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Development of an optimized protocol for studying the interaction of filamentous bacteriophage with mammalian cells by fluorescence microscopy

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Abstract

Filamentous bacteriophage has been proposed as a vehicle that can carry and deliver therapeutics into mammalian cells for disease treatment, thus a protocol for imaging phage-cell interaction is essential. Because high signal intensity is necessary to study the mechanism of interaction between filamentous bacteriophage and mammalian cells, it is important to optimize the procedure for fluorescence labeling of phage in order to understand such interaction. Here, we describe a procedure that gives intense fluorescence labeling and can show interactions between fd-tet bacteriophage selected from phage libraries and mammalian cells (SKBR-3 and MCF-10A). The indirect labeling of phage with dye-conjugated antibody and cytoskeleton associated proteins was significantly enhanced in the presence of a cross-linking reagent called dithiobissuccinimidylpropionate (DSP) as shown by qualitative and quantitative fluorescence microscopy. The use of DSP resulted in high signal intensity in fluorescence imaging of phage-cell complex. The DSP cross-linker is believed to preserve soluble unbound proteins for fluorescence imaging. The interaction between the phage and mammalian cells was further confirmed by scanning electron microscopy (SEM).

Keywords

Bacteriophage; Dithiobissuccinimidylpropionate; Fluorescence Microscopy; DAPI; Immunofluorescence; Scanning Electron Microscopy

INTRODUCTION

Filamentous bacteriophage (also called phage) is a virus that specifically infects bacteria and is non-toxic to human beings. Earlier studies showed that phage display technology can be successfully employed to identify affinity selected peptides that bind selectively to a variety of targets (Ladner and others, 2004; Lowman, 1997). The previous review (Smith and Petrenko, 1997) showed that phage libraries can be envisioned as populations of replicable and mutable chemicals. Thus, the solvent-exposed amino acid residues on the surface of filamentous phage can be assumed as free chemicals in a solution. One of the most important applications of phage display is identifying ligands for unknown receptors from whole cells in a culture flask (Abbineni and others, 2009; Shukla and Krag, 2005) to whole organ in an *in vivo* living mouse (Pasqualini and Ruoslahti, 1996). Recently the application

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of phage display technology in nanoscience has gained considerable attention, leading to a new field of science called phage nanobiotechnology. Phage nanobiotechnology has a wide range of applications from synthesizing oriented bio-nanomaterials (Mao and others, 2004) to humidity sensors (Liu and others, 2009) and from drug discovery (Schumacher and others, 1996) to tumor treatment including targeted delivery to cancer cells (Ngweniform and others, 2009), molecular imaging (Kelly and others, 2006; Manchester and Singh, 2006), and cancer treatment (Arap and others, 2002). Recently it was proved that phage can also act as a biosensing receptor for analyte detection (Zhu and others, 2008). Therefore, the understanding of the interaction between phage and mammalian cells is highly essential and a new method needs to be developed to image such interaction and satisfy this rapidly developing field.

Regardless of the type of target being used for affinity selection procedure, the relative avidity and binding assessment of the isolated phage clones were typically assessed by Enzyme Linked Immuno Sorbent Assay (ELISA) (Rhyner and others, 2003), fluorescence associated cell sorting (FACS) (Knurr and others, 2003) and fluorescence microscopy (David and others, 2004). The fluorescence assessment is usually performed by either labeling the phage directly or indirectly with fluorochromes. There are, however, circumstances in which fluorescence signal intensity from the selected phage and its target is relatively weak. Since the phage studies are gaining importance in medical application, it is highly essential to optimize the protocol to track, visualize, and measure the fluorescence intensity of phage inside the mammalian cells. Therefore here we report the development of a new protocol to study the interaction between the filamentous bacteriophage and mammalian cells.

In the present study, we introduce a protocol for labeling phage particles in mammalian cells using a well characterized phage selected against breast cancer SKBR-3 cells from our previous study (Abbineni and others, 2009). The selected phage has no affinity to our control normal breast cells, MCF-10A. Here we show that commercially available fluorochromes, such as Alexa fluor 488 conjugated to anti pVIII, readily label the major coat protein (called pVIII) of fd-tet bacteriophage and the signal intensity of the binding is significantly enhanced in the presence of a cross-linker called dithiobissuccinimidylpropionate (DSP).

MATERIALS & METHODS

Reagents

All the reagents and solutions used are shown in Table 1. All the chemicals used were purchased from Sigma (unless otherwise stated).

Mammalian cell culture

SKBR-3 breast cancer cell line and MCF-10A normal breast cells were purchased from American Type Culture Collection (ATCC), Rockville, MD. Both cell types were cultured according to the suppliers instructions (Soule and McGrath, 1986; Trempe, 1976). Briefly, the SKBR-3 cells were grown in McCoy's modified medium (Ekerljung and others, 2008) supplemented with fetal bovine serum (FBS) and gentamycin. The cells were cultured in tissue culture dishes at 37°C in 5% CO₂ and humid air. MCF-10A cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Helena and others, 2008) supplemented with FBS and gentamycin. Once the cells reached 90% confluence, cells were removed with 0.25% trypsin/2mM EDTA. For experimental procedures, 1×10⁴ cells in 2 ml of suitable medium were seeded in six-well plates containing 25-mm round cover glasses. The cells were allowed to attach and spread for two days. The phase contrast microscope equipped

with Hamamatsu camera and Scion software were used to capture digital images of the live SKBR-3 cells and MCF-10A cells that are shown in Figure 1a & b. The cells on cover glasses were rinsed with Hank's Buffered Salt Solution (HBSS) (Safiejko-Mrocza and Bell, 1996) and processed as follows.

Phage propagation & purification

Phage display libraries were used to isolate phage that specifically binds to SKBR-3 cells. The detailed procedure for affinity selection of SKBR-3 cells is described elsewhere (Abbineni and others, 2009). Briefly phage suspension was obtained by using overnight culture of K91 BlueKan cells. Two tubes of 3 ml bacterial culture was incubated with phage in a 1 liter LB medium on a shaking incubator at 37°C overnight with suitable antibiotics. The overnight culture was first centrifuged at 2400 g for 10 min at 4°C using Beckman Coulter™ High performance centrifuge, and then the supernatant was collected and re-centrifuged at 6700 g for 10 min at 4°C. PEG/NaCl solution was added and the suspension was stored in refrigerator for overnight. Phage precipitate was collected at 10100 g for 60 min at 4°C, dissolved in Tris buffered saline (TBS) and centrifuged at 10100 g for 10 min until clear. A sample (10 µl) of appropriate phage dilution was taken on a copper grid and examined under transmission electron microscope (TEM) (as shown in figure 2a), and a drop of phage sample was placed on mica and examined under atomic force microscope (AFM) (as shown in figure 2b)

Cross linking and extraction procedure

Cells on cover glasses were treated according to one of the three procedures outlined in Table 2. The following procedures were used to differentiate the intensity of fluorescence labeling between fd-tet bacteriophage on SKBR-3 cells and MCF-10A cells: a) Using bifunctional protein cross-linker (XL) DSP (Pierce) and permeabilization with Triton X-100; b) Paraformaldehyde (PFA) fixation and permeabilization with Triton X-100 detergent under stabilizing conditions; c) PFA fixation and permeabilization with organic solvent (ethanol). All three procedures were compared and an optimal protocol is developed as shown in Table 3.

Fluorescence labeling of cells

Phage bound to SKBR-3 cells was labeled with Alexa flour 488 (Invitrogen) conjugated to anti-pVIII antibody (Amersham). SKBR-3 and MCF-10A cell nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI). To determine the intensity of F-actin when phage was bound to SKBR-3 cell, the F-actin was labeled with phalloidin conjugated to rhodamine (Sigma) (data not shown).

Sample preparation for fluorescence and SEM imaging

Fluorescence Microscopy—SKBR-3 cells were cultured on cover glasses and incubated with SKBR-3 selected phage (1×10^{10} Pfu) for 1 h at 37°C. The cells were washed thoroughly with HBSS, and one of the three extraction procedures, as indicated in Table 2, was followed. After an additional incubation with Alexa flour conjugated antibody, samples were washed, mounted on microscope slides using mounting medium, and observed under fluorescence microscope. All samples were examined using a Zeiss Universal epifluorescence microscope equipped with Olympus oil immersion DApo UV objectives. Digital images of cells were collected using Olympus DP-70 camera and software. The quantitative fluorescence intensity associated with ten individual cells from each procedure was measured by using image analysis software at constant exposure time.

Scanning Electron Microscopy (SEM)—SKBR-3 cells were cultured on cover glasses and incubated with SKBR-3 selected phages for various time points (5 min, 10 min, 15 min, 30 min, 45 min and 1 h), and then fixed in 2.5% glutaraldehyde in sodium cacodylate-sucrose buffer for 15 min at room temperature. After fixation, the cover glasses were rinsed twice with cacodylate buffer and post fixed with 1% osmium tetroxide (OsO₄) in cacodylate buffer for 30 min at 4°C. The samples were washed twice and dehydrated in increasing concentrations of ethanol. Samples from 100% ethanol were dried using critical point drying from liquid CO₂ and coated with gold using a sputter coater. Samples were examined in a JSM-840 scanning electron microscope (JEOL) at 20KV.

RESULTS

The developed procedure for studying the interaction of phage with mammalian cells is based on the extraction of attached cells using a detergent in the presence of the cross-linker DSP. Extraction of the cells with ethanol and Thbss, consisting of 0.5% Triton X-100 in HBSS in the absence of DSP, resulted in the detachment of most SKBR-3 and MCF-10A cells from the substratum. The cells were completely retained and the cell extensions were completely preserved in the presence of DSP. Therefore, fluorescence labeling of phage is relatively strong in the presence of the cross-linker, preserving the intracellular soluble molecules and attachment of cells to the substratum (Figure 3b).

The commonly used method for labeling phage with a fluorescent dye is to fix the cells first in either paraformaldehyde or glutaraldehyde buffered solution and add the antibody conjugated dye to the phage treated and fixed cells. No cross-linkers were used in any of the previously reported phage labeling studies. However, we obtained very strong fluorescence signal in the presence of the cross-linker DSP compared to previously used methods. To determine which method is optimal, we compared the fluorescence labeling intensity of phage in the presence and absence of DSP using solvent and detergent permeabilization and fixation in PFA. The fluorescent images obtained indicate that the intensity of fluorescence signal is increased greatly when the phage treated cells are incubated with the cross-linker DSP and with detergent extracted in the presence of the cross-linker DSP (Figure 3 a, b). The control samples obviously contained no signal from Alexa fluor 488, indicating that the phage is not specific for normal MCF-10A cells (Figure 4 b,d,f). The results of conventional staining procedure of labeling phage when interacted with SKBR-3 and MCF10A cells are shown in Figure 3 c,d and 4 c,d respectively. In general, phage treated with Alexa flour 488 conjugated antibody is labeled more intensely in the presence of DSP. Figure 3 (a,b) shows the fluorescence intensities of SKBR-3 cells labeled with Alexa flour 488 and DAPI after cross linking with DSP, which is a water-insoluble N-hydroxysuccimide ester (NHS ester). We also compared the effectiveness of fluorescently labeled antibody binding in the absence of the cross-linker under two extraction procedures (Thbss vs EtOH) (Figure 3 c–f). Extraction with 0.5% Triton X-100 gave a significant increase in the intensity of Alex flour 488 and DAPI labeling over that obtained with EtOH, whereas the highest intensity of labeling was obtained with cross-linker DSP. The order of increasing Alexa flour 488 and DAPI labeling was EtOH<Tsb<DSP. These different extraction and cross-linking procedures also affected the patterns of F-actin labeling (data not shown). Figure 4 shows the labeling of normal breast cells MCF-10A under similar conditions stated above.

Use of a cross-linker has proved to be very useful for preserving the cellular soluble molecules and attachment of cells to the substratum (Safiejko-Mroccka and Bell, 1996). In agreement with earlier reports (Safiejko-Mroccka and Bell, 1996), we noticed the detachment of SKBR-3 and MCF-10A cells in the presence of 0.5% Triton X-100 detergent in HBSS and in the absence of the cross- linker DSP. The structure of the mammalian cells

was completely retained in the presence of the cross-linker DSP when extracted with Triton-HBSS.

Intensity of phage labeling in the presence and absence of the cross-linker DSP was quantified in all three labeling methods. The fluorescence associated with ten individual cells from each procedure was measured by using digital image analysis. The quantitative results are shown in Figure 5. Intensity of phage labeling in the presence of the cross-linker is increased ~2 fold compared to the phage labeling when the cells are permeabilized with Triton X-100. In comparison with other extraction procedure such as ethanol, the phage labeling in the presence of the cross-linker DSP is increased by ~2.7 fold.

We further used SEM to confirm the binding of filamentous bacteriophage to SKBR-3 cells. As the SKBR-3 selected phage from the phage library is specific for SKBR-3 cells, we observed phage binding to SKBR-3 cells. No phage is found associated with control normal MCF-10A cells under electron microscope because of its non-specificity (data not shown). Under the electron microscope we found that affinity selected phage is bound to SKBR-3 cells (Figure 6a). But no phage is observed on the cells when the SKBR-3 cells are treated with wild type phage (Figure 6b). We have incubated the SKBR-3 cells with filamentous bacteriophage at various time points such as 5 min, 10 min, 15 min, 30 min, 45 min and 1 h at 37°C. However, phage binding to the cell surface was only observed at 15 min incubation time and not observed at other time points. The possible reason for not observing the phage interactions at other time points could be that phage is not bound strongly enough to the cells before 15 min to withstand the multiple step preparation procedure. In addition, internalization of phage most probably takes place between 15 and 30 minutes of incubation, and SEM imaging provides only morphological information from the sample surface.

DISCUSSION

Here we report that the use of bifunctional protein cross-linker DSP increases the fluorescence intensity of phage labeling and preserves the intracellular proteins and cell attachment during fixation. The high affinity SKBR-3 selected phage was selected from f8/8 landscape phage display libraries described elsewhere (Abbineni and others, 2009). Fluorescence imaging was performed to assess the best procedure for phage labeling. Paraformaldehyde and other related reagents (such as glutaraldehyde) are widely used as chemical cross linkers for fixing the cellular structures for microscopic examination before labeling with antibodies and fluorescent dyes (Rinnerthaler and others, 1988). Treating the cells with paraformaldehyde results in the formation of covalent linkages with exposed amino and imino groups from proteins, creating a methylene bridge between amino acids in close proximity. However, the use of paraformaldehyde is limited in the presence of short intermolecular contacts. In case of immuno-fluorescence studies involving dye labeling, the cells need to be permeabilized with solvent or extracted with detergent for the antibody or other probes to enter or penetrate into the cells (Lindroth and others, 1992). It has been shown previously that bifunctional protein cross-linking reagents significantly improve structural preservation during immunolabelling (Safiejko-Mroczka and Bell, 1996). Therefore, we have chosen to use a cross-linker, DSP, to stabilize cellular components before extracting with detergents. When the plasma membrane has to be removed for dye conjugated antibody penetration in order to bind to its intracellular epitope, cross-linkers such as DSP are the best choice to cross link intracellular components because aldehydes form relatively coarse cross-linking and limit solubilization of membrane proteins (Bell and others, 1988; Bell and others, 1989). DSP is a water insoluble, cross-linking NHS-ester that acylates free primary and secondary aliphatic amino groups present between amino acids in proteins within organic or aqueous medium (Lomant and Fairbanks, 1976; Xiang and others, 2004). Intense phage labeling in the presence of DSP compared to other procedures is

attributed to the ability of DSP to cross the intact plasma membrane of the living cells and begin cross-linking proteins before membrane permeabilization. This enables the soluble and membrane bound proteins to be stabilized before the extensive extraction of cell membrane by a detergent, providing excellent accessibility for antibodies to their intracellular epitopes. With regard to extraction procedures in the presence of Triton X-100 and ethanol, the fluorescence labeling is relatively poor because there is no stabilization and preservation of soluble intracellular molecules provided by the membrane permeable cross-linker.

We have labeled phage indirectly with monoclonal antibody for evaluation of cell-phage labeling under different extraction conditions. Phage labeling can be done by two different methods (Turnbough, 2003). Phage can be labeled either directly with fluorochrome using protein labeling kits following the manufacturer's instructions or indirectly with dye labeled monoclonal antibodies (mAb) that binds to one of the components of phage. In our experiments the phage is indirectly labeled with Alexa fluor 488. The phage suspension is incubated with cells and the resultant cell-phage complex is treated with Alexa fluor 488 labeled monoclonal antibody that binds to capsid protein (pVIII) of filamentous bacteriophage. Alexa fluor 488 dye contains a succinimidyl ester that reacts with the exposed primary amine present in monoclonal antibody. The interaction between the monoclonal antibody labeled dye and pVIII of phage is a standard antigen-antibody interaction.

CONCLUSION

The use of DSP as an efficient protein cross-linker improves the preservation of soluble intracellular and attachment proteins and increases the intensity of fluorescence labeling of phage bound to mammalian cells. A comparative summary of procedures performed by various procedures in the presence and absence of the cross-linker is shown in Table 2. The application of this technique can greatly improve the strength and specificity of fluorescence labeling of cells and associated phage particles or other intracellular or surface bound structures.

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Figure 1.
Phase contrast images of a) SKBR-3 breast cancer cells and b) MCF-10A normal breast cells. (40x objective)

Figure 2.

a) TEM image of SKBR-3 selected fd-tet filamentous bacteriophage (scale bar 25 nm) b) AFM image of SKBR-3 selected fd-tet bacteriophage in TBS (scale bar 1 μm)

Figure 3.

Fluorescence labeling of breast cancer cells SKBR-3 after interaction with filamentous phage using different procedures. Nuclear (DAPI) (a) and phage (Alexa) (b) staining in the presence of the cross-linker (protocol 1); nuclear (c) and phage (d) staining after 0.5% Triton x-100 extraction (protocol 2); and nuclear (e) and phage (f) staining after 70% ethanol extraction (protocol 3).

Figure 4. Fluorescence staining of normal breast cells MCF-10A and filamentous phage using different procedures. Nuclear (DAPI) (a) and phage (Alexa) (b) staining in the presence of cross linker (protocol 1); nuclear (c) and phage (d) staining after 0.5% Triton x-100 extraction (protocol 2); and nuclear (e) and phage (f) staining after 70% ethanol extraction (protocol 3).

Figure 5.
Histogram showing the relative fluorescence intensity of SKBR-3 cells labeled with Alexa flour 488 conjugated antibody after cross-linking, extraction in Triton X-100 and ethanol.

Figure 6.

Scanning electron microscopic images of SKBR-3 cells and filamentous phage. (a) Filamentous bacteriophage bound to SKBR-3 cells when incubated for 15 min (inset, mag (30,000)). (b) Control SKBR-3 cells treated with wild type bacteriophage (no phage was found on cell surface at high magnification). It should be noted that phage is coated by metals for imaging purpose and may probably form bundles on the cell surface. Thus the size of the phage structures shown here does not reflect that of individual phage particle.

Table 1

Reagents and solutions

Abbreviation	Reagent or Solution
Anti pVIII-Alexa	anti-pVIII labeled Alexa flour 488 (Invitrogen): 10 μ M stock solution in sterile water diluted 1:5 in PBS
BSA	Bovine serum albumin
BSA-TBS	1% BSA in Tris-buffered saline containing 0.12% sodium azide, pH.7.6
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium (ATCC)
DSP	Dithiobis(succinimidylpropionate), 1mM: 20mg/ml stock solution in DMSO diluted 1:50 (prepared fresh) (Pierce)
EtOH	Ethanol, absolute
Gelvatol	20g Gelvatol (Monsanto; St Louis, MO), 40ml glycerol, 80ml PBS-A
Glutaraldehyde	2.5% glutaraldehyde
Glycine-PBS-A	0.1M glycine in PBS-A
Glycine-HBSS	0.1M glycine in HBSS
HBSS	Hanks' balanced salt solution, pH 7.2 (Safiejko-Mrocza and Bell, 1996)
OsO ₄	Osmium tetroxide
PFA	4% paraformaldehyde: 20% aqueous stock solution diluted to 4% in either HBSS or PBS
Pfu	Plaque forming units
PBS-A	Phosphate-buffered saline, calcium and magnesium-free: 137mM NaCl, 3mM KCl, 0.5 mM Na ₂ HPO ₄ 1.5mM KH ₂ PO ₄ , pH 7.4
TBS	Tris-buffered saline: 150mM NaCl, 0.12% sodium azide, 20mM Tris, pH 7.6
Thbss	0.5% Triton X-100 detergent in HBSS

Table 2

Cross Linking and extraction procedures

Protocol	Extraction procedure
XL-PFA (protocol 1)	Treat cells with phage solution for 1 h, 37°C; wash in HBSS, 10 min, 37°C; 1 mM freshly prepared DSP in HBSS, 10 min, 37°C; DSP/Thbss, 10 min, 37°C; Thbss, 5 min at 37°C; PFA in HBSS, 15 min, 37°C; HBSS, 5 min at 37°C; Glycine HBSS, 5min at 37°C; TBS, 5min; 1% BSA/TBS, 5min; Alexa flour conjugated antibody, 45min ; 1% BSA/TBS,TBS, DAPI, 3min; PBS-A, 5 min; mount in gelvatol.
PFA-Thbss (protocol 2)	Treat cells with phage solution for 1 h, 37°C; wash in HBSS, 10 min, 37°C; PFA in HBSS, 15 min, 37°C; HBSS, 5 min at 37°C;Thbss, 5 min at 37°C; PBS-A, 5 min; Alexa flour conjugated antibody, 45 min ; PBS-A; DAPI, 3 min; PBS-A, 5 min; mount in gelvatol.
PFA-EtOH (protocol 3)	Treat cells with phage solution for 1 h, 37°C; wash in HBSS, 10 min, 37°C; PFA in HBSS, 15 min, 37°C; HBSS, 5 min at 37°C; Glycine HBSS, 5min at 37°C; HBSS, 5 min at 37°C; 70% ethanol, 5 min at 37°C; TBS, 5 min; 1% BSA/TBS, 5 min; Alexa flour conjugated antibody, 45 min ; 1% BSA/TBS,TBS, DAPI, 3 min; PBS-A, 5 min; mount on gelvatol.

Table 3

Flow chart showing the optimized protocol for visualization of phage interaction with mammalian cells (SKBR-3 and MCF-10A)