Optimization of Doxorubicin Conjugation to Bacteriophages Displaying Anti-MICA for Targeted Cancer Chemotherapy
การหาสภาวะที่เหมาะสมในการเชื่อมต่อยาต้านมะเร็งดอกโซรูบิซิน เข้ากับแบคทีริโอฟาจที่มีการแสดงออกของแอนติบอดีต่อ MICA เพื่อการรักษามะเร็งแบบพุ่งเป้าด้วยเคมีบำบัด

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ABSTRACT

Conventional chemotherapeutic drugs in cancer therapy are distributed nonspecifically where they affect both tumor and normal tissues lead to over dose and highly cytotoxic to normal cells. In this study, doxorubicin carrying phages targeting to major histocompatibility complex class I chain related A (MICA) antigens was developed to improve specificity and decrease dose of cancer drug. MICA is upregulated and expressed on almost originated epithelial cancer cells which can be targeted by anti-MICA antibodies on phage particles. Doxorubicin conjugation to phage coat proteins by using 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide EDC chemistry was optimized by varying pH and different ratios of EDC and doxorubicin. Flow cytometry was brought to analyse doxorubicin on phage particle The ability of DOX-conjugated phages (DOX-Phage) to bind MICA was evaluated by ELISA. The dialysis method was performed to determine the releasing of doxorubicin from phage particles for 72 hours. The optimal pH to conjugate doxorubicin to phage particles was pH 5 with the ratio of EDC and doxorubicin of 40:1. The result from flow cytometry shown that 98.8 % of phage particle could be conjugated to doxorubicin After conjugation, DOX-Phage remained the binding activity against MICA antigens as shown by ELISA. The releasing amount of doxorubicin was highest at pH 5 which was 43.53 μg. In conclusion, the application of EDC chemistry is effective to conjugate doxorubicin and major coat proteins of bacteriophages

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without destroying binding activity of MICA antibodies. The drug releasing result indicated that doxorubicin conjugated phages should be able to release the drug at the target sites when were endocytosed into the cells but not in the circulation. Thus, DOX-carrying bacteriophages targeting MICA has been developed but further characterization will be needed regarding MICA targeting cytotoxicity of cancer cells.

บทคัดย่อ

การรักษาโรคมะเร็งด้วยการใช้ยาต้านมะเร็งในการแพทย์แผนปัจจุบันยังมีผลข้างเคียงต่อเนื้อเยื่อปกติ และต้องใช้ยาในปริมาณมาก ในการศึกษาครั้งนี้ได้พัฒนารูปแบบตัวขนส่งยาที่จับอย่างจำเพาะกับเนื้อเยื่อมะเร็งที่เป็นเป้าหมายขึ้นเพื่อลดผลข้างเคียงที่เกิดกับเนื้อเยื่อปกติ ลดปริมาณการใช้ยาและเพิ่มประสิทธิภาพในการเข้าทำลายเซลล์เป้าหมาย โดยใช้แบคทีริโอฟาจเป็นตัวขนส่งยาไปยังเซลล์เป้าหมายที่มีการแสดงออกของ major histocompatibility complex class I chain related A (MICA) ซึ่งมีการแสดงออกเพิ่มขึ้นบนเซลล์มะเร็งโดยเฉพาะที่มีต้นกำเนิดมาจากเซลล์ผิว (epithelium) โดยติดยาต้านมะเร็งดอกโซรูบิซินเข้ากับโปรตีนของแบคทีริโอฟาจด้วยปฏิกิริยาทางเคมีโดยใช้สาร 1-Ethyl-3- [3-dimethylaminopropyl] -carbodiimide (EDC) โดยศึกษาสภาวะที่เหมาะสมด้วยการทำปฏิกิริยาในค่าพีเอชต่าง ๆ และปรับสัดส่วนของ EDC และยาที่ใช้ในการติด พบว่าสภาวะที่เหมาะสม คือ การทำปฏิกิริยาที่พีเอช 5 โดยใช้สัดส่วนของ EDC ต่อยา เท่ากับ 40:1 ผลโฟลไซเมทรีชี้ให้เห็นว่าแบคทีริโอฟาจสามารถติดยาดอกโซรูบิซินได้ร้อยละ 98.8 จากผลการทดสอบความสามารถในการจับกับแอนติเจน MICA โดยวิธี ELISA พบว่าแบคทีริโอฟาจที่ติดยายังสามารถจับกับแอนติเจน MICA ได้ การทดสอบการปลดปล่อยยาจากตัวแบคทีริโอฟาจโดยวิธี dialysis เป็นเวลา 72 ชั่วโมง พบว่ามีการปลดปล่อยยามากที่สุดที่ พีเอช 5 (43.53 μg) จากการศึกษาครั้งนี้สรุปว่าสามารถใช้สาร EDC ในการติดยาดอกโซรูบิซินเข้ากับโปรตีนของแบคทีริโอฟาจได้อย่างมีประสิทธิภาพและการติดยาดังกล่าวไม่ได้ทำลายความสามารถในการจับแอนติเจนของ anti-MICA นอกจากนี้ผลการศึกษาการปลดปล่อยยาชี้ให้เห็นว่าฟาจน่าจะสามารถปลดปล่อยยาได้ดีที่เซลล์เป้าหมายเมื่อเข้าสู่เซลล์ไม่ใช่ในกระแสเลือด ดังนั้นการศึกษานี้ได้พัฒนาการติดยาบนแบคทีริโอฟาจเพื่อใช้ในการในการฆ่าเซลล์มะเร็งแบบพุ่งเป้าต่อ เซลล์ที่มีการแสดงออกของ MICA อย่างไรก็ตามยังต้องมีการศึกษาเพิ่มเติม เพื่อประเมินการฆ่าเซลล์มะเร็งที่มีการแสดงออกของ MICA อย่างพุ่งเป้า

Key Word: Targeted chemotherapy, Bacteriophage, Doxorubicin, MICA
คำสำคัญ : เคมีบำบัดแบบพุ่งเป้า แบคทีรีโรฟาจ ดอกโซรูบิซิน MICA

Introduction

Monoclonal antibodies (mAbs) coupled to a highly toxic agents are becoming an interested platform to anticancer treatments. The combination of specificity of mAbs with enhanced tumor-killing power of toxic effector molecules lead to discrimination between the tumor and normal tissues, resulting in reduced administration of drug concentration and fewer toxic side effect than the treatment of only chemotherapeutic drugs.However, only

small amount of drugs or cytotoxic agents could be conjugated to antibodies leading to low treatment efficiency. Filamentous phages have been used as vehicles for drug delivery because a payload of drug could be conjugated [1-3] Thus, in combination with targeting ability, effective drug delivery could be reach at the target sites.

Major histocompatibility complex class I chain related A or MICA [4, 5] is a ligand to the activating natural killer group 2 member D (NKG2D) receptor [6], upon engaging leading to cytolysis of target cells. MICA is not normally expressed on normal cells but its expression was induced in cancer cells especially of epithelial origins [7]. Thus, MICA is a good candidate for targeting molecules in cancer chemotherapy. We have previously produced monoclonal antibodies against MICA [8] and phages displaying these antibodies with improved binding affinities have been produced [9]. These mateirals are essential for developing MICA targeting chemotherapy.

Thus, in this study, we aimed to optimize the conditions for conjugation of anti-cancer drug (doxorubicin) to major coat proteins (g8p) of filamentous bacteriophages carrying anti-MICA antibodies on minor coat proteins (g3p) attempting for cancer targeting. Then, the binding activities against MICA antigens of doxorubicin conjugated phages carrying anti-MIC antibodies (DOX-phage) were evaluated. The drug-carrying phages specific to MICA antigens might be an effective model that can be further improved or used for cancer therapeutic applications.

Methodology

1. Materials

1.1 Phages displaying anti-MICA antibodies

Two phages displaying anti-MICA antibodies, WW2G8 and WW9B8 [9] which display monoclonal anti-MICA derived from WW2G8 and WW9B8 monoclones, respectively [8]. These phages were used for conjugation with anticancer drug, doxorubicin.

1.2 Soluble MICA antigen (sMICA)

Soluble MICA antigens (sMICA) were produced from an eukariotic expression clone carrying a full length coding sequence of the *MICA*009* gene with inserted stop codon before the transmembrane portion to produce a soluble formas described previously [8]

2. Methods

2.1 Conjugation of doxorubicin to M13 phages using the EDC chemistry

The application of 1-Ethyl-3- [3-dimethylaminopropyl]carbodiimide (EDC) chemistry was performed to conjugate doxorubicin to the phage major coat protein g8p contains 3 carboxylic animo acids (Glu2, Asp4 and Asp5), in reactions of pH 4-6 [1-3]. The optimal concentration and ratio of carboxylate groups on phage particles: amine groups on doxorubicin molecules : concentration of EDC were calculated using at ratio as 1 mole COOH : 1 mole amine : 4 moles EDC as follows: phage particles have 3 carboxylate groups on g8p coat protein which about 2700 copies/phage, thus

Phage 1 x 10^{12} pfu = 8,100x10^{12} COOH molecules

= 8.1x10^{15} COOH molecules

= 100 ul of phage 10^{13} pfu

(1 phage particle ≈ 8,100 COOH molecules)

DOX.HCl (MW=580) = amine group

Using doxorubicin as excess ≈ 10^{17} molecules

1 mole = 6.02x10^{23} molecules

DOX 8.1x10^{17} molecules = 8.1x10^{17}/6.02x10^{23}

= 1.34x10^{-6} mole

gram = [1.34x10^{-6}]x580

≈ 770 μg

Stock 5 mg/ml = 154 μl

EDC (MW=191.7)

EDC 1 mole : DOX.HCl 1 mole = 1.34x10^{-6} mole

gram = 1.34x10^{-6}x191.7

= 2.57x10^{-4}

≈ 257 μg

EDC 4 mole: DOX.HCl 1 mole = 1028 μg (1x)

EDC 40 mole: DOX.HCl 1 mole = 10280 μg (10x)

The reagents and conditions for doxorubicin conjugation to phages are phages (1 x 10^{12} pfu), doxorubicin 770 μg, 0.1 M sodium citrate buffer pH 4, 5 or 6, 0.75 M NaCl 345 μl, EDC aqueous solution 1,028 μg and 10,280 μg

In the experiment, doxorubicin was mixed with phages and 0.1 M sodium citrate buffer pH 4, 5 or 6, and 0.75 NaCl into a 1.5 ml tube. Then, the EDC solution was added by repeated 4 times at 0, 30, 60, and 90 mins at room temperature with gentle stirring (10 rpm) for 2 hours. The dialysis step was performed to separate the conjugated phages from the reaction solution. The two dialysis steps against 1,000 ml of steriled 0.3 M NaCl each for 24 hours. Then, the Dox-phages were precipitated by adding 4% PEG/3% NaCl on ice for 1 hour, and were centrifuged at 9000 rpm, 4°C, for 20 mins. The supernatant was discarded and the pellet was resuspended with 1,000 μl of PBS pH 7 for the next experiments [14]

2.2 Evaluation of MICA binding activities of doxorubicin conjugated phages

The evaluation of MICA binding activies was performed according to the previous study [9]. In brief, the indirect ELISA was performed to evaluate the

binding activities of Dox-phage against MICA after conjugation. The sMICA antigens were coated on each well of micro plate with 5 μg in carbonate buffer pH 9.6 and incubated at 4°C overnight. The solution was discarded and then washed with PBST 3 times, followed by adding blocking solution, 5% skim milk and incubated at room temperature for 2 hours. The blocking solution was discarded and washed with PBST 3 times followed by adding the 1011 pfu of Dox-phage incubated at room temperature for 1 hour and then discarded. For this experiment, protein extraction of mocked transfection was used as a negative control. After 3 times washing with PBST, the anti-M13 conjugated HRP (GE Healthcare, Germany) (diluted 1:15000 in blocking solution) were added to each well and incubated at room temperature for 1 hour. The antibody solution was washed 3 times with PBST followed by adding TMB substrate (Kirkegaard & Perry Laboratories, Inc,USA) per well containing substrate A and substrate B at 1:1 (v/v) ratio, and then incubated for 20 mins in the dark at room temperature. After the blue color was developed, then the stop reaction solution (2 N H_2PO_4) was added to each well, the yellow color was developed. The absorbance (optical density; O.D.) at 450 nm was measured by Sunrise ELISA plate reader (Tecan, Austria) with Magellen7 software (Tecan, Austria) [9].

2.3 Detection of doxorubicin conjugation on bacteriophage coat protein

The 2 sample, naked phage and Dox-phage approximately10^7 phage particles were stained with 2 μg/ml of anti-M13-FITC (antibodies-online, Germany) then incubated on ice 1 hour. Doxorubicin on phage coat proteins has excitation at 488 nm with emission integrated above 530 nm (could detect as PerCP chanel)and fluorescent signal of anti-M13-FITC were analyzed by using BD FACSCanto II Flow cytometer (BD, USA) [10-12]. The all samples data were analyzed by BD FACSDiva software.

2.4 Releasing of doxorubicin from phage particles *in vitro*

In vitro drug releasing profile of doxorubicin conjugated phages was determined by the dialysis method [13,14]. The doxorubicin conjugated phages of 1x 10^{13} particles were precipitated with 4% PEG/3% NaCl (v/v) on ice for 30 mins and centrifuged at 9,000 rpm, 4°C for 20 mins, and then the supernatant was removed. Thephages were resuspended with 1 ml of PBS pH 4, 5 and 7.4 and each loaded into a dialysis tube (M.W. 7,000), followed by submerged into 10 ml PBS pH 4, 5 and 7.4 in 50 ml tubes, respectively. Then the tubes were shaken continuously at 37°C, 100 rpm and 100 ul of sample outside of dialysis membrane were withdrawn and replaced by an equivalent amount of PBS buffer at 0, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144, 168, 194 and 218 hour time intervals. The standard curve of doxorubicin concentration was obtained from the measuring of dilution series of doxorubicin concentrations ranged from 0, 1, 2, 5, 10, 25, 50 and 100 μg/0.1 ml. The amounts of released drug and doxorubicin standards were analyzed with UV-VIS spectrophotometer (Shimadzu,

Japan) at 485 nm [14]. The accumulated amounts of doxorubicin released at different time points after compared to the standard curve were calculated according to the followed equation [15].

$$W_n = V_T \times C_n + V_s \times {}^{n}\sum_{n-1} C_{n-1}$$

Where, W_n = accumulated amount of drug released (μg)

V_T = total volume of dissolution medium (ml)

V_s = sampling volume (ml)

C_n = calculated concentration of samples n compared to standard curve (ug)

Results

1. Conjugation of doxorubicin on phage g8p coat proteins using the EDCchemistry

1.1 The mild acidic pH for conjugation of doxorubicin and phage coat proteins

EDC can mediate the coupling between substances containing carboxylates and amines with the highest yield at pH 4.0-6.0 [2]. The conjugation reactions included phage particles, doxorubicin (solution), 0.75 M NaCl, EDC aqueous solution and 0.1 M sodium citrate buffer. The 0.1 M sodium citrate buffer was used as acidic pH buffer with varied pH at 4, 5 and 6. After conjugation and dialysis, the solution reactions of each pH were precipitated and the supernatants and pellets were collected for testing of the specific binding to sMICA antigens. There were only samples from the reaction at pH 5 (Figures 1) that binding activities to sMICA remained intact, particularly in the pellet part (Table 1). Thus, the optimal pH for conjugation of doxorubicin to the phage particles with maintaining reactive activities against MICA is pH 5.

1.2 Concentration of EDC for conjugation of doxorubicin and phage coat proteins

The EDC chemistry is the most favored carbodiimide and frequently used as crosslinking agent for biochemical conjugations. For the conjugation of doxorubicin and phages, the EDC concentration of 1x (1,028 μg) and 10x (10,280 μg) were tested. The reaction solution using 10x EDC showed deeper red color of doxorubicin than the reaction of 1x EDC after dialysis. The results suggested that doxorubicin could be conjugated to phage coat protein better when using 10x EDC (Figure 2).

2. Detection of doxorubicin conjugation on bacteriophage coat protein

Doxorubicin could be conjugated to phage particles indicated by drug auto-fluorescence (PerCP) and FITC. The percentages of conjugation of doxorubicin on phage particles were evaluated. Most of phages (98.8%) were double positively stained (drug auto-fluorescence (PerCP) and FITC) by flow cytometry (Figure 3)

3. Releasing of doxorubicin from phage particles *in vitro*

The releasing and accumulation of

doxorubicin from conjugated phages was conducted with a horizontal shaker and submerged dialysis bag that contained conjugated phages in PBS at different pH 4, 5 and 7 at 37°C. The standard curve of doxorubicin concentrations was generated by OD measurement at 485 nm (Figure 4). Doxorubicin was released from phages and accumulated in solution buffer at acidic pH (4 and 5) more than the neutral condition (pH 7) and at pH 5 the doxorubicin was released at the highest amount followed by those of pH 4 and pH 7 which was 43.53, 34.38 and 26.47 μg, respectively (Figure 5). The doxorubicin was released at a higher rate from 0 hour to 72 hours at pH5 and pH7 and quite stable. At pH4, the higher rate was from 0 hour to 168 hours and quite stable at 168 hours (Figure 5).

Discussion and conclusion

Conjugation of doxorubicin anticancer drug to phage g8p coat proteins was performed by using EDC chemistry at mild acidic pH condition. The ratio of carboxylate groups on phage coat proteins (glu2, asp4 and asp5), amine groups on doxorubicin molecules and concentration of EDC were calculated as caboxylate 1 mole: excess amine 1 mole: EDC 4 mole as previous study [1, 2, 16]. The pH of 0.1 M sodium citrate buffer and concentration of EDC were also optimized. For pH buffer, mild acidic pH ranges, pH 4, 5 and 6 were chosen as candidate conditions. After conjugation, the excess doxorubicin molecules that were not conjugated to phage coat proteins were removed by the dialysis method against 0.75 M NaCl. It was found that the reaction condition at pH 5 and 6 have darker solution. However, this factor was not enough for choosing the optimal pH for conjugation reaction. Therefore, the binding activity to targeted sMICA antigens was evaluated, by precipitated with 4% PEG/ 3%NaCl (v/v) then collected the supernatant and pellet for testing binding activity by the ELISA assay. It was found that doxorubicin conjugated phages at pH 5 could bind specifically to sMICA better than others condition, with almost of phage particles contained in the pellet part. We also optimized the concentration of EDC chemistry which were1x (4 mole EDC per doxorubicin 1 mole) and 10x (40 mole EDC per doxorubicin 1 mole). Adding of EDC into the reactions was divided into 4 time points (0, 30, 60, 90 mins) with a total reaction time of 2 hours. It was found that the solution of reaction containing 10x EDC had deeper red color than using 1x EDC, which might indicate that the higher amount of doxorubicin molecules conjugated to phage coat proteins. From the results above, we decided to use the 0.1 M sodium citrate buffer pH 5 and 10x EDC for the conjugation reaction consistent with other studies [1, 2, 17, 18].

After conjugation reaction, detection of doxorubicin molecules on phage particles was performed by flow cytometry assay was used to detect the signal of doxorubicin molecules due to auto fluorescent property which can be excited and emission. The

percentage of phage particles conjugated with doxorubicin was approximately 98.8%.

The release of doxorubicin from phage particles by the dialysis method was performed. At acidic pH, the drug molecules were released faster than at neutral pH. The releasing rate was high from 0-72 hours and then it appeared more stable. The accumulated amount of doxorubicin was highest at pH 5 which was 43.53 μg. This indicated that doxorubicin conjugated phages should be able to release the drug at the target sites not in the circulation and the reagent should be kept at neutral pH. Conjugation of doxorubicin to phage g8p coat proteins by using EDC chemistry is one approach to construct the targeted therapeutic nanoparticles with no affect to binding moiety of phage particles. The process of conjugation is easy by adding EDC to the reaction at mild acidic pH. However, amide bond between drugs and phage coat proteins is also sensitive to mild acidic pH as seen in releasing experiment. This is an advantage of these particles when reaching to tumor site with acidic pH environment and was internalized into cells by endocytosis.

In conclusion, this study demonstrated the condition for conjugation of doxorubicin on phage particles displaying anti-MICA without affecting their MICA binding activities. The highest releasing of doxorubicin at pH 5 indicated that Dox-phage able to release the drug at the target sites not in the circulation. The linkages between conjugated doxorubicin molecules and phage proteins were sensitive to acidic pH. This targeting platform required further development for use as drug targeted carriers.

References

1. Bar H, Yacoby I, Benhar I. Killing cancer cells by targeted drug-carrying phage nanomedicines. BMC Biotechnol 2008; 8: 37-44.
2. Yacoby I, Shamis M, Bar H, Shabat D, Benhar I. Targeting antibacterial agents by using drug-carrying filamentous bacteriophages. Antimicrob Agents Chemother 2006; 50: 2087-97.
3. Yacoby I, Bar H, Benhar I. Targeted drug-carrying bacteriophages as antibacterial nanomedicines. Antimicrob Agents Chemother 2007; 51: 2156-63.
4. Leelayuwat C, Townend DC, Degli Esposti MA, Abraham LJ. Dawkins RL. A new polymorphic and multicopy MHC gene family related to nonmammalian class I. Immunogenetics 1994; 40: 339-51.
5. Bahram S, Bresnahan M, Geraghty DE, Spies T. A second lineage of mammalian major histocompatibility complex class I genes. Proc Natl Acad Sci U S A. 1994; 91:6259-63.
6. Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, Spies T. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science. 1999; 285: 727-9.

7. Groh V, Rhinehart R, Secrist H, Bauer S, Grabstein KH, Spies T. Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB. Proc Natl Acad Sci U S A. 1999; 96: 6879-84.
8. Wongsena W, Sconocchia G, Cho HS, Chang CC, Wang X, Klumkrathok K, et al. Production and characterization of monoclonal antibodies against MHC class I chain related gene A (MICA). Tissue Antigens 2008; 72: 431-40.
9. Phumyen A, Jumnainsong A, Leelayuwat C. Improved binding activity of antibodies against major histocompatibility complex class I chain-related gene A by phage display technology for cancer-targeted therapy. *J Biomed Biotechnol.* 2012; 2012: 1-8.
10. Krishan A. Flow cytometric monitoring of drug resistance in human tumor cells. Methods Cell Sci 2002; 24: 55-60.
11. Krishan A, Sauerteig A, Wellham LL. Flow cytometric studies on modulation of cellular adriamycin retention by phenothiazines. Cancer Res 1985; 45: 1046-51.
12. Krishan A. Monitoring of cellular resistance to cancer chemotherapy: drug retention and efflux. Methods Cell Biol 2001; 64: 193-209.
13. Jayant S, Khandare JJ, Wang Y, Singh AP, Vorsa N, Minko T. Targeted sialic acid-doxorubicin prodrugs for intracellular delivery and cancer treatment. Pharm Res 2007; 24: 2120-30.
14. Cai L, Niu G, Hu Z, Jin W, Wang J, Sun L. Polybutylcyanoacrylate magnetic nanoparticles as carriers of adriamycin. J Drug Target 2009; 17: 200-6.
15. Lin WJ, Wu TL. Modification of the initial release of a highly water-soluble drug from ethyl cellulose microspheres. J Microencapsul 1999; 16: 639-46.
16. King HD, Yurgaitis D, Willner D, Firestone RA, Yang MB, Lasch SJ, et al. Monoclonal antibody conjugates of doxorubicin prepared with branched linkers: A novel method for increasing the potency of doxorubicin immunoconjugates. Bioconjug Chem 1999; 10: 279-88.
17. Tomlinson R, Heller J, Brocchini S, Duncan R. Polyacetal-doxorubicin conjugates designed for pH-dependent degradation. Bioconjug Chem 2003; 14: 1096-106.
18. King HD, Dubowchik GM, Mastalerz H, Willner D, Hofstead SJ, Firestone RA, et al. Monoclonal antibody conjugates of doxorubicin prepared with branched peptide linkers: inhibition of aggregation by methoxytriethyleneglycol chains. J Med Chem 2002; 45: 4336-43.

Table 1 MICA binding activities of doxorubicin conjugated phages at different pH conditions.

Dox-phage at pH conditions	OD at 450 nm
pH 4 supernatant	0.056
pH 4 pellet	0.068
pH 5 supernatant	0.291
pH 5 pellet	1.498
pH 6 supernatant	0.074
pH 6 pellet	0.066
Negative control (mocked-protein)	0.056

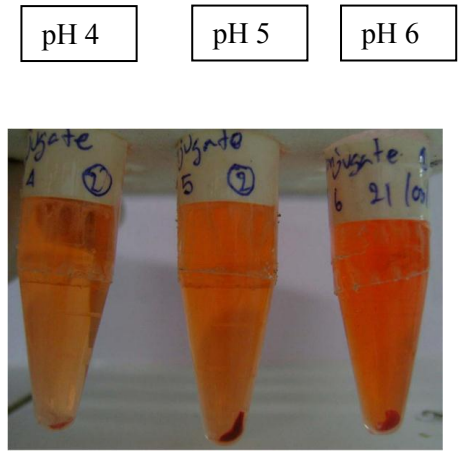

Figure 1 Conjugation of doxorubicin to phage coat protein by using EDC in different pH conditions. pH4, pH 5 and pH 6 represent the conjugations of doxorubicin at each pH of 0.1 M sodium citrate buffer. After conjugation,dialysis, and centrifugation at 14,000 rpm at 4°C for 20 mins, a red pellet at the buttom of the tube can be observed. It should be noted that at pH 5 a bigger pellet is evident.

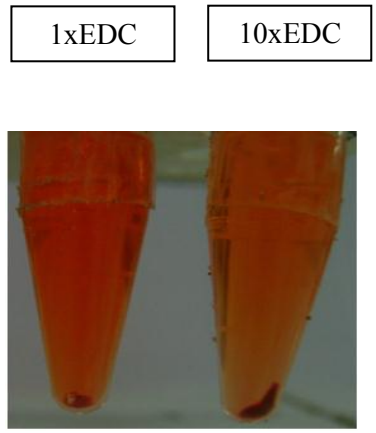

Figure 2 Conjugation of doxorubicin to phage particles by using different concentrations of EDC. The concentrations of EDC with 1x and 10x were evaluated. The red precipitation of 10x EDC was darker than using 1x EDC in the reaction.

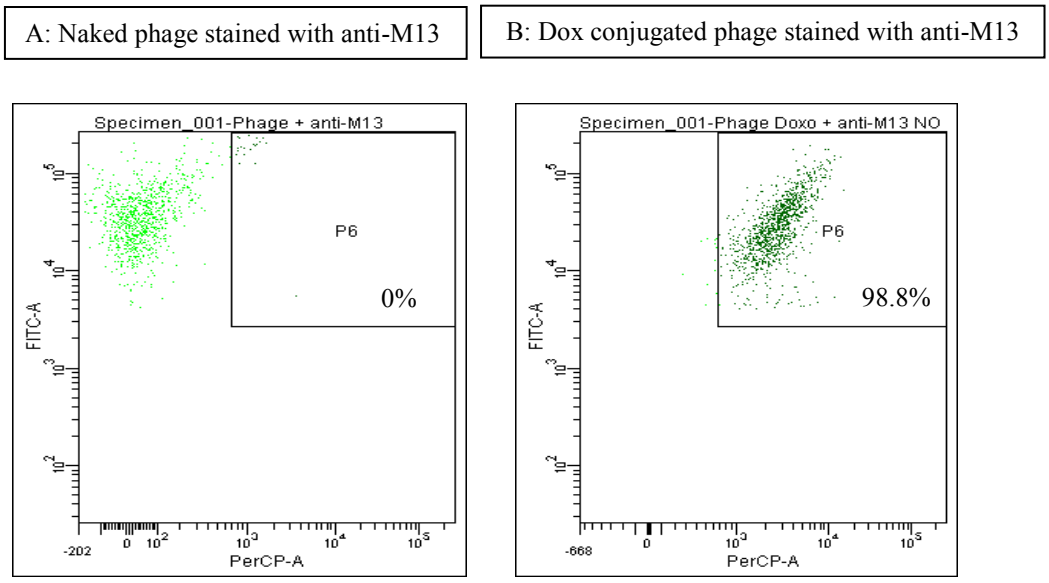

Figure 3 Detection of doxorubicin conjugation on bacteriophage coat protein. A: naked phages stained with FITC conjugated anti-M13, B: double positively stained (drug auto-fluorescence (PerCP) and FITC) indicated that doxorubicin could be conjugated on phage coat proteins.

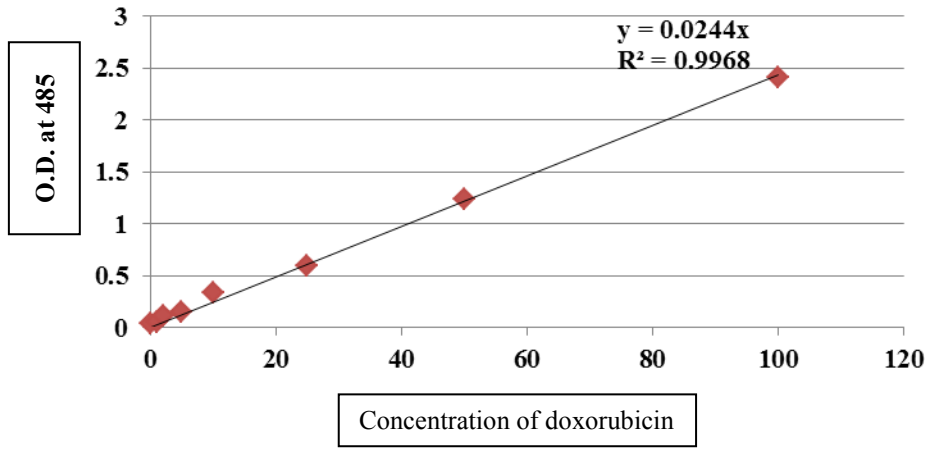

Figure 4 The standard curve of doxorubicin concentrations. The concentrations of doxorubicin were from 0, 1, 2, 5, 10, 25, 50 and 100 μg whose the absorbance were measured by UV-vis spectrophotometer at 485 nm. This standard curve was used to estimate the doxorubicin released from conjugated phage particles.

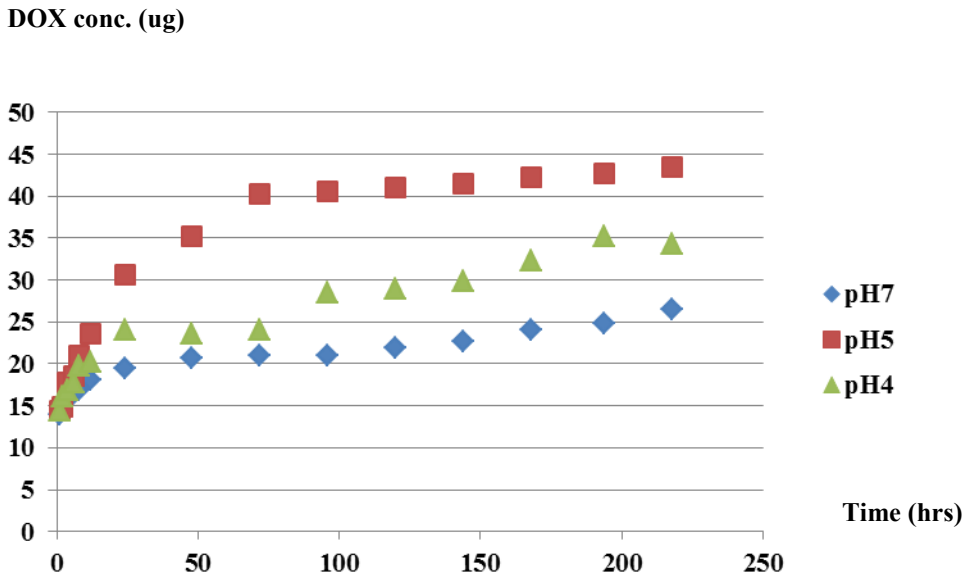

Figure 5 The accumulation of released doxorubicin from phage particles at different pH conditions. Doxorubicin was released from phages and accumulated in solution buffer at acidic pH (4 and 5) higher than in neutral condition (pH 7). At pH 5, the doxorubicin was released at the highest amount. The doxorubicin was accumulated at a higher rate in the initial steps of experiment from 0 to 72 hours.