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Selective staining and eradication of cancer cells by protein-carrying DARPin-functionalized liposomes

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ABSTRACT

Since their discovery, liposomes have been widely employed in biomedical research. These nano-size spherical vesicles consisting one or few phospholipid bilayers surrounding an aqueous core are capable of carrying a wide variety of bioactive compounds, including drugs, peptides, nucleic acids, proteins and others. Despite considerable success achieved in synthesis of liposome constructs containing bioactive compounds, preparation of ligand-targeted liposomes comprising large quantities of encapsulated proteins and capable of affecting pathological cell still remains a big challenge. Here we described a novel method for preparation of small (80-90 nm in diameter) unilamellar liposomes containing very large quantities (thousands of protein molecules per liposome) of heme-containing cytochrome c, highly fluorescent mCherry and highly toxic PE40 (the fragment of *Pseudomonas aeruginosa* exotoxin A). Efficient encapsulation of the proteins was achieved through electrostatic interaction between positively charged proteins (at pH lower than pI) and negatively charged liposome membrane. The proteoliposomes containing large quantities of mCherry or PE40 and functionalized with designed ankyrin repeat protein (DARPin)_9-29, which targets human epidermal growth factor receptor 2 (HER2) were shown to specifically stain and kill in sub-nanomolar concentrations HER2-positive cells, overexpressing HER2, respectively. Specific staining and eradication of the receptor-positive cells demonstrated here makes the DARPin-functionalized liposomes carrying large quantities of fluorescent and/or toxic proteins a promising candidate for tumor detection and therapy.

Keywords: Cancer, liposomes, DARPin, mCherry, exotoxin A, apoptosis, cryo-TEM.

1. Introduction

Since their discovery [1] liposomes have been widely used in biomedical research (for reviews, see [2-4]). The ability to encapsulate a wide variety of small molecules,

as well as biopolymers, such as nucleic acids and proteins, their high bio-compatibility, low toxicity, tunable size and lipid composition made liposomes a unique carrier for the delivery of bioactive compounds to pathological cells and tumors. In addition, covalent attachment of ligands, such as antibodies, proteins, carbohydrate polymers as well as peptides, sugars and other small bioactive molecules, to the liposome membrane enabled specific delivery of the lipid vesicles to target cells (for reviews, see [2, 5]).

Designed ankyrin repeat proteins (DARPins), a novel class of non-IgG scaffolds based on naturally occurring ankyrin repeats [6] have been shown to bind to protein targets with specificity and affinity exceeding those of antibodies [6-9]. DARPins are small (13-20 kDa), highly soluble in water, very stable and lack cysteine residues. A number of DARPin molecules that specifically bind to human epidermal growth factor receptor 2 (HER2) [7-10] overexpressed in breast cancer and ovarian cells, as well as conjugates between the DARPins and toxic proteins have been synthesized and shown to specifically eliminate HER2-positive cells [11-13].

Preparation of DARPin-functionalized liposomes using "click chemistry" [14] and the carbodiimide-mediated coupling reaction [15] has been recently reported. The liposomes were shown to specifically bind to target cells through corresponding receptors overexpressed on the surface of the cells. To the best of our knowledge, no data have been reported so far on specific delivery of proteins encapsulated inside DARPin-functionalized liposomes to target cells.

Here we reported a novel method for preparation of unilamellar liposomes (80-90 nm in diameter) containing thousands of encapsulated protein molecules per vesicle. These protein-containing liposomes were functionalized with DARPin_9−29 that specifically binds to HER2 receptors overexpressed in SK-BR-3 and SK-OV-3 cells. The DARPin-functionalized liposomes containing large amounts of mCherry were shown to specifically stain HER2-positive cells, and the liposomes loaded with a fragment of *Pseudomonas* exotoxin A, PE40, which strongly inhibits elongation of peptides in eukaryotic cells [16], proved capable of specifically killing the cells.

2. Materials and methods

Unless otherwise stated, reagents and chemicals were obtained from Sigma-Aldrich (USA) and were used without further purification.

2.1. Preparation of DARPin_9-29

The *E. coli* BL21(DE3) strain cells were transformed with the plasmid, pDARP. Fresh transformants (one colony per ml) were inoculated in 100 ml of auto-induction TBP-5052 medium [17] containing 0.1 g/l ampicillin and grown under aerobic at 25 C. After reaching OD_{600} of 12-14, cells were harvested by centrifugation at 6000 g for 10 min at 4ºC. The pellet was resuspended in 40 ml of lysing buffer: 20 mM sodium phosphate (Na-Pi), 500 mM NaCl, pH 7.5, 50 μg/ml lysozyme, placed into an ice bath and sonicated 30 times for 10 s with 10 s intervals on a VibraCell (Sonics, USA) ultrasonic disintegrator. Cell debris was removed by centrifugation at 15000 *g* for 20 min at 4°C. The clarified lysate containing his-tagged DARPin_9-29 was passed through a 0.22-μm filter and applied onto a Ni^{2+}-NTA (GE Healthcare, USA) column equilibrated in: 20 mM Na-Pi, pH 7.5, 500 mM NaCl containing 30 mM imidazole. The column was washed with ~ 20 ml of the above buffer containing 30 mM imidazole. The protein was eluted from the column by increasing imidazole

concentration from 100 to 500 mM. The yield of the purified protein was 70 mg per liter of culture. The purified DARPin may still contain minor amounts of the improperly folded protein that can self-aggregate into high molecular weight complexes. Purification of the individual protein from the aggregates was conducted on a Sepharose CL-2B column (10x35 mm) equilibrated with 100 mM potassium-phosphate (K-Pi), pH 7.5. The void volume fraction containing the aggregates was discarded and the fraction eluted in the total volume, containing the monomeric protein, was collected and analyzed using 12% polyacrylamide gel electrophoresis under denaturing and reduced conditions (Fig. S1). The protein was stored at 4 C until used.

2.2. Preparation of PE40 and mCherry

E. coli strain BL21(DE3) cells were transformed with the plasmids pPE40 or p-mCherry essentially as described earlier [18]. Freshly grown transformant (one colony per 1 ml) of each of the above proteins was inoculated in 100 ml of a medium containing: 2 mM $MgSO_4$, 2 mM $MgCl_2$, 5 mM KH_2PO_4, 45 mM K_2HPO_4, 100 mM NaCl, 1% yeast extract, 1% tryptone and 0.1 g/l ampicillin and left to grow in a 2000-ml flask at 37°C. When OD_{600} of 0.5 was reached, 1 mM isopropyl-D-thiogalactopyranoside was added to the cell suspension. After 6 hours of induction, the cells were cooled and harvested by centrifugation at 6000g for 10 min at 4°C. The pellet was resuspended in 40 ml of: 20 mM Na-Pi, 500 mM NaCl, pH 7.5, 30 mM imidazole, placed into an ice bath and sonicated 30 times for 10 s with 10 s intervals on a VibraCell (Sonics, USA) ultrasonic disintegrator. Cells debris was removed by centrifugation at 15000 *g* for 30 min at 4°C. The supernatant containing his-tagged PE40 or mCherry was passed through 0.22-μm filter and applied onto a Ni^{2+}-NTA (GE Healthcare, USA) column equilibrated with: 20 mM Na-Pi, pH 7.5, 500 mM NaCl containing 30 mM imidazole. The column was washed with ~ 20 ml of the above buffer. mCherry was eluted from the column with the buffer supplemented with 150 mM imidazole. PE40 was eluted with the buffer supplemented with 250 mM imidazole. The proteins eluted from the column may still contain minor amounts of aggregates. Purification of the individual proteins from the aggregates was conducted on a Sepharose CL-2B column (10x35 mm) equilibrated with 100 mM K-Pi (pH 7.5) and 1 mM EDTA. The void volume fractions were discarded and the fractions eluted in the total volume (containing the monomeric proteins) were collected, and analyzed using 12% polyacrylamide gel electrophoresis under denaturing and reduced conditions (Fig. S1). The proteins were stored at 4 C until used. The yields of PE40 and mCherry were 12 and 25 mg per liter of culture respectively.

2.3. Preparation of cytochrome c-encapsulated liposomes

The phospholipid suspension was prepared by swelling 0.2 g of L-α-Phosphatidylcholine granules (Avanti Polar Lipids, Soy 40%, 341602) in 10 ml of distilled water for 16-20 hours at ambient temperature. The suspension was further filtered through a 0.8 micron syringe filter and stored at 4 C until used.

0.5 ml of the phospholipid suspension was mixed with 0.5 ml of horse heart cytochrome c solution (2-40 mg/ml) in 20 mM K-Pi (pH 7.5). The protein-lipid suspension was frozen and thawed 5 times and subsequently extruded 19 times through a 100 nm pore size polycarbonate membrane at ambient temperature using Avanti Mini Extruder. The extrusion yields unilamelar liposomes with the average

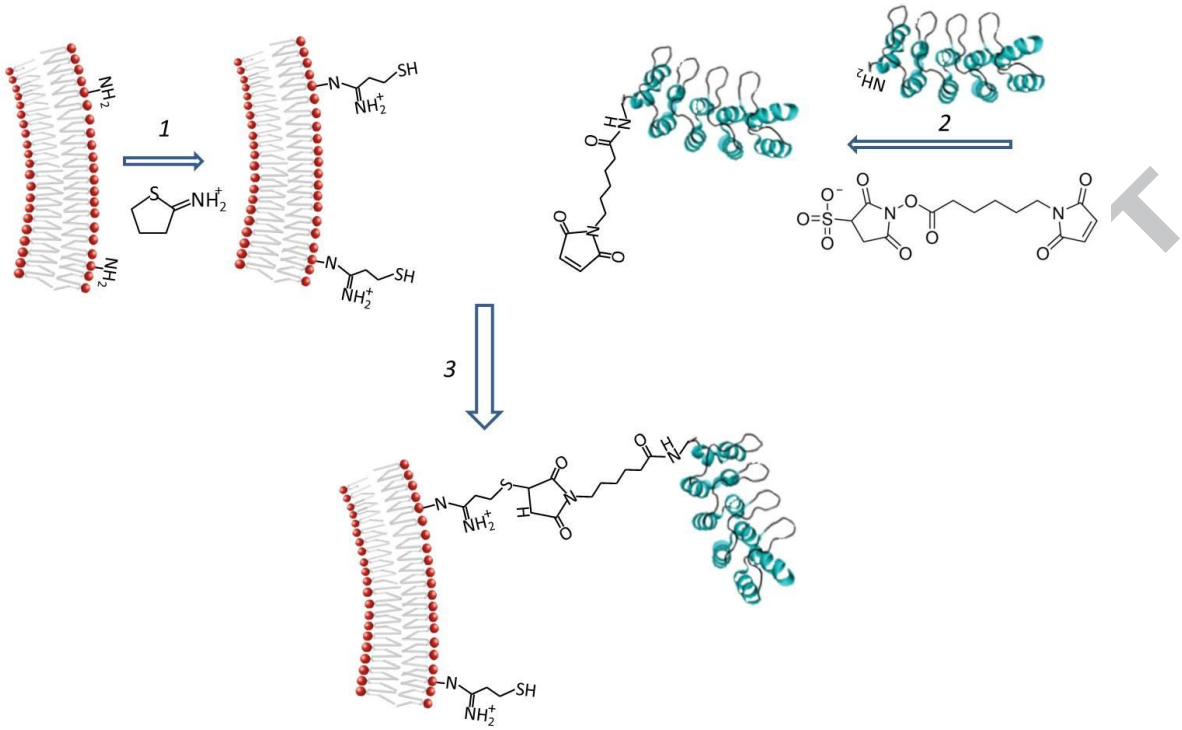
diameter of 80-90 nm (see Fig. S3), SUVs. The liposomes were separated from the excess of cytochrome c on a Sepharose CL-2B column (10x35 mm) equilibrated with 10 mM K-Pi (pH 7.5) containing 0.2 M NaCl. At high ionic strength the protein dissociates from the outer surface of the liposome membrane. Chromatography on the Sepharose CL-2B column enabled to completely separate the liposomes from the protein not associated with the vesicles. The liposome fraction, collected in the void volume, was stored at 4 C until used.

2.4. Preparation of mCherry- and PE40-encapsulated liposomes

The phospholipid suspension was prepared as described in the previous paragraph for the cytochrome c-containing liposomes. The phospholipid suspension (2 mg/ml) was mixed with either mCherry (4 mg/ml) or PE40 (4 mg/ml) in 20 mM MES-Na (pH 6.0). The protein-lipid suspension was frozen and thawed 5 times and subsequently extruded 19 times through a 100 nm pore size polycarbonate membrane at ambient temperature using Avanti Mini Extruder. The liposomes were separated from the excess of the proteins on a Sepharose CL-2B column (10x35 mm) equilibrated with 100 mM K-Pi (pH 8.0) and 1 mM EDTA. At neutral (mild basic) pH the protein molecules dissociate from the outer surface of the liposome membrane. Chromatography on the Sepharose CL-2B column enabled complete separation of the dissociated proteins from the liposomes. The latter were eluted in the void volume of the column. The liposome fraction was collected and stored at 4 C until used.

2.5. Covalent coupling of DARPin_9-29 to liposomes

Covalent attachment of DARPin_9-29 to the outer surface of the liposome membrane included the following steps: 1 - modification of liposomes by 2-iminothiolane (Traut's reagent), the reaction that introduces SH-groups to primary amine-containing phospholipids; 2 - modification of amino groups of the DARPin by sulfo-EMCS (N-ε-maleimidocaproyl-oxysulfosuccinimide ester), a hydrophilic amine- and sulfhydryl-crosslinker; 3 - coupling of the sulfo-EMCS-treated protein to the Traut's reagent-treated liposomes. The schematic drawing illustrating the process is presented in Fig. 1.



Liposomes containing mCherry or PE40 were prepared as described 2.4. The proteoliposomes were incubated with 6 mM Traut's reagent at ambient temperature for 1 hour in 100 mM K-Pi (pH 8.0) containing 1 mM EDTA. Incubation with Traut's reagent leads to the attachment of a residue terminated by SH-group to primary amino groups of the amine-containing phospholipids composing the liposome membrane (reaction "1" in Fig. 1). The phospholipid mixture used here (soy PC, 40%, from Avanti Polar Lipids, USA) consisted of about 16% phosphatidylethanolamine, making it possible to introduce many SH-groups to the surface of a liposome. The incubation was passed through a NAP-5 desalting column (GE Healthcare, USA) equilibrated in 100 mM K-Pi (pH 7.5). The void volume fraction (~0.5 ml) was collected and kept on ice.

DARPin_9-29 (2-4 mg/ml) purified as described above was incubated with a 10-fold molar excess (1-2 mM) of sulfo-EMCS for 45 min under ambient conditions. This heterobifunctional amine-to-sulfhydryl linker, containing the succinimide (which specifically reacts with primary amino groups) and the maleimide (which reacts with thiols) moieties at opposite ends of the molecule, covalently binds to DARPin_9-29 through one of primary amino groups on the surface of the protein. The protein lacks SH-groups, thus leaving the maleimide group of the linker available for further reaction with the Traut's reagent-treated liposomes (reaction "2" in Fig. 1). The DARPin modification should occur simultaneously with treatment of the liposomes with Traut's reagent in order to avoid oxidation of SH-groups introduced to the bilayer. The incubation was passed through a NAP-5 desalting column (GE Healthcare, USA) equilibrated in 100 mM K-Pi (pH 7.5). The sulfo-EMCS-treated DARPin was mixed with equal volume of the Traut's reagent-treated liposomes and incubated for 2 hours under ambient conditions. The non-bound DARPin was separated from the liposomes on a Sepharose CL-2B column (10x35 mm) equilibrated with 20 mM K-Pi (pH 7.5). The void volume fraction was collected and stored at 4 C until used.

2.6. Cell cultures

Cell lines SK-BR-3 (human breast adenocarcinoma, ATCC number HTB-30), SK-OV-3 (human ovarian adenocarcinoma, ATCC number HTB-77) and CHO-K1 (Chinese hamster ovary, ATCC number CCL-61) were cultured in complete McCoy's 5A medium with 10% (v/v) heat-inactivated fetal calf serum (Thermo Scientific), 100 U/ml penicillin/streptomycin (Paneco) and 2 mM L-glutamine (PanEco, Russia) in a humidified atmosphere with 5% CO_2 at 37°C. To prevent removal of receptors from the cell surface upon subcultivation, the cells were detached from the support with trypsin-free Versen (PanEco) solution.

2.7. Cell viability assay

To study cytotoxicity of PE40-encapsulated liposomes functionalized with DARPin_9-29, DARPin-PE40-SUVs, SK-BR-3, SK-OV-3 and CHO cells (2-3x10^4 cells per well) were inoculated onto 96-well plates (Corning, USA) and cultured overnight. Then, the growth medium in the wells was replaced with the medium containing DARPin-PE40-SUVs and the cells were incubated at 37°C in a 5% CO_2 atmosphere for 72 hours. Cell viability was estimated essentially as described in [19]. Briefly, the medium was replaced by McCoys 5A solution containing 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and the cells were incubated for 1 hour at 37°C. The medium was subsequently replaced with 100 µl of DMSO. Absorbance of formazan formed in the incubation was measured at 570 nm using Infinite M1000 (Tecan, Switzerland) monochromator-based microplate reader. The A_{570} value obtained for the liposome-treated cells was divided by the control (A_{570} for non-treated cells) to determine relative cell viability. All experiments were performed in triplicate. The half-maximal inhibitory concentration (IC_{50}) was calculated using nonlinear regression analysis in GraphPad 6 software.

2.8. Preparation of FITC-labeled PE40.

For the sake of visualization, PE40 lacking visible fluorescence was covalently labeled with Fluorescein isothiocyanate (FITC). For that the protein (5 mg/ml,) was incubated with the dye (0.63 mM) in 0.1 M phosphate buffer (pH 8.5) for 1 hour. The dye-protein conjugate was purified from the unbound FITC on NAP-5 desalting column (GE Healthcare, USA) equilibrated in 0.1 M phosphate buffer (pH 7.5). The void volume fraction was collected and used for preparation of DARPin-PE40-FITC-SUVs as described above (see section 2.4. and 2.5)

2.9. Confocal microscopy

SK-BR-3 and CHO cells were cultured on were inoculated in glass-bottom dishes (WillCo Well) overnight in 5% CO_2 at 37°C. To study interaction of DARPin-functionalized SUVs containing either mCherry or PE40 with HER2-receptors, 0.3 nM of the proteoliposomes were added to each well and left for 15 min at 4 C. The cells were subsequently washed twice with the medium and visualized immediately using a laser scanning microscope (Zeiss LSM-710-NLO, Germany). It is essential to avoid heating the cells during the measurements.

Internalization of the proteoliposomes into the cells was conducted as follows. The cells were incubated with 0.3 nM of the proteoliposomes for 15 min at 37 C. The

cells were subsequently washed twice with the medium and incubated further for 1 hour at 37°C. Hoechst 33342 (2 nM) was then added and the cells nuclei were stained for 10 min at 37°C. The cells were finally washed twice with the medium and visualized on the confocal microscope. Images of cells containing mCherry, PE40-FITC and Hoechst 33342 were obtained by sequential measurements of mCherry and the dyes emission. mCherry was excited at 561 nm; the protein emission was measured in the 565-700 nm range. FITC conjugated to PE40 was exited at 488 nm; the emission of the dye was measured in the 497-562 nm range. Hoechst 33342 was exited at 700 nm using femtosecond laser; the emission was monitored at 400–600 mn. The 63× oil Plan-Apochromat objective was used in order to obtain high-quality images.

2.10. Measurement of phosphatidylserine (PS) content in the outer leaflet of the plasma membrane

The appearance of phosphatidylserine in the outer leaflet of the plasma membrane is an indicator of early stages of cell apoptosis [20, 21]. To measure PS in the outer membrane leaflet, SK-BR-3 cells were inoculated in a 6-well plate (10^5 cells per well) and were grown overnight at 37 C. DARPin-PE40-SUVs (0.3 nM) were added to the wells and the incubation was conducted for 27 hours at 37 C. The cells were detached from the wells with the Versen solution, concentrated by centrifugation and washed with phosphate-buffered saline. Phosphatidylserine in the outer leaflet of the plasma membrane was determined using FITC-conjugated annexin V (Invitrogen, USA) as follows. The cells were resuspended in 100 µl of annexin V-binding buffer (Invitrogen, USA); 5 µl of FITC-conjugated annexin V and 1 µl of propidium iodide, PI (Invitrogen, USA), were added to the cell suspension. After incubation under ambient temperature in darkness for 15 min 400 µl of annexin V-binding buffer were added and the cells were analyzed on a NovoCyte 3000 (Bioscience, USA) flow cytometer. FITC fluorescein was excited with a blue laser at 488 nm and the emission of the dye was detected using a 530/30 bandpass filter. PI was excited at 561 nm; the emission of the dye was measured using a 615/20 bandpass filter. SK-BR-3 cells treated with 1 mM $CaCl_2$ for 3 hours were used as a positive control for apoptosis. The necrotized cells were prepared by 10-min treatment of the cells at 75°C.

2.11. Measurement of DNA fragmentation content in cells.

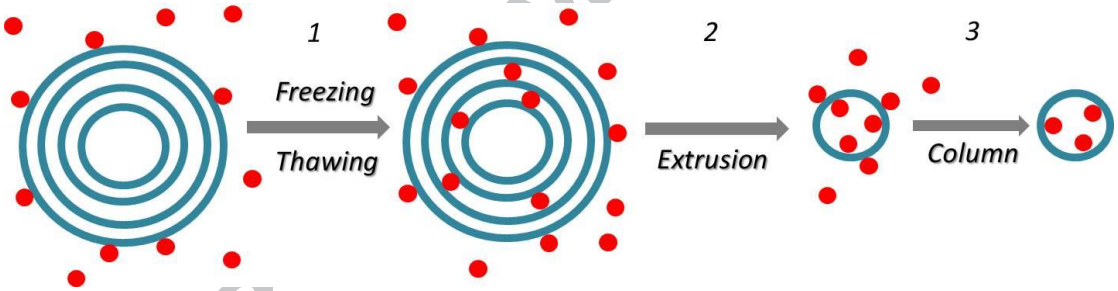
Approximately 10^5 SK-BR-3 cells were seeded in each well of a 6-well plate and incubated on the substrate overnight; DARP-PE40-SUVs (0.2 nM) were added to the cells. After 18 hours incubation with the proteoliposomes at 37°C the cells were detached from the substrate, washed, harvested, and fixed with ice-cold 70% ethanol at 4°C. After the fixation, cells were washed with phosphate buffered saline (PBS) and incubated with 50 µg/ml propidium iodide (PI) and 200 µg/ml RNase A for 30 min in darkness on ice. The DNA fragmentation flow cytometry analysis was performed with the NovoCyte 3000 device (Biosciences, USA) and analyzed using NovoExpress software. The DNA content was estimated by measuring fluorescence of the nucleic acid-intercalated PI. The dye was excited with at 561 nm; the fluorescence emission was recorded using a 586/20 nm band filter. Cells treated with 1 mM $CaCl_2$ were used as an apoptotic control. Necrotic cells were produced by incubating the cells for 10 min at 75°C.

2.12. Cryo-TEM Measurements

A drop (3.5 µl) of a liposome suspension was applied to a glow discharged TEM grid (300-mesh Cu grid) coated with a holey carbon film (Lacey substrate, Ted Pella, Ltd.). The excess liquid was blotted, and the specimen was vitrified by a rapid plunging into liquid ethane pre-cooled with liquid nitrogen using Vitrobot Mark IV (FEI). The vitrified samples were transferred to a cryo-holder (Gatan model 626), and examined at −177 C using a FEI Tecnai 12 G^2 Spirit TWIN TEM. The images were recorded (4Kx4K FEI Eagle CCD camera) at 120 kV in low-dose mode. TIA (Tecnai Imaging & Analysis) software was used to record the images and to measure the thickness of the liposome's membrane.

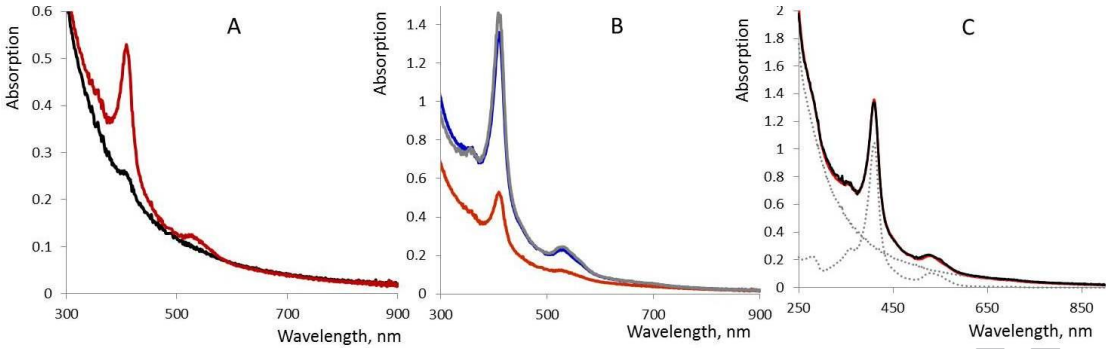
3. Results

Three proteins: heme-containing cytochrome c, highly fluorescent mCherry and highly toxic PE40 were encapsulated in liposomes composed of natural (soybean) phospholipids by the procedure shown schematically in Fig. 2. The encapsulation method included: electrostatic binding of proteins to phospholipid membranes; freeze-thawing of the proteolipid suspension; extrusion of the suspension through a 100 nm pore size polycarbonate membrane; and size-exclusion column chromatography separation of liposomes from the non-encapsulated protein.



3.1. Cytochrome c encapsulation in liposomes

Cytochrome c was encapsulated in liposomes, using the approach illustrated in Fig. 2 (see also Materials and Methods). The absorption spectrum of the cytochrome-containing liposomes is presented in Fig. 3A (red curve). Absorption peaks with maxima at 410 and 530 nm above the continuously rising curve (corresponding to light scattering by liposomes) are characteristic of cytochrome c. Extrusion of the proteolipid mixture under high ionic strength conditions resulted in almost complete disappearance of the peaks (Fig. 3A, black curve). This strongly indicates that electrostatic binding to the phospholipid membrane is required for efficient encapsulation of the protein into liposomes. Freeze-thawing of the protein-phospholipid mixture prior to the extrusion leaded to more than 3-fold increase in the amount of liposome-encapsulated cytochrome c (compare blue and red curves in Fig. 3B). The increase of cytochrome c concentration in the extrusion mixture from 4 to 20 mg/ml had almost no effect on the amount of the liposome encapsulated protein (Fig. 3B, blue and grey curves).



The spectrum of the proteoliposomes (Fig. 3C, black curve) was successfully simulated (Fig. 3C, red curve) by summing the light-scattering spectrum of 1.6 mg/ml liposomes and the absorption spectrum of 9.7 µM cytochrome c (Fig. 3C, dotted grey curves). Since molar concentration of liposomes in 1.6 mg/ml suspension is equal to ~1.8 nM (see "Estimation of molar concentration of the lipid vesicles" and Figs. S2 and S3), the cytochrome to liposome molar ratio, which corresponds to the number of cytochrome c molecules per liposome, was equal to (9,700 nM/1.8 nM) ~5400.

In order to prove that cytochrome c is encapsulated within the lipid vesicles we studied the kinetics of the protein reduction by ascorbic acid, which cannot penetrate the lipid bilayer. The reduction was followed by monitoring the absorption at 550 nm which is characteristic of the reduced form of the protein (its heme group). As seen in Fig. S4A, only a minor portion (less than 5 %) of the protein was reduced following 20 min incubation of the proteoliposomes with 1 mM ascorbic acid (notice slight growth of absorption at 550 nm indicated by the arrow in Fig. S4A). Addition of 0.5 % Triton X-100, that dissolves the liposome membrane, led to almost complete reduction of the protein within a minute (Fig. S4B). These results clearly demonstrated that the cytochrome c molecules are separated from the bulk by the liposome membrane.

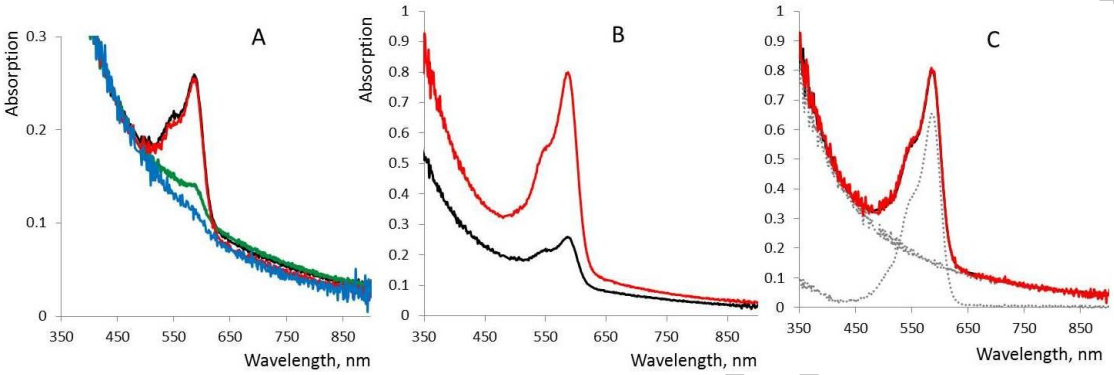
Cryo-TEM enables direct imaging of the liposomes and thus evaluation of their shape, diameter and membrane thickness. Cryo-TEM images of liposomes loaded with cytochrome c molecules (Fig. S5B), showed that the average thickness of the liposome's membrane is slightly larger (7.2 ± 2.0 nm) than that measured for the empty liposomes (5.0 ± 0.8 nm, Fig. S3A, S5A). This may indicate that protein molecules are adsorbed on the liposome's inner membrane surface. In addition, while the inside of the empty liposomes (the intra-liposome cavity) looks the same as the background, some electron density is observed inside the cytochrome c-containing vesicles (Fig. S5B). These observations are consistent with the very large number of cytochrome c molecules that we succeeded to encapsulate per liposome.

3.2. mCherry encapsulation in liposomes

The same method of encapsulation (see Fig. 2) was used for preparation of mCherry-loaded liposomes. mCherry is characterized by strong absorption and fluorescence in the visible range of the spectrum and can be easily monitored by absorption and fluorescent spectroscopies. The protein is slightly negatively charged at neutral pH (the expected pI is ~ 6.01, as calculated by ExPasy [22]) and thus should not bind to the liposome membrane by means of electrostatic forces at pH 7.5.

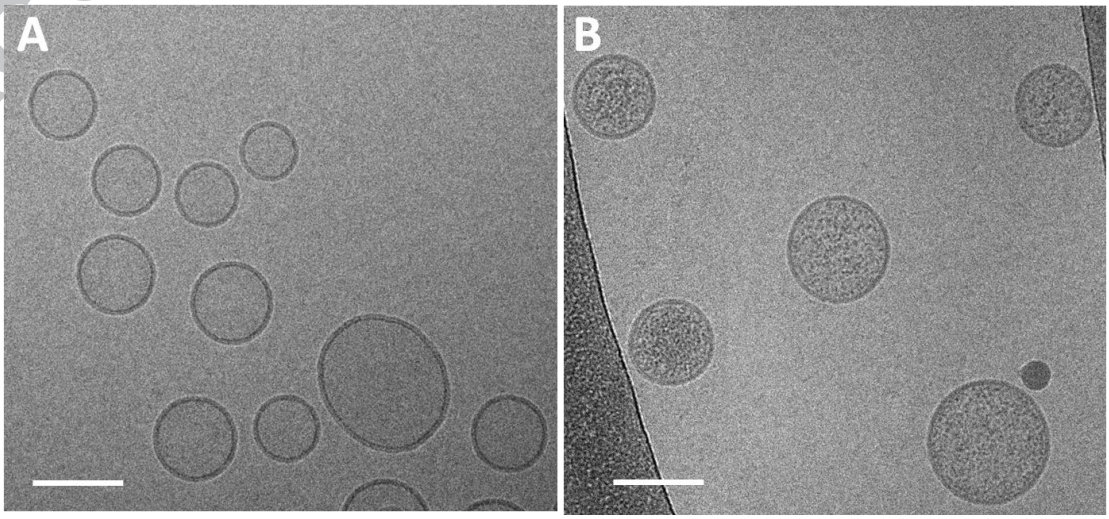
As expected, almost no mCherry was bound to liposomes when the vesicles were prepared at neutral pH (Fig. 4A, blue curve). The extrusion under mild acidic conditions (pH 4.0-6.0), however, yielded liposomes containing noticeable amounts of the protein (Fig. 4A, black and red curves). The amount of the encapsulated protein at

pH 6.0 can be strongly reduced by addition of 0.2 M NaCl to the extruded mixture (Fig. 4A, green curve). As in the case of cytochrome c (Fig. 3B), the efficiency of mCherry incorporation into the liposomes can be considerably increased by freeze-thawing of the phospholipid-protein mixture prior to extrusion (Fig. 4B, black and red curves).



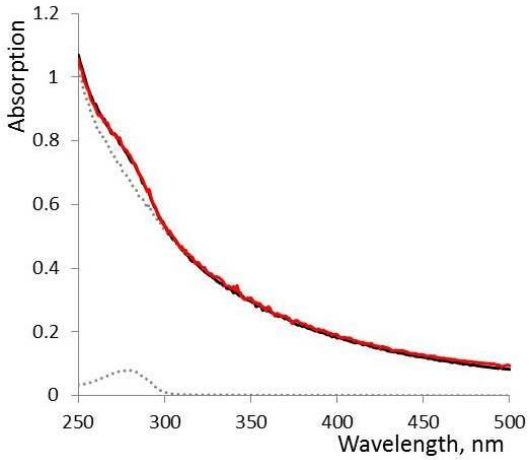
The spectrum of mCherry containing liposomes (Fig. 4C, black curve) can be fairly simulated (Fig. 4C, red curve) by summing spectral curves corresponding to ~1.7 nM liposomes (molar concentration of the vesicles is 1.55 mg/ml suspension, see Supplementary materials) and 9 µM mCherry (Fig. 4C, dotted grey curves). The mCherry to liposome molar ratio, which corresponds to the number of protein molecules in a liposome is thus equal to (9,000 nM/ 1.7 nM) 5300.

Cryo-TEM images of mCherry-containing liposomes that have been transferred to pH = 7.5 and kept at neutral pH for 18 hours (Fig. 5B) showed high electron density inside the liposomes (in the intra-liposome cavity) pointing at high protein concentration in the vesicles. Note that mCherry-containing liposomes that were kept at pH = 6 (Fig. S5C) appeared with a thicker membrane (10 ± 3 nm on average) relative to that of the empty liposomes (5.0 ± 0.9 nm) and in some of them a chain of dark and grey spots is seen adjacent to the inner membrane surface (Fig. S5C, white arrow). These results are consistent with electrostatic attraction of the encapsulated mCherry protein to the membrane at mild acidic pH and dissociation of the protein from the membrane at neutral pH.



3.3. PE40 encapsulation in liposomes

Encapsulation of PE40 in liposomes was conducted as described in Materials and Methods under mild acidic conditions. The protein-phospholipid mixture was freeze-thawed 5 times prior to the extrusion. Since PE40 does not absorb light in the visible range of the absorption spectrum, quantitation of the protein by absorption spectroscopy is more challenging and less accurate than quantitation of cytochrome c or mCherry. Molar concentration of the protein in the liposome fraction was estimated at 280 nm using extinction coefficient of 44,140 $M^{-1}cm^{-1}$ (calculated by ExPasy [22]). Analysis of the proteoliposome spectrum conducted as described in Figs. 3C and 4C for cytochrome c- and mCherry-containing liposomes, respectively, revealed that the average number of the toxin molecules per liposome is approximately equal to 2000 (see Fig. 6).

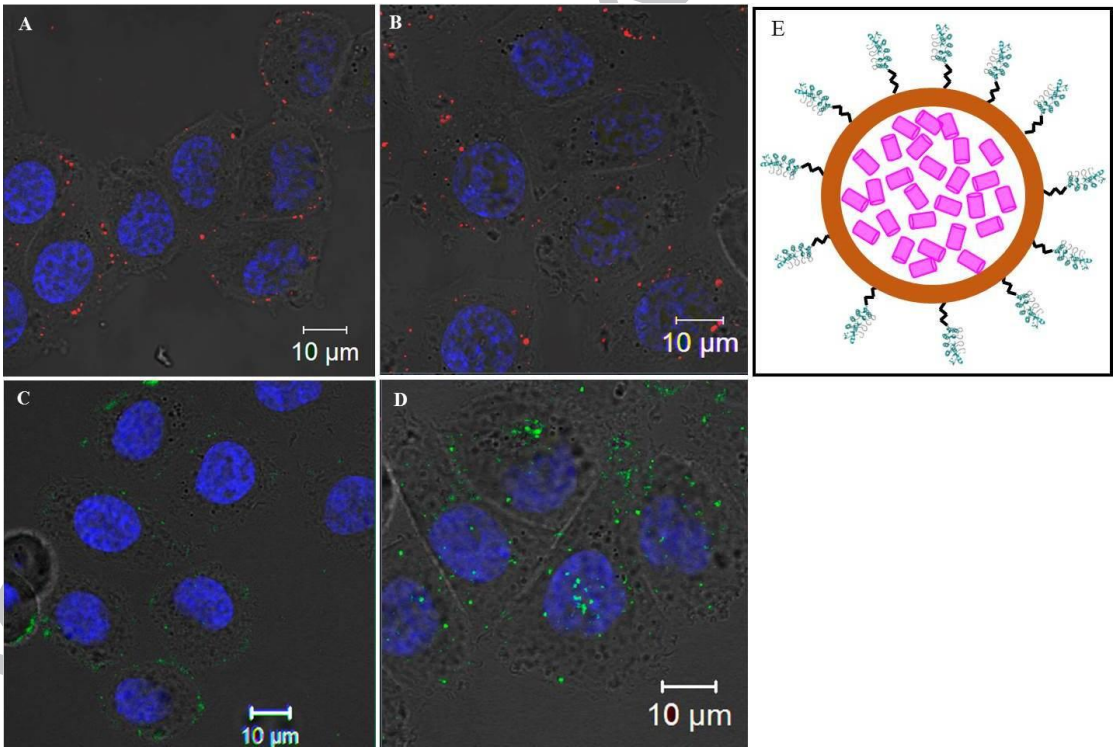


3.4. Covalent coupling of DARPin_9-29 to the liposome membrane

The protein containing liposomes were functionalized with DARPin_9-29, which binds with very high affinity to HER2 receptors overexpressed in breast and ovarian cancer cells. The protein was covalently attached to the outer surface of the liposomes as described in details in Materials and Methods and illustrated in Fig. 1. To test the efficiency of the method, we examined coupling of mCherry to the surface of empty liposomes. This colorful protein can be easily quantified by absorption spectroscopy in contrast to DARPin_9-29 which does not absorb light in the visible region. The spectrum of the mCherry-liposome conjugate (Fig. S6, red curve) was simulated by summing light-scattering spectrum of 0.52 mg/ml (or ~ 0.6 nM, see Supplementary materials) liposomes and absorption spectrum of 0.6 μM mCherry (Fig. S6, dotted grey curves). This result, showing that about 1000 protein molecules are attached to a liposome, proves the efficiency of the coupling method. Incubation of liposomes that haven't been treated with Traut's reagent with the protein (either treated or not treated with sulfo-EMCS) followed by separation of the reactants using size-exclusion chromatography did not lead to appearance of the absorbance band centered around 585 nm (which is indicative of mCherry) in the liposome fraction.

3.5. Interaction of DARPin_9-29-functionalized mCherry- or PE40-encapsulated liposomes with HER2-positive cells

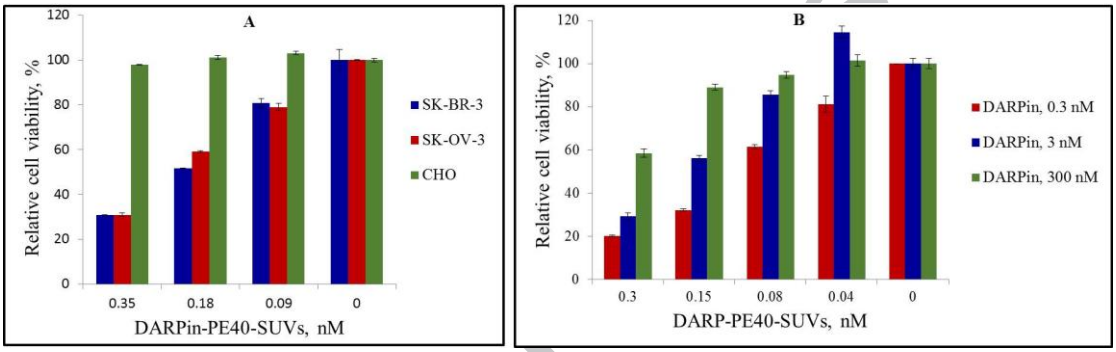
The DARPin-functionalized proteoliposome constructs, DARPin-mCherry-SUVs or DARPin-PE40-SUVs, containing encapsulated mCherry or PE40 respectively, functionalized with DARPin_9-29 (see schematic drawing in Fig. 7E) was prepared as described in Materials and Methods. To prove that the proteoliposome constructs interact specifically with HER2-receptors on the surface of the cell, DARPin-mCherry-SUVs (0.3 nM) or DARPin-PE40-FITC-SUVs (0.3 nM) were incubated with HER2-positive SK-BR-3 cells for 15 min at 4°C. The cells were washed twice with the cold medium and analyzed by confocal microscopy. As seen in Fig. 7A and C, treatment of SK-BR-3 cells with either of the above conjugates at 4°C led to the appearance of bright red (corresponding to mCherry) or green (corresponding to FITC-labeled PE40) fluorescent spots on the cell surface; no fluorescence was detected either in the cytoplasm or in the nuclei. Staining is specific with respect to HER2-positive SK-BR-3 cells; no fluorescence was detected when HER2-negative CHO cells were treated with either DARPin-mCherry-SUVs or DARPin-PE40-FITC-SUVs under identical conditions (Fig. S7). Treatment of SK-BR-3 cells with DARPin-PE40-FITC-SUVs and DARPin-m-Cherry-SUVs conjugates at 37 °C led to the appearance of bright green and red fluorescent spots in the cytoplasm, respectively (see Figure 7B and D).



3.6. The effect of DARPin-PE40-SUVs on cell viability

HER2-positive, SK-BR-3 and SK-OV-3 cells, as well as HER2-negative, CHO cells were incubated with sub-nanomolar concentrations of DARPin-PE40-SUVs, for 72 hours at 37°C. As shown in Fig. 8A the proteoliposomes strongly affected viability of both types of HER2-positive cells. IC_{50} values estimated using nonlinear regression analysis were equal to 0.17 0.02 and 0.21 0.02 nM for SK-BR-3 and SK-OV-3 cells respectively. In contrast, HER2-negative CHO cells (Fig. 8A) were not affected by the

DARPin-PE40-SUVs. Incubation of both types of HER-positive cells types with empty, DARPin_9-29-functionalized liposomes not carrying PE40, did not lead to cell death (Fig. S8), pointing out that the observed harmful effect on cells is due to PE40 rather than to the cargo vesicles themselves. In order to confirm that binding of DARPin-PE40-SUVs to the cell occurs through interaction of the DARPin moieties with HER2 receptors on its surface, the effect of added DARPin_9-29 (which competes with the liposome-bound DARPin for binding to HER2 receptor) on the proteoliposome-induced cell death was studied. As shown in Fig. 8B DARPin_9-29 strongly protects SK-BR-3 cells from the damage caused by DARPin-PE40-SUVs. The protection is concentration-dependent; strong protection was achieved at concentration of the DARPin as low as 300 nM (Fig. 8B). This clearly demonstrates that HER2 is involved in binding of the proteoliposome construct and its internalization into SK-BR-3 cells.



It is known that wild-type PE40 induces apoptosis in target cells [23-25]. In order to evaluate the mechanism of cell death induced by DARPin-PE40-SUVs, we stained the construct-treated cells with Annexin V-FITC and PI (Fig. S9). The staining is specific with respect to phosphatidylserine located in the outer leaflet of the cell membrane [26]. Translocation of phosphatidylserine from the inner to the outer leaflet of the membrane is one of the earliest events in apoptosis; therefore the staining is indicative of apoptotic mechanism of cell death [20, 21]. The staining revealed that about 55% of SK-BR-3 cells underwent early (PI^-AnxV^+) and late (PI^+AnxV^+) apoptosis 27 hours after addition of DARPin-PE40-SUVs. Similar percent was measured for control apoptotic cells (Fig. S9).

DNA fragmentation in mammalian cells is a hallmark of apoptosis. Analysis of DNA fragmentation by flow cytometry thus provides important information about the mechanism of cell death. We have demonstrated that treatment with DARPin-PE40-SUVs for 18 hours leads to strong fragmentation of DNA (about 37% of Sub-G1 fraction) in SK-BR-3 (Fig. S10). Taken together the results on the phosphatidylserine translocation and those on the DNA fragmentation suggest that the death of DARPin-PE40-SUV-treated HER2-positive cells involves apoptosis rather than necrosis.

4. Discussion

In this study we have developed a novel method for encapsulation of large quantities of proteins in liposomes. As many as thousands of protein molecules were incorporated into a 80-90-nm (in diameter) liposome. Concentrations of the encapsulated colorful proteins, cytochrome c and mCherry, are so high that even rather diluted suspensions (1 mg/ ml or less) of the proteoliposomes were intensely colored

(Fig S11). We have demonstrated that electrostatic binding of proteins to the phospholipid membrane is required for the efficient encapsulation. Large quantities (about 5000 molecules per liposome) of the basic protein, cytochrome c, can be encapsulated into lipid vesicles at neutral pH (Fig. 3), while mild acidic proteins, mCherry and PE40, carrying negative charge at pH 7, can be encapsulated in noticeable quantities only at pH lower than 6 (Fig. 4A). At neutral pH the yield of encapsulation is very low due to lack of binding of the above negatively charged proteins to the liposome membrane. The same is true for the encapsulation of the proteins at high ionic strength; the electrostatic interactions under high ionic strength conditions are not strong enough to attract and keep the proteins bound to the bilayer. Interestingly, reduction of pH from 6 to 4 had no effect on encapsulation of mCherry into the vesicles (Fig. 4A). The isoelectric point of the protein is about 6, meaning that the net charge of mCherry is close to zero at pH 6. The positive charged areas on the surface, however, can anchor the protein molecule to the membrane. The Tag sequence attached to the N-terminus of mCherry and PE40, which consist of 6 histidine residues and carries positive charge (pK of the histidine's imidazole is ~6) can serve as an anchoring group connecting the proteins to the membrane. Proteins containing Tag sequences composed mainly of Lys and Arg residues can be engineered. These sequences (positively charged at neutral pH) should anchor acidic and neutral proteins to the liposome membrane under physiological pH, thus extending the applicability of the encapsulation method.

The encapsulation efficiency was significantly increased when the phospholipid-protein mixture was freeze-thawed several (5-10) times prior to extrusion (Figs. 2B and 4B). This result is consistent with the earlier observation on the effect of freeze-thawing on encapsulation of human growth hormone in liposomes [27].

Using the method described here (see Fig. 2) we succeeded to encapsulate roughly 5000 cytochrome c or mCherry molecules inside a 80-90-nm liposome. This number was shown to be independent of cytochrome c concentration in the range from 4 to 20 mg/ml (Fig. 3B) pointing out for for saturation of the phospholipid membrane with the protein. Cytochrome c is a roughly spherical molecule with a diameter of approximately 3 nm [28]. If bound to the inner surface of the liposome membrane, the encapsulated molecules should be spaced very closely together forming a layer on the surface of the membrane. The same is true for mCherry (identification number in PDB is 2h5q), a 4 nm long and 3 nm wide molecule. Indeed, cryo-TEM images showed that the membrane of the cytochrome c- and mCherry-encapsulated liposomes is thicker than the membrane of empty SUVs (see Fig. S5). When the m-Cherry-encapsulated liposomes where kept at pH 7.5, high electron density was observed inside the liposome cavity rather than on its membrane (see Fig. 5B). The different location of the encapsulated mCherry at neutral and slightly acidic pH is consistent with the expected (electrostatic) interaction of the protein (charged negatively at neutral pH and slightly positive under mild acidic conditions) with the negatively charged liposome membrane. Note that small proteins composed of light atoms are generally invisible in TEM unless highly ordered or extremely concentrated. Thus, the fact that we see additional density in the cryo-TEM images of the proteoliposomes in comparison to the empty liposomes further supports our conclusion that large amounts of the proteins are encapsulated in the vesicles.

DARPin_9-29 was covalently coupled to the membrane of liposomes carrying mCherry or PE40 through a relatively long (about 1.7 nm) flexible linker (Fig. 1). The linker is long enough, allowing the DARPin to attain spatial orientation that favors high-affinity binding to HER2.

DARPin-mCherry-SUVs and DARPin-PE40-FITC-SUVs were shown to specifically bind to the membrane of HER2-positive SK-BR-3 cells (Fig. 7A and C) at low (4 C) temperature. Further incubation at 37 C leads to internalization of the proteoliposomes into the cells (Fig. 7 B and D). CHO cells lacking HER2 have not been stained by the proteoliposome constructs (Fig. S7). These results clearly demonstrate that specific interaction with HER2 receptors (through DARPin_9-29 molecules) is a prerequisite for internalization of the DARPin functionalized liposomes into the cell. The results are also consistent with the receptor mediated energy-dependent (temperature-dependent) endocytosis mechanism of internalization.

DARPin-PE40-SUVs were shown to eradicate HER2-positive SK-BR-3 and SK-OV-3 cells; viability of HER2-nehative CHO cells was not affected by the proteoliposome construct (Fig. 8A). This, along with specific staining of SK-BR-3 cells with DARPin-PE40-SUVs (Fig. 7 C and D) suggest that the construct binds to the cell membrane through high affinity interaction of the liposome-conjugated DARPin moieties with HER2 receptor molecules on the cell surface followed by internalization of the construct into the cell. Strong protection of SK-BR-3 cells from DARPin-PE40-SUVs by DARPin_9-29 (Fig. 8B) confirms the above suggestion.

Flow cytometry analysis of phosphatidylserine externalization (Fig. S9) along with the analysis of DNA cleavage (Fig. S10) suggest that treatment with DARPin-PE40-SUVs leads to apoptosis in SK-BR-3 cells. This result is in line with earlier observations showing that wild-type PE, as well as a number of PE-based immunotoxins induce apoptosis in target cells [13, 29, 30].

Utilization of liposome constructs containing large quantity of encapsulated hydrophilic proteins and functionalized with DARPins may pave the way to the development of new promising avenues for theranostics of cancer. More advanced constructs containing toxic and/or colorful proteins of various types and functionalized with DARPins capable of targeting various receptors can be synthesized. These constructs will target and kill different types of pathological cells leaving healthy ones untouched.

Acknowledgements

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Figure captions

Fig. 1. A scheme presenting DARPin_9-29 attachement to the liposome membrane. 1 - Modification of primary amino groups of the liposome membrane by Traut's reagent; 2 - attachment of sulfo-EMCS to one of the primary amino groups of the DARPin; 3 – covalent attachment of the sulpho-EMCS-modified DARPin to the Traut's reagent-treated membrane. The length of a spacer arm connecting the protein to the membrane is equal to sum of sulfo-EMCS (9.1 angstrom) and Traut's reagent (8.1 angstrom) spacer arms lengths, ~ 17 angstroms.

Fig. 2. Schematic illustration of the method used for preparation of protein-containing liposomes. Positively charged protein molecules (red spheres) electrostatically bind to the surface of the outer membrane (light blue circle) composing the multilamelar proteolipid structure. 1 - Freeze-thawing of the proteolipid mixture enables the protein

molecules to get into the onion-shaped structure; 2 – extrusion of the proteolipid mixture through a 100 nm pore size membrane filter yields unilamelar vesicles containing the encapsulated protein; 3 – size-exclusion column chromatography conducted under high ionic strength conditions completely separate of the liposomes from the non-encapsulated protein.

Fig. 3. Absorption spectroscopy of cytochrome c-containing liposomes. A – The proteoliposomes were prepared by extruding a mixture of 2 mg/ml phospholipids and 4 mg/ml cytochrome c (red curve) containing 0.2 M NaCl (black curve) through a 100 nm pore size membrane filter. The non-encapsulated protein was separated from the liposomes by size-exclusion chromatography as described in Materials and Methods. The suspension hasn't been freeze-thawed prior to the extrusion. B – A mixture of 4 (blue curve) or 20 mg/ml (grey curve) cytochrome c with 2 mg/ml phospholipids was freeze-thawed 5 times, subsequently extruded through a 100 nm pore size membrane filter and separated from the non-encapsulated protein as described in Materials and Methods. Red curve is the same as red curve in A. C – black curve is the same as blue curve in B; red curve is a sum of spectral curves corresponding to 9.7 μM cytochrome c and 1.6 mg/ml liposomes (dotted grey curves). The extinction coefficient of oxidized cytochrome c at 410 nm is equal to 106 $mM^{-1} \cdot cm^{-1}$.

Fig. 4. The effect of pH and ionic strength on mCherry encapsulation in liposomes. A- The proteoliposomes we prepared at different pH conditions by extruding a mixture of 2 mg/ml phospholipids and 4 mg/ml mCherry in: 20 mM Na-Acetate, pH 4.0 (red curve), 20 mM MES-Na, pH 6.0 (black curve), 20 mM K-Pi, pH 7.5 (blue curve) or 20 mM MES-Na, pH 6.0 containing 200 mM NaCl (green curve) through a 100 nm pore size membrane filter. B – The protein-lipid mixture in 20 mM MES-Na (pH 6.0) (black curve) was freeze-thawed 5 times prior to extrusion (red curve). C – Black curve is the same as red one in B; red curve is a sum of spectral curves corresponding to 9.0 μM mCherry (the extinction coefficient of the protein at 587 nm is equal to 72,000 $M^{-1}cm^{-1}$ [31]) and 1.55 mg/ml liposomes (dotted grey curves).

Fig. 5. Cryo-TEM images of: A - "empty" liposomes and B - mCherry-containing liposomes at pH = 7.5. Scale bar = 100 nm.

Fig. 6. Absorption spectroscopy of PE40-containing liposomes. The proteoliposomes were prepared by extrusion a mixture of 2 mg/ml phospholipids and 4 mg/ml PE40 through a 100 nm pore size membrane filter (red curve). The suspension was freeze-thawed 5 times prior to extrusion. Black curve is a sum of spectral curves corresponding to 2.3 μM PE40 and 0.93 mg/ml liposomes (dotted grey curves).

Fig. 7. Interaction of DARPin-mCherry-SUVs and DARPin-PE40-SUVs with HER2-positive cells. SK-BR-3 cells were incubated with DARPin-mCherry-SUVs (A, B) or DARPin-PE40-FITC-SUVs (C, D) at 4°C (A, C) or 37°C (B, D), as described in Materials and Methods. Confocal fluorescent images of the cells were acquired at excitation wavelengths of 561 nm (A, B) or 488 nm (C, D). Superimposed images of the cells in blue-green (C, D) and blue-red (A, B) fluorescence channels are presented. Nuclei were stained with Hoechst 33342. E – schematic representation of DARPin-mCherry-SUVs.

Fig. 8. Cytotoxicity of DARPin-PE40-SUVs. A - HER2-positive SK-BR-3 and SK-OV-3 cells and HER2-negative CHO were incubated with DARPin-PE40-SUVs (concentrations indicated on the axes) at 37°C for 72 hours. Cell viability was measured as described in Materials and Methods. B - SK-BR-3 cells were incubated with DARPin-PE40-SUVs (concentrations indicated on the axes) and 0.3 (red bars), 3 (blue bars) and 300 nM (green bars) DARPin_9-29 at 37°C for 72 hours. Cell viability was estimated as described in Materials and Methods. Statistical analyses were performed using one-way analysis of variance (ANOVA). The difference between HER2-positive and HER2-negative cells was statistically significant ($p<0.01$). Bars indicate SD for 3 individual experiments.

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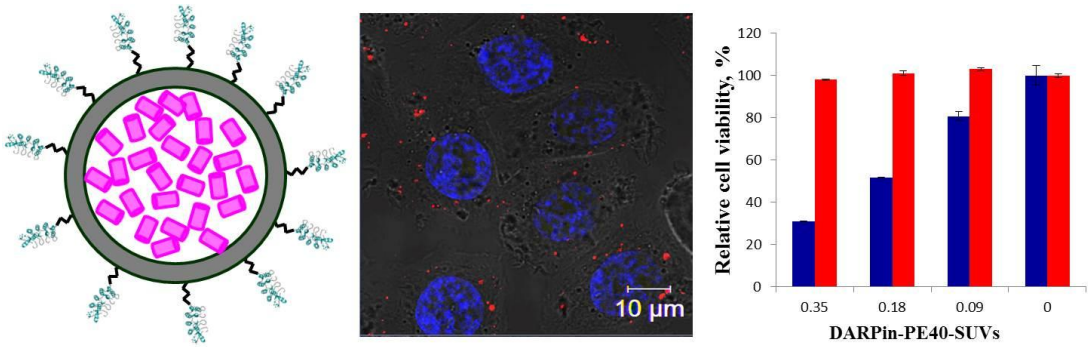
Graphical abstract



The proteoliposomes containing large quantities of highly fluorescent protein, mCherry or a fragment of *Pseudomonas* exotoxin A, PE40 and functionalized with the designed ankyrin repeat protein (DARPin), which targets human epidermal growth factor receptor 2 (HER2) were shown to specifically stain and kill in sub-nanomolar concentrations human breast adenocarcinoma cells, overexpressing HER2, respectively.