Specific targeting and delivery of virus envelope-coated nanoparticle cargoes into receptor-bearing cells and subcellular compartments

A. Moore[*], A. Kolokoltsov[*], D. Boehning[**], T. Albrecht[*] and R. Davey[*,**]

[*]Department of Microbiology and Immunology, [**]Department of Neuroscience and Cell biology,
University of Texas Medical Branch
4.138 MRB, 301 University Blvd, Galveston, Texas, USA.
amoore@utmb.edu, aakoloko@utmb.edu, [**]dfboehni@utmb.edu, talbrech@utmb.edu
correspondence should be addressed to: radavey@utmb.edu (409) 772 4915

1 ABSTRACT

Viruses are highly efficient nanoscale machines that not only target specific cell types but also penetrate the cell membrane to deliver the virus genome into the cytoplasm. Recently, we described a simple and highly scalable method to modify the surface of nanoparticles with the membranes of murine leukemia viruses (MLV) and showed that these can deliver a simple cargo into receptor bearing cells (Deniger et al., Nano Lett. 2006 Nov;6(11):2414-21). However, the entry route and subcellular destination of the nanocargoes was not determined. Here we use state-of-the-art microscopy techniques on live cells to visualize the penetration and delivery of the cargo to different subcellular compartments. We show that the MLV membrane-coated nanoparticles are specifically captured on the surface of receptor bearing cells from which they are rapidly taken up into early endosomes. We predict that coating of nanoparticle cargoes with virus envelopes will create a biologically compatible capsule to enhance circulation and delivery of nanoscale drug formulations to specific cells and subcellular compartments.

Keywords: delivery, virus, membrane proteins, endosome, trafficking

2 INTRODUCTION

An important promise of nanotechnology for medicine is the specific delivery of drugs. By conveying agents directly into cells, dosages can be lowered. Drugs can also be delivered to tissues, such as the brain, that would otherwise be inaccessible. To achieve this goal, methods need to be developed to target nanoscale drug capsules to specific cells and overcome the biological barriers imposed by vasculature as well as the cell membrane itself. After binding, the nanocapsule needs to be taken up into the cell. One pathway is through endocytosis but if this route is used the capsule still needs to escape into the cell cytosol before being deposited into the destructive environment of the lysosome. Progress toward these goals, has focused on targeting cells by identifying new ligands through phage display, aptamer selection and other methods. To achieve release from endosomes some have used pH-sensitive lipids that react to the lower pH environment of the mature endosome to cause endosome lysis. However, more work to make each step more efficient and precise is still needed.

Virus envelope proteins (envs) are hydrophilic proteins that are embedded in the virus membrane and project from the surface of enveloped viruses. Envs bind cell surface receptors that can stimulate trafficking of the virus into the cell. Envs also sense and react to their environment by undergoing conformational changes resulting in membrane fusion between the virus and cell surface or endosomal membrane. The most common environmental trigger is the lowering of endosomal pH. Alternatively, retroviruses like MLV and HIV respond to other stimuli that are poorly understood, such as change in redox potential [1]. In both cases, the outcome is very specific release of the virus capsid into the cell cytosol, without causing a breach in the target membrane. These attributes have made harnessing the potential of virus envs a goal of our research.

Much early work on the study of virus membrane fusion mechanism involved the production of virosomes from viruses such as Influenza A and Vesicular stomatitis virus. Typically, detergents were used to dissolve the virus membrane and then reconstitute the virus envs into artificial membranes. However, this method was inefficient, producing mostly denatured envs, that had lost membrane fusion potential and required careful tailoring to each virus. Furthermore, most attempts to translate this work to other viruses has proved very difficult or ineffective as most virus envs are labile to detergent treatment.

Recently, we described a new, simple method for coating nanoscale particles (NPs) with purified virus membranes. This method is likely to be applicable to other virus types and since no detergent is used, the envs are maintained in an active state. The method uses extrusion of NPs with virus membranes purified free from other cellular contaminants. We have previously shown that this highly scalable technique results in coating the NP with a thin film of virus membrane. The method was demonstrated with MLV. MLV is a useful model virus since it has been

extensively characterized for gene therapy use. Furthermore, different MLV strains exist that use distinct, well characterized receptor proteins.

Here we further describe the interactions of ecotropic MLV membrane-coated NPs with cells bearing its receptor protein, mCAT-1. By use of live, high speed, dual color video microscopy we demonstrate that coated NPs efficiently bind to cells and these are then taken up by endocytosis. NPs are seen associating with the early endosome marker protein Rab5. In contrast, little uptake is seen in Caveolae in 293 cells.

3 MATERIALS AND METHODS

3.1 Cell lines and virus production

Moloney murine leukemia virus was used for all experiments. This is an ecotropic MLV that infects cells expressing the mCAT-1 protein. Virus was collected by harvesting culture supernatants from CL-1 cells (gift from Dr. J. Cunningham, Harvard Medical School). These cells are a derivative of NIH3T3 fibroblasts that were infected with MLV. Clonal cell lines, making high titered MLV, were then isolated. Virus was concentrated and purified by pelleting through 20% (w/v) sucrose in 10 mM HEPES buffer, pH 7.4. The virus pellets were resuspended in Dulbecco's phosphate buffered saline (PBS), pH 7.4 (0.01 volumes of culture volume).

3.2 Preparation of virus membranes

The method is illustrated in Fig. 1. The virus membranes from MLV were separated off virus cores by osmotic shock in 1 mM EDTA and 10 mM HEPES, pH 7.4, followed by sonication. Sonication was ten times in 1 second pulses using a Branson E-Module Ultrasonicator at full power. This was then followed by sonication in a water bath three times for 10 seconds. Sucrose was added to 0.25 M and virus cores were pelleted by centrifugation at 20 000 g for 10 minutes at 4°C. The supernatant containing the virus membranes was removed and centrifuged at 100 000 g for one and a half hours at 4°C. The membrane pellet was then resuspended in PBS.

3.3 Preparation of virus membrane-coated fluorescent nanoparticles

An aliquot of the viral membrane preparations was diluted to 200 µL with PBS before being passed twenty times though an Avanti mini-extruder (Avanti Polar Lipids, Inc., CA) using a Whatman 0.2 µm polycarbonate membrane filter (Fisher Scientific, NH) flanked on each side by a filter support (Anvanti Polar Lipids, Inc., CA). 2 µL of NP stock (5% solids w/v, red fluorescent nanospheres, #F8801, Invitrogen, CA) was then incubated with extruded membranes for 15 minutes before being passed a further twenty times through the extruder. The

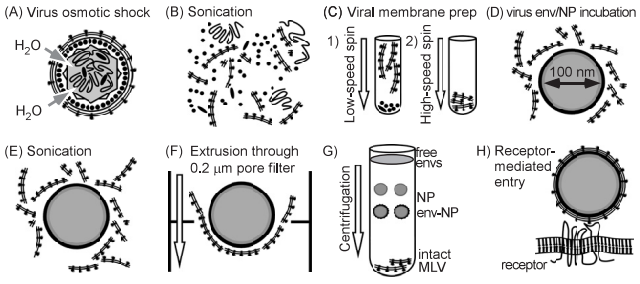

Fig. 1. Method for purification of virus membranes and coating of NPs. Figure is modified from Deniger et al., 2006. 10^8 virus particles were used per preparation. These were harvested from culture supernatants and pelleted through sucrose to eliminate cell debris and serum proteins. (A) Virus was osmotically shocked in hypotonic buffer and then (B) disrupted by sonication. (C) Intact virus and viral cores were pelleted by 10,000 xg centrifugation in 0.25M sucrose. The buoyant virus membranes were then pelleted by 100,000 xg centrifugation. (D) After a brief incubation (E) and second sonication (F) the virus membranes were extruded on 100 nm diameter NPs. (G) coated NPs were separated from intact virus membranes and NPs by dextran gradient centrifugation. Coated NPs concentrate as a band below unmodified particles. (H) cell interaction was then analyzed.

coated NPs were then centrifuged through a dextran gradient (3-24% w/v 70 kDa dextran) at 100 000 g for 1.5 hours at 10°C. The band containing the virus membrane-coated NPs was extracted and diluted five-fold in PBS. NPs were then pelleted at 50 000 g for 2 h.

As a control, NPs were coated with purified lipids instead of virus membranes. For this, 2.5 mg of bovine polar lipid (Avanti Polar Lipids, Inc., CA) in chloroform was dried overnight under vacuum. The dried lipid was overlayed with argon gas and then then resuspended to 1 mL with PBS and extruded with NPs as above.

3.4 Incubation of coated particles with cells and microscopy

HEK 293 cells stably expressing mCAT-1 were transfected with 2 µg Cav-GFP or 2 µg Rab5-GFP and 8 µg of carrier plasmid by calcium phosphate. The cells were grown on laminin (Mouse laminin 1 protein, Trevigen, Inc., MD)-coated 25 mm circular coverslips (Fisher Scientific, NH). The coverlips were then placed on a heated microscope stage at 37°C. Glass tubes, 0.5 cm in diameter, were sealed onto the coverslip using silicone vacuum grease on the tube rim. The entire slip was immersed in Krebs Medium (pH 7.2, 25 mM HEPES and 2mM $CaCl_2$). 10 µL of virus-NP prep was then added to the center of each glass tube and imaged either immediately or after 10, 30 or 60 minutes. Images were acquired using a TE2000 Nikon inverted microscope fitted with a 100x oil immersion

objective (SuperFluor, Nikon) and a Cool-SNAP HQ cooled CCD camera (Roper Scientific, GA). Excitation light wavelength was switched between 492 (for GFP) and 572 nm (for red fluorescent NP) using an automated high-speed filter wheel and controller (Lambda 10-2, Sutter, CA) Exposure time was 150 ms per wavelength and 5 seconds between each pair of exposures. Images were acquired using MetaFluor (Molecular devices) and sequences compiled and analyzed using WCIF ImageJ software.

4 RESULTS

The NPs used in this work are commercially available, polystyrene, highly fluorescent particles, of 100 nm in diameter. These were chosen as the particle is similar in size to a native virus core and are visible as a bright point of light by epifluorescence microscopy. These NPs were coated with purified virus membranes taken from ecotropic (Moloney strain) MLV (Fig. 1). Unlike other attempts to reconstitute the function of virus envs, detergents were avoided altogether. Instead, coating was by mechanical extrusion. The purified, coated particles have been previously characterized by electron microscopy and shown to consist of single (majority) and sometimes double or triple particles joined by thin membraneous films [2]. Here we wanted to know if the coated NPs behaved similarly to live virus, binding to and penetrating into live cells.

To visualize the coated NPs interacting with cells, NPs coated with either virus membranes or pure lipids alone were incubated with 293 cells expressing mCAT-1 (acts as the ecotropic MLV receptor). Cells were also made to express endosomal marker proteins tagged with green fluorescent protein (GFP). Rab5 and caveolin-1 were chosen as markers and are present on early endosomes and caveolae respectively. The placement of the GFP tag in each expression construct has been previously shown to not disrupt the function of each marker protein [3].

From microscope images, NPs coated with virus membranes were visible and could be seen attaching immediately, upon addition to cells (Fig. 2). Attachment was evident from particle tracking; rapid Brownian motion of the NP abruptly ceased and a slower, more directed migration followed. Sometimes NPs attached to cell projections but mostly attachment appeared to be at the cell body. NP association was strictly dependent on the presence of virus membranes as cells incubated with NPs coated with bovine brain lipids showed no significant cell interaction (not shown). In our previous work we have shown a requirement for the mCAT-1 receptor before good interaction can take place.

Next, association with GFP-tagged Rab5 or caveolin containing vesicles was measured. Distinct colocalization with Rab-5 was apparent. After 10 minutes, approximately 5-10 NPs were seen per cell but this increased 5 to 10-fold after 1 h. Live microscopy revealed that over a 10 minute period at least 70% of the NPs were either stably or transiently associated with Rab5. In multiple cases Rab5+ vesicles were seen in the process of associating with the NP (Fig. 3A). Often, this appeared to be through coalescence of two small vesicles that surrounded the NP. In many cases association lasted for more than 5 minutes (Fig. 3B), after

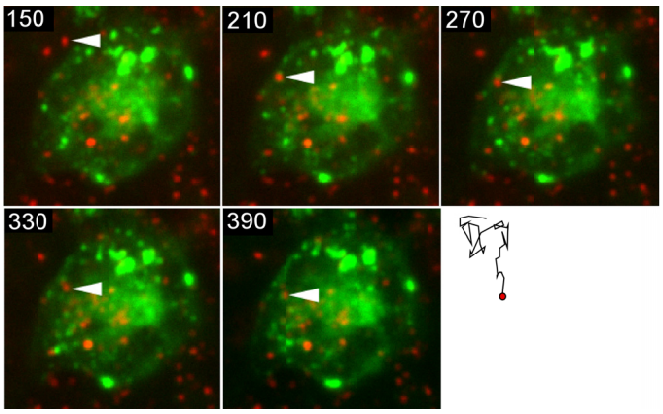

Fig. 2. Tracking of NP attachment to live cells. Recombinant GFP-tagged Rab5 protein (labels early endosomes) was expressed in cells using plasmid transfection. Vesicle movement is seen in a series of frames (times indicated are in seconds and are from start of imaging only) taken of a single representative cell (diameter 15 μm). NPs are red and Rab5+ vesicles are green. Virus membrane coated-NPs had been preincubated with cells for 1 hour. Many NPs were already internalized but a free NP (arrowhead) is seen attaching to (second frame) and entering the cell. The movement of the particle was tracked and its path is shown at lower right. Preceding attachment, rapid motion was observed which ceased after cell contact. The particle then followed a slower, directed path, inward.

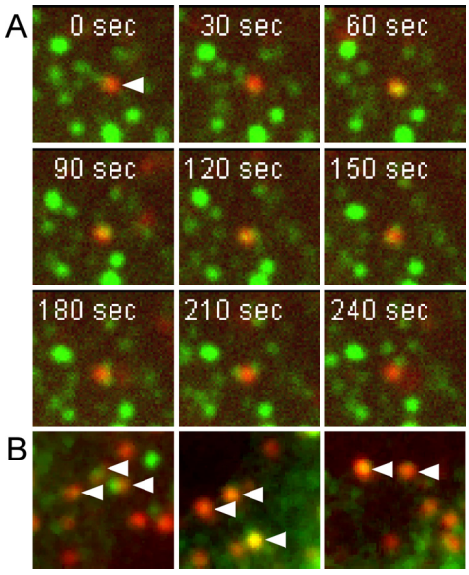

Fig. 3. NP association with Rab5-labeled vesicles. Coated NPs were incubated with cells expressing virus receptor for 1 hour. Cells were then imaged (times given in seconds for image sequence). (A) In multiple cases vesicles labeled with Rab5-GFP (green) were seen associating with NPs. A representative NP is shown (red, indicated by arrowhead) in the sequence. (B) Many NPs appeared to remain stably associated with Rab5 for the duration of the 10 minute sequence. NPs with distinct co-association are indicated by arrowheads.

which the Rab5 separated from the NP or additional Rab5 associated with the NP. Additional markers, such as for the late endosome, will be needed to determine the fate of the NP after Rab5 detachment.

Interestingly, little association was seen with GFP-tagged caveolin. This may be because 293 cells are reported to lack caveolae, and while vesicles were evident, they may not be functional. Alternatively, although caveolae have been associated with mCAT-1 they may not participate in uptake of virus. We are presently testing Hela cells, which have functional caveolae, to help to resolve this issue.

5 DISCUSSION

In this work we tested the potential of NP delivery into the cellular endocytic pathway by coating with virus membranes. MLV have been extensively studied and used as gene therapy vectors [4] since cells lacking receptors are not efficiently infected while receptor positive cells are highly susceptible (virus titers are 10 compared to 10^7 respectively). Ecotropic MLV naturally binds and infects mouse cells. Human cells can be infected after making them express the mCAT-1 protein. Alternatively, the same MLV can infect human cell subsets when the virus envelope protein is modified by introduction of a variety of ligands, including erythropoietin [5], c-Kit [6], or Protein A and antibodies [7]. Harnessing the cell targeting and membrane penetration potential of the MLV envelope protein will greatly assist in design of an efficient drug or vaccine delivery platform.

Since detergents are destructive toward virus envs we have chosen to use a simple and highly scalable technique, where virus is first disrupted by sonication and the purified virus membranes are then coated onto NPs by extrusion. This process mimics the natural budding of viruses from the cell membrane. From our previous work we know that little virus remains in the membrane preparation [2]. For use in the clinic, residual virus could be inactivated by gamma irradiation or unlike many other virus systems, genome-free MLV particles can be generated that are no longer a threat for infection. The latter can be achieved since MLV particles can be made recombinantly and bud without requiring a viral RNA [8].

The mode of membrane association to NPs is not fully understood at this time. We expect that the carboxylic acid motifs used to enhance the hydrophilic properties of the NP may play a role. This may be due to interaction with lipid head groups in the virus membrane or through the virus env c-termini. In future work we will address the mode of interaction by testing the effect of NP surface modification on coating efficiency. This work will involve characterization of NP coats by cryoelectron microscopy.

We show that the extrusion-coating method using MLV membranes successfully targets NPs and delivers them into the cell. This is apparently through a Rab5-dependent pathway. Rab5 is a well characterized marker of the early endosome that acts as a molecular switch controlling endosome trafficking from the cell surface to early endosomes. The kinetics of Rab5 with endocytosed ligands has been heavily studied using epidermal growth factor (EGF). Rab5 transiently associates with EGF containing vesicles, usually remaining attached for 2 min or more [9]. The coated NPs therefore appear to behave as a conventional endocytosed ligand.

The ability of ecotropic MLV envs to access early endosomes and deliver an NP to this compartment was not entirely expected. Originally it was thought that retroviruses like MLV and HIV enter cells at the cell surface, independent of endocytosis. However, recent work by our lab with MLV and by others with HIV, has supported a role for endocytosis [10]. Others have shown an association of the ecotropic MLV receptor with caveolae. However, the strong association of NPs with Rab5 and not caveolin in the present work, indicates that caveolae may not be prominent for virus infection. Instead, an alternative route, either via clathrin-coated vesicles or one of the clathrin/caveolin-independent pathways may be important for bringing virus to early endosomes. The virus membrane coated NPs provide a new tool to define this path in live cells.

At time of submitting this manuscript we do not know if extrusion can be used to coat NPs with membranes of other viruses. We will address this question in the immediate future by looking at several viruses harvested from infected cells as well as viruses made using recombinant technology. These will differ from MLV by receptor usage and cell tropism and comparison of their behavior to the MLV-derived NPs will be important for determining the full potential of this new technology for delivering nanoscale cargoes, drugs and vaccine candidates to specific cells.

REFERENCES

[1] A. Gallina, T.M. Hanley, R. Mandel, M. Trahey, C.C. Broder, G.A. Viglianti, and H.J. Ryser, J Biol Chem 277 50579-88 2002.

[2] D.C. Deniger, A.A. Kolokoltsov, A.C. Moore, T.B. Albrecht, and R.A. Davey, Nano Lett 6 2414-21 2006.

[3] R.L. Roberts, M.A. Barbieri, K.M. Pryse, M. Chua, J.H. Morisaki, and P.D. Stahl, J Cell Sci 112 (Pt 21) 3667-75 1999.

[4] K.W. Culver, W.R. Osborne, A.D. Miller, T.A. Fleisher, M. Berger, W.F. Anderson, and R.M. Blaese, Transplant Proc 23 170-1. 1991.

[5] N. Kasahara, A.M. Dozy, and Y.W. Kan, Science 266 1373-6. 1994.

[6] T. Yajima, T. Kanda, K. Yoshiike, and Y. Kitamura, Hum Gene Ther 9 779-87 1998.

[7] C.K. Tai, C.R. Logg, J.M. Park, W.F. Anderson, M.F. Press, and N. Kasahara, Hum Gene Ther 14 789-802 2003.

[8] D. Muriaux, J. Mirro, K. Nagashima, D. Harvin, and A. Rein, J Virol 76 11405-13 2002.

[9] O.J. Driskell, A. Mironov, V.J. Allan, and P.G. Woodman, Nat Cell Biol 9 113-20 2007.

[10] J. Daecke, O.T. Fackler, M.T. Dittmar, and H.G. Krausslich, J Virol 79 1581-94 2005.