รายงานวิจัยฉบับสมบูรณ์

โครงการ พาหะนำส่งระดับนาโนจากแบคทิริโอฟาจที่ถูกดัดแปลงเพื่อการนำส่งอย่างมีเป้าหมายของดีเอ็นเอวัคซีนชนิดกิน

โดย ดร. ธีรพงศ์ ยะทา

มิถุนายน 2560

สัญญาเลขที่ TRG5880185 (P-15-51267)

รายงานวิจัยฉบับสมบูรณ์

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ศูนย์นาโนเทคโนโลยีแห่งชาติ

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและ
ศูนย์นาโนเทคโนโลยีแห่งชาติ

Abstract

Project Code : TRG5880185 (P-15-51267)

Project Title : Engineered M13 Bacteriophage Nanocarriers for Targeted Oral Delivery of DNA Vaccine

Investigator : Dr. Teerapong Yata, National Nanotechnology Center

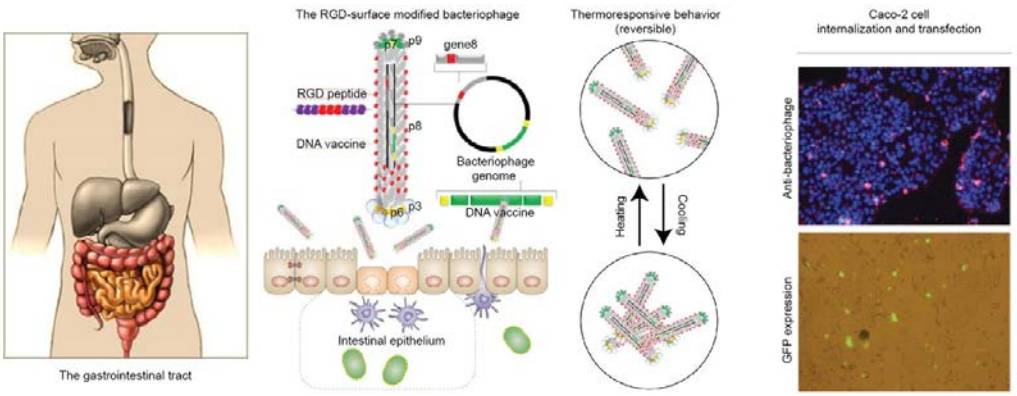
E-mail Address : teerapong@nanotec.or.th

Project Period : 2 years (1 July 2015 to 30 June 2017)

The use of the gastrointestinal tract as a site for the local delivery of DNA vaccines is an exciting prospect. In order to obtain an effective, cheap, safe, and easy-to-administer vector, we developed a new generation of filamentous bacteriophage. This particular bacteriophage was genetically engineered to display integrin receptor-binding peptides and carry a mammalian DNA cassette. One unanticipated observation is the thermoresponsive behavior of engineered bacteriophage. This finding has led us to simplify the isolation method to purify bacteriophage particles from cell culture supernatant. *In vitro* model of the human intestinal follicle-associated epithelium demonstrated that bacteriophage particles were able to cross the human intestinal barrier. Our results also showed that in contrast to non-surface modified, the RGD-modified bacteriophage was successfully used to deliver a transgene to mammalian cells. We also confirmed the adjuvant's ability of the engineered bacteriophage to induce nitric oxide production by macrophages. In conclusion, our study demonstrated the possibility of using bacteriophage for gene delivery to target cells within the gastrointestinal tract.

Keywords: bacteriophage, thermoresponsive, DNA vaccine, gastrointestinal tract, nanocarrier

Graphical abstract



บทคัดย่อ (ภาษาไทย)

รหัสโครงการ : TRG5880185 (P-15-51267)

โครงการ : พาหะนำส่งระดับนาโนจากแบคทีริโอฟาจที่ถูกดัดแปลงเพื่อการนำส่งอย่างมีเป้าหมายของดีเอ็นเอวัคซีนชนิดกิน

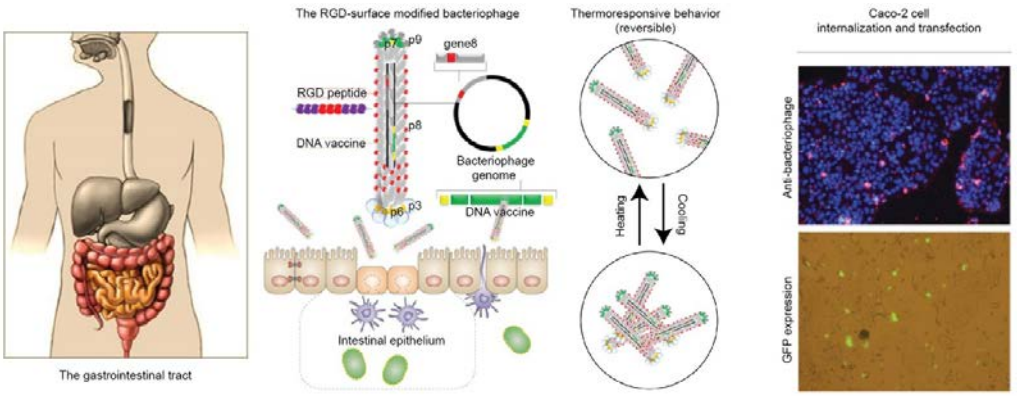
ผู้วิจัย/สังกัด : ดร.ธีรพงศ์ ยะทา/ศูนย์นาโนเทคโนโลยีแห่งชาติ

อีเมลล์ : teerapong@nanotec.or.th

ระยะเวลาโครงการ : 2 ปี (1 กรกฎาคม 2558 ถึง 30 มิถุนายน 2560)

การนำส่งดีเอ็นเอวัคซีนผ่านทางเดินในระบบอาหารโดยการกิน เป็นวิธีการที่น่าสนใจเพื่อให้บรรลุวัตถุประสงค์ดังกล่าวจึงมีความจำเป็นอย่างยิ่งในการพัฒนาระบบนำส่งที่มีประสิทธิภาพ ราคาถูก และปลอดภัย ด้วยเหตุนี้ผู้วิจัยจึงได้พัฒนาพาหะนำส่งระดับนาโนจากแบคทีริโอฟาจที่ถูกดัดแปลงให้มีความเหมาะสมในการทำหน้าที่นำส่งวัคซีนผ่านทางการกิน โดยแบคทีริโอฟาจได้ถูกดัดแปลงลักษณะทางพันธุกรรมให้มีการสร้างเปปไทด์อาร์จีดีบนผิวเปลือกหุ้มของแบคทิริโอฟาจที่ทำหน้าที่ช่วยในการจับกับโปรตีนอินทิกรินบนผิวเซลล์และการเข้าไปในเซลล์เป้าหมายในระบบทางเดินอาหาร นอกจากนี้แบคทิริโอฟาจยังถูกดัดแปลงให้สามารถบรรจุและนำส่งชิ้นส่วนดีเอ็นเอที่จะใช้เป็นส่วนของดีเอ็นเอวัคซีน หลังการดัดแปลงดังกล่าว เราพบคุณสมบัติพิเศษที่ไม่ได้คาดหมายของแบคทิริโอฟาจที่ถูกดัดแปลง คือ การตกตะกอนได้เองของอนุภาคเมื่อลดอุณหภูมิให้เย็นลง ด้วยพฤติกรรมพิเศษนี้ทำให้ผู้วิจัยสามารถแยกอนุภาคของแบคทิริโอฟาจได้โดยง่ายจากน้ำเลี้ยงเชื้อเจ้าบ้านที่ใช้ในการผลิต ซึ่งเดิมต้องเติมสารเคมีช่วยในการตกตะกอน นอกจากนี้ยังพบว่าแบคทิริโอฟาจสามารถผ่านเยื่อบุทางเดินอาหารจากการศึกษาความสามารถในการซึมผ่านด้วยแบบจำลองเซลล์ในระบบทางเดินอาหาร ที่สำคัญคือแบคทิริโอฟาจที่ถูกดัดแปลงสามารถส่งถ่ายยีนเข้าไปในเซลล์ของสัตว์เลี้ยงลูกด้วยนมได้สำเร็จเมื่อเทียบกับแบคทิริโอฟาจไม่ถูกดัดแปลงที่ไม่สามารถทำได้ เรายังพบว่าแบคทิริโอฟาจมีความสามารถในการกระตุ้นเซลล์ในระบบภูมิคุ้มกัน จึงมีความเป็นไปได้ที่จะใช้อนุภาคของแบคทิริโอเฟจที่ถูกดัดแปลงดังกล่าวในการนำส่งดีเอ็นเอวัคซีนเข้าสู่เซลล์เป้าหมายในระบบทางเดินอาหาร

คำสำคัญ: แบคทิริโอฟาจ; การตอบสนองต่ออุณหภูมิ; ดีเอ็นเอวัคซีน; ระบบทางเดินอาหาร.



Engineered Bacteriophage Nanocarrier for Gene Therapy in Gastrointestinal Diseases

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and Teerapong Yata*[1]

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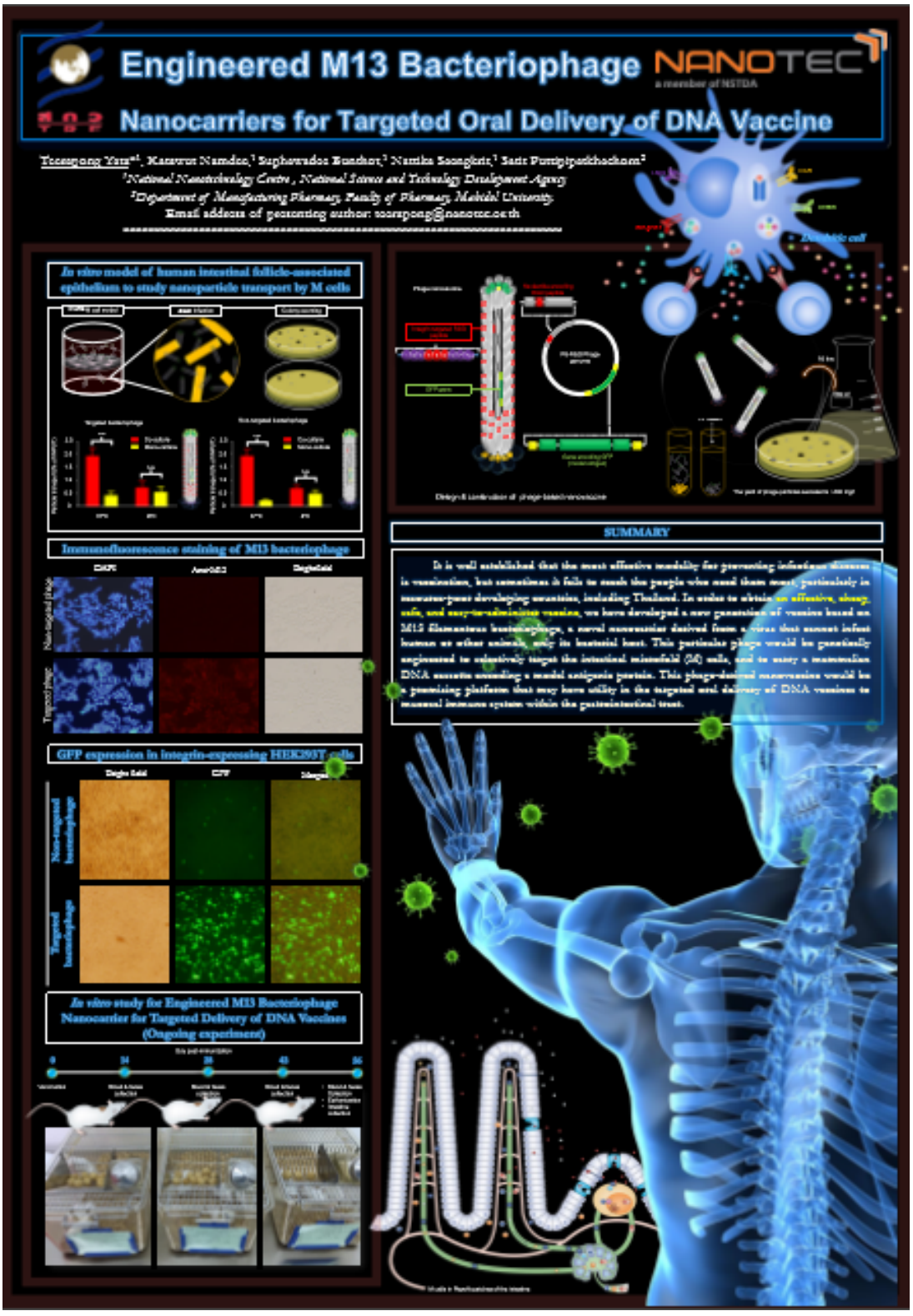
The use of the gastrointestinal (GI) tract as a site for the local delivery of gene therapeutics or vaccines is an exciting prospect. A number of GI diseases that might be treatable or preventable by gene therapy include inflammatory bowel diseases (IBD), intestinal cancers, and GI infections.[1] In order to obtain an effective, cheap, safe, and easy-to-administer vector, we developed a new generation of filamentous bacteriophage, a novel class of nanomaterial derived from a virus that cannot infect human or other animals, only its bacterial host. This particular bacteriophage was genetically engineered to display integrin receptor-binding (RGD) peptides and carry a mammalian DNA cassette. Similar physical and morphological properties were observed in both surface-modified and non-surface modified bacteriophage as confirmed by zeta potential and transmission electron microscopy. *In vitro* model of the human intestinal follicle-associated epithelium demonstrated that bacteriophage particles were able to cross the human intestinal barrier. Moreover, engineered bacteriophage showed a high resistance towards degradation under various conditions. Our results also showed that in contrast to non-surface modified, the RGD-modified bacteriophage was successfully used to deliver a model gene to mammalian cells in both two-dimensional (2D) and three-dimensional (3D) cell culture systems. In conclusion, our study demonstrated potential use of bacteriophage as a scaffold for constructing nanocarriers that may have utility in the oral delivery of gene therapeutics or vaccines to target cells within the gastrointestinal tract.

References:

1. Martin J. O'Neill, Ludovic Bourre, Silvia Melgar and Caitriona M. O'Driscoll. *Drug Discov. Today*, **2015**, 16, 203-218.


Engineered M13 Bacteriophage
NANOTEC
a member of NSTDA
Nanocarriers for Targeted Oral Delivery of DNA Vaccine
Teerapong Yata[1], Kasorn Namdee,[1] Supheradee Bundhoo,[1] Nattika Saengkrit,[1] Sarit Pimpipatchachaem[2]
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In vitro model of human intestinal follicle-associated epithelium to study nanoparticle transport by M cells
Immunofluorescence staining of M13 bacteriophage
DAPI
Alexa488
Bright field
Non-targeted phage
Targeted phage
GFP expression in integrin-expressing HEK293T cells
Bright field
GFP
Merged
Non-targeted bacteriophage
Targeted bacteriophage
In vitro study for Engineered M13 Bacteriophage Nanocarrier for Targeted Delivery of DNA Vaccines (Ongoing experiment)
SUMMARY
It is well established that the most effective modality for preventing infectious diseases is vaccination, but sometimes it fails to reach the people who need them most, particularly in resource-poor developing countries, including Thailand. In order to obtain an effective, cheap, safe, and easy-to-administer vaccine, we have developed a new generation of vaccine based on M13 filamentous bacteriophage, a novel nanomaterial derived from a virus that cannot infect human or other animals, only its bacterial host. This particular phage would be genetically engineered to selectively target the intestinal microfold (M) cells, and to carry a mammalian DNA cassette encoding a model antigenic protein. This phage-derived nanovaccine would be a promising platform that may have utility in the targeted oral delivery of DNA vaccines to mucosal immune system within the gastrointestinal tract.

Manuscript/Current status: Under Review; Cell Press;Molecular Therapy Nucleic Acids IF:6.3

Title

Thermoresponsive bacteriophage-based nanocarriers designed for oral delivery of DNA vaccine

Authors

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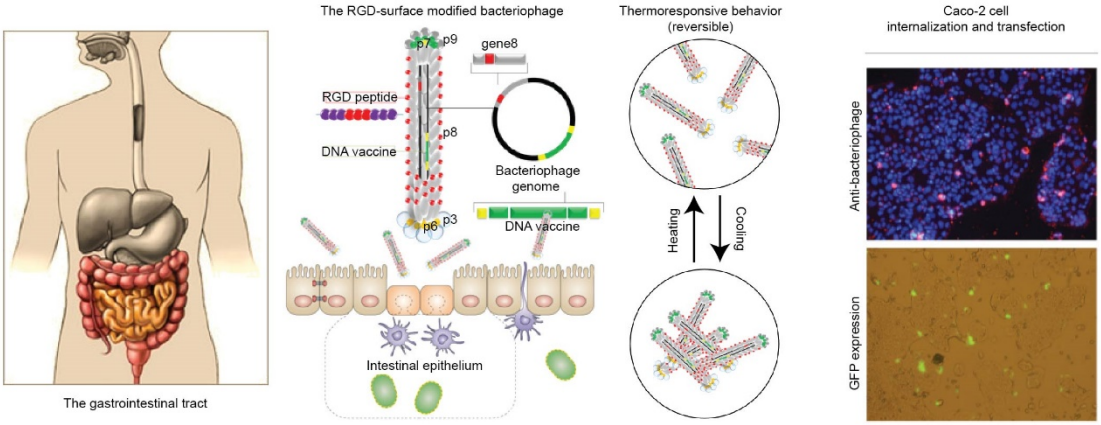
Abstract

The use of the gastrointestinal tract as a site for the local delivery of DNA vaccines is an exciting prospect. In order to obtain an effective, cheap, safe, and easy-to-administer vector, we developed a new generation of filamentous bacteriophage. This particular bacteriophage was genetically engineered to display integrin receptor-binding peptides and carry a mammalian DNA cassette. One unanticipated observation is the thermoresponsive behavior of engineered bacteriophage. This finding has led us to simplify the isolation method to purify bacteriophage particles from cell culture supernatant. *In vitro* model of the human intestinal follicle-associated epithelium demonstrated that bacteriophage particles were able to cross the human intestinal barrier. Our results also showed that in contrast to non-surface modified, the RGD-modified bacteriophage was successfully used to deliver a transgene to mammalian cells. We also confirmed the adjuvant's ability of the engineered bacteriophage to induce nitric oxide production by macrophages. In conclusion, our study demonstrated the possibility of using bacteriophage for gene delivery to target cells within the gastrointestinal tract.

Keywords: bacteriophage, thermoresponsive, DNA vaccine, gastrointestinal tract, nanocarrier



1. Background

DNA vaccination, also referred to as genetic immunisation, involves the introduction of a DNA molecule, generally a circular plasmid with an antigen-encoding gene, into a host where the expression of an antigenic protein in host cells can elicit both humoural and cellular immune response.[1] DNA vaccines offer significant advantages over traditional attenuated, killed, or subunit vaccines. They are cheaper and easier to produce than recombinant protein vaccines and do not require complete knowledge of the pathogen. They are stable at room temperature and therefore easy to transport and store.[2]

Most commercial vaccines available today are delivered by injection, intramuscular or subcutaneous routes, with problems of safety, patient acceptability and morbidity. Recently, needle-free vaccine delivery has received considerable attentions.[3-5] Orally administered vaccine is of particular interest due to its easy handling, low cost of production and induction of mucosal immunity.[6] Most importantly, the development of safe and cheap carriers capable of efficient and selective delivering DNA vaccine to target cells is of important.

Recently a number of investigations have focused on designing bio-inspired

nanocarriers, such as bacteriophage.[7] Filamentous bacteriophage, a novel class of nanomaterial, has been exploited for vaccine delivery vector.[8] A vast majority of previous reports have utilized filamentous bacteriophage as immunogen carriers for boosting immune response against peptides or proteins displayed on their surface.[8] Bacteriophage have several advantages that make them well suited for developing vaccine-delivery platforms. The particulate nature of phage particle attracts antigen-presenting cells (APC). Phage itself serves as a strong adjuvant and thus promotes excellent immunity against antigens being delivered by phage.[9, 10] Additionally, unmethylated CpG motifs of the phage genome act as immunostimulants, which further enhance immunity.[11] It has been shown that bacteriophage-based vaccine can access both the MHC class I and II, which activates both cell-mediated and humoural immunity.[12] The natural stability of phage particles makes them easy and cheap to process and purify by simple centrifugation steps. Importantly, they are very thermostable and resist to degradation, making them ideally suited for shipping, storage, and delivery without requiring refrigeration.[13]

In this study, we used filamentous bacteriophage to construct an ideal vehicle for delivering DNA vaccine by oral route. In particular, we genetically engineered (i) the bacteriophage genome to carry a mammalian DNA cassette encoding a protein or antigen of interest, and (ii) the major coat protein p8 to express at its N-terminus the **integrin receptor-binding (RGD) peptide** able to target bacteriophage particles to appropriate cells in the gastrointestinal tract.

Results

Design and Construction of RGD-surface modified bacteriophage

We generated the RGD-bacteriophage by introducing the integrin-binding (RGD) peptide to the N-terminus of each copy of the major coat protein p8 as schematically shown in **Figure 1**. To engineer this new bacteriophage, we used the modified fUSE5-MCS vector in which the multiple cloning site has been inserted into the intergenic region of fUSE5 vector as previously described.[14] The first genetic engineering step was to introduce the RGD peptide between residues Gly3 and Asp4 of the N-terminal region of the major coat protein p8 using site-directed mutagenesis. The correct insertion of the RGD nucleotide sequence of the resultant vector was confirmed by sequencing analysis. Next the transgene cassette (GFP reporter gene) was inserted into the multiple cloning site of the engineered vector. Finally, a positive clone was used for bacteriophage propagation followed by purification (See **materials and methods**).

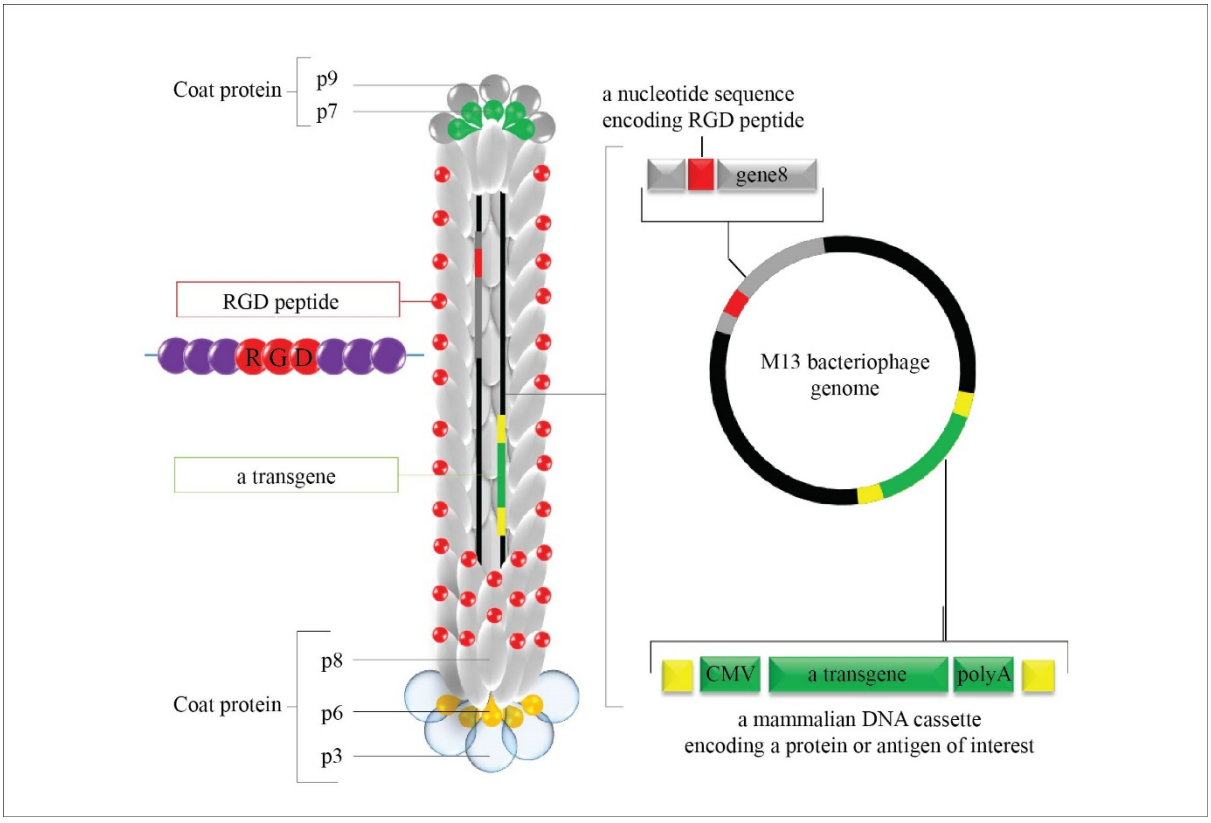


Figure 1 Schematic representation of the RGD-surface modified bacteriophage nanocarrier. The vector displays the RGD peptide on the p8 major coat protein and a protein-encoding gene cassette inserted in the bacteriophage genome.

Physicochemical and biological characterization of bacteriophage nanocarriers

We first investigated the charge characteristic of bacteriophage particles by measuring their ζ-potential. Similar to the NS-bacteriophage, the results show the negative charge of the RGD-bacteriophage at physiological pH (**table1**). However, the RGD-bacteriophage showed an increase of the average size of complexes possibly due to precipitation or formation of large particle aggregates of the RGD-bacteriophage solution. **Figure 2a** shows that the RGD-bacteriophage sample had a wider particle size distribution range. Taken together, these results indicate that RGD-bacteriophage particles are incorporated physically together to form larger aggregated.

One unanticipated finding was Thermo-responsive aggregation behavior of bacteriophage nanocarriers. The RGD-bacteriophage was studied according to its reversible thermoresponsive aggregation behavior in water as shown in **Figure 2b**. The RGD-bacteriophage, but not NS-bacteriophage, spontaneously precipitated from water upon cooling. Upon increasing the temperature to 25°C and 37°C, collapse of the aggregates could be clearly observed and the solution of the RGD-bacteriophage became transparent instantly. This kind of behavior was not observed for the NS-bacteriophage. Also turbidity measurements (**Figure 2c**) revealed significant differences in the solution behavior of the RGD- and the NS-bacteriophage. A 50 mg/mL solution of the RGD-bacteriophage appeared to be turbid (OD600 = X) at 4°C,

indicating the presence of large aggregates. Upon increasing the temperature to 25°C and 37°C, optical density dropped sharply to 0.

We also evaluated the effect of coat protein mutation on the infectivity of engineered bacteriophage vectors. Bacteriophage concentration was measured by spectrophotometric quantification of DNA and protein. After being adjusted to an identical bacteriophage particle number as previously described[15], clones were assayed for infectivity by counting colonies (colony forming units; cfu) formed on LB agar plates. There was a difference in their ability to infect *E.coli* bacteria compared to the NS-bacteriophage, as shown in **Figure 2d**. The RGD-bacteriophage yielded lower number of colonies (**Figure 2e**). This suggests that the insertion of a RGD sequence on wild type p8 can affect its ability to infect *E.coli* host cells.

Table 1 Physical characterization of bacteriophage nanocarriers

Samples	ζ-potential (mV)	Average diameter (nm)	PDI
NS	-18.13±0.29	141.40±1.63	0.46±0.01
RGD	-19.53±0.58	210.17±3.30	0.49±0.01

PDI = Polydisperse Index

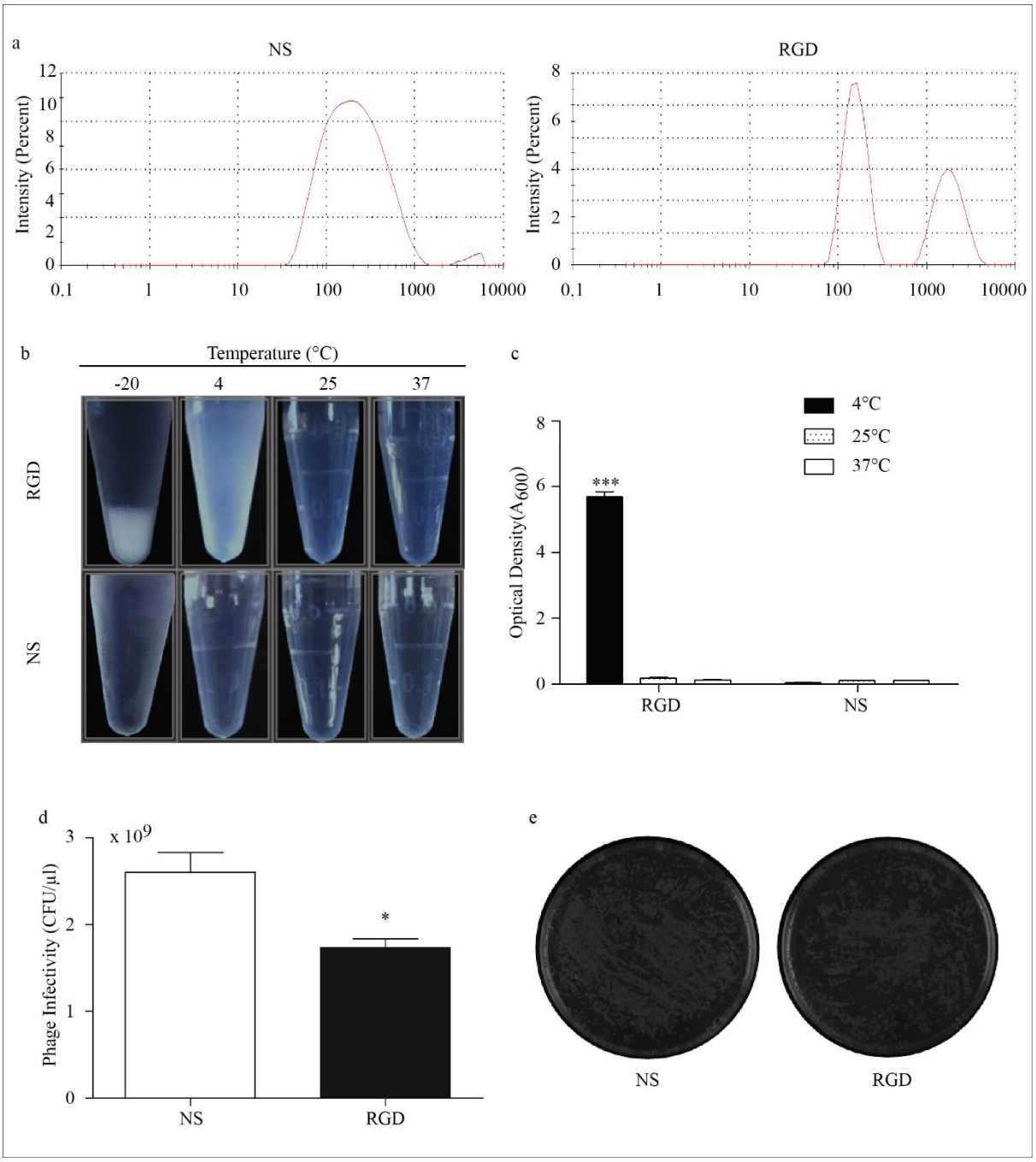


Figure 2. **Physicochemical and biological properties of bacteriophage nanocarriers**. (**a**) Size distribution of the NS- and the RGD-bacteriophage. (**b**) Photographs of a 50 mg/mL aqueous solution of the RGD- and NS-bacteriophage at different temperatures. (**c**) Optical density of the RGD- and NS- bacteriophage at different temperatures. (**d**) Infectivity (colony forming units/µl) was calculated from the number of colonies that grew on the plates overnight. (**e**) LB-agar plates showing the colony formation between the NS-

and the RGD-bacteriophage. One representative plate of each bacteriophage are shown. Data represent the mean + SEM of triplicate samples from one representative experiments of three, significant difference; n.s.-not significant, * p<0.05, ** p<0.01, ***p<0.001 (Two-way ANOVA)

Cell surface α_V integrin receptors binding and transgene delivery characteristics of the engineered bacteriophage nanocarrier

We validated the function of the RGD targeting ligand displayed on the p8 major coat protein by assessing binding to cells expressing integrin receptors. Immunofluorescence using antibodies against the bacteriophage coat protein was performed on highly integrin-expressing HEK293T cells.[16] As shown in **Figure 3a**, we demonstrated targeting capabilities of the RGD-bacteriophage, indicating that the display of RGD peptide is functional. The NS-bacteriophage showed background signal only.

To examine that the RGD-bacteriophage can deliver transgenes into mammalian cells, we also carried out cell transfection experiments on HEK293 cells. Analysis of GFP expression showed GFP expression in cells transfected with the RGD-bacteriophage (**Figure 3b**). Low GFP expression was observed in the NS-bacteriophage-transfected cells (**Figure 3b**). The data prove that the RGD-bacteriophage successfully mediate transgene expression in mammalian cells superior to the NS-bacteriophage.

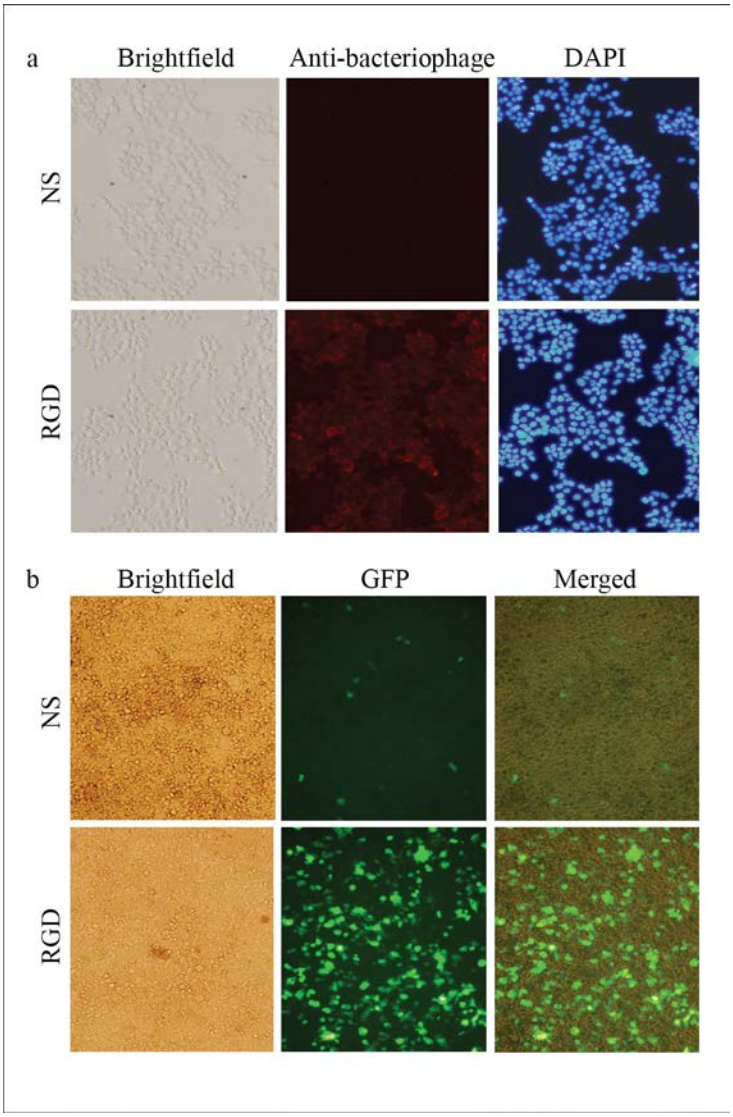


Figure 3. Evaluation of the targeting of mammalian cells by the engineered bacteriophage nanocarrier. (**a**) Immunofluorescence-based bacteriophage binding assay. Cultured HEK293T cells were incubated with the RGD- or NS-bacteriophage. The red color represents fluorescence from bacteriophage staining, and the blue color shows fluorescence of DAPI-stained cell nuclei. (**b**) GFP expression observed after transfection of HEK293T cells with the RGD- or NS-bacteriophage.

Targeting of gene delivery to intestinal cells by the engineered bacteriophage nanocarrier

We first studied cell viability, tight junction *protein (F-actin) distribution* and the presence of normal nuclei in the Caco-2 cell line. No cytotoxicity was observed in the concentration ranges of the NS- or RGD-bacteriophage tested (**Figure 4a**). As shown in **Figure 4b**, normal morphology of viable cells (green fluorescence) treated with the same concentration of the NS- or RGD-bacteriophage. Cells were also treated with Alexa Fluor 584-conjugated phalloidin and DAPI to examine actin filaments and cell nuclei, respectively **(Figure 4b)**. Phalloidin staining did not reveal alterations in the actin cytoskeleton in treatments with the RGD-bacteriophage and in cells treated with the NS-bacteriophage. Cell nuclei showed no signs of condensation or fractionation, again indicating no cytotoxicity or cell death.

Next we evaluated the binding capacity of engineered bacteriophage by using caco-2 cells known to express high levels of the integrin receptors [17] Fluorescence microscopy revealed that a large number of the RGD-bacteriophage can bind to cell surfaces, whereas no bacteriophage particles were observed on cell surfaces incubated with the NS-bacteriophage (**Figure 4c**).

Then, we investigated the RGD-bacteriophage-mediated gene delivery to caco-2 cells. At day 5 post transfection, representative microscopic imaging of caco-2 as shown in **Figure 4d**, revealed GFP expression in cells transfected with the RGD-bacteriophage compared with the NS-bacteriophage. No GFP expression was observed in cells treated with the NS-bacteriophage. These data confirm that the introduction of RGD peptide resulted in the RGD-bacteriophage that significantly boosted gene delivery to human intestinal cells.

We *next* assessed the ability of engineered bacteriophage to translocate across the cell layer by using *in vitro* model of the human intestinal follicle-associated epithelium

The RGD- or NS-bacteriophage was applied to *in vitro* co-culture (as schematically shown in **Figure 4e**), and measured each titer of bacteriophage translocated across the cell layer (**Figure 4f**). As shown in **Figure 4g and h**, the RGD-bacteriophage revealed similar transcytosis efficacy compared with the NS-bacteriophage having no RGD peptide insert.

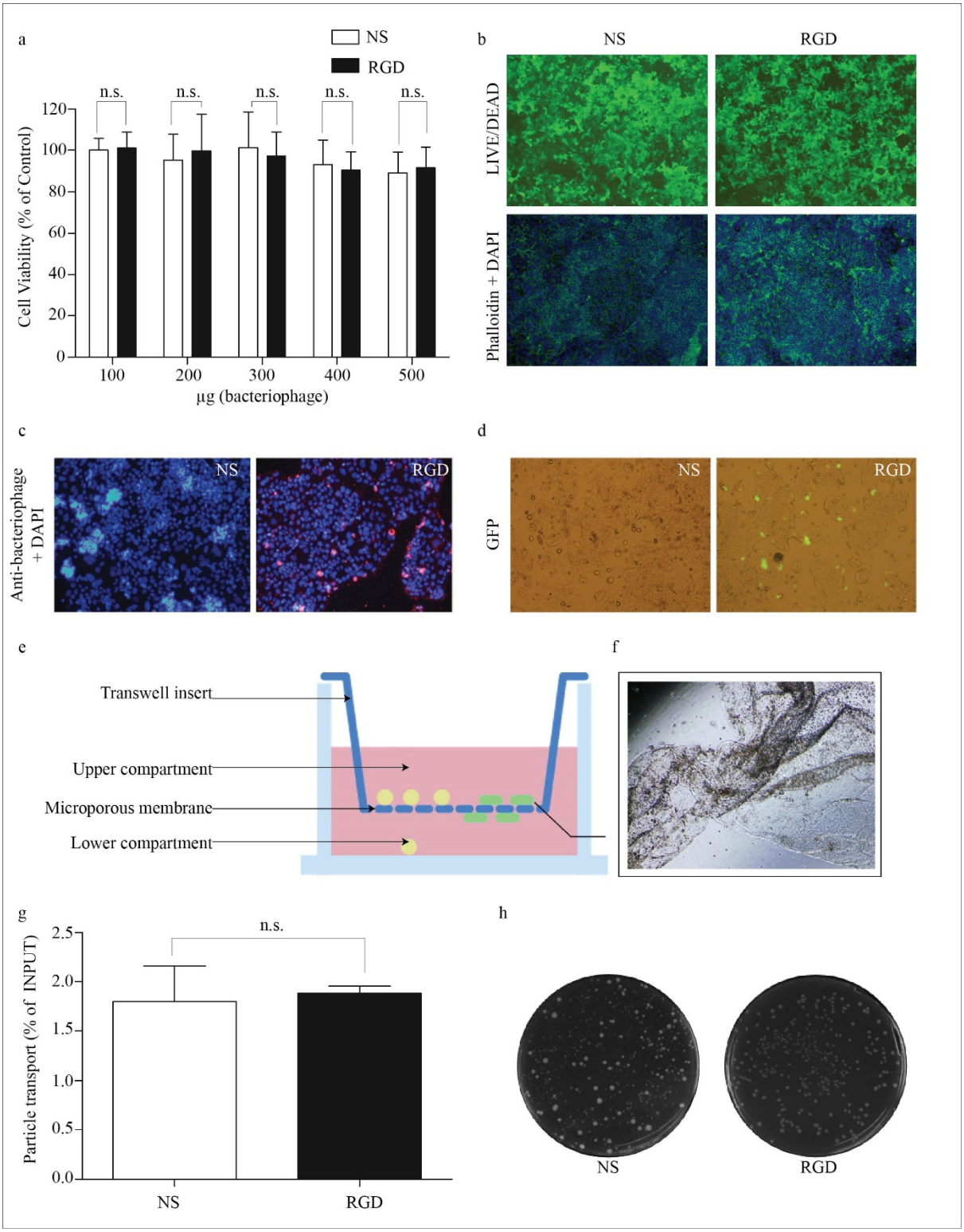


Figure 4. Targeting of gene delivery to intestinal cells by the engineered bacteriophage nanocarrier. (**a**) Cytotoxicity of bacteriophage nanocarriers at various concentration in caco-2 cells. Cells were treated with increasing doses (µg/well) of the RGD- or NS- bacteriophage. Following 24 hr incubation, cell

viability was determined by the CellTiter-Glo® cell viability assay. (**b**) Morphological characteristics of Caco-2 cells visualized under the fluorescence microscope. Cells were also stained with the reagents in the LIVE/DEAD® Cell Viability/Cytotoxicity Assay Kit, Alexa Fluor 584-conjugated phalloidin and DAPI, and visualized under the fluorescence microscope.

(**c**) Fluorescent microscopy of Caco-2 cells after treated with the RGD- or NS-bacteriophage. Cells were immunofluorescently stained for bacteriophage particles (red). **(d)** Analysis of transfection efficiency mediated by the engineered bacteriophage nanocarrier in caco-2 cells. GFP expression was observed after transfection of caco-2 cells with the RGD- or NS-bacteriophage.

(e) Experimental setting to study the bacteriophage translocation across the cell layer by using caco-2/Raji B co-culture system.

Cells were co-cultured on Transwell filters before the addition of bacteriophage particles to the apical compartment. Later, the lower compartment was collected and processed for bacteriophage quantification. **(f)** The cell layer of caco-2/Raji B intestinal cell co-culture system. **(g)**. Total bacteriophage titer translocated across the human intestinal follicle-associated epithelium. The bacteriophage population transported through the cell layer was quantified using the *E.coli* infection method and counting colony forming units. Data represent the mean + SEM of triplicate samples from one representative experiments of three, significant difference; n.s.-not significant, * $p<0.05$, ** $p<0.01$, ***$p<0.001$ (t-test). (**h**) LB-agar plates showing the colony formation between the NS-bacteriophage and the RGD-bacteriophage. One representative plate of each bacteriophage are shown.

NO production by macrophage stimulated with engineered bacteriophage *nanoccariers*

In the present study, NO was used as an intercellular mediator produced in RAW264.7 cells in order to determine the adjuvant property of bacteriophage nanocarriers. After a 24 hr incubation, unstimulated macrophages secretes a background level of nitrite of about 2.8 μM in the culture medium (**Figure 5**). After treatment with LPS (100 ng/mL) for 24 hr, nitrite concentration in the supernatant increased remarkably by about 43.1 μM. Interestingly, when cells were incubated with the RGD- or NS-bacteriophage, the amount of nitrite in the medium was similar to that in the LPS-treated cells, as shown in **Figure 5**. This finding suggests that activation of Raw264.7 cells by the bacteriophage can cause NO production.

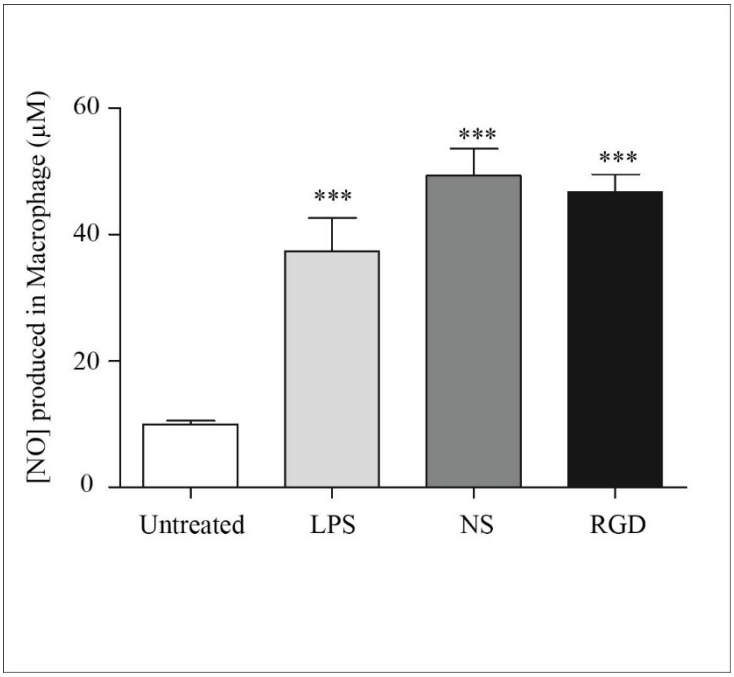


Figure 5. Nitric oxide production in RAW264.7 cells incubated with bacteriophage nanocarriers. Lipopolysaccharide (LPS), a component from the outer membrane of gram-negative bacteria, was used as a positive control. Data are presented as the mean±SEM from replicates and are from at least

three independent experiments (one-way ANOVA with tukey's post hoc test, n.s.-not significant, * $p<0.05$, ** $p<0.01$, ***$p<0.001$).

Discussion

One approach to targeted vaccine delivery takes advantage of elevated levels of receptor expression in the appropriate cells.[18] For example, the integrin receptor which serve as receptors for RGD-containing protein have been found to be expressed on epithelium of small intestine [19], antigen presenting cells of the immune system [20], or dendritic cells [21], and microfold (M)-cell of intestinal epithelium [22] A number of previous studies have demonstrated that RGD-labelled nanovaccines particularly concentrated in the mucosal membrane of the gastrointestinal tract, thus making it a promising strategy for oral vaccination.[23-26]

Major (pVIII) coat proteins of bacteriophage provide the ability to display a wide range of foreign peptides of differing sizes. Therefore, it is possible to design novel bacteriophage constructs depending on which foreign proteins need to be displayed on the outer capsid. In this study, we proved the possibility of constructing bacteriophage-based nanocarrier, showing that moieties displayed on the bacteriophage capsid remain intact and functional by displaying the RGD ligand and carrying a mammalian DNA cassette expressing the reporter gene. We demonstrated that the presence of the RGD sequence on the coat protein of the engineered bacteriophage mediated a stronger uptake of these nanocarriers and the gene expression in targeted cells expressing the integrin receptor.

Although the display of the RGD peptide on p8 protein of engineered bacteriophage slightly affected their ability to infect *E.coli* bacteria, the yield of the engineered bacteriophage was considered as high titers and within a

workable range. Our finding is in agreement with a previous study showing that the display of peptides on wild type p8 affects their ability to infect bacteria.[27]

Another unexpected advantage of the RGD-surface modified bacteriophage is the spontaneous precipitation at low temperature. This kind of behavior was not observed for the non-surface modified bacteriophage. In fact, the standard method to isolate and purify bacteriophage particles from the supernatant of bacteria host cell culture is the use of PEG/NaCl solution for precipitation.[28-30] Having shown that lowering the temperature causes the precipitation of the RGD-bacteriophage solution, the separation of particles from the cell culture supernatant could be carried out without the addition of PEC/NaCl solution. This simple and economical protocol for bacteriophage isolation is well-suited for large-scale production.

As aforementioned in the introduction, oral DNA vaccine delivery systems must be capable of delivering DNA vaccine to the appropriate cells. DNA vaccine vectors could adhere to gastrointestinal epithelia, be transported across the intestinal barrier by M-cells and transfect epithelial and/or immune cells in the gut associated lymphoid tissues (GALT), a specialized tissue which comprises the majority of T cells and a significant B cell compartment. [31, 32] either directly or through antigen transfer.[33] Using the host cellular machinery, the DNA vaccine enters the nucleus of transfected cells and initiates the production of antigen protein. As a proof of concept, the engineered bacteriophage that displays RGD peptide, could be effectively internalised by integrin-expressing cells. Most importantly, the RGD-surface modified bacteriophage can mediate transgene expression in integrin-expressing cells superior to the non-surface modified bacteriophage.

However, we observed no apparent advantage to using non-surface modified or RGD-surface modified bacteriophage to improve the transport across the *in vitro* human intestinal barrier. A possible explanation for our observation in the present study is the nature of bacteriophage and their ability to cross intestinal barrier. In fact, bacteriophage can pass the intestinal wall and migrate to gut-associated lymphoid tissue.[31, 32]

We also investigated whether the engineered bacteriophage nanocarrier was toxic for the human intestine. Our results suggested that the engineered bacteriophage is safe and biocompatible. In fact, bacteriophages have long been administered to humans; for example, its therapeutic use to treat pathogenic bacterial infection[34], and certain bacteriophage preparations have recently been approved by the Food and Drug Administration of USA as antibacterial food additives[35].

Another significant aspect of a vaccine is its adjuvant ability to enhance the body's immune response. Adjuvants enhance immunity to vaccines by a variety of mechanisms, namely increased local inflammation, stimulation of the proliferation of non-specific lymphocytes, and the prolonged persistence of the antigen.[36] It has been suggested that bacteriophage serves as a strong adjuvant and boost the immune response; and this is taken advantage of in applications in vaccination.[9, 10] The result of the present study indicates that the engineered bacteriophage has a high potential to induce NO production in macrophages, confirming the adjuvant's ability of the engineered bacteriophage.

In conclusion, oral vaccination provides both social and economic advantages, especially in developing countries. We expected from this study an efficient, cheap, and safe delivery platform that target the intestinal cells.

This bacteriophage-derived nanovaccine would be a promising platform that may have utility in the targeted oral delivery of DNA vaccines to mucosal immune system within the gastrointestinal tract.

Materials and Methods

Materials

The fUSE5-MCS vector and a mammalian DNA cassette containing a Green Fluorescent Protein (GFP) gene were kind gift of Dr. Amin Hajitou. The human embryonic kidney (HEK293T), the human colonic Caco-2, the human B-cell lymphoma (Raji) and the murine macrophage RAW264.7 cell lines were obtained from American Type Culture Collection (ATCC). LB Broth (Miller), tetracycline hydrochloride, rabbit anti-bacteriophage antibody, 4', 6-Diamidine-2'-phenylindole dihydrochloride (DAPI) and lipopolysaccharide (LPS) were purchased from Sigma. The Dulbecco's modified eagle medium (DMEM), Roswell Park Memorial Institute (*RPMI*) *1640 medium,* antibiotics, and fetal bovine serum (FBS) were purchased from Gibco. LIVE/DEAD® Viability/Cytotoxicity kit, goat anti-rabbit AlexaFluor 647-conjugated secondary antibody, and Alexa Fluor 488 phalloidin were obtained from Invitrogen. CellTiter-Glo® Luminescent Cell Viability assay system and Steady-Glo® Luciferase assay system were provided by Promega.

Construction and production of bacteriophage vectors

To generate bacteriophage-derived vectors for oral delivery of DNA vaccine, the bacteriophage was genetically engineered to display copies of the RGD peptide, on the p8 major coat protein, and to carry a mammalian gene cassette encoding a cytomegalovirus promoter-driven transgene expression. Briefly, the RGD peptide-encoding nucleotide was introduced into gene8 using

site-directed mutagenesis after which the mammalian DNA cassette containing a GFP gene was inserted in the multiple cloning site of the fUSE5-MCS vector. Subsequently, bacteriophage particles were generated and purified from culture supernatants of host bacteria (*Esherichia coli*) by using polyethylene glycol (PEG)/NaCl precipitation method as described previously.[14] Bacteriophage particles were sterile-filtered through 0.45-µm filters, then titrated using spectrophotometry method and expressed as mg/ml.Throughout this paper, the abbreviation RGD and NS will be used to refer to the RGD-surface modified and non-surface modified bacteriophage, respectively.

Size and charge measurement

Nanosizer was used for measuring the particle size (diameter) and ζ-potential charge of RGD or NS bacteriophage in batch mode at 25°C in a quartz microcuvette.

Infectivity assay

The bacteriophage particle number was calculated from measuring protein and DNA by spectrophotometry (adapted from protocol by George P. Smith *et al.).* An aliquot of bacteriophage solutions being previously adjusted to an identical particle number was incubated with host *E.coli*. The infected cells were plated onto LB plates containing 40 µg/ml tetracycline. Infectivity (colony forming units/µl) was calculated from the number of colonies that grew on the plates overnight.

Cell culture

HEK293T and Caco-2 cell lines were maintained in complete D-MEM medium supplemented with 10% Fetal Bovine Serum (FBS), Penicillin (100 units/ml), Streptomycin (100 µg/ml) and L-glutamine (2 mM). RAW264.7 and Raji cells

were grown in RPMI 1640 supplemented with 10% FBS, 100 units/ml penicillin, 100 units/ml streptomycin and 2 mM L-glutamine. Cells were grown at 37˚C in a humid atmosphere of 5% CO_2.

In vitro cytotoxicity

In vitro cytotoxicity of bacteriophage was evaluated by CellTiter-Glo® Luminescent Cell Viability assay system. Cells were seeded and cultured in medium containing RGD- or NS-bacteriophage with different concentrations. The cytotoxicity of bacteriophage was examined 24 h post incubation. Cells were also stained with the reagents in the LIVE/DEAD® Cell Viability/Cytotoxicity Assay Kit. Cells were also stained with Alexa Fluor 584-conjugated phalloidin and DAPI.

Immunofluorescence staining

Cells were incubated with bacteriophage vectors for 4 hours, washed with PBS, and fixed in 4% paraformaldehyde (PFA). After sample preparation by fixation, permeabilization and blocking, cells were then incubated for 1hr at room temperature with rabbit anti-bacteriophage antibody. For secondary staining, cells were incubated with goat anti-rabbit AlexaFluor 647-conjugated secondary antibodies and with DAPI for 1hr in darkness at room temperature. Images were obtained with a fluorescent microscope.

Cell transfection and examination of GFP gene expression

Bacteriophage was directly added to the cell supernatant and incubated at 37°C. Transfection efficiency as determined by the expression of the GFP reporter gene was assessed using a Nikon Eclipse TE2000-U fluorescence microscope. Photographic images were obtained by using 4X or 10X magnification and fluorescent setting.

In vitro model of the human intestinal follicle-associated epithelium

Caco-2/Raji co-cultivation was established in the transwell system with a procedure previously described.[37] RGD- or NS-bacteriophage diluted in cell culture medium was introduced into the apical chamber of transwells. The basolateral solution was collected after 4h incubation. The number of bacteriophage particles was quantified using the *E.coli* bacterial infection method and counting colony forming units, as previously described.

The measurement of nitric oxide production

The RAW264.7 cells were pre-incubated with RGD- or NS- bacteriophage for 30 min at 37°C. As a positive control, macrophages were cultured with LPS. Following a 24 h incubation at 37°C, supernatants were mixed with an equal volume of Greiss reagent and incubated at room temperature for 10 min. Using $NaNO_2$ to generate a standard curve, nitrite production was measured by an absorbance reading at 540 nm (A_{540}).

Statistical analysis

GraphPad Prism software (version 5.0) was used to perform statistical analyses. Data were presented as mean±standard error of the mean (s.e.m). P values were generated by t-test, one-way or two-way ANOVA, considered significant when <0.05 and denoted as follows: *p < 0.05, **p <0.01 and ***p < 0.001.

Author Contributions

T.Y. was involved in the design and supervision of the construction and physicochemical characterizations. T.Y., K.N., and S.B. were involved in conducting physicochemical experiments. T.Y., N.S., S.P., A.H. and K.R. were involved in the design and supervision of the biological assays. T.Y., K.N., and M.K. were involved in conducting the biological experiments. T.Y. performed

the statistical analyses and wrote the manuscript text. All authors reviewed the manuscript.

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Disclosure

The authors report no conflicts of interest in this work.

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