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Optimization of Landscape Phage Fusion Protein-Modified Polymeric PEG-PE Micelles for Improved Breast Cancer Cell Targeting

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Abstract

Amphiphilic landscape phage fusion proteins with high affinity and selectivity towards breast cancer MCF-7 (Michigan Cancer Foundation-7) cells self-assemble with polymeric PEG-PE conjugates to form mixed micelles (phage-micelles) capable of cancer cell-targeted delivery of poorly-soluble drugs. While the PEG corona provides the stability and longevity to the micelles, its presence is a potential steric difficulties for the interaction of phage fusion protein with cell surface targets. We attempted to address this problem by controlling the length of the PEG block and the phage fusion protein quantity, selecting the optimal ones to produce a reasonable retention of the targeting affinity and selectivity of the MCF-7-specific phage fusion protein. Three PEG-PE conjugates with different PEG lengths were used to construct phage- and plain-micelles, followed by FACS analysis of the effect of the PEG length on their binding affinity and selectivity towards target MCF-7 cells using either a MCF-7 cell monoculture or a cell co-culture model composed of target cancer MCF-7 cells and non-target, non-cancer C166 cells expressing GFP (Green Fluorescent Protein). Both, the length of PEG and quantity of phage fusion protein had a profound impact on the targetability of the phage-micelles. Phage-micelles prepared with PEG_{2k}-PE achieved a desirable binding affinity and selectivity. Incorporation of a minimal concentration of phage protein, up to 0.5%, produced maximal targeting efficiency towards MCF-7 cells. Overall, phage-micelles with PEG_{2k}-PE and 0.5% of phage protein represent the optimal formulation for targeting towards breast cancer cells.

Keywords: Drug delivery; Polymeric micelles, PEG-PE; Phage display; Landscape phage fusion protein; Tumor targeting; Breast cancer

Introduction

Lipophilic compounds account for more than 40% of new drug candidate molecules. Despite their potent pharmacological activity, therapeutic application of these molecules is limited due to their poor solubility and low bioavailability. To overcome this limitation, many efforts have been made to develop effective drug delivery systems in order to enhance solubility of hydrophobic drugs [1-4].

One of promising delivery systems is the polymeric micelle. Nano-scale micellar particles can be formed by the self-assembly of amphiphilic molecules in an aqueous environment, with hydrophobic fragments forming the core of a micelle and with the hydrophilic parts forming a micellar corona [5,6]. The hydrophobic core of a micelle has been used to encapsulate a variety of sparingly-soluble therapeutic and diagnostic agents [5-9]. Micellar drug delivery systems substantially increase the bioavailability of poorly-soluble pharmaceuticals and protect them from destructive factors upon parenteral administration [10]. Their nanometre-sizes (typically, between 5 and 50 nm) allow micellar drugs to passively target tumor sites via the Enhanced Permeability and Retention (EPR) effect [11].

The tumor-targeting efficiency of micelle-encapsulated drugs can be further enhanced by introducing targeting ligands into a micellar formulation to allow for active targeting of tumors [6,12]. In the ongoing efforts in the search for targeting ligands, peptide-mediated tumor targeting has become a fast growing field [13], since peptides show multiple advantages, including lesser susceptibility to clearance by the Mononuclear Phagocyte System (MPS), less immunogenicity, and better tumor penetration when compared with antibodies [14,15]. The molecular mechanism behind this phenomenon is that peptide receptors are over-expression in a wide spectrum of tumors [16].

Combinatorial technologies, such as phage display techniques, have made targeting ligands available in a high throughput fashion [17,18] .

Recently, we identified MCF-7 breast- and prostate cancer–specific phage fusion proteins and demonstrated their abilities for actively-targeted delivery of the liposomal doxorubicin [19-22]. However, the effectiveness of the use of liposomes for delivery of water-insoluble drugs is far from ideal. Both, the limited drug encapsulation capacity and the potentially unstable packaging of hydrophobic drugs within the lipid bilayers of liposomes are major concerns [5,23]. Additionally, the *in vivo* premature release of a liposome-loaded hydrophobic drug could represent other issues [23]. We proposed recently the use of PEG-PE-based micelles for the solubilization of hydrophobic drugs [12] and explored the utility of phage fusion proteins in targeting delivery of micelles carrying poorly-soluble drugs. This approach is based on the amphiphilic nature of the phage fusion protein, which ensures its ability to assemble spontaneously with micelle-forming polymers, such as PEG-PE conjugates, resulting in the formation of mixed micelles capable of delivery of water-insoluble drug to specific tumor cells (Figure 1).
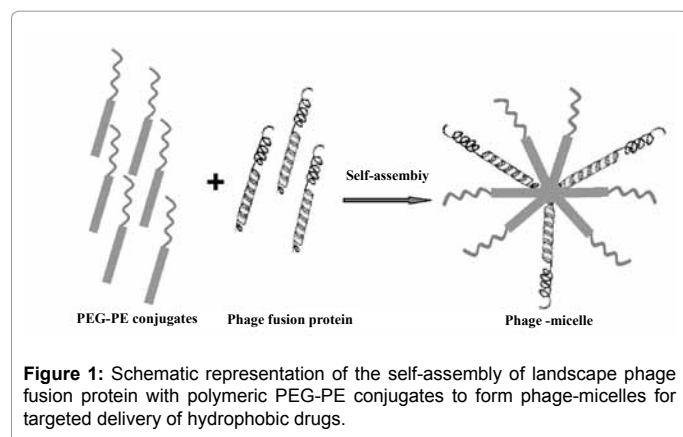
However, the presence of a PEG corona in the mixed micelles could potentially set up a steric barrier between the targeting phage fusion

Figure 1: Schematic representation of the self-assembly of landscape phage fusion protein with polymeric PEG-PE conjugates to form phage-micelles for targeted delivery of hydrophobic drugs.

peptides and molecular receptors on the surface of target cells. In this study, we sought to address this problem by controlling the length of the PEG block and phage protein quantity, selecting parameters, which produce an optimal balance between the target affinity and selectivity of the micelle-incorporated phage protein.

Materials and Methods

Materials and reagents

1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)2000] (ammonium salt; PEG_{2000}-PE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)750] (ammonium salt; PEG_{750}-PE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)5000] (ammonium salt; PEG_{5000}-PE), and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt, Rho-PE) were purchased from Avanti Polar Lipids Inc (Alabaster, AL). Paclitaxel (PCT) was from Cedarburg Pharmaceuticals (Beverly, MA). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Pittsburgh, PA). Sodium cholate was from Sigma (St.Louis, MO). Cell Titer Blue assay* kit was from Promega (Madison, WI). Fluor Mounting Medium was from Trevigen Inc (Gaithersburg, MD). MCF-7 human breast adenocarcinoma (HTB-22™) cells and C166-GFP (CRL-2583™) mouse yolk sac endothelial cells were obtained from the ATCC (*American Type Culture Collection)* (Manassas, VA). All cells were grown as recommended by the ATCC at 37°C, 5% CO_2.

Phage fusion protein

Phage selection and phage protein purification have been carried out as described by us previously [19].

Preparation of micellar formulations

Phage-micelles were prepared with compositions shown in Table 1. Briefly, to make rhodamine-labeled micelles, 5 mM of PEG-PE was mixed with traces of rhodamine-PE in chloroform. To form phage-micelles, after evaporation of chloroform, the PEG-PE film formed was hydrated with MCF-7-specific-phage fusion proteins dissolved in 10 mM of sodium cholate, followed by vortexing and overnight dialysis against Phosphate Buffered Saline (PBS), pH 7.4 to remove the detergent. To form plain micelles, the PEG-PE film formed after evaporation was hydrated with PBS, pH 7.4, followed by vortexing and overnight dialysis against PBS, pH 7.4.

FACS analysis of the uptake of phage-micelles by MCF-7 Cells

MCF-7 cells were grown in 12.5 cm² flasks in MEM with 10% serum until 70-80% confluence. Cells were incubated with 75 μM of rhodamine-labeled plain- or phage-micelle formulations for 1 h. After washing 3 times with PBS, pH 7.4, cells were detached and collected by centrifugation. The cell pellets were resuspended in 200 μl of PBS with 4% paraformaldehyde, followed by flow cytometry (FACS) analysis. A right shift on the x-axis of the histogram plot indicated the cellular binding of the rhodamine-labeled micelles.

FACS Analysis of Selective Binding of Phage-Micelles with Target MCF-7 Cells in a Co-culture Model

Target MCF-7 cells were co-cultured with non-target C166 endothelial cells expressing GFP in a 1:1 ratio and seeded in 12.5cm² flasks in MEM with 10% serum. After co-culture until 70-80% confluence, cells were incubated with 75 μM of rhodamine-labeled plain- or phage-micelle formulations for 1 h. The cells were washed 3 times with PBS (pH 7.4), detached, and collected by centrifugation. The cell pellets were resuspended in 200 μl of PBS with 4% paraformaldehyde, followed by the FACS analysis. After acquiring data displayed as dot plots, the dot plots were inserted into four regions (R1, R2, R3, and R4). The cellular binding of the rhodamine-labeled micelles was detected as a right shift of the cell population on the x axis (FL2-H, Red). The percent cell-associated micelles were calculated as follows:

For MCF-7 cells,

The percent cells with associated micelles = R3 / (R1+R3) × 100.

For C166-GFP cells,

The percent of cells with associated micelles = R4 / (R2+R4) × 100.

The binding affinity was determined by the percent of MCF-7 cells with associated micelles.

The binding selectivity was defined as the percent of MCF-7 cells with associated micelles divided by the percent of C166-GFP cells with associated micelles as follows:

The binding selectivity = [R3 / (R1+R3)] / [R4 / (R2+R4)].

Fluorescence microscopy analysis of selective binding of optimized phage-micelles to target MCF-7 cells

MCF-7 and C166-GFP cells were co-cultured on 6-well plates for 24h at 37°C, and treated with 75 μM of rhodamine-labeled phage-micelles in MEM with 10% serum for 30 min at 37°C. After washing 3 times with PBS, the coverslip was mounted onto a glass slide over the fluorescence mounting medium. The images were acquired by a fluorescence microscope (Nikon, Japan) at 40 × magnification with FITC or TRITC filters.

Cytotoxicity

After MCF-7 cells were cultured in 96-well microplates to 50-60% confluence, cells were treated for 72h with paclitaxel (PCT)-loaded, optimized PEG_{2k}-PE phage-micelles and different controls, including free PCT in DMSO, PCT-loaded PEG_{2k}-PE plain micelles, and drug-free, optimized phage-micelles. The concentration of paclitaxel used in different treatments with drug-containing formulations is equivalent to 1.76 μM. Cells were then washed once with PBS, pH 7.4, and incubated with fresh complete medium (100 μl/well) along with the Cell Titer Blue assay reagent (20 μl/well) for 2 h at 37°C. The fluorescence intensity was measured using a multi-detection microplate reader (Bio-Tek, Winooski, VT) with 525/590 nm excitation/emission wavelengths.

Table 1: Micellar formulations.

The use of micelles	Designations	PEG length	PEG-PE (mM)	Phage Protein (% w/w)
*Optimization of PEG length	Phage PEG5K-PE micelles	5K	5	0.5
	Phage PEG2K-PE micelles	2K	5	0.5
	Phage PEG750-PE micelles	750	5	0.5
	Plain PEG5K-PE micelles	5K	5	0
	Plain PEG2K-PE micelles	2K	5	0
	Plain PEG750-PE micelles	750	5	0
*Optimization of phage protein quantity	Plain PEG2K-PE micelles	2K	5	0
	Phage PEG2K-PE micelles	2K	5	0.125
	Phage PEG2K-PE micelles	2K	5	0.25
	Phage PEG2K-PE micelles	2K	5	0.5
	Phage PEG2K-PE micelles	2K	5	1

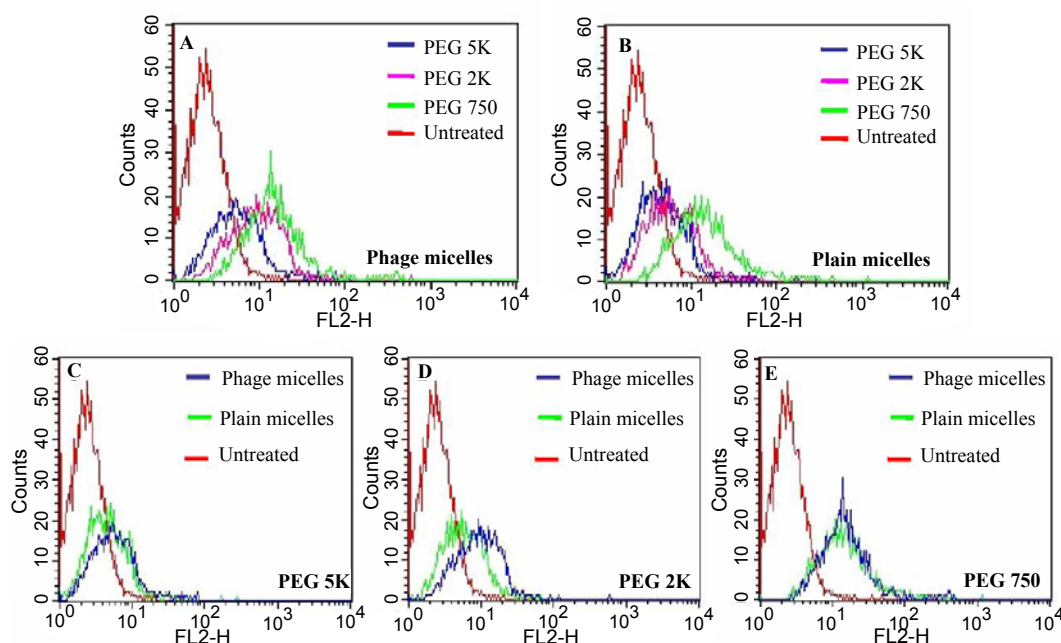*Trace rhodamine-PE was added to formulations

Figure 2: FACS analysis of the effect of different PEG length on the binding affinity of micellar formulations towards target MCF-7 cells. MCF-7 breast cancer cells were treated for 1h with either plain micelles or phage-micelles constructed from PEG_{750}-PE, PEG_{2k} -PE, or PEG_{5k} -PE conjugates, followed by FACS analysis of cell-associated micelles as indicated by an increase in red fluorescence intensity (FL2-H).

Results

Optimization of the PEG Length

We have used three PEG species with different lengths to construct plain- and phage-micelles, and compared their binding affinity towards target MCF-7 cells. As expected, a decrease in the length of PEG chain increased target cell association of phage-micelles in the order of PEG_{750} > PEG_{2k} > PEG_{5k} (Figure 2A). Control plain micelles with varying PEG chain also showed their binding affinity to MCF-7 cells in the order of P EG_{750} > PEG_{2k}> =PEG_{5k} (Figure 2B). However, the modification of PEG_{5k} micelles (Figure 2C) and PEG_{750} (Figure 2E) micelles with MCF-7 specific phage fusion protein showed less advantage in tumor-cell targeting compared to non-modified plain micelles. In contrast, the phage protein-modified PEG_{2k}-PE micelles (Figure 2D) showed a significantly enhanced target cell binding compared to plain PEG_{2k}-PE micelles.

To investigate the binding selectivity of different micelle formulations towards target MCF-7 cancer cells compared to non-target, non-cancer cells, we have designed a co-culture assay, in which target cancer MCF-7 cells were co-grown with non-target, non-cancer endothelial cells, C166 cells expressing GFP (C166-GFP). FACS analysis of the co-culture revealed two distinct cell populations. One cell population corresponding to higher green fluorescence intensity, as indicated on the Y-axis (FL1-H) of the dot plot, was C166-GFP cells. The other cellular population with lower green fluorescence intensity was MCF-7 cells (Figure 3A). After Treatment of this co-culture with different micelles followed by the FACS analysis, we found that plain- and phage- PEG_{750}-PE micelles had the highest binding to target cells but also to non-target cells, indicating a poor selective binding to tumor cells (Figure 3B and Figure 3E), while micelles with PEG_{5k}-PE showed the best targeting selectivity but a compromised binding affinity (Figure 3D and Figure 3G). Micelles with PEG_{2k}-PE had an optimal balance
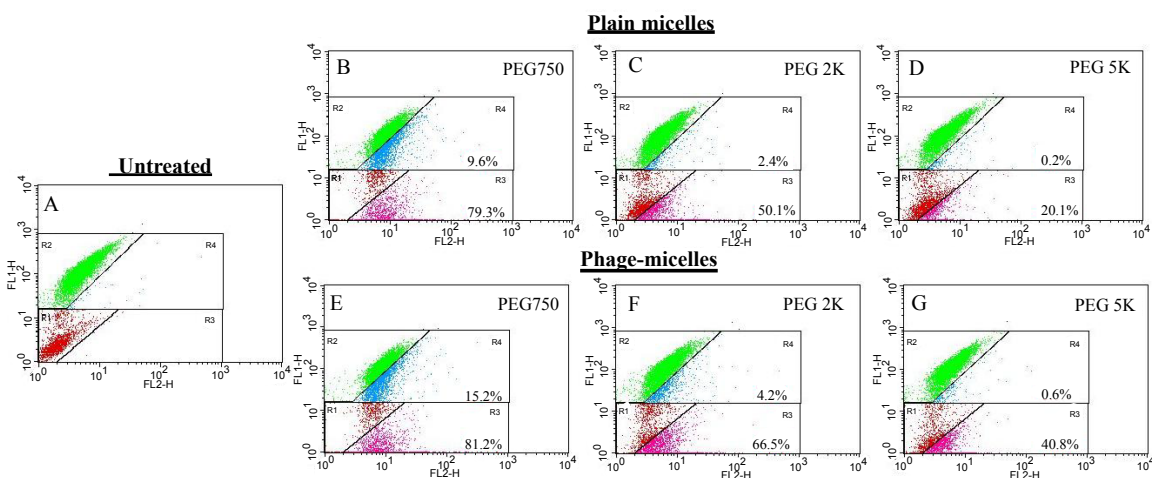
Figure 3: FACS analysis of cellular binding of micellar formulations prepared with different PEG blocks in a co-culture system. A co-culture composed of target MCF-7 cells and non-target C166-GFP cells was treated for 1h with either plain micelles or phage-micelles constructed from PEG$_{750}$-PE, PEG$_{2K}$-PE, or PEG$_{5K}$-PE conjugates, followed by FACS analysis of cell-associated micelles as indicated by the red fluorescence increase. The dot plots were bounded into four regions (R1, R2, R3, and R4). FL1-H (green); FL2-H (red); (A) A representative dot plot showing the untreated co-culture of MCF-7 cells and C166-GFP cells. Red dots in region R1, the location of untreated MCF-7 cells; green dots in region R2, the location of untreated C166-GFP cells; (B-D) Plain micelle-treated co-culture of MCF-7 cells and C166-GFP cells. Pink dots in region R3, plain-micelle-associated MCF-7 cells; blue dots in region R4, plain-micelle-associated C166-GFP cells; (E-G) MCF-7-targeted phage-micelle-treated co-culture of MCF-7 and C166-GFP cells. Pink dots in region R3, phage-micelle-associated MCF-7 cells; blue dots in R4, phage-micelle-associated C166-GFP cells.
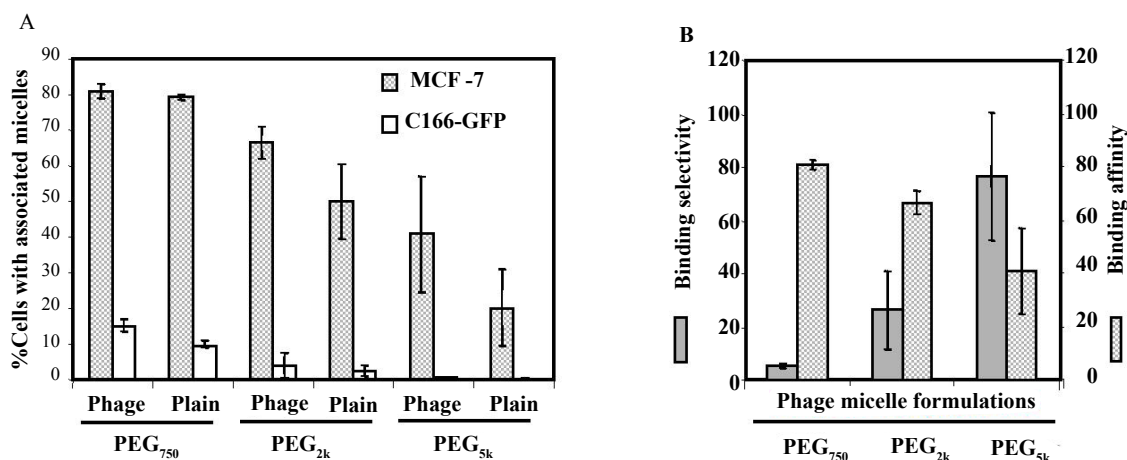
Figure 4: Quantitative analysis of the effect of PEG length on the binding affinity and selectivity of micellar formulations after FACS analysis in a co-cultured cell model. (A) The percent of cells with associated micelles indicated as the cell population shifted to the region with higher red fluorescence intensity in the dot plot acquired by FACS analysis. (B) The binding affinity defined as the percent of MCF-7 cells with associated micelles divided by the total MCF-7 cells tested; The binding selectivity defined as the percent of MCF-7 cells with associated micelles divided by the percent of C166-GFP cells with associated micelles (mean ± SD, n=9).

between the binding affinity and selectivity (Figure 3C, Figure 3F and Figure 4).

Optimization of phage protein quantity

We defined an optimal phage protein quantity as one providing the maximal binding affinity with a minimum of phage fusion protein used. The incorporation of MCF-7-targeting phage fusion protein up to 0.25% by weight into PEG$_{2k}$-PE micelles resulted in a limited increase in target cell-association compared to the plain PEG$_{2k}$-PE micelles, but the modification of plain micelles with 0.5% phage fusion protein provided a pronounce enhancement in target MCF-7 cell-binding,

but the further increase in phage protein quantity from 0.5% to 1% by weight did not result in any noticeable increase in the binding (Figure 5). Therefore, the optimal phage protein quantity to be used in phage-micelles is 0.5% by weight.

Targetability and cytotoxicity of the optimized phage-micelle formulation

After treatment of the co-culture composed of target MCF-7 and non-target C166-GFP with rhodamine-labeled phage-micelles, the overlay fluorescence micrograph clearly showed the lack of co-localization between red and green fluorescence, indicating that the

Figure 5: Effect of phage protein quantity on the binding affinity of micelle formulations towards target MCF-7 cells (Mean ± SD, n=6).
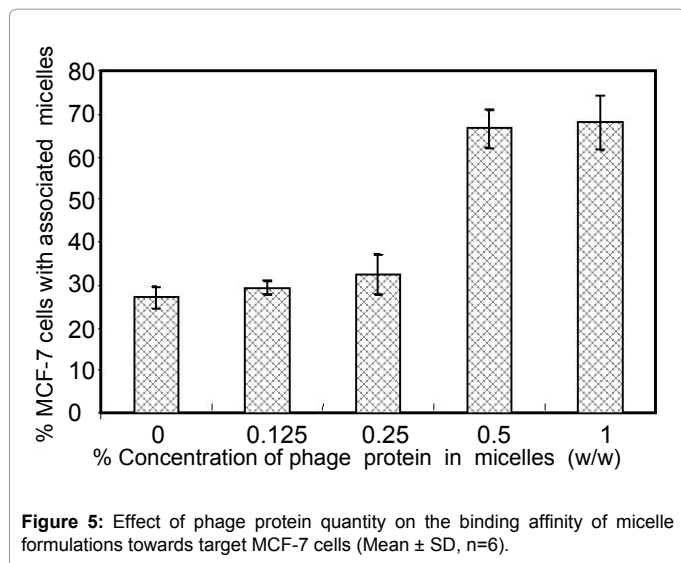
optimized phage-micelles preferentially bind to target MCF-7 cancer cells rather than to co-cultured C166-GFP cells (Figure 6A).

The treatment with the optimized phage-micelles loaded with paclitaxel at a PCT concentration of 1.76 µM for 72 h, led to 51.3 % MCF-7 cell death. The estimated IC_{50} of the phage micelle-loaded with paclitaxel is 1.63 µM (as PCT). The significantly lower tumor cell killing is seen however in the groups treated with both, free paclitaxel dissolved in DMSO and paclitaxel in plain PEG_{2k}-PE micelles at the equivalent concentration of PCT tested, with MCF-7 cell death level at 18.7% and 23.8%, respectively (Figure 6B). The drug-free, optimized phage-micelles produced negligible tumor cell killing.

Discussion

Combining of passive tumor targeting of pharmaceutical nanocarriers with tumor cell recognition systems represents a sophisticated strategy for targeted delivery of anti-cancer drugs to specific tumor cells [24]. Within this approach, a novel self-assembled micellar system, composed of PEG-diacyllipid micelles and phage-derived fusion proteins, has recently been designed for tumor-targeted delivery of water-insoluble drugs, such as paclitaxel [12].

The PEG-diacyllipid micelles prepared from amphiphilic polymer conjugates of polyethylene glycol (PEG) and diacyllipids (PE) have served as drug carriers for the solubilization of water-insoluble drugs and for tumor passive targeting [5,6,10]. Compared with conventional amphiphilic polymer micelles, the use of diacyllipid moieties (PE) as hydrophobic blocks, offers better particle stability as a result of a considerable contribution of the two fatty acid acyls within the PE blocks that increase hydrophobic interactions within the micelle's core. On the other hand, highly water-soluble PEG chains effectively provide steric protection and physiological stability for various nanoparticles in biological media. PEG chains protect nanoparticles from the clearance by the MPS system, and from other possible undesirable interaction with blood components. In the blood stream, PEGylated particles have a longer circulation time, which additionally promotes drug accumulation within target sites, such as tumors, via the EPR effect. Consequently, PEG-PE micelles loaded with a variety of poorly-soluble drugs can deliver their payload into tumors in mice with a greater efficiency [25,26].

It has been recognized for some time that PEGylation could also influence drug delivery negatively [27,28]. The presence of the PEG corona covering drug carriers produces a steric hindrance, affecting the interaction of drug carriers with target cells [28]. Particularly, PEG blocks within the micelles modified with phage fusion protein could have interfered with the interaction of targeting phage fusion protein with tumor cell-surface receptors.

An important method to solve this PEG dilemma is to control PEG length to induce a proper balance between the micellar stability and its targetability. Early studies have shown the effect of PEG length on micellar size and thermodynamic stability [5]. Generally, PEG-PE conjugates with PEG blocks with a molecular weight from 1000 to 15000 Dalton are able to form stable nanoparticles. With an increase in the length of PEG form 750 to 2000 to 5000 Dalton, particle sizes are increased and the Critical Micellar Concentration (CMC) of PEG-PE micelles is decreased [5]. Further studies have demonstrated that PEG length also dictates the *in vivo* behaviour of PEG-PE micelles. Micelles formed with PEG_{5K}-PE have a longer circulation time, with less uptake by normal tissue compared to micelles prepared from a shorter PEG-PE conjugate, and a higher accumulation in tumors compared to non-target tissues, such as muscle, as observed in a Lewis lung carcinoma-bearing experimental mice [29,30]. Our results have further demonstrated the shielding effect produced by the PEG chain.
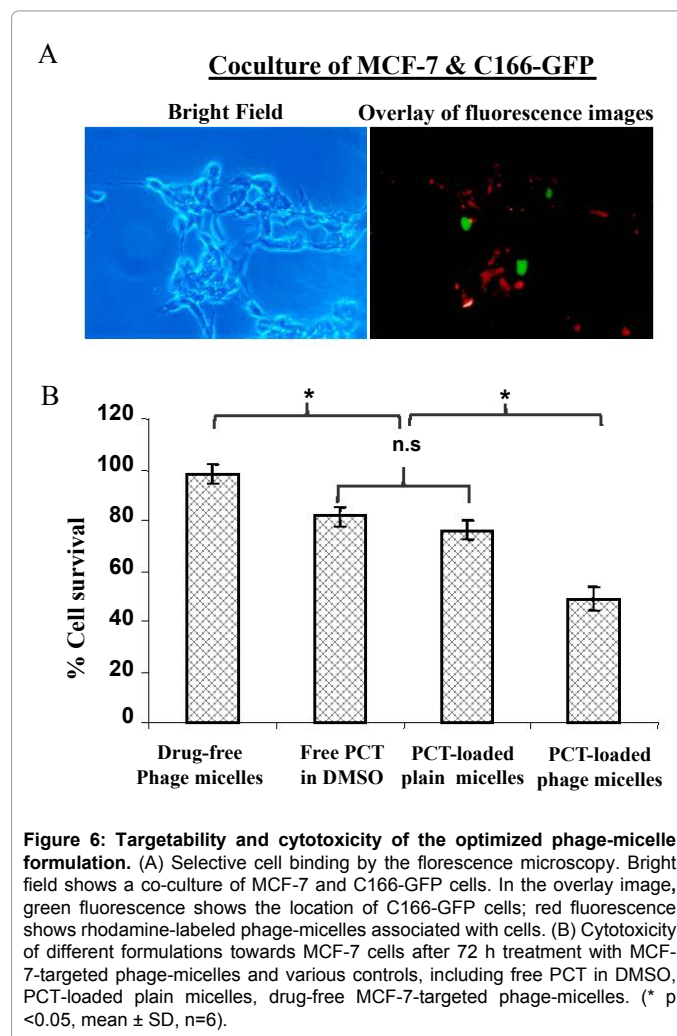
Figure 6: Targetability and cytotoxicity of the optimized phage-micelle formulation. (A) Selective cell binding by the florescence microscopy. Bright field shows a co-culture of MCF-7 and C166-GFP cells. In the overlay image, green fluorescence shows the location of C166-GFP cells; red fluorescence shows rhodamine-labeled phage-micelles associated with cells. (B) Cytotoxicity of different formulations towards MCF-7 cells after 72 h treatment with MCF-7-targeted phage-micelles and various controls, including free PCT in DMSO, PCT-loaded plain micelles, drug-free MCF-7-targeted phage-micelles. (* p <0.05, mean ± SD, n=6).

The longer the PEG block used, the weaker the interaction of PEG-PE micelles with tumor cells. On the other hand, micelles prepared from PEG-PE conjugates with longer versions of PEG have better targeting selectivity towards tumor cells compared to non-target, non-cancer cells.

Within the objective of this study, to develop an optimized self-assembling system for active tumor targeting, we used the whole landscape phage fusion protein (the targeting peptide fused to phage major coat protein PVIII), the hydrophobic phage major coat protein part of which interacts with diacyllipid-PE to form the micellar core. Targeting peptides of the whole protein, together with PEG block of PEG-PE conjugates, built up the micellar corona. An ideal phage-micelle formulation should contain an appropriate PEG block, which can efficiently shield hydrophobic segments (PE and phage major coat protein PVIII), and, at the same time, exposes the targeting peptides to a maximally possible extent. During optimization of the PEG length and phage protein quantity used to formulate the targeted phage-micelles, we found that only PEG_{2K}-PE increased binding affinity of phage-micelles towards targeted cells, implying that PEG_{750} was too short to hide the hydrophobic segments, while PEG_{5K} was too long to allow for the exposure of targeting ligands. In accordance with this observation, PE G_{750} -PE micelles showed pronounced non-specific binding to non-target C166-GFP cells. Interestingly, the increase in phage protein quantity in PEG_{2K}-PE phage-micelles was not necessarily favourable for targeting, suggesting that the mixed micellar aggregations can only accommodate a certain amount of phage fusion protein within stable particles with a maximal tumor cell targeting. Similar phenomenon was also observed when the phage protein was used to modify liposomes [31].

Loading hydrophobic drug paclitaxel into the optimized formulation with PEG_{2K}-PE and 0.5% of phage fusion protein (by weight) forms stable nanoparticles. Incorporation of phage protein into PEG_{2K}-PE micelles improves MCF-7 tumor cell targeting and cytotoxicity of the micellar paclitaxel [12]. Similarly, we have earlier observed the specific tumor cell targeting and enhanced tumor cell killing when liposomes are modified with phage fusion protein for the delivery of water-soluble amphiphilic drugs, such as doxorubicin hydrochloride [19].

The PEG_{2K}-DSPE used in the preparation of the optimized phage-micelles has been approved as an excipient by the U.S. Food and Drug Administration, and it is generally safe, biocompatible and relatively nontoxic [32,33]. Thorough investigation on the metabolism and excretion of PEG and PEG-biological molecule conjugates suggests the less safety concerns associated with their utility in chronic and acute administration even with high concentration [34]. In this study, drug-free PEG-PE micelles modified with phage fusion protein showed no toxicity towards MCF-7 at the concentration of PEG-PE up to 48 μM, providing evidence for the safe use of the phage-micelle formulation.

In summary, phage-micelles created by the self-assembly of phage fusion protein and PEG-PE specifically bind to targeted tumor cells. Both length of PEG and quantity of phage fusion protein have a profound impact on the micellar targetability. PEG_{2K}-PE and phage protein quantity of 0.5% (w/w) constitute a formulation with an optimized balance between tumor cell-binding affinity and selectivity.

Acknowledgements

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