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The architecture of ligand attachment to nanocarriers controls their specific interaction with target cells

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Abstract

Surface architecture of pharmaceutical nanocarriers (using polymeric micelles as an example) and the length of the spacer group through which specific ligand is attached to the carrier surface determine the interaction of ligand-bearing nanocarrier with cells. We have prepared surface-modified polyethyleneglycol–phosphatidylethanolamine (PEG–PE) micelles containing TATp attached to PEG–PE with a PEG block longer or shorter (TATp–PEG₁₀₀₀–PE or TATp–PEG₃₄₀₀–PE) than the PEG block in the main micelle-forming material (PEG₇₅₀–PE and/or PEG₂₀₀₀–PE). The length of the PEG spacer in TATp–PEG–PE should allow for a non-hindered interaction of TATp with the cell surface, but it should not be too long to allow for the conformational “folding in” of TATp moiety inside the PEG globule making it unable to interact with the cells. The “folding in” of the ligand attached to an unnecessary long PEG spacer was further supported by the fluorescence resonance energy transfer (FRET) study between fluorescently labeled lipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE) inserted into the core of PEG₇₅₀–PE micelles and micelle-incorporated rhodamine-labeled TATp–PEG–PE. Micelles containing rhodamine-labeled TATp–PEG–PE with the longest PEG spacer (3400 Da) demonstrated strongly enhanced quenching of NBD-PE fluorescence with rhodamine-TATp confirming the “folding in” of TATp moiety into PEG globule bringing it closer to the micelle core-incorporated NBD.

Keywords

Polymeric micelles; polyethyleneglycol–phosphatidylethanolamine; cell-penetrating peptide; TAT peptide; intracellular delivery

Introduction

Various *in vitro* and *in vivo* studies have proved the potential of cell-penetrating peptides (CPPs), including TAT peptide (TATp) and oligoarginines, for the intracellular delivery of different cargoes. TATp-mediated cytoplasmic uptake of polymers (Nori et al. 2003; Hyndman et al. 2004), bacteriophages (Paschke et al. 2005), plasmid DNA (Torchilin et al. 2001, 2003; Kleemann et al. 2005), magnetic nanoparticles (Dodd et al. 2001; Zhao et al. 2002; Nitin et al. 2004), liposomes (Cryan et al. 2006; Sawant et al. 2006; Gupta et al. 2007), and micelles (Sawant et al. 2006; Sethuraman et al. 2007, 2008) has been reported. Successful intracellular

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delivery requires direct contact between the surface-attached CPPs on the pharmaceutical nanocarrier and the cell surface (Torchilin et al. 2001; Levchenko et al. 2003). Actually, this is true and generally applicable to any ligand-bearing carrier for targeted delivery. Reasonable assumption is usually made that such ligand should be sufficiently distant from the carrier surface or the surface of any coating applied to the carrier (such as sterically protecting PEG coating) to allow for the required unhindered interaction with the cell. A natural practical consequence from such assumption is using long spacer groups to attach ligands to nanocarriers. To which extent this assumption is correct and how long the spacer arm should really be?

We prepared and studied the *in vitro* PEG–PE polymeric micelles modified with TATp moieties. TATp–PEG–PE conjugates with different PEG-spacer lengths were used allowing or not allowing for direct TATp-to-cell interaction. The specific objective of the present work was to study the effect of PEG-spacer length in TATp–PEG–PE on the TATp-mediated uptake of the nanocarriers (micelles) by cells.

Materials and methods

Materials

Cell line, BT20 (human breast adenocarcinoma), was purchased from the ATCC (Manassas, VA, USA). Cell culture media, Dulbecco's modified Eagle's medium (DMEM), heat-inactivated fetal bovine serum (FBS), and concentrated solutions of sodium pyruvate and penicillin/streptomycin stock solutions were purchased from Cellgro (Herndon, VA, USA). TAT-peptide (TATp-NH₂) (11-mer: TyrGlyArgLysLysArgArgGlnArgArgArg) and TAT-cysteine peptide (TATp-Cys) (12-mer: CysTyrGlyArgLysLysArgArgGlnArgArgArg) were synthesized by Tufts University Core Facility (Boston, MA, USA). 1,2-Distearoyl-*sn*-glycero-3-phos-phoethanolamine-*N*-[methoxy (poly(ethylene glycol))-750 or -2000] (PEG₇₅₀-PE or PEG₂₀₀₀-PE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rh-PE), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE) were from Avanti Polar Lipids (Alabaster, AL, USA). Carboxytetramethyl rhodamine succinimidyl ester (Rh-NHS) was from Pierce (Rockford, IL, USA). All other chemicals were analytical grade preparations from Sigma (St Louis, MO, USA).

Synthesis of TATp–PEG–PE conjugates

TATp–PEG₁₀₀₀-PE was synthesized using a heterobifunctional NHS–PEG₁₀₀₀-Mal derivative, DOPE, and TATp-cysteine as in Kale et al. (2007a, 2007b). TATp–PEG₃₄₀₀-PE was synthesized by conjugating TATp to *p*-nitrophenyl-PEG₃₄₀₀-PE (pNP-PEG₃₄₀₀-PE; Torchilin et al. 2001). In brief, a solution of 5mg of TATp-NH₂ in 1ml chloroform supplemented with 10 µl of triethylamine (TEA) was incubated with 12 mg of pNP-PEG₃₄₀₀-PE in 0.6 ml chloroform overnight with stirring at room temperature (RT). Organic solvents were removed by rotary evaporation and freeze-drying. To the resultant film, 1ml of deionized water was added and vortexed to remove the film from the flask. To purify the TATp–PEG₃₄₀₀-PE from unconjugated TATp-NH₂ and pNP, the solution was dialyzed using cellulose ester dialysis membrane MWCO 2000 (Spectrum Medical Industries, Rancho Dominguez, CA, USA) at RT against water.

Synthesis of rhodamine-labeled TATp–PEG–PE for fluorescence resonance energy transfer (FRET) studies

For labeling TATp–PEG₁₀₀₀-PE or TATp–PEG₃₄₀₀-PE with rhodamine, 0.34µmol of TATp–PEG–PE in 2ml dry chloroform was mixed with 5 molar excess of Rh-NHS and 5µl of TEA.

The mixture was stirred for 24 h at RT under argon. Organic solvent was removed using rotary evaporator and freeze-drying to form a thin film, which was rehydrated and vortexed to ensure the formation of Rh-TATp-PEG-PE suspension. The Rh-TATp-PEG-PE conjugate was purified by dialysis against water using MWCO 1000 dialysis bag followed by freeze-drying to a constant weight. The product was dissolved in dry chloroform at 2 mg/ml and stored under argon at -80°C .

Preparation of micelles

For micelle preparation, a mixture of PEG-PE (750 and/or 2000 Da PEG blocks) and TATp-PEG-PE (1000 or 3400 Da PEG blocks) was prepared in chloroform at a molar ratio of 97.5:2.5. Chloroform was removed by rotary evaporation and freeze-drying. The film was hydrated with HEPES-buffered saline (HBS, pH 7.4) at room temperature and vortexed for 5 min. For cell interaction study, 0.5 mol% of the fluorescent probe, Rh-PE, was added to the micelle composition. For FRET experiments, micelles were prepared with varying quantities of Rh-TATp-PEG-PE and 1 mol% of the micelle core-incorporated fluorescent probe, NBD-PE (for compositions see Table I). Micelle size was measured using a Coulter N4 Plus Submicron Particle Analyzer (Coulter Corporation, Miami, FL, USA).

Interaction of TATp-nanocarriers with cells

BT20 cells were grown on cover slips placed in six-well tissue culture plates. After the cells reached a confluence of 60–70%, the plates were washed with DMEM and the cells were treated with various Rh-PE-labeled micelle (0.2 mg/ml of the micelle-forming material) samples (with and without TATp) in DMEM. After 1 h, the medium was removed and the plates were washed with sterile PBS (pH 7.4). Individual cover slips were mounted cell-side down onto fresh glass slides with PBS. Cells were viewed with a Nikon Eclipse E400 microscope under bright light or under epifluorescence with rhodamine-/TRITC filter.

FRET studies of TATp behavior on the surface

FRET between two fluorescent probes was used to follow the spatial behavior of TATp attached to a spacer arm. FRET results in the quenching of the donor fluorescence. Since the extent of FRET depends on the distance between the donor and the acceptor molecules, the interaction between the “outer” peptide-attached and core lipid-anchored probes can be monitored following the changes in fluorescence intensities. The fluorescent lipid NBD-PE (1 mol%) and Rh-TATp-PEG-PE (varying quantities) were incorporated into the micelles. The NBD-PE fluorescence intensity at the excitation wavelength of 460 nm and the emission wavelength of 534 nm was monitored at the fixed lipid concentration of 1 mg/ml using F-2000 fluorescence spectrometer (Hitachi, Japan).

Results and discussion

It was assumed that for efficient interaction of the nanocarrier-attached TATp with the cell, TATp moiety should be located “above” the carrier surface. Thus, in case of PEG-PE micelles, TATp was attached to the distal PEG tip of some PEG-PE moieties, and these moieties contained longer PEG block than the main body of micelle-forming PEG-PE unimers. Micelles were prepared using PEG₇₅₀-PE or PEG₂₀₀₀-PE or the mixture of PEG₇₅₀-PE and PEG₂₀₀₀-PE as main micelle-forming components with the addition (depending on the micelle composition) of either TATp-PEG₁₀₀₀-PE or TATp-PEG₃₄₀₀-PE. In some samples, micelles have also been prepared with TATp attached to the micelle core via a shorter PEG spacer (1000 Da) than PEG moiety in micelle-forming PEG-PE (2000 Da); see Table I and Figure 1 for micelle compositions and hypothetical architecture. All micelles had the size within the 10–20 nm range.

Preparations with TATp located “above” PEG blocks of the micelle-forming PEG–PE were expected to demonstrate an increased cell uptake due to assumed unhindered contact of TATp with cells.

TATp-mediated interaction of nanocarriers with cells in vitro

The importance of unhindered contact of TATp with cells for successful intracellular delivery of TATp-modified nanocarriers has already been demonstrated by us with the “smart” pH-sensitive micelles and liposomes (Sawant et al. 2006). Such micelles were composed of PEG₇₅₀–PE, pH-sensitive detachable PEG₂₀₀₀–Hz–PE, and TATp–PE. These micelles, when intact (at pH 8.0), exhibited minimal cell uptake due to the shielding of the TATp function. Their incubation at a lowered pH (5.0) facilitating the removal of PEG₂₀₀₀ blocks due to breaking of the pH-sensitive hydrazone bond resulted in increasingly strong interaction of the micelles with the cells because of deshielding of the TATp function. However, the role of the length of the spacer group via which TATp was attached to micelles was not specifically investigated.

The interaction of different micelle preparations with BT20 cells is shown in Figure 2. Micelles composed of PEG₇₅₀–PE or PEG₂₀₀₀–PE demonstrated limited uptake by the cells (Figure 2a,b); however, the addition of the TATp–PEG₁₀₀₀–PE component to PEG₇₅₀–PE micelles resulted in the expected strong interaction with the cells compared with plain micelles (Figure 2c). Gradual replacement of PEG₇₅₀–PE in the micelle composition with PEG₂₀₀₀–PE component (at the same molar fraction of TATp–PEG₁₀₀₀–PE) resulted in gradual decrease in TATp-mediated micelle interaction with cells because of eventual shielding of TATp function by the longer PEG chains (Figure 2d–f). This interaction remains significant for the micelles containing 30 mol% of PEG₂₀₀₀–PE (Figure 2d), but decreases dramatically for the micelles containing 50% or composed of only PEG₂₀₀₀–PE (Figure 2e,f).

Somewhat different pattern was observed for the micelles with TATp–PEG₃₄₀₀–PE component (same molar fraction as TATp–PEG₁₀₀₀–PE). Such micelles (built of PEG₇₅₀–PE or PEG₂₀₀₀–PE main component) demonstrated minimal cell interaction similar to that for the plain micelles (Figure 2g,h). This result looks quite surprising since we assumed that the TATp function attached to a long PEG spacer should be unhindered and provide easy contact of TATp moiety with the cell surface.

The most probable explanation of this phenomenon comes from the behavior of soluble polymers in aqueous solutions. Soluble polymers with a flexible main chain (and PEG is a very good example of such polymers) in aqueous solutions have a strong tendency to fold into a statistical globule rather than maintain a stretched conformation (Jannink et al. 1990). In this case, TATp moiety attached to the relatively long PEG₃₄₀₀ chain has a good chance to be “folded into” the PEG globule thus being removed from the “above-the-surface” position closer to the inner part of the nanocarrier (micelle core in this case). As a result of such “self-shielding”, TATp moiety loses its ability to directly interact with the cell surface. At the same time, shorter PEG₁₀₀₀ spacer does not have the length sufficient for the folding into the statistical globule, and TATp moiety always remain protruded from the nanocarrier surface and available for the interaction with the cell surface (unless it is “shielded” by surrounding longer PEG chains). To check this hypothesis, we have performed fluorescence quenching experiments.

FRET experiments

To gain information on the “folding” of TATp from the “above-the-surface” position closer to the micelle core when attached to a longer PEG spacer using FRET approach, we have utilized the NBD-PE probe (with no spacer between PE and NBD) as the micelle core-incorporated

donor probe (it should be incorporated into the core via its lipid anchor with the fluorescent part of the molecule located also in the core or very close to it). Rhodamine attached to the TATp moiety of TATp-PEG-PE conjugate served as acceptor probe. One can expect that with no PEG-spacer folding, Rh-labeled TATp should remain distant from the core-incorporated NBD, while if the PEG spacer is long enough to allow for the chain folding, TATp moiety could get inserted into the micelle corona, and TATp-attached Rh group should come closer to the NBD group and cause the higher quenching of NBD fluorescence.

NBD-PE-labeled (fixed 1 mol%) PEG₇₅₀-PE micelles containing varying quantities of Rh-TATp-PEG₁₀₀₀-PE or Rh-TATp-PEG₃₄₀₀-PE were used. The NBD-PE fluorescence intensity was measured at the fixed concentration of the micelle-forming components of 1 mg/ml. The data in Figure 3 clearly show that with the increase in TATp-PEG-PE content, micelles containing Rh-TATp-PEG₃₄₀₀-PE demonstrate significant quenching of NBD-PE fluorescence compared with that in case of Rh-TATp-PEG₁₀₀₀-PE-containing micelles.

As was already mentioned, the extent of FRET strongly depends on the distance between the donor and the acceptor molecules, and theoretically, assuming a fully extended conformation of PEG spacer at any length, the NBD-PE fluorescence should be less affected in case of Rh-labeled TATp attached to longer PEG₃₄₀₀-PE than to shorter PEG₁₀₀₀-PE. The fact that we observe quite opposite result clearly confirms the folding phenomenon of longer PEG spacer bringing TATp moiety (and fluorescent tag attached to it) into the inner part of the micelle corona more close to the micelle core compartment where the NBD-PE is located. This namely phenomenon affects also the interaction of such micelles with the cells.

Our result shows the importance of the proper choice of the length of spacer group for the attachment of various specific ligands to the surface of pharmaceutical nanocarriers to allow for the optimal surface architecture and thus the effective interaction with target cells.

Abbreviations

CPP, cell-penetrating peptide
 FRET, fluorescence resonance energy transfer
 PEG-PE, polyethyleneglycol-phosphatidylethanolamine
 PEG₇₅₀-PE or PEG₂₀₀₀-PE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy (poly(ethylene glycol))-750 or -2000]
 pNP-PEG₃₄₀₀-PE, *p*-nitrophenyl-PEG₃₄₀₀-PE
 DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine
 TATp-PEG₁₀₀₀-PE or TATp-PEG₃₄₀₀-PE, TAT peptide conjugated to 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy (poly(ethylene glycol))-1000 or -3400]
 Rh-TATp-PEG₁₀₀₀-PE or Rh-TATp-PEG₃₄₀₀-PE, rhodamine-labeled TAT peptide conjugated to 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy (poly(ethylene glycol))-1000 or -3400]
 NBD-PE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl)
 Rh-PE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl)
 Rh-NHS, carboxytetramethyl rhodamine succinimidyl ester

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Figure 1.

Schematic representation of the TATp-containing micelles: (a) PEG₇₅₀-PE: TATp-PEG₁₀₀₀-PE micelles, (b) PEG₇₅₀-PE: TATp-PEG₃₄₀₀-PE micelles, (c) PEG₇₅₀-PE: PEG₂₀₀₀-PE: TATp-PEG₁₀₀₀-PE micelles, (d) PEG₂₀₀₀-PE: TATp-PEG₁₀₀₀-PE micelles, and (e) PEG₂₀₀₀-PE: TATp-PEG₃₄₀₀-PE micelles. 1, PEG₇₅₀-PE; 2, TATp-PEG₁₀₀₀-PE; 3, TATp-PEG₃₄₀₀-PE; 4, PEG₂₀₀₀-PE.

Figure 2.

In each panel, the left figure shows the bright field and the right figure shows the fluorescent microscopy of BT20 cells treated with (a) Rh-PE:PEG₇₅₀-PE micelles, (b) Rh-PE: PEG₂₀₀₀-PE micelles, (c) Rh-PE: PEG₇₅₀-PE: TATp-PEG₁₀₀₀-PE micelles, (d) Rh-PE: PEG₇₅₀-PE: PEG₂₀₀₀-PE (30 mol%): TATp-PEG₁₀₀₀-PE micelles, (e) Rh-PE: PEG₇₅₀-PE: PEG₂₀₀₀-PE (50 mol%): TATp-PEG₁₀₀₀-PE micelles, (f) Rh-PE: PEG₂₀₀₀-PE: TATp-PEG₁₀₀₀-PE micelles, (g) Rh-PE: PEG₇₅₀-PE: TATp-PEG₃₄₀₀-PE micelles, and (h) Rh-PE: PEG₂₀₀₀-PE: TATp-PEG₃₄₀₀-PE micelles. Magnification 40 × objective.

Figure 3. Fluorescence intensity of NBD-PE containing micelles with rhodamine-labeled TATp-PEG₃₄₀₀-PE or TATp-PEG₁₀₀₀-PE, $n = 3$, ★ $p < 0.05$.

Table I

Compositions of micelles used in the study.

Sr. No.	Micelle composition (mol%)			
	PEG-PE main component	Fluorescent label	PEG-PE second component	TAT-component
1.	PEG ₇₅₀ -PE (99.5)	Rh-PE (0.5)	—	—
2.	PEG ₇₅₀ -PE (97.0)	Rh-PE (0.5)	—	TATp-PEG ₁₀₀₀ -PE (2.5)
3.	PEG ₇₅₀ -PE (97.0)	Rh-PE (0.5)	—	TATp-PEG ₃₄₀₀ -PE (2.5)
4.	PEG ₇₅₀ -PE (99.0–95.0)	NBD-PE (1.0)	—	Rh-TATp-PEG ₁₀₀₀ -PE (0–4)
5.	PEG ₇₅₀ -PE (99.0–97.0)	NBD-PE (1.0)	—	Rh-TATp-PEG ₃₄₀₀ -PE (0–2)
6.	PEG ₇₅₀ -PE (67.0)	Rh-PE (0.5)	PEG ₂₀₀₀ -PE (30)	TATp-PEG ₁₀₀₀ -PE (2.5)
7.	PEG ₇₅₀ -PE (47.0)	Rh-PE (0.5)	PEG ₂₀₀₀ -PE (50)	TATp-PEG ₁₀₀₀ -PE (2.5)
8.	PEG ₂₀₀₀ -PE (99.5)	Rh-PE (0.5)	—	—
9.	PEG ₂₀₀₀ -PE (97.0)	Rh-PE (0.5)	—	TATp-PEG ₁₀₀₀ -PE (2.5)
10.	PEG ₂₀₀₀ -PE (97.0)	Rh-PE (0.5)	—	TATp-PEG ₃₄₀₀ -PE (2.5)