

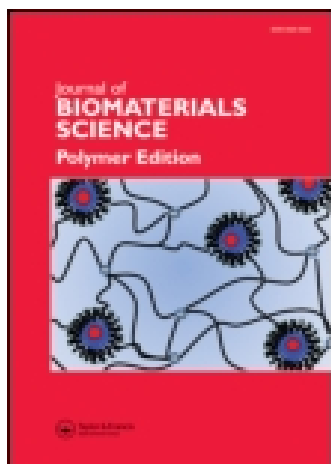
This article was downloaded by: [University of York]

On: 18 October 2014, At: 08:55

Publisher: Taylor & Francis

Informa Ltd Registered in England and Wales Registered Number: 1072954

Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



## Journal of Biomaterials Science, Polymer Edition

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/tbsp20>

### Transfection of Immortalized Keratinocytes by Low Toxic Poly(2-(Dimethylamino)Ethyl Methacrylate)-Based Polymers

Nancy Van Overstraeten-Schlögel <sup>a</sup>, Yong Ho-Shim <sup>b</sup>,  
Virginie Tevel <sup>c</sup>, Sébastien Bontems <sup>d</sup>, Philippe Dubois <sup>e</sup>  
& Martine Raes <sup>f</sup>

<sup>a</sup> Research Unit of Cell Biology, University of Namur, B-5000 Namur, Belgium.  
[nancy.vanoverstraeten@fundp.ac.be](mailto:nancy.vanoverstraeten@fundp.ac.be)

<sup>b</sup> Laboratory of Polymeric and Composite Materials  
Center of Innovation and Research in Materials and  
Polymers, University of Mons-Hainaut, B-7000 Mons,  
Belgium

<sup>c</sup> Research Unit of Cell Biology, University of Namur,  
B-5000 Namur, Belgium

<sup>d</sup> Laboratory of Virology and Immunology, GIGA-R,  
University of Liège, B-4000 Liège, Belgium

<sup>e</sup> Laboratory of Polymeric and Composite Materials  
Center of Innovation and Research in Materials and  
Polymers, University of Mons-Hainaut, B-7000 Mons,  
Belgium

<sup>f</sup> Research Unit of Cell Biology, University of Namur,  
B-5000 Namur, Belgium

Published online: 13 Apr 2012.

To cite this article: Nancy Van Overstraeten-Schlögel , Yong Ho-Shim , Virginie Tevel , Sébastien Bontems , Philippe Dubois & Martine Raes (2012) Transfection of Immortalized Keratinocytes by Low Toxic Poly(2-(Dimethylamino)Ethyl Methacrylate)-Based Polymers, Journal of Biomaterials Science, Polymer Edition, 23:6, 739-761, DOI: [10.1163/092050611X559430](https://doi.org/10.1163/092050611X559430)

To link to this article: <http://dx.doi.org/10.1163/092050611X559430>

PLEASE SCROLL DOWN FOR ARTICLE

Taylor & Francis makes every effort to ensure the accuracy of all the information (the "Content") contained in the publications on our platform. However, Taylor & Francis, our agents, and our licensors make no representations or warranties whatsoever as to the accuracy, completeness, or suitability for any purpose of the Content. Any opinions and views expressed in this publication are the opinions and views of the authors, and are not the views of or endorsed by Taylor & Francis. The accuracy of the Content should not be relied upon and should be independently verified with primary sources of information. Taylor and Francis shall not be liable for any losses, actions, claims, proceedings, demands, costs, expenses, damages, and other liabilities whatsoever or howsoever caused arising directly or indirectly in connection with, in relation to or arising out of the use of the Content.

This article may be used for research, teaching, and private study purposes. Any substantial or systematic reproduction, redistribution, reselling, loan, sub-licensing, systematic supply, or distribution in any form to anyone is expressly forbidden. Terms & Conditions of access and use can be found at <http://www.tandfonline.com/page/terms-and-conditions>

# Transfection of Immortalized Keratinocytes by Low Toxic Poly(2-(Dimethylamino)Ethyl Methacrylate)-Based Polymers

Nancy Van Overstraeten-Schlögel<sup>a,\*</sup>, Yong Ho-Shim<sup>b</sup>, Virginie Tevel<sup>a</sup>,  
Sébastien Bontems<sup>c</sup>, Philippe Dubois<sup>b</sup> and Martine Raes<sup>a</sup>

<sup>a</sup> Research Unit of Cell Biology, University of Namur, B-5000 Namur, Belgium

<sup>b</sup> Laboratory of Polymeric and Composite Materials Center of Innovation and Research in Materials and Polymers, University of Mons-Hainaut, B-7000 Mons, Belgium

<sup>c</sup> Laboratory of Virology and Immunology, GIGA-R, University of Liège, B-4000 Liège, Belgium

Received 2 August 2010; accepted 2 February 2011

## Abstract

Skin carcinoma are among the most spread diagnosed tumours in the world. In this study, we investigated the transfection of immortalized keratinocytes, used as an *in vitro* model for skin carcinoma, using antisense technology and poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA)-based polymers, with original architecture and functionalities. We tested PDMAEMA polymers with different structures: linear, with two (DEA-PDMAEMA) or three (TEA-PDMAEMA) arms. The cytotoxicity of these polymers was assessed over a wide range of apparent  $M_n$  (from 7600 to 64 600). At a N/P ratio of 7.38, cytotoxicity increases with the  $M_n$ . Keratinocytes were transfected with a fluorescent oligonucleotide and then analyzed by flow cytometry. For the three architectures tested, the percentage of transfected cells and abundance of internalized oligonucleotide were closely related to the  $M_n$  of the polymer. Confocal microscopy and FACS analyses showed a wide spread fine granular distribution of the oligonucleotide up to 3 days post-transfection. Then, we assessed the silencing efficiency of the polymers, targeting GFP in GFP expressing keratinocytes. The maximal silencing effect ( $\pm 40\%$ ) was obtained using a DEA-PDMAEMA polymer ( $M_n = 30\,300$ ). These results suggest that PDMAEMA-based polymers can be efficiently used to transfect immortalized keratinocytes and, thus, open new perspectives in the therapy of skin carcinoma.

© Koninklijke Brill NV, Leiden, 2012

## Keywords

Keratinocytes, skin carcinoma, antisense oligonucleotide, transfection, 2-(dimethylamino)ethyl methacrylate, hostasol

## 1. Introduction

Skin cancers are among the most frequent diagnosed tumours in the world. Among skin cancers, basal cell carcinoma are the most spread type [1]. Their formation

\* To whom correspondence should be addressed. E-mail: nancy.vanoverstraeten@fundp.ac.be

results from constitutional and/or inherited factors combined with environmental factors. Skin carcinoma are usually treated by surgical excision [2], radiotherapy [3–5], irradiation [6] or dynamic phototherapy [7]. However, these treatments are painful and invasive. Skin carcinoma occurs in the epidermis made up by constantly regenerating keratinocytes. Keratinocytes proliferate in the basal layer and then move upwards, beginning a differentiation program in the suprabasal layers that culminates at the surface into fully differentiated dead cells of the cornified layer [8]. In skin carcinoma, keratinocytes display an anarchistic proliferation characteristic of the cancerous phenotype. Evidence suggests that there are also numerous tumour suppressor genes involved in skin carcinogenesis [9–11]. Identification of activated oncogenes and anti-apoptotic genes in cancer cells allows considering such genes as targets in gene therapy. In this regard, antisense oligonucleotides (ASO) can be used as a promising tool to efficiently silence a specific gene [12–16].

Recent advances in biotechnology have improved gene-delivery technology to achieve safe and effective delivery of nucleic acids into cells for gene therapy. Because of their high efficiency, viral vectors are often used as gene delivery tools [17–19]. However, safety, efficiency and producibility are serious concerns for clinical applications [20]. To overcome these issues, non-viral vectors with enhanced efficiency and safety are required.

Vectors such as cationic lipids and polymers have been widely studied [21–23]. Among those, cationic polymers, such as poly(L-lysine) (PLL) and polyethylenimine (PEI), are the best known [24]. PEI displays higher efficiency than PLL, but with significant drawbacks including poor biocompatibility and non biodegradability. Indeed, validation of these non-viral vectors in clinical trials requires minimal interactions with biological components. The nucleic acid–vector complexes must be small enough for renal elimination and inert towards blood constituents while maintaining structure and efficiency of the nucleic acids trapped inside [25].

Most of the investigated polycations, such as PDADMAC or PDMAEMA, show interesting transfection efficiencies [26–28]. The successful transfection of cells with PDMAEMA polymers based polyplexes is assigned to their ability to destabilize endosomes, as well as to dissociate easily from the DNA once delivered in the cytosol [28]. The mechanism of internalization of these polyplexes has been shown to proceed by both caveolae- and clathrin-dependent pathways [29].

Among the studies investigating new non-viral gene delivering tools a general focus on the delivery of a plasmidic DNA is observable [30–32]. However, as we aim for a gene silencing effect and not the expression of a foreign gene, we tried to transfect an antisense oligonucleotide. Plasmidic DNA carriers are not always very effective with oligonucleotides. Indeed, PLL can provide a poor efficiency, while PEI displays a higher efficiency but with significant drawbacks, including poor biocompatibility and non-biodegradability, whereas the validation of these non-viral vectors in clinical trials requires minimal interactions with biological components. The formed cationic polymer/gene complexes, the so-called polyplexes, must be small enough in size for renal elimination and inert towards blood constituents

while maintaining structure and efficiency of the nucleic acids trapped inside [25]. Therefore, designing a polymer for specific oligonucleotide delivery is of high interest and can find numerous applications in antisense therapy.

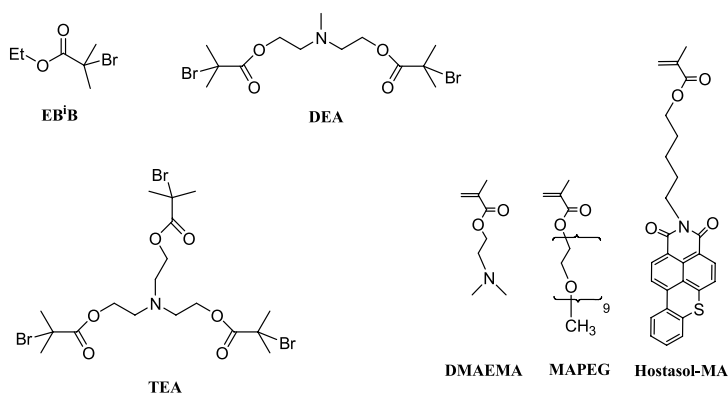
Here, we developed a new strategy for transfecting keratinocytes based on antisense technology using original non-viral polymeric vectors. Polymeric vectors present the advantage compared to cationic lipids, that they are much more versatile, offering the possibilities of varying their composition, architecture and size in a controlled way. We synthesized novel PDMAEMA-based architectures by ATRP [33] and compared them with linear PDMAEMA polymers. We investigated the efficiency of vectorisation of these polymers and their ability to ensure the efficiency of an ASO to silence the green fluorescent protein (GFP) in immortalized keratinocytes. We also analyzed the cellular uptake and intracellular trafficking of the polyplexes. Our data show that a two-arm DEA-PDMAEMA with a  $M_n$  of about 30 000 was the most effective polymer.

## 2. Materials and Methods

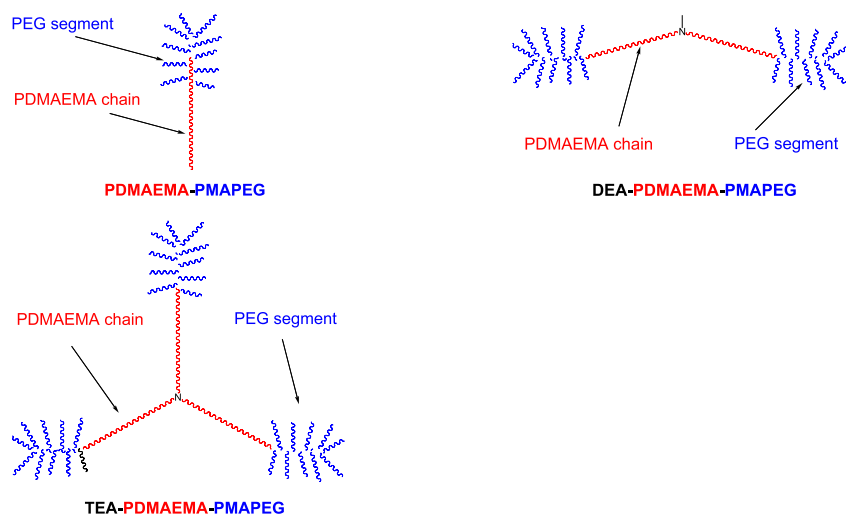
### 2.1. Synthesis of PDMAEMA-Based (Co)Polymers

#### 2.1.1. Materials

The mono-, di- and trifunctional initiators used in this study, i.e., ethyl 2-bromoisobutyrate (EB<sup>1</sup>B), *bis*( $\alpha$ -bromoisobutyryl) *N*-methyl diethanolamine (DEA), *tris*( $\alpha$ -bromoisobutyryl) triethanolamine (TEA), and their PDMAEMA homo-polymers and palm-tree like co-polymers with  $\alpha$ -methoxy,  $\omega$ -metacrylate poly(ethylene glycol) (MAPEG) (Figs 1 and 2; Table 1) were prepared by atom transfer radical polymerization (ATRP) using copper(I) bromide (CuBr) ligated with 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA, 97%; Aldrich) as catalytic complex following a procedure recently reported [34].



**Figure 1.** Alkylbromide (multi)functional initiators and methacrylate monomers used for the (co)polymers preparation.



**Figure 2.** Schematic representation of palm-tree-like co-polymers. This figure is published in colour in the online edition of this journal, which can be accessed via <http://www.brill.nl/jbs>

**Table 1.**

Characterization of the PDMAEMA-based (co)polymers

Code	Initiator	$M_n$ (GPC)	$M_w/M_n$	Composition	MAPEG (mol%)
L1	EB <sup>i</sup> B	7600	1.24	PDMAEMA	0
L2	EB <sup>i</sup> B	11 200	1.24	PDMAEMA	0
L3	EB <sup>i</sup> B	22 900	1.31	PDMAEMA	0
L4	EB <sup>i</sup> B	45 400	1.25	PDMAEMA	0
L5	EB <sup>i</sup> B	64 600	1.30	PDMAEMA	0
D2	DEA	10 100	1.52	DEA-PDMAEMA	0
D3	DEA	30 300	1.48	DEA-PDMAEMA	0
T3	TEA	31 600	1.44	TEA-PDMAEMA	0
T4	TEA	44 000	1.59	TEA-PDMAEMA	0
F4	EB <sup>i</sup> B	52 500	1.55	PDMAEMA-st-Hos	0
LP	EB <sup>i</sup> B	24 200	1.50	PDMAEMA-b-PMAPEG	11
DP	DEA	54 100	1.46	DEA-PDMAEMA-b-PMAPEG	12
TP	TEA	61 500	1.52	TEA-PDMAEMA-b-PMAPEG	14

*N,N*-dimethylamino-2-ethyl methacrylate (DMAEMA, from Aldrich, 98%) was passed through a column of basic alumina to remove the stabilising agents and stored under nitrogen atmosphere at  $-20^{\circ}\text{C}$ . Ethyl 2-bromoisobutyrate (EB<sup>i</sup>B, from Aldrich, 98%), 2-bromoisobutyryl-bromide (Br<sup>i</sup>BuBr, Aldrich), HMTETA and copper bromide (CuBr, 98%; Fluka) were used without further purification. Benzothioxantheno-3,4-dicarboxylic anhydride (Hostasol-anh, Clariant) was used as received. Hostasol-methacrylate monomer (2-(8-methacryloyloxy-3,6-dioxao-

tyl)thioxantheno[2,1,9-dej]isoquinoline-1,3-dione (Hostasol-MA)) (Fig. 1) was prepared from the precursor Hostasol-anh as described in the literature [35].

### 2.1.2. Method

To prepare PDMAEMA (e.g., L4 in Table 1), to a dried glass-tube was charged with copper(I) bromide (CuBr) (17 mg, 0.12 mmol) and a magnetic stir bar. The tube was closed with a three-way stopcock capped by a rubber septum and purged by three repeated vacuum/nitrogen cycles. Separately, in a dried flask, HMTETA (22.4 mg, 0.115 mmol) and (6 ml, 35.6 mmol) were introduced and degassed of oxygen by nitrogen sparging for 5 min before transferring the mixture into a glass tube placed in a oil bath maintained at 30°C. Finally, the degassed initiator (EB<sup>1</sup>B) was added to the tube with a previously degassed syringe. The polymerization was stopped after 3 h by immersing the tube into a liquid nitrogen bath and the polymer was selectively recovered by precipitation in a sevenfold volume excess of cold heptane and dried until constant weight. Monomer conversion was 94%, determined by <sup>1</sup>H-NMR. The catalyst was removed by passing through a column of basic alumina *via* THF or toluene solution. Homo-polymers PDMAEMA were prepared by the same method controlling the ratio of the initiator. DEA and TEA homo-polymers were prepared also by this method except the initiator were DEA and TEA instead of EB<sup>1</sup>B.

For the co-polymerization with poly(ethylene glycol)  $\alpha$ -methoxy,  $\omega$ -methacrylate (PMAPEG), after homo-polymerization of DMAEMA by ATRP as described, previously nitrogen-sparged MAPEG/THF solution (1:1, v/v; 0.97 M) was added with a degassed syringe into the glass reaction tube and co-polymerization was conducted for 16 h under an atmosphere of nitrogen. The co-polymerization was stopped by immersing the tube into a liquid nitrogen bath and the co-polymers isolated by following the aforementioned purification procedure. LP (Table 1) was recovered with 89% yield by this procedure.

### 2.1.3. Synthesis of Fluorescent PDMAEMA-st-Hostasol Co-Polymers for Oligonucleotide Tracking

CuBr (3.8 mg, 0.026 mmol) and a magnetic bar were introduced in a dried glass-tube. The tube was then closed with a three-way stopcock capped by a rubber septum and purged by three repeated vacuum/nitrogen cycles. Separately, in a dried flask, HMTETA (11.8 mg, 0.051 mmol), DMAEMA (1.4 ml, 11.5 mmol), Hostasol-MA (52.0 mg, 0.115 mmol) and 2 ml THF (5 M) were introduced and oxygen-degassed by bubbling with nitrogen for 5 min before transferring the mixture into the glass-tube placed in a oil bath maintained at 60°C. Finally, degassed EB<sup>1</sup>B was added to the tube with a previously degassed syringe. The polymerisation was stopped after 16 h by immersing the tube into a liquid nitrogen bath and the polymer was selectively recovered by precipitation from a sevenfold volume excess of cold heptane and dried until constant weight. Monomer conversion was determined by <sup>1</sup>H-NMR. The catalyst was removed by passing through a column of

basic alumina *via* THF solution. After purification 0.94 mol% of the Hostasol-MA was detected in the co-polymer (F4, Table 1) with UV-Vis spectroscopy.

#### 2.1.4. Characterisation

The molar fraction of MAPEG in the co-polymers was determined by  $^1\text{H-NMR}$  as previously reported [34] using a Bruker AMX-300 MHz spectrometer at room temperature in  $\text{CDCl}_3$  enriched with 0.03% TMS. Gel-permeation chromatography (GPC) was performed in THF + 2 wt%  $\text{NEt}_3$  at 35°C using a Polymer Laboratories liquid chromatograph equipped with a PL-DG802 degasser, an isocratic HPLC pump LC 1120 (flow rate 1 ml/min), a Marathon auto sampler (loop volume 200  $\mu\text{l}$ , solution concentration 1 mg/ml), a PL-DRI refractive index detector and three columns: a PL gel 10  $\mu\text{m}$  guard column and two PL gel Mixed-B 10  $\mu\text{m}$  columns. Poly(methyl methacrylate) standards were used for calibration. The amount of Hostasol-MA in the co-polymer was measured by UV-Vis spectroscopy (ethanol, 25°C, Varian Cary 50 Scan) using the calibration graph presented as supporting data.

#### 2.2. Cells and Reagents

Immortalized human keratinocytes (IHK), ectopically expressing the catalytic subunit of telomerase and characterized by inactivated  $\text{p16}^{\text{INK-4A}}$  gene (N-hTERT keratinocytes), were a kind gift of Prof. J. Rheinwald (Division of Dermatology, Department of Medicine and Harvard Skin Disease Research Center). IHK cells were grown at 37°C in a humidified atmosphere with 5%  $\text{CO}_2$  in Epilife Medium (Cascade Biologics) supplemented with HKGS (Cascade Biologics). Cells were diluted ( $10^6$  cells in a T75 flask) before reaching 80% confluence in order to maintain exponential growth. In order to assess the silencing induced by ASO, an IHK cell line (IHK-MAZ-GFP) stably expressing a destabilized form of GFP protein [36] was created using a lentiviral approach. For this, the MAZ-GFP gene was amplified by PCR from the pMAZ-ODC vector (a generous gift from Prof. B. Epe) and cloned into the pLenti6/V5-D-Topo vector (Invitrogen). The generation of the IHK-MAZ-GFP cell line was performed using the 293FT (Invitrogen) cells following manufacturer's instruction. Blasticidin (20  $\mu\text{g/ml}$ , Sigma-Aldrich) was used for the selection of the positively transduced cells. Transduction efficiency was assessed by visualisation of the GFP-positive cells by fluorescence microscopy. The addition of the proteasome inhibitor MG132 (Sigma-Aldrich) 4 h prior to observation demonstrated that the turn-over of the GFP-MAZ protein is clearly dependent on this degradation pathway.

#### 2.3. Oligonucleotides and siRNAs

In order to assess the vectorisation efficiency of the polymers, fluorescent oligonucleotides WA488 and WA647 were synthesized and purified by Eurogentec, by grafting respectively Alexa 488 and Atto 647 to the following oligonucleotide: 5'-CCCTTGCTCACCAT-3'. An antisense oligonucleotide against the green fluorescent protein was synthesized and purified by Eurogentec. The sequence and loca-



tion of the oligonucleotides are W21\*: 5'-CTCCTCGCCCTTGCTCACCAT-3' (nucleotides 1–21). An oligonucleotide consisting of the scrambled sequence W21sc\* (5'-TCCATCGTTACCGTCCTCCCC-3') was used as a negative control. In order to normalise the results obtained by quantitative RT-PCR, we used a scrambled oligonucleotide IL8sc\* (sequence: 5'-GTGATTGAGAGTGGACCACACTGCGC CAAC-3'). All the oligonucleotides used were phosphorothioate modified. We also used siRNAs for highlighting the efficiency of the polymers used in this study. The synthetic anti-GFP siRNAs used were designed as follows: sense, AACU UCAGGGUCAGCUUGC; and antisense, GCAAGCUGACCCUGAAGUU (19-mer) [37]. As control, we used the non-targeting siRNA from Eurogentec.

#### 2.4. Preparation of Polyplexes and Transfection of IHK Cells

The day before transfection, cells were trypsinized and resuspended in medium before being transferred to 6-well plates at a density of  $12 \times 10^4$  cells/well in a final volume of 2 ml. The commercial agent used as reference for assessing the efficiency of the transfection by polymers was the SuperFect (Qiagen) used following the manufacturer's instructions. SuperFect (6  $\mu$ l) was added to 3  $\mu$ l oligonucleotide in 200  $\mu$ l OptiMEM (Gibco). The mixture was left to stand for 10 min at room temperature before being added onto the cells. SuperFect was shown to be the best commercial agent for assessing the efficiency of vectorisation in the IHK cells, but for demonstrating a silencing effect following the transfection of an antisense oligonucleotide, Oligofectamine (Invitrogen) was the best performing commercial agent. In both cases, we used optimal concentrations of mixtures according to the manufacturer instructions to transfect the immortalised keratinocytes. The transfection mixtures with Oligofectamine were prepared following the manufacturer's instructions. Briefly, 10  $\mu$ l of a solution of oligonucleotides (20  $\mu$ M) was diluted in 175  $\mu$ l OptiMEM and 2  $\mu$ l Oligofectamine was diluted in 15  $\mu$ l OptiMEM. After 10 min of incubation at room temperature, the two solutions were gently mixed, followed by a second incubation of 20 min at room temperature. The medium was removed from the wells of the 6-well plate and 800  $\mu$ l Epilife-HKGS medium was added. The diluted complex was then overlaid and cells were incubated at 37°C under a humidified atmosphere. After 4 h, the medium containing the mixture was removed and fresh medium was added. Transfection experiments with polymers were carried out as follows. After dilution of 4  $\mu$ g oligonucleotide in 400  $\mu$ l OptiMEM medium, the homo-polymer was gently added to the solution. After 30 min of incubation, the diluted polyplex was overlaid on the cultured cells for 24 h. Medium was changed after 4 h. In the case of a ternary complex, the co-polymer was supplemented to the solution containing the homo-polymer and the oligonucleotide after the 30 min incubation. Then the mixture was let for another 30 min at room temperature before being added into the cells-containing wells.

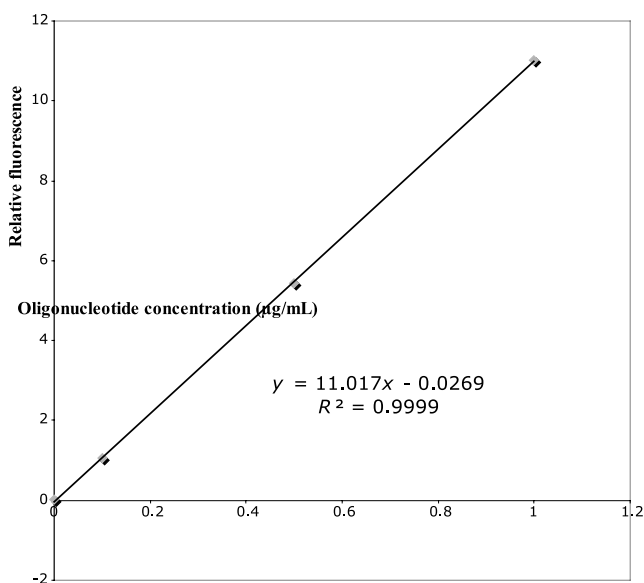
### 2.5. Checking of Complex Formation by Fluorescent Dye Exclusion Assay

For verifying the formation of a complex between our polymers and the 14-mer oligonucleotide, we used the Quant-iT™ OliGreen® kit (Invitrogen), especially designed to monitor the presence of an ssDNA in a complex solution. Briefly, we formed complexes with different w/w ratios (1:1, 2:1, 3:1 or 10:1) in OptiMem medium. After 30 min at room temperature, 800 µl TE medium was added. The aqueous working solution of the Quant-iT™ OliGreen® reagent (1 ml) was added to each sample. After incubation for 5 min in the dark at room temperature the fluorescence of each sample was measured.

As recommended in the manufacturer's instructions, a standard curve with the 14-mer oligonucleotide was made (Fig. 3).

### 2.6. Determination of Polymer Cytotoxicity (MTT)

Cell proliferation was measured one day post-transfection using MTT (Sigma) at a final concentration of 2.5 mg/ml. Cells were incubated for 4 h with the MTT solution, then the medium was removed and a lysis solution (30% SDS/*N,N*-dimethyl-formamide, 2:1 (v/v), pH 4.7 (80% acetic acid + 1 M HCl, 9:1)) was added. After 1 h at 37°C, the developed colour of the formazan derivative was read in a photometer at 570 nm using the lysis solution as blank. The optical density (OD) of the blank was withdrawn from the OD of the different samples which was reported to the control (non-transfected) cells.



**Figure 3.** Standard curve obtained with the Quant-iT™ OliGreen® kit.

### 2.7. Determination of the Vectorisation Efficiency by Flow Cytometry

Cells were transfected with the fluorescent oligonucleotide WA488 as described and were trypsinized and washed in PBS + 1% bovine serum albumin (BSA). Then cells were resuspended in PBS + 1% BSA and analyzed for their fluorescence. Flow cytometric data were acquired on a FACS Calibur flow cytometer (BD Biosciences) and analyzed by the Cellquest software (BD Biosciences). Mean channel fluorescence values (MCFR) were calculated by reporting the mean fluorescence values of the samples to the control. The percentage of transfected cells was calculated by gating the fluorescent population compared to the control.

### 2.8. Intracellular Trafficking Study and Image Analysis

Cells were seeded on coverslips the day before transfection with the fluorescent oligonucleotides. WA488 was used to monitor the persistence over time of the transfected oligonucleotide, whereas WA647 was used for co-localisation experiments allowing the discrimination of the fluorescence associated with the oligonucleotide from the fluorescence associated with the polymer. At 24 h post-transfection, cells were fixed for 10 min with 4% paraformaldehyde. After 3 washes in PBS, coverslips were mounted in Mowiol (Kuraray) and processed for confocal microscopy (Leica). Three specimens of each group were chosen and at least 4 randomly chosen fields were analyzed.

### 2.9. RT-PCR Determination of GFP mRNA

At 24 h post-transfection with the W21\* ASO (anti-GFP), total RNA was extracted with the RNeasy total RNA isolation system (Promega) and 500 ng were used in reverse transcription using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instruction. Quantitative RT-PCR was directly performed with the ice-cooled cDNA by monitoring the increase in fluorescence of incorporated SYBR Green dye (Applied Biosystems) with an ABI PRISM 7000 sequence detector system (Applied Biosystems). Specific primers for GFP were designed using the Primer express software (Applied Biosystems): forward, 5'-CCTACGGCGTGCAGTGCT-3'; reverse, 5'-AGATGGTGCCTCCTGGAC-3'; and GAPDH: forward, 5'-ACCCACTCCTCCACCTTTGAC-3'; reverse, 5'-ACCCACTCCTCCACCTTTGAC-3'. In order to improve the reproducibility of the silencing experiments, IHK-GFPd cells were used between passages 5 and 15. We also used a scrambled ASO sequence (IL8 scramble) to normalise the results obtained by quantitative RT-PCR. Indeed, the comparison with a single housekeeping gene can lead to relatively large errors in a significant proportion [38]. Therefore, we decided to use another sequence to introduce another normalization factor for the GFP RNA level in our samples.

### 3. Results

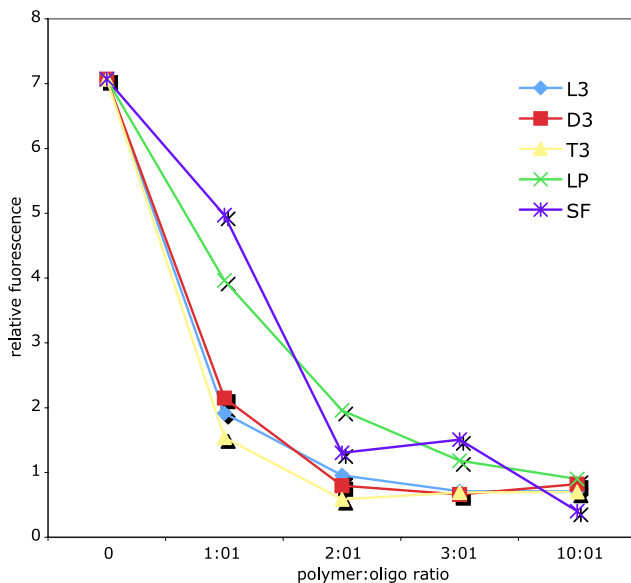
#### 3.1. Formation of Polyplexes Based on the Tested PDMAEMA Polymers

To ensure the formation of stable polyplexes with our PDMAEMA of different architectures and a 14-mer oligonucleotide, we performed a fluorescent dye exclusion assay with the Quant-iT™ OliGreen® kit. As the correlation coefficient of the standard curve was reliable, we analysed complexes formed with L3, D3, T3, LP or the SuperFect with the described method (Fig. 4).

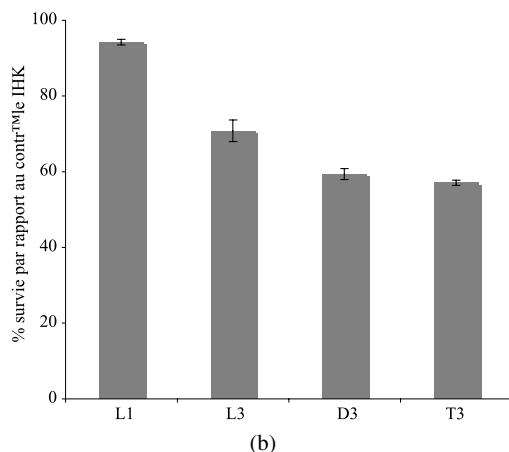
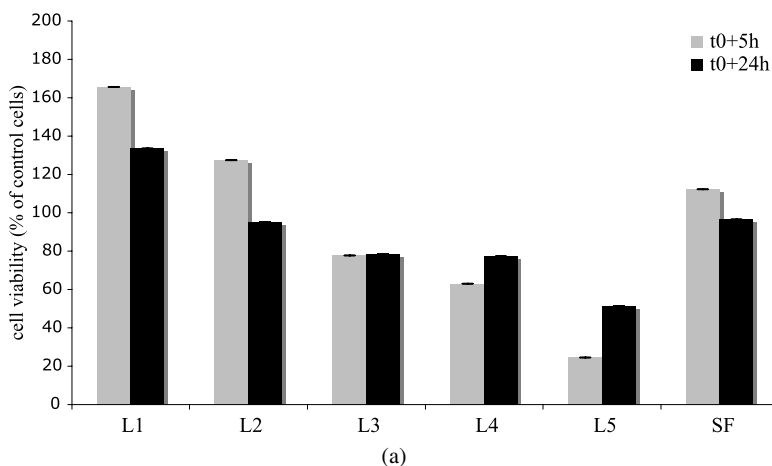
We observed a similar complexation between the linear, two- or three-arm PDMAEMA polymers and the oligonucleotide for every ratio tested. For the w/w ratio of 2:1, a poor difference between the tested PDMAEMA and the commercial agent SuperFect was highlighted. Therefore, complexes between the PDMAEMA of different architectures and an oligonucleotide can be formed at every w/w ratio tested.

#### 3.2. Cytotoxicity of the PDMAEMA Homo-Polymers

In the first series of experiments, we analyzed the cytotoxicity of the polyplexes after transfection of the IHK cells with a 14-mer oligonucleotide using linear PDMAEMA polymers. One day after seeding, the IHK cells were transfected using linear PDMAEMA polymers within a broad range of average  $M_n$  at the optimal N/P ratio of 7.38, corresponding to a ratio of 3:1 (w/w) (Fig. A.1 in the Appendix).



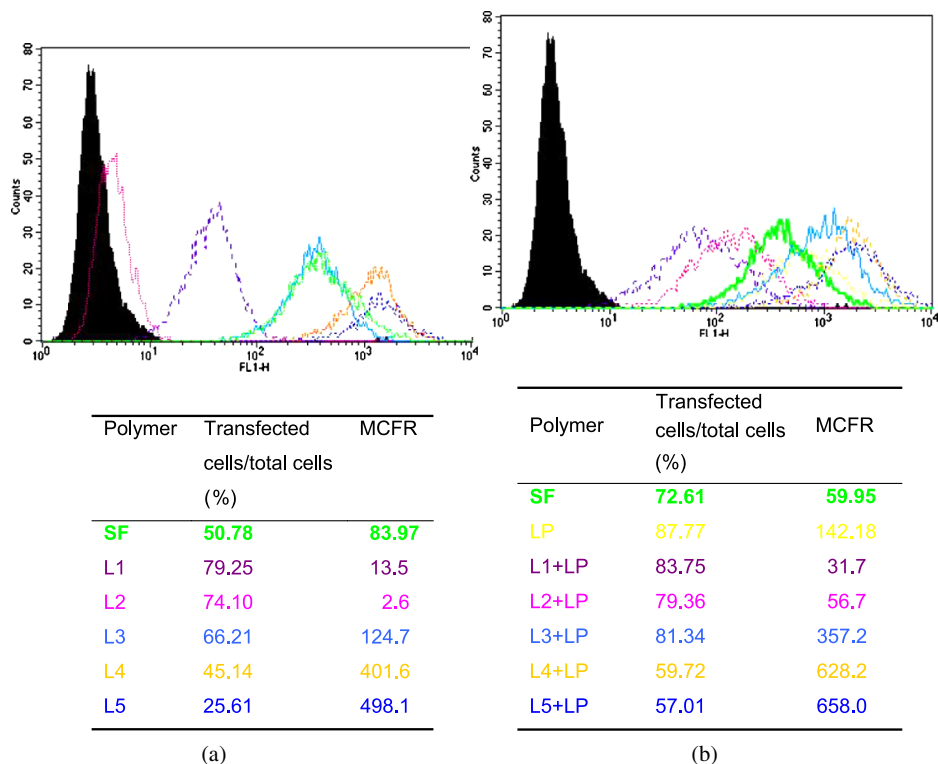
**Figure 4.** Checking of the presence of the oligonucleotide inside different complexes by dye exclusion assay. Complexes of different polymer/oligonucleotide (w/w) ratio were produced and the presence of the oligonucleotide in the complexes was assessed by dye exclusion. This figure is published in colour in the online edition of this journal, which can be accessed via <http://www.brill.nl/jbs>



**Figure 5.** Cytotoxicity of the linear or branched PDMAEMA polymers. Cells were seeded ( $15 \times 10^3$  cells/cm<sup>2</sup>) and were transfected the following day by linear PDMAEMA polymers (N/P = 7.38) of different  $M_n$  (a) or by DEA or TEA-initiated PDMAEMA polymers of  $M_n \approx 26\,000$  (b). MTT assay was performed 5 (grey columns) and 24 h (black columns) post-transfection (a) or 24 h post-transfection (b). Data represent the mean  $\pm$  SEM of 3 experiments;  $P < 0.01$ .

We also compared the cytotoxicity of the linear PDMAEMA *versus* branched DEA or TEA-initiated PDMAEMA architectures at a  $M_n \approx 26\,000$ . Cytotoxicity was assessed by the MTT test 5 and/or 24 h after transfection (Fig. 5a and b). In parallel, we transfected IHK cells using SuperFect and assessed the cytotoxicity.

MTT assay revealed a cell viability for SuperFect-transfected cells of more than 80%. The MTT test revealed that the cytotoxicity of the polyplexes was closely related to the  $M_n$  of the polymer used. Indeed, we noticed that the higher the  $M_n$ , the higher the toxicity. Nevertheless, for a  $M_n \leq 45\,000$ , the viability of IHK cells was more than 60%. For a  $M_n \approx 26\,000$ , the architecture did not significantly influence the cytotoxicity.



**Figure 6.** Effect of the polymer  $M_n$  on the oligonucleotide vectorisation efficiency. Cells were transfected with WA488 fluorescent oligonucleotide using different PDMAEMA polymers ( $N/P = 7.38$ ), with the  $M_n$  ranging from 7600 to 64 699 (see Table 1 for details), without (a) or with (b) PDMAEMA-*b*-PMAPEG co-polymers consecutively added. Cells were collected for flow cytometry analysis 24 h post-transfection. This figure is published in colour in the online edition of this journal, which can be accessed *via* <http://www.brill.nl/jbs>

### 3.3. Effect of the Polymer $M_n$ on the Vectorisation Efficiency

We then tested several linear PDMAEMA polymers of different  $M_n$  for their capacity to vectorize IHK cells, i.e., their ability to internalize a genetic material. IHK cells were transfected with the Alexa 488-labelled oligonucleotide (WA488), using different PDMAEMA polymers with a  $N/P$  ratio of 7.38. At 24 h post-transfection, we analyzed the efficiency of vectorisation by flow cytometry (Fig. 6a). For sake of comparison, several commercial agents, i.e., FuGene, SuperFect, LipofectAmine and Oligofectamine, all recognized for their high efficiency in transfection, were also tested (data not shown). The best one proved to be SuperFect that was further used as reference.

FACS analyses showed that the internalisation of the fluorescent oligonucleotide is highly dependent on the  $M_n$  of the PDMAEMA polymer used for vectorisation. Indeed, the higher the  $M_n$  of the polymer, the better the incorporation of the oligonucleotide, estimated by the MCFR, proved to be, but also the lower the num-

ber of transfected cells observed. We also assessed different N/P ratios, 2.46 and 14.76. At the N/P ratio of 2.46, no cytotoxicity was observed for any of the linear polymers tested, but the vectorisation efficiency was low. On the contrary, with N/P = 14.76 all polymers displayed a higher cytotoxicity, but an increased efficiency (data not shown). As the addition of a P(DMAEMA-*b*-MAPEG) co-polymer has been reported to improve the vectorisation efficiency and hemocompatibility of the polyplexes [39], we added a palm-tree-like co-polymer to pre-formed PDMAEMA-oligonucleotide polyplexes and transfected IHK cells with the ternary polyplexes (P(DMAEMA-*b*-MAPEG) + PDMAEMA + WA488). Internalisation of the fluorescent oligonucleotide was used to assess the efficiency of vectorisation (Fig. 6b). The formation of ternary polyplexes slightly improved the vectorisation efficiency with low- $M_n$  polymers while it did not affect the efficiency observed with high- $M_n$  polymers. However, the addition of a P(DMAEMA-*b*-MAPEG) polymer increased the cytotoxicity of the polyplexes (Fig. A.1). Taken together, our results on cytotoxicity and vectorisation efficiency, suggest that PDMAEMA polymers in the range 22 900–45 400 seem the most suited for a compromise between acceptable cytotoxicity and efficient vectorisation.

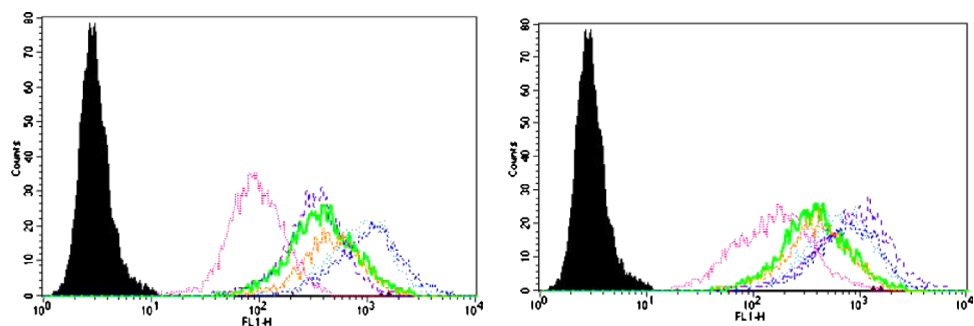
### 3.4. Effect of the Architecture on Vectorisation Efficiency

In order to improve the vectorisation efficiency, we used PDMAEMA based polymers with different architectures. Two- (DEA-) or three- (TEA-) arm PDMAEMA polymers were tested for transfecting IHK cells with the Alexa 488-labelled oligonucleotide and the efficiency of vectorisation analyzed by flow cytometry (Fig. 7a).

As for the linear PDMAEMA polymers, the internalisation of the fluorescent oligonucleotide was closely related to the  $M_n$  of the branched PDMAEMA-based polymers used for vectorisation. For polymers with a  $M_n$  of around 30 000, DEA-initiated polymer D3 seemed to be slightly more efficient than the TEA-polymer T3. Tertiary complexes (see Section 2) including P(DMAEMA-*b*-MAPEG) co-polymers added in a second step to branched DEA- or TEA-PDMAEMA-based polyplexes, did not seem to really improve the vectorisation (Fig. 7b), which is in agreement with the data on linear PDMAEMA (Fig. 4b). Finally, we also tried to find out if a direct grafting of MAPEG segments on linear, two- or three-arm PDMAEMA polymers could have a beneficial effect on the vectorisation efficiency (Fig. 7c). No architecture effect was noticed after vectorisation with the palm-tree-like linear, two- or three-arms polymers as all polymers provided comparable efficiencies.

### 3.5. Effects of the Polymer on the Persistence of Transfected Oligonucleotides

In order to assess the persistence of the oligonucleotides vectorized by PDMAEMA or DEA-initiated PDMAEMA or TEA-initiated PDMAEMA polymers, we performed a flow cytometry analysis of the transfected cells after 24, 48 or 72 h (Fig. 8).

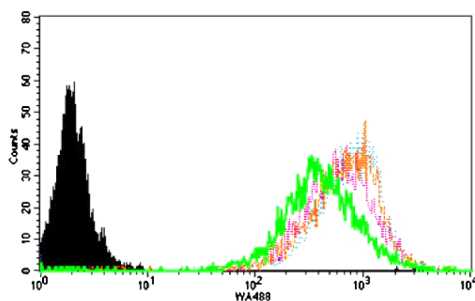


Polymer	Transfected cells/total cells (%)	MCFR
SF	72.61	59.95
L3	55.13	124.58
D2	84.32	13.45
D3	65.47	120.40
T3	51.79	66.16
T4	61.53	149.36

(a)

Polymer	Transfected cells/total cells (%)	MCFR
SF	72.61	59.95
L3+LP	81.68	140.25
D2+LP	85.39	25.69
D3+LP	75.87	93.87
T3+LP	63.39	58.99
T4+LP	66.59	110.58

(b)

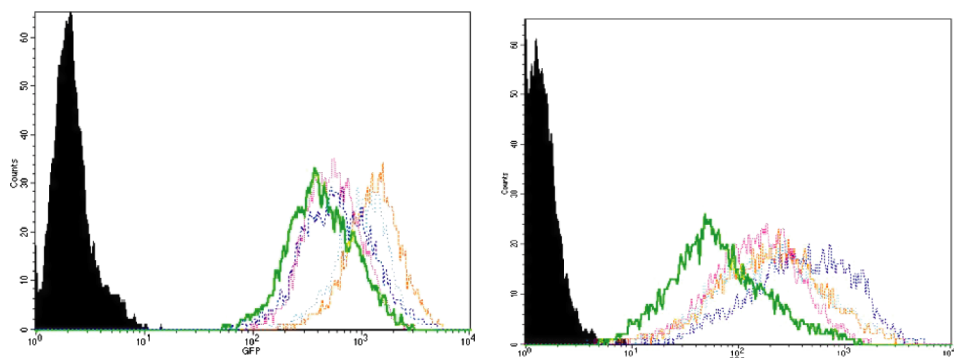


Polymer	Transfected cells/total cells (%)	MCFR
SF	42.72	101.36
LP	82.77	142.18
DP	18.27	143.70
TP	14.77	153.70

(c)

**Figure 7.** Effect of polymer architecture on oligonucleotide vectorisation efficiency. Cells were transfected with the WA488 fluorescent oligonucleotide using either (a) different DEA/TEA-PDMAEMA polymers ( $N/P = 7.38$ ) alone or (b) combined with PDMAEMA-*b*-PMAPEG polymers or (c) DEA/TEA-PDMAEMA-*b*-PMAPEG polymers (see Table 1 for details). Cells were collected for flow cytometry analysis 24 h post-transfection. This figure is published in colour in the online edition of this journal, which can be accessed *via* <http://www.brill.nl/jbs>



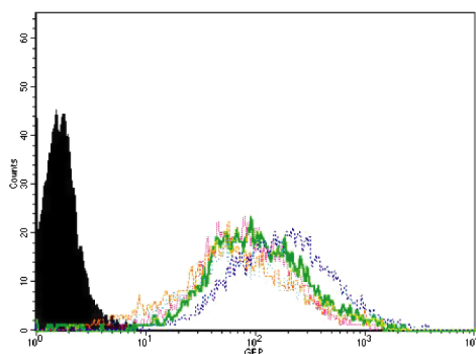


Polymer	Transfected cells/total cells (%)	MCFR
SF	90.42	164.72
L3	87.33	197.96
D3	62.90	353.28
T3	71.68	456.88
LP	90.17	247.33

Polymer	Transfected cells/total cells (%)	MCFR
SF	91.00	66.15
L3	92.25	125.48
D3	92.57	216.98
T3	92.45	203.13
LP	91.43	371.37

(a)

(b)



Polymer	Transfected cells/total cells (%)	MCFR
SF	87.95	61.83
L3	84.75	46.70
D3	77.07	62.37
T3	83.87	54.16
LP	87.57	102.63

(c)

**Figure 8.** Persistence of the oligonucleotide into the transfected cells. Cells were transfected with the WA488 fluorescent oligonucleotide using either Superfect (SF), PDMAEMA (L3) or DEA-PDMAEMA (D3) or TEA-PDMAEMA (T3) polymers (N/P = 7.38) or the PDMAEMA-b-MAPEG co-polymer (LP) at (a) 24, (b) 48 and (c) 72 h post-transfection. Flow cytometry analysis of cells transfected with SuperFect (—), L3 (—), D3 (···), T3 (----) or LP (- - -). This figure is published in colour in the online edition of this journal, which can be accessed via <http://www.brill.nl/jbs>

The data suggest that most of the transfected oligonucleotides (60%) remained inside the cells up to 3 days post-transfection, whereas cells transfected with SuperFect only retained 25% of the fluorescent oligonucleotides 2 days after transfection. As previously, we observed a similar efficiency of the polymers L3, D3 and T3 at 24 h post-transfection. Over the following 24 h, this trend remained stable. However, we could observe a quicker decrease in efficiency of the polymers L3, D3 and T3 compared to the polymer LP, especially after 72 h.

Flow cytometry analyses provide data on the percentage of cells efficiently transfected and on the quantity of oligonucleotides internalized but do not provide information about the distribution of oligonucleotides inside the cells. Therefore, confocal microscopy analyses were performed on the cells transfected with the fluorescent WA488 oligonucleotide using linear, DEA- or TEA-initiated PDMAEMA polymers after 1, 2 or 3 days to highlight the localisation and distribution of the internalized polyplexes (Fig. 9).

After 24 h, the fluorescent oligonucleotide was detected inside the cells and was distributed into many fluorescent dots scattered throughout the cytoplasm for most cells. After 48 and 72 h, fluorescence was still observed inside the transfected immortalized keratinocytes, confirming the flow cytometry observations. Altogether, these data confirm that oligonucleotides remain in the cells up to 3 days after vectorisation with either PDMAEMA, DEA-PDMAEMA or TEA-PDMAEMA polymers, with an improved persistence compared with SuperFect.

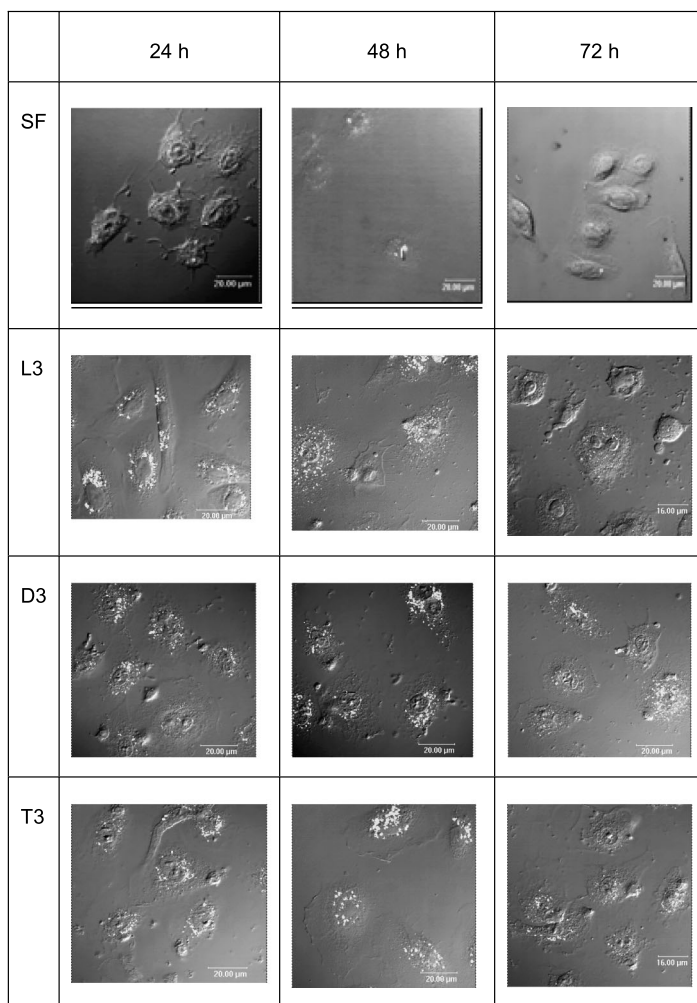
### 3.6. Intracellular Distribution of Fluorescent Polymers and Oligonucleotides

As biological activity is closely depending on the cellular uptake, intracellular stability of the polyplexes and bioavailability of the oligonucleotide, it is crucial to monitor the uptake and trafficking. Therefore, we vectorized an Atto 647-labelled oligonucleotide using a hostasol-labelled PDMAEMA polymer and analyzed the distribution of their respective fluorescence by confocal microscopy 1, 2 and 3 days after the transfection (Fig. 10).

At 24 h post-transfection, the polymer and the oligonucleotide partially co-localized in the cell, but there are also clearly small aggregates of oligonucleotides devoid of the polymer fluorescent signal, suggesting the disruption of some of the polyplexes with the release the oligonucleotides. However, at 48 h post-transfection aggregates of polymers and oligonucleotides were still observable. After 3 days, confocal images confirmed that oligonucleotide associated fluorescence was still present intracellularly, but less polyplexes were observed, which confirms the previous data on the persistence of oligonucleotides vectorized by PDMAEMA polymers.

### 3.7. Effects of the Polymers on the Silencing Efficiency of Anti-GFP ASO

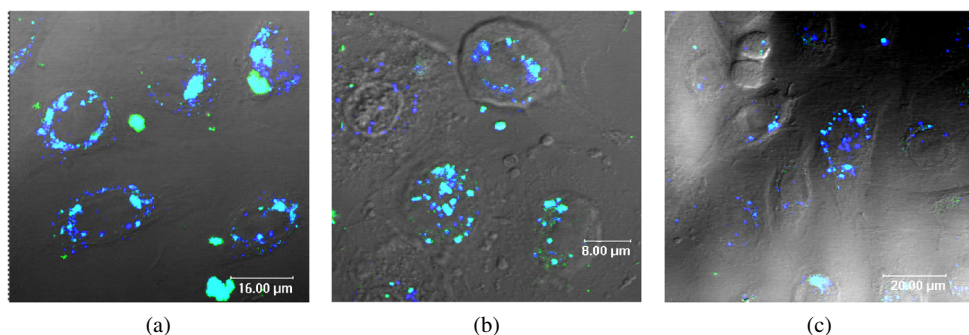
Up to now, we showed that PDMAEMA polymers with different architectures were able to efficiently vectorise an ASO inside keratinocytes. Finally, it was of importance to demonstrate that the oligonucleotides remained active inside the cell.



**Figure 9.** Analysis by confocal microscopy of cells transfected with the WA488 fluorescent oligonucleotide using either PDMAEMA (L3), DEA-PDMAEMA (D3) or TEA-PDMAEMA (T3) polymers ( $N/P = 7.38$ ). The intracellular distribution of the transfected oligonucleotide was investigated 24, 48 or 72 h post-transfection. This figure is published in colour in the online edition of this journal, which can be accessed via <http://www.brill.nl/jbs>

Therefore, we used a keratinocyte cell line expressing a destabilized GFP and we transfected anti-GFP ASO with linear (L3), DEA-initiated (D3) or TEA-initiated (T3) PDMAEMA polymers, with a comparable  $M_n$  and at a  $N/P$  ratio of 7.38. Ternary complexes were not tested considering their high toxicity (Fig. A.1). At 24 h post-transfection, we analyzed the *GFP* mRNA abundance by quantitative RT-PCR (Fig. 11a).

Although SuperFect proved to be highly efficient for vectorising the oligonucleotides, it appeared to be poor in achieving GFP silencing, contrary to Oligofec-



**Figure 10.** Analysis by confocal microscopy of cells transfected with a fluorescent polymer (F4 (green) and an Atto 647-labelled oligonucleotide (WA647) (blue) after 24 (a), 48 (b) or 72 h (c). Co-localization appears in turquoise. This figure is published in colour in the online edition of this journal, which can be accessed *via* <http://www.brill.nl/jbs>

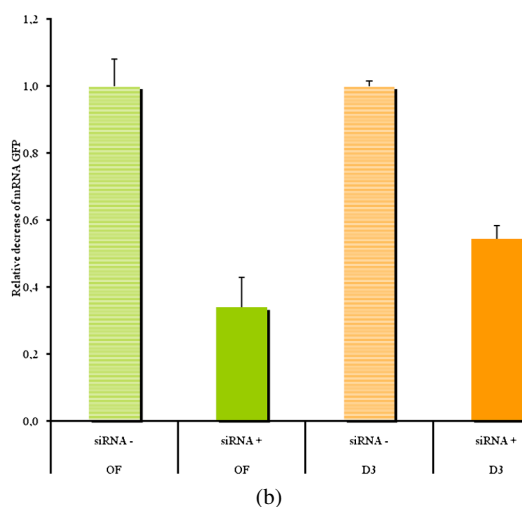
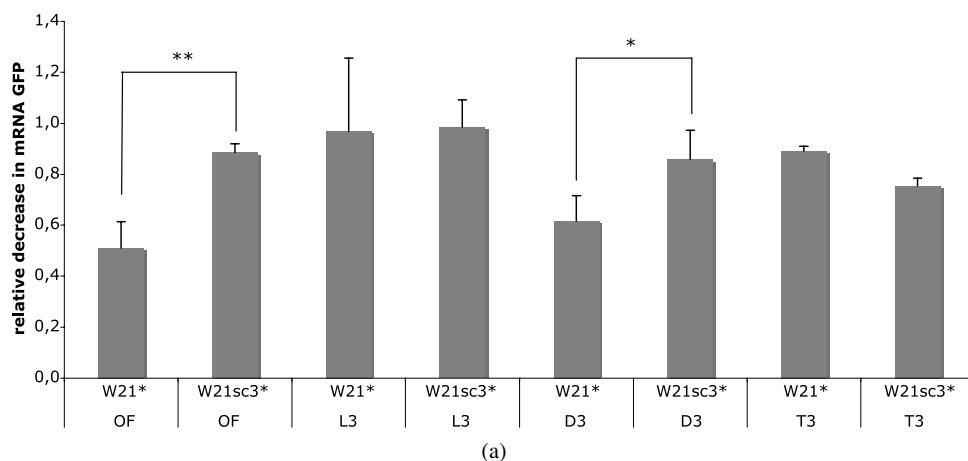
tamine (data not shown) which was used as reference in this part of the study. D3, the DEA-initiated PDMAEMA polymer with a  $M_n$  of 30 300 gave the best results with a reproducible GFP silencing of about 20–40%, which was comparable to the silencing obtained with Oligofectamine. A limited silencing was observed when the cells were transfected with the scrambled sequence (17%). No such silencing effect was observed with the linear (L3) or TEA-PDMAEMA (T3) polymer.

In order to correlate the decrease in mRNA GFP level with the GFP expression at the protein level, we measured the mean fluorescence of the transfected populations by flow cytometry. In order to compare the decrease of GFP protein abundance after transfection using the different polymers, we reported the mean GFP fluorescence of IHK-GFPd cells transfected with the ASO GFP on the mean fluorescence of IHK-GFPd cells transfected with a scrambled sequence. We confirmed that the DEA-initiated PDMAEMA polymer D3 was able to ensure a silencing effect of 33%, whereas the PDMAEMA polymer L3 provided 13% of silencing and the TEA-initiated polymer T3 showed only 2% of silencing. On the other hand, the transfection by Oligofectamine achieved 11% of GFP silencing. These data confirmed that the D3 polymer was the best transfection agent for maintaining the biologic activity of an antisense oligonucleotide in IHK cells (Table 2).

Finally, the D3 DEA-polymer was also used to vectorise siRNA, as shown in Fig. 11b, with a reduction of about 50% of the GFP mRNA, confirming the versatility of this polymer and its ability to vectorise short nucleic acid sequences.

#### 4. Discussion

In this study, we investigated the transfection of immortalized keratinocytes, used as an *in vitro* model for skin carcinoma. The aim of this work was to develop a safe and efficient non-viral gene-delivery system focused on ASO. Therefore, various PDMAEMA-based (co)polymers with different lengths and architectures were synthesized and characterized in order to be able to correlate the polymer proper-



**Figure 11.** Relative reduction of *GFP* mRNA (%) in IHK-dGFP cells induced 24 h post-transfection by (a) antisense (W21\*) targeting *GFP* or scrambled (W21sc3\*) oligonucleotides using different polymers (L3, D3 or T3). Data are given as the mean  $\pm$  SEM ( $n = 3$ ); \*\* $P = 0.01$ ; \* $P = 0.05$ . This experiment is representative of 4. (b) Cells were transfected with anti-*GFP* or non-targeting siRNA using D3. Oligofectamine was used to compare the efficiency of the transfection by the PDMAEMA polymers. This experiment is representative of 2. This figure is published in colour in the online edition of this journal, which can be accessed via <http://www.brill.nl/jbs>

**Table 2.**

Mean fluorescence of IHK-GFPd cells transfected with antisense or scramble oligonucleotide using linear (L3), two- (D3) or three-arm (T3) PDMAEMA polymer

	L3	D3	T3	Oligofectamine
W21*	80.43	102.31	101.37	85.11
W21sc3*	92.33	151.32	102.79	94.58

ties with their efficiency not only to vectorise ASO, but also to ensure the silencing effects of the vectorized ASO in immortalized keratinocytes. Together, our data demonstrated the effectiveness of PDMAEMA-based polymers for transfecting keratinocytes. First, we observed a significant influence of the molecular weight of the polymer tested on the toxicity. Indeed, we showed that the cellular viability was closely related to the  $M_n$  of the polymer used. As described previously [28, 40, 41], we confirmed that a  $M_n$  around 30 000–40 000 ensures a good compromise between low cytotoxicity and efficient vectorisation. We can assume that the molecular weight of the polymer provides a good estimation of the size of the corresponding complexes [42]. In the case of keratinocytes, the use of a PDMAEMA-b-PMAPEG co-polymer, whatever the architecture, strongly increased the cytotoxicity. It was shown that the primary mode of toxicity associated to polymers with high  $M_w$  is membrane destabilisation [40].

An AFM study has previously highlighted that the  $M_w$ , the N/P ratio or the cationic distribution of the condensing agent can strongly affect the dimension of the condensate [43]. For instance, it was shown for PEI that low N/P ratios (N/P = 3) support low DNA condensation, whereas high N/P ratios (N/P > 6) result in large amounts of free PEI causing cytotoxicity [44]. Therefore, we investigated different N/P ratios. In the case of the different PDMAEMA polymers studied here, it appeared that an N/P ratio of 7.38 was the optimal ratio for conciliating efficiency of vectorisation and low cytotoxicity. This N/P ratio corresponded to a weight ratio of 3:1, which was shown to be appropriate for the stability of the complexes [45]. Using the PDMAEMA-b-PMAPEG co-polymer LP in addition to the pre-formed polyplex, we observed that the N/P ratio was important but the weight ratio of the polymer, the co-polymer and the oligonucleotide was even more important, as at a N/P ratio of 7.4 only the weight ratio of 1.5:0.5:0.5 was not cytotoxic, whereas at a ratio of 3:1:1 we observed 75% mortality (data not shown). However, the cellular viability after transfection is highly dependent on the cell line studied. Indeed, the IHK seemed highly sensitive to transfection, while SiHa cells, in immortalized keratinocytes expressing the HPV virus, transfection by polymers with N/P ratios up to 15 did not affect cell viability.

Analyses by confocal microscopy and flow cytometry revealed that in cells transfected with the polymers, oligonucleotides remained inside the keratinocytes up to three days after the transfection, which is significantly better than with SuperFect, the commercial agent used for sake of comparison. Indeed, we observed by confocal microscopy that 24 h after transfection polyplexes were disrupted in the cytoplasm and oligonucleotides were still detectable after 3 days. This is in agreement with the silencing experiments showing that the antisense oligonucleotide was effective 24 h after transfection.

Our data also clearly show that the best polymer for vectorisation was not necessarily the best polymer for maintaining the biological activity of the transfected oligonucleotide, in our case the silencing effect. As previously shown for plasmidic DNA [46], even if polymers with a broad range of molecular weights are

able to ensure an efficient vectorisation, the gene expression associated with the cellular uptake is not necessarily as efficient. Therefore, even if the linear, two- or three-arm PDMAEMA polymers provided comparable vectorisation properties, the DEA-initiated PDMAEMA polymer D3 with a  $M_n$  of 30 300 was clearly the most efficient in ensuring the silencing effect of the transfected anti-GFP oligonucleotide, as evidenced both at the *GFP* mRNA and protein levels. This polymer was also effective in vectorising functional siRNA.

Our results suggest that the best architecture of the PDMAEMA tested was the DEA-initiated polymer D3 with a  $M_n$  of 30 300. This polymer had a rigid core with two mobile arms. We assume that this architecture provided less steric obstruction than the three-arm polymers. Indeed, in the TEA-initiated PDMAEMA polymer, the rigid star in the core could form a large three dimensional net making release of the trapped oligonucleotides more difficult. On the other hand, the linear polymer was less efficient than the DEA polymer because it may form winds into a ball and nodes that could impair the release of the oligonucleotide. Reports have also shown that the stereochemistry of polymers used for transfection could affect the biological properties of the vectorised material [47]. Thus, the two-arm polymer structure seems to combine efficient binding to the oligonucleotide and endosomal release.

## 5. Conclusion

In summary, we demonstrated that the two-arm PDMAEMA polymer of  $M_n = 30\,000$  can effectively transfect immortalized keratinocytes with antisense oligonucleotides paving the way to novel skin carcinoma therapeutic applications.

## Acknowledgements

This work was supported by the WALEO 2 Program ‘CARCINOM’ financed by DGTRE (Région Wallonne). D. M. Haddleton (Warwick University, UK) is warmly thanked for kindly providing authors with the Hostasol-MA precursor. We thank Prof. B. Epe from the Johannes Gutenberg University of Mainz (Germany) for providing us the pMAZ-ODC vector. We thank the FNRS-FRS for the financial support for the confocal microscope in the URBC (Namur). This work was partially supported by the ‘Région Wallonne’ and the European Commission in the frame of ‘Phasing-out Hainaut’: ‘Materia Nova’ program, by the Belgian Federal Government Office of Science Policy (SSTC-PAI 6/27).

## References

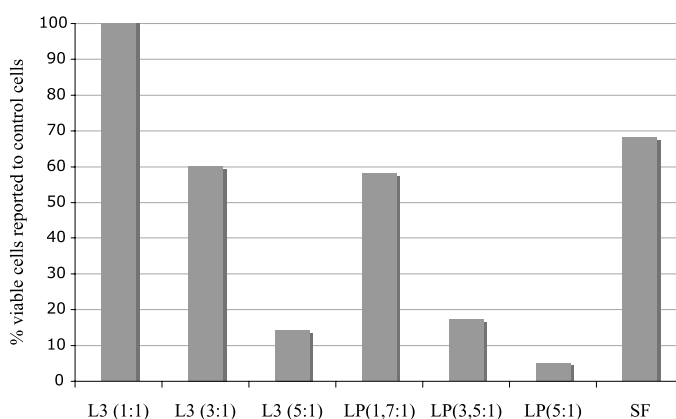
1. R. Marks and R. J. Motley, *Drugs* **50**, 48 (1995).
2. J. P. Shah and Z. Gil, *Oral Oncol.* **45**, 394 (2009).
3. E. Calitchi, Y. Kirova and J. P. Le Bourgeois, *Cancer Radiother.* **2**, 556 (1998).

4. Y. M. Kirova, F. Feuilhade, E. Calitchi, Y. Otmezguine, E. Belembaogo and J. P. Le Bourgeois, *Cancer Radiother.* **2**, 381 (1998).
5. Y. M. Kirova, E. Belembaogo, H. Frikha, E. Haddad, E. Calitchi, E. Levy, P. Piedbois and J. P. Le Bourgeois, *Radiother. Oncol.* **46**, 19 (1998).
6. N. Yamamoto, C. Ikeda, T. Yakushiji, T. Nomura, A. Katakura, T. Shibahara and J. E. Mizoe, *Bull. Tokyo Dent. Coll.* **48**, 177 (2007).
7. E. C. Tapajos, J. P. Longo, A. R. Simioni, Z. G. Lacava, M. F. Santos, P. C. Morais, A. C. Tedesco and R. B. Azevedo, *Oral Oncol.* **44**, 1073 (2008).
8. R. L. Eckert, J. F. Crish and N. A. Robinson, *Physiol. Rev.* **77**, 394 (1997).
9. P. Boukamp, *Carcinogenesis* **26**, 1657 (2005).
10. P. Erb, J. Ji, E. Kump, A. Mielgo and M. Wernli, *Adv. Exp. Med. Biol.* **624**, 283 (2008).
11. C. L. Benjamin, V. O. Melnikova and H. N. Ananthaswamy, *Adv. Exp. Med. Biol.* **624**, 265 (2008).
12. H. Heemskerck, C. L. de Winter, G. J. van Ommen, J. C. van Deutekom and A. Aartsma-Rus, *Ann. NY Acad. Sci.* **1175**, 71 (2009).
13. J. Furukawa, C. J. Wraight, S. M. Freier, E. Peralta, L. M. Atley, B. P. Monia, M. E. Gleave and M. E. Cox, *Prostate* **70**, 206 (2010).
14. S. Akhtar, M. D. Hughes, A. Khan, M. Bibby, M. Hussain, Q. Nawaz, J. Double and P. Sayyed, *Adv. Drug Deliv. Rev.* **44**, 3 (2000).
15. J. Ji, M. Wernli, S. Buechner and P. Erb, *J. Invest. Dermatol.* **120**, 1094 (2003).
16. J. J. Shieh, K. T. Liu, S. W. Huang, Y. J. Chen and T. Y. Hsieh, *J. Invest. Dermatol.* **129**, 2497 (2009).
17. J. C. Tseng, T. Granot, V. Digiacomio, B. Levin and D. Meruelo, *Cancer Gene Ther.* **17**, 244 (2010).
18. E. E. Thacker, M. Nakayama, B. F. Smith, R. C. Bird, Z. Muminova, T. V. Strong, L. Timares, N. Korokhov, A. M. O'Neill, T. D. de Gruijl, J. N. Glasgow, K. Tani and D. T. Curiel, *Vaccine* **27**, 7116 (2009).
19. M. Huang, Z. Chen, S. Hu, F. Jia, Z. Li, G. Hoyt, R. C. Robbins, M. A. Kay and J. C. Wu, *Circulation* **120**, S230 (2009).
20. C. E. Thomas, A. Ehrhardt and M. A. Kay, *Nature Rev. Genet.* **4**, 346 (2003).
21. F. J. Xu, Y. Ping, J. Ma, G. P. Tang, W. T. Yang, J. Li, E. T. Kang and K. G. Neoh, *Bioconjug. Chem.* **20**, 1449 (2009).
22. J. Chen, C. Wu and D. Oupicky, *Biomacromolecules* **10**, 2921 (2009).
23. M. Ma, F. Li, F. J. Chen, S. X. Cheng and R. X. Zhuo, *Macromol. Biosci.* **10**, 183 (2010).
24. L. L. Farrell, J. Pepin, C. Kucharski, X. Lin, Z. Xu and H. Uludag, *Eur. J. Pharm. Biopharm.* **65**, 388 (2007).
25. M. Morille, C. Passirani, A. Vonarbourg, A. Clavreul and J. P. Benoit, *Biomaterials* **29**, 3477 (2008).
26. F. J. Verbaan, P. Klein Klouwenberg, J. H. van Steenis, C. J. Snel, O. Boerman, W. E. Hennink and G. Storm, *Int. J. Pharm.* **304**, 185 (2005).
27. Y. Z. You, D. S. Manickam, Q. H. Zhou and D. Oupicky, *J. Control. Rel.* **122**, 217 (2007).
28. P. van de Wetering, J. Y. Cherng, H. Talsma, D. J. Crommelin and W. E. Hennink, *J. Control. Rel.* **53**, 145 (1998).
29. M. A. van der Aa, U. S. Huth, S. Y. Hafele, R. Schubert, R. S. Oosting, E. Mastrobattista, W. E. Hennink, R. Peschka-Suss, G. A. Koning and D. J. Crommelin, *Pharm. Res.* **24**, 1590 (2007).
30. C. H. Lin, Y. C. Hsiao and M. D. Shau, *Int. J. Pharm.* **393**, 136 (2010).
31. V. Incani, X. Lin, A. Lavasanifar and H. Uludag, *ACS Appl. Mater. Interfaces* **1**, 841 (2009).



32. L. E. Prevette, D. G. Mullen and M. M. Banaszak Holl, *Mol. Pharm.* **7**, 2370 (2010).
33. S. B. Lee, A. J. Russell and K. Matyjaszewski, *Biomacromolecules* **4**, 1386 (2003).
34. Y. Shim, F. Bougard, O. Coulembier, R. Lazzaroni and P. Dubois, *Eur. Polym. J.* **44**, 3715 (2008).
35. R. A. Limer, A. K. Rullay, V. San Miguel, C. Peinado, S. Keely, E. Fitzpatrick, S. Carrington, D. Brayden and D. Haddleton, *React. Funct. Polym.* **66**, 51 (2006).
36. N. Kitsera, A. Khobta and B. Epe, *Biotechniques* **43**, 222 (2007).
37. S. H. Kim, H. Mok, J. H. Jeong, S. W. Kim and T. G. Park, *Bioconjug. Chem.* **17**, 241 (2006).
38. J. Vandesompele, K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe and F. Speleman, *Genome Biol.* **3**, RESEARCH0034 (2002).
39. S. Piroton, C. Muller, N. Pantoustier, F. Botteman, S. Collinet, C. Grandfils, G. Dandrifosse, P. Degee, P. Dubois and M. Raes, *Pharm. Res.* **21**, 1471 (2004).
40. J. M. Layman, S. M. Ramirez, M. D. Green and T. E. Long, *Biomacromolecules* **10**, 1244 (2009).
41. W. T. Godbey, K. K. Wu and A. G. Mikos, *J. Biomed. Mater. Res.* **45**, 268 (1999).
42. M. A. Wolfert, E. H. Schacht, V. Toncheva, K. Ulbrich, O. Nazarova and L. W. Seymour, *Hum. Gene Ther.* **7**, 2123 (1996).
43. L. Liu, Y. L. Yang, C. Wang, Y. Yao, Y. Z. Ma, S. Hou and X. Z. Feng, *Colloids Surfaces B* **75**, 230 (2010).
44. C. G. Koh, X. Kang, Y. Xie, Z. Fei, J. Guan, B. Yu, X. Zhang and L. J. Lee, *Mol. Pharm.* **6**, 1333 (2009).
45. F. Dai, P. Sun, Y. Liu and W. Liu, *Biomaterials* **31**, 559 (2010).
46. S. Srinivasachari, Y. Liu, L. E. Prevette and T. M. Reineke, *Biomaterials* **28**, 2885 (2007).
47. Y. Liu and T. M. Reineke, *Bioconjug. Chem.* **17**, 101 (2006).

## Appendix



**Figure A.1.** Cytotoxicity of the linear PDMAEMA polymer L3 or palm-tree PDMAEMA-b-PMAPEG co-polymer LP. Cells were seeded ( $15 \times 10^3$  cells/cm<sup>2</sup>) and were transfected the following day with homo- or co-polymer at different ratios (w/w). SuperFect (SF) was used as control. MTT assay was performed 24 h post-transfection. Data represent one experiment out of 3.