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Liposomes derivatized with tetrabranched neurotensin peptides *via* click chemistry reactions†

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Liposomes decorated with neurotensin tetramers are obtained by using a post-liposomal derivatization method in which a click-chemistry reaction between liposomes containing azido functions on the external surface, DOPC/$(C18)_2$-Peg9-N_3 (90/10), and branched neurotensin peptides modified for the presence of a C≡C triple-bond [$(NT8\text{-}13)_4$-alkyne] is performed. Results show that the post liposomal derivatization method is very efficient and that click-chemistry procedures are very attractive for nanoparticle functionalization. A structural characterization of liposomes has been performed by dynamic light scattering (DLS) measurements. The hydrodynamic radii of pure DOPC and mixed DOPC/$(C18)_2$-Peg9-N_3 and DOPC/$(C18)_2$-Peg-triazole-$(NT8\text{-}13)_4$ liposomes (at 90 : 10 molar ratio) are 61 ± 21 nm, 60 ± 22 nm and 82 ± 43 nm, respectively. A very efficient doxorubicin loading has been observed, specially for DOPC/$(C18)_2$-Peg9-N_3 liposomes, with a drug loading content of 90%.

Introduction

Nowadays, nanoscopic (10–500 nm) systems such as polymeric micelles, dendrimers, liposomes and inorganic nanoparticles that incorporate therapeutic agents, diagnostic probes and molecules for selective targeting are emerging as the next generation of multifunctional nanomedicines for improving the therapeutic outcome of drug therapy.[1] The motivations for the widespread application of nanomedicine depend on the potential advantages of nanosystems with respect to conventional small molecule-based therapy: high payload capacity, reduced toxicity to healthy tissue, and improved antitumor efficacy.[2] Moreover, nanosystems can selectively extravasate into tumor tissue more rapidly than drugs at low molecular weight and can remain in circulation for an extended period of time when injected intravenously.[3,4] This phenomenon, identified as ''enhanced permeability and retention (EPR) effect'',[5] represents a form of selective delivery termed 'passive targeting'. More recently, several authors have highlighted the importance of using targeted liposomes functionalized with bioactive peptides to realize an active targeting to the cancerous organs.[6–12] Many synthetic strategies have been established by modifying liposome surfaces with targeting peptide sequences.[13] The obvious goal of each approach is to achieve high coupling efficiency, leaving the bioactive peptide far away from the liposome surface and in its right specific conformation ready for binding to the target. The main difference between the different approaches concerns the introduction of the bioactive peptide on the liposome surface: the peptide should be introduced directly during liposome formulation by using a peptide amphiphile, or after liposome preparation by modifying the liposome surface. Peptide coupling after liposome preparation involves the introduction of suitable activated functional groups into the external side of the liposomes for covalent or non-covalent binding of the peptide. Functional groups commonly used are: (a) amines for the amine-*N*-hydroxy-succinamide coupling method, (b) maleimide for Michael addition, (c) azides for Cu(I)-catalyzed Huisgen cycloaddition (CuAAC), (d) biotin for non-covalent interaction with avidin or triphosphines for Staudinger ligation.[13] The synthetic strategy to adopt should be opportunely selected in order to increase the reproducibility and the yield of the coupling reaction.

We previously reported the structural characterization and the *in vitro* behaviour of DOPC liposomes containing the cytotoxic drug Doxorubicin (Doxo) functionalized on the external surface with linear or tetrabranched form of truncated neurotensin peptide NT(8-13).[14,15] This C-terminal NT peptide fragment is well known for its ability to mimic the NT peptide in receptor binding.[16,17] Target liposomes were prepared by mixing together commercial DOPC phospholipids and synthetic $(C18)_2$-L(NT8-13) or $(C18)_2$Lys$(NT8\text{-}13)_4$ peptide amphiphiles (PAs). The selective internalization of NT-derivatized liposomes, compared to nude liposomes, was tested in HT29 human colon adenocarcinoma and TE671 human rhabdomyosarcoma by confocal microscopy. Cytotoxicity of NT4-liposomal doxorubicin increased four-fold

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with respect to untargeted DOPC-Doxo liposomes in both cell lines. Results from fluorescence-activated cell sorting (FACS) analysis are in line with these results, resulting in a fluorescence signal increase of the NT4 liposomes as compared to the nude analogues. Moreover, liposomes decorated with the branched peptide showed a better profile in drug delivery, with respect to liposomes decorated with the corresponding monomeric peptide.

This achievement might be seen as a general approach to increase the exposure of carriers on the surface of the nanoparticles, which is a frequently wanted matter. The use of multimeric peptides as decorating units of nanoparticles has, eventually, the advantage of increasing the amount of hydrophilic carrier to be exposed to target cells. Recently, peptide tetramerization approach for liposome delivery has been successfully applied also for peptides selected by phage display (αvβ6-specific H2009.1 peptide).[18] Besides, the presentation of peptides in a grouped shape facilitates binding and fusion of nanoparticles to cells.

Here, we describe the preparation of liposomes decorated with NT tetramers obtained by using a click-chemistry reaction between liposomes containing azido functions on the external surface and branched neurotensin peptides modified for the presence of a C≡C triple-bond [$(NT8\text{-}13)_4$-alkyne].[19,20] Structural characterization and drug loading capability of targeted liposomes are also reported.

Results and discussion

Design and synthesis of liposomes decorated with branched NT8-13

Surface functionalization of supramolecular aggregates such as liposomes and micelles with bioactive peptides can be achieved introducing peptide amphiphiles (PAs) directly during the formulation, in the lipid film preparation step. PAs can be synthesized in solid phase by coupling one or more alkyl chains to a functional group of the peptide. Nevertheless, the solid phase peptide synthesis (SPPS) approach works well only for short and hydrophilic peptide sequences. More complexes and hydrophobic sequences, in which the low solubility of the peptide is combined with the high hydrophobicity of the alkyl chains, require the development of alternative methodologies for synthesis, purification and collection of pure compounds.[21] Moreover, when a dimeric or a tetrameric form of the peptide is required to increase its biological activity (*i.e.* neurotensin), the final yield of the purified amphiphilic product could significantly decrease. In these cases, PAs should be synthesized by using a divergent approach or by combining solid phase and solution approaches. As alternative to the PA preparation, different strategies for liposomes or micelles bioconjugation can be exploited.[13,22] Peptide coupling can be performed after liposomes assembly by using activated functional groups introduced into the external side of the liposomes, according to a post-liposomal derivatization method. Functional groups should remain available on the surface for an efficient chemical ligation of the peptide. The application of click chemistry is particularly appealing because of its regiospecificity, chemoselectivity, and tolerance to a wide variety of other functional groups. The external surface of preformed DOPC liposomes containing 10 mol% of the $(C18)_2$-Peg9-N_3 azido-derivative could be modified with four copies of the NT8-13 peptide [$(NT8\text{-}13)_4$] by using click reaction in aqueous solution with stoichiometric amounts of tetrameric peptide $(NT8\text{-}13)_4$ bearing a propargylglycine moiety [$(NT8\text{-}13)_4$-alkyne].

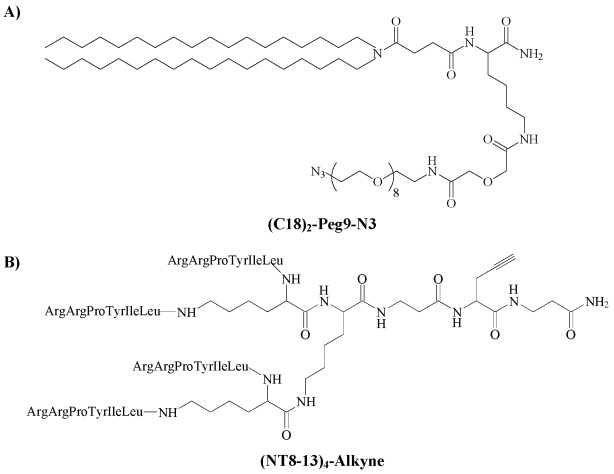
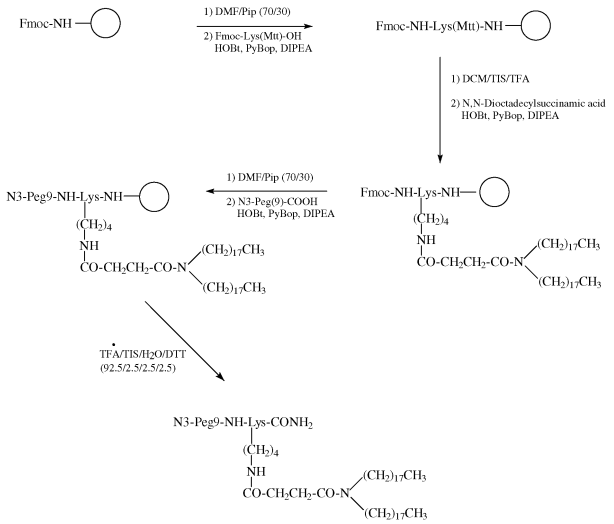


Fig. 1 Schematic representation of $(C18)_2$-Peg9-N_3 and $(NT8\text{-}13)_4$-alkyne.

Both the reactants used in the click-chemistry process [$(NT8\text{-}13)_4$-alkyne and $(C18)_2$-Peg9-N_3], schematized in Fig. 1, were synthesized by SPPS with Fmoc/*t*Bu chemistry according to standard protocols. Chemical synthesis of $(C18)_2$-Peg9-N_3 was performed on rink amide resin, according to Scheme 1. As previously reported for other amphiphilic azido-derivatives, no reductive side reactions were observed during the cleavage step of the product from the resin.[23] $(NT8\text{-}13)_4$-alkyne was synthesized on Novasyn TGR resin with a low functionalization degree. Low density of anchorage functional groups is suitable to avoid interactions



Scheme 1 Scheme for the solid-phase synthesis of the $(C18)_2$-Peg9-N_3 monomer. The rink-amide resin is schematically represented as an empty circle.

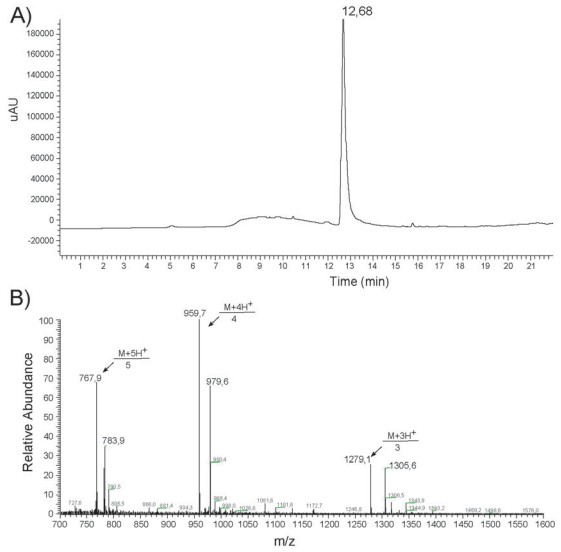


Fig. 2 Analytical characterization by LC-MS of $(NT8\text{-}13)_4$-alkyne. (A) HPLC profile; (B) MS spectrum showing peaks at 1279.1 ($[M + 3H^+]/3$), 959.7 ($[M + 4H^+]/4$) and 767.9 ($[M + 5H^+]/5$) and their corresponding satellite peaks, representing the $[M + H^+ + 2K^+]/3$, the $[M + 2H^+ + 2K^+]/4$ and the $[M + 3H^+ + 2K^+]/5$, respectively.

between the growing peptide chains thus increasing synthetic yields. Four copies of NT8-13 peptide were assembled, step by step, on a core matrix of a branched lysine scaffold. The core matrix also contains the alkyne functional group for the presence of a propargylglycine residue introduced between the solid support and the lysine residue. The high soluble crude product was easily purified by RP-HPLC chromatography and obtained in good yields (Fig. 2). Hence, both reactants were collected in good yields (85% and 50%, respectively) after HPLC-RP purification, and analyzed by mass spectrometry (MS-ESI), ^1H and ^{13}C NMR spectroscopy (for $(C18)_2$-Peg9-N_3), and HPLC to confirm compound identities and their purity.

Liposome formulation and characterization

Mixed DOPC/$(C18)_2$-Peg9-N_3 (90/10) liposomes were obtained according to standard procedures[14] in which a lipid film of the two monomers in the chosen ratio was hydrated by addition of 10 mM HEPES buffer at pH 7.4, sonicated, and extruded through a polycarbonate membrane with 100 nm pore size. The number of functional groups present on the liposomal surface was checked by using 7-nitrobenzofurazan (NBD)-labeled Pra fluorescent derivative as previously described.[21] Results confirm that about 50% of the azido functions are present on the external surface of the liposome. The bioconjugation reaction was carried out after liposome formulation. This strategy allows us to have the bulky peptide derivative only on the liposome external surface, leaving the liposome inner compartment empty, thus available to load a large amount of Doxorubicin. Click chemistry reaction was carried out on DOPC/$(C18)_2$-Peg9-N_3 liposomes at 1×10^{-3} M concentration in 10 mM HEPES buffer, by adding $CuSO_4 \cdot 5H_2O$, ascorbic acid, and the peptide derivative 1 equivalent of $(NT8\text{-}13)_4$-alkyne with respect to the azido moiety, as schematized in Fig. 3. After click chemistry reaction, liposomes were separated from

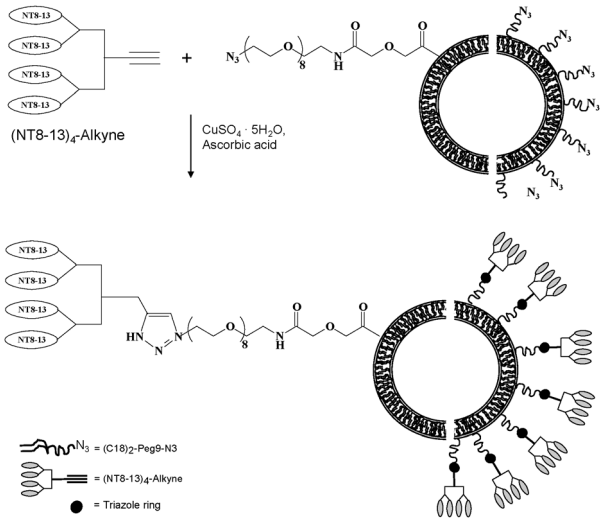


Fig. 3 Coupling of the $(NT8\text{-}13)_4$-alkyne tetramer to the azide-functionalized liposomes by a click-chemistry reaction.

free alkyne-peptides and from copper ions by gel-filtration and the conjugation efficiency was calculated by the RP-HPLC calibration curve as the ratio of unbound $(NT8\text{-}13)_4$-alkyne to its total amount. The results confirm that the yield of the reaction was higher than 95% after 12 h at room temperature. The liposomal fraction was then treated with a Triton solution 10× and $(C18)_2$-Peg9-triazole-$(NT8\text{-}13)_4$ was isolated and characterized by LC-MS and UV-Vis spectroscopy (Fig. 4). The product isolated at 17.15 minutes in a preparative HPLC course corresponds the product of the click reaction, with a typical UV/Vis spectrum in which there are three absorbance peaks: the peak at 210 nm relative to the amide bond, the peak at 267 nm due to four tyrosine residues present in the tetrabranched

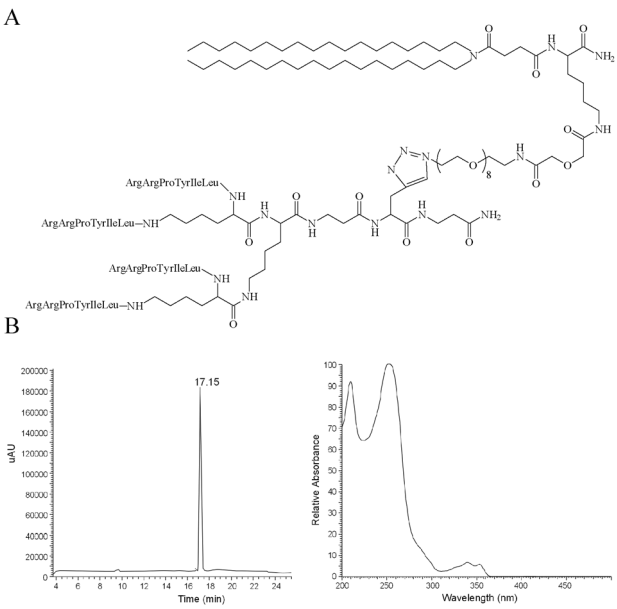


Fig. 4 (A) Schematic representation of $(C18)_2$-Peg9-triazole-$(NT8\text{-}13)_4$ and (B) its HPLC profile and the UV/Vis spectrum.

neurotensin, and the peak at 350 nm of the triazole ring. Finally, the mass spectrum confirms the identity of the product.

The size of pure DOPC liposomes, mixed DOPC/$(C18)_2$-Peg9-N_3 (90/10) and DOPC/$(C18)_2$-Peg9-triazole-$(NT8\text{-}13)_4$ (90/10) liposomes, *i.e.* before and after click-chemistry reaction, was assessed by dynamic light scattering (DLS). Measurements were performed at $\theta = 173^\circ$ on liposomes at 2×10^{-4} M concentration in 10 mM HEPES buffer at pH 7.4. Hydrodynamic radius (RH) distribution functions of liposomes are reported in Fig. 5 and RH, diffusion coefficients and polydispersity index (P.I.) of all systems are summarized in Table 1. Inspection of Fig. 5 shows that all liposome solutions present a monomodal distribution due to a translational diffusion process, which could be attributed to liposome aggregates. The hydrodynamic radii of pure DOPC and mixed DOPC/$(C18)_2$-Peg9-N_3 and DOPC/$(C18)_2$-Peg-triazole-$(NT8\text{-}13)_4$ liposomes (at 90 : 10 molar ratio) are 61 ± 21 nm, 60 ± 22 nm and 82 ± 43 nm, respectively. As expected, the size of mixed DOPC/$(C18)_2$-Peg9-N_3 liposomes is quite similar to the size of pure DOPC liposomes, thus confirming that the introduction of a small amount of the synthetic monomer does not influence the size of the DOPC liposomes.[22] After click chemistry reaction, a structural evolution of liposomes occurs (see Fig. 5) and an increase of diameter from 60.0 nm to 82.1 nm was observed for DOPC/$(C18)_2$-Peg-triazole-$(NT8\text{-}13)_4$ liposomes, as expected for the presence of the tetrameric NT peptides well exposed on the outer liposome surface (Table 1).

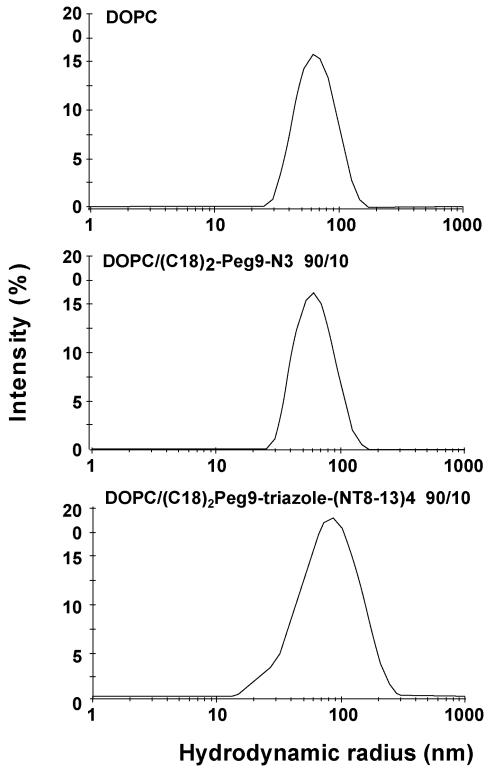


Fig. 5 DLS spectra of DOPC/$(C18)_2$-Peg9-N_3 (90/10) at 25 °C before and after the functionalization by click-chemistry reaction. DLS spectrum of pure DOPC liposomes is also reported for comparison.

Doxorubicin encapsulation

The cytotoxic drug doxorubicin (Doxo) was loaded into pure DOPC liposomes and into mixed liposomes before (DOPC/$(C18)_2$-Peg9-N_3) and after (DOPC/$(C18)_2$-Peg-triazole-$(NT8\text{-}13)_4$) their functionalization by click-chemistry reaction. In all systems, drug encapsulation was performed using the ammonium gradient method[24] and an initial drug(wt)/lipid(wt) ratio of 0.100. The Doxo loading content values (DLC), reported in Table 2, were calculated by fluorescence measurements with subtraction of the amount of free doxorubicin, eluted by gel filtration, from the total amount of initial doxorubicin. The Doxo loading contents (DLC) defined as the weight ratio of the encapsulated Doxo *versus* the amount of the amphiphilic molecules forming liposomes were 0.095 for pure DOPC liposomes and 0.090 for mixed DOPC/$(C18)_2$-Peg9-N_3 liposomes corresponding to 95% and 90%, respectively, of the used doxorubicin. Only a slight decrease of DLC is found when $(C18)_2$-Peg9-N_3 is co-assembled with DOPC forming liposomes. In contrast, DOPC/$(C18)_2$-Peg-triazole-$(NT8\text{-}13)_4$ liposomes, in which the outer liposomal surface is partially hindered by four copies of the neurotensin peptides, show a lower DLC value of 0.078 (78% of the used Doxo).

This value is higher with respect to that found for DOPC/$(C18)_2$Lys$(NT8\text{-}13)_4$ liposomes (DLC of 0.065 corresponding to 65% of the used Doxo) prepared by using $(C18)_2$Lys$(NT8\text{-}13)_4$ peptide amphiphile directly during the formulation step.[15] In fact, in the latter case, both inner and outer liposome surfaces

Table 2 Drug loading content (DLC, defined as the ratio between weight of encapsulated Doxo and the total weight of amphiphilic molecules forming liposomes), for the different formulations. In all experiments an initial drug(wt)/lipid(wt) ratio of 0.100 was used

Systems	DLC
DOPC	0.095
DOPC/$(C18)_2$-Peg9-N_3	0.090
DOPC/$(C18)_2$-Peg9-triazole-$(NT8\text{-}13)_4$	0.078
DOPC/$(C18)_2$Lys$(NT8\text{-}13)_4$	0.065[a]

[a] Value from ref. 15.

Table 1 Structural parameters (hydrodynamic radii, diffusion coefficients and polydispersity indexes) obtained from dynamic light scattering measurements for the studied systems

Systems	R_H/nm	$D \times 10^{12}$ m^2 s^{-1}	P.I. ± S.D.
DOPC	62 ± 21	3.3 ± 1.1	0.079 ± 0.002
DOPC/$(C18)_2$-Peg9-N_3	60 ± 22	3.3 ± 1.2	0.095 ± 0.005
DOPC/$(C18)_2$-Peg9-triazole-$(NT8\text{-}13)_4$	82 ± 43	2.4 ± 1.2	0.240 ± 0.015

are obstructed by NT tetramers, thus comporting a space decrease of the inner liposomal compartment. The above reported data suggest that a very efficient loading procedure to obtain Doxo liposomes covered by tetrameric NT peptides could be based on a two-step process: in a first step doxorubicin should be loaded in DOPC/$(C18)_2$-Peg9-N_3 liposomes (90% of loading efficiency), and, then, in a second step Doxo loaded liposomes could be derivatized by click chemistry reaction with tetrameric NT peptides.

Conclusions

Liposomes decorated with bioactive peptides can be prepared by using several synthetic approaches: an approach consists of the self-assembling process of phospholipids with peptide amphiphiles (PAs), whereas the other method needs a post-modification of the liposomes, introducing the bioactive peptides on the external liposome surface. In first approximation, for basic studies, both approaches are always possible, but when the final objective is the preparation of a new liposomal drug for clinical studies or for the market, the scaling up process should be taken into consideration. In these cases the choice of one procedure with respect to the other one is a critical decision and it depends on a preventive study of the properties of the peptide sequence (length and hydrophobicity). When the peptide required for binding is represented by poorly soluble sequences or by branched peptide forms, the post-liposomal derivatization method should be preferred. By using the post-liposomal derivatization method two different compounds should be synthetically prepared: an amphiphilic compound modified on its hydrophilic head with a reactive moiety, and a peptide derivative containing the other reactant function on its N- or C- terminal end. The synthesis of two different molecules could be more efficient, in terms of yield and final purity, with respect to the synthesis of a single, but very complex, amphiphilic molecule containing both a bioactive peptide and the hydrophobic moiety based on hydrocarbon chains. This is especially true when high amounts are requested. We have described the preparation of liposomes decorated with neurotensin tetramers obtained by using the post-liposomal derivatization method based on a click-chemistry reaction between liposomes containing azido groups on the external surface and branched neurotensin peptides derivatized with an alkyne function [$(NT8\text{-}13)_4$-alkyne]. Using this procedure we have obtained DOPC/$(C18)_2$-Peg9-triazole-$(NT8\text{-}13)_4$ liposomes, very similar to the already published DOPC/$(C18)_2$Lys$(NT8\text{-}13)_4$ liposomes that showed to be very efficient target selective drug delivery systems for tumour cells expressing neurotensin receptors,[15] with the additional advantage of a higher doxorubicin loading content with respect to the previous system.

Results show that for branched peptides, also if short peptide sequences are employed, as for the truncated NT form (NT8-13), the post liposomal derivatization method is very efficient. Moreover, the obtained data indicate that click-chemistry based reactions are very attractive procedures to be employed in nanoparticle functionalization. Finally, the post-liposomal derivatization method allows us to obtain liposomes in which bioactive peptides are present only on the external surface, leaving more space in the liposome inner compartment thus allowing a very high drug loading content.

Experimental

Materials and instrumentation

Fmoc-protected amino acid derivatives, coupling reagents, and resins (rink amide *p*-methylbenzhydrylamine MBHA and NovaSyn® TGR) were purchased from Calbiochem–Novabiochem (Laufelfingen, Switzerland). Fmoc-L-propargylglycine (Fmoc-Pra-OH) was purchased from Neosystem (Strasbourg, France). The 14-azido-5-oxo-3,9,12-trioxa-6-azatetradecan-1-oic acid (N_3-Peg(9)-COOH) was purchased by Iris Biotech GmbH (Marktredwitz, Germany). (8*S*,10*S*)-10-(4-amino-5-hydroxy-6-methyl-tetrahydro-2*H*-pyran-2-yloxy)-6,8,11-trihydroxy-8-(2-hydroxyacetyl)-1-methoxy-7,8,9,10-tetra hydro-tetracene-5,12-dione; (doxorubicin HCl) was purchased from Sigma-Aldrich. 1,2-Dioleoyl-*sn*–*glycero*-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). *N,N*-Dioctadecylsuccinamic acid was synthesized according to a published procedure.[25] All other chemicals were commercially available by Sigma-Aldrich, Fluka (Buchs, Switzerland), or LabScan (Stillorgan, Dublin, Ireland) and were used as received, unless otherwise stated. Preparative HPLC was carried out on a LC8 Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a UV lambda-Max Model 481 detector using a Phenomenex (Torrance, CA) C18 (300 Å, 250 × 21.20 mm, 5 μ) column eluted with H_2O/0.1% TFA (A) and CH_3CN/0.1% TFA (B) from 5–70% over 15 min at a flow rate of 20 mL min^{-1}. Purity and identity were assessed by analytical LC-MS analyses by using Finnigan Surveyor MSQ single quadrupole electrospray ionization (Finnigan/Thermo Electron Corporation San Jose, CA), column: C18-Phenomenex eluted with H_2O/0.1% TFA (A) and CH_3CN/0.1% TFA (B) from 5–70% over 30 min at a flow rate of 0.8 mL min^{-1}. UV measurements were performed on a UV/vis Jasco V-5505 spectrophotometer equipped with a Jasco ETC-505T Peltier temperature controller with a 1 cm quartz cuvette (Hellma). Fluorescence spectra were recorded at room temperature on a Jasco Model FP-750 spectrofluorophotometer in a 1.0 cm path length quartz cell. Equal excitation and emission bandwidths were used throughout the experiments, with a recording speed of 125 nm min^{-1} and automatic selection of the time constant. The liposomes were extruded using a mini-extruder purchased from Avanti Polar Lipids. ^1H NMR and ^{13}C NMR spectra were recorded on a Varian 400 MHz spectrometer (Palo Alto, CA).

Synthesis of azide-Peg9-Lys(C(O)CH_2CH_2C(O)N

$(C_{18}H_{37})_2$)-amide $(C18)_2$-Peg9-N_3. The $(C18)_2$-Peg9-N_3 monomer was synthesized in the solid phase under standard conditions by using the Fmoc/*t*Bu strategy. Rink-amide MBHA resin (0.51 mmol g^{-1}, 0.1 mmol, 0.196 g) was used as a polymeric support. The Fmoc protecting group on the resin was removed by a DMF–piperidine (70 : 30) mixture. Fmoc-Lys(Mtt)-OH (0.125 g, 0.2 mmol) was activated with PyBop (1 equiv.) and HOBt (1 equiv.) and DIPEA (2 equiv.) in DMF and coupled to the resin, while stirring the suspension for 1 h. The solution was

filtered and the resin was washed four times with DCM; the Mtt protecting group on the Nε-amino function of lysine was removed by treatment with DCM–TIS–TFA (94 : 5 : 1, 5.0 mL) for 2 min. This procedure was repeated several times until the solution became colorless. The resin was washed three times with DCM and three times with DMF. Then, *N,N*-dioctadecylsuccinamic acid (0.249 g, 0.4 mmol) was coupled according to the previously published procedure.[26] Successively, after Fmoc removal, N_3-Peg(9)-COOH (2 equiv.) was coupled under standard conditions in DMF for 1 h to the Nα terminus of the lysine residue. The product was removed from the resin by treatment with TFA containing TIS (2.5%), dithiothreitol (DTT, 2.5%), and water (2.5%) under vortexing for 2 h. The crude product was slowly precipitated at 0 °C by adding water drop-wise. The precipitate was washed several time with small portions of water and lyophilized in order to remove the solvent. The white solid was recrystallized from MeOH/H_2O and recovered with high yields (>85%). The product was identified by MS (ESI+) and NMR spectroscopy.

$(C18)_2$-Peg9-N_3. ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$ 50 : 50): d = 4.1 (m, 4H; OCH_2CONH, OCH_2CONH), 3.9 (m, 1H; CH Lysα), 3.70 (s, 28H; OCH_2CH_2O), 3.4 (m 2H; $N_3CH_2CH_2O$,), 3.25–3.20 (m, 8H; N–CH_2 $NHCH_2CH_2O$), 3.20 (m, 2H; CH_2Lysε, 2H; $N_3CH_2CH_2O$), 2.64–2.49 (m, 4H; $NHCOCH_2CH_2CO$), 1.74 (m, 2H; CH_2Lysβ), 1.55 (m, 2H; CH_2Lysδ), 1.45 (m, 2H; CH_2Lys4), 1.40 (m, 4H; RCH_2CH_3), 1.25 (m, 60H; CH_2 aliphatic), 0.88 ppm (t, 6H; CH_3);

^{13}C NMR (100.512 MHz, $CDCl_3/CD_3OD$ 50 : 50): d = 173.98 ($CONH_2$), 173.27 (CONH), 171.94 ($CH_3(CH_2)_{17}NCO$), 170.55 (CONH), 72.0 ($COCH_2O$), 71.0 (CH_2N_3), 70.4 (OCH_2CH_2O), 52.4 ($NH(CH_2)_4CH$), 51.0 ($NHCH_2(CH_2)_3CH$), 48.6 ($CH_3(CH_2)_{16}CH_2N$), 46.8 ($CH_3(CH_2)_{15}CH_2CH_2N$), 39.3 ($NH(CH_2)_3CH_2CH$), 32.4–32.0 ($NHCH_2(CH_2)_2CH_2CH$), 29.2($NCOCH_2CH_2CONH$), 30.2–27.5 ($CH_3CH_2(CH_2)_{14}CH_2CH_2N$), 23.17 ($CH_3CH_2(CH_2)_{16}$), 14.60 ppm ($CH_3CH_2(CH_2)_{16}$)MS (ESI+): m/z (%) calcd for C_{68} $H_{132}N_8O_{14}$ [M − H^+]:1284.5; found: 1285.6 (100).

Synthesis of $(NT8\text{-}13)_4$-alkyne

$(NT8\text{-}13)_4$-alkyne was synthesized in the solid phase under standard conditions by using the Fmoc/*t*Bu strategy. Novasyn TGR resin (0.24 mmol g^{-1}, 0.1 mmol, 0.420 g) was used as a polymeric support. Fmoc-protected amino acids (4 equiv. relative to resin loading) were coupled according to the PyBop/HOBt/DIPEA method: Fmoc amino acid (1 equiv.), PyBOP (1 equiv.), HOBt (1 equiv.) and DIPEA (2 equiv.) in DMF. The Fmoc protecting group was removed with 30% piperidine in DMF (v/v). All couplings were performed twice for 1 h. Fmoc-Pra-OH was coupled once for 45 min with 2 equivalents of PyBop/HOBt and 2 equivalents of DIPEA. At the end of the core peptide synthesis, the four copies of NT8-13 peptide were synthetized by adding amino acids step by step. The tetrabranched peptide was fully deprotected and cleaved from the resin with TFA containing triisopropylsilane (2.5%), and water (2.0%) as scavengers, at room temperature for 2 h, and then precipitated with ice-cold ethyl ether, filtered, dissolved in water, and lyophilized. The crude alkyne derivative was purified by RP-HPLC. Purity and identity were assessed by analytical LC-MS analyses. The final product was obtained in 70% yield and analyzed by LC-MS: Rt = 12.68; MS (ESI+): m/z (%) calcd [M − H^+]: 3835; found: [M + $3H^+$]/3 = 1279.1; [M + $4H^+$]/4 = 959.7; [M + $5H^+$]/5 = 767.9

Liposome preparation and DLS characterization

All solutions were prepared by weight and buffered at pH 7.4 using 10 mM HEPES buffer. The pH was controlled using a pH meter (MeterLab PHM220). Mixed aggregates of DOPC/$(C18)_2$-Peg9-N_3 (at 90 : 10 molar ratio) were prepared as previously reported.[14] Briefly, the two amphiphiles were dissolved in a small amount of MeOH/$CHCl_3$ (50 : 50); subsequently a thin film of amphiphiles was obtained by evaporating the solvent by slowly rotating the tube containing the solution under a stream of nitrogen. Lipid film was hydrated by addition of 10 mM HEPES buffer (pH 7.4), sonicated for 30 min and extruded 21 times through a polycarbonate membrane (100 nm pore size) by using a mini-extruder purchased from Avanti Polar Lipids (USA, Canada). After dilution of samples at 2.0×10^{-4} M concentration and centrifuged at room temperature at 13 000 rpm for 5 min, hydrodynamic radii (R_H) were measured by Dynamic light scattering. DLS measurements were carried out using a Zetasizer Nano ZS (Malvern Instruments, Westborough, MA) that employs a 173° backscatter detector. Other instrumental settings are measurement position (mm): 4.65; attenuator: 8; temperature 25 °C; cell: disposable sizing cuvette. The number of azido functions present on the outer surface of the liposomes was determined by using NBD-labeled Pra. After click-chemistry reaction, the amount of NBD-Pra bound to the liposome surface was estimated by fluorescence spectroscopy by using the calibration curve obtained by measuring the fluorescence emission at λ = 530 nm.

Procedure for "Click" reaction

The Click reaction was carried out on DOPC/$(C18)_2$-Peg9-N_3 liposomes at 1×10^{-3} M concentration in HEPES buffer. The reaction was carried out by adding 4.4 equiv. of $CuSO_4 \cdot 5H_2O$, 6.7 equiv. of ascorbic acid, and 1 equiv. of the peptide derivative with respect to the azido moiety. In particular, solutions containing $CuSO_4 \cdot 5H_2O$ (40 mM, solution A), ascorbic acid (57 mM, solution B), and the alkyne-modified peptide (1 mM, solution C) were freshly prepared in water. Solution A (16.5 μL), solution B (17.5 μL), and solution C (0.150 mL) were added to a suspension of azido-functionalized liposomes in HEPES buffer (3.0 mL). The concentration of solution C was determined by measuring the absorbance at 267 nm (ε_{Tyr} = 5400) on a UV/Vis Jasco V-5505 spectrophotometer. The reaction mixture was stirred at 40 °C for 30 min and successively left overnight at room temperature. After the conjugation step the peptide-bound liposomes were separated from the unbound peptide using Sephadex G-50 (Amersham Biosciences) column pre-equilibrated with HEPES buffer. The number of $(NT8\text{-}13)_4$ molecules effectively linked to the liposomal surface was determined by using the RP-HPLC calibration curve. The percentage of functionalization was calculated as the ratio of bound NT8-13 to their total amount. The click-chemistry product, $(C18)_2$-Peg9-triazole-$(NT8\text{-}13)_4$, was isolated by HPLC after treatment of the liposomal fraction with Triton 10X (10 μL); it was then characterized by RP-HPLC and UV-Vis spectroscopy. Rt: 17.15 min; MS (ESI+): m/z (%) [M − H^+]:5119; found: [M + $3H^+$]/3 = 1707.1; [M + $4H^+$]/4 = 1280.1; [M + $5H^+$]/5 = 1024.8.

Doxorubicin loading

Doxorubicin was remote-loaded in pure DOPC, in mixed DOPC/$(C18)_2$-Peg9-N_3 (90/10) liposomes, and in mixed DOPC/$(C18)_2$-Peg9-triazole-$(NT8\text{-}13)_4$ (90/10) liposomes, by using the ammonium sulfate gradient method.[24] Briefly, a liposomal solution (1 mM) was prepared as above reported in an ammonium sulfate solution (250 mM) at pH 5.5. Liposomes were eluted on a Sephadex G-50 column pre-equilibrated with HEPES buffer (10 mM) at pH 7.4, then 68 μL of doxorubicin from an aqueous stock solution (1.78×10^{-3} M) were added to 1.0 mL of liposomes in order to reach a drug weight/lipid weight ratio of 0.100. The suspension was stirred for 30 min at 60 °C and subsequently unloaded Doxo was removed by gel-filtration on a Sephadex column. The Doxo concentration in all experiments was determined by UV-Vis spectroscopy using calibration curves obtained by measuring absorbance at λ = 480 nm. The drug loading content (DLC, defined as the weight ratio of encapsulated Doxo *versus* the amphiphilic molecules forming liposomes) was quantified by subtraction of the amount of removed Doxo from the total amount of loaded Doxo.

Abbreviations

DIPEA	*N,N*-Diisopropylethylamine
DLS	Dynamic light scattering
DMF	Dimethylformammide
DOPC	Dipalmitoyl phosphatidyl glycerol
Doxo	Doxorubicin
DTT	Dithiothreitol
ESI-MS	Electrospray ionization-mass spectrometry
FACS	Fluorescence-activated cell sorting
Fmoc	9-Fluorenylmethoxycarbonyl
HOBt	1-Hydroxybenzotriazole
MBHA resin	4-Methylbenzhydrylamine resin
Mtt	4-Methyltrityl
Pip	Piperidine
Pra	Propargylglycine
PyBOP	Benzotriazole-1-yl-oxytrispyrrolidinophosphonium hexafluoro phosphonate
RP-HPLC	Reverse phase-high performance liquid chromatography
SPPS	Solid-phase-peptide-synthesis
TFA	Trifluoracetic acid
TIS	Triisopropylsilane

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