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TAT peptide-based micelle system for potential active targeting of anti-cancer agents to acidic solid tumors

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Abstract

A novel drug targeting system for acidic solid tumors has been developed based on ultra pH sensitive polymer and cell penetrating TAT. The delivery system consisted of two components: 1) A polymeric micelle that has a hydrophobic core made of Poly(L-lactic acid) (PLLA) and a hydrophilic shell consisting of Polyethylene Glycol (PEG) conjugated to TAT (TATmicelle), 2) An ultra pH sensitive diblock copolymer of poly(methacryloyl sulfadimethoxine) (PSD) and PEG (PSD-*b*-PEG). The anionic PSD is complexed with cationic TAT of the micelles to achieve the final carrier, which could systemically shield the micelles and expose them at slightly acidic tumor pH. TATmicelles had particle sizes between 20 to 45 nm and their critical micelle concentrations were 3.5 mg/L to 5.5 mg/L. The TATmicelles, upon mixing with pH sensitive PSD-*b*-PEG, showed slight increase in particle size between pH 8.0 and 6.8 (60–90 nm), indicating complexation. As the pH was decreased (pH 6.6 to 6.0) two populations were observed, one that of normal TAT micelles (45 nm) and the other of aggregated hydrophobic PSD-*b*-PEG. Zeta potential measurements showed similar trend substantiating the shielding/deshielding process. Flowcytometry and confocal microscopy showed significantly higher uptake of TAT micelles at pH 6.6 compared to pH 7.4 indicating shielding at normal pH and deshielding at tumor pH. The flowcytometry indicated that the TAT not only translocates into the cells but is also seen on the surface of the nucleus. These results strongly indicate that the above drug loaded micelles would be able to target any hydrophobic drug near the nucleus.

Keywords

TAT specificity; tumor pH; pH-sensitive polymer; micellar carrier

Introduction

Tumor targeting of a cytotoxic agent refers to the passive accumulation of nano-scaled drug carriers to solid tumors, followed by active internalization into tumor cells [1]. The internalization of drug, either alone or along with a carrier, is required for cell death because most cytotoxic drugs act intracellularly [2,3]. Important considerations of nano-sized carriers for improved passive tumor targeting include the surface property, shape and size for a given tumor. Surface PEGylation of the carriers is regarded as the gold standard for longer residence time in the blood and improved biocompatibility [4]. Spherical nanocarriers with diameters from 40 to 300 nm are typically used for passive targeting [5].

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Active targeting carriers have either monoclonal antibodies (mAb) [6], binding fragments [7] specific to a tumor associated surface antigen or a ligand binding to its corresponding receptor on the tumor cell surface. It has been clearly established that active targeting results in higher accumulation of carriers in tumors [8] albeit mAb or ligand conjugated to carriers may not guarantee long-range interactions with tumor cells. The term 'active targeting' is a misnomer as the carriers do not actively seek their target, in this case the tumor areas, but exert specific interactions with tumor cells only upon contact.

Most therapeutic systems rely on the receptor mediated endocytotic pathway for internalization into cells. This pathway leads to the entrapment and, to a large extent, degradation of transported biomolecules in lysosomes. The use of cell penetrating peptides, like the HIV peptide TAT, has the advantage of avoiding this pathway and taking the cargo directly into the cell. TAT-mediated cytoplasmic uptake of drug conjugated polymers [9,10], plasmid DNA [10], bacteriophages [11], magnetic nanoparticles of about 10–20 nm in diameter [12] and even liposomes having a diameter of 200 nm [13] has been documented in the literature [14–17]. Until now the mechanism of internalization of TAT peptide is unclear. However a number of evidence show that the internalization does not necessarily involve the presence of a specific cellular receptor or transporter as uptake seems to be temperature and cell type independent [18–21]. There appears to be two kinds of mechanism involved depending upon the size of the cargo. Smaller molecules attached to TAT seem to transduce directly into cells by the energy independent electrostatic interactions and hydrogen bonding [22] but larger cargos get into the cells by the energy dependent macropinocytosis pathway [23]. For over a decade various attempts have been made to make use of this versatile tool but only recently have some progress been made [24,25]. The main hurdle has been to bestow specificity and target the TAT peptide to the place of interest.

The delivery system is a 'smart micellar nanoplatform' that can possibly hide the non specific TAT peptide in normal body. It involves assimilation of two components: 1) chemotherapeutic polymeric micelles – consisting of polyethylene glycol (PEG) outer shell, with TAT attached to the PEG and a hydrophobic core made of poly(L-lactic acid) into which any chemotherapeutic can be incorporated 2) The TAT shield – an ultra pH sensitive smart block copolymer PSD-*b*-PEG (Poly sulfonamide). Physical mixing of the two components forms the final carrier. When the PSD-*b*-PEG polymer is mixed with the TAT micelle, the TAT peptides which are positively charged get shielded by the negatively charged PSD of the block copolymer.

A model of the proposed drug delivery system is shown in figure 1. The model is proposed to have a final size of about 50 – 200 nm after drug loading, and having a PEG shell it has a strong possibility of preferentially accumulate in the tumor tissue by Enhanced Permeation and Retention Mechanism (EPR) [26]. The PSD that is negatively charged at pH 7.4 has been shown to become neutral below pH 7.0 [27] (Extracellular tumor pH). Hence deshielding of TATmicelle is triggered by the lower pH of the tumor milieu [28,29]. As a consequence, at the tumor site the TATmicelles will be exposed to the surrounding. The TAT would then help target the drug loaded micelle into the cells and nucleus where the cytotoxic effect takes place.

Another possible advantage of this carrier system could be attributed to its potential to kill the so called 'cancer stem cells'. These cells, which have been implicated for tumor relapse and metastasis, are very difficult to kill with conventional methods and active targeting. They are usually buried deep inside the tumors and are extremely difficult to reach with conventional therapies [30]. More importantly, these tumor cells have a different phenotype compared to other cancer cells, so active targeting, which is usually designed for a particular antigen (present in predominant variety of tumor cells), will be ineffective against them [31]. On the other hand

this micelle based delivery system uses TAT for internalization, so in the acidic environment as TAT is exposed there is a potential for penetrating into these cells and kill them.

2. Materials and Methods

2.1 Materials

Sulfadimethoxine [4-amino-N-(2,6-dimethoxy-4pyrimidinyl)benzenesulfonamide] (SD), N-hydroxysuccinimide (HOSu) and dicyclohexyl carbodiimide (DCC) were purchased from Aldrich chemical Co. (Milwaukee, WI, USA) and used without further purification. α -Hydroxy- ω -carboxymethyl poly(ethylene oxide) (PEG monoacid) was synthesized and purified by Zalipsky's method [32]. Methacryloyl chloride (Aldrich) was distilled under reduced pressure (10 mmHg) at 30°C; dimethyl sulfoxide (DMSO, Aldrich) was purified by vacuum distillation at 75°C at 12 mmHg. 2, 2'-Azobisisobutyronitrile (AIBN) (Aldrich) was recrystallized in methanol twice. TAT labeled with FITC was synthesized at protein synthesis core facility at the University of Utah. All other chemicals were of reagent grades and were used without further purification.

2.2 Synthesis of pH sensitive PSD-b-PEG

The synthesis of poly(methacryloyl sulfadimethoxine) (PSD) is described in our previous report [33]. Briefly, methacryloyl SD was prepared in 20 ml of acetone/water (1:1) and sodium hydroxide (0.01 N). SD was first dissolved in the acetone/water mixture in the presence of NaOH, then methacryloyl chloride (10 mmol) was added drop wise into the mixture with simultaneous stirring at 0°C. The precipitated product was filtered and washed with distilled water three times. A white powder was obtained after drying *in vacuo* at room temperature for three days.

Semitelechelic SD polymer was prepared by free radical solution polymerization of methacryloyl SD in the presence of 2-aminoethanethiol (0.15 mmol) as a chain transfer agent in DMSO. AIBN (0.2 mol% for monomer) was added as an initiator. Methacryloyl SD, 2-aminoethanethiol and AIBN were dissolved in 80 ml of DMSO. The mixture was degassed by freeze-thaw cycles. The round-bottom flask containing reaction mixture was sealed under reduced pressure and immersed in an oil bath and kept at 60°C for 20 hours. After polymerization the content was poured into deionized (DI) water to precipitate out the product. The polymer was collected by filtration. Unreacted monomers were eliminated by dissolving dried polymer in 10-fold excess methanol. Reactions were confirmed and monitored with IR and ¹H NMR spectra. The molecular weight was determined to be 3 KDa (PDI = 1.16) using MALDI-TOF mass spectroscopy. Thus PSD is an oligomer rather than a polymer.

For the coupling of PSD with PEG monoacid (2,000 Da), PEG was activated in 30 ml tetrahydrofuran (THF) by HOSu with a molar ratio of PEG:HOSu:DCC = 1:2:2. The PEG was dissolved in 30 ml THF with HOSu and stirred, then SDM was added (mole ratio 1:1 with PEG) and the reaction continued for one hour. DCC was then added and the mixture was allowed to react for 12 hours. The contents were poured into 300 ml n-hexane for precipitation of the conjugate. Monomers and other impurities were removed by dissolving the polymer in DMSO and dialyzing (MWCO: 3 KDa) against water for 3 days. The conjugation was confirmed by mass spectrometry.

2.3 Synthesis of micelle components PLLA-b-PEG and PLLA-b-PEGmal

The block copolymer was synthesized using standard ring opening polymerization of L-Lactide and mono hydroxy PEG with staneous octoate as catalyst [34]. Briefly, first to remove any trace of water, the starting materials (2.5 mmol of poly(ethylene glycol)-monomethyl ether 5 kDa and 2 kDa and 70 mmol of L-lactide) were each dissolved in 150 ml toluene separately

in a round-bottomed flask. Thirty milliliters of toluene was distilled off using a water separator at 120 °C under nitrogen atmosphere. The water-free solutions were united in a three-neck flask and a precisely weighed amount of 100 mg stannous 2-ethylhexanoate (1 wt% of L-lactide) was added and then the mixture was refluxed for 24 h under nitrogen atmosphere at 110 °C. Upon completion of the reaction, the mixture was precipitated in 10 fold ice cold ethyl ether. The precipitated polymer was then filtered with an aspirator and dried in a desiccator at room temperature at a pressure of less than 0.1 mbar using a RV5 two stage vacuum pump from Edwards, Crawley, West Sussex, UK. After drying for three days, the polymer was ground under liquid nitrogen in a mortar to obtain a free flowing powder. The molecular weight and composition of the final polymer was determined using ¹H-NMR spectroscopy [35].

Similar procedure was employed to synthesize PLLA-*b*-PEGmal. Poly(ethylene glycol)-maleimide ether was used instead of poly(ethylene glycol)- mono methyl ether as the starting material and was purchased directly from Nektar Therapeutics Inc., San Carlos, CA. The maleimide group on the PEG was used to conjugate TAT using a thio ether bond.

2.4 Preparation of polymeric micelles

The polymeric micelles were formed by using diafiltration method. PLLA-*b*-PEGmal was dissolved in dioxane and PLLA-*b*-PEG was dissolved in DMSO. 0.5, 2, 4, 7 and 10 wt% PLLA-*b*-PEGmal solutions were mixed with PLLA-*b*-PEG solution and dialyzed in phosphate buffer saline (PBS) for 3 days. To form micelles and remove the organic solvents the PBS was changed every 1 hr, the first 6 h and then once every 8 h after that. The initial concentration of the polymers in the organic solvents was adjusted such that the final concentration of polymer in the micelle was approximately 1 g/L. The micelles were stable in aqueous solution for over 1 month (stored at 4°C). The stability was measured by determining if there was any change in particle size of the micelles with time using light scattering technique. The solutions were subsequently lyophilized after filtering through a 0.8 µm syringe filter. The yield (69.3 wt.%) of micelles was calculated by weighing the freeze-dried micelle powder.

2.5 Conjugation of TAT to polymeric micelles to form TATmicelles

The TAT peptide [FITC-Gly-Cys-(Gly)₃-Tyr-Gly-Arg-(Lys)₂-(Arg)₂-Gln-(Arg)₃] was conjugated to the maleimide on the PEG of the micelles via a thioether linkage. The polymeric micelles in PBS, having maleimide groups on the outside of the shell, were mixed with a little molar excess of TAT peptide solution at pH of 7.2 under nitrogen atmosphere. The reaction mixture was stirred overnight in dark at room temperature. The TAT conjugated micelles were then separated from unreacted TAT by using a PD10 column in PBS. The various fractions obtained were analyzed with HPLC and fluorescence spectroscopy to confirm conjugation and purity of product. The analyses showed 96% TAT conjugated to the micelles and about 4% free TAT.

A potential way to load the anticancer hydrophobic drug doxorubicin into the system is outlined below. First doxorubicin HCl (DOX HCl) will be stirred with twice the number of mole of TEA in DMSO overnight to obtain the DOX base. The TATmicelle in the mean time would be lyophilized to solid powder form. This powder would then be dissolved in DMSO and stirred along with DOX base for 3 h. The solution mixture would then be transferred into a pre swollen dialysis membrane with appropriate MW cutoff. Dialysis will be carried out against PBS buffer at pH 7.4 for 1 day with exchange of medium several times. The amount of DOX loaded will be determined by first dissolving the DOX loaded TATmicelles in DMSO and measuring the UV absorbance of the DOX loaded micelles at 481 nm.

2.6 pH-sensitivity studies of PSD-b-PEG using titration and light transmittance

15 mg of PSD-*b*-PEG was dissolved in 2 ml 0.1 N NaOH solution and titrated against 0.1 N HCl. The pH and optical transmittance of the solution after each NaOH addition was measured. The pH was measured with a Corning pH meter and the transmittance was measured at 500 nm using Varian Cary 1E UV/Vis spectrometer. The transmittances at different pHs were expressed as percentage relative to the transmittance at pH 12.0.

2.7 Determination of critical micelle concentration (CMC) of plain and TATmicelles

The CMC of the micelles was estimated by fluorescence spectroscopy using pyrene, a hydrophobic fluorescence probe that preferentially partitions into the hydrophobic core of the micelle. CMC values were obtained by monitoring the changes in the ratio of the pyrene excitation spectra intensities at $\lambda = 333$ nm (I_{333}) for pyrene in water and $\lambda = 336$ nm (I_{336}) for pyrene in the hydrophobic medium (THF) within the micelle core. The micellar solutions were diluted to obtain a concentration range from 2 to 1×10^{-4} g/L. Pyrene solution in THF (1.2×10^{-3} M) was added to doubly distilled water to give a pyrene concentration of 12×10^{-7} M, and THF was removed using a rotary evaporator at 30 °C. The pyrene solution was mixed with block copolymer solutions to obtain copolymer concentrations from 1 to 5×10^{-5} g/L. The pyrene concentration of the samples was 6.0×10^{-7} M. All the samples were sonicated for 10 min and were allowed to stand for 1 day before the fluorescence measurements. The fluorescence measurements were performed using Shimadzu RF5301PC fluorescence spectrometer. The CMC values were calculated as the polymer concentration corresponding to the onset of the increase in the ratio of I_{336}/I_{333} . The ratio is the intersection of the horizontal line with an almost constant value of the ratio I_{336}/I_{333} and the steep upward line of the sigmoidal curve.

2.8 Particle size and zeta potential determination of various micelles

Zeta potential and particle size were measured using laser light scattering technique on a Malvern Zetasizer 3000HS (Malvern Instruments, Worcestershire, UK) equipped with He-Ne laser (633 nm). The TATmicelle and PSD-*b*-PEG complexes were made by mixing the two components at individual pHs which contained a 10 mM NaCl concentration.

2.9 Flowcytometry studies

Human breast adenocarcinoma (MCF-7) cells were obtained from Korean Cell Line Bank (KCLB). They were maintained in RPMI-1640 medium with 2 mM L-glutamine, 1 % penicillin-streptomycin and 10 % fetal bovine serum in a humidified incubator at 37°C and 5 % CO₂ atmosphere.

The cells (5×10^4 cells/ml) grown as a monolayer were harvested by 0.25% (w/v) trypsin-0.03% (w/v) EDTA solution and were seeded in 12 well plates at a density of 2.5×10^5 cells per well and incubated for 24 h. Medium with serum was replaced with 1 ml media of pH 6.6 and 7.4 having no serum, one hour before addition of various complexes. The test samples: TATmicelles and TATmicelles complexed with PSD-*b*-PEG, were added (2% TAT on the surface of the micelles and 20 μ l of 0.780 mg/mL micelles, concentration about 4 times the CMC) into the well plates and were incubated for 15, 30, 60 and 120 minutes. The incubation solution was then removed and the cells were washed with 20 mM PBS. The cells on the surface of the plate were detached by using trypsin solution and then fixed with 2.5% glutaraldehyde before flow cytometry analysis. The fluorescence of the TATmicelles internalized into the cells was measured by using FACSCAN flow cytometer (Becton Dickinson Inc., USA). The excitation wavelength was set at 488 nm and measured with the FL1 channel.

2.10 Confocal microscopy

The analysis of intracellular distribution of TATmicelles was carried out on MCF-7 cells grown on a Lab-Tek^R II chamber slide (Nalge Nunc International, Naperville, IL). Two kinds of experiments were performed: 1) The internalization of TATmicelles with and without complexation with pH sensitive PSD-*b*-PEG as a function of pH, 2) Internalization kinetics and localization of TATmicelles without complexation. For the first, cells were incubated with plain TATmicelles or TATmicelles complexed with pH sensitive polymer at pHs 6.6 or 7.4 and incubated for 4 h. Then the cells were treated with a drop of anti-fade mounting media (5% n-propyl gallate, 47.5% glycerol and 47.5% Tris-HCl pH 8.4) before covering them with a cover slip. For the internalization kinetics experiments, the test samples and experimental conditions were the same as that of flow cytometry. After washing the cells with PBS and before the mounting of the cover slip, a nuclear stain dye TOPRO-3 and a drop of anti-fade were added and allowed to set in for 1 minute. All the samples were dried at room temperature for one hour before sealing the cover slips with Premount. A laser scanning Olympus FluoviewTM series confocal microscope equipped with Argon and krypton lasers providing excitation energy at 488 for the double-labeled specimens (FITC on TAT and TOPRO-3) and 60x, N.A. 1.35 oil immersion lens was utilized for obtaining the confocal micrographs.

2.11 Statistical Analysis

All the experiments were repeated at least three times with a minimum sample size of three. Student's t-test was used, using STATA statistical software (StataCorp LP, Texas), to test for statistical difference between samples. Comparisons having 'p' value ≤ 0.05 were considered significant.

Result and Discussions

The aim of this paper is to demonstrate the feasibility of utilizing a pH sensitive polymer in a targeted carrier system. The ability to convert the non specific yet highly versatile TAT peptide into specific targeting to tumor cells is explored here.

3.1. pH-sensitivity of PSD-PEG

We first tested the pH sensitivity and apparent pK_b of the sulfonamide polymer PSD conjugated to PEG. The apparent pK_b of the polymer was found to be 7.0 (Fig 2a) as it was observed that addition of small amounts HCl at this point did not decrease the pH. This may be because the negatively charged sulfonamide groups in the polymer abstracted the protons released by HCl. Figure 2b shows the % light transmittance of the polymer. It is clearly seen that PSD-*b*-PEG shows very sharp decrease in transmittance at about pH 7.0. The sulfonamide group is ionized at high pH because the strong electronegativity of the oxygen atoms at the sulfonyl group draws electrons from the sulfur atom, which in turn pulls the electrons from the nitrogen atom (Fig 2c). This results in nitrogen pulling electrons from the N-H bond and thus releasing the proton. The pK_b of the sulfonamide compound is governed by dimethoxypyrimidine group, which here is the electron withdrawing group.

In this study, the sulfonamide part (PSD) of the pH sensitive polymer has a low degree of polymerization ($DP \approx 7$) and very narrow polydispersity ($PDI = 1.1$) due to which all the chains of the polymer experience similar forces. This may be the main reason for the polymer to show such sharp transition. In general, the sulfonamide polymers have a much sharper transition than polymers based on carboxylic end groups [36,37]. It should be noted here that this transition of the PSD-*b*-PEG polymer is ideally suited for tumor targeting.

3.2. Characterization of plain and TATmicelles

Polymeric micelles were prepared from three different molecular weights of PLLA-*b*-PEG and were tested for stability and size to determine the optimum size that would be suitable for testing of the shielding and deshielding concept. The CMC values and size measurement showed good stability and optimized size for assembling a carrier system and are comparable to the micelles prepared by other researchers[38,39] (Fig 3a and Table 1). The attachment of TAT on the outside of the micelles did not change either the CMC or size of the micelles significantly (Fig 3b).

PLLA12k-*b*-PEG5k was chosen for further studies because of its larger size. It was thought that it would be easier to study the attachment/detachment phenomenon of pH sensitive polymer with the larger micelles. Also 2% surface TATmicelles were chosen, over other percentage conjugations micelles, for further studies because TAT attachment above 2% showed higher population of secondary micelles (above 300 nm) and precipitated after 15 days (compared to 1 month for 2%).

3.3 Shielding/deshielding of TATmicelles with pH sensitive polymer

Before testing the shielding/deshielding of the TATmicelles with pH sensitive polymer, the TATmicelles' stability at different pHs was tested. The micelles were incubated in solutions between pHs 8.0 to 6.0 for 24 h and tested for particle size changes. The data did not show any significant difference in size at the various pHs tested (data not shown). Particle size evaluation of PLLA12k-*b*-PEG5k TATmicelles, after complexation with pH sensitive PSD-*b*-PEG, showed slight increase in size between pH 8.0 and 6.8 (60–90 nm) indicating complexation (Fig 4a). The increase in size may be due to attachment of the copolymer and reduction in surface charge of the micelles.

Between pH 6.6 to 6.0 two populations were observed, one about 40 nm in size that corresponds to TATmicelles and the other about 200nm, which could be that of aggregated hydrophobic PSD-*b*-PEG. Zeta potential measurements showed similar trend, substantiating the shielding/deshielding process. From pH 8.0 to 6.8 the zeta potential was around zero indicating complete shielding of TAT and from 6.6 to 6.0 the zeta potential was increased to 6.0 mV, which is the same as the zeta potential measured for TATmicelle alone (Fig 4b), implicating the deshielded TAT. The TAT peptide has high positive charge still the zeta potential of the micelles is low, this is attributed to the low surface density of TAT conjugated to the micelle surface.

3.4 In vitro characterization of TATmicelles complexed with PSD-b-PEG

3.4.1 Kinetics of TATmicelles uptake into MCF-7 cells—The MCF-7 adenocarcinoma cell lines were tested for micelle shielding/deshielding and uptake. Figure 5 shows the flowcytometry data for various micelles taken up at different time points. It is seen that the TATmicelles enter the cells very quickly as even at 15 min of incubation these micelles show higher FL-1 reading than PMC (pH sensitive polymer and TATmicelle complexes) (Fig 5a). At 30 min of incubation lot more TATmicelle have been taken up (indicated by increase in area of FL-1 curve) but micelles complexed with pH sensitive polymer have still not entered the cells (Fig 5b). The area under the curve of FL-1, which denotes the total amount of cells having fluorescence, for TATmicelles is about 10 times more than that of PMC (x-axis being logarithmic). It is interesting to note that the TATmicelles show similar histogram for both pHs 7.4 and 6.6 indicating that the pH does not affect internalization.

The micelles complexed with pH sensitive PSD-*b*-PEG at pH 6.6 have internalized at 1 h of incubation (Fig 5c). At pH 6.6 the PSD of pH sensitive PSD-*b*-PEG polymer becomes neutral and detaches from TAT of the micelles. This detachment enables, the now exposed TAT, to interact with the cells and internalizes the micelles. The complexed micelles incubated at pH

7.4, on the other hand, show lower fluorescence. This may be because the pH sensitive negatively charged PSD-*b*-PEG is still complexed with the positively charged TAT and is preventing it from interacting with the cells. Similar trend is observed at 2 h also. Even though the PSD-*b*-PEG polymer is able to shield the TAT effectively at pH 7.4, the deshielding at pH 6.6 is not complete. This may be due to various reasons: for instance, hindrance by the separated PSD-*b*-PEG on the surface of the cells, and/or incomplete detachment of the sulfonamide groups from the TAT. Various techniques are being employed to improve this deshielding process, such as changing the molecular weight of PSD and use of a different sulfonamide.

3.4.2 Internalization studies of the micelle complex with confocal microscopy—

The flow cytometry data are more quantitative than confocal microscopy; however they do not clarify whether the micelles are on the surface or are actually internalized. Confocal microscopy with these micelles shows that the micelles are not on the surface but have internalized into the cells (fig 6). The shielded and unshielded TATmicelles were tested for internalization into cells at pHs 7.4 and 6.6. The micrographs of the unshielded TATmicelles show internalization into the cells both at pH 7.4 and 6.6 (Figs 6a and b). The TATmicelles complexed with pH sensitive PSD-*b*-PEG, on the other hand, do not internalize at pH 7.4, indicating shielding (Fig 6c). At pH 6.6 internalization is clearly seen (Fig 6d) indicating deshielding. These data strongly support the results indicated by the flow cytometry experiments. The confocal micrographs show uniform distribution of fluorescence indicating macro pinocytosis entry mechanism rather than endocytosis which should have shown pockets of fluorescence inside the cells. In recent years, researchers are of the opinion that the TAT enters the cells through caveolae mediated macro pinocytosis [23,40] and our results support this theory.

To go a step beyond the entry of the TATmicelles into the cells, the TATmicelles nuclear localization was studied. Experiments were performed by incubating MCF-7 cells with only fluorescent TATmicelles and staining the nucleus with TOPRO-3 dye. These cells were then analyzed with dual label confocal microscopy. Figure 7 shows the micro graphs of the results. The green stain (Fig 7a) is the fluorescence of FITC attached on TAT and the red stain is that of TOPRO-3 showing the nucleus. Figure 7c shows the superimposed micrograph of both the images. The yellow color on the nucleus clearly indicates co-localization of green and red, which strongly suggests TAT and its cargo are localized at the nucleus. Figure 7 provides a more compelling case for non-endocytotic pathway because of fluorescence seen at nucleus. The carrier does not have an endosome escaping component; if it had entered the cells via endocytosis, there was greater chance for the carriers to have degraded or exocytosed out of the cells. It would have been very hard for the TATmicelles to translocate to the nucleus if a receptor mediated entry mechanism was involved; macro pinocytosis seems to be a more likely mechanism.

Conclusion

In this study, we describe a carrier system that modifies the non specific TAT, which could be used to effectively target the tumor areas that provide an acidic profile. The designed micelle system was able to effectively distinguish a small difference in pH and internalize into cells. The TAT attached to the micelles helped translocate the micelles not only into the cells but also near the nucleus. Further testing of the delivery system is underway, using chemotherapeutic agents both *in-vitro* and *in-vivo*, to evaluate its potential to kill tumors.

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References

1. Minko T, Dharap SS, Pakunlu RI, Wang Y. Molecular targeting of drug delivery systems to cancer. *Curr Drug Targets* 2004;5:389–406. [PubMed: 15134222]
2. Kim R. Recent advances in understanding the cell death pathways activated by anticancer therapy. *Cancer* 2005;103:1551–1560. [PubMed: 15742333]
3. Wardwell NR, Massion PP. Novel strategies for the early detection and prevention of lung cancer. *Semin Oncol* 2005;32:259–268. [PubMed: 15988680]
4. Crawford J. Clinical uses of pegylated pharmaceuticals in oncology. *Cancer Treat Rev* 2002;28(Suppl A):7–11. [PubMed: 12173409]
5. Demeneix B, Hassani Z, Behr JP. Towards multifunctional synthetic vectors. *Curr Gene Ther* 2004;4:445–455. [PubMed: 15578993]
6. Richter M, Zhang H. Receptor-targeted cancer therapy. *DNA Cell Biol* 2005;24:271–282. [PubMed: 15869404]
7. Spiridon CI, Guinn S, Vitetta ES. A comparison of the in vitro and in vivo activities of IgG and F(ab')₂ fragments of a mixture of three monoclonal anti-Her-2 antibodies. *Clin Cancer Res* 2004;10:3542–3551. [PubMed: 15161714]
8. Normanno N, Bianco C, Strizzi L, Mancino M, Maiello MR, De Luca A, Caponigro F, Salomon DS. The ErbB receptors and their ligands in cancer: an overview. *Curr Drug Targets* 2005;6:243–257. [PubMed: 15857286]
9. Nori A, Jensen KD, Tijerina M, Kopeckova P, Kopecek J. Subcellular trafficking of HPMA copolymer-Tat conjugates in human ovarian carcinoma cells. *J Control Release* 2003;91:53–59. [PubMed: 12932637]
10. Hyndman L, Lemoine JL, Huang L, Porteous DJ, Boyd AC, Nan X. HIV-1 Tat protein transduction domain peptide facilitates gene transfer in combination with cationic liposomes. *J Control Release* 2004;99:435–444. [PubMed: 15451601]
11. Paschke M, Hohne W. A twin-arginine translocation (Tat)-mediated phage display system. *Gene* 2005;350:79–88. [PubMed: 15794923]
12. Nitin N, LaConte LE, Zurkiya O, Hu X, Bao G. Functionalization and peptide-based delivery of magnetic nanoparticles as an intracellular MRI contrast agent. *J Biol Inorg Chem* 2004;9:706–712. [PubMed: 15232722]
13. Fretz MM, Mastrobattista E, Koning GA, Jiskoot W, Storm G. Strategies for cytosolic delivery of liposomal macromolecules. *Int J Pharm* 2005;298:305–309. [PubMed: 15941633]
14. Wang J, Dai H, Yousaf N, Moussaif M, Deng Y, Boufelliga A, Swamy OR, Leone ME, Riedel H. Grb10, a positive, stimulatory signaling adapter in platelet-derived growth factor BB-, insulin-like growth factor I-, and insulin-mediated mitogenesis. *Mol Cell Biol* 1999;19:6217–6228. [PubMed: 10454568]
15. Riedel H, Yousaf N, Zhao Y, Dai H, Deng Y, Wang J. PSM, a mediator of PDGF-BB-, IGF-I-, and insulin-stimulated mitogenesis. *Oncogene* 2000;19:39–50. [PubMed: 10644978]
16. Chikh G, Bally M, Schutze-Redelmeier MP. Characterization of hybrid CTL epitope delivery systems consisting of the Antennapedia homeodomain peptide vector formulated in liposomes. *J Immunol Methods* 2001;254:119–135. [PubMed: 11406158]
17. Troy CM, Shelanski ML. Down-regulation of copper/zinc superoxide dismutase causes apoptotic death in PC12 neuronal cells. *Proc Natl Acad Sci U S A* 1994;91:6384–6387. [PubMed: 8022792]
18. Magzoub M, Kilk K, Eriksson LE, Langel U, Graslund A. Interaction and structure induction of cell-penetrating peptides in the presence of phospholipid vesicles. *Biochim Biophys Acta* 2001;1512:77–89. [PubMed: 11334626]
19. Derossi D, Calvet S, Trembleau A, Brunissen A, Chassaing G, Prochiantz A. Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent. *J Biol Chem* 1996;271:18188–18193. [PubMed: 8663410]
20. Fahraeus R, Lane DP. The p16(INK4a) tumour suppressor protein inhibits alphavbeta3 integrin-mediated cell spreading on vitronectin by blocking PKC-dependent localization of alphavbeta3 to focal contacts. *Embo J* 1999;18:2106–2118. [PubMed: 10205165]

21. Sharma SK, Ramsey TM, Chen YN, Chen W, Martin MS, Clune K, Sabio M, Bair KW. Identification of E2F-1/Cyclin A antagonists. *Bioorg Med Chem Lett* 2001;11:2449–2452. [PubMed: 11549444]
22. Vives E, Richard JP, Rispal C, Lebleu B. TAT peptide internalization: seeking the mechanism of entry. *Curr Protein Pept Sci* 2003;4:125–132. [PubMed: 12678851]
23. Wadia JS, Stan RV, Dowdy SF. Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat Med* 2004;10:310–315. [PubMed: 14770178]
24. Lewin M, Carlesso N, Tung CH, Tang XW, Cory D, Scadden DT, Weissleder R. Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells. *Nat Biotechnol* 2000;18:410–414. [PubMed: 10748521]
25. Sawant RM, Hurley JP, Salmaso S, Kale A, Tolcheva E, Levchenko TS, Torchilin VP. “SMART” drug delivery systems: double-targeted pH-responsive pharmaceutical nanocarriers. *Bioconjug Chem* 2006;17:943–949. [PubMed: 16848401]
26. Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *J Control Release* 2000;65:271–284. [PubMed: 10699287]
27. Kang SI, Bae YH. pH-Induced solubility transition of sulfonamide based polymers. *J Control Release* 2002;80:145–155. [PubMed: 11943394]
28. van Sluis R, Bhujwalla ZM, Raghunand N, Ballesteros P, Alvarez J, Cerdan S, Galons JP, Gillies RJ. In vivo imaging of extracellular pH using ¹H MRSI. *Magn Reson Med* 1999;41:743–750. [PubMed: 10332850]
29. Sethuraman VA, Na K, Bae YH. pH-responsive sulfonamide/PEI system for tumor specific gene delivery: an in vitro study. *Biomacromolecules* 2006;7:64–70. [PubMed: 16398499]
30. Soltysova A, Altanerova V, Altaner C. Cancer stem cells. *Neoplasma* 2005;52:435–440. [PubMed: 16284686]
31. Ponti D, Zaffaroni N, Capelli C, Daidone MG. Breast cancer stem cells: an overview. *Eur J Cancer* 2006;42:1219–1224. [PubMed: 16624548]
32. Zalipsky S, Seltzer R, Menon-Rudolph S. Evaluation of a new reagent for covalent attachment of polyethylene glycol to proteins. *Biotechnol Appl Biochem* 1992;15:100–114. [PubMed: 1550659]
33. Han SK, Na K, H BY. Sulfonamide based pH-sensitive polymeric micelles: physicochemical characteristics and pH-dependent aggregation. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 2003;214:49–59.
34. Lucke A, Tessmar J, Schnell E, Schmeer G, Gopferich A. Biodegradable poly(D, L-lactic acid)-poly(ethylene glycol)-monomethyl ether diblock copolymers: structures and surface properties relevant to their use as biomaterials. *Biomaterials* 2000;21:2361–2370. [PubMed: 11055283]
35. Kataoka K, Harada A, Nagasaki Y. Block copolymer micelles for drug delivery: design, characterization and biological significance. *Adv Drug Deliv Rev* 2001;47:113–131. [PubMed: 11251249]
36. Bibby DC, Davies NM, Tucker IG. Poly(acrylic acid) microspheres containing beta-cyclodextrin: loading and in vitro release of two dyes. *Int J Pharm* 1999;187:243–250. [PubMed: 10502630]
37. Ramkissoon-Ganorkar C, Liu F, Baudys M, Kim SW. Modulating insulin-release profile from pH/thermosensitive polymeric beads through polymer molecular weight. *J Control Release* 1999;59:287–298. [PubMed: 10332061]
38. Lin WJ, Chen YC, Lin CC, Chen CF, Chen JW. Characterization of pegylated copolymeric micelles and in vivo pharmacokinetics and biodistribution studies. *J Biomed Mater Res B Appl Biomater* 2006;77:188–194. [PubMed: 16245284]
39. Lee J, Cho EC, Cho K. Incorporation and release behavior of hydrophobic drug in functionalized poly(D, L-lactide)-block-poly(ethylene oxide) micelles. *J Control Release* 2004;94:323–335. [PubMed: 14744484]
40. Kaplan IM, Wadia JS, Dowdy SF. Cationic TAT peptide transduction domain enters cells by macropinocytosis. *J Control Release* 2005;102:247–253. [PubMed: 15653149]

Figure 1.

Schematic model for the proposed drug delivery system: the carrier system consists of two components, a PLLA-*b*-PEG micelle conjugated to TAT and a pH sensitive diblock polymer PSD-*b*-PEG. a) At normal blood pH, the sulfonamide is negatively charged, and when mixed with the TAT micelle, shields the TAT by electrostatic interaction. Only PEG is exposed to the outside which could make the carrier long circulating; b) when the system experiences a decrease in pH (near tumor) sulfonamide loses charge and detaches, thus exposing TAT for interaction with tumor cells.

Figure 2. pH response of the polymer PSD-*b*-PEG. a) Titration curve of the polymer showing the apparent pK_b ; b) Percentage transmittance of the polymer; c) Chemistry of sulfonamide pH transition. [For a) and b) $n = 3$]

Figure 3.

a) Particle sizes of different MW micelles as determined by light scattering. The sizes do not change significantly between room temperature and 37 °C ($n = 3$, $p > 0.2$) and b) Sizes of PLLA12k-*b*-PEG5k micelles with different amounts of TAT attached ($n = 3$).

Figure 4.

Particle size and zeta potential data of PMC (pH sensitive polymer + TATmicelles complex) at various pHs. (a) Particle size of the complex from pH 8.0 to pH 6.0. From pH 8.0 to 6.8 unimodal particle distributions was observed, indicating PSD-*b*-PEG complexed with the micelles, 6.6 to 6.0 shows bimodal particle distribution, the bigger population could be the aggregates of the neutral PSD-*b*-PEG ($n = 3$). (b) Zeta potential studies indicate similar trend, the particles show near zero zeta potential between pH 8.0 and 6.8 that indicate complete complexation of PSD-*b*-PEG and positive potential from pH 6.6 to 6.0 indicating decomplexation ($n = 3$).

Figure 5.

Flow cytometry histograms of TATmicelles and PMC (PSD-*b*-PEG Polymer TATmicelles Complex) internalized into MCF-7 cells at pHs 7.4 and 6.6. Incubation times a) 15, b) 30, c) 60 and d) 120 minutes. (Control histograms are of cells incubated with plane micelles)

Figure 6. Confocal micrographs of MCF-7 cells incubated for 2 h with TATmicelles and PMC. a) and b) TATmicelles at 7.4 and 6.6 respectively; c) and d) PMC at pH 7.4 and 6.6 respectively. The micrographs clearly show the shielding at pH 7.4 and deshielding at pH 6.6.

Figure 7.

Dual label confocal micrographs of MCF-7 cells incubated with TATmicelles. a) Cells stained with green of FITC attached to TAT in the micelles; b) Nucleus stained red with TOPRO-3; c) Superimposed image of the two micro graphs. The yellow color showing the localization of TAT at the nucleus.

Table 1

Characterization of various micelles

	Molecular weight (kDa)	Polydispersity index	Yield (%)	Plain micelles (CMC)	TA Micelles (CMC)	Particle size of plain micelles (nm)
PLL/A2k- <i>b</i> -PEG2k	4	1.43	84	4.583	3.745	25
PLL/A3k- <i>b</i> -PEG5k	8	1.57	89	5.331	4.960	43
PLL/A12k- <i>b</i> -PEG5k	17	1.97	91	3.847	3.792	51
PSD3k- <i>b</i> -PEG2k	5	1.23	76			