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Non-Covalent Functionalization of Carbon Nanovectors with an Antibody Enables Targeted Drug Delivery

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Abstract

Current chemotherapeutics are characterized by efficient tumor cell-killing and severe side effects mostly derived from off target toxicity. Hence targeted delivery of these drugs to tumor cells is actively sought. We previously demonstrated that poly(ethylene glycol)-functionalized carbon nanovectors are able to sequester paclitaxel, a widely used hydrophobic cancer drug, by simple physisorption and deliver the drug for killing of cancer cells. The cell-killing when these drug-loaded carbon nanoparticles were used was equivalent to when a commercial formulation of paclitaxel was used. Here we show that by further mixing the drug-loaded nanoparticles with Cetuximab, a monoclonal antibody that recognizes the epidermal growth factor receptor (EGFR), paclitaxel is preferentially targeted to EGFR+ tumor cells *in vitro*. This supports progressing to *in vivo* studies. Moreover, the construct is unusual in that all three components are assembled through non-covalent interactions. Such non-covalent assembly could enable high-throughput screening of drug/antibody combinations.

Keywords

drug delivery; nanovectors; paclitaxel; targeted; non-covalent

An increasing number of drug candidates with high therapeutic efficacy have low water solubility, posing a challenge for *in vivo* delivery.[1] Moreover, even when these drugs can be delivered, often systemic toxicity both severely compromises the patient's quality of life and is dose limiting for the therapy. Paclitaxel (PTX) is a classic example of a water-insoluble drug with high therapeutic efficacy and severe off target toxicity. In an FDA-approved commercial formulation of PTX, (Taxol®, Bristol-Myers-Squibb, Princeton, NJ, USA) the drug is solubilized in ethanol and a polyethoxylated castor oil, Cremophor EL® (Cremophor). The use of Cremophor as the excipient for PTX is well-known to cause

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Supporting Information. Dot and western blot results, a comparison between flow cytometry and luciferase activity analysis, evaluation of PEG-OCB and PEG-GONRs are available free of charge *via* the Internet at http://pubs.acs.org.

significant allergic reactions, including anaphylaxis. Consequently, patients are pre-medicated with antihistamines and corticosteroids in order to prevent potentially life-threatening hypersensitivity reactions.[2] One solution to this problem has been the sequestering of PTX in albumin, and the commercial formulation is called Abraxane®.[3] Though milder, significant side effects remain, such as sensory neuropathy.[3] It is noteworthy that both commercial formulations of PTX involve the non-covalent sequestration of the unmodified drug, likely due to both the ease of preparing this class of formulations and the fact that covalently modifying the PTX can alter its efficacy.

Numerous efforts have been made to find alternative excipients for PTX that would result in an increased therapeutic index.[4–8] Nanovectors, nanoparticles capable of transporting and delivering one or more bioactive molecules, are an emerging class of drug delivery platforms and have been evaluated as excipients for PTX.[9–10] In recent reports, we have more fully discussed alternative delivery platforms for PTX and recent uses of nanovectors.[11–12] These reports demonstrated that a variety of poly(ethylene glycol)-functionalized carbon nanovectors are able to sequester PTX by simple physisorption and deliver the drug for killing of cancer cells *in vitro*. The cell-killing when these drug-loaded carbon nanoparticles were used was equivalent to when Taxol (PTX/Cremophor) was used. One of the formulations we developed, PTX sequestered on PEGylated hydrophilic carbon clusters (PTX/PEG-HCCs; note that in our notation the slash "/" represents a non-covalent association and the dash "-" a covalent association) was evaluated for the treatment of an orthotopic xenograft model of head and neck squamous cell carcinoma (HNSCC). Treatment with PTX/PEG-HCCs resulted in tumor growth delay equivalent to that obtained when PTX/Cremophor was used. Preliminary studies suggested that the PEG-HCCs were not toxic.[11]

Nevertheless, similar to the commercially available formulations, PTX/PEG-HCCs provide a systemic therapy with little to no bias for the accumulation of the PTX in the tumor versus the rest of the organism. Active targeting has been widely recognized as a promising method for the delivery of both nanoparticles and small molecules to tumors.[13–15] This technique involves conjugating a therapeutic payload to targeting ligands that selectively bind the tumor cells of interest. Approximately 90% of HNSCCs overexpress the epidermal growth factor receptor (EGFR) and this is correlated with worse clinical outcomes.[16–17] Cetuximab (Erbitux, ImClone Systems) (Cet) is an IgG monoclonal antibody that exclusively binds to EGFR with high affinity and blocks the normal function of the receptor.[18–20] Cet is the most widely studied EGFR targeting agent and is approved by the Food and Drug Administration for the treatment of patients with HNSCC.[21–22] Cet has been used to target a variety of non-carbonaceous nanoparticles to EGFR+ cells, while EGF has been used for the same purpose with carbon nanotubes functionalized with cis-platin.[23–26] In each of those hitherto published cases, the targeting moiety was covalently attached to the nanoparticle. Since Cet can inhibit the growth of EGFR+ cells and radiosensitize them,[16,19–20] while EGF will stimulate the growth of EGFR+ cells, we chose to further functionalize the PTX/PEG-HCCs with Cet to prepare a targeted formulation. Surprisingly, we found that this functionalization can occur by simply mixing Cet with the PTX/PEG-HCCs to prepare Cet/PTX/PEG-HCCs wherein both the drug and the antibody are physisorbed on the amphiphilic carbon core of the nanovectors. Herein, we demonstrate that Cet/PTX/PEG-HCCs is a stable formulation that targets the delivery of PTX to EGFR+ cells *via* binding to the EGFR. The success of this formulation both sets the stage for further testing *in vivo* and establishes a potential platform for *in vitro* screening of antibody/drug combinations where no covalent linking of the antibody or drug to the nanovector is required.

Results and Discussion

PEG-HCCs were prepared from single-walled carbon nanotubes (SWCNTs) as recently described by using a strongly oxidizing chemical treatment that cuts the tubes to <40 nm in length and heavily oxidizes the framework, resulting in the loss of spectral signatures characteristic of a tubular structure.[27–29] Hence, we refer to these extremely small, highly oxidized carbon particles as HCCs and not nanotubes as there is little resemblance between the two. As in our previous reports, all concentrations given in this manuscript will be for the carbon cores of the PEG-HCCs as this can be directly measured by ultraviolet-visible (UV-Vis) spectroscopy. The carbon cores of the PEG-HCCs are amphiphilic with domains of unfunctionalized carbon and domains of highly oxidized carbon.[11] It was envisioned that the Cet, which itself possesses both hydrophobic and hydrophilic domains, could be sequestered by the PEG-HCCs. To investigate this interaction, solutions were prepared with varying amounts of Cet combined with PEG-HCCs (100 μg/ml) in water (Figure 1a). These samples were prepared for SDS-PAGE gel (7%) analysis by heating them to 95 °C in SDS sample buffer. The gel analysis then demonstrated that no free antibody was observed when the concentration of Cet used was ≤ 62 μg/mL (Figure 1b). Since gel electrophoresis is not a very sensitive detection method, there might be residual free Cet in the 62 μg/mL sample. A band that we speculate is Cet/PEG-HCCs was observed at the top of the gel and increased in intensity as the concentration of Cet was increased up to 62 μg/mL, and then the intensity remained constant as the concentration of Cet was increased further. Thus the loading capacity was estimated to between 0.31 and 0.62 μg of Cet per μg of PEG-HCCs.

The EGFR binding ability of Cet/PEG-HCCs as compared to free Cet was next investigated. A dot blot confirmed that EGFR binding was maintained in the Cet/PEG-HCCs (Figure S1). Immunoprecipitation was performed wherein either Cet, Cet/PEG-HCCs or PEG-HCCs were mixed with protein A agarose, and then protein extracts from EGFR- cells (MCF-7) or EGFR+ cells (OSC-19) were passed over each mixture. After washing, the bound proteins were eluted and run on a gel. Both Cet/PEG-HCCs and free Cet selectively pulled down nothing from the EGFR- cells and a ~170 kDa protein from EGFR+ cells (Figure 2a), which was identified as EGFR by staining with an anti-EGFR antibody (Santa Cruz sc-03). It was clear that less EGFR was pulled down by Cet/PEG-HCC compared to free Cet (Figure 2b). Nevertheless, the EGFR binding efficacy of Cet/PEG-HCCs was sufficient to proceed to *in vitro* studies.

As previously described, PEG-HCCs were loaded with PTX by dropwise addition of PTX in methanol (5 mg in 1 mL) to a rapidly stirring solution of PEG-HCCs in water (100 mg/L concentration of core HCCs, 5 mL).[11–12] The solution was bath-sonicated, concentrated to 3 mL by rotary evaporation to remove the methanol and then diluted back to the original volume (5 mL) with water, providing a translucent solution of PTX/PEG-HCCs. The formulation for targeted drug delivery was prepared by adding Cet in buffered saline to a solution of PTX/PEG-HCCs (final concentrations: Cet 35 μg/mL, PTX 1 mg/mL, PEG-HCCs 100 μg/mL) and briefly vortexing the solution (Figure 3a). The ability to deliver the therapeutic of interest to targeted cells in preference to other bystander cells was evaluated using a competitive cell-killing assay (Figure 3b) in which OSC-19-luc cells that had been transfected to stably express green fluorescent protein (GFP) and luciferase (Luc) were co-cultured with the breast cancer cell line MCF-7 that does not express EGFR, GFP or Luc (See Figure S2 for EGFR expression). The co-cultures were treated for 3 d with either Cet, PEG-HCCs, PTX/PEG-HCCs, Cet/PTX/PEG-HCCs, PTX/Cremophor or Cet/PTX/Cremophor. The survival of each cell type was measured by flow cytometry using the GFP expression to differentiate the two cell populations and the ratio was normalized to the control treatment (Figure 3b). Only the Cet/PTX/PEG-HCCs were able to selectively kill the EGFR+ cells; for all of the other treatments there was no statistical difference in the ratio of

surviving EGFR+ to EGFR− cells as compared to the control. The fact that the Cet/PTX/Cremophor did not show any selectivity indicates that at the concentrations used, there is no synergistic effect of treating with Cet and PTX. The Cet/PTX/PEG-HCCs formulation is effective due to a targeting effect, not a dual therapy effect.

In order to have a technique for rapidly analyzing changes in conditions, a modified protocol was also used in which the OSC-19-luc cells were co-cultured with NIH-3T3 cells and treatment was carried out for 2 days. A comparison of analysis techniques indicated that measuring just OSC-19-luc survival by luciferase activity gave comparable indication of targeting as when flow cytometry was used to measure both cell populations (Figures S3 and S4). Since measuring luciferase activity was significantly faster, we chose this technique to evaluate changing conditions and because both the flow cytometry and luciferase studies demonstrated that using a PTX concentration of 3 nM gave the clearest indication of targeting, all the data in Figure 4 is presented only for [PTX] = 3 nM. A range of Cet to PTX/PEG-HCCs ratios was evaluated using this protocol (Figure 4a). In good agreement with the loading capacity we had established by SDS-PAGE gel analysis, it was found that a ratio of 35 μg of Cet to 100 μg of the carbon core of the PEG-HCCs was most effective at targeting the delivery of PTX to the EGFR+ cells. Several other carbon nanovectors that we recently described [12] were also evaluated briefly, but the PEG-HCCs proved to be the most effective (Figure S5). The mechanism of targeting was probed by pretreating the cells with EGF to block the EGFR. Pretreatment with EGF had no effect on treatment with PTX/PEG-HCCs, but the pretreatment completely eliminated the increased OSC-19-killing of Cet/PTX/PEG-HCC, supporting the hypothesis that this formulation is targeted by interacting with the EGFR (Figure 4b). When administered *in vivo*, the Cet/PTX/PEG-HCCs will be in the presence of many other proteins in the blood stream. In order to test the stability of the Cet binding to the PEG-HCCs in the presence of exogenous protein, the Cet/PTX/PEG-HCCs were mixed, prior to cell treatment, with a 1,000× excess of albumin relative to Cet. This solution was then allowed to stand at RT for varying lengths of time before being administered to the cells. The targeting effect of the Cet remained even after being in the presence of the excess albumin for 6 h, but exposure for 24 h eliminated the targeting effect (Figure 4c). This stability is encouraging and sufficient to proceed to *in vivo* studies.

Cet/PTX/PEG-HCCs were also tested for the targeted killing of another EGFR+ cell line, Krib-1. The Krib-1 cells, an osteosarcoma cell line, were stably transfected to express Luc and were compatible with being co-cultured with NIH-3T3 cells. Thus, targeted cell-killing was evaluated by Luc activity in co-cultures of Krib-1 and NIH-3T3 cells following treatment, in analogy to the experiments above with OSC-19-Luc cells. The use of Cet/PTX/PEG-HCCs resulted in a significant decrease in the survival of the Krib-1 cells (Figure 5a). In a separate experiment, the modularity of the system was evaluated by using a different antibody, Panitumumab (Pan), which recognizes the EGFR. While Cet is a chimeric monoclonal antibody, Pan is fully human. Pan/PTX/PEG-HCCs were prepared in the same manner as the Cet/PTX/PEG-HCCs and the ratio between Pan and the PTX/PEG-HCCs was empirically optimized using the competitive cell-killing assay with OSC-19-Luc and NIH-3T3 cells (Figure 5b). The Pan/PTX/PEG-HCCs were effective for the targeted delivery of PTX (compare Pan = 0 to Pan = 6 μg/mL), and indicative of the differences between the antibodies a different ratio was found to be best for the Pan/PTX/PEG-HCCs as compared to the Cet/PTX/PEG-HCCs.

Conclusion

PEG-HCCs are a versatile platform for drug delivery. We previously reported encouraging preliminary toxicity studies in mice and that the PEG-HCCs could be non-covalently loaded with PTX and enable the delivery of PTX *in vitro* and *in vivo*. In this report, we have

demonstrated that by simply mixing the PTX/PEG-HCCs with an antibody, such as Cet, the antibody will non-covalently associate with the nanovectors to produce a formulation capable of targeted drug delivery *in vitro*. The interaction between the Cet and the PEG-HCCs is relatively robust as it is able to withstand heating to 95 °C in the presence of SDS and being incubated in the presence of a large excess of albumin at RT for 6 h. The binding specificity of the Cet for EGFR is retained. Targeted delivery of the PTX to EGFR+ cells was demonstrated in two different cell lines using the Cet/PTX/PEG-HCCs. In one of the cell lines, EGF-pretreatment was used to validate that the targeting occurred *via* binding to the EGFR. Finally, the formulation strategy is facile and modular, as the antibody can be easily switched. As an example, Pan was used instead of Cet and efficacy was maintained. The *in vitro* results presented here support progressing to *in vivo* studies. In addition, because the formulation process is so simple and modular, the PEG-HCCs may be used to rapidly screen different antibody/drug combinations for targeted delivery.

Materials and Methods

General

PEG-HCCs and PTX/PEG-HCCs were prepared as previously described.[11] Cet (C225/Erbitux) was obtained from Imclone (New York, NY). Panitumumab (Vectibix) was obtained from Amgen Inc. (Thousand Oaks, CA). OSC-19 was purchased from the Health Science Research Resources Bank (Osaka, Japan). MCF-7 cells were provided by Dr. Francois-Xavier Claret (Department of Systems Biology, MD Anderson Cancer Center). NIH-3T3 cells were purchased from ATCC (Manassas, VA). OSC-19 cells were retrovirally infected with the green fluorescent protein and the luciferase gene (OSC-19-luc) as described previously.[30] All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, L-glutamine, sodium pyruvate, nonessential amino acids, and a 2-fold vitamin solution (Life Technologies, Inc., Grand Island, NY). Adherent monolayer cultures were maintained on plastic plates and incubated at 37 °C in 5% carbon dioxide and 95% oxygen. The integrity of all maintained cell lines was clearly established using short tandem repeat genomic profiling. The cultures were *Mycoplasma*-free and maintained for no longer than 12 weeks after they were recovered from frozen stocks.

Statistical Methods

For Figure 3c, a paired t-test was used to determine that the Cet/PTX/PEG-HCCs were statistically significantly different from each of the other treatments. ANOVA analysis was used for Figures 4 and 5, with $p<0.001$.

Preparation of Cet/PEG-HCCs—Varying amounts of a solution of Cet (2 mg/mL) were added to PEG-HCCs (carbon core concentration 0.2 μg/mL) in DI water to produce final solutions of 250 μL with varying concentrations of Cet and 0.1 μg/mL of PEG-HCCs. The solutions were vortexed briefly.

Preparation of Pan/ or Cet/PTX/PEG-HCCs—1 h prior to use, varying amounts of a solution of Pan or Cet (2 mg/mL) was added to PTX/PEG-HCCs (carbon core concentration 0.2 μg/mL) in DI water to produce final solutions of 1 mL with varying concentrations of Cet and 0.1 μg/mL of PEG-HCCs. The solutions were vortexed briefly and then stored at RT until use.

Incubation with Albumin—Albumin [724 μg/mL] was added to Cet/PTX/PEG-HCCs [Cet 35 μg/mL, PTX 1 mg/mL, PEG-HCCs 100 μg/mL]. The mixture was stored at RT for 2, 4, 6 or 24 h and then used.

SDS-PAGE analysis of Cet/PEG-HCCs—Binding efficiency between Cet and PEG/HCCs was assessed by SDS-PAGE under reducing conditions. A 7% gel was utilized for optimal separation of PEG-HCC from the Cet components.

Immunoprecipitation studies—Whole cell lysates of OSC-19 and MCF-7 cells were obtained by lysing cells in Cell Lysis buffer (50 mM Tris, pH 8.0, 0.1 mM EDTA, 1 mM DTT, 12.5 mM $MgCl_2$, 0.1 M KCl, 20% glycerol) supplemented with protease inhibitors [leupeptin (0.5%), aprotinin (0.5%) and PMSF (0.1%)]. 500 μg cell lysates were mixed with various amounts of Cet (36 μg/mL), Cet/PEG-HCCs (36 μg/mL Cet, 100 μg/mL PEG-HCCs), PEG-HCCs (100 μg/mL) or vector in a final volume of 250 μL and incubated for 2 h at 4 °C. The mixtures were then incubated for 1 h with 5 μL Protein A agarose beads at RT and washed 4× in lysis buffer. Protein samples were separated by SDS-PAGE under reducing conditions and electrophoretically transferred to a PVDF membrane overnight at 4 °C in transfer buffer (25 mM Tris-HCl, pH 7.6, 190 mM glycine, 20% HPLC-grade methanol). The samples were analyzed for EGFR with rabbit anti-EGFR polyclonal antibody (Santa Cruz sc-03). The membranes were then incubated with goat-anti-rabbit IgG horseradish peroxidase (HRP), developed using the Amersham ECL detection system and exposed to X-ray film.

Cell survival of OSC-19-Luc mono-culture following treatment—Cells were grown for 24 h in a 96-well plate (2000 cells/well in 100 μL of 10% serum containing media) at 37 °C in 5% CO_2 and 95% air before treating with drugs. Then 100 μL of PTX/Cremophor [PTX 1 mg/mL], PTX/PEG-HCCs [PTX 1 mg/mL, PEG-HCCs 0.1 μg/L] or Cet/PTX/PEG-HCCs [Cet 35 μg/mL, PTX 1 mg/mL, PEG-HCCs 100 μg/mL] was added; each treatment was added to six wells. The cells were incubated for 1, 4, 24, or 72 h at 37 °C, then MTT solution (25 μL of 2 mg/mL) was added in each well. After incubation for 2 h, the media was removed by careful aspiration to avoid suctioning of the cells. Then, 100 μL of DMSO was added to each well in order to lyse the cells and to solubilize the colored crystals. Finally, the plates were analyzed using an ELISA plate reader at a wavelength of 570 nm to measure optical density (OD) of each well, which was proportionate to the number of viable cells in each well. The percent of viable cells relative to the control was obtained by dividing the average OD for the treated wells by the OD for the control wells. All *in vitro* experiments were performed in triplicate. The results are expressed as mean ±SE.

Cell survival of co-cultures of EGFR+ and EGFR- cells following treatment, with and without EGF pretreatment—A solution was prepared containing an equal number of EGFR+/Luc+ cells (either OSC-19-luc which was also GFP+ or Krib-1) and EGFR-/Luc- (either MCF-7 or NIH-3T3) cells. 250 μL of this solution was added to each well of a 24 well plate (7500 cells/well). The cells were incubated for 23 h at 37 °C in 5% CO_2 and 95% air. At this point, if EGF pretreatment was being evaluated, EGF (100 ng/mL) was added to each well and the cells were then returned to the incubator for 1 h. Otherwise, the cells simply remained in the incubator for 1 h. Then 250 μL of each treatment was added to six wells and the plate was incubated for 48 h. The treatments were Cet [35 μg/mL], PEG-HCCs [100 μg/mL], PTX/Cremophor [PTX 1 mg/mL], Cet/PTX/Cremophor [Cet 0 to 480 μg/mL, PTX 1 mg/mL], PTX/PEG-HCCs [PTX 1 mg/mL, PEG-HCCs 100 μg/mL], Cet/PTX/PEG-HCCs [Cet 35 μg/mL, PTX 1 mg/mL, PEG-HCCs 100 μg/mL] and Pan/PTX/PEG-HCCs [Pan 0 to 35 μg/mL, PTX 1 mg/mL, PEG-HCCs 100 μg/mL]. Cell survival was determined by either flow cytometry analysis or bioluminescence.

Bioluminescence analysis was performed by adding 120 μl of an aqueous solution of D-luciferin (Caliper Life Sciences) at 150 μg/ml to each well 2 min prior to imaging. Cell culture plates containing the luciferase-expressing cells were imaged using the Xenogen

IVIS 200 Imaging System (Caliper Life Sciences, Hopkinton, MA). Then, bioluminescence was quantified using Living Image software 3.2 (Caliper Life Sciences). The results are expressed as mean ±SE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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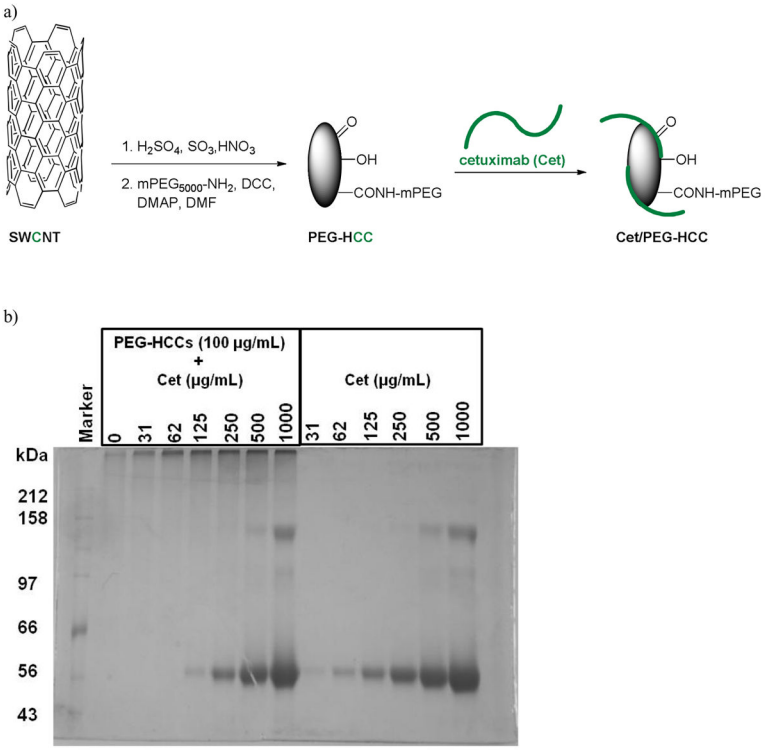

Figure 1.

a) Preparation of Cet/PEG-HCCs, beginning from SWCNTs. Note that for clarity, only one of each functional group type is shown on each particle, while the particle surfaces are actually well covered with similar functionalities.[11] b) SDS-PAGE gel of Cet/PEG-HCCs and free Cetuximab at different concentration of Cet (from 31 to 1,000 μg/mL) to study the binding efficiency between Cet and PEG-HCCs. The concentration of PEG-HCCs was constant at 100 μg/mL. Note that for Cet concentrations below 62 μg/mL, no free Cet is observed for the Cet/PEG-HCCs. The band at 50 kDa corresponds to the heavy chain of Cet and the band at 150 kDa corresponds to the unreduced Cet.

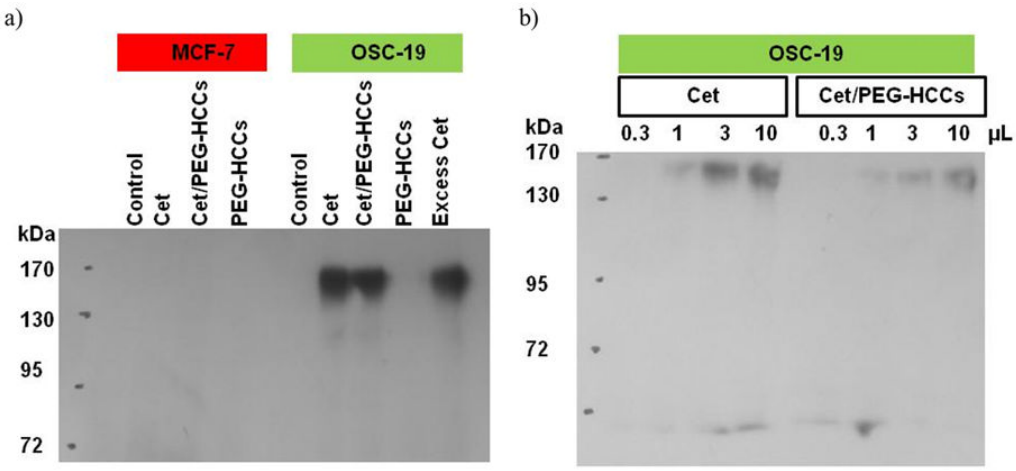

Figure 2.

a) Immunoprecipitation of an EGFR- cell line (MCF-7) and an EGFR+ cell line (OSC-19) using Cet [36 μg/mL], Cet/PEG-HCCs [36 μg/mL of Cet, 100 μg/mL of PEG-HCCs] and PEG-HCCs [100 μg/mL] alone. As a positive control, the immunoprecipitation was also performed with an excess of Cet (200 μg/mL). Cet, Cet/PEG-HCCs and the positive control all selectively pulled down EGFR. b) Immunoprecipitation of an EGFR+ cell line (OSC-19) with varying amounts of Cet [36 μg/mL] or Cet/PEG-HCCs [36 μg/mL of Cet, 100 μg/mL of PEG-HCCs]. The free Cet is more effective than the Cet/PEG-HCCs at pulling down EGFR. Note: The two gels were run under slightly different conditions so the same ~170 kDa band appears in different locations in the two images.

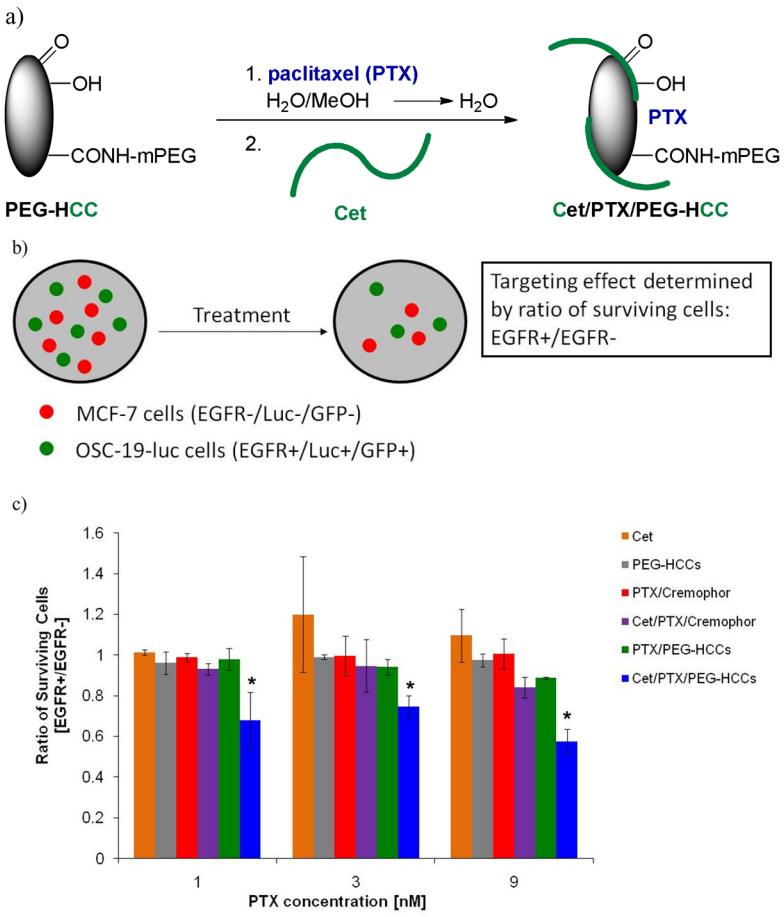

Figure 3.

a) Preparation of Cet/PTX/PEG-HCCs by non-covalently sequestering first the PTX and then the Cet against the carbon core of the PEG-HCC. Note that for clarity, only one of each functional group type is shown on each particle, while the particle surfaces are actually well covered with similar functionalities. b) A competitive cell-killing assay was developed wherein EGFR+ and EGFR- cells were co-cultured and treated with either Cet, PEG-HCCs, PTX/PEG-HCCs, Cet/PTX/PEG-HCCs, PTX/Cremophor or Cet/PTX/Cremophor. Successful targeting was indicated by a reduction in the ratio of surviving EGFR+ cells to EGFR− cells, as compared to the control. c) The ratio of surviving cells (EGFR+/EGFR−) as measured by flow cytometry following each treatment. Only the Cet/PTX/PEG-HCCs selectively kill EGFR+ cells. * $p < 0.05$.

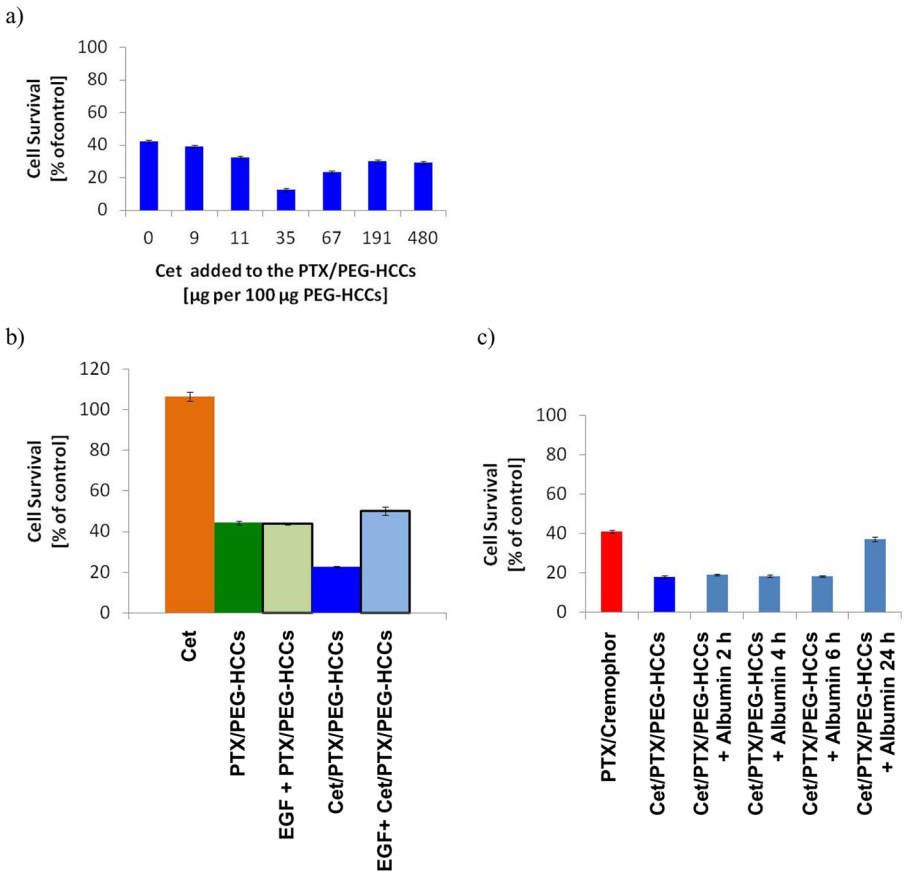

Figure 4.

a) Optimization of the ratio between Cet and PTX/PEG-HCCs evaluated by competitive cell-killing. In all cases, the concentration of PTX is 3 nM so there is always a high level of cell death. ANOVA $p<0.001$ b) Pretreating with EGF has no effect on the non-targeted formulation but completely blocks Cet targeting. ANOVA $p<0.001$ c) Cet/PTX/PEG-HCCs is stable in the presence of a 1,000× excess of albumin for between 6 and 24 h. ANOVA $p<0.001$

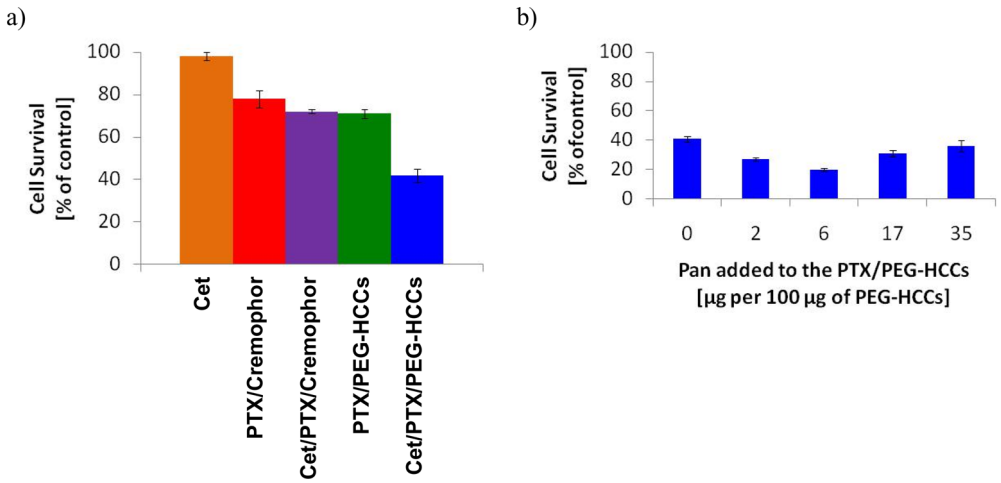

Figure 5.

a) A co-culture of Krib-1 (EGFR+/Luc+) and NIH-3T3 (EGFR−/Luc−) cells was treated with various formulations, and the Cet/PTX/PEG-HCCs statistically significantly decreased the survival of the Krib-1 cells. ANOVA $p<0.001$ b) A co-culture of OSC-19-Luc (EGFR+/Luc+) and NIH-3T3 (EGFR−/Luc−) was treated with PTX/PEG-HCCs functionalized with Pan. Differing from when Cet was used, the optimal ratio of antibody per 100 μg of the carbon core of the PTX/PEG-HCCs shifted from 35 to 6 μg (compare to Figure 4a). ANOVA $p<0.001$.