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RESEARCH PAPER

Optimizing Adenoviral Transduction of Endothelial Cells under Flow Conditions

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ABSTRACT

Purpose To target adenoviral vectors to cells of the vasculature and shielding vectors from inactivation by the immune system.

Methods Complexes of reporter gene expressing adenoviral vectors with positively charged magnetic nanoparticles were formed by electrostatic interaction in presence or absence of additional negatively charged poly(ethylene glycol)-based polymer. Transduction of HUVEC was analyzed *in vitro* under flow. Protection from inactivation by the immune system was analyzed by pre-incubation of AdV and complexes with neutralizing antibodies and subsequent reporter protein analysis of infected cells.

Results Physical association of AdV with MNP and polymers was demonstrated by radioactive labelling of components and co-sedimentation in a magnetic field. Ad-MNP+/−polymer resulted in efficient transduction of HUVEC, depending on MOI and flow rate in presence of magnetic field, whereas no transduction was observed without complex formation with MNP or in absence of magnetic field. Association with MNP did result in protection from neutralizing antibodies, with slightly increased protection provided by the polymer.

Conclusions Complex formation of AdV with MNP is a viable means for targeting of vectors to areas of magnetic field gradient. Additional coating with polymer might proof useful in protection from inactivation by the immune system.

KEY WORDS adenoviral vectors · endothelial cells · gene transfer · magnetic nanoparticles · shielding

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ABBREVIATIONS

AdV	adenoviral vectors
CMV	cytomegalovirus
eGFP	enhanced green fluorescent protein
HUVEC	human umbilical cord venous endothelial cells
Luc	firefly luciferase
MNP	magnetic nanoparticles
MOI	multiplicity of infection
PBS	phosphate buffered saline
PEG	poly(ethylene glycol)
PEI	poly(ethylene imine)
PFU	plaque forming units
PM	polymer P6YE5C
VP	virus particles

INTRODUCTION

Recombinant human adenoviruses have been used as gene transfer vectors for decades and are among the most commonly used vectors in pre-clinical and clinical trials for *in vivo* gene transfer. Adenoviruses infect dividing as well as resting cells and do not integrate actively into the host genome, thus eliminating the problem of insertional mutagenesis. Consequently gene expression is transient, which is considered advantageous in therapeutic approaches where only short term gene expression is needed, as in the case of expression of growth factors. Despite their high transduction efficiency *in vitro* and their extensive use in animal models, transduction of primary tissues with adenoviral vectors remains challenging. Targeting of adenoviral vectors to the vascular endothelial system thus poses two major challenges: accumulation of therapeutically relevant doses of adenoviral vectors to the site of interest and protection of vectors from inactivation by the immune system.

Adenoviral vectors induce both humoral as well as cellular immunity in a host, allowing for long term protection against a given serotype. Different approaches to reduce AdV immunity have been described including the construction of gutless vectors that reduce the risk of background expression of viral genes (1,2) and thus induction of the cellular immune system. Alternatively genetic modifications of capsid components have been used as well as shielding AdV by chemical means from attack by neutralizing antibodies and non-specific interaction with blood components (3–5).

Different approaches have been employed to increase target organ transduction and thus de-target the liver. These methods encompass capsid modifications, the use of bi-specific antibodies (6) as well as transcriptional targeting (7,8). Capsid modifications have been achieved by genetic (9–11), chemical and combined genetic and chemical means. Genetic approaches involved transcriptional targeting or modification of fiber, amongst others the elimination of CAR-binding domain and replacement by peptides that result in targeting of AdV to endothelial cells (12,13). Chemical modifications representing a more general way of de-targeting — stealthing — have been achieved by covalently binding polymers based on e.g. poly(ethylene glycol) (14–16) or poly[N-(2-hydroxypropyl)methacrylamide] (17) to the capsid of adenoviruses. PEGylation lead to retention of AdV infectivity in the lung, while escaping inactivation by neutralizing antibodies *in vitro* and *in vivo* and reducing off-target gene expression in the liver. Depending on the chemical type of PEG and the degree of PEGylation used, some scientists observed massive reduction in infectivity *in vitro*, which could in turn be overcome by magnetically forcing PEGylated vectors onto cells (18) or binding of e.g. αv integrin specific peptide RGD or endothelial cell specific antibody (19). Stealthing with poly[N-(2-hydroxypropyl)methacrylamide] resulted in abrogation of natural host tropism, which was reverted to endothelial cell specific expression by subsequent grafting of endothelial cell specific peptide SIGYPLP to the polymer (20) or binding of VEGF or FGF-2 (17). Additionally coating of AdV with poly[N-(2-hydroxypropyl) methacrylamide] resulted in evasion of antibody neutralization. A further dimension has been added recently by combining genetic and chemical capsid modifications allowing for specific covalent binding of polymers to specific, genetically introduced reactive groups in fiber, protein IX or hexon protein (21,22).

Additionally non-covalent methods have been used to enhance adenoviral gene transfer (5,23,24). Adenoviruses were engineered by coating polymers and lipids via electrostatic interactions (24). However expression varied with cell lines and agent used and toxicity was observed at high concentrations.

A physical way of enhancing viral and non-viral gene transfer *in vitro* and *in vivo* by magnetic force has been developed by our group (25,26). Adenoviral vector particles can be associated with different, in general positively charged magnetic nanoparticles by electrostatic interaction. Exposure to a magnetic field not only allows for infection of cells normally not susceptible to adenovirus infection (enhancement of gene transfer) (25,27), but also allows for locally defined gene transfer (25).

Complexes of MNP and AdV have been used to enhance transduction of endothelial as well as smooth muscle cells (28) and K562 suspension cells (29) *in vitro* and have been used to target AdV transduction to a restricted area in the brain (30), lung and intestine (31) in mouse models.

Furthermore we have used charged PEG-based polymers for shielding of non-viral vectors in different approaches e.g. for tissue regeneration (32–35). In this manuscript we combined both methods for targeting of endothelial cells under flow conditions *in vitro*: the complex formation of adenoviral vectors with magnetic nanoparticles for localization of gene transfer to areas of high magnetic field gradients and shielding of adenoviral vectors with the negatively charged polymer P6YE5C were analyzed under flow conditions.

MATERIALS AND METHODS

Adenoviral Vectors

The construction and purification of the recombinant human adenoviral vector AdmCMVeGFPLuc has been described elsewhere (27). In essence the vector carries the eGFPLuc fusion gene under control of the murine CMV promoter. The expression cassette replaces the E1-region and was rescued by cotransfection with pBHG10loxΔE1,E3Cre (36) (purchased from Microbix, Canada) into 293 cells using the calcium phosphate precipitation method (37). Plaques were isolated, plaque purified and expanded on 293 cells (Microbix, Canada). Adenoviral vector was purified by double cesium chloride gradient centrifugation and subsequent desalting with PD-10 Column Sephadex G-25M (GE Healthcare, Munich, Germany). Titers were determined by endpoint dilution assay to obtain biological titer (pfu/ml) and photometrically to obtain physical titer (VP/ml) (27). The ratio of physical to biological titer was approximately 200. MOIs are given as biological titers. However, complex formation was based on corresponding physical titers.

Magnetic Nanoparticles

Core-shell-type magnetic nanoparticles SOMag6-125 had an iron oxide core with an average crystallite size of 6.8 nm

and a silica oxide coating with surface phosphonate groups formed by condensation of tetraethyl orthosilicate and 3-(trihydroxysilyl)propylmethyl-phosphonate as previously described (38) followed by secondary surface decoration with 25 kD branched poly(ethylene imine) at w/w PEI-to-iron ratio of 12.5%. The mean hydrodynamic diameter and zeta potential of the MNPs suspended in ddH_2O were measured to be 96±23 nm and 40.2±2.6 mV, respectively. Saturation magnetization per unit of iron weight at 298 K was found to be 94 emu/g iron.

Complexes were formed in PBS by electrostatic interaction of negatively charged adenovirus and positively charged MNPs. Complexes were formed with 5 or 10 fg Fe MNP/VP in a volume of 400 µl PBS for 20 min at room temperature. Complexes were used either directly in experiments or further incubated with polymer. When appropriate, the volume was increased with PBS or complete medium.

Polymer

The negatively charged polymer P6YE5C consists of a thiol reactive dicarboxylic acid derivative for the copolymerisation of diamino PEG chains with an average molecular weight of 6000 Da. Subsequently the branched anionic peptide YE5C $(Ac\text{-}YEEEEE)_2K\text{-}ahx\text{-}C$ was reacted with the PEG polymer backbone via disulfide bonds as originally described by Finsinger *et al.* (32), with slight modifications described recently (34).

P6YE5C was assembled with complexes of adenoviral vectors and MNP by electrostatic interactions in volumes of 400 µl PBS for 30 min at room temperature. When appropriate, the volume was increased with PBS or complete medium.

Physical Characterization of Complexes

The mean hydrodynamic diameter and surface charge of complexes of adenoviral vector, MNP and polymer were determined using a Malvern Zetasizer Nano Series 3000 HS (Malvern Instruments GmbH, Herrenberg, Germany). After complex formation the volume was increased to 1 ml with PBS and samples were immediately analyzed in folded capillary cells for surface charge and subsequently for size.

Magnetophoretic mobility of complexes was determined photometrically at 360 nm in a total volume of 500 µl PBS as described previously (39). Clearance velocity under the influence of a defined gradient magnetic field was determined and normalized to the initial optical density. The rate constant K was calculated from sedimentation curves using GraphPad Prism 5.

Determination of Physical Interaction of Ad-MNP and Polymers

1.5×10^{11} VP of AdeGFPLuc were labelled with 7.4 MBq ^{125}I (Hartmann Analytic, Braunschweig, Germany) in IodoGen cap tubes (Thermo Fisher Scientific, Bonn, Germany) for 25 min at room temperature, while slowly agitating. Unbound radiotracer was removed by PD-10 column (GE health care, Munich, Germany) and samples were collected. Fraction 5, containing 9.8×10^{10} virus particles (determined by UV absorbance) and an activity of 1933 kBq per ml was used for binding studies.

At the same time 6.24 mg P6YE5C was labelled with 18.5 MBq ^{131}I (PerkinElmer, Rodgau, Germany) as described above. Unbound radiotracer was removed by PD-10 column (GE health care, Munich, Germany) eluting with water and samples were collected. Fractions were analyzed for incorporation of ^{131}I by gamma counting and photometrically at $OD_{280\ nm}$ for polymer concentration with $\varepsilon=2560\ M^{-1}$. The product fraction containing 2453 pmol/µl of P6YE5C and 3.8 MBq/ml was diluted 1:10 and used for binding studies.

Complexes were produced by adding 4.3 µl of labelled AdeGFPLuc, 2.3 µl of unlabelled AdV (5.8×10^8 VP), in 300 µl PBS. 20 min later 500 pmol of labelled polymer was added to each sample and non-labelled polymer added to obtain the higher concentrations. 150 µl were transferred to U-bottom 96-well plate and magnetically sedimented for 1 h at room temperature. 80 µl of supernatant of sedimented samples and 80 µl of non-sedimented samples were measured for ^{131}I and ^{125}I in a gamma counter (Wallac, Turku, Finland). After decay and spill over correction percentage of sedimented radioactivity was calculated.

Infectivity Assays

Human umbilical cord venous endothelial cells were obtained from Promocell (Heidelberg, Germany) or were a kind gift of Katrin Zimmermann and Alexander Pfeiffer (Department of Pharmacology, University of Bonn) and were cultured in EGM-2 Bullet Kit medium (Promocell) according to the manufacturer's instructions. Infectivity assays under static conditions were performed in 24-well plates with 2.5×10^5 cells/well. AdV or complexes (400 µl) were added to the medium and samples were placed on magnets arranged in a 24-well format (OZ Biosciences, Marseille, France) for 20 min at 37°C. Controls were treated the same way, but were not exposed to magnetic fields. Twenty four hours post infection samples were visualized by fluorescence microscopy (Zeiss Axiovert 135, Zeiss Axiovision). Samples were lyzed and the specific luciferase activity was determined as described elsewhere (32). Results were analyzed by one-way ANOVA using GraphPad Prism 5.

Serum Inactivation

AdV-MNP complexes with or without polymer were treated with ^{125}I labelled serum of sero-positive donor for Ad5. Labelling of serum was performed as described above with 18.5 MBq ^{125}I (Hartmann Analytic, Braunschweig, Germany) in IodoGen cap tubes (Thermo Fisher Scientific, Bonn, Germany) for 25 min at room temperature, while slowly agitating. Unbound radiotracer was removed by PD-10 column (GE health care, Munich, Germany) with PBS as diluent and samples were collected. This resulted in a 1.9-fold dilution of the sample as determined photometrically at OD 280 nm. Thirty µl aliquots were incubated for 1 h at 37°C with complexes consisting of 2×10^9 VP and 5 µg Fe of SO-Mag6-125 or 2×10^9 VP, 5 µg Fe of SO-Mag6-125 and 2 nmol P6YE5C in a total volume of 30 µl, respectively. This corresponded to a final serum concentration of 27% during incubation. Complexes were magnetically sedimented for 30 min 55 µl supernatants were collected, pellets washed with 150 µl PBS and sedimented again. Supernatants were combined and pellets resuspended in 100 µl PBS for gamma counting.

AdV or complexes of AdV were pre-incubated with serial dilutions of heat-inactivated (30 min 56°C) serum of donor sero-positive for Ad5 for 1 h at 37°C. Subsequently serum-treated and non-treated controls were used to infect HUVEC cells in 24-wells (2.5×10^5 cells/well) in the presence of magnetic plates for 20 min at 37°C. The specific luciferase activity was determined 24 h post infection. Luciferase activity was expressed as % of untreated control. Additionally we infected A549 (ATCC, LGC standards GmbH, Germany) cells in 96-well plates (2×10^4 cells/well) with serum-treated samples and non-treated controls in absence of magnetic field for 90 min and proceeded as described above.

Erythrocyte Inactivation Assay

Complex formation was as described above (500 VP/cell, 10 fg Fe/VP and 1000 pmol of P6YE5C negative charges/10^9 VP) in a volume of 100 µl PBS. AdV or AdV complexes were incubated with 25 µl of freshly isolated human erythrocytes (5×10^9 cells/ml) or serial dilutions thereof and were subsequently used to infect A549 cells in absence of magnetic field (40). Cells were washed three times with PBS to remove erythrocytes, before fresh complete medium was added. Luciferase assay was performed 24 h post infection.

In Vitro Flow Model

The ibidi perfusion sets for high and low flow rates were purchased from ibidi (ibidi GmbH, Martinsried, Germany), and the ibidi pump system with fluidic unit were used for analysis of AdV infection under flow conditions. All media, slides and tubes were equilibrated in a 37°C humidified incubator 1 day prior to experiment. Tissue culture treated ibidi µ-slides I 0.4 (ibidi GmbH, Martinsried, Germany) were seeded with HUVEC at 3×10^5 cells/slide 1 day prior to experiment.

Complexes were produced as described above. Meanwhile 12 ml complete medium were equilibrated in the flow system for 5 min at a flow rate of 5 ml/min without slide chamber attached. Complexes were added and the system was equilibrated for another 5 min. After connecting the slide to the flow system, the slide was placed on three magnets (Nd-Fe-B Neodelta with 1080–1150 mT; d=6 mm, h=5 mm) (IBS Magnet, Berlin; Germany) placed in a row in a holder for 96-well magnets in strictly alternating polarization. Flow was continued for 15 min at room temperature at the indicated rates. Subsequently slides were removed from magnets and cells were washed five times with complete medium. Twenty four hours post infection cells were analyzed as described above. Additionally before lysis of cells eGFP expression was detected macroscopically using a combined phospho- and fluorescence imager FLA 2000 (raytest, Straubenhardt, Germany) and AIDA software (raytest, Straubenhardt, Germany) for evaluation. 1D evaluation was used to show position of fluorescence expression in the centre path of the flow chamber. 2D evaluation was used to quantify eGFP expression and was expressed as arbitrary units (a.u.)/mm^2.

RESULTS

Previously we have used magnetofection to localize and enhance adenoviral gene transfer (25,27) and charged polymers for shielding of non-viral vectors (32). Thus we thought to combine both methods and used the negatively charged polymer P6YE5C for shielding of adenoviral vectors associated with magnetic nanoparticles.

Characterization of Complexes

Complexes of adenovirus, MNP and polymer were produced and size and zeta-potential were determined. Upon complex formation by electrostatic interaction, size increased from 80 nm of virus and 160 nm of MNP in buffer containing salt to approx. 600 nm (Fig. 1a). This size was not significantly altered by addition of increasing concentrations of negatively charged polymer P6YE5C. At the same time, the zeta-potential of complexes changed from positive (+8 mV) of AdV-MNP complexes without polymer to near electro-neutrality at 500 and 1000 pmol of P6YE5C/10^9 VP (+1.6 mV and −0.46 mV, respectively)

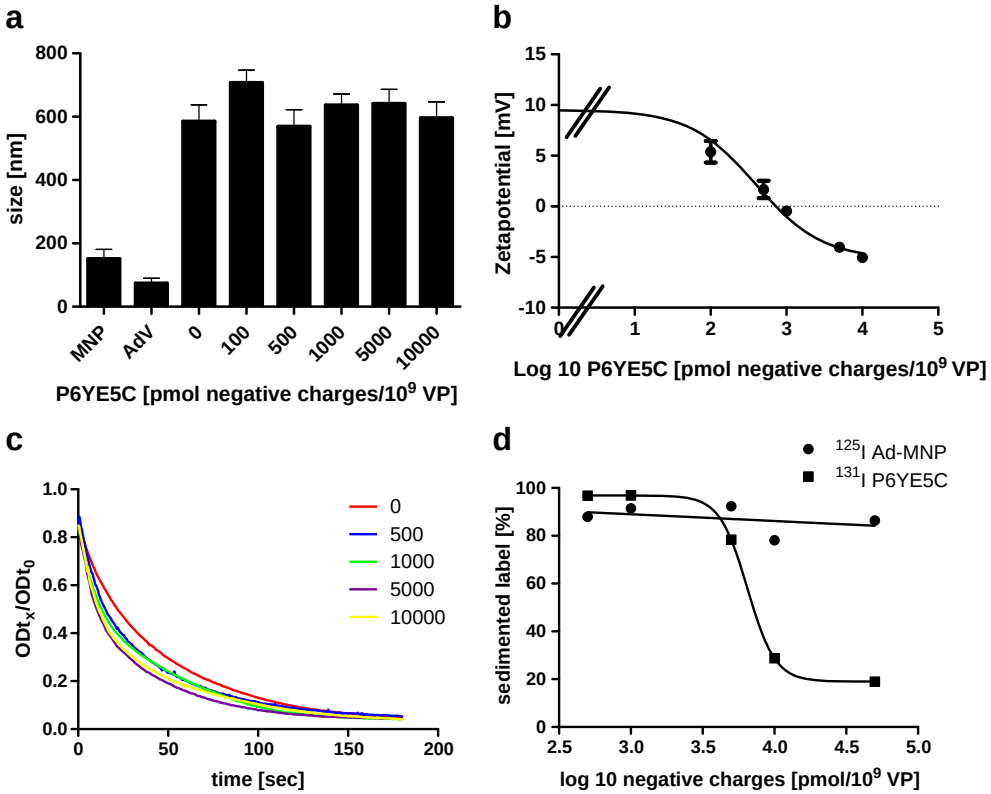


Fig. 1 Physical characterization of adenoviral complexes. (**a**) The hydrodynamic diameters of MNP SO-Mag6-125 and AdeGFPLuc (V) alone or in complex (0) and complexes with increasing concentrations of polymer (500, 1,000, 5,000, 10,000 pmol negative charges/10^9 VP) were determined. Whereas the size of complex of AdV with MNP increased, no further size increase was observed after addition of polymer. (**b**) The zeta potential of AdeGFPLuc SO-Mag6-125 complexes of with increasing polymer concentrations were determined. At 500 and 1000 pmol P6YE5C/10^9 VP near neutral values were obtained. (**c**) The magnetophoretic mobility of complexes in absence (0) and presence of polymers were analyzed. (**c**) Exemplary data set. Numbers indicate pmol negative charges of P6YE5C/10^9 VP. (**d**) Physical association of adenoviral vectors with magnetic nanoparticles and polymer. Complexes of SO-Mag6-125 (MNP) and radioactively labelled AdeGFPLuc (^{125}I-Ad) were formed as described and further incubated with increasing concentrations of ^{131}I-labelled polymer P6YE5C. Complexes were magnetically sedimented, resulting in co-sedimentation of polymer at 500 to 5,000 pmol/10^9 VP concentrations with the Ad-MNP complexes. Higher polymer concentrations resulted in excess of P6YE5C that was not associated with complexes.

with further decrease to negative surface charges with higher polymer concentrations (Fig. 1b). No precipitation or instability of complexes was observed when polymer was added to achieve neutral surface charge (1000 pmol P6YE5C/10^9 VP; Fig. 1a).

Additionally the magnetophoretic mobility of the complexes was compared for AdV MNP complexes with and without polymer (Fig. 1c). The addition of polymers did not interfere with sedimentation, but rather increased the magnetic responsiveness of the complexes. From these first order decay curves the rate constants K [s^{-1}] were calculated. Increasing values indicate an increased magnetic responsiveness (Table I). Calculation of the average number of MNP/VP in complexes from the magnetic responsiveness data according to Tresilwised *et al.* (26) resulted in ≈37,000 MNP and ≈48,000 MNP, with ≈10.4 and 13.6 VP in complexes for AdV-MNP and AdV + MNP + PM at 1,000 pmol P6YE5C/10^9 VP, respectively, taking into account the hydrodynamic diameter and core size of complexes and magnetization of the nanoparticles.

Physical Association of AdV with MNP and Polymer

The physical association of adenoviruses with magnetic nanoparticles SO-MAG6-125 is described in detail in the accompanying paper by Mykhaylyk *et al.* and virus binding is 80–90% at 5 and 10 fg Fe/VP (41). Therefore we analyzed the co-sedimentation of radioactively ^{125}I-labelled adenoviral vector and ^{131}I-labelled Polymer PY6E5C in complexes with SO-MAG6-125 at 10 fg Fe/VP by magnetic force. At 500 and 1,000 pmol negative charges per 10^9 VP, 80–90% of adenoviral vector and 96% of polymer were co-sedimented by magnetic force (Fig. 1d). At 5,000 pmol the 78% of polymer were sedimented by the Ad-MNP complexes, indicating excess of unbound

Table I Rate Constants (K) for AdV-MNP Complexes in Magnetic Field

	P6YE5C [pmol negative charges/10^9 VP]				
	0	500	1000	5000	10000
K [s^{-1}]	0.022	0.034	0.027	0.036	0.033

polymer. Higher concentrations of polymer resulted in 70–80% unbound PY6E5C that was not sedimentable by magnetic force.

Infectivity of AdV Complexes of Endothelial Cells under Static Conditions

HUVEC cells were infected with AdeGFPLuc in complex with MNP SOMag 6–125 and the P6YE5C polymer. In general, complex formation lead to increased luciferase expression (Table II). Except for 1,000 pmol negative charges of P6YE5C/10^9VP ($p<0.05$) no significant differences in gene expression were observed as compared to AdV-MNP complexes. No difference in gene expression was observed at MOI 5 (pfu/cell) under static infection (Table II). In absence of magnet luciferase expression was detectable, but low and was not influenced by addition of polymer to complexes (Table II).

Shielding from Serum Inactivation

The protection of AdV from inactivation by serum components was analyzed by pre-incubation of complexes of AdV with MNP and complexes of AdV with MNP and P6YE5C polymer with non-heat-inactivated ^{125}I-labelled serum of a donor with neutralizing anti-adenovirus serotype 5 antibodies for 60 min. ^{125}I labelled serum co-sedimenting with complexes indicates interaction with serum components. Polymer-coating resulted in a co-sedimentation of radioactively labelled serum with 22,364±5,150 cpm and 18,902±1,325 cpm for AdV + MNP and AdV + MNP + PM, respectively, indicating a slight, but not significant ($p=0.32$) inhibition of interaction with serum components (Fig. 2a). The measured radioactivity corresponds to 0.73% (corresponding to 11.9 µg serum solutes) and 0.64% (corresponding to 10.4 µg serum solutes) of the applied dose, respectively.

Subsequently the ability to shield AdV from neutralizing antibodies was analyzed by pre-incubation of the naked AdV, complexes of AdV with MNP, complexes of AdV with MNP and P6YE5C polymer with serial dilutions of heat-inactivated serum of a donor with neutralizing anti-adenovirus serotype 5 antibodies for 60 min and subsequent infection of HUVEC cells. Protection of AdV was evaluated by luciferase expression (Fig. 2b). The data were normalized to luciferase expression in cells infected with the respective complexes that were not treated with serum (100%). The dose response curves shown in Fig. 2b were fitted with a logistic function to find serum concentration resulting in 50% inhibition of virus infectivity (IC50). These resulted in IC50 values of 0.078±0.013%, 0.289±0.056%, 0.255±0.029% and 0.483±0.058% for AdV, AdV + MNP, AdV + MNP + PM at 500 and 1,000 pmol negative charges of P6YE5C/10^9VP, respectively. This means that complex formation with SO-Mag6-125 results in a 3.7 fold enhanced protection *vs.* AdV alone. Addition of polymer P6YE5C at 500 pmol negative charges/10^9 VP did not result in enhanced protection, whereas 1,000 pmol negative charges/10^9 VP lead to a 6.2 fold enhanced protection against neutralizing antibodies *vs.* AdV alone. Thus P6YE5C polymer adds another 1.7 fold increase in protection against serum components as compared to AdV + MNP complexes. To analyze if the slight protection seen might result from differences in infectivity of hard to transduce HUVEC, we infected A549 cells as described before (Fig. 2c). Even A549 cells were more easily infected

Table II Reporter Gene Expression of HUVEC Infected under Static Conditions

MOI	2.5		5	
complexes	+ magnet	−magnet	+ magnet	−magnet
AdV	0.03±0.002	0.03±0.001	0.14±0.009	0.24±0.035
AdV-MNP-0	3.01±0.11	0.38±0.02	18.49±1.67	5.06±0.24
AdV-MNP-500	2.54±0.48	0.80±0.05	17.14±0.47	4.91±0.38
AdV-MNP-1000	2.00±0.06	0.54±0.04	17.66±1.49	3.84±0.18
AdV-MNP-5000	2.96±0.20	0.75±0.04	18.11±1.34	4.91±0.39
AdV-MNP-10000	3.95±0.27	0.82±0.03	17.02±2.11	5.67±0.36

Infections were carried out with adenovirus alone (AdV) or complexed with MNPs (AdV-MNP-0) or modified with increasing P6YE5C-to-VP ratios (500, 1,000, 5,000, 10,000 pmol negative charge/10^9 VP) with or without magnetic field application

Data are: Specific firefly luciferase activity [ng/µg total protein]; mean ± SEM, $n=3$

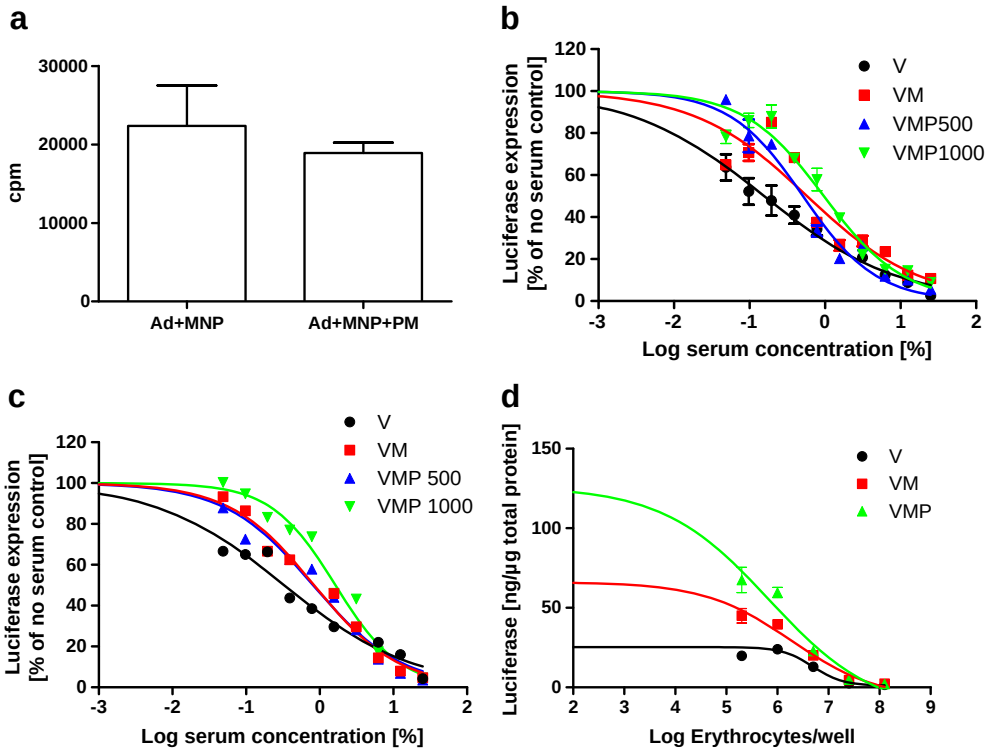


Fig. 2 Shielding of adenoviral vectors from serum inactivation. (**a**) Complexes of AdV with SO-Mag6-125 or AdV + SO-Mag6-126 + P6YE5C were incubated with ^{125}I-labelled serum (not heat-inactivated) and subsequently magnetically co-sedimented. ^{125}I serum co-sedimenting with complexes indicates interaction with serum components. Addition of polymer does not result in enhanced protection against serum, when complement system is not inactivated by heat, as compared to MNP. (**b**) Adenoviral vectors alone (V) or in complex with SO-Mag6-125 (VM) and increasing concentrations of polymer (VMP 500, 1,000 pmol P6YE5C negative charges/10^9 VP) were pre-incubated with serial dilutions of heat-inactivated serum. Subsequently samples were used to infect HUVEC and 24 h post infection specific luciferase activity was determined. To account for differences in infectivity, values were expressed as percentage of sample not treated with serum. Complex formation with MNP results in reduced inactivation of infectivity by serum, with further protection at 1,000 pmol P6YE5C negative charges/10^9 VP. (**c**) Same as (**b**), but infectivity was analyzed on A549 cells. (**d**) Adenoviral vectors alone (V) or in complex with SO-Mag6-125 (VM) and polymer (VMP 1,000 pmol P6YE5C negative charges/10^9 VP) were pre-incubated with serial dilutions of freshly isolated human erythrocytes and were subsequently used to infect A549 cells. 24 h post infection luciferase expression was determined. Complex formation resulted in higher gene expression, but did not prevent inhibition by erythrocytes.

with AdV + MNP and AdV + MNP + PM complexes in absence of magnetic field. For A549 cells dose response curves shown in Fig. 2c were fitted with a logistic function to find serum concentration resulting in 50% inhibition of virus infectivity (IC50), which were determined to be 0.329%, 0.841%, 0.816% and 1.635% for AdV, Ad + MNP, Ad + MNP + 500 PM and Ad + MNP + 1,000 PM, respectively. Thus addition of MNP resulted in 2.6-fold enhanced protection and 5.0-fold enhanced protection of Ad + MNP + PM 1000 *vs.* AdV alone.

Interaction of AdV and Complexes with Erythrocytes

The ability of complexes to prevent binding of AdV with erythrocytes was investigated by incubation of AdV, Ad + MNP or Ad + MNP + PM with freshly isolated human erythrocytes prior to infection of A549 cells. Binding of Ad to SO-MAG6-125 or in complex with P6YE5C polymer at 1,000 pmol negative charges/10^9 VP did not result in enhanced protection from erythrocyte inactivation at high red blood cell concentrations (Fig. 2d). However at lower and intermediate erythrocyte concentrations complex formation with MNP increased overall gene expression levels compared to naked AdV. The highest reporter gene expression was observed in the presence of polymer.

Infection of HUVEC Cells under Flow Conditions

Infection of HUVEC with adenoviral vectors and different complexes under flow conditions was analyzed with ibidi slides and the ibidi flow kit. eGFP fluorescence from eGFP- firefly luciferase fusion protein was analyzed by fluorescence imaging (Fig. 3a) and fluorescence microscopy (Fig. 3d). Localization of complexes was visible by eye as brown staining (data not shown) and transduction of HUVEC was localized to the positions of the three magnets placed underneath the flow chamber. eGFP-expression was

highest in areas of high magnetic field gradient, that is to say at the rims of the magnets as demonstrated in the overview fluorescent images, detail fluorescent micrographs and analysis of relative fluorescence along the centre of the path (Fig. 3b). Due to the geometry of the magnets and configuration of magnetic fields, accumulation and thus transduction and gene expression is highest at the middle position of magnets. Quantification of fluorescence images resulted in similar eGFP fluorescence intensity values (a.u./mm^2), ranging from 15.6±1.2 to 18.5±1.83 irrespective if polymer was added to AdV-MNP complexes or not with 17.4±1.6 a.u./mm^2 for AdV-MNP-1000 (Fig. 3c).

These results were confirmed by firefly luciferase assay, which analyses the pool of both uninfected and infected cells after lysis (Fig. 3e). In absence of magnetic nanoparticles AdV lead to background expression of 0.017±0.007 ng luciferase per µg total protein in HUVEC cells under flow conditions, which correlates with poor infectivity even under static conditions (Table II). At MOI of 5 pfu/cell and an intermediate flow rate of 5 ml/min luciferase expression raised 80-fold to 1.337±0.083 ng luciferase per µg total protein when AdV was associated with MNP SO-Mag6-125 (Fig. 3e). No further change was observed when P6YE5C polymer at 500 to 5,000 pmol negative charge per 10^9 VP

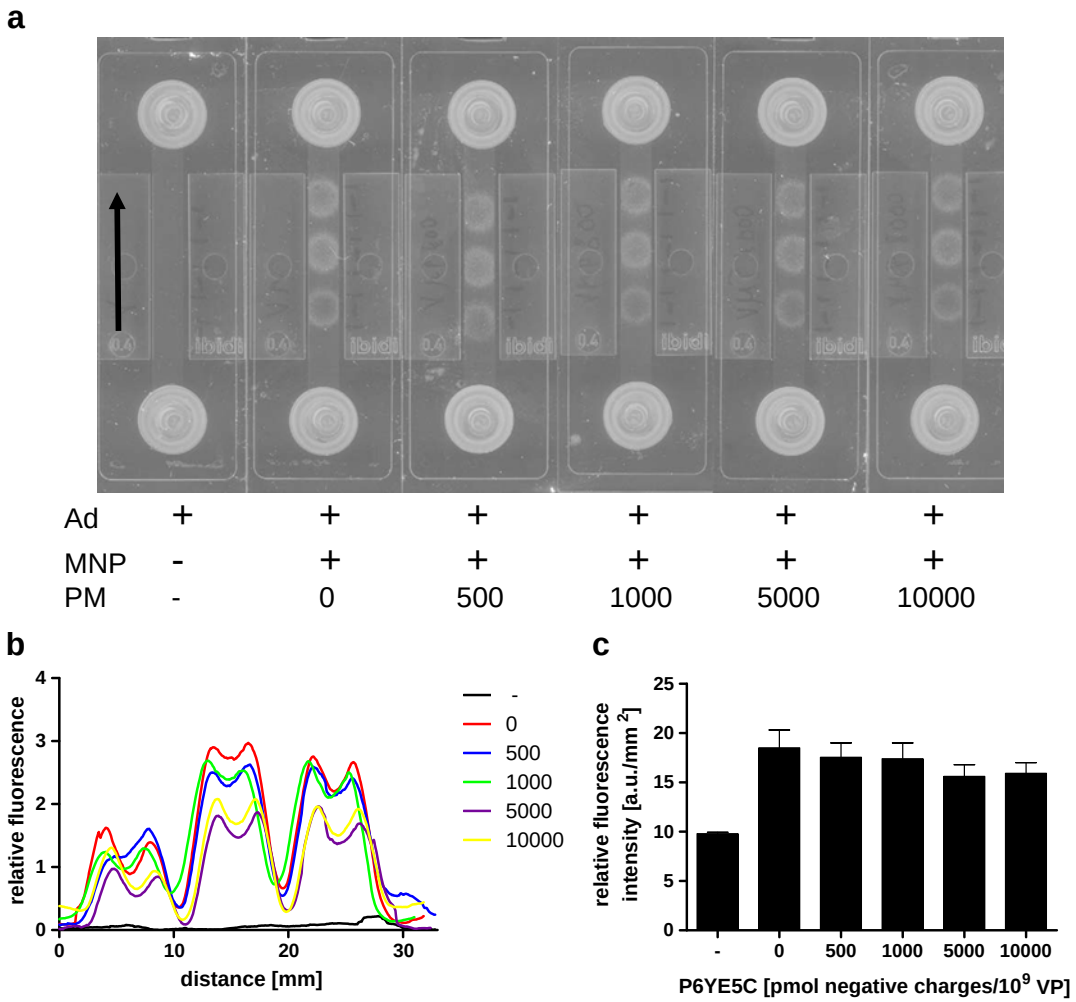
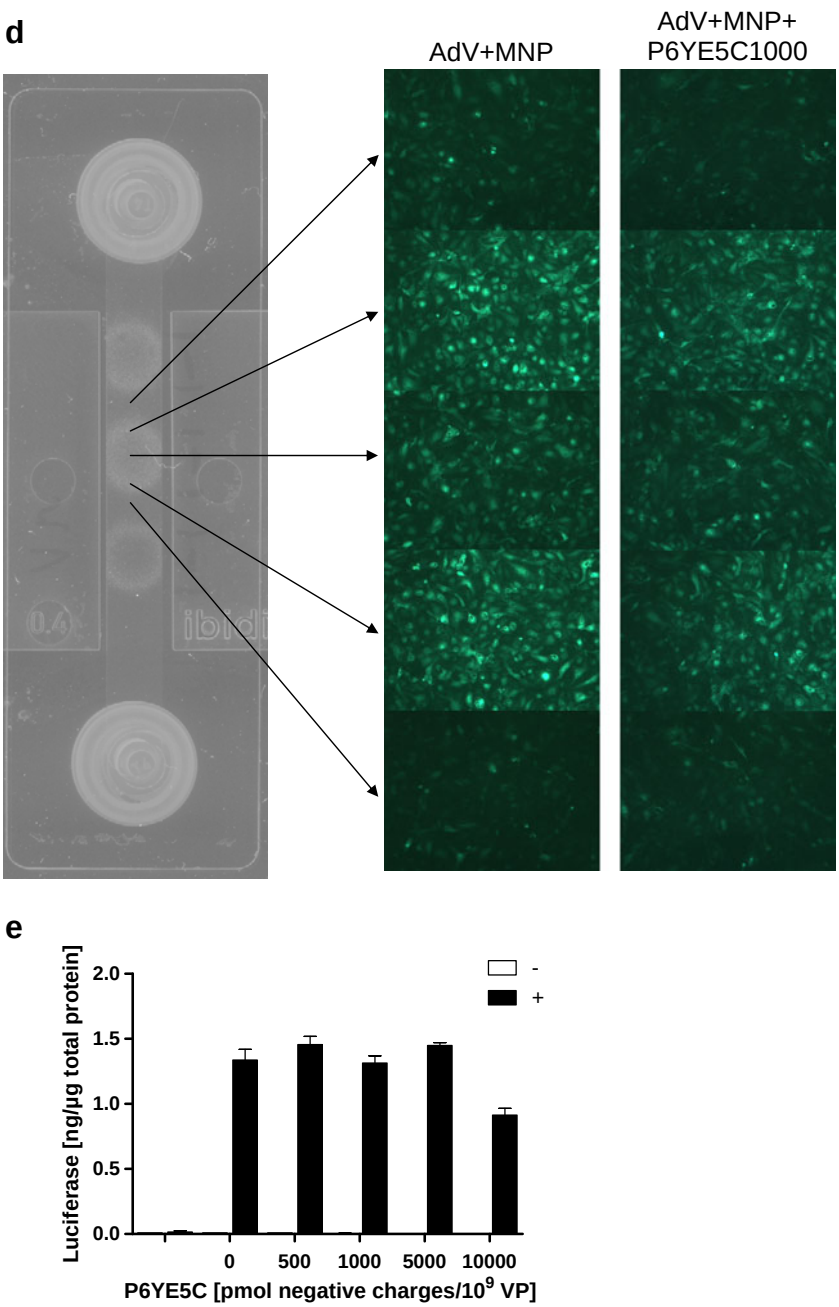


Fig. 3 Transduction of HUVEC under flow. HUVEC grown in an ibidi slide were exposed to AdV (4×10^8 VP) in a total volume of 12.5 ml for 15 min alone (Ad), as complexes with MNP SO-Mag6-125 (0) at 5 fg Fe/VP or with shielding polymer P6YE5C (500, 1,000, 5,000, 10,000 pmol negative charges/10^9 VP) at a flow rate of 5 ml/min. 24 h post infection reporter gene expression was visualized by fluorescence imager (**a**). The *arrow* indicates flow direction. The positions of the three magnets are seen as bright spots in the flow chamber. (**b**) Data from (**a**) quantitated for relative fluorescence along the centre path of each flow chamber, demonstrate the highest gene expression at rims of magnets, which coincide with the highest magnetic field gradients. Additionally the fluorescence is highest for the centre magnet for each sample containing MNP. (**c**) Quantification of eGFP-expression areas resulted in similar values irrespective if polymer was added to AdV + MNP complexes or not. (**d**) Examples of fluorescence micrographs for AdeGFPLuc + SO-Mag6-125 (AdV + MNP) and complexes shielded with 1000 pmol P6YE5C (right) from areas as indicated by *arrows*. (**e**) Quantification of reporter gene expression by luciferase assay of transduced HUVEC under flow conditions 24 h post infection. Data are given as means and SEM; $n=3$. Black bars (+): with magnets, white bars (−): no magnets. Specific luciferase activity is increased in presence of magnetic field when MNP are present, but is not influenced by addition of polymer, except at the highest concentration tested.

Fig. 3 continued



was included in the complex formation. Addition of higher polymer-to-virus ratios slightly reduced luciferase expression. Furthermore no luciferase expression was observed in absence of magnetic field in any of the complexed or native AdV formulations (Fig. 3e, light bars).

Luciferase expression was also dependent on virus MOI (Fig. 4a). At complex formation the ratio of MNP and polymer to virus particles was kept constant. As expected, the highest expression was obtained at MOI 50 (pfu/ml) in presence of MNP without or with polymer. For uncomplexed AdV only at the highest MOI of 50 a very low expression of 0.22 ± 0.01 ng luciferase per µg total protein was detectable (Fig. 4a).

Transgene expression in infected HUVEC was also dependent on the flow rate of virus containing solution (Fig. 4b). At low and intermediate flow rates of 2.5 ml/min and 5 ml/min, corresponding to shear stress of 3.29 and 6.58 dyn/cm^2, and shear rates 329/s and 658/s, respectively, values between 4.81 ± 0.74 and 6.32 ± 0.44 ng luciferase/µg total protein were obtained. At higher flow rates of 10 ml/min, corresponding to 13.16 dyn/cm^2 and a shear rate of 1316/s, transduction of HUVEC was still possible, but decreased by a factor of 1.6 for non-shielded complexes as compared to a flow rate of 5. This might indicate a suboptimal retention of complexes and thus association with HUVEC depending on the magnetic moment of the

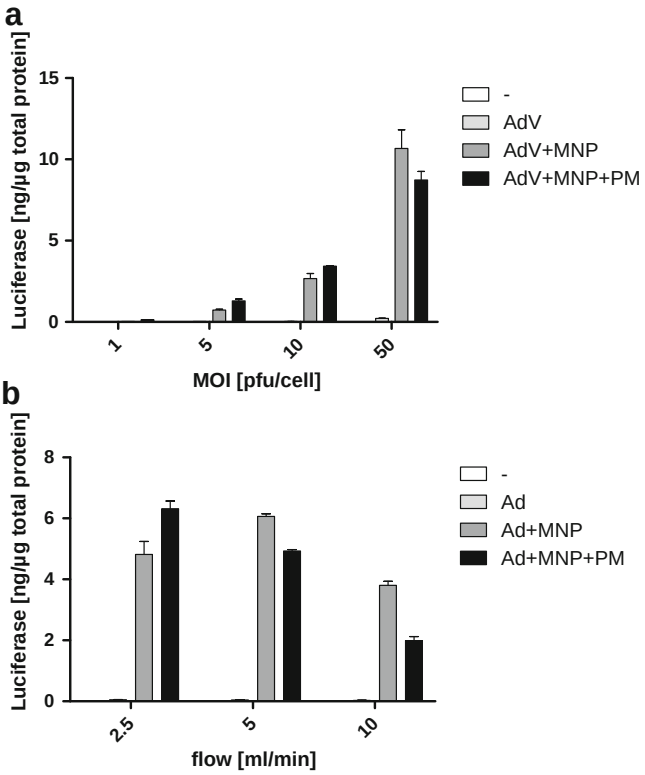


Fig. 4 Dependence of Ad infection under flow on virus dose and flow rate. (**a**) HUVEC were infected with increasing MOI of 5, 10 and 50 pfu/cell in a total volume of 12.5 ml. Ratios of 5 fg Fe/VP and 1000 pmol/10^9 VP remained constant for the experiments. A flow rate of 5 ml/min was applied. Luciferase expression increases with increasing MOI. (**b**) HUVEC were infected at MOI 10 with complexes formed with 5 fg FE/VP in presence or absence of 1000 pmol P6YE5C/10^9 VP. Flow rates varied from 2.5 ml/min to 10 ml/min. Luciferase extracts were harvested 24 h post infection. Luciferase expression is highest at flow rate 2.5 and 5 ml/min. Data are given as means and SEM; $n = 3$.

complex. Polymer coated complexes resulted in a decrease in the reporter gene expression by a factor of 2.5 at the same flow rate, indicating either reduced retention or instability of complexes at high shear stress.

DISCUSSION

Major challenges while targeting adenoviral vectors (AdV) to the vascular endothelial system are accumulation of therapeutically relevant doses of vectors to the site of interest and protection of vectors from inactivation by blood components and the immune system. The aim of this study was to target AdV to endothelial cells, measure inactivation by serum components and analyze transduction efficiency under flow condition.

Adenoviral magnetofection has been used to enhance gene transfer *in vitro* and *in vivo* (25,30,31). In mouse models, authors were able to demonstrate localized adenovirally mediated gene expression to specific areas e.g. in the brain, intestine and lung.

Enhancement of adenoviral transduction of endothelial cells by magnetofection under static conditions has been shown before: Chorny *et al.* (28,42) demonstrated magnetic targeting of BAEC and smooth muscle cells using AdV coupling to MNP via antigen-antibody interactions. Although using a different mode of interaction of AdV with MNP our data corroborate these data.

As mentioned before, covalently bound PEG has been used to shield Adv and has resulted in loss of AdV infectivity, depending on the type and ratio of PEGylation (18,19). We were interested in whether the PEG-based negatively charged polymer P6YE5C was suitable to associate with AdV magnetic complexes and to confer shielding against interaction with serum components. P6YE5C has been developed as a shielding component for nonviral vectors and is associated by electrostatic as opposed to covalent bonding (32). A range of polymer concentrations was analyzed. Physical characterization of our complexes revealed that after addition of polymers magnetization and magnetophoretic mobility slightly increased (Fig. 1c), however no significant size increase of complexes was measured by laser light scattering after modification with the polymer (Fig. 1a). Therefore, and based on the fact that in the absence of a magnetic field the polymer coated complexes did not sediment by gravitation, we speculate, that addition of polymer results in complexes that have increased density. This can also be concluded from the MNP/VP ratios calculated from magnetophoretic mobility measurements for shielded and unshielded complexes that are essentially equal.

An essential finding was that electroneutral magnetic complexes comprising the entire virus dose and being stable in size can be obtained by adding negatively charged polymer (Fig. 1b and d).

Based on these physical characteristics we expected that the polymer may provide additional stability against the inactivating interactions of serum and erythrocytes. In fact, we had previously shown, that complex formation of AdV with PEI-coated MNP resulted in some protection from serum-inactivation (43). Surprisingly, we did not observe any protective activity of the shielding polymer against serum inactivation (Fig. 2a). Our data however suggest that association of AdV with MNP and shielding polymer provides protection from neutralizing antibodies. MNP alone resulted in a 3.7-fold increase in IC50 value (Fig. 2b), while additional modification with P6YE5C polymer furthermore increased the IC50 1.7-fold. In these experiments non-covalently bound polymer was used at concentrations that yielded electroneutral surface of the particles. Data were compared to negatively charged AdV and positively charged complexes of AdV and SO-Mag6-125 MNP.

Carlisle *et al.* recently reported, that human erythrocytes display Coxsackie virus-adenovirus receptor (CAR) on their surface, thereby presenting a mechanism for sequestration of Ad from blood in natural as well as therapeutic settings and that stealthing AdV can prevent inactivation of AdV by erythrocytes (40). We therefore analyzed if MNP and more importantly addition of the polymer P6YE5C can shield AdV from erythrocyte inactivation. Consistently we observed highest reporter gene expression levels with the polymer coated complex at intermediate erythrocytes concentrations, when compared to naked virus or MNP associated AdV. However, the relative inactivation levels on the various vector compositions indicate that there is in fact no protection against sequestration by erythrocytes. Since SO-MAG6-125 are PEI coated, incubation of complexes with red blood cells might disrupt interaction with AdV, rendering it accessible to erythrocyte inactivation. Whether covalent binding of components could protect from erythrocyte sequestration of AdV will have to be investigated.

As mentioned earlier covalently bound PEG resulted in loss of AdV infectivity, depending on the type and ratio of PEGylation (18,19). Complexing of AdV with MNP or MNP and electrostatically bound polymer resulted in protection from serum components (Fig. 2b) but, in contrast to the results obtained with covalent PEG shielding of AdV (18,19) did not prevent infectivity under static culture conditions (Table II). In absence of magnetic field and without polymer this could be attributed to the positive charge of complexes (Fig. 1b), allowing or enhancing interaction of complexes with negatively charged cells as compared to negatively charged AdV on negatively charged cell surface even in absence of a magnetic field. However, adding P6YE5C to the magnetic complex to obtain an overall neutral surface of the complex did not reduce infectivity as compared to samples without polymer (Table II). Since association of the polymer with magnetic complex had been verified by co-sedimentation of Ad-MNP and polymer (Fig. 1d) it can be speculated, that either shielding is not complete or that complexes sediment onto cells during incubation time, although there was no clearance of complex suspension in absence of magnet over a period of 30 min (data not shown).

Most importantly we demonstrate in an *in vitro* flow system, that enhancement of adenovirally mediated gene transfer of endothelial cells is possible under flow conditions, provided, AdV are associated with magnetic nanoparticles and a magnetic field is applied. Under flow conditions in absence of magnet no reporter gene expression was observed independent on use of polymer, whereas eGFP and luciferase expression increased when flow chambers were exposed to magnetic fields as compared to AdV alone. eGFP expression allowed for visualization of gene transfer to areas of high magnetic field gradient (Fig. 3a–d). Due to enhancement of magnetic fields by neighbouring magnets eGFP expression was highest at the centre magnet. Quantification of gene expression by luciferase assay clearly demonstrated that complexes of AdV and MNP SO-Mag6-125 with or without polymer P6YE5C resulted in ≈80 — fold increase compared to AdV alone (Fig. 3e).

With the given dimensions of the slides, flow rates of 2.5 ml min^{-1} converting into 2.1 cm s^{-1}, 5 ml min^{-1} converting into 4.2 cm s^{-1} and 10 ml min^{-1} converting into 8.3 cm s^{-1} were analyzed. The first two rates are well above the range of blood capillaries with ≈0.5–1 mm s^{-1} (44–46), thus covering flow rates in medium sized vessels. A flow rate of 10 ml min^{-1} converting into 8.3 cm s^{-1} was chosen to be in the range of blood flow in major arteries (femoral artery ≈10 cm s^{-1})(46). Thus the flow rates allowing for transduction of HUVEC as model for endothelial cells indeed cover physiological flow rates in larger size vessels.

To our knowledge this is the first systematic investigation of infection of AdV-MNP complexes under flow conditions.

Recently Danielsson *et al.* (47) analyzed the interaction of unmodified AdV and PEG-modified AdV with whole human blood in a tubing system under circulation. The aim of the study was not gene transfer under flow conditions, but rather simulation of interaction of AdV with molecules and cells in a more physiological setting. PEGylation prevented clot formation, resulted in reduced binging to blood cells and reduced complement activation: However, one of the results of this study was that although PEGylation, especially 20 K-PEGylated Ad, results in protection from blood components in mice, interaction with human blood is not as well prevented. This study stressed the importance of analysis of such interactions under continuous flow and shear stress

To this extent the *in vitro* flow system described here might also add to a better understanding of the behaviour of AdV complexes and their infectivity.

Perez *et al.* (48) have used the clustering of AdV with iron oxide-based, dextran coated MNP with virus specific antibodies to detect low levels of AdV in cell lysates and serum by magnetic resonance imaging. Huh *et al.* (49) covalently attached "manganese-doped" iron oxide nanoparticles to AdV to image gene transfer to CAR-positive cells by magnetic resonance imaging as well as by eGFP fluorescence. Since only few nanoparticles were bound per virus particle and no magnetic field was applied, this method resulted in specific labelling and imaging of CAR-positive cells, only. The accompanying manuscript by Mykhaylyk *et al.* (41) describes the usefulness of SO-Mag6-11.5, a MNP with the same core decorated with 11.5% PEI exhibiting good adenovirus and lentivirus binding properties in magnetic resonance imaging of cells. Efficiency of AdV associated SO-Mag6-125 for magnetic resonance imaging remains to be demonstrated.

CONCLUSION

Magnetofection is a useful tool for targeting adenoviral vectors to endothelial cells under flow conditions, since complexes can withstand shear stress similar to values characteristic for medium sized vessels. The method is versatile, since it does not need genetic modification of AdV and allows for localized gene transfer to otherwise refractory cells. The modification of the magnetic AdV complexes with PEG-based polymer via electrostatic interaction does not interfere with infectivity and provides a slightly improved shielding from serum components as compared to MNP alone. If covalently bound PEG-based polymer could further improve shielding will have to be investigated.

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