Bio-mimetic Nanostructure Self-assembled from Au@Ag Heterogeneous Nanorods and Phage Fusion Proteins for Targeted Tumor Optical Detection and Photothermal Therapy

Fei Wang[1], Pei Liu[1], Lin Sun[2], Cuncheng Li[2,*], Valery A. Petrenko[3,*], and Aihua Liu[1,*]

[1] Laboratory for Biosensing, Qingdao Institute of Bioenergy & Bioprocess Technology, and Key Laboratory of Biofuels, Chinese Academy of Sciences, 189 Songling Road, Qingdao 266101, China, and University of Chinese Academy of Sciences, 19A Yuquan Road, Beijing 100049, China

[2] Key Laboratory of Chemical Sensing & Analysis in Universities of Shandong, School of Chemistry and Chemical Engineering, University of Jinan, Jinan 250022, China

[3] Department of Pathobiology, Auburn University, 269 Greene Hall, Auburn, Alabama 36849-5519, United States

Corresponding authors.

E-mails: liuah@qibebt.ac.cn (A.H.L.); chm_licc@ujn.edu.cn (C.C.L.); petreva@auburn.edu (V.A.P.).

Supplementary Information

Instrumentation. Transmission electron microscopic (TEM) images were obtained using an H-7650 transmission electron microscope (Hitachi, Japan) with an accelerating voltage of 80 kV. Samples for TEM analysis were prepared by being unstained or negatively stained with 1% uranyl acetate. The UV-Vis-NIR absorption spectra were collected using DU800 scanning spectrophotometer (Beckman Coulter, Inc., U.S.) over the range from 300 to 1100 nm and deionized water was used as reference. Zeta potential (ζ) of samples dispersed in deionized water was measured at 25 ℃ for three times using a Malvern Zetasizer (Malvern Instruments Ltd., Worcestershire, UK). Fluorescence spectra were measured using FluoroMax-4 fluorescence spectrophotometer (HORIBA Jobin Yvon, USA). The confocal fluorescent images were taken by the Fluo View^{TM} FV1000 (OLYMPUS) confocal laser scanning biological microscopy with 543 nm excitation wavelength.

Cell Culture. The target cell (SW620) and control cells (HEK293T and HepG2) were cultured in the 30 cm^2 culture flask or 96-well culture plate containing DMEM medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FBS. All cells were cultured at 37 ℃ under a humidified incubator with 5% CO_2.

Selection of SW620 Cell-Binding Phages. The f8/8 landscape phage library was screened to isolate SW620 cell-binding phage particles. The procedures of biopanning, preparation of starved cells and amplification of phages have been described previously[1, 2]. Briefly, the primary library (~10^{11} virions in 50 μl blocking buffer of serum-free DMEM medium containing 0.5% BSA) was added to an empty microwell of the 96-well cell culture plate and incubated for 1 h at room temperature (RT). Then, the solution containing unbound phage particles was transferred to serum-treated well and incubated for 1 h at RT. At the same

time, HepG2 cells were washed twice with DMEM medium and incubated at 37 ℃ in serum-free DMEM medium which would be removed immediately before adding the phages. The solution containing unbound phages was transferred to HepG2 cells and incubated for 1 h at RT. Meanwhile, HEK293T cells were washed and incubated as HepG2. Phage particles that did not bind to HepG2 cells were transferred to HEK293T cells and incubated for 1 h at RT. Phage particles that have been depleted against control cells were transferred to SW620 cells washed with serum-free DMEM medium and incubated for 1 h at RT. Following incubation, SW620 cells were washed 6 times with washing buffer (0.5% BSA, 0.1% Tween 20 in serum-free DMEM) to remove phages that did not bind to SW620 cells. Phages bound to the surface of SW620 cells were collected by incubating with 100 μl of elution buffer (200 mM glycine-HCl buffer, pH 2.2, containing 1 mg/ml BSA and 0.1 mg/ml phenol red) for 10 min on ice. The eluate was neutralized with 19 μl of 1 M Tris-HCl buffer (pH 9.1) and concentrated to about 80 μl with a Centricon 30-kDa unit in a centrifuge tube. To recover SW620 cell-internalizing phages, cells were washed with DMEM medium and treated with 0.25% trypsin in phosphate buffered solution (PBS) buffer (containing 1 mM ethylene diamine tetraacetic acid, EDTA) for 5 min at 37 ℃. Cells were transferred to a centrifuge tube and centrifuged at 1000 rpm for 5 min. The supernatant were removed and cell pellets were dissolved in 200 μl of the lysis buffer (10 mM Tris-HCl buffer containing 2% sodium deoxycholate and 2 mM EDTA, pH 8.0). Output phages from the cell surface and internalizing fractions were tittered in *E. coli* (K91 BlueKan) host bacterial cells, and then amplified and purified for use as the input for the next round as described[3]. The detailed procedures of selection in the subsequent rounds were the same as described above but

without preliminary depletion of the library. The results of each round of selection were presented as the ratio of output to input phages. After 3 rounds of selection, different phage monoclones collected from the cellular surface and from internalizing fractions were randomly picked up and their genome region encoding guest-peptides were PCR amplified, detected by 1% agarose gel electrophoresis and sequenced to reveal the peptide sequences binding with the target cancer cells.

Isolation of Peptide-Fused PVIII Coat Protein from SW620 Cell-Specific Phage. The major coat proteins pVIII of the SW620 cell-specific landscape phage were extracted by phenol following a procedure reported with modification[4]. Briefly, 400 μl phage solution was mixed with equal volume of redistilled phenol (saturated with Tris-HCl, pH 8.0) and vigorously shaken for 8 min. The mixture was centrifuged at 3,000 g for 10 min. Due to different solubility of DNA and hydrophobic coat proteins, the phage DNA was dissolved in the upper Tris-HCl buffer and pVIII coat proteins were dissolved in the mixture of phenol and methanol (1:2). So, the upper aqueous layer containing phage DNA was aspirated and the phenol layer was re-extracted 4 times with equal volume of 10 mM Tris-HCl (pH 8.0). Subsquently, the phenol layer containing phage proteins was diluted with two volumes of methanol and vigorously shaken. The mixture in 1.2 ml was progressively dialyzed against 2 L of a mixture of methanol and Tris-HCl (1:1), a mixture of methanol and Tris-HCl (1:3), Tris-HCl buffer and deionized water for 12 h for each step. Finally, the solution was freeze-dried to power and the preparations of the dry phage coat protein were stored at -20 ℃.

Cytotoxicity Test of PMHNRs. The cytotoxicity of PMHNRs was evaluated by the test with

MTT, which can be reduced to purple formazan by the mitochondria in living cells. The cell viability was calculated based on the absorption of formazan. Stock solutions of PMHNRs (5 mg/ml) were diluted to different concentrations using serum-free DMEM medium. All three kinds of cells were seeded in 96-well culture plates and cultivated to the density of 4×10^3 cells/well in DMEM medium with 10% FBS overnight at 37 ℃. Then cells were washed twice with PBS buffer and treated with 50 μl PMHNRs of different concentration for 12 h. Then cells were incubated with 100 μl MTT (0.5 mg/ml) for 4 h. Followed by the supernatant was removed and replaced by 100 μl of DMSO. The formazan crystals were dissolved completely after the samples were agitated for 10 min. The optical absorption of purple formazan was measured by the Biotek plate reader at 510 nm. Cells treated with nanoparticles are defined as experimental groups and cells without nanoparticles as the control. The cell viability was calculated as the ratio of the optical density (OD) obtained from experimental groups to the OD value obtained from the control group.

Additional Figures:

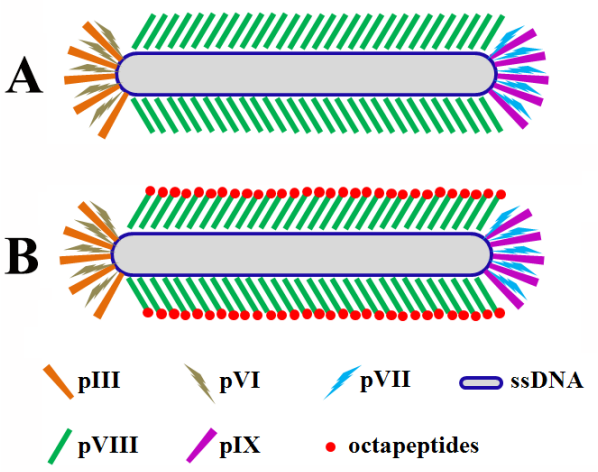

Figure S1. This scheme depicts the structure of fd phage (A) and f8/8 landscape phage (B). The single-stranded DNA (ssDNA) of fd phage is surrounded by 2,700 copies of pVIII major coat protein. Five copies of each pIII and pVI minor coat protein are at one tip, and pIII and pVI minor coat proteins are at the other tip. The f8/8 landscape phage was constructed based on the fd-tet phage, and the random octapeptides were displayed on all 4,000 copies of pVIII major coat protein.

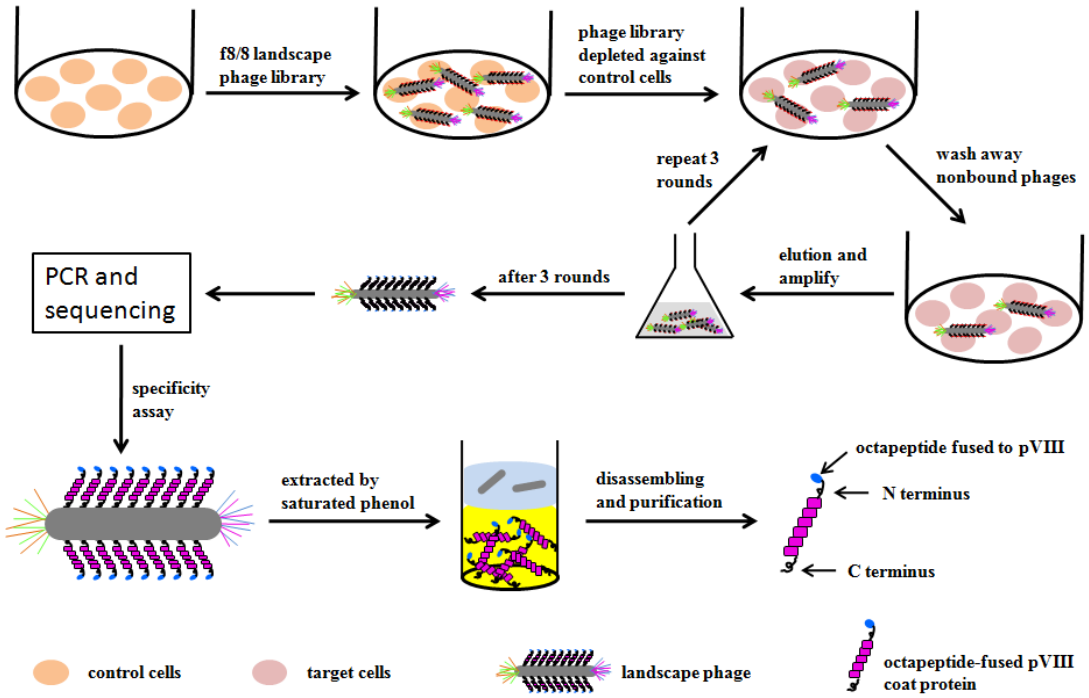

Figure S2. Schematic illustration of the selection of SW620 cell-binding phages and the isolation of pVIII coat proteins fused with SW620 cell-specific octapeptide. The f8/8 landscape phage library was first depleted against control cells, and then incubated with the target SW620 cells. Unbounded phages were washed away. Bounded phages were eluated and amplified as the input sublibrary for the next round. The depleted phage library was biopanned against SW620 cells for 3 times, and then the SW620 cell-binding phage monoclones were selected for sequencing. The SW620 cell-specific phage was finally identified by the specifictiy assay. The obtained SW620 cell-specific phage was amplified and the octapeptide-fused pVIII coat proteins were extracted by saturated phenol. Based on the different solubility, coat proteins were soluble in the phenol layer, while the ssDNA in the upper aqueous layer was discarded. There are about 4,000 copies of pVIII coat protein in a individual phage, so lots of octapeptide-fused pVIII coat proteins can be finally isolated and purified from the SW620 cell-specific phage.

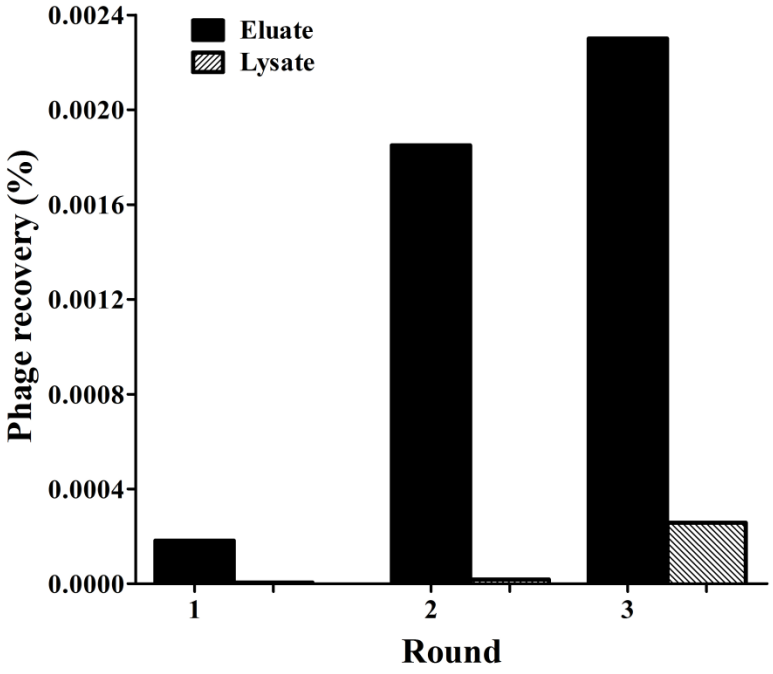

Figure S3. Phage recovery of biopanning. The recovery rate was calculated as the ratio of output phages to input phages.

Table S1. Amino acid sequences of selected phages

phage	Peptide sequences	Frequency
C1[a,b]	DDAGNRQP	4
C2[a,b]	DFAPVDGQ	5
C3[b]	DHTASWST	1

Phage clones were picked up in the final round of selection from different fraction of SW620 cells. The nucleotide acid of the displayed peptide in the *gVIII* was amplified by PCR, sequenced and translated to the corresponding amino acids sequences.

[a]eluate; [b]lysate.

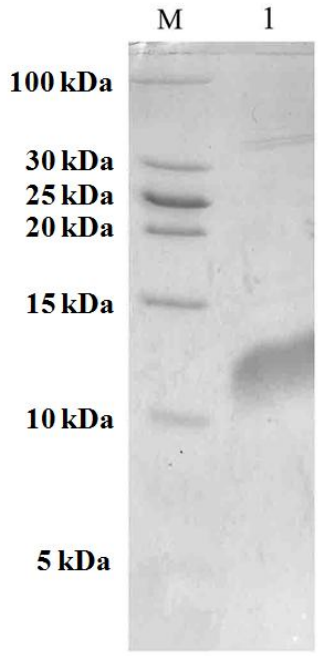

Figure S4. 16% Tricine-SDS-PAGE of purified coat proteins of C1 phage. Lane M, protein marker; Lane 1, isolated proteins.

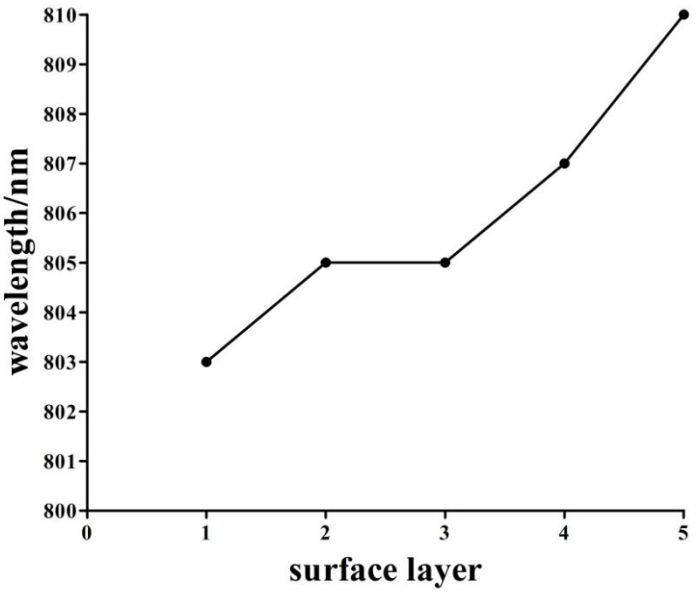

Figure S5. The absorption peak in the NIR region was systematically red-shifted as the surface layer of the Au@Ag heterogenous NR added during the self-assembling process. The surface layer 1 to 5 was corresponding to Au@Ag@PDDA NR, Au@Ag@PDDA@PSS NR, Au@Ag@PDDA@PSS@R6G NR, Au@Ag@PDDA@PSS@R6G@PSS NR and PMHNR, respectively.

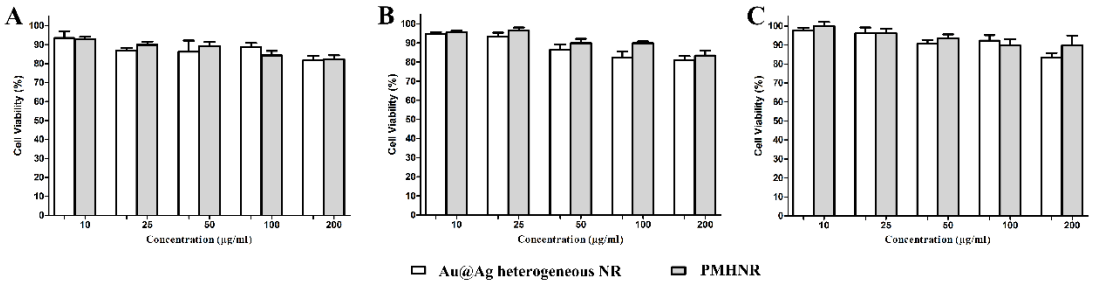

Figure S6. Cell viability was quantified by MTT assay. HepG2 (A), HEK293T (B) and SW620 cells (C) were incubated with different concentrations of Au@Ag heterogeneous NRs or PMHNRs.

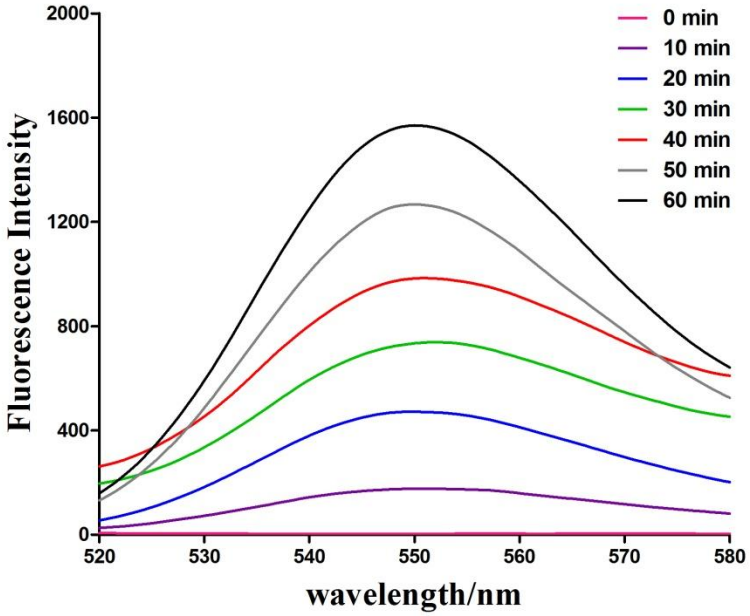

Figure S7. Fluorescence spectra of PMHNRs exposed to an 808 nm laser with the power of 100 mW in 5 min interval for different irradiation time.

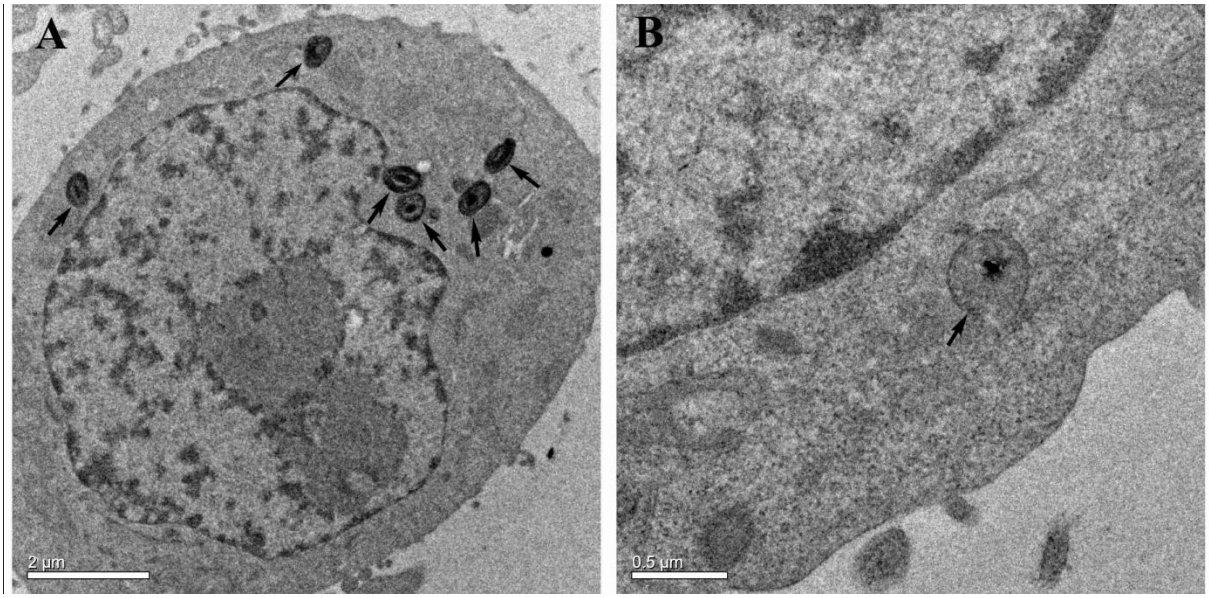

Figure S8. Ultrathin TEM images of SW620 cells incubated with PMHNR for 4 h. Arrows denote the PMHNR uptaken by vesicles.

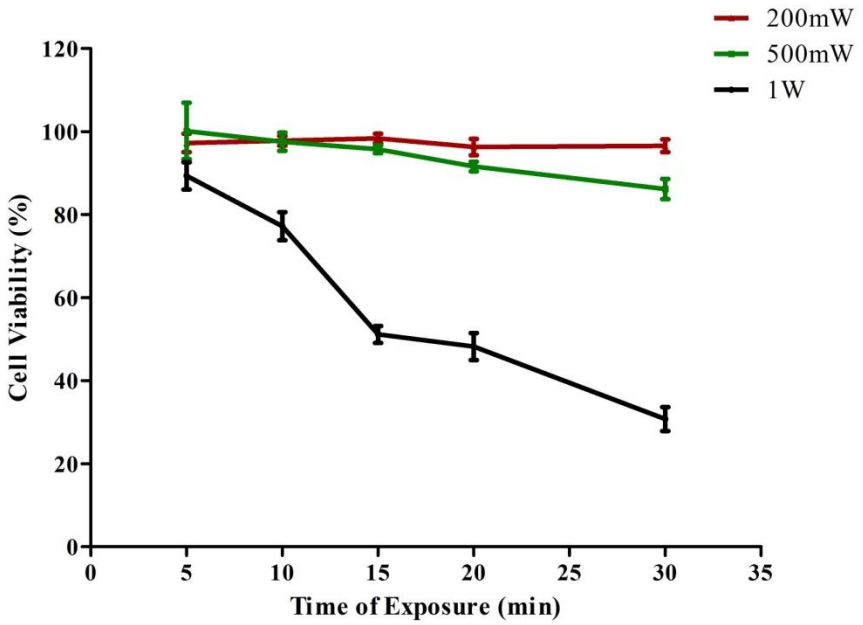

Figure S9. The cell viability of SW620 cells incubated with PMHNRs as a function of light intensity and exposure time.

References:

1. Abbineni, G., Modali, S., Safiejko-Mroczka, B., Petrenko, V.A. & Mao, C.B. Evolutionary selection of new Breast cancer cell-targeting peptides and phages with the cell-targeting peptides fully displayed on the major coat and their effects on actin dynamics during cell internalization. *Mol. Pharm.* **7**, 2369-2369 (2010).
2. Jayanna, P.K., Bedi, D., Deinnocentes, P., Bird, R.C. & Petrenko, V.A. Landscape phage ligands for PC3 prostate carcinoma cells. *Protein Eng. Des. Sel.* **23**, 423-430 (2010).
3. Fagbohun, O.A. et al. Landscape phages and their fusion proteins targeted to breast cancer cells. *Protein Eng. Des. Sel.* **25**, 271-283 (2012).
4. Knippers, R. & Hoffmann-Berling, H. A coat protein from bacteriophage Fd .I. hydrodynamic measurements and biological characterization. *J. Mol. Biol.* **21**, 281-292 (1966).