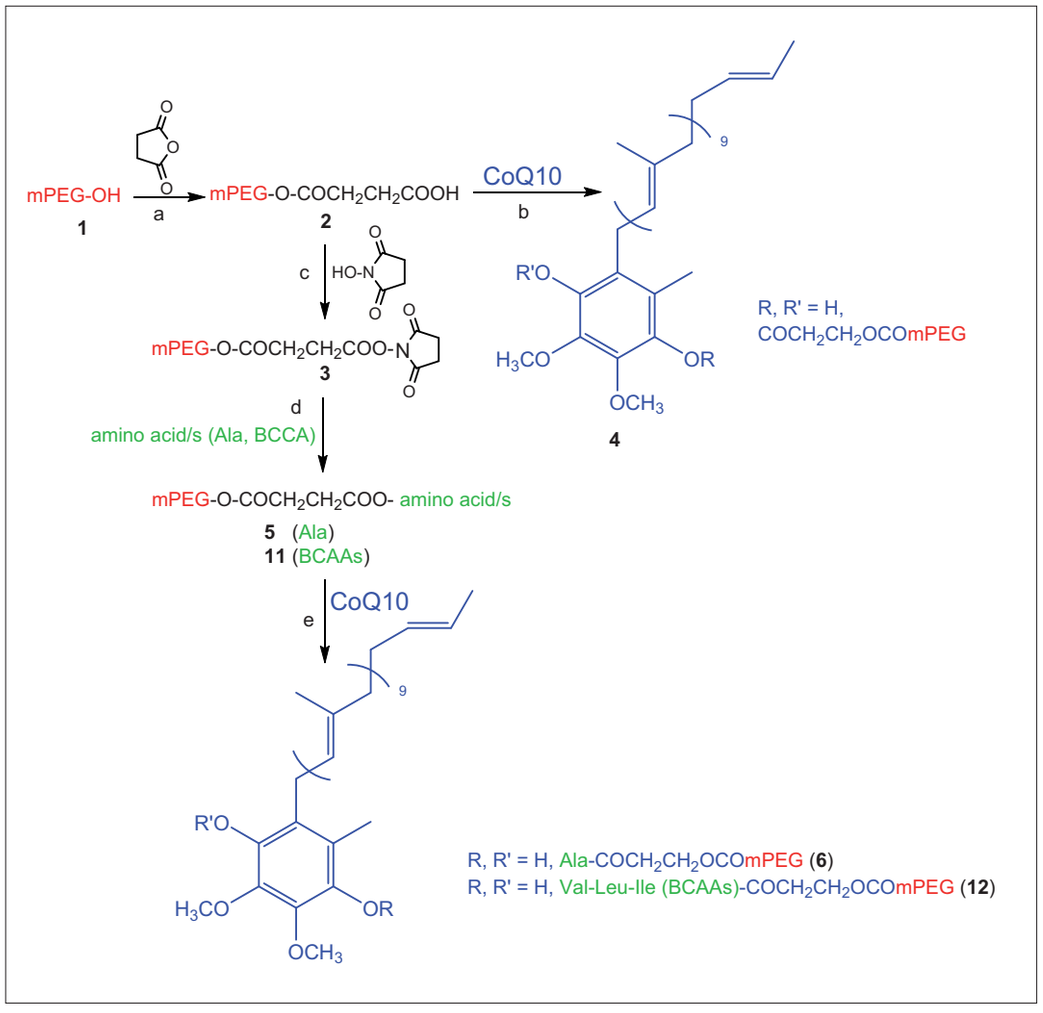

Fig. 2: Synthetic route of mPEG-succinyl-CoQ_{10} (**4**) and mPEG-succinyl-amino acidyl-CoQ_{10} conjugates (**6**, **12**). Reagents and conditions: (a) **1**, Toluene, Succinic anhydride, 6 h, 150 ºC, 85%; (b) **2**, CoQ_{10}, DCC, DMAP [4-(dimethylamino) pyridine], rt, 5 h, 79%; (c) **2**, NHS, DCC, CH_3CN, 24 h, 93%; (d) **3**, DMF, 0 ºC, Ala, $NaHCO_3$, 24 h, 60%; (e) **5** (**11**), CoQ_{10}, DMF, DCC, DMAP, 0 ºC, 24 h, 60% (**6**), 58% (**12**).

Generally, the design of a prodrug involves the optimization of drug release. This synthetic polymer is one of the most widely used for the preparation of prodrugs on the basis of its chemical-physical and biological properties (Veronese 2001). The use of PEG prodrugs and the *in vivo* release of the conjugated drugs have been extensively studied and many practical applications have been reported. The main advantages of PEGylation are the increased solubility of the prodrug that leads to a better distribution of the parent drug, an improvement of the half-life time and a reduction of the side effects of bioactive molecules (Greenwald et al. 2003). Moreover, the conjugation to a polymer of a biologically active molecule is one of several methods to modify and control the pharmacokinetics, biodistribution and often the side effects of these compounds.

The terminal hydroxyl group of methoxy PEG (mPEG) was activated by a succinyl group, which provides end carboxyl group to link with bioactive molecules and was proved non-toxic to body. In the current study, we synthesized mPEG-CoQ_{10} conjugates (**4**, **6**, **12**, **Fig. 2**, **2a**) with succinyl and various amino acids as spacers in order to improve the pharmacokinetics of CoQ_{10} for clinical applications. Alanine and valine, leucine and isoleucine (BCAAs) were conjugated to mPEG by amide linkage and to CoQ_{10} by an ester bond. Branched chain amino acids (BCAAs), which are released along with CoQ_{10}, have received much attention in recent years as 'anti-fatigue' elements, with a benefical effect for human health and with a possible facilitating effect on the absorption of CoQ_{10}. In this work, CoQ_{10} modified by $mPEG_{5000}$ through succinyl and amino acids (Ala, BCAAs) was developed, and the characterization and preliminary *in vitro* release kinetics of the water soluble mPEG and mPEG-amino acidyl-CoQ_{10} conjugates were investigated. The synthesized mPEG conjugates were characterized by FT-IR and ^{1}H NMR. To our knowledge, this is the first report on the synthesis and on *in vitro* release kinetics evaluation of PEG conjugate as a prodrug system for CoQ10. The antioxidant activity of the synthesized conjugates was also evaluated by DPPH assay.

2. Investigations, results and discussion

2.1. Chemistry

The aim of our investigation was to develop and characterize a new drug delivery system for CoQ_{10} to improve its pharmacokinetics. CoQ_{10} has received increasing attention in recent years for preventing or attenuating human diseases such as cardiovascular disorders and neurodegenerative diseases (e.g. parkinsonism) and for its radical-scavenging activities (Littarru and Tiano 2010). Nowadays, this compound is widely used as a food nutraceutical ingredient and drug. However, the extremely low solubility and oral bioavailability of CoQ_{10} greatly limits its application in nutraceutical formulations. Polyethyleneglycol (PEG) conjugates usually have better pharmacokinetic behavior and water solubility and, in general, are more efficient in drug targeting. To our knowledge, no

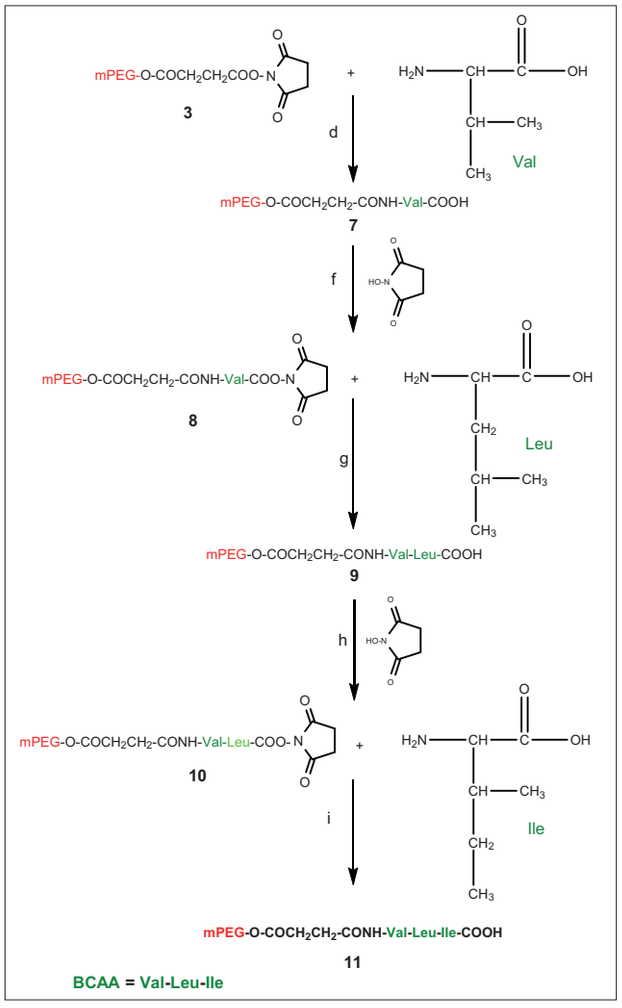

Fig. 2a: Synthetic route of mPEG-succinyl-val-leu-ile (BCAAs) (**11**). Reagents and conditions: (d) **3**, DMF, 0 ºC, Val, $NaHCO_3$, 24 h, 45%; (f) **7**, NHS, DCC, CH_3CN, 24 h, 91%; (g) **8**, DMF, 0 ºC, Leu, $NaHCO_3$, 24 h, 52%; (h) **9**, NHS, DCC, CH_3CN, 24 h; (i) **10**, DMF, 0 ºC, Ile, $NaHCO_3$, 24 h, 58%.

covalently-bound PEG-CoQ_{10} conjugates have been synthesized. Furthermore, the conjugation of PEG moiety to CoQ_{10} through a relatively labile bond, such as an ester bond, which can be cleaved under physiological conditions, leads the PEG unit to be only an inert, polar vehicle for CoQ_{10}.

mPEG-Succinyl-CoQ_{10} (**4**) and mPEG-Succinyl-CoQ_{10} conjugates with amino acids (**6**, **12**) as spacers were synthesized in order to study the role of PEG conjugation as a method for improving the solubility and bioavailability of CoQ_{10}. An ester bond link CoQ_{10} to PEG since esters can be hydrolyzed in the presence of pancreatic secretions to regenerate the parent CoQ_{10}. For the synthesis of CoQ_{10} prodrugs **4**, **6** and **12** $mPEG_{5000}$-succinic acid (**2**) was the building block. The $mPEG_{5000}$-succinic acid (**2**) was prepared by the reaction of PEG_{5000}-OMe (**1**) with succinic anhydride in toluene. The attachment of **2** to CoQ_{10} (ubiquinol) was performed by means of DCC and DMAP as coupling agents to give the mPEG-succinyl-CoQ_{10} (**4**) with a satisfactory yield (79%) (**Fig. 2**). The prepared PEG-CoQ_{10} and PEG-amino acidyl-CoQ_{10} conjugates were characterized by FT-IR and ^{1}H NMR. In the FT-IR spectrum of mPEG-Succinyl-CoQ_{10} (**4**) peaks were observed at 3400 cm^{-1} (OH) and at 2863 cm^{-1} ($-CH_2$) due to the linking with CoQ_{10} and to methylenes from PEG, respectively. The peaks at 1738-1750 cm^{-1} were indicative of the characteristic absorptions of ester groups. The peaks at 1450-1600 cm^{-1} were attributed to the benzene ring and the peak at 965 cm^{-1} is a typical absorption peak of *trans* olefin which confirms the presence of CoQ_{10}. The ^{1}H NMR spectrum of the adduct showed the presence of the methylene protons, ascribed to the PEG backbone (δ = 3.55 – 3.90 ppm), together with the signals at 1.64, 1.97 and 2.0-2.23 ppm due to the methyl and methylene protons adiacent to the double bonds of CoQ_{10}. The percentage of the conjugated drug was evaluated by spectroscopic details and by alkaline hydrolysis followed by HPLC analysis and estimated to be near 88% (w/w). The absence of any free drug was also verified.

Besides, we designed two PEG-CoQ_{10} conjugates (**6**, **12**) with succinyl and various amino acids as spacers. Alanine and valine, leucine and isoleucine (BCAAs) were conjugated to mPEG by amide linkage and to CoQ_{10} by an ester bond. Amino acids are biocompatible and essential to health; in this study, alanine was selected as linking arm for the synthesis of the conjugate **6** since it is a key component of the glucose biosynthetic pathway, while CoQ_{10} exhibits well known antioxidant properties. This leads to a synergistic effect since the oxidation events might be reduced in muscle after an intense physical exercise and, at the same time, ergogenic material, glucose, increases by the cycle alanine-glucose (Mizuno et al. 2008). Recent studies suggest that free radicals are involved in the failing myocardium and may be important contributors to major adverse cardiovascular events so CoQ_{10} through its antioxidant effects, may reduce oxidative stress, which is known to adversely affect, in particular, left ventricular ejection fraction and may stabilize calcium-dependent channels in the myocardium, enhancing effective ATP synthesis (DiNicolantonio et al. 2015).

On the basis of the above considerations mPEG-succinyl-alanine-CoQ_{10} (**6**) was synthesized. The terminal carboxyl group of the initial building block $mPEG_{5000}$-succinic acid (**2**) was activated by reaction with NHS to give the active ester **3**. The reaction of the compound **3** with alanine in DMF led to mPEG-succinyl-alanine (**5**). The attachment of **5** to CoQ_{10} (ubiquinol) was performed by means of DCC and DMAP as coupling agents to give the mPEG-succinyl-alanine-CoQ_{10} (**6**) with a satisfactory yield (60%) (**Fig. 2**). In the FT-IR spectrum of **6** peaks were observed at 3410 cm^{-1} (OH) and at 2865 cm^{-1} ($-CH_2$) because of linking with CoQ_{10} and methylenes from PEG, respectively. The peaks at 1738-1750 cm^{-1} were attributed to the characteristic absorptions of ester groups. From 1580 cm^{-1} to 1680 cm^{-1} there was strong amide peak and relatively strong absorption of approximately 1110 cm^{-1} due to C-N bond and to the integrity of PEG backbone. The peaks at 1450-1600 cm^{-1} were attributed to the benzene ring and the peak at 965 cm^{-1} is a typical absorption peak of *trans* olefin and also confirms the presence of CoQ_{10}. The ^{1}H NMR spectrum of the adduct indicated the presence of the methylene protons, ascribed to the PEG backbone (δ = 3.44 – 3.79 ppm). The doublet at 1.58 ppm was due to the methyl group of alanine, the multiplet at 5.30 ppm ascribed to the olefinic protons of CoQ_{10} confirm the synthesis of the compound **6** (**Fig. 3**). The percentage of the conjugated drug was evaluated by spectroscopic details and by alkaline hydrolysis

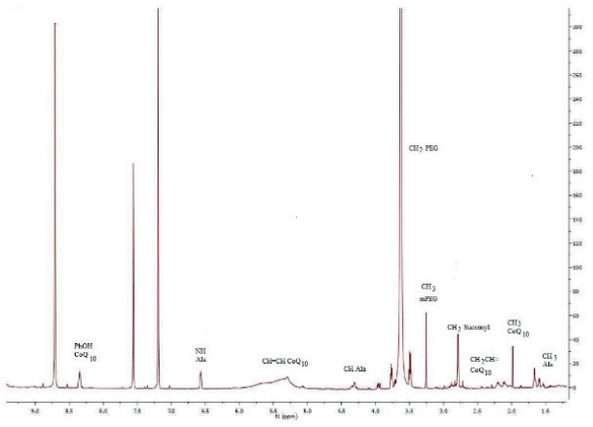

Fig. 3: ^{1}H NMR spectrum (C_5D_5N, 500 MHz) of mPEG-succinyl-Ala-CoQ_{10} (**6**).

followed by HPLC analysis and estimated to be near 86% (w/w). The absence of any free drug was also verified.

Besides, the readly available active ester **3**, synthesized as described above, was the building block for the synthesis of mPEG-succinyl-valine-leucine-isoleucine-CoQ^{10} (**12**) conjugate. Valine, leucine and isoleucine are known as branched-chain amino acids (BCAAs) and are essential for humans who can only receive them from the diet. BCAAs have received increasing attention in recent years as special dietary supplements for sport nutrition since they are expected to promote maintaining the skeletal muscle and its regeneration. Since BCAAs show immunoactivating properties, the interest in their nutritional and medical value has increased (Calder 2006). On the other hand, CoQ^{10} has a crucial role in the cellular bioenergetics; it is a cofactor in the electron transport chain of the mitochondria so the co-administration of BCAAs and CoQ_{10} gradually released from PEG may be considered a challenge on the basis of the possible synergistical facilitating effects on the absorption of CoQ_{10} (Zhao et al. 1991). On the basis of the above consideration BCAAs were linked to PEG backbone by active esters **3** for valine, **8** for leucine and **10** for isoleucine to give the synthon mPEG-succinyl-BCAAs (**11**). Figure 2a illustrates the method applied for the synthesis of mPEG conjugated BCAAs. The carboxyl end group of mPEG-succinyl-valine (**7**) was activated with NHS to give the active ester **8**. The coupling with leucine led to mPEG-succinyl-valine-leucine (**9**) whose terminal carboxyl group was activated again with NHS to give the active ester **10**. The reaction of **10** with isoleucine gave the synthon mPEG-succinyl-valine-leucine-isoleucine (**11**). The attachment of **11** to CoQ_{10} (ubiquinol) was performed by means of DCC and DMAP as coupling agents to give the mPEG-succinyl-valine-leucine-isoleucine-CoQ_{10} (**12**) with a satisfactory yield (58%) (**Fig. 2**). In the FT-IR spectrum of **12** peaks were observed at 3410 cm^{-1} (OH) and at 2865 cm^{-1} (-CH_2) because of linking with CoQ_{10} and methylenes from PEG, respectively. The peaks at 1738-1750 cm^{-1} were attributed to the characteristic absorptions of ester groups. From 1580 cm^{-1} to 1680 cm^{-1} there was strong amide peak and relatively strong absorption of approximately 1110 cm^{-1} due to C-N bond and to the integrity of PEG backbone. The peaks at 1450-1600 cm^{-1} were attributed to the benzene ring and the peak at 965 cm^{-1} is a typical absorption peak of *trans* olefin and also indicates the presence of CoQ_{10}. The ^1H NMR spectrum of the adduct confirmed the presence of the methylene protons, ascribed to the PEG backbone (δ = 3.45 – 3.79 ppm). The multiplets at 0.83-0.95 ppm, 1.11 ppm ascribed to the methyl groups of valine, leucine and isoleucine, the multiplet at 5.23-5.38 ppm due to the olefinic protons of CoQ_{10} together with the broad singlets at 6.54 and 8.4 ppm ascribed to the amidic bonds confirm the synthesis of the compound **12**. The percentage of the conjugated drug was evaluated by spectroscopic details and by alkaline hydrolysis followed by HPLC analysis and estimated to be near 86% (w/w). The absence of any free drug was also verified.

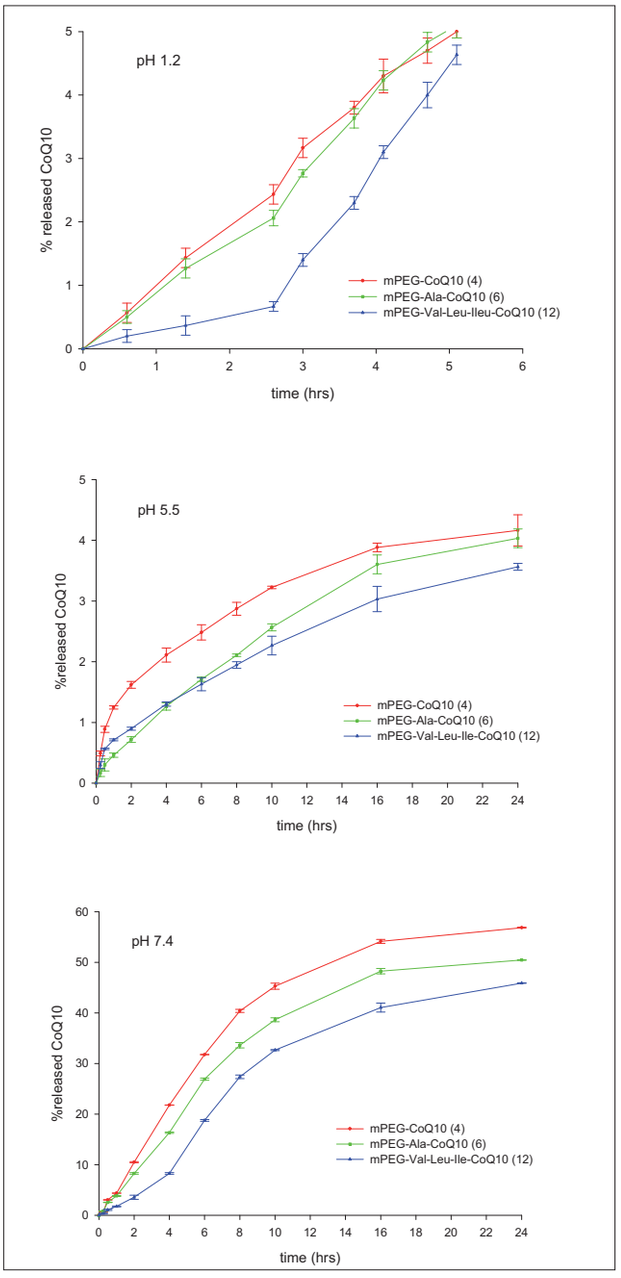

Fig. 4: Hydrolysis of the three conjugates **4**, **6** and **12** in buffer solutions, at 37±0.1 °C. Each experiment was carried out in triplicate and expressed as the mean value ± S. D.

2.2. In vitro drug release studies

To better verify the stability of mPEG conjugates (**4**, **6** and **12**) as potential prodrugs (for oral administration) they were investigated by incubation under different environmental conditions. The method includes a sample pretreatment to ensure all CoQ_{10} is present in the fully oxidised state (Orozco et al. 2007).The incubation of conjugates in solution at different pH values demonstrated their stability. Less than 5% of CoQ_{10} (ubiquinone) was released over 6 h from the three polymeric conjugates at pH 1.2 and 5.5, while the release of CoQ_{10} was faster at pH 7.4, but it did not overcome the values of 57%, 50% and 46% for mPEG-succinyl-CoQ_{10} (**4**), mPEG-succinyl-alanine-CoQ_{10} (**6**) and mPEG-succinyl-valine-leucine-isoleucine-CoQ_{10} (**12**) after 24 h, respectively (**Fig. 4**). Since the capacity of porcine esterase to catalyze the hydrolysis of ester bonds is well-known (Greenwald et al. 2003), the possibility that all the synthesized adducts may be good substrates for this enzyme (in which drug molecules are linked to a polymeric backbone by ester linkages) was therefore evaluated. As shown in **Fig. 5**, at pH 8 approximately 79%, 72% and 68% of the linked drug was released from the polymeric conjugates **4**, **6** and **12**, respectively, within 24 h in the presence of esterase; while less than 50% was released in the absence of the enzyme. In addition, the structure of the released drug was verified by EI-MS analysis. The molecular ion at *m/z* 865 and the fragments at *m/z* 850 and at *m/z* 834, due to the loss of the methyl and methoxy group, confirm the presence of CoQ_{10}. These results show the capacity of mPEG-succinyl-CoQ_{10} and mPEG-succinyl-amino acidyl CoQ_{10} to release free drug on the basis of the hydrolytic activity of esterase.

2.3. Free radical scavenging activity

The free radical scavenging activity was assayed spectrophotometrically using the DPPH method (Blois 1958; Choi et al. 2002; Brand-Willians et al. 1995).The activity of the synthesized conju-

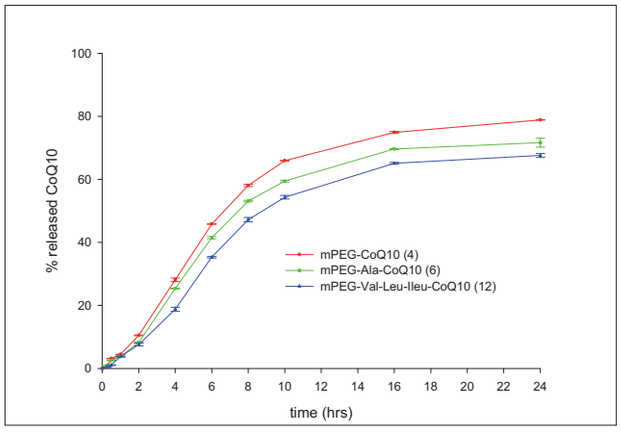

Fig. 5: Hydrolysis of the three conjugates **4**, **6** and **12** in buffer solution, at 37±0.1°C at pH 8 in presence of porcine esterase. Each experiment was carried out in triplicate and expressed as the mean value ± S. D.

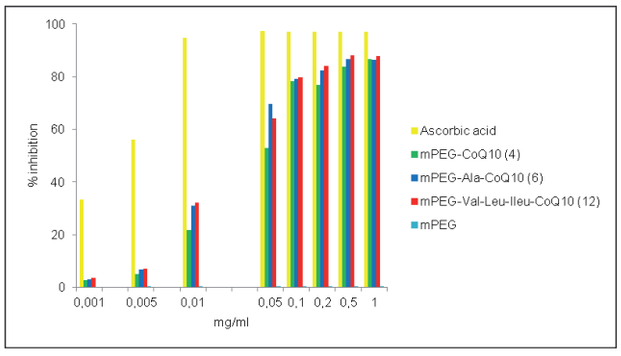

Fig. 6: Free radical scavenging activity of the conjugates **4**, **6** and 12.

gates mPEG-succinyl-CoQ_{10} (**4**), mPEG-succinyl-alanine-CoQ_{10} (**6**) and mPEG-succinyl-valine-leucine-isoleucine-CoQ_{10} (**12**) was measured at concentrations of 0.001-1 mg/mL and the results were shown in **Fig. 6**. The free radical scavenging activity was expressed as SC_{50}, the concentration needed to reduce 50% of DPPH. The conjugates **4**, **6** and **12** possess a phenolic hydroxyl group that shows significant radical scavenging activity with SC_{50} of 36 µg/mL (**4**), 25 µg/mL (**6**) and 22 µg/mL (**12**).

2.4. Conclusions

On the basis of our previous investigations we studied mPEG-based support as a new drug delivery system for CoQ_{10}. Three types of new PEG-CoQ_{10} and PEG-amino acidyl-CoQ_{10} conjugates using alanine and BCCAs (valine, leucine and isoleucine) as amino acids were prepared using succinic acid as spacer. These conjugates are expected to improve the pharmacokinetics of CoQ_{10} and increase uptake of these essential amino acids with the release of CoQ_{10}. The conjugates were characterized by FT-IR and ^{1}H NMR. The *in vitro* release behaviors were studied in buffer solution at different pH values and in the presence of esterase. The results demonstrated that more CoQ_{10} was released from PEG at pH 7.4 and in the presence of proteolytic enzymes. Therefore, the three novel PEG conjugates may be used for oral administration, enhancing the controlled release and thereby improving the pharmacokinetics of CoQ_{10}. In addition, the conjugates showed a significant free radical scavenging activity by DPPH assay maintaining the antioxidant properties of CoQ_{10} in its reduced form (ubiquinol) which, once gradually released *in vivo*, is able of recycling and regenerating other antioxidant such as tocopherol and ascorbate.

3. Experimental

3.1. General

CoQ_{10} was obtained from Sigma-Aldrich (St. Louis, Mo, USA). Ubiquinol was purchased from Finetech® Industry Limited (Hubei, China). Analytical grade $mPEG_{5000}$, chemical grade succinic anhydride, dicyclohexylcarbodiimide (DCC), 1-hydroxy-benzotriazole (HOBT), N,N-4-dimethylaminopyridine (DMAP) and alanine, valine, leucine and isoleucine were all of industrial grade and purchased by Fluka (Milano, Italy). Esterase from porcine liver-ammonium sulfate suspension ≥ 150 units/mg protein (biuret) was purchased from Sigma-Aldrich (St. Louis, Mo, USA). All solvents were of analytical grade and dried over 4Å molecular sieves. The purity of the synthesized compounds was checked by TLC on Merck Silicagel 60 F_{254} plates. TLC plates were analyzed by visualization under UV light (254 nm), exposition to iodine vapors or by spraying with 0.2% ninhydrin in ethanol. The spectrophotometric UV/VIS analyses were performed on a Helios β UNICAM spectrophotometer. IR spectra were obtained on a FT-IR Nicolet Magna 550 instrument using KBr as the sample holder and samples were scanned from 400 to 4000 cm^{-1}. ^{1}H NMR data were acquired using a Varian (500 MHz) Fourier transform spectrometer, in $CDCl_3$ or C_5D_5N. Proton chemical shifts were referenced to the residual signal of protonated $CDCl_3$ at δ 7.24 or δ 8.71 when C_5D_5N was used. ESI mass spectra were recorded using a API Perkin Elmer (voltage + 5600 with orifice 90 and/or 120) mass spectrometer.

The amount of CoQ_{10} in the *in vitro* release test was determined by high-performance liquid chromatography. HPLC analyses were carried out with a Spectra System® P2000 HLPC connected to a variable-wavelength UV detector (Thermo Separation Products Inc.). A Gemini C18 (5 µm, 250 mm x 4.6 mm i.d., Phenomenex) column was used. The mobile phase was a mixture of acetonitrile – water – tetrahydrofuran (55 : 5 : 45, v/v). The detection wavelength was 275 nm and the flow rate was 2.0 mL/min. The sample injection volume was 20 µL. Quantification was performed on the basis of linear calibration plot of peak area against concentration. Each curve is based on seven concentrations of the standard. Identification of CoQ_{10} was made by comparison of the retention time with that of pure standard.

3.2. Synthesis of mPEG-succinic acid (2)

$mPEG_{5000}$ (**1**) (10 g, 2 mmol) was dissolved in toluene (50 mL) and dried by azeotropic distillation; succinic anhydride (0.8 g, 8 mmol) was added and the mixture was stirred for 6 h at 150 ºC. The mixture was cooled, taken up in CH_2Cl_2 and the polymer precipitated by ether. The product was recrystallized twice from EtOH to give the compound **2** as a white powder (8.6 g, 85%); TLC (BuOH/AcOH/ H_2O 4:1:1) showed only one peak and no succinic anhydride; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.60-2.63 (m, CH_2COO), 3.35 (s, 3H, CH_3O of mPEG), 3.55-3.68 (m, CH_2 of mPEG), 4.20-4.25 (q, CH_2 of mPEG); FT-IR (KBr): υ = 1736, 1243, 1110 cm^{-1}.

3.3. Synthesis of mPEG-succinyl-CoQ_{10} (4)

CoQ_{10} (Ubiquinol) (0.675 g, 0.78 mmol) and DCC (105 mg, 0.51 mmol) were added in small portions, under nitrogen, to a solution of mPEG-succinic acid (**2**) and DMAP (12.2 mg, 0.10 mmol) in dry dichloromethane (20 mL) and the mixture was stirred for 5 h at room temperature. The precipitate of dicyclohexylurea was filtered and the filtrate concentrated *in vacuo* and precipitated by ether. The mPEG-Succinyl-CoQ_{10} was recrystallized twice from EtOH to give the compound **4** as a white powder (1.83 g, 79%). The absence of the free drug in the adduct was confirmed by HPLC; ^{1}H NMR (500 MHz, C_5D_5N): δ = 1.64 (s, 27H, CH=CH-CH_3 of CoQ_{10}), 1.97 (s, 3H, CH=CH-CH_3 of CoQ_{10}), 2.0-2.23 (br m, 23H, CH_2CH=, CH_3Ph of CoQ_{10}), 2.78 (m, CH_2COO of mPEG), 3.23 (d, 2H, Ph-CH_2CH=CH of CoQ_{10}), 3.47 (s, 3H, CH_3O of mPEG), 3.55-3.90 (br m, CH_2 of mPEG, 2 CH_2O-Ph of CoQ_{10}), 4.26-4.35 (q, CH_2 of mPEG), 5.28 (m, 10H, CH=CH of CoQ_{10}), 6.57, 8.31 (br s, PhO*H* of CoQ_{10}); FT-IR (KBr): υ = 3400, 2863, 1738- 1750, 1450-1600, 1243, 1110, 965 cm^{-1}.

3.4. Synthesis of mPEG-succinyl-amino acidyl-CoQ_{10} conjugates

mPEG-Succinyl-Amino Acidyl-CoQ_{10} conjugates were synthesized according to the route shown in Figure 2. Selected amino acids were alanine, valine, leucine and isoleucine.

3.5. Synthesis of mPEG-succinyl-alanine (5)

mPEG-Succinic acid (**2**) (3 g, 0.59 mmol) was dissolved in dry dichloromethane (25 mL) and cooled to 0 ºC. DCC (0.24 g, 1.18 mmol) and a precooled solution of NHS (0.136 g, 1.18 mmol) in acetonitrile (10 mL) were slowly added to the dichloromethane solution and the mixture was stirred for 24 h at room temperature. The obtained suspension was filtered and 10% acetic acid aqueous solution (10 mL) was added into the filtrate to remove excess DCC. After agitating for 30 min, the mixture was steadied for a while and the organic phase was separated, washed off with saturated brine until neutral, dried by anhydrous sodium sulfate, filtered, concentrated in *vacuo* and the product was precipitated by ether. The $mPEG_{5000}$ active ester was recrystallized twice from EtOH to give the compound **3** as a white powder (2.85 g, 93%); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.74, 2.92 (t, 8H, CH_2 of NHS), 2.79 (m, CH_2- of succinyl), 3.34 (s, 3H, CH_3O of mPEG), 3.44-3.77 (m, CH_2 of mPEG), 4.21-4.26 (q, CH_2 of mPEG).

Compound **3** (2 g, 0.38 mmol) was dissolved in DMF (20 mL) and cooled to 0-5 °C. Alanine (0.14 g, 1.52 mmol) dissolved in 1 mol·L^{-1} $NaHCO_3$ aqueous solution (5 mL) was then added. The mixture was kept at room temperature for 24 h, and then the pH of mixture was adjusted to 2 by 1 mol·L^{-1} hydrochloric acid. The mixture was then extracted by dichloromethane (10 mL x 3) and the organic phase was washed by saturated brine to neutral, dried with sodium sulfate, filtered, concentrated and the product

was precipitated by ether. The mPEG-succinyl-alanine was recrystallized twice from EtOH to give the compound **5** as a white powder (1.2 g, 60%); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.40 (d, *J*= 7.14 Hz, 3H, C*H*$_3$ of Ala), 2.50-2.67 (t, 4H, C*H*$_2$- of succinyl), 3.35 (s, 3H, C*H*$_3$O of mPEG), 3.40-3.80 (m, C*H*$_2$ of mPEG), 4.22 (q, C*H*$_2$ of mPEG), 4.49 (q, *J*= 6.14, 7.45 Hz, 1H, chiral H from Ala), 6.57 (d, *J*= 5.49 Hz, 1H, N*H* of Ala). Alanine was replaced by valine to prepare compound **7** with a similar preparation method starting from compound **3** (5 g, 0.96 mmol) and valine (0.45 g, 3.84 mmol). The mPEG-succinyl-valine was recrystallized twice from EtOH to give compound **7** as a white powder (2.5 g, 49%); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.89 (dd, *J*= 6.99, 6.92 Hz, 6H, 2C*H*$_3$CH of Val), 2.16 [m, 1H, C*H*(CH$_3$)$_2$ of Val], 2.52-2.65 (dt, 4H, C*H*$_2$- of succinyl), 3.32 (s, 3H, C*H*$_3$O of mPEG), 3.37-3.81 (br m, C*H*$_2$ of mPEG), 4.20 (q, C*H*$_2$ of mPEG), 4.45 (d, 1H, chiral H from Val), 6.26 (d, 1H, N*H* of Val).

3.6. Synthesis of mPEG-succinyl-alanine-CoQ$_{10}$ (6)

mPEG-succinyl-alanine (**5**) (1 g, 0.20 mmol) was dissolved in dry dichloromethane (15 mL) and cooled to 0-5 ºC. A solution of CoQ_{10} (ubiquinol) (0.69 g, 0.80 mmol) in DMF (5 mL), under nitrogen, DCC (62 mg, 0.30 mmol) and DMAP (30.4 mg, 0.25 mmol) were mixed under stirring for 24 h at room temperature. The suspension was filtered and the pH of filtrate was adjusted to 3 with a 2% hydrochloric acid aqueous solution. The excess DCC in the filtrate was decomposed by 20 mL of a 10% acetic acid aqueous solution. The filtrate was then extracted by dichloromethane (10 mL x 3) and the organic phase was washed with saturated brine, dried by anhydrous sodium sulfate, filtered, concentrated and the product was precipitated by ether. The mPEG-succinyl-alanine-CoQ_{10} was recrystallized twice from EtOH to give compound **6** as a white powder (0.74 g, 60%). The absence of the free drug in the adduct was confirmed by HPLC; ^{1}H NMR (500 MHz, C_5D_5N): δ = 1.58 (d, *J*= 7.12 Hz, 3H, C*H*$_3$ of Ala), 1.66 (s, 3H, CH=CH-C*H*$_3$ of CoQ_{10}), 1.98 (s, 30H, CH=CH-C*H*$_3$, C*H*$_3$-Ph of CoQ_{10}), 2.12-2.22 (br m, 20H, C*H*$_2$CH= of CoQ_{10}), 2.79 (m, C*H*$_2$ of succinyl), 3.26 (br s, 5H, Ph-C*H*$_2$ of CoQ_{10}, C*H*$_3$O of mPEG), 3.44-3.79 (br m, C*H*$_2$ of mPEG, 2 C*H*$_2$O-Ph of CoQ_{10}), 3.97 (q, C*H*$_2$ of mPEG), 4.31 (q, *J*= 6.13, 7.44 Hz, 1H, chiral H of Ala), 5.30 (m, 10H, C*H*=C*H* of CoQ_{10}), 6.57, 8.34 (br s, PhO*H* of CoQ_{10}, N*H* of Ala); FT-IR (KBr): υ = 3410, 2865, 1738- 1750, 1580-1680, 1110, 965 cm^{-1}.

3.7. Synthesis of mPEG-succinyl-valine-leucine-isoleucine (9)

mPEG-succinyl-valine (**7**) (2.5 g, 0.47 mmol) was dissolved in dry dichloromethane (25 mL) and cooled to 0 ºC. DCC (0.2 g, 0.94 mmol) and a precooled solution of NHS (0.11 g, 0.94 mmol) in acetonitrile (10 mL) were slowly added to the dichloromethane solution and the mixture was stirred for 24 h at room temperature. The obtained suspension was filtered and 10% acetic acid aqueous solution (10 mL) was added into the filtrate to remove excess DCC. After agitating for 30 min, the mixture was steadied for a while and the organic phase was separated, washed off with saturated brine until neutral, dried by anhydrous sodium sulfate, filtered, concentrated in *vacuo* and the product was precipitated by ether. The mPEG$_{5000}$-succinyl-valine active ester was recrystallized twice from EtOH to give compound **8** as a white powder (2.3 g, 91%); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.93, 1.0 (dd, *J*= 6.97, 6.93 Hz, 6H, 2C*H*$_3$CH of Val), 2.0-2.40 [m, 5H, C*H*$_2$ of NHS, C*H*(CH$_3$)$_2$ of Val], 2.41-2.64 (dt, 4H, C*H*$_2$- of succinyl), 2.81 (br m, 4H, C*H*$_2$ of NHS), 3.35 (s, 3H, C*H*$_3$O of mPEG), 3.40-3.90 (br m, C*H*$_2$ of mPEG), 4.20 (q, C*H*$_2$ of mPEG), 4.46 (d, 1H, chiral H from Val), 6.29 (d, 1H, N*H* of Val).

Compound **8** (2.3 g, 0.43 mmol) was dissolved in DMF (20 mL) and cooled to 0-5 °C. Leucine (226 mg, 1.72 mmol) dissolved in 1 mol·L^{-1} $NaHCO_3$ aqueous solution (5 mL) was then added. The mixture was kept at room temperature for 24 h, and then the pH of mixture was adjusted to 2 by 1 mol·L^{-1} hydrochloric acid. The mixture was then extracted by dichloromethane (10 mL x 3) and the organic phase was washed by saturated brine to neutral, dried with sodium sulfate, filtered, concentrated and the product was precipitated by ether. The mPEG-succinyl-valine-leucine was recrystallized twice from EtOH to give compound **9** as a white powder (1.21 g, 52%); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.90, 0.93 [dd, 12H, (C*H*$_3$)$_2$CH of Val and Leu), 1.28 [m, 1H, C*H*(CH$_3$)$_2$ of Leu], 1.72 (m, 2H, C*H*$_2$ of Leu), 1.89 [m, 1H, C*H*(CH$_3$)$_2$ of Val], 2.44-2.69 (m, 4H, C*H*$_2$- of succinyl), 3.34 (s, 3H, C*H*$_3$O of mPEG), 3.43-3.76 (br m, C*H*$_2$ of mPEG), 4.20 (q, C*H*$_2$ of mPEG), 4.41, 4.44 (m, 2H, chiral H from Val and Leu), 5.45, 6.28 (br s, 2H, N*H* of Val and Leu).

Isoleucine was conjugated to mPEG-succinyl-valine-leucine (**9**) (1.21 g, 0.22 mmol) via active ester (**10**) with the similar preparation method reported above. The active ester **10** was employed in the next step without purification.

The mPEG-succinyl-valine-leucine-isoleucine was recrystallized twice from EtOH to give compound **11** as a white powder (607 mg, 50%); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.83-0.95 [m, 15H, 2(C*H*$_3$)$_2$CH of Val and Leu, C*H*$_3$ of Ile), 1.16 [d, 3H, C*H*$_3$CH of Ile], 1.22 [m, 1H, C*H*(CH$_3$)$_2$ of Leu], 1.46 [m, 2H, C*H*$_2$CH$_3$ of Ile], 1.94 [m, 3H, C*H*$_2$ of Leu, C*H*(CH$_3$)$_2$ of Val], 2.19 [m, 1H, C*H*(CH$_3$) of Ile], 2.44, 2.65 (m, 4H, C*H*$_2$- of succinyl), 3.34 (s, 3H, C*H*$_3$O of mPEG), 3.42-3.80 (br m, C*H*$_2$ of mPEG), 4.20 (m, C*H*$_2$ of mPEG), 4.24, 4.41 (m, 3H, chiral H from Val, Leu and Ile), 6.29, 6.61 (br s, 3H, N*H* from Val, Leu and Ile).

3.8. Synthesis of mPEG-succinyl-valine-leucine-isoleucine-CoQ$_{10}$ (12)

mPEG-succinyl-valine-leucine-isoleucine (**11**) (600 mg, 0.11 mmol) was dissolved in dry dichloromethane (10 mL) and cooled to 0-5 ºC. A solution of CoQ_{10} (ubiquinol) (381 mg, 0.44 mmol) in DMF (5 mL), under nitrogen, DCC (46 mg, 0.22 mmol) and DMAP (20 mg, 0.17 mmol) were mixed, and stirring for 24 h at room temperature. The suspension was filtered and the pH of filtrate was adjusted to 3 with a 2% hydrochloric acid aqueous solution. The excess DCC in the filtrate was decomposed by 10 mL of a 10% acetic acid aqueous solution. The filtrate was then extracted by dichloromethane (10 mL x 3) and the organic phase was washed with saturated brine, dried by anhydrous sodium sulfate, filtered, concentrated and the product was precipitated by ether. mPEG-succinyl-valine-leucine-isoleucine-CoQ_{10} was recrystallized twice from EtOH to give compound **12** as a white powder (410 mg, 58%).^{1}H NMR (500 MHz, C_5D_5N): δ = 0.83-0.95 [m, 15H, 2(C*H*$_3$)$_2$CH of Val and Leu, C*H*$_3$ of Ile), 1.11 [m, 3H, C*H*$_3$CH of Ile], 1.20-1.33 [m, 3H, C*H*(CH$_3$)$_2$ of Leu, C*H*$_2$CH$_3$ of Ile], 1.58 (m, 3H, C*H*$_2$ of Leu), 1.66 (br m, 30H, CH=CH-C*H*$_3$ of CoQ_{10}), 1.98 (s, 3H, C*H*$_3$-Ph of CoQ_{10}), 2.10 (m, 20H, C*H*$_2$CH=CH of CoQ_{10}), 2.19 [m, 2H, C*H*(CH$_3$)$_2$ of Ile, C*H*(CH$_3$)$_2$ of Val], 2.40-2.65 (m, 4H, C*H*$_2$- of succinyl), 3.13 (d, 2H, Ph-C*H*$_2$ of CoQ_{10}), 3.26 (s, 3H, C*H*$_3$O of mPEG), 3.45-3.79 (br m, C*H*$_2$ of mPEG, 2 C*H*$_2$O-Ph of CoQ_{10}), 3.96 (m, C*H*$_2$ of mPEG), 4.26-4.31 (m, 3H, chiral H from Val, Leu and Ile), 5.23-5.38 (m, 11H, C*H*=C*H* of CoQ_{10}), 6.54, 8.40 (br s, 3H, N*H* from Val, Leu and Ile); FT-IR (KBr): υ = 3410, 2865, 1738- 1750, 1580-1680, 1110, 965 cm^{-1}.

3.9. Conjugates stability in simulated extra-cellular fluid and in simulated gastric medium

The study was carried out incubating the compounds **4**, **6** and **12** (10 mg/mL) in phosphate buffer solution (10 mM NaH_2PO_4, 0.15 M NaCl) pH 7.4, in phosphate/citrate buffer (0.2 M) pH 5.5 and artificial gastric juice (Aditya et al.2013) (2 g NaCl, 3.2 g pepsin powder, 80 mL HCl 1 M in 1 L of water) pH 1.2. The solutions were incubated at 37±0.1ºC. Samples (100 µl) were taken at suitable intervals and 800 µl of mobile phase, 100 µl of $FeCl_3$ (1 % solution in ethanol) were added. The samples were sonicated for 15 min, centrifuged at 12,000 rpm for 5 min and the supernatant filtered (Millipore, 45 µm). 20 µl of each sample was analyzed by RP-HPLC on the basis of formation of free CoQ_{10} (ubiquinone). The quantity of CoQ_{10} released by hydrolysis was quantified by HPLC method previously described. Every experiment was repeated in triplicate.

3.10. Enzymatic hydrolysis

The hydrolytic stability of the drug-polymer linkage of the polymeric conjugates to esterase was assessed in 0.08 M Tris buffer, 0.1 M $CaCl_2$ at pH 8 with an adduct 10 mg/mL. Samples of 900 µl of mPEG-succinyl-CoQ_{10} (**4**), mPEG-succinyl-alanine-CoQ_{10} (**6**) and mPEG-succinyl-valine-leucine-isoleucine-CoQ_{10} (**12**) solutions were added to 100 µl of a porcine esterase solution. The solutions were incubated at 37±0.1ºC. Samples (100 µl) were taken at suitable intervals and 800 µl of mobile phase, 100 µl of $FeCl_3$ (1% solution in ethanol) were added. The samples were sonicated for 15 min, centrifuged at 12,000 rpm for 5 min and the supernatant filtered (Millipore, 45 µm). 20 µl of each sample was analyzed by RP-HPLC on the basis of formation of free CoQ_{10} (ubiquinone) (Ferreiro-2012). The quantity of CoQ_{10} released by hydrolysis was quantified by HPLC method previously described. As a control, analogous experiments were performed by adding the buffer solution without the enzyme to the conjugate solutions. Every experiment was repeated in triplicate.

3.11. DPPH discoloration assay

Sample stock solutions (10 mg/mL) were diluted from 0.001 mg to 1 mg/mL final concentrations in 70% methanol. A volume of 0.5 mL of 0.2 mM DPPH ethanol solution was added to 1 mL of each sample solution at different concentrations. These were shaken well by vortex and allowed to react at room temperature. The absorbance values were measured after 10 min at 525 nm by UV/Vis spectrophotometer.

The free-radical scavenging activity of samples was calculated according to the following formula:

DPPH radical scavenging activity (%) = [^{1} – (*Abs* sample – *Abs* blank)/ *Abs* control] x 100 where *Abs* sample is the absorbance of the experimental sample, *Abs* blank is the absorbance of the blank and *Abs* control is the absorbance of the control.

As a blank, 70% methanol (0.5 mL) and sample solution (1.0 mL) were used. DPPH solution (0.5 mL, 0.2 mM) with 70% methanol (1.0 mL) was used as a negative control. Ascorbic acid (standard solution) was used as a positive control. Each treatment was replicated three times.

Conflicts of interest: None declared.

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