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ORIGINAL ARTICLE

Synthetic peptide-labelled micelles for active targeting of cells overexpressing EGF receptors

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Abstract

The goal of nanomedicine is to transport drugs to pathological tissues, reducing side effects while increasing targeting and efficacy. Aggregates grafted by bioactive molecules act as the active targeting agents. Among bioactive molecules, peptides, which are able to recognize overexpressed receptors on cancer cell membranes, appear to be very promising. The aim of this study was to formulate analog peptide-labeled micelles enabled to potentially deliver highly hydrophobic drugs to cancer cells overexpressing epidermal growth factor (EGF) receptor (EGFR). The selected synthetic peptide sequences were anchored to a hydrophobic moiety, aiming to obtain amphiphilic peptide molecules. Mixed micelles were formulated with Pluronic® F127. These micelles were fully characterized by physico-chemical methods, estimating the critical micellar concentration (CMC) by fluorescence. Their sizes were established by dynamic light scattering (DLS) analysis. Then, micelles were also tested in vitro for their binding capacity to human hepatocellular carcinoma (HCC) cell lines overexpressing EGFR.

Keywords Peptide · Peptide backbone structures · Drug delivery · Peptide self-assembling carriers · Active targeting receptors · Binding peptides · Epidermal growth factor · Epidermal growth factor receptor · Hepatocellular carcinoma

Introduction

In drug administration, two major limits are well-known: drug degradation and lack of selectivity. The latter is a drawback that can cause numerous side effects in healthy tissues. In addition, decreased drug bioavailability and lower percentages of drugs accumulated in pathological tissues are often observed. Another tricky issue is the poor aqueous solubility of many drugs. Drug loading onto nanocarriers can prevent these inconveniences and also facilitate delivery to unhealthy tissues by increasing the blood circulation time.

In recent years, several types of carriers, such as micelles, albumin core–shell nanoparticles (NPs) and nanostructured lipid carriers (NLC), have been developed to efficiently deliver hydrophobic drugs, including conventional chemotherapeutic agents, e.g., paclitaxel (PTX) (Liggins and Burt 2002; Tang et al. 2018, Upponi et al. 2018), and targeted agents, e.g., Sorafenib (Cervello et al. 2017; Bondi et al. 2015) and erlotinib (Hsu et al. 2018).

A promising nanomedicine-based technology is the use of polymeric micelles, which have a core–shell structure incorporating poorly soluble drugs that defends them from inactivation in biological fluids (Torchilin, 2001). Due to their small particle size (< 100 nm), polymeric micelles exhibit many advantages that can overcome previously described drawbacks: better targeting ability, longer circulation in vivo and more effective drug delivery (Varela-Moreira, et al. 2017; Oerlemans et al. 2010; Trubetskoy 1999).

More recently, many systems have been assessed in clinical trials as carriers for hydrophilic and hydrophobic anticancer chemotherapeutics (Varela-Moreira et al. 2017; Cabral and Kataoka 2014). Among the polymeric nanocarriers, the most broadly investigated have been amphiphilic block copolymers (Kozlov et al. 2000; Chiappetta and Sosnik 2007; Mohammadi et al. 2017): poly(ethylene

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oxide)–poly(propylene oxide) (PEO–PPO–PEO), containing a hydrophobic region (poly-propyleneoxide, PPO) and two hydrophilic portions (poly-ethyleneoxide, PEO). At low concentration, this peculiar structure can self-assemble into spherical micelles. In addition, the PEO sterically stabilizes the micelles and makes them more resistant to opsonization and to uptake by the macrophages of the reticuloendothelial system (RES), allowing them to circulate in the blood for longer (Moghimi et al. 1993). These characteristics widely fit with non-ionic surfactant Pluronic® F127 (PF127), which in aqueous solution is able to aggregate in micelles (Kabanov et al. 2002). Physico-chemical techniques have established the properties of these micelles, such as size, shape and temperature-changing behavior dependence (Basak and Bandyopadhyay 2013). These properties allow NPs to accumulate within solid tumors in a passive targeting process due to the enhanced permeability and retention (EPR) effect. This effect is a consequence of the abnormal features of tumor blood vessels and the poorly developed lymphatic system, which limits drainage from tumor tissue (Matsumura and Maeda 1986).

However, passive targeting suffers from several limitations. Targeting cells within a tumor is not always possible because some drugs cannot diffuse effectively, and the random nature of the approach makes the process difficult to control. To overcome these bottlenecks, binding bioactive molecules could further improve drug targeting, and among these bioactive molecules are antibodies (Song et al. 2010), sugars (Liang et al. 2006) and peptides (Accardo et al. 2014). In particular, peptides offer many advantages, providing unlimited sequence/structure opportunities and binding with high selectivity and affinity to cancer-related targets. Furthermore, peptides possess an enhanced extravasation behavior, a fast diffusion tendency, and low immunogenicity (Langer and Beck-Sickinger 2001). They can be selected by screening phage display libraries, or they can be designed by breaking the insight 3D sequence and structure–activity relationship (SAR). More recently, numerous peptide-decorated micelles have been formulated to target tumors overexpressing G protein-coupled receptors (Accardo et al. 2014). These micelles have been characterized to deliver contrast agents for imaging (Accardo et al. 2004, 2013) with accurate physico-chemical analysis. They were tested and loaded with different types of poorly soluble drugs (Zhang et al. 2012; Jaskula-Sztul et al. 2016). Epidermal growth factor (EGF) analogs can be used as shuttle vectors to deliver NP-loaded drugs to tumor cells.

Nowadays, it is known that EGF plays a pivotal role in cell growth (Hackel et al. 1999), differentiation (McCawley et al. 1997) and migration (Blume-Jensen and Hunter 2001). Its positive signaling has been found to cause increased proliferation, decreased apoptosis and enhanced tumor cell motility and angiogenesis (Woodburn 1999). The EGF receptor (EGFR; also known as ErbB1 and Her1) is a transmembrane protein which is overexpressed in a wide variety of human cancers of epithelial origin, including breast, lung, gastric, colorectal, prostate, pancreatic, ovarian and liver cancers (Rocha-Lima et al. 2007). Above all, liver cancer is the second highest cause of cancer-related death worldwide. In hepatocellular carcinoma (HCC), a cancer type for which therapeutic options in advanced stages are quite limited, the over-activation of the EGF/EGFR pathway has been associated with an aggressive phenotype, intrahepatic metastasis, and poor patient survival (Bohl et al. 2002). Therefore, EGFR represents an important potential molecular target for novel therapeutic approaches to HCC.

This evidence pushed researchers, more than 10 years ago, to investigate the possibility of attaching EGF to 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC)/cholesterol (Chol)/1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[amino(polyethyleneglycol)-2000 (DSPE-PEG2000) liposomes. Biological results showed EGFR-specific cellular binding in cultured human glioma cells (Berasain and Avila 2014). This useful development led to intensified efforts toward developing EGF peptide analogs, decorated with liposomes or metal NPs. A twelve-residue peptide fragment of EGF, referred to as GE11 (YHWYGYTPQNVI), was successfully identified using phage display library screening (Li et al. 2005). Computational studies have turned this binding to the extracellular domain into the EGF binding pocket.

This sequence was selected to label different types of nanostructures, such as liposomes (Song et al. 2008), NPs (Cheng et al. 2011) and micelles (Liu and Chien 2015). The GE11 peptide was conjugated with a polyethylene glycol lipid (DSPE-PEG2000) and then mixed with 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (EPC)/Chol/DSPE–PEG2000 preformed liposome, by following a post-insertion method. This aggregate is able to cross membranes by endocytosis in vitro when mediated by EGFR overexpressing cancer cells (H1299 cells). In vivo, it accumulated in xenograft tumor tissues (Song et al. 2008). Moreover, compared to untargeted conjugates, the GE11 labeling gold NP (Au-NP)–Pc 4 showed tenfold improved selectivity for the glioma brain tumor. This delivery system demonstrates the capacity to deliver hydrophobic therapeutic cargos to hard-to-reach cancers (Cheng et al. 2011). GE11 decorated PLGA-PEG NPs have been reported to enhance DNA internalization within cells and to achieve higher cellular transfection in cells with high EGFR expression (Liu and Chien 2015). More recently, the co-delivery of hydrophobic docetaxel (DTX) and pH-sensitive curcumin (CUR) prodrug has been employed for the treatment of prostate cancer (Jin et al. 2017).

Later, the peptide LARLLT (referred to as D4) of EGF was generated using computer-assisted design (CAD) (Song et al. 2009). This sequence binds the extracellular domain

I away from the EGF binding pocket. Due to the fact that this peptide is easier to synthesize than GE11, it was conjugated with DSPE-PEG2000, obtaining similar results to GE11 (Song et al. 2009). Moreover, small peptides that are able to strongly and specifically bind to EGFR are preferable for their minimal immunogenicity and proliferative effects. Recently, Vicente's group coupled this sequence to organic chromophores, suggesting that it may have the ability to bind to cells overexpressing EGFR (Fontenot et al. 2016; Zhao et al. 2017). Despite these considerations in the literature, though, only one example of a supramolecular aggregate has been reported (Song et al. 2009).

In the present study, we formulated new mixed micelles based on PF127 by adding amphiphilic molecules functionalized with the D4 sequence to produce a new vehicle for potentially shuttling hydrophobic antitumor drugs. Moreover, pluronic micelles are able to transport hydrophilic drugs, such as doxorubicin, to multidrug-resistant cancer cells (Alakhova et al. 2013). Therefore, this formulation is the first D4 peptide-labelled system with wide potential capability of reaching selected cancer cells. The active targeting micelles were tested in vitro for their binding capacity to human HCC cell lines overexpressing EGFR.

Materials and methods

Materials

Protected *N*α-(9-fluorenylmethoxycarbonyl) (Fmoc) amino acid derivatives, coupling reagents and Rink-amide MBHA resin were purchased from Calbiochem-Novabiochem (Laufelfingen, Switzerland). Fmoc-8-amino-3,6-dioxaoctanoic acid (Fmoc-AdOO-OH) was purchased from Neosystem (Strasbourg, France). *N*,*N*-Dioctadecylsuccinamic acid was synthesized according to the literature (Schmitt et al. 1994). All other chemicals were commercially available from Sigma–Aldrich, Fluka (Buchs, Switzerland) or Lab-Scan (Stillorgan, Ireland) and were used as received unless stated otherwise. PF127 and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt) (Rhod PE) were acquired from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). All solutions were prepared by weight and solubilized in double distilled water.

Preparative high-performance liquid chromatography (HPLC) was carried out on an LC8 Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with an ultraviolet (UV) lambda-Max Model 481 detector using a Phenomenex (Torrance) C4 (300 Å, 250 mm × 21.20 mm, 5 µm) column. The column was eluted by H_2O/0.1% trifluoroacetic acid (TFA) with a linear gradient elution of CH_3CN/0.1% TFA from 20% to 95% over 25 min at a 20 mL/min flow rate. Liquid chromatography coupled to mass spectrometry (LC–MS) analyses were performed using Finnigan Surveyor MSQ single quadrupole electrospray ionization (Finnigan/Thermo Electron Corporation, San Jose, CA, USA). UV measurements were performed with a 1-cm quartz cuvette (Hellma, Müllheim, Germany).

Peptide conjugate synthesis

Both amphiphilic peptides, (D4-$(AdOO)_2(C18)_2$ and scD4 $(AdOO)_2(C18)_2$), were synthesized using standard solid-phase Fmoc procedures (Carpino and Han 1972). The Rink amide MBHA resin (substitution 0.90 mmol/g) was used as the solid-phase support, and the synthesis was performed at a scale of 0.2 mmol. All couplings were performed once for 1 h with an excess of 2 equivalents per single amino acid. Fmoc deprotection was carried out with a 30% solution of piperidine in dimethylformamide (DMF). When the synthesis was complete, the Fmoc N-terminal protecting group was removed and two residues of the Fmoc-AdOO-OH linker and *N*,*N*-dioctadecylsuccinamic acid were sequentially condensed as previously reported (Accardo et al. 2007).

The amphiphilic peptide was then fully deprotected and cleaved from the resin with TFA and 2.0% (v/v) water and 2.0% (v/v) using triisopropyl silane (TIS) as scavengers, at room temperature, and then precipitated with ice-cold water. The precipitate was then dissolved in a water/CH_3CN (80/20 v/v) mixture and then lyophilized. The crude peptide derivative was purified by RP-HPLC. The purity and identity were assessed through LC–MS analysis. The physico-chemical data for both peptides are reported as follows:

LARLLT D4$(AdOO)_2(C18)_2$: R_t 28.5 min; MS (ESI): 1579.1 *m*/*z* calcd.: 1579.06 m/z [M + H^+].
RTALLL scD4$(AdOO)_2(C18)_2$: R_t 29.5 min; MS (ESI): *m*/*z*: 1579.1 *m*/*z* calcd.: 1579.06 m/z [M + H^+].

Aggregate formulation and dynamic light scattering (DLS) characterization

Pure PF127 micelles were prepared by weighing 20.0 mg of the copolymer and then adding 1.00 g of water to obtain an aqueous solution at 2% (w/w). Mixed PF127/D4$(AdOO)_2(C18)_2$ and PF127/scD4$(AdOO)_2(C18)_2$ micelles were prepared by adding 2.5% (w/w) of the amphiphilic EGF peptide analog to PF127. The two amphiphilic compounds were dissolved in a small amount of MeOH/$CHCl_3$ (50:50, v/v). A thin film of amphiphiles was obtained by evaporating the solvent, slowly rotating the tube containing the solution under a steady stream of nitrogen. The film was hydrated (1 mL) for 5 min by vortexing, and after that it was used without further treatment. The same procedure was followed to obtain fluorescence-labeled micelles by adding 1‰ w/w of Rhod-PE.

The mean diameter was measured using a Zetasizer Nano ZS (Malvern Instruments, Westborough, MA, USA) with a 173° backscatter detector. The other instrumental settings were as follows: measurement position (mm): 4.65; attenuator: 8; temperature: 25 °C; cell: disposable sizing cuvette. The concentration of the DLS samples was 1.0 mM, and afterward they were centrifuged at room temperature at 13,000 rpm for 5 min. For each batch, the hydrodynamic radius and size distributions were the means of three measurements, and the values were calculated as the means of three different batches.

Cell cultures and fluorescence analysis

Four human HCC cell lines, HepG2, Hep3B, Huh7 and PLC/PRF/5, were employed in this study. HepG2 and Hep3B cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Huh7 and PLC/PRF/5 cells were a gift from Prof. M. Levrero (Sapienza University of Rome, Italy) and Prof. O. Bussolati (University of Parma, Italy), respectively. Cells were cultured in RPMI 1640 (SIGMA, Milan, Italy) containing 10% FCS (Gibco, Invitrogen Corporation, Carlsbad, CA, USA) and maintained at 37 °C at 5% CO_2. All cell lines were authenticated as previously reported (Augello et al. 2018).

For fluorescence analysis, HepG2 cells (20,000/well) and PLC/PRF/5 cells (10,000/well) were seeded in chamber slides. After 24 h, cells were treated for 3 h with PF127/D4$(AdOO)_2(C18)_2$/Rhod-PE. PF127/Rhod-PE and PF127/scD4$(AdOO)_2(C18)_2$/Rhod-PE were used as controls. All aggregates were sonicated on ice for 10 min before use. After treatment, cells were washed 3 times with phosphate buffer saline (PBS) and fixed for 15 min with 3.7% paraformaldheyde. Then, slides were mounted using Vectashield containing DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) to stain cellular nuclei, and cells were observed using a Leica fluorescence microscope.

Western blotting and immunofluorescence

Total cell lysates were obtained using RIPA buffer (Cell Signaling Technologies Inc., Beverly, MA, USA). Extracted total proteins were used, and Western blotting analysis was performed, as previously reported (Cusimano et al. 2015), to evaluate different expression levels of EGFR in HCC cell lines. Rabbit polyclonal antibody (SC-120) specific for human EGFR was purchased from Santa Cruz (Dallas, TX, USA). EGFR expression levels were normalized using the quantified level of β-actin expression, using anti-β-actin antibody (SIGMA).

For immunofluorescence analysis, cells were plated in chamber slides and, 24 h later, cells were fixed and incubated for 1 h with anti-EGFR antibody. Slides were mounted using Vectashield mounting medium containing DAPI (Vector Laboratories, Inc.). Images were acquired by Leica fuorescence microscope.

Results and discussion

Monomer design and synthesis

The D4 sequence contains both a hydrophobic part and a polar amino acid. Therefore, two units of dioxoethylene linkers (AdOO) were introduced on the N-terminal side to increase hydrophilicity and maintain appropriate exposure on the external aggregate surface. Two main features prompted the choice of ethoxyl linkers: the ability, in vivo, to decrease aggregate clearance, and the lack of electrical charges, which normally increase repulsion in the hydrophilic shell on the aggregate surface. A hydrophobic moiety containing two C18 aliphatic chains was added to the second linker to promote interactions with the hydrophobic surfactant moiety through van der Waals forces. The distearoyl moiety allows stronger interactions compared to shorter and/or unsaturated chains. In general, the addition or insertion of peptide sequences onto the N-terminal moiety may alter its biological activity. The scrambled amino acid sequence RTALLL (scD4) was designed as a negative control for the biological assays. This sequence was selected, in turn, to reproduce the scrambled sequence used in the literature (Song et al. 2009). It is noteworthy to consider that the Arg residue on the C-terminus could induce a specific penetration through the membrane owing to the presence of a positive charge at physiological pH.

As previously stated, the D4$(AdOO)_2(C18)_2$ and scD4$(AdOO)_2(C18)_2$ (see Fig. 1) peptide derivatives were synthesized through solid phase methods on a Rink amide resin using the standard Fmoc procedure. At the end of monomer assembly, the two monomers were both cleaved using standard trifluoroacetic acid mixtures to ensure the complete removal of all protecting groups of the amino acid side chains. The crude products were purified by preparative reversed-phase HPLC on a C4 column and/or recrystallized up to a maximum final purity of 90%. Finally, they were isolated in 25–35% yields in lyophilized form. Their molecular masses were determined by MS ESI mass spectroscopy (Supplementary material).

Micelle formulation and DLS measurements

The surfactant Pluronic PF127 (see Fig. 1) was chosen for several reasons. The applicability of Pluronic is mainly driven by its self-assembly to form micelles in aqueous solution (Raval et al. 2017). Pluronic copolymers are potent sensitizers multi-drug resistant (MDR) cells that intensify

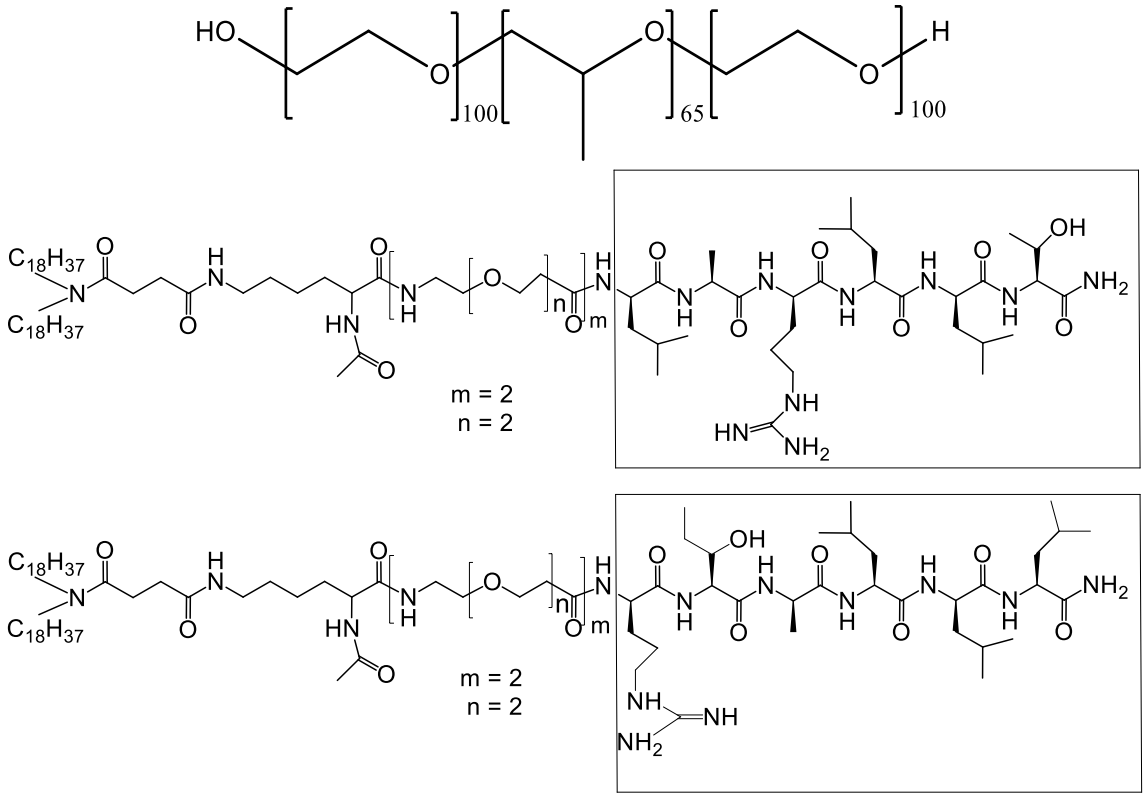

Fig. 1 Chemical structures of Pluronic PF127 (upper), D4(AdOO)$_2$(C18)$_2$ (middle), and scD4(AdOO)$_2$(C18)$_2$ (lower). The amino acid sequences of D4 and scD4 are reported in the boxes

the cytotoxic activity of drugs. Moreover, the US Food and Drug Administration (FDA) has approved triblock copolymer for biomedical applications. PF127 was preferred to other pluronics because it exhibits a longer PEO chain and forms micelles at room temperature. These properties allow researchers to use the same system to transport both hydrophobic and hydrophilic drugs at the same time, producing stable micelles suitable for in vivo application.

In physiological solution (0.9% aqueous solution in NaCl), micelles are produced by a hydrophobic core, comprising oxypropylene units, and by a hydrophilic sphere, made up of oxyethylene units. Peptide amphiphilic molecules were then added to obtain mixed micelles. In particular, two formulations of this supramolecular aggregate were made with equal percentages (2.5% w/w) of D4(AdOO)$_2$(C18)$_2$ or scD4(AdOO)$_2$(C18)$_2$. Fluorescence aggregates were achieved by adding a modest mass amount (1‰) of Rhod-PE.

The choice of the amount of amphiphilic peptide added was suitable to guarantee a sufficient number of bioactive molecule units able to interact with the receptors. The fact that a higher amount might have induced interactions between the positive charges of arginine residues present on peptide chains was taken into account. Moreover, amphiphilic peptides might have excessively influenced the structure of the aggregate driven by PF127, so another aggregate was formulated without the bioactive molecule to use these micelles as a control in biological tests.

DLS measurements were performed to assess the size of the micelles (data are reported in Table 1). The diagrams show a monomodal distribution for both mixed aggregates (Fig. 2). In agreement with previously reported data (Basak and Bandyopadhyay 2013; Sharma and Bhatia 2004), the mean hydrodynamic diameter of the whole system at 25 °C provides a value of 25 nm (Table 1). The addition of both amphiphilic peptides increased the Gaussian distribution of the aggregates. The amino acid sequence did not influence the diameter of the mixed aggregate. Therefore, in the scD4-labeled micelle, the positive charges on arginine are sufficiently distant from the core. This issue allows us to suppose

Table 1 Structural parameters, mean hydrodynamic diameter (nm) and polydispersity index, (PI), obtained from DLS measurements for the examined systems

System	Diameter (nm)	PI
PF127/Rhod-PE	27.0	0.083
PF127/D4(AdOO)$_2$(C18)$_2$/Rhod-PE	24.4	0.25
PF127/scD4(AdOO)$_2$(C18)$_2$/Rhod-PE	25.0	0.20

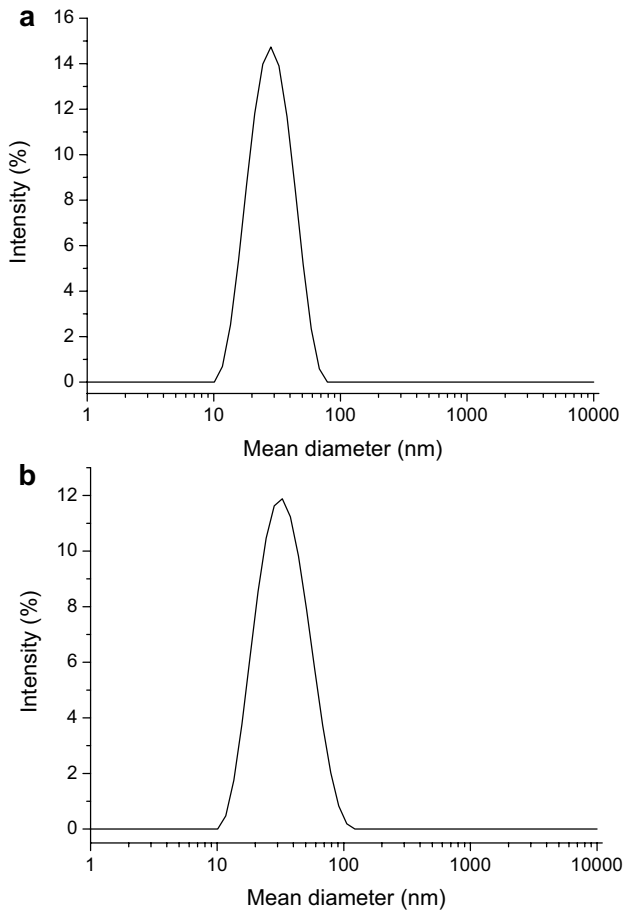

Fig. 2 DLS micelles **a** PF127/scD4(AdOO)$_2$(C18)$_2$ 97/2,5/Rhod-PE (upper); **b** PF127/D4(AdOO)$_2$(C18)$_2$ 97/2,5/Rhod-PE (lower)

that the peptide chains are detached from the micelle surface and are able to interact with EGFRs.

The stability of both systems in solution was verified by carrying out DLS measurements over 48 h while storing the samples at room temperature.

EGFR expression in HCC cells

The basal expression levels of EGFR were examined in 4 human HCC cell lines: HepG2, Hep3B, Huh7, and PLC/PRF/5 (Fig. 3). Three HCC cell lines exhibited different expression levels of the protein, with PLC/PRF/5 cells expressing the highest levels, while HepG2 showed almost no expression (Fig. 3a). Western blotting results were confirmed by immunofluorescence analysis (Fig. 3b). In HepG2 cells, no evident expression of EGFR was observed, whereas in PLC/PRF/5, a clear cell membrane localization was evident.

Based on these results, for subsequent experiments, we chose HepG2 and PLC/PRF/5 cells as EGFR negative and positive HCC models, respectively.

Selectivity of PF127/D4(AdOO)$_2$(C18)$_2$ in binding to HCC cells expressing EGFR

We used fluorescence analysis to investigate the specificity of PF127/D4(AdOO)$_2$(C18)$_2$ binding to HCC cells expressing EGFR by taking into account the presence of Rhod-PE in mixed micelles. As shown in Fig. 4, after in vitro exposure at 37 °C for 3 h, PF127/D4(AdOO)$_2$(C18)$_2$Rhod-PE binding to high-EGFR expressing PLC/PRF/5 cells was significantly higher than to EGFR-negative HepG2 cells. In addition, micelles decorated with a scrambled peptide (PF127/scD4(AdOO)$_2$(C18)$_2$Rhod-PE) showed no binding to HCC cells, whether positive or negative for EGFR expression.

These results highlighted the selectivity of the D4 peptide and subsequent specificity of micelles in targeting EGFR-postive HCC cells.

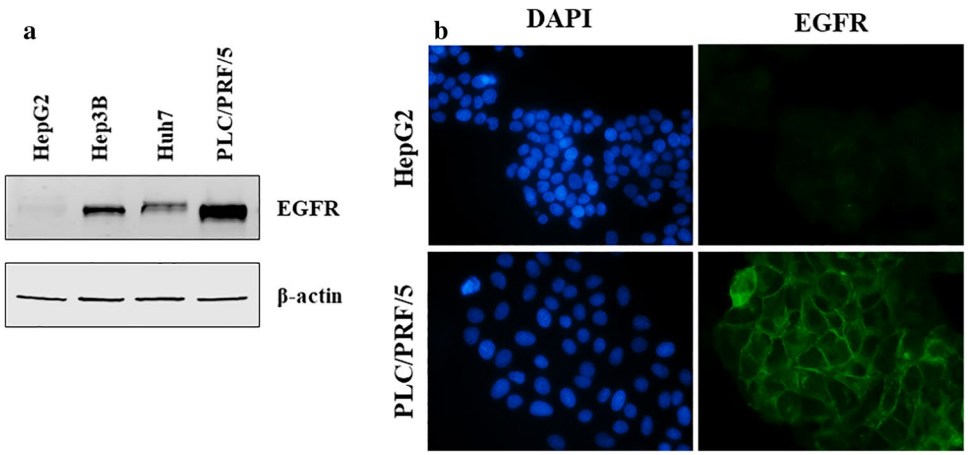

Fig. 3 Expression of EGFR in HCC cell lines. **a** Immunoblotting evaluation of EGFR expression in four HCC cell lines. Equal amounts of protein loading were determined using β-actin as internal control. **b** Immunofluorescence analysis of EGFR protein expression in HepG2 and PLC/PRF/5 cells. Cell nuclei were stained with DAPI. Magnification = × 20

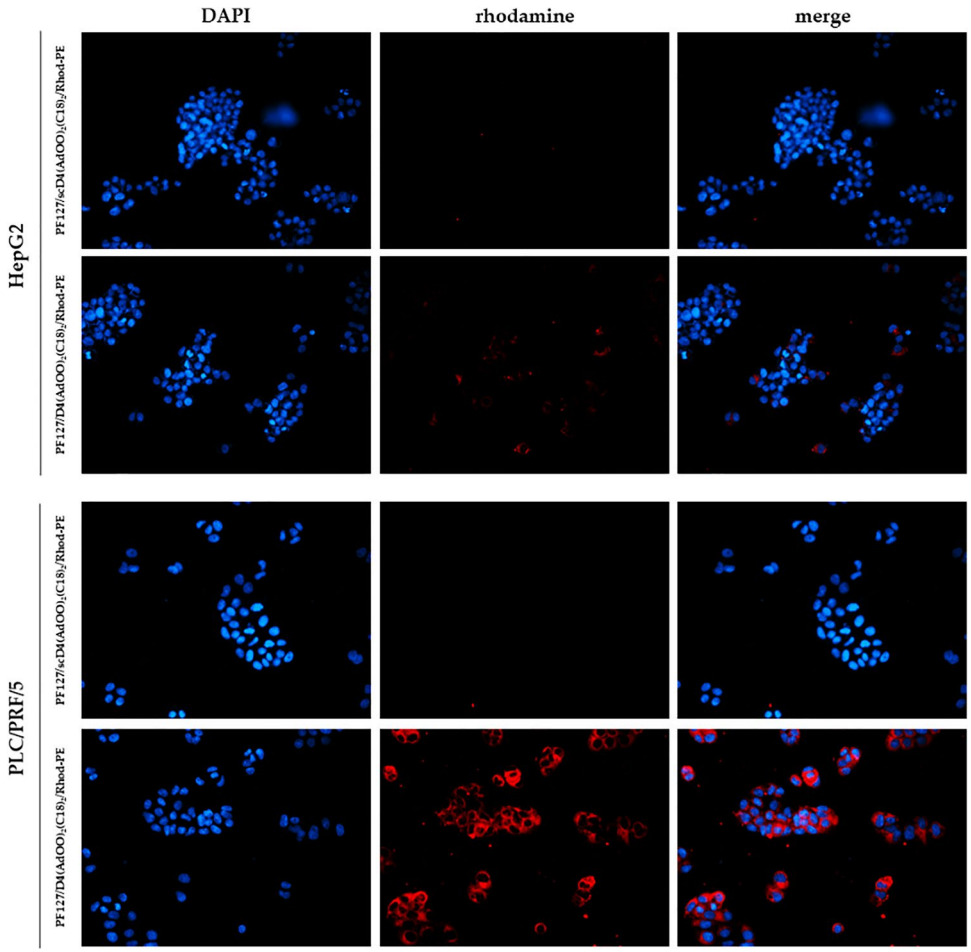

Fig. 4 Fluorescence microscopy images of HepG2 and PLC/PRF/5 cells after in vitro exposure to PF127/D4$(AdOO)_2(C18)_2$/Rhod-PE (4.0 mg/0.1 mg/6 × 10^5 Rho-PE mol) or PF127/scD4$(AdOO)_2(C18)_2$/Rhod-PE (4.0 mg/0.1 mg/6 x 10^5 Rho-PE mol) at 37 °C for 3 h. Images from left to right show: cell nuclei stained with DAPI (blue); fluorescent micelles bound to cells (red); overlays of the two images (merge). Magnification = × 20

Conclusion

The D4 amphiphilic peptide was prepared by conjugating an ethoxilic linker to a distearoyl tail. This monomer, mixed with PF127, attaches to micelles, allowing them to enhance EGFR binding affinity as a targeting ligand. The cellular binding of D4 peptide-conjugated micelles was more efficient in PLC/PRF/5 cancer cells overexpressing EGFR than in HepG2 tumoral cells with low EGFR expression. Moreover, in cells highly expressing EGFR, the D4-conjugated micelles exhibited higher cellular binding than the D4 scrambled peptide-labeled micelles. These results allowed us to demonstrate that the N-terminal moiety of the sequence can be modified and conjugated to ethoxilic linkers without losing receptor affinity. Moreover, although PF127 endows a long PEO chain, it does not hide the peptide sequence that is well-exposed on its hydrophilic shell. The low uptake of the scrambled peptide confirms that the interaction with the receptor is not driven by random contacts between amino acids. All of this experimental evidence led us to conclude that these aggregates can be loaded with hydrophobic and/or hydrophilic drugs, and they can deliver their payload selectively to cancer cells. Further perspectives are very promising due to the broad range of applications for drugs nanocarriers. They can be loaded with specific drugs, either conventional chemotherapy (such as doxorubicin, paclitaxel and cisplatin.) or molecular targeted agents (such as erlotinib and sorafenib.) which have been used as antitumor agents in different cancer types, including HCC.

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Compliance with ethical standards

Conflicts of interest The authors declare that there are no conflicts of interest.

Research involving human participants and/or animals The research does not involve human participants and/or animals.

Informed consent Informed consent was obtained from all individual participants included in the study.

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