Video Article

# Vascular Gene Transfer from Metallic Stent Surfaces Using Adenoviral Vectors Tethered through Hydrolysable Cross-linkers

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## Abstract

In-stent restenosis presents a major complication of stent-based revascularization procedures widely used to re-establish blood flow through critically narrowed segments of coronary and peripheral arteries. Endovascular stents capable of tunable release of genes with anti-restenotic activity may present an alternative strategy to presently used drug-eluting stents. In order to attain clinical translation, gene-eluting stents must exhibit predictable kinetics of stent-immobilized gene vector release and site-specific transduction of vasculature, while avoiding an excessive inflammatory response typically associated with the polymer coatings used for physical entrapment of the vector. This paper describes a detailed methodology for coatless tethering of adenoviral gene vectors to stents based on a reversible binding of the adenoviral particles to polyallylamine bisphosphonate (PABT)-modified stainless steel surface via hydrolysable cross-linkers (HC). A family of bifunctional (amine- and thiol-reactive) HC with an average $t_{1/2}$ of the in-chain ester hydrolysis ranging between 5 and 50 days were used to link the vector with the stent. The vector immobilization procedure is typically carried out within 9 hr and consists of several steps: 1) incubation of the metal samples in an aqueous solution of PABT (4 hr); 2) deprotection of thiol groups installed in PABT with tris(2-carboxyethyl) phosphine (20 min); 3) expansion of thiol reactive capacity of the metal surface by reacting the samples with polyethyleneimine derivatized with pyridyldithio (PDT) groups (2 hr); 4) conversion of PDT groups to thiols with dithiothreitol (10 min); 5) modification of adenoviruses with HC (1 hr); 6) purification of modified adenoviral particles by size-exclusion column chromatography (15 min) and 7) immobilization of thiol-reactive adenoviral particles on the thiolated steel surface (1 hr). This technique has wide potential applicability beyond stents, by facilitating surface engineering of bioprosthetic devices to enhance their biocompatibility through the substrate-mediated gene delivery to the cells interfacing the implanted foreign material.

## Video Link

The video component of this article can be found at http://www.jove.com/video/51653/

## Introduction

The effectiveness of gene therapy as a therapeutic modality is hampered by the poor targeting capacity of gene therapy vectors[1,2]. The lack of proper targeting results in sub-therapeutic levels of transgene expression at the target location and leads to a wide dissemination of vectors to non-target organs[3], including those responsible for mounting immune responses against both the vector and encoded therapeutic product[4,5]. One potential means to offset the promiscuity of transduction and to promote targeting is to introduce gene vectors at the desired location in a form that precludes their free dissemination via blood and lymph. Typically, such efforts rely on a locally injectable delivery systems comprising of either viral or non-viral vectors admixed with fibrin, collagen or hyaluronic acid hydrogel matrices[6-10] that are capable of transiently sustaining gene vectors at the injection site by physically entrapping them in a polymeric network.

Another generally accepted paradigm for localized gene therapy utilizes immobilization of gene vectors on the surface of implanted prosthetic devices[11,12]. Permanent medical implants (endovascular, bronchial, urological and gastrointestinal stents, pacemakers, artificial joints, surgical and gynecological meshes, *etc.*) are used yearly in tens of millions of patients[13]. While generally effective, these devices are prone to complications that are inadequately controlled for by current medical practices[14-17]. Implantable prosthetic devices present a unique opportunity to serve as proxy platforms for localized gene therapy treatment. From the pharmacokinetic standpoint, surface derivatization of medical implants with relatively low input doses of gene vectors results in achieving both high local concentrations of gene vectors on the implant/tissue interface and slowing the kinetics of their elimination from this location. As a consequence of protracted residence and enhanced uptake by the targeted cell population, vector immobilization minimizes spread of the gene vector. Thus the inadvertent inoculation of non-target tissues is reduced.

Surface tethering of gene vectors on implantable biomaterials (also termed as substrate-mediated gene delivery or solid phase gene delivery) has been implemented in cell culture and animal experiments using both specific (antigen-antibody[18-20], avidin-biotin[21,22]) and non-specific[23-26] (charge, van der Waals) interactions. The covalent attachment of vectors to the surface of the implanted device has been previously considered as non-functional since excessively strong bonds with the surface preclude vector internalization by target cells. Recently it was demonstrated that this limitation can be overcome through the use of spontaneously hydrolysable cross-linker used as the tethers between the modified

 

metallic surface of the stent and capsid proteins of the adenoviral vector[27,28]. Moreover, the vector release rate and time course of transgene expression *in vitro* and *in vivo* can be modulated with the use of hydrolysable cross-linkers exhibiting different kinetics of hydrolysis[28].

The present paper provides a detailed protocol for the reversible covalent attachment of adenoviral vectors to activated metal surface and introduces a useful experimental setup for studying ensuing transduction events *in vitro* in cultured smooth muscle and endothelial cells and *in vivo* in the rat carotid model of stent angioplasty.

## Protocol

## 1. Preparation of Cy3-labeled Adenovirus for the Release Experiments

1. Suspend 2 x $10^{12}$ particles of $Ad_{empty}$ (approximately 2 x $10^{11}$ infective units) in 650 µl of carbonate/bicarbonate buffer (CBB; pH 9.3).
2. Dissolve the content of 1 vial (0.2 mg) of amine-reactive fluorescent dye ($Cy3(NHS)_2$) in 1 ml CBB to a final concentration of 0.2 mg/ml.
3. Add 100 µl of the dye solution to virus suspension, vortex for 5 sec and incubate for 1 hr at 28 °C with shaking (100-200 rpm).
4. Equilibrate a 20 ml Sepharose 6B column with PBS. Add 750 µl of Cy3-labeled Ad suspension drop-wise to the center of the gel bed.
5. After viral suspension permeates the resin, add 5 ml of PBS. Discard the eluate.
6. Add 0.5 ml of PBS and collect the eluate in a labeled glass container. Repeat this step 10x collecting a total of ten 0.5 ml fractions.
7. Assay the collected fractions by spectrophotometry (260 and 280 nm) for viral DNA content.
8. Pool the fractions containing more than 10% of total (sum of F1 through F10) optical density (OD) at 260 nm. Note: Typically the fractions F3, F4, F5, and F6 are pooled and mixed.
9. Re-assay the mixed pooled fractions by spectrophotometry (260 and 280 nm) and assay by fluorometry ($550_{ex}/570_{em}$ nm) for viral DNA content and Cy3 labeled capsid protein, respectively. Note: A $Cy3(NHS)_2$ calibration curve covering the $10^{-9}$-$10^{-13}$ mol/L range is prepared and fluorimetrically assayed on the same plate as the pooled fractions.
10. Calculate the labeled virus yield and labeling density. Assume that 1.19 x $10^{12}$ virus particles/ml corresponds to 1 OD unit for a 1 cm path length[29]. Use the formula: Yield = $[(OD_{260nm}/1.19)*10^{12}*V/\text{input Ad amount}]*100$, where, $OD_{260nm}$ is the optical density of the pooled formulation at 260 nm and V is the volume of the pooled formulation (ml) to calculate Ad recovery yield (%). Use the formula: Labeling $\text{Density}=C*6.02*10^{23}/(OD_{260nm}/1.19)*10^{12}$, where C is Cy3 concentration of the pooled formulation (moles/ml) and $OD_{260nm}$ is the optical density of the pooled formulation at 260 nm to calculate average Cy3 labeling density . NOTE: A typical recovery of labeled virus is >70% of the input dose. The labeling density is 600-800 fluorophore molecules per single virus particle.
11. Aliquot Cy3-labeled $Ad_{empty}$ into smaller portions (typically 5 x $10^{11}$ particles) suitable for the individual release experiments. NOTE: Current protocol specifies the use of Cy3-labeled Ad solely in the release experiment. All transduction studies are carried out with non-labeled vectors.

## 2. Activation of Metal Samples

1. Wash the stainless steel mesh disks or stainless steel stents consecutively in isopropanol (5 min x 2) and chloroform (5 min x 2) at 55 °C with shaking (100 rpm). Remove the solvent.
2. Heat the samples for 30 min at 200 °C.
3. Dissolve polyallylamine bisphosphonate with installed latent thiol groups (PABT[27,28,30]) in water (1-2% w/v) at 72 °C with shaking (100 rpm). Adjust pH to 4.5-5 with $KHCO_3$.
4. Incubate metal samples in the PABT solution for 2-4 hr at 72 °C with shaking (100-200 rpm). NOTE: The procedure may be paused at this point. The samples are stable for 3 days at 4 °C.
5. Rinse the samples thrice with double distilled water (DDW).
6. Expose the samples to tris(2-carboxyethyl) phosphine (TCEP; 15 mg/ml in 0.1 M acetic buffer) for 15 min at 28 °C with shaking (100-200 rpm).
7. Rinse 5x in degassed DDW.
8. Expose the samples to 2% polyethyleneimine with installed pyridyldithio groups ($PEI_{PDT}$[27,28,30]) in degassed DDW in an argon atmosphere for 2 hr at 28 °C with shaking (100-200 rpm). NOTE: The procedure may be paused at this point. The samples are stable for 2 weeks at 4 °C.
9. Rinse the samples three times with DDW.
10. Expose the samples to 10 mg/ml dithiothreitol/DDW to convert pyridyldithio groups to thiols.
11. Rinse 5x again in degassed DDW.

## 3. Adenovirus Activation and Metal Surface Immobilization

1. Suspend 5 x $10^{11}$ particles of either $Ad_{eGFP}$ or $Ad_{Luc}$ (approximately 5 x $10^{10}$ infective units) in 487.5-495 µl of carbonate/bicarbonate buffer (CBB; pH 9.3). Note: For the release studies, use 5 x $10^{11}$ of Cy3 labeled $Ad_{empty}$ particles (adjust volume to 487.5-495 µl with CBB).
2. Dissolve HC with varying hydrolysis rates[28] [(rapidly ($t_{1/2}$ = 5 d), intermediately ($t_{1/2}$ = 12 d) and slowly ($t_{1/2}$ = 50 d) HC, *i.e.* RHC, IHC or SHC; **Figure 1**] or non-hydrolysable cross-linker (NHC), sulfo-LC-SPDP in CBB at 20 mM.
3. Immediately add 5-12.5 µl of cross-linker solution to the virus suspension for a total volume of 500 µl (200-500 µM final concentration of cross-linker), vortex and incubate for 1 hr at 28 °C with shaking (100-200 rpm).
4. Equilibrate a 20 ml Sepharose 6B column with degassed 5 mM EDTA/PBS (EPBS). Add dropwise 500 µl of cross-linker-modified Ad suspension to the center of the resin bed.
5. Add 5 ml of degassed EPBS. Discard the eluate.
6. Add 0.5 ml of degassed EPBS and collect the eluate in a labeled glass container. Repeat this step 10 times collecting a total of ten 0.5-ml fractions.
7. Determine OD of the collected fractions using spectrophotometry at 260 and 280 nm and convert OD to virus titers (1.19 x $10^{12}$/ml corresponds to 1 OD)[29].

8. Pool the fractions containing >10% of the eluted virus (Note: Typically, the fractions F3-F6). Repeat the spectrophotometric titer assay for the pooled suspension.
9. Transfer viral suspension to the vial with the activated metal samples (as per 2.11). Incubate in an argon atmosphere for 1 hr at 28 °C with shaking (100-200 rpm). Note: The Ad-tethered metal samples obtained in this step are further used in the subsequent release and transduction experiments described in the protocol sections 5-7.

## 4. Quantification of Surface-associated Ad Vector by PCR

1. Prepare meshes formulated with surface-immobilized $Ad_{eGFP}$ tethered via NHC, SHC, IHC and RHC (n=3 for each type) as described in sections 2 and 3.
2. Use a QIAamp DNA Micro kit to isolate viral DNA. Place the meshes individually into 1.5-ml plastic tubes containing 200 µl of mixture composed of 180 µl of ATL buffer and 20 µl of proteinase K (both from the kit). Add known amount of $Ad_{eGFP}$ particles (as standards for the calibration curve) into the separate tubes containing the same mixture. Incubate at 56 °C overnight without shaking.
3. Add 200 µl of AL buffer (from the kit), mix by vortexing, add 200 µl of 100% ethanol, and incubate at room temperature for 5 min.
4. Transfer the mixture from each tube onto an individual MinElite column (from the kit) and spin at 8,000 rpm for 1 min. Rinse the mesh-containing tubes with 180 µl of fresh ALT buffer, add onto respective columns and spin at 8,000 rpm for 1 min. Discard the eluates.
5. Add 500 µl of AW1 buffer (from the kit) into the columns and spin at 8,000 rpm for 1 min. Discard the eluates.
6. Add 500 µl of AW2 buffer (from the kit) into the columns and spin at 8,000 rpm for 1 min.
7. Discard the eluates.
8. Spin at 14,000 rpm for an additional 3 min until the columns are completely dry. Discard the eluates.
9. Add 50 µl of MilliQ-grade water into the columns. Spin at 14,000 rpm for 5 min. Collect 50 µl of eluate from each column into individual collection tube (from the kit).
10. Prepare PCR Master Mix solution by combining multiplies of the following (12.5 µl of Power Sybr Green PCR Master Mix, 0.63 µl of 10 µM eGFP sense primer [5'- ACG TAA ACG GCC ACA AGT TC -3'], 0.63 µl of 10 µM eGFP anti-sense primer [5'- AAG TCG TGC TGC TTC ATG TG -3'], 6.3 µl of MilliQ-grade water). Multiply these volumes by the number of planned reactions including "no DNA" controls.
11. Load 5 µl of $Ad_{eGFP}$ DNA (from step 4.8) and 20 µl of PCR Master Mix into triplicate wells of a MicroAmp Optical 96-well Reaction plate. Seal the plate with MicroAmp Optical Adhesive Film. Spin the plate at 1,000 rpm for 1 min to eliminate air bubbles.
12. Place the plate into the receptacle of a 7500 Real-Time PCR engine. In the main menu of the 7500 System SDS Software (v1.4 or higher) select "Create new experiment". Click "next". In the "New experiment wizard" screen choose Sybr Green from a scroll-down menu and click "add". Click "next" to get a layout of the plate. Highlight all wells to be analyzed, select Sybr Green. Highlight consecutively no DNA control wells, $Ad_{eGFP}$ DNA standards and unknowns, and mark them using respective designations from the scroll-down menu.
13. Switch to the instrument tab. Select "add dissociation phase", change the well volume from default 50 µl to 25 µl. Click start.
14. Analyze PCR results using after checking the QC summary for outliers and other irregularities.

## 5. Release Kinetics of Hydrolysable Cross-linker-tethered Vector Particles from the Model Steel Mesh

1. Wash the mesh samples derivatized with Cy3-labeled Ad via RHC, IHC, SHC and NHC (as per 3.9) in 1% BSA/PBS (1 hr x 3) with shaking (100-200 rpm).
2. Using sterile fine forceps, place the meshes into individual wells of a 96-well plate prefilled with 200 µl of elution buffer (0.1% BSA/0.1% Tween-20/PBS).
3. Take fluorescent images of a central part of each mesh. Record the settings of the microscope and the CCD camera used for image acquisition.
4. Assay the plate fluorimetrically ($550_{ex}/570_{em}$) in well-scan mode with maximum reduction. Use the wells with non-derivatized meshes as a background control.
5. Incubate the plate at 37 °C with shaking (50 rpm).
6. At predetermined times (1-30 days range) aspirate the elution buffer without disturbing the meshes and add 200 µl of fresh elution buffer. Repeat steps 4.3-4.5 after replacing the buffer.

## 6. Transduction of Cultured Cells by Mesh-immobilized Ad Vectors

1. Wash the $Ad_{eGFP}$- or $Ad_{Luc}$-derivatized meshes thrice with sterile PBS for 5 min with shaking (100 rpm).
2. Using fine sterile forceps remove the mesh disks one by one and individually place them into the wells of a 96-well plate with the cell type of interest in the log phase of growth.
3. Incubate the cells for 24 hr at 37 °C, in 5% $CO_2$.
4. Use the respective non-terminal endpoint assay (fluorescent microscopy, fluorometry for eGFP or bioluminescence imaging for luciferase) to determine the extent and the spatial distribution of gene expression in the wells.
5. Optionally, substitute medium for PBS to increase sensitivity of the fluorometry assay (485/535 nm) if low eGFP expression is expected. Note: If a fluorometer is equipped with well-scan capability, read the plate in a well-scan mode to assess the spatial distribution of eGFP-expressing cells in the wells. Exchange PBS for medium after completing fluorometry.
6. Image the transduced cells in the wells with $Ad_{eGFP}$-eluting meshes (both underlying and outlying the mesh) using the FITC filter set. Take representative images at 40-200X magnification. Record the exact settings of the fluorescent microscope and the CCD camera used for the acquisition of images.
7. Add 5 µl of luciferin stock in PBS (10 mg/ml) directly to wells with $Ad_{Luc}$-eluting meshes to a final concentration of 500 µg/ml and incubate at 37 °C and 5% $CO_2$ for 10 min prior to bioluminescence imaging. Aspirate media and replace with luciferin-free media after imaging.

8. Repeat endpoint assays at predetermined times (up to 2 weeks) to study the kinetics of reporter gene expression following substrate-mediated gene transfer.

## 7. Validation of Preserved Transduction Capacity at Delayed Time Points

1. Prepare the $Ad_{eGFP}$ derivatized meshes using RHC, IHC, SHC and NHC for vector tethering (as per 2.1-2.11 and 3.1-3.9) and individually place the meshes in the wells of a 96-well plate with 60-80% confluent bovine aortic endothelial cells (BAEC).
2. Analyze transduction of cultured BAEC with mesh-immobilized $Ad_{eGFP}$ at 1 and 2 days post-mesh placement using fluorescence microscopy and fluorometry (as per 6.4-6.5).
3. 48 hr after commencement of transduction wash the mesh-containing wells with PBS twice.
4. Add 200 µl of 0.25% trypsin/EDTA to each well and incubate for 15 min at 37 °C with shaking (100 rpm). Wash 3x with PBS.
5. Use fluorescent microscopy and fluorometry to ascertain complete removal of all eGFP-positive cells associated with the meshes.
6. Seed freshly passaged BAEC into the wells with partially released meshes at a 60-80% initial seeding density (2-2.7 x $10^4$ cells/well).
7. Incubate the plate at 37 °C and 5% $CO_2$ for predetermined periods of time (1-10 days) prior to assessment of “new” transduction events by fluorescent microscopy and fluorometry.

## 8. Ad-eluting Stent Deployment in the Rat Carotid Model of Stent Angioplasty

1. All animal procedures described in this protocol conform to Federal regulations on laboratory animal use and were approved by the IACUC of the Children’s Hospital of Philadelphia. To adhere to aseptic surgical conditions all instruments are autoclave sterilized. To assure continuous sterility a bead sterilizer is employed between use of the instruments in up to five consecutive animals.
2. Prepare $Ad_{Luc}$-eluting stents according to 2.1-2.11 and 3.1-3.9. Store the virus-derivatized stents in sterile PBS at 4 °C for no longer than 24 hr prior to use.
3. Anesthetize male Sprague-Dawley rats (400-450 g) with IP injection of ketamine (100 mg/kg), and xylazine (5 mg/kg). Determine the depth of anesthesia by paw pinch response and muscle tonus. Apply ophthalmic vet ointment to prevent dryness of cornea and sclera. Note: The inhalation anesthesia with 4% and 2% isoflurane (1 L/min) for inducing and maintaining anesthesia, respectively, is possible but impedes free access to the neck region of the animal and thus is not recommended for an inexperienced user.
4. Reassess depth of anesthesia by toe pinch. Shave and aseptically prep neck and upper chest region. Administer antibiotic (cefazolin; 20 mg/kg; IM), analgesic (meloxicam; 0.5 mg/kg; SC) and saline (10 ml/kg; SC). Catheterize the tail vein with a 24 G catheter and administer heparin (200 IU/kg; IV). Note: A dose of 10 mg/kg (IM) enrofloxacin (Baytril) can be used instead of cefazolin. Avoid use of antibiotics lacking significant activity against Gram-positive bacteria. 10-15 mg/kg carprofen (Rimadyl) may be used instead of meloxicam as a preemptive analgesic. Narcotic analgesics (*e.g.*, morphine, buprenorphine) should be avoided because of their respiration-depressing properties.
5. Perform a midline incision through the skin and neck fascia. Use blunt dissection techniques to isolate the left external carotid artery. Tie-off the external carotid artery at the most distal approachable site. Apply a sliding temporary ligature to the origin of the internal carotid artery.
6. Make a 2-mm arteriotomy incision in the left external carotid artery.
7. Insert a 2-French Fogarty catheter into the common carotid artery through the incision in the external carotid artery. Inflate the tip of catheter with saline and pass 3x from the aortic arch to the carotid bifurcation in order to denude the endothelium.
8. Slide a piece of tubing (1.04 mm OD, 0.99 mm ID) over the Fogarty catheter and into the common carotid artery. Withdraw the Fogarty catheter.
9. Mount and crimp an $Ad_{Luc}$-derivatized stent over the balloon of a 1.5 mm diameter angioplasty catheter. Insert the stent through the Teflon tubing and advance it into the mid-section of the common carotid artery. Avoid rubbing the stent against the tube or vessel wall.
10. Deploy the stent at 12 atm for 30 sec and withdraw the angioplasty catheter.
11. Tie-off the external carotid artery proximal to the arteriotomy site and release the temporary ligature on the internal carotid artery.
12. Repair the operative wound in layers with running 4.0 Vicryl suture and staple the skin.
13. Recover the animal on a warming pad until ambulatory and return to its isolated cage. While no signs of pain or discomfort are typically exhibited by the post-operative animals beyond the first 12 hr after the procedure, consider extension of meloxicam therapy (0.5 mg/kg, SC daily) for 72 hr.

## 9. Bioluminescence Imaging of Arterial Gene Expression

1. At predetermined time points (1 day – 3 weeks range) after gene-eluting stent deployment in the common carotid artery, anesthetize the rat using isoflurane inhalation anesthesia (2-4% isoflurane in oxygen).
2. Remove the surgical staples, aseptically prepare the site and reopen the operative wound. Using blunt dissection, re-gain access to the left common carotid artery and separate it from the vagus nerve and adjacent connective tissue.
3. Prepare a mixture of 50 mg/ml Luciferin in PBS and 25% Pluronic F-127 in PBS (1:4 v/v) and store it on ice. Note: This formulation presents as a viscous solution at 4 °C and immediately turns to gel upon contact with tissue at 37 °C.
4. Apply 200 µl of a chilled Luciferin/Pluronic mixture directly to the exposed segment of the common carotid artery and verify gel solidity.
5. Place the animal in the supine position in the imaging chamber of the IVIS-Spectrum apparatus and maintain isoflurane anesthesia with a face mask.
6. In the acquisition control panel window (Living Image, version 4.2 or higher) choose the position “B” (6.6 cm camera to object distance) and binning factor “medium” from the dropdown menus entitled “field of view” and “binning”, respectively. Type in “2.5 cm” in the subject height box. Choose “min” as a unit of time in the “exposure time” box, and choose a numerical value of “2”.
7. Three min after application of the Luciferin/Pluronic gel, start image acquisition by clicking the “Acquire” button on the screen. NOTE: Image acquisition time can vary from 1 to 6 min depending on the anticipated signal strength.
8. After acquiring the image, wash the gel off with saline and dab the periarterial space with sterile gauze and cotton applicators.
9. Close the wound with Vicryl suture and staple the skin.
10. Recover the animal and return to its cage. Repeat imaging at later time points to study the time course of arterial expression brought about by the stent-immobilized Ad vectors.

11. Alternatively, perform imaging following a systemic administration of luciferin. Anesthetize and prep the animal as per 9.1-9.2. Catheterize the tail vein with a 24 G catheter and secure catheter with surgical tape.
12. Prepare a solution of luciferin in PBS (50 mg/ml) and inject 1 ml of the solution through the catheter over a 10 sec interval. One min after injection start image acquisition as per 9.7-9.8. Note: A faster injection rate (<10 sec) can provoke seizure activity and respiratory arrest. The animal must be euthanized if seizures or respiratory arrest occur during imaging.
13. Follow step 9.10.

## Representative Results

### Vector Release Experiments

Tethering of adenoviral vectors to the surface of implants, including interventional devices such as endovascular stents, approximates the vector to the disease site, partially obviating the lack of vectors' physical targeting. However, to be able to achieve therapeutic effects via the transduction of target tissue, the vector must be released from the surface (**Figure 2**). The use of hydrolysable cross-linkers was hypothesized to allow 1) effective attachment of vectors to polybisphosphonate-modified, thiol-installed metal implants and 2) sustained release mediated by hydrolysis of the cross-linker.

The number of genomic copies of $Ad_{eGFP}$ vector associated with the mesh disks by the end of the derivatization procedure is determined by PCR of viral DNA with eGFP-specific primers (**Figure 3**). Depending on the specific cross-linker used, the amount of bound Ad vector should vary between $3.5 \times 10^9$ and $5.7 \times 10^9$ particles/mesh. This amount is in fair correspondence with the estimated maximal capacity ($2.5 \times 10^9$) of a single mesh disk with a total area of 0.25 $cm^2$ to accommodate 100-nm Ad particles in a monolayer arrangement. Since the starting amount of Ad vector at the step of cross-linker modification is $5 \times 10^{11}$ particles, only ~1% of the modified vector is eventually immobilized on the PABT/$PEI_{PDT}$-derivatized stainless steel surface.

Both the rate of in-chain ester hydrolysis and the number of links between the vector and the biomaterial are expected to affect the overall kinetics of Ad vector release from the surface.

To facilitate the release experiments, adenoviral particles can be pre-labeled with Cy3 fluorophore (Protocol; section 1) prior to cross-linker modification and surface immobilization (Protocol; section 3). The decrease of surface-associated fluorescence over time assessed concurrently by fluorometry (**Figure 4A**) and fluorescence microscopy (**Figure 4B**) can then be used as an indirect method for monitoring vector release into physiological buffer. Ad vectors attached through RHC and IHC linkages should demonstrate significantly faster release in comparison with their SHC- and NHC-attached counterparts. In the given example, a 45% and a 39% loss of surface-associated vector was observed with RHC and IHC-tethered vector by day 30, respectively, while less than 20% of tethered vector was observed to be released in SHC and NHC-immobilized samples (**Figure 4A**).

Additionally, Ad vectors immobilized by using different concentrations of the same HC and thus presumably having differing numbers of tethers between the vector and metal substrate should have dissimilar kinetics of vector release. Indeed, vector modification using 0.1 mM RHC resulted in significantly faster release rates than observed for vector immobilized with 0.5 mM RHC (**Figure 4A**). The fluorescence microscopy data (**Figure 4B**) correlates well with the fluorometry-based quantitative vector release analysis, demonstrating a faster and more profound decrease of surface-associated fluorescence with mesh samples formulated using IHC-, RHC- and especially low concentration RHC-tethered vectors in comparison with their NHC- and SHC-tethered counterparts.

### *In vitro* Transduction with Mesh Immobilized Ad Vectors

By being compatible with both quantitative expression analysis (fluorometry) and spatial observations (fluorescence microscopy), $Ad_{eGFP}$ is the preferred reporter vector to monitor transduction in SMC and endothelial cell cultures treated with both free and mesh-immobilized Ad vectors. Chemical modification of Ad capsid with RHC (**Figure 5**) as well as with other cross-linkers (not shown) at concentrations exceeding 75 µM significantly impairs transduction effectiveness of non-immobilized vector *in vitro*, most probably due to masking and reconfiguring of fiber knob domains utilized by Ad for binding to the cells through the Coxsackie-Adenovirus receptors (CAR). However, the role of knob-CAR interactions is less vital for the transduction with substrate immobilized vector since the vector is retained in the vicinity of targeted cells by tethering to substrate. In cell culture experiments regardless of the HC type, mesh-associated Ad vectors are consistently capable of spatially restricting transduction events to cells located within 300-500 µm from the mesh borders (**Figures 6A** and **6C**). Typically the peak levels of transgene expression are achieved 3-5 days after the placement of $Ad_{eGFP}$-loaded meshes into SMC (**Figures 6A** and **6B**) or BAEC (**Figures 6C** and **6D**) cultures. At the same vector load, peak eGFP expression levels increase in the following sequence: NHC-, SHC-, IHC- and RHC-tethered vector, reflecting the differences in their respective release kinetics profiles (**Figure 4**). Furthermore, more durable transgene expression is observed in cells treated with IHC-formulated meshes in comparison to cells treated with the RHC-formulated counterparts (**Figure 6D**).

The utilized cell culture system presents a convenient set-up for the investigation of delayed transduction events brought about by vector particles released from the metal surface at or later than 48 hr after mesh placement. In this application, after determining eGFP expression by fluorometry and fluorescence microscopy at 48 hr after commencement of transduction, BAEC are trypsinized and aspirated out of the wells without disturbing the meshes. Freshly passaged non-transduced BAEC are then re-plated over the partially released meshes. "New" transduction events caused by the virus particles released from the mesh carrier after the 2 day time point are then assessed by fluorescence microscopy and fluorometry (**Figures 7A** and **7B**). Robust eGFP expression demonstrating the characteristic spatial restriction to a mesh locale is typically observed in these "new" cultures (**Figure 7**), confirming a well-preserved transduction capacity of the mesh-bound Ad vectors even after 48 hr exposure to complete cell culture medium at 37 °C.

### *In vivo* Studies

To examine potential effects related to the use of different HC on arterial transduction, animals implanted with $Ad_{Luc}$-eluting stents prepared with RHC-, IHC-, SHC- and NHC-modified vector underwent bioluminescent imaging 1 and 8 days after stent deployment (**Figure 8**). The imaging

was carried out following local perivascular administration of luciferin (2 mg) co-formulated with Pluronic gel. This formulation is a viscous fluid when chilled on ice, but undergoes immediate phase transition to gel when brought in contact with tissue at 37 °C. Preliminary experiments (not shown) demonstrated that perivascular delivery of luciferin results in more stable and reproducible luminescence signals in $Ad_{Luc}$-transduced vascular tissue when compared to systemic (intraperitoneal or intravenous) luciferin administration.

If virus tethering to the stent and the stent deployment surgery were technically sound, a well-defined signal corresponding to the stented segment of rat carotid artery is emitted and recorded. One day after stent implantation, animals treated with RHC-formulated $Ad_{Luc}$ stents typically exhibit the highest luminescence signal, followed by the rats receiving IHC- and SHC- formulated stents (**Figures 8A** and **8B**). No perceptible signal is observed in the group of rats implanted with stents prepared using NHC-tethered $Ad_{Luc}$, underscoring the importance of unimpeded vector release from the stent for effective transduction of vascular tissue (**Figures 8A** and **8B**). By day 8, the average intensity of luciferase expression with RHC-formulated stents drops several-fold, while it increases 1.4- and 1.8-fold with IHC- and SHC-formulated stents, respectively (**Figures 8A** and **8B**). This finding is consistent with the hypothesis of the more durable vascular transduction with gene-eluting stents exhibiting a slower kinetics of vector release from the stent surface.

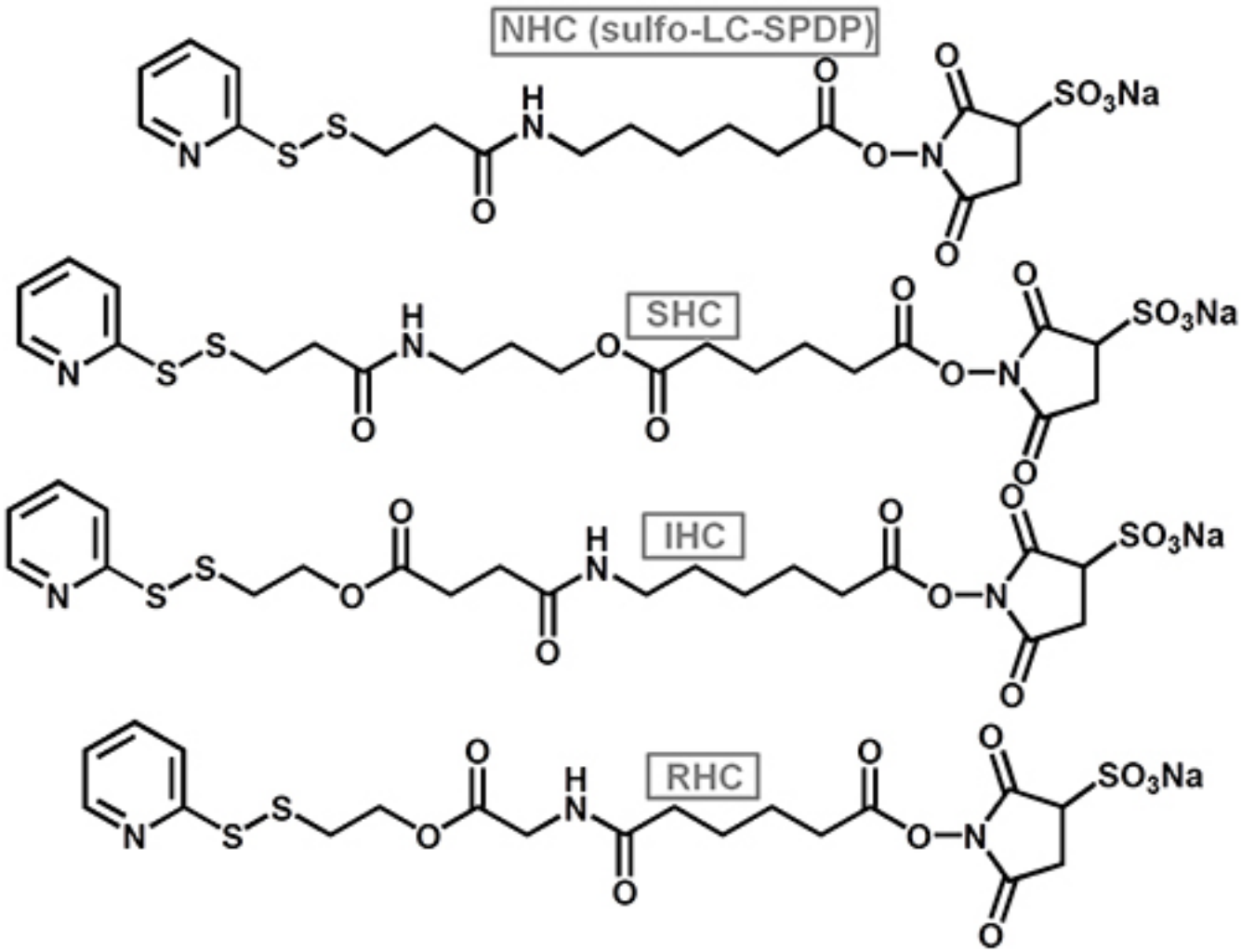


**Figure 1. Structural formulas of cross-linkers (NHC, SHC, IHC and RHC) used throughout the described studies** (modified with permission[28]).

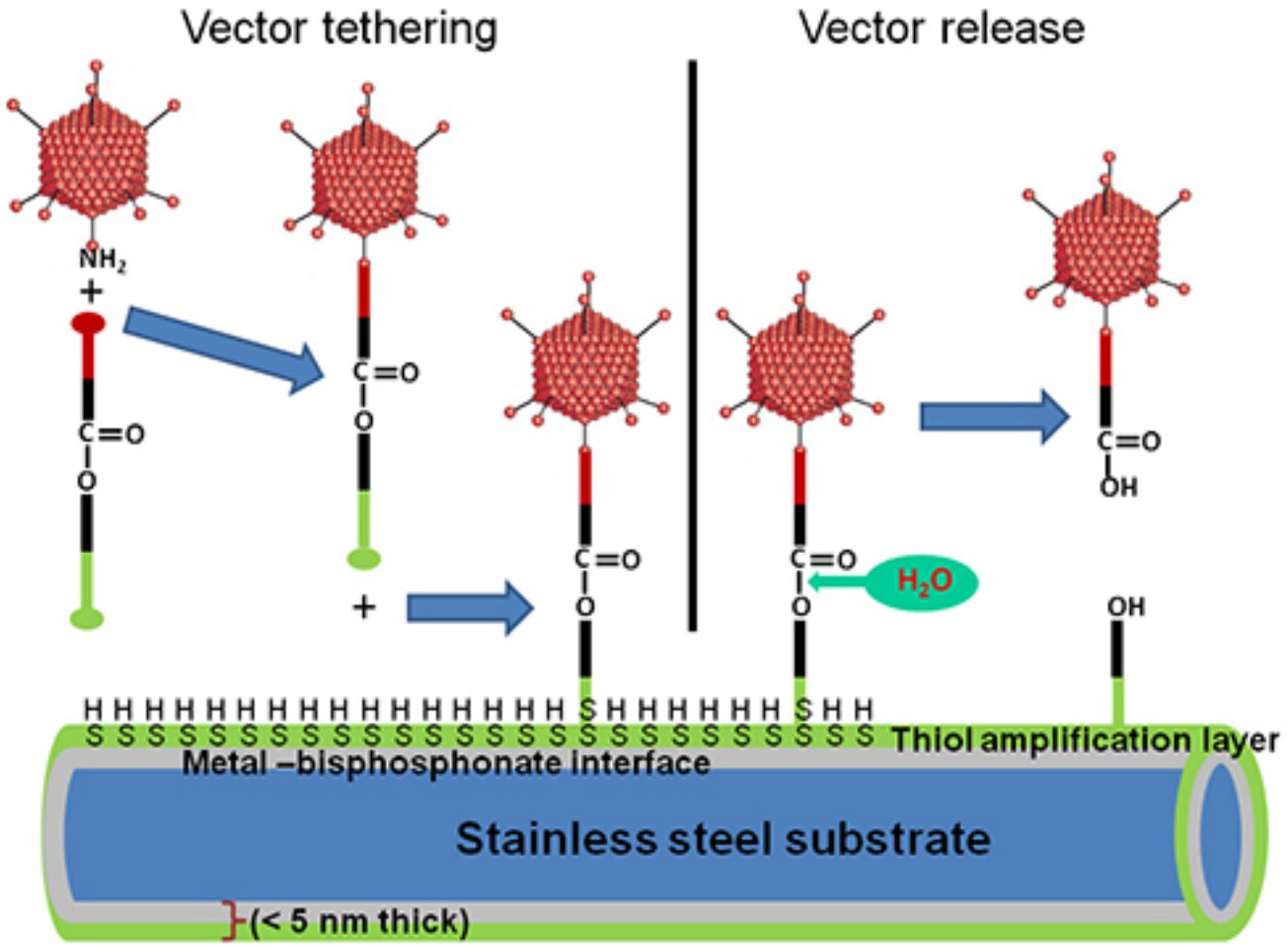


**Figure 2. A scheme representing Ad vector tethering to a PABT/PEI$_{PDT}$-modified stainless steel surface via a HC and subsequent release of the vector upon cross-linker hydrolysis** (modified with permission[28]).

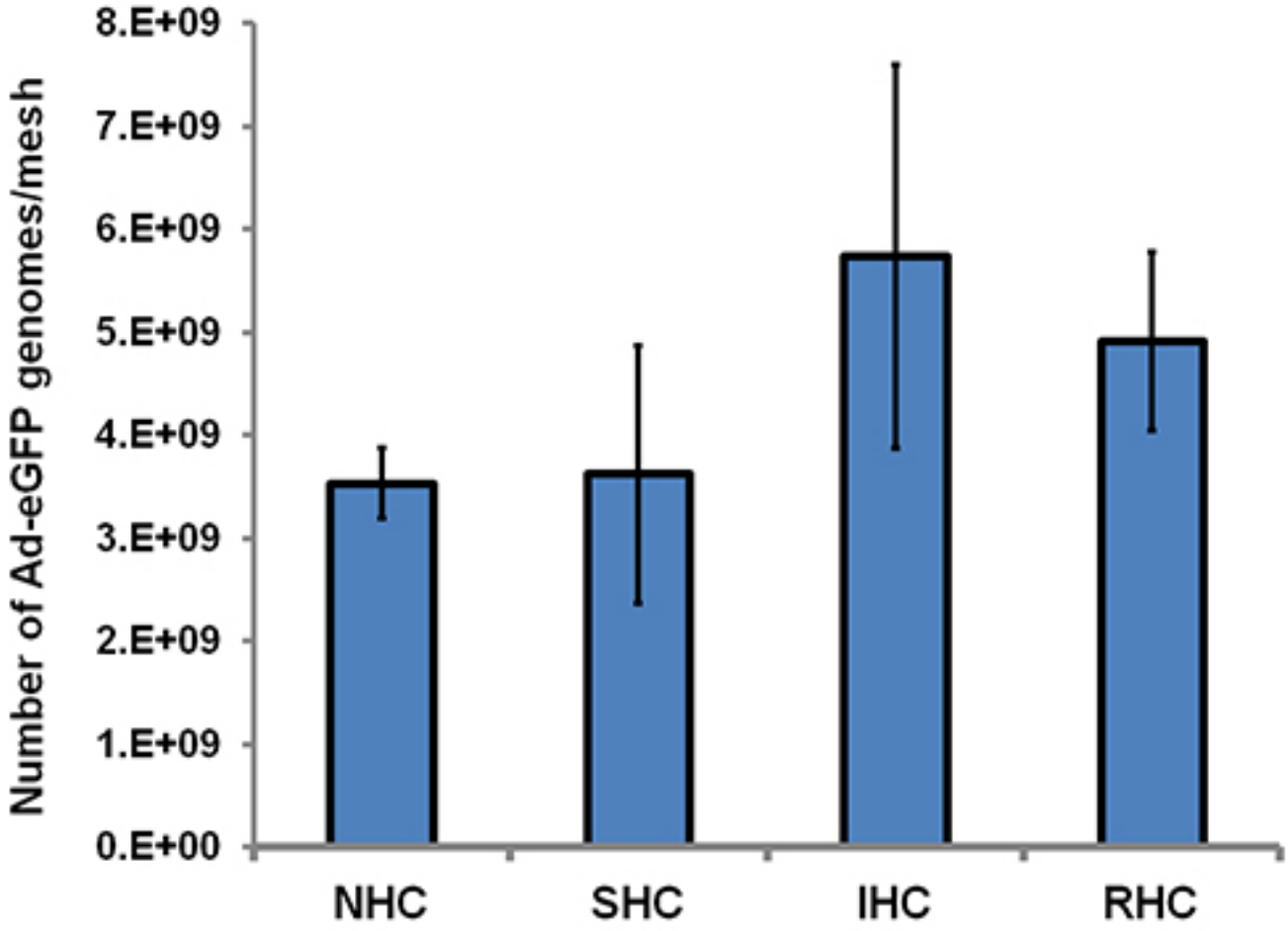


**Figure 3. PCR-based quantification of $Ad_{eGFP}$ immobilized via cross-linker tethering on the surface of $PABT/PEI_{PDT}$-modified stainless steel meshes.** The stainless steel meshes were formulated with $Ad_{eGFP}$ immobilized via RHC, IHC, SHC, or NHC (n = 3 for each condition). After proteinase K treatment, viral DNA was eluted and purified using MinElute columns. Amplification of viral DNA using eGFP-specific primers was carried out in a 7500 Real-Time PCR engine and was detected with Sybr Green. Data normalization was based on a calibration curve prepared with a known amount of non-immobilized $Ad_{eGFP}$(modified with permission[28]).

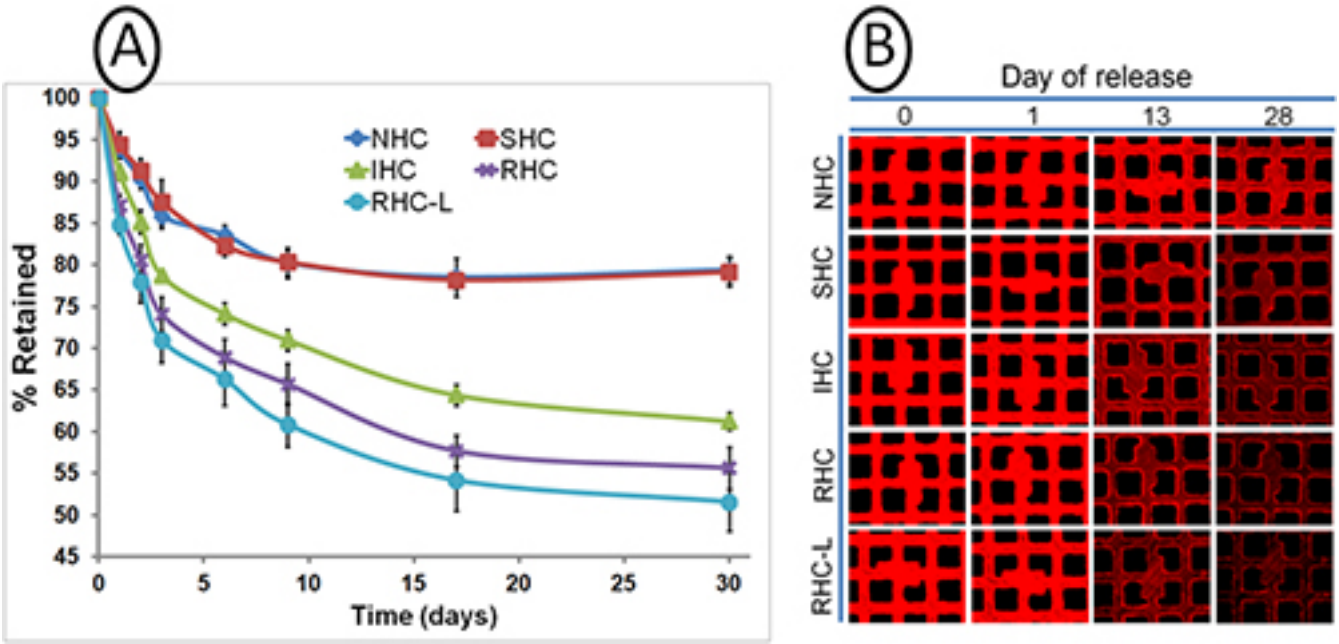


**Figure 4. Release kinetics of Ad vector tethered to metal substrates with HC.** Stainless steel meshes were derivatized with ~2 x $10^9$ Cy3-labeled Ad particles attached to the PABT/$PEI_{PDT}$-modified surface after modification with 500 µM NHC, SHC, IHC, RHC and 100 µM RHC (designated as RHC-L). The release of fluorescently labeled Ad-particles was studied at the indicated time points by (**A**) well-scan fluorometry at 550/570 nm and (**B**) fluorescent microscopy (rhodamine filter set; original magnification 200X). Fluorometry results are presented as means ± SEM, *n* = 8-10; $p < 0.001$ for all comparisons between the NHC and SHC vs IHC, RHC and RHC-L groups were determined by Anova with a post-hoc Tukey test (modified with permission[28]).

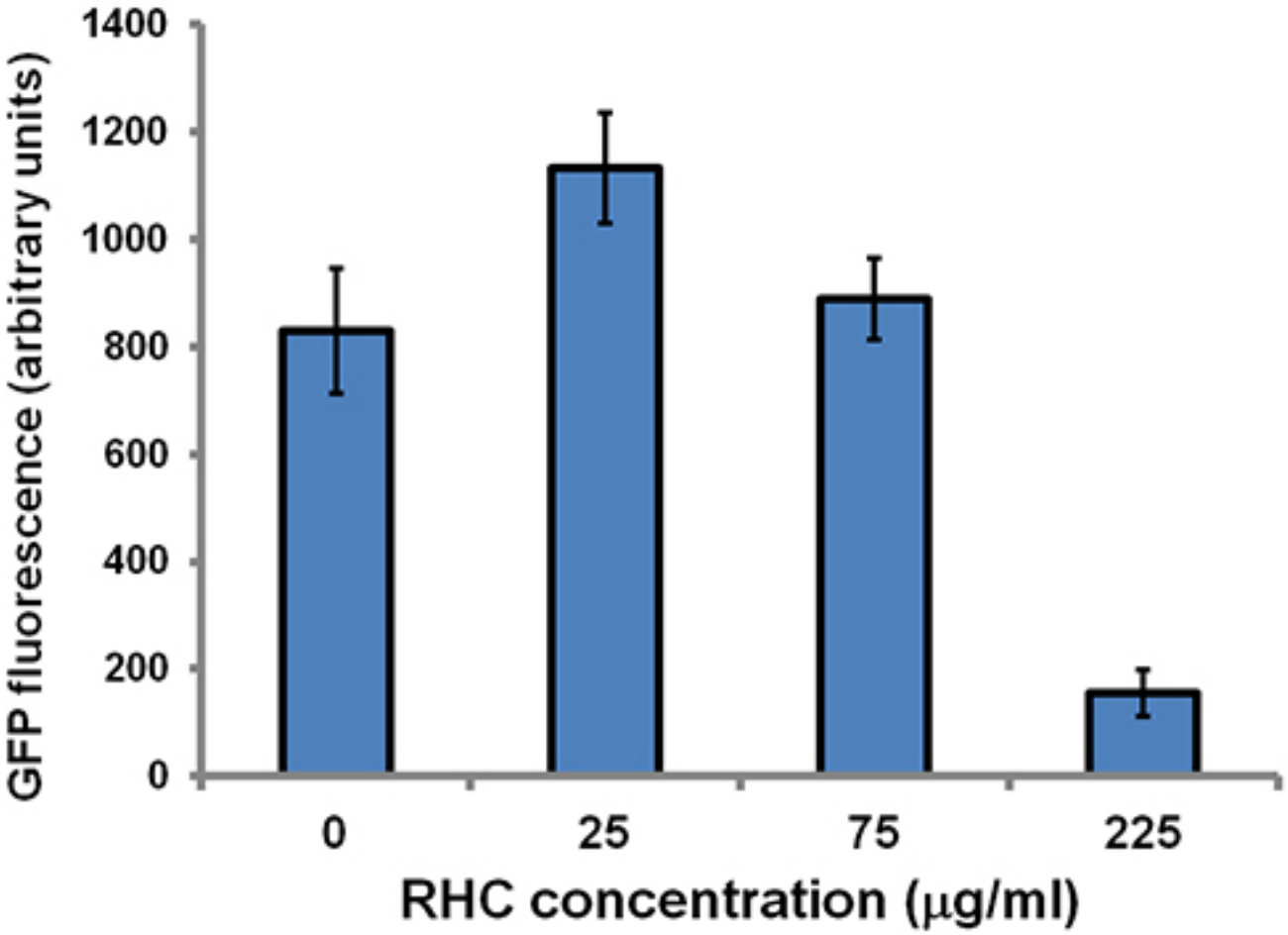


**Figure 5. Transduction effectiveness of non-immobilized $Ad_{eGFP}$ following modification with RHC.** Rat embryonic aorta-derived SMC (A10 line) were transduced at a MOI of 1,000 with either unmodified $Ad_{eGFP}$ or vector modified with RHC as indicated. A reporter expression was determined fluorometrically (485/535 nm) 48 hr after transduction (modified with permission[27]).

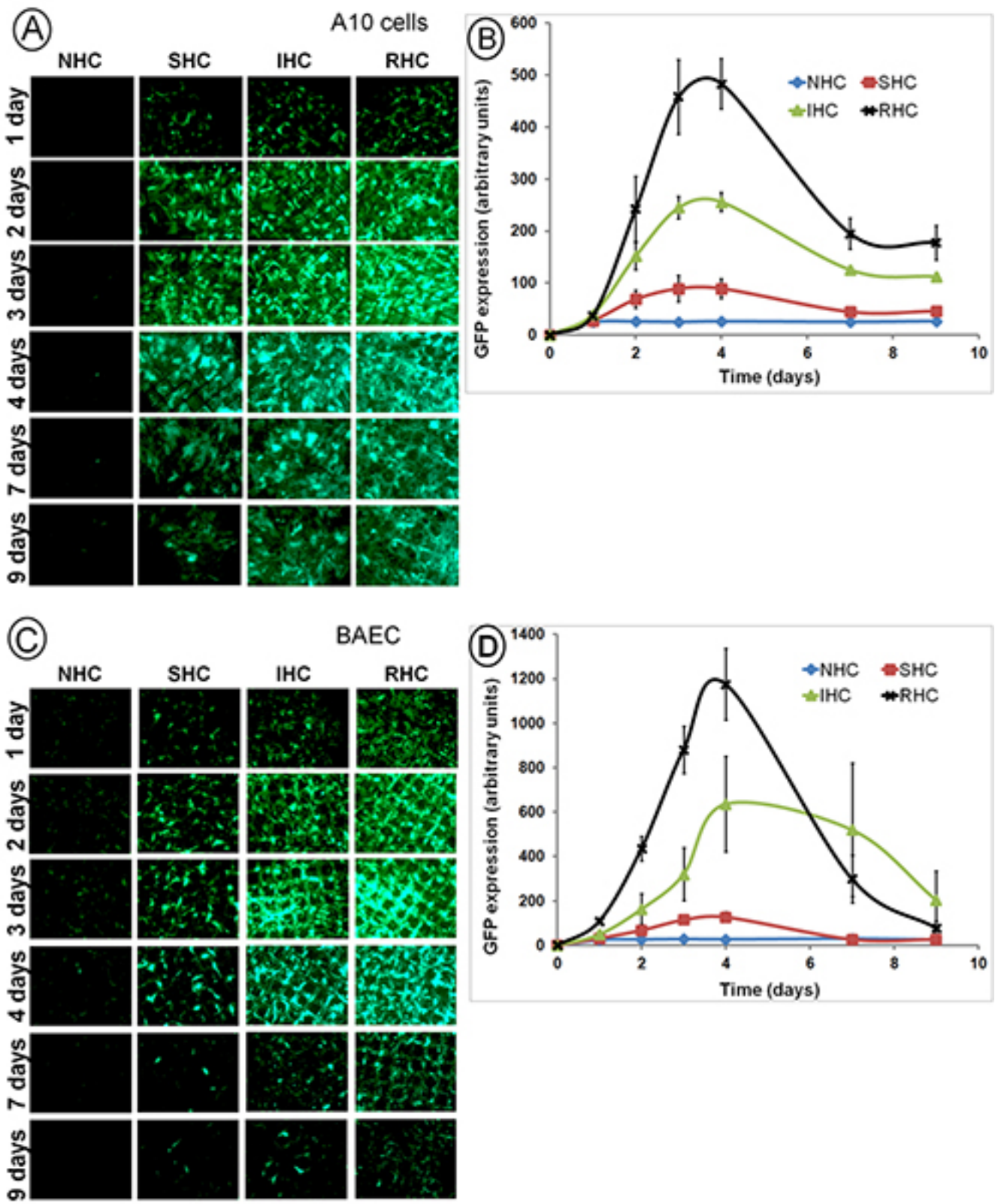


**Figure 6. Transduction of cultured SMC and BAEC with Ad vector immobilized to stainless steel meshes with HC.** Meshes were derivatized with ~2 x $10^9$ $Ad_{eGFP}$ particles appended via NHC, SHC, IHC, and RHC. Meshes were then individually placed on top of sub-confluent A10 (**A**, **B**) and BAEC (**C**, **D**) monolayers. Transduction (expressed as eGFP expression levels) of cells treated with Ad vector-tethered meshes was assessed by fluorescence microscopy (**A**, **C**; FITC filter set; original magnification 100X) and well-scan fluorometry (**B**, **D**). Fluorometry results are presented as means ± SEM, *n* = 4 (modified with permission[28]). Please click here to view a larger version of this figure.

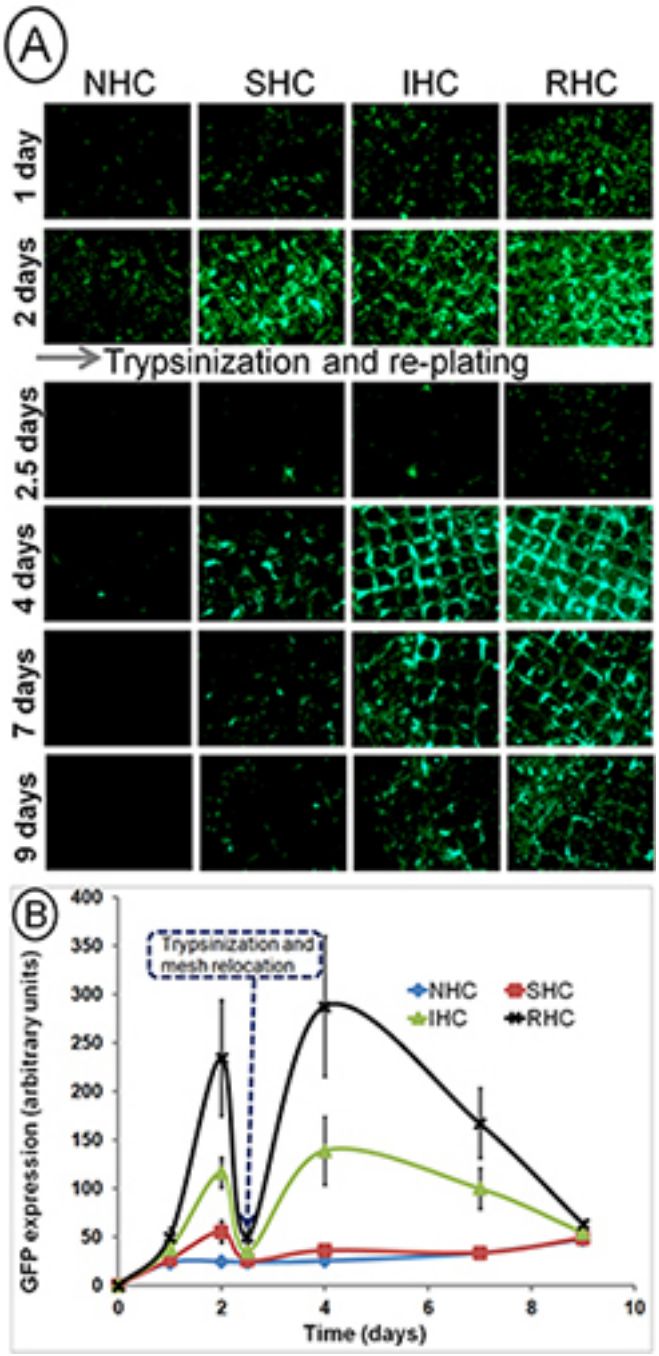


**Figure 7. Transduction competence of substrate immobilized Ad vectors at delayed time points.** $Ad_{eGFP}$ was immobilized on steel meshes through NHC, SHC, IHC or RHC tethers. The meshes were placed on the subconfluent BAEC monolayers. Two days after placement, BAEC were trypsinized and removed without disturbing the meshes. New, non-transduced BAEC were then seeded over the meshes. $Ad_{eGFP}$ transduction competency was measured by eGFP expression using fluorescent microscopy (**A**; FITC filter set; original magnification 100X) and fluorometry (**B**). Measurements were taken at indicated days, representative images were used and fluorometry results are means ± SEM, *n* = 4 (modified from with permission[28]). Please click here to view a larger version of this figure.

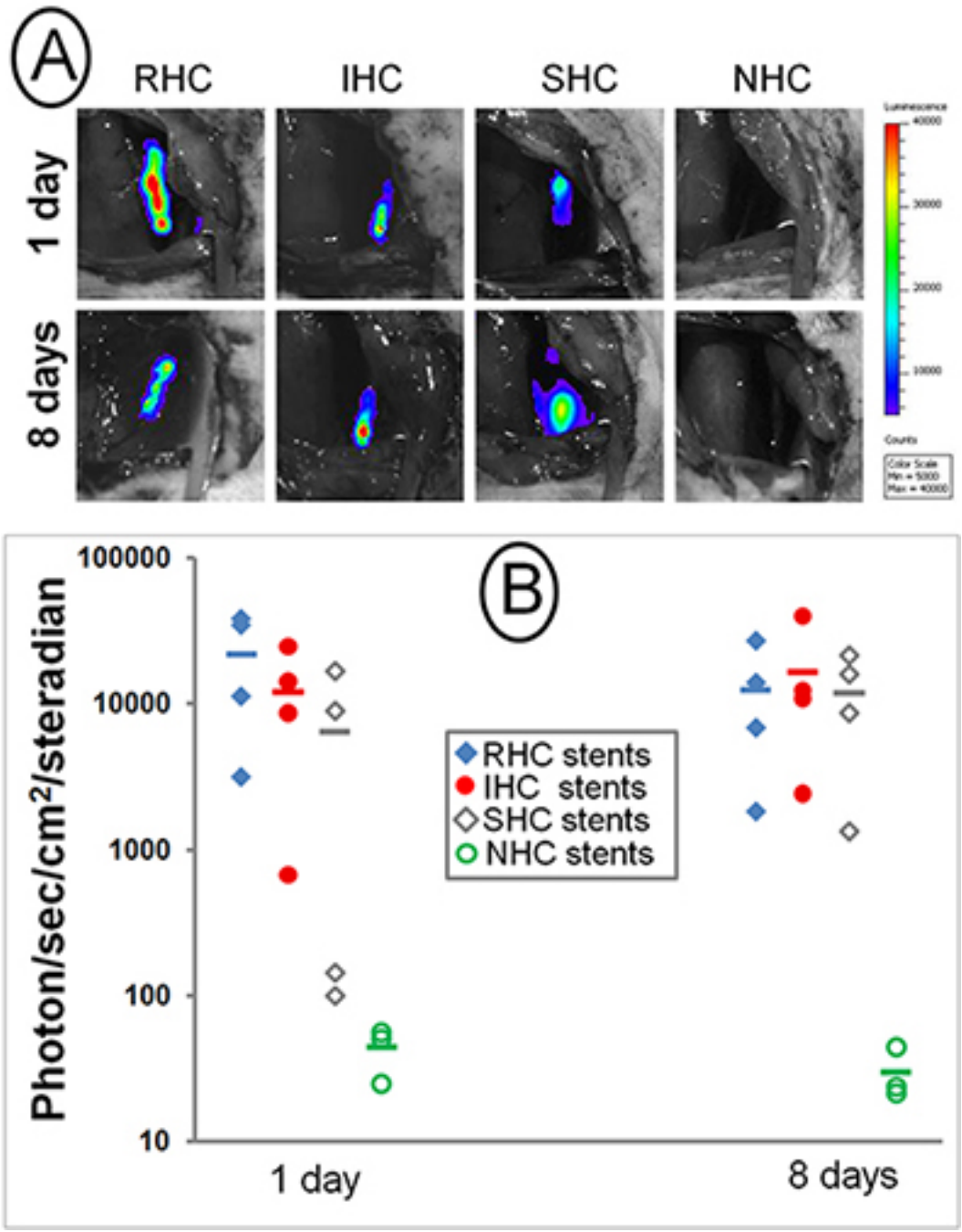


**Figure 8.Transduction efficiency of stent-immobilized $Ad_{Luc}$ *in vivo*.** $Ad_{Luc}$ ($1.3 \times 10^{10}$ particles) was tethered to endovascular stents via NHC, SHC, IHC or RHC (n = 3-4 for all groups). Transduction efficiency of $Ad_{Luc}$ eluted from stents was measured by bioluminescence imaging (IVIS Spectrum) 1 and 8 days post stent deployment (**A**), and plotted using a logarithmic scale (**B**) (modified from with permission[28]). Please click here to view a larger version of this figure.

## Discussion

The presented protocol describes an operational method for substrate mediated gene delivery achieved through reversible attachment of adenoviral vectors to coatless stainless steel surfaces. While developed for the specific purpose of stent-based gene therapy of vascular restenosis, this technique has much broader applications in the areas of biomaterials, biomedical implants and gene therapy.

Although presented studies have solely utilized stainless steel as a prototypical metal substrate, PABT binds other metal alloys such as cobalt/chromium and nitinol with comparable affinity (not published). The indiscriminant interaction of PABT with metals significantly expands the number of potential biomedical applications for this technology[31]. Moreover, the use of HC-tethered gene vectors, albeit requiring other methods of thiol installation onto material surface, is feasible with other types of biomaterials.

While Ad gene vectors achieve robust transduction in many human cell types and tissues, their clinical significance is limited by strong inflammatory and immune responses elicited by *Adenoviridae*[4]. To this end, prolonged (4 months) arterial expression with gene eluting stents formulated using HC modification of luciferase-expressing adeno-associated viral vectors was recently shown (not published).

The methodology is robust. Small deviations from the main protocol do not generally lead to significant alterations of gene expression *in vitro* and *in vivo*. Nevertheless, several critical issues that may affect outcomes were identified.

First, as the name suggests, hydrolysable cross-linkers are cleavable upon reaction with water due to hydrolysis of the in-chain ester. Moreover, the amine-reactive moiety, *N*-sulfosuccinimidyl is hydrolysable as well. Cross-linkers should be stored under an argon atmosphere at -20 °C to preserve their activity. For the same reasons, after preparing solutions of HC (protocol section 3.2) proceed immediately with the addition of HC solutions to the virus preps.

Second, thiol groups that are formed on the metal surface during several intermediate steps of the presented bioconjugation sequence are rapidly oxidized by ambient air oxygen. To prevent this, keep the vulnerable metal samples submerged in degassed water at all times.

Third, a perfect alignment of the mesh with the bottom of the well is required for successful transduction. Do not bend mesh disks during handling.

Fourth, avoid excessive rubbing of gene-eluting stents against the Teflon sheath and vessel wall during stent insertion to prevent dislodging of surface-associated Ad vectors.

Covalent attachment of gene vector to the surface of implantable biomaterials has an apparent advantage of better control over the vector release kinetics than realizable with non-covalent tethering of the vectors. In the setting of gene delivery from stent platform most studies report complete release of viral and non-viral vectors within 1-7 days after stent deployment[32-34] . On the other hand, Ad vector immobilization via HC demonstrated release of only 20-45% of the vector load in the first month (**Figures 4A** and **4B**). Moreover, a partial modulation of release and transduction kinetics is achievable with the use of different HC exhibiting dissimilar hydrolysis kinetics or by varying the concentration of HC employed for virus surface modification. Additionally, a concurrent co-modification of the vector with different cross-linker molecules, although not explored in the present study, provides another opportunity for fine-tuning vector release and transduction.

## Disclosures

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