Synthesis, Characterization, and Evaluation as Transfection Reagents of Ampholytic Star Copolymers: Effect of Star Architecture

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Five star polymers based on the positively ionizable hydrophilic 2-(dimethylamino)ethyl methacrylate (DMAEMA) and the hydrophobic but hydrolyzable tetrahydropyranyl methacrylate (THPMA) were prepared by group-transfer polymerization (GTP) using ethylene glycol dimethacrylate (EGDMA) as the coupling agent. In particular, four isomeric star copolymers (one heteroarm, two star block, and the statistical star), all with a 3:1 DMAEMA:THPMA molar ratio, plus one star homopolymer of DMAEMA, with degrees of polymerization of the arms equal to 15, were synthesized. After star polymer preparation and preliminary characterization, the THPMA units were hydrolyzed to negatively ionizable hydrophilic methacrylic acid (MAA) untis, thus yielding star polyampholytes. All the star polyampholytes as well as the commercially available transfection reagent SuperFect were evaluated for their ability to transfect human cervical HeLa cancer cells with the modified plasmid pRLSV40 bearing the enhanced green fluorescent protein (EGFP) as the reporter gene. The transfection efficiency was affected by star architecture. The $DMAEMA_{15}$-*star*-MAA_5 polyampholyte presented the highest transfection efficiency of all the star polymers tested but lower than that of SuperFect at its optimum conditions. All four star copolymers showed decreased toxicity compared to the DMAEMA star homopolymer for the same amounts of star polymer tested and also compared to the SuperFect at its optimum conditions.

Introduction

DNA transfection, the delivery of exogenous DNA into cells, is a topic of intense research because it can be used to study gene function or develop potential therapeutic approaches against several diseases.[1] In addition to deactivated viruses and cationic lipids, several cationic polymers have been investigated as DNA transfer vehicles.[2−14] These positively charged polymers bind electrostatically and condense the negatively charged DNA into smaller structures of lower negative or even zero charge, thus enabling a more effective transfection.[15,16] The main polymer characteristics determining transfection efficiency are polymer molar mass (MM), the p*K* values of the positively ionizable groups in the polymers, and polymer architecture. An interesting polymer architecture that has presented good transfection characteristics is that of "activated" dendrimers, combining a dense polymer structure with some flexibility.[11,12]

Recently, we developed an easier-to-synthesize and inexpensive polymer system that mimics the structural characteristics of "activated" dendrimers and presents an equally high transfection performance. In particular, we synthesized cationic star homopolymers of 2-(dimethylamino)ethyl methacrylate (DMAEMA),[17] a monomer unit bearing a tertiary amine weak base with a p*K* of 7, falling within the p*K* range for optimal transfection.[13] From the several star homopolymers prepared, the one with the shortest arm length was identified as the optimal transfectant.[17] In a subsequent study, we tried to further optimize the transfection performance of star polymers by combining DMAEMA with a second monomer, the nonionic, hydrophilic, and biocompatible methoxy hexa(ethylene glycol) methacrylate (HEGMA), whose presence in some star copolymer samples improved transfection by reducing cell toxicity.[18]

In the present study we wished to employ a different strategy for improving transfection performance by combining DMAEMA with a negatively ionizable, rather than a nonionic, monomer. Polymers combining positively charged with negatively charged units are called polyampholytes[19,20] and present several interesting physicochemical features, such as the isoelectric point, which is the pH of zero net charge.[21] The negative charge contributed by the units of the comonomer would weaken the electrostatic binding between the negatively charged DNA and the positively charged DMAEMA units in the complex and facilitate release of DNA in the cytoplasm. The selected comonomer was methacrylic acid (MAA), which was introduced into the star copolymers in a "protected" form as tetrahydropyranyl methacrylate (THPMA). Unlike MAA, THPMA is compatible with group-transfer polymerization (GTP),[22−26] the controlled polymerization employed for the synthesis of the star copolymers. Ethylene glycol dimethacrylate (EGDMA) was the agent used for the coupling of the arms. The resulting star copolymers were characterized in solution and subsequently evaluated in terms of their ability to transfect human cervical HeLa cancer cells.

Experimental Section

Materials. DMAEMA (monomer), EGDMA (coupling agent), 1-methoxy-1-trimethylsiloxy-2-methyl propene (MTS, initiator), tetrabutylammonium hydroxide, benzoic acid, calcium hydride (CaH_2), 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH, free-radical inhibitor), basic alumina, and potassium metal were all purchased from Aldrich, Germany. The monomer THPMA was in-house synthesized by the catalytic esterification of MAA with 100% excess 3,4-dihydro-2H-pyran at 55 °C[27] using a modification of the procedure reported by Hertler.[28] Figure 1 shows the chemical structures and names of the monomers,

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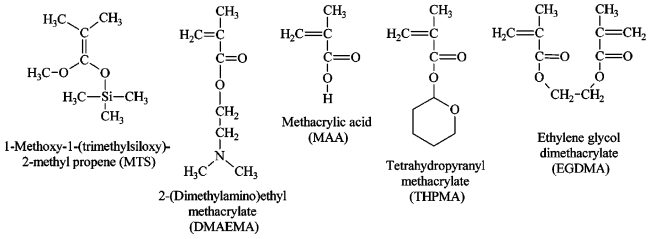

Figure 1. Chemical structures and names of the initiator, monomers, and coupling agent used for the star polymer synthesis.

coupling agent, and initiator used for the preparation of the star copolymers. Sodium metal was purchased from Fluka, Germany. Tetrahydrofuran (THF) was purchased from Labscan, Ireland, and used as both the polymerization solvent (reagent grade) and the mobile phase in chromatography (HPLC grade). Dulbecco's phosphate-buffered saline (D-PBS) without calcium chloride and magnesium chloride, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, glutamine, penicillin, and streptomycin were purchased from Invitrogen, U.K., and used for the cell culture experiments. Finally, SuperFect transfection reagent was purchased from Qiagen, Germany.

Methods. The monomers, DMAEMA and THPMA, and the coupling agent, EGDMA, were passed twice through basic alumina columns to remove inhibitors and protonic impurities. They were subsequently stirred over CaH_2 for 3 h in the presence of added free-radical inhibitor DPPH, kept in the refrigerator, and finally distilled prior to use. The initiator was distilled once prior to polymerization but was neither contacted with CaH_2 nor passed through basic alumina columns because of the risk of hydrolysis. The polymerization catalyst was tetrabutylammonium bibenzoate (TBABB), synthesized by reaction of tetrabutylammonium hydroxide and benzoic acid, as described by Dicker and co-workers.[24] The dried catalyst powder was stored in a round-bottom flask under vacuum until use. The polymerization solvent was dried by refluxing it for 3 days over a potassium/sodium amalgam. All glassware was dried overnight at 120 °C and assembled hot under dynamic vacuum prior to use.

Star Polymer Synthesis. A typical polymerization procedure is detailed below which describes the synthesis of the star polymer with DMAEMA-*b*-THPMA arms of degree of overall polymerization (DP) of 20. Freshly distilled THF (45 mL), MTS (0.70 mL, 0.60 g, 3.4 mmol), and DMAEMA (8.7 mL, 8.1 g, 52 mmol) were syringed in this order into a 100 mL nonthermostated (~27 °C) round-bottom flask kept under an inert nitrogen atmosphere containing a small amount (~20 mg, 40 μmol) of TBABB and fitted with a rubber septum. The reactor temperature rose quickly from 27.0 to 49.0 °C. After 5 min the exotherm abated and a 0.1 mL aliquot of the reaction solution was extracted for GPC analysis. Subsequently, THPMA monomer (2.9 mL, 2.9 g, 17 mmol) was added. The temperature increased from 36.4 to 42.9 °C. After extraction of a 0.1 mL aliquot from the reaction solution for GPC analysis, EGDMA (2.6 mL, 2.7 g, 14 mmol) was added rapidly and the temperature increased from 35.6 to 45.4 °C. When the exotherm abated, another 0.1 mL aliquot was withdrawn for GPC analysis. The resulting star copolymer was recovered by precipitation in *n*-hexane and dried under vacuum at room temperature for 3 days. Star copolymers of different architectures were prepared by varying the sequences of monomer and coupling agent additions but keeping the DMAEMA to THPMA molar ratio constant at 3 (25% mol THPMA). A DMAEMA star homopolymer of arm DP equal to 15 was also prepared with the sequential addition of initiator, DMAEMA, and EGDMA.

Characterization in Organic Solvents. *Gel Permeation Chromatography (GPC).* Molar masses (MMs) and molar mass distributions (MMDs) of the DMAEMA−THPMA star polymers and their linear precursors were obtained by gel permeation chromatography (GPC) using a single Polymer Laboratories PL-Mixed "D" column. The mobile phase was THF (without any modifier, such as triethylamine or pyridine) delivered at a flow rate of 1 mL min^{-1} using a Polymer Laboratories PL-LC1120 isocratic pump. The refractive index signal was measured using an ERC-7515A refractive index detector supplied by Polymer Laboratories. The calibration curve was based on eight narrow MM (630, 2600, 4250, 1300, 22 650, 50 000, 128 000, and 260 000 g mol^{-1}) linear poly(methyl methacrylate) (PMMA) standards supplied by Polymer Laboratories, which provided relatively accurate MM calculations for the linear polymer arms but only qualitative estimates for the MMs of the star polymers.

Proton Nuclear Magnetic Resonance Spectroscopy (^1H NMR). A Bruker 300 MHz instrument was used to acquire the proton NMR spectra of the synthesized THPMA monomer (in $CDCl_3$), the DMAEMA−THPMA star copolymers (in $CDCl_3$), and the final DMAEMA−MAA star copolymers (in d_6-DMSO). In all cases, the residual nondeuterated solvent ($CHCl_3$ or DMSO) was used as an internal reference for chemical shift calibration.

Star Polymer Hydrolysis. The THPMA units of the star copolymers were hydrolyzed to MAA units using HCl. In particular, a 2 M HCl solution was added to aqueous dispersions of the DMAEMA−THPMA star copolymers. The mixture was left to react under stirring for 2 weeks at room temperature. Subsequently, the resulting DMAEMA−MAA star polyampholyte solutions were neutralized by the addition of the appropriate volume of a 2 M NaOH solution, purified by dialysis against deionized water, and lyophilized.

Aqueous Solution Characterization. Aqueous solutions of the DMAEMA−MAA star copolymers and the DMAEMA star homopolymer were characterized in terms of their p*K*s and isoelectric points, cloud points, hydrodynamic diameters, and absolute MMs using hydrogen-ion titration, turbidimetry, dynamic light scattering (DLS), and static light scattering (SLS), respectively.

Hydrogen-Ion Titrations. Aqueous star polymer (1% w/w) solutions containing 0 and 150 mM NaCl were titrated between pH 2 and 12 using a standard NaOH (0.5 M) solution under continuous stirring. The pH was measured using a Corning PS30 portable pH meter. The pH range of any increased turbidity was visually observed and noted.

Turbidimetry. A single-beam Lambda 10 Perkin-Elmer UV/vis spectrometer was used for the turbidity measurements of salt-free solutions. The polymer solution (1% w/w) was placed in a 10 mm pathlength quartz cuvette containing a small magnetic bar set in motion with the aid of a miniature magnetic stirrer. A small temperature probe was immersed in the upper part of the solution which was heated from 20 to 90 °C. The optical density at 500 nm and the temperature were monitored using the software package TempLab (version 1.56) along with UVWinLab (version 2.7). The cloud point was taken as the temperature where the first large increase in optical density occurred.

Dynamic Light Scattering (DLS). A 90 Plus Brookhaven DLS spectrophotometer equipped with a BI9000 correlator and a 30 mW red diode laser operating at 673 nm was used for the DLS measurements at an angle of 90° and at room temperature to determine the hydrodynamic diameters of the star polymers in aqueous solution. Five 2-min runs were performed for each star polymer aqueous solution (1% w/w), and the data were averaged. The data were processed using multimodal size distribution (MSD) analysis based on non-negatively constrained least squares (NNLS). Prior to the DLS measurements, the star polymer aqueous solutions were filtered through 0.45 μm PTFE syringe filters and left at rest for approximately 1 h so that any air bubbles could escape.

Static Light Scattering (SLS). A BIMwA Brookhaven instrument equipped with a 30 mW red diode laser operating at 673 nm was employed for the SLS experiments to determine the absolute weight-average of MMs (M_ws) of the star polymers. Scattered intensities were measured at seven different angles, 35°, 50°, 75°, 90°, 105°, 130°, and 145°, and different star polymer concentrations, depending on their MMs. Toluene (refractive index = 1.4740, Rayleigh ratio = 1.090×10^{-5}) was used as the calibration liquid, and water was used as the normalization solvent. Zimm plots were constructed from which the M_ws were obtained as the common intercept of the zero-angle and zero-polymer-concentration extrapolation straight lines.

Evaluation of Star Polymers as Transfection Reagents. *Plasmid DNA Preparation.* Plasmid pRLSV40 EGFP was prepared by cloning the enhanced green fluorescent protein (EGFP) gene in pRLSV40 (Promega). The construct was grown in *E. coli* DH5a competent cells and purified using HiSpeed Plasmid Max Kit (Qiagen, Germany). The DNA concentration of the construct was determined by measuring the optical density at 260 nm (Eppendorf Biophotometer).

Preparation of Star Polymer/Plasmid DNA Complexes. Complexes of star polymer and plasmid DNA were prepared by adding the solution of the star polymer (3 mg mL^{-1} in deionized and sterilized water) to a plasmid DNA solution in serum-free DMEM. An amount of 15−210 μg of each star polymer was mixed with 3 μg of pRLSV40-EGFP plasmid DNA in a total volume of 100 μL. A time period of 30 min was allowed for complexation to take place before adding 400 μL of complete DMEM (DMEM supplemented with glutamine, 10% fetal calf serum, penicillin, and streptomycin). The final 500 μL of solution was then added to the cells. A similar procedure was followed for the commercially available transfection reagent SuperFect at the optimum SuperFect:plasmid DNA mass ratio of 12:1 following the manufacturer's instructions.

Cell Culture. Cells from the human cervical cancer cell line HeLa were cultured in complete DMEM (see previous paragraph). Before performing a transfection experiment, the cells were grown to 40−60% confluency at 37 °C in a humidified atmosphere containing 5% CO_2.

Transfection Studies. Transfection experiments were performed with HeLa cells using the pRLSV40 EGFP plasmid. Cells were seeded at a concentration of 5×10^4 in 12-well plates (40−60% confluency) 24 h before transfection. On the day of the transfection, the cells were washed with PBS and incubated at 37 °C for 3 h with 500 μL of the complex solution per well. After removal of the transfection complexes, 2 mL of fresh complete DMEM was added and the cells were further cultured for 48 h. Floating dead cells were removed by washing with PBS, and the remaining living cells were detached from the plate using a trypsin solution. An inverted fluorescence microscope (Zeiss Axiovert 25) along with a hemocytometer were used to count the total number of living cells and the number of fluorescent cells (expressing the EGFP gene). Living cells were counted under visible light, while fluorescent cells were counted under an excitation wavelength of 488 nm. The number of living cells in the untransfected control well, where neither polymer nor plasmid was added, was also counted. The *transfection efficiency* was calculated as the number of fluorescent cells in a well divided by the total number of cells in the same well.[17,18] The *overall transfection efficiency* was calculated as the number of fluorescent cells divided by the total number of cells in the untransfected control well.[17,18] This latter definition also takes into account the viability of the cells. Finally, the *cell viability* was calculated as the total number of living cells divided by the total number of cells in the untransfected control well.[7,17,18] Each transfection experiment was repeated approximately five times, and the average of the results (transfection efficiency, cell viability, and overall transfection efficiency) is reported.

Results and Discussion

Synthetic Strategy. The synthesis of the arms of the star copolymers was accomplished by the GTP of DMAEMA and THPMA monomers, initiated by a monofunctional initiator. Subsequently, coupling took place in situ by the polymerization of a bifunctional methacrylate, EGDMA, which led to the interconnection of the "living" linear chains at one end to give "arm-first" star copolymers.[17,29−32] In one case, the THPMA monomer was introduced after the sequential addition of DMAEMA and the coupling agent, resulting in formation of a DMAEMA−THPMA heteroarm "in−out" star copolymer.[17,29,30]

The schematic representation of the procedure for the synthesis of the "arm-first" star copolymer $DMAEMA_{15}$-*b*-$THPMA_5$-*star* is shown in Figure 2, where white and black indicate the DMAEMA and THPMA segments, respectively, while asterisks denote the active polymerization sites. The sequential addition of the two monomers, DMAEMA and THPMA, in the first two steps led to the preparation of a linear diblock copolymer. The synthesis was concluded by the addition of the cross-linker, EGDMA, in a 4-fold molar excess with respect to the initiator to yield the "arm-first" star-block copolymer. At this point, if more monomer were added, new arms would grow from the core of the "arm-first" star outward, resulting in an "in−out" star copolymer.

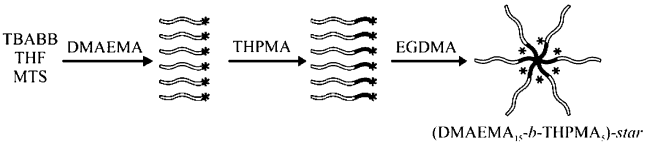

Figure 2. Schematic representation of the synthetic procedure used for the preparation of the "arm-first" star copolymer $DMAEMA_{15}$-*b*-$THPMA_5$-*star*. The DMAEMA units are depicted white, while the THPMA units are colored black.

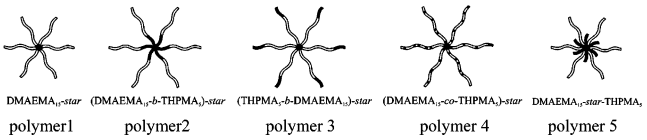

Figure 3. Schematic representation of the different architectures of the cationic star polymers synthesized in this study. The DMAEMA units are depicted white, while the THPMA (MAA) units are colored black.

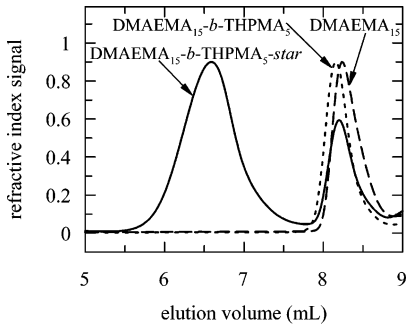

Figure 4. Gel permeation chromatograms of the "arm-first" star block copolymer $DMAEMA_{15}$-*b*-$THPMA_5$-*star* and its two linear precursors.

Figure 3 shows all the star polymer structures synthesized in this study. The DMAEMA units are illustrated in white, while the THPMA (MAA) units are in black, similar to Figure 2. The first structure (polymer 1) represents a DMAEMA star homopolymer with DP of the arm equal to 15. The other four polymers are isomeric structures with 75% mol DMAEMA and 25% mol THPMA. Polymers 2 and 3 are "arm-first" star copolymers whose arms are block copolymers with DMAEMA in the outer part in the former case and close to the core of the star in the latter. The fourth structure corresponds to a statistical star copolymer, $DMAEMA_{15}$-*co*-$THPMA_5$-*star*, with the same overall arm DP as the previous three copolymers. The last structure, polymer 5, is the heteroarm star copolymer $DMAEMA_{15}$-*star*-$THPMA_5$ which is the only "in−out" star copolymer with the arms being homopolymers of one of the two monomers. It is important to point out that the number of arms at the cross-links is not 6 or 12, as indicated in Figure 3, but much higher, typically between 30 and 89.[17,30,31]

Confirmation of Polymer Structure. *Polymer Size.* Figure 4 shows the GPC chromatograms of the "arm-first" star block copolymer $DMAEMA_{15}$-*b*-$THPMA_5$-*star* and its two linear

Table 1. Molar Mass Characteristics of the Star Polymers

no.	polymer theoretical formula	theoretical MM[a]	GPC results[b] M_n	M_p	M_w/M_n
1	$DMAEMA_{15}$	2458	2280	2780	1.11
	$DMAEMA_{15}$-*star*	c	46 600	59 800	1.55
2	$DMAEMA_{15}$	2458	2630	3150	1.09
	$DMAEMA_{15}$-*b*-$THPMA_5$	3308	3210	3680	1.09
	($DMAEMA_{15}$-*b*-$THPMA_5$)-*star*	c	60 700	67 700	1.33
3	$THPMA_5$	950	1270	1280	1.03
	$THPMA_5$-*b*-$DMAEMA_{15}$	3308	3450	3670	1.07
	($THPMA_5$-*b*-$DMAEMA_{15}$)-*star*	c	51 600	58 000	1.27
4	$DMAEMA_{15}$-*co*-$THPMA_5$	3308	3080	3560	1.10
	($DMAEMA_{15}$-*co*-$THPMA_5$)-*star*	c	48 500	56 200	1.32
5	$DMAEMA_{15}$	2458	2160	2610	1.10
	$DMAEMA_{15}$-*star*	c	35 500	48 100	1.39
	$DMAEMA_{15}$-*star*-$THPMA_5$	c	40 700	52 800	1.40

[a] Mass from initiator fragment (100 g mol^{-1}) included. [b] In THF using PMMA MM standards. [c] It cannot be calculated because the number of arms is not known a priori.

precursors. The MMD of the linear DMAEMA homopolymer is narrow and unimodal, as expected. The MMD of the linear diblock copolymer $DMAEMA_{15}$-*b*-$THPMA_5$ is also narrow and unimodal without any traces of linear homopolymer. The MMD of the "arm-first" star copolymer is broad and bimodal, containing a small amount of unattached linear chains (free arms) that corresponds to the diblock copolymer. The GPC chromatograms of all "arm-first" star polymers also exhibited two peaks, one due to the star polymer and the second due to a low fraction (<15%) of the constituting linear polymer, as observed previously for star polymers synthesized by GTP.[17,18,31] Incomplete incorporation of the linear polymers into the stars is due to increased solution viscosity, lower chain mobility, and possible chain termination/chain transfer,[33] also observed previously for both anionic[34] and "living" cationic[35] "arm-first" star polymer synthesis.

The number-average MMs, M_ns, and polydispersity indices (PDIs, M_w/M_n) of all star polymers and their precursors, as determined by GPC, are shown in Table 1. The GPC M_ns of the linear precursors were slightly higher than their theoretical MMs due to partial initiator deactivation. The GPC M_ns of the "arm-first" star polymers ranged between 35 500 and 60 700 g mol^{-1} and were 15−23 times higher than the values of their corresponding linear polymers. The M_n of the "in−out" star copolymer, $DMAEMA_{15}$-*star*-$THPMA_5$, is 40 700 g mol^{-1}, higher than that of the corresponding "arm-first" star polymer precursor, as expected. We wish to stress at this point that because of the use of linear calibration standards, the M_ns of the star polymers determined by GPC are only rough estimates of the true MMs. Accurate measurements of the M_ws of the star polymers were performed using SLS and are discussed later. The PDIs of the linear precursors were equal to or lower than 1.11, while the PDIs of the star polymers were equal to or lower than 1.55.

Star Polymer Composition. The ^1H NMR spectra of the star copolymers (before the hydrolysis) confirmed that these copolymers had the expected composition in DMAEMA and THPMA. The star compositions were determined from the spectra by comparing the area of the peak of the methine proton in the THPMA units at 5.9 ppm and of the six azamethyl protons in the DMAEMA units at 2.3 ppm. The experimentally calculated star copolymer compositions are presented in Table 2. In the same table the theoretical star copolymer compositions are also shown. All star copolymer compositions were close to the theoretically expected values but slightly enriched in DMAEMA. Complete hydrolysis of the THPMA units to MAA units in the star copolymers was also confirmed by ^1H NMR,

Table 2. Composition Analysis of the Star Polymers

no.	polymer formula	% mol composition in THPMA experimental, ^1H NMR	theoretical
1	$DMAEMA_{15}$-*star*	0	0
2	($DMAEMA_{15}$-*b*-$THPMA_5$)-*star*	23	25
3	($THPMA_5$-*b*-$DMAEMA_{15}$)-*star*	23	25
4	($DMAEMA_{15}$-*co*-$THPMA_5$)-*star*	22	25
5	$DMAEMA_{15}$-*star*-$THPMA_5$	21	25

which indicated the absence of the methine proton in the THPMA at 5.9 ppm.

Aqueous Solution Properties. *Effective pKs and Isoelectric Points.* The effective p*K*s of the ionizable units were calculated from the hydrogen-ion titration curves as the pH at 50% ionization. For the DMAEMA star homopolymer, the effective p*K* value of the DMAEMA units was readily calculated to be 6.9 and 7.5 at NaCl concentrations of 0 and 150 mM, respectively. The former value is in agreement with previous investigations on salt-free solutions of DMAEMA star homopolymers where the p*K* was found to be in the range between 6.6 and 7.0.[17] Due to the low MAA content in the star copolymers (25% mol), the part of the titration curve corresponding to the MAA units was discernible only in some samples, where a value of 5.0 was determined. The effective p*K* values for the DMAEMA units in the polyampholyte stars were determined to be 7.7 and 8.0 in salt-free and 150 mM NaCl-containing aqueous solutions, respectively.

With the exception of the statistical star copolymer, all other salt-free star polyampholyte solutions (1% w/w) presented an increased turbidity (but no precipitation) between pH 7.7 and 8.5, indicating that their isoelectric points lay in this range. Given the polyampholyte composition (25% mol MAA) and the experimentally determined effective p*K*s of 7.7 and 5 for DMAEMA and MAA, respectively, the theoretically expected isoelectric point is about 8.2,[21] in reasonable agreement with the observed turbidity range. It is noteworthy that highly diluted star polyampholyte solutions, as the ones used in transfection, did not show any signs of increased turbidity at any pH range. Moreover, star polyampholyte solutions at a 150 mM NaCl concentration did not present turbidity at any pH value, even at the isoelectric point, due to the screening of the electrostatic attractions by the salt. Due to the complete neutralization of all the charges, both salt-free and 150 mM NaCl-containing DMAEMA star homopolymer aqueous solutions precipitated above pH 9.

Cloud Points. None of the star polymer aqueous solutions (even the salt-free) presented a clear cloud point between 20 and 90 °C (solution pH between 6.3 and 7.0). However, the DMAEMA star homopolymer presented a slight increase in turbidity (~0.2 au) at 30 °C. The star copolymers did not present any increase in turbidity probably because of the presence of the highly hydrophilic and ionic MAA units.

Hydrodynamic Diameters. The hydrodynamic diameters of aqueous solutions of the star polymers measured at room temperature are displayed in Table 3. The same table shows the upper limit of the size of the star polymers calculated for fully stretched arms. The calculation involved doubling the arm contour length, which was calculated by multiplying the DP of the arm times 0.252 nm, the contribution of one monomer repeat unit.[36] All samples presented 2−3 peaks in their hydrodynamic diameter distributions, corresponding to star polymer and star polymer aggregates.

The size at the maximum of the most populated peak in each sample appears in bold. In most cases, this size corresponded to star polymer aggregates. The star polymer aggregates were

Table 3. Hydrodynamic Diameters of the Star Polymers in 1% w/w Aqueous Solutions

		hydrodynamic diameter (nm)	
no.	polymer formula	Experimental[a]	theoretical limit
1	$DMAEMA_{15}$-*star*	10.0, **43.2,** 283.7	8.1
2	($DMAEMA_{15}$-*b*-MAA_5)-*star*	**10.5,** 24.8	10.7
3	(MAA_5-*b*-$DMAEMA_{15}$)-*star*	5.8−7.0, **22.5,** 158.0	10.7
4	($DMAEMA_{15}$-*co*-MAA_5)-*star*	5.7, **25.1**	10.7
5	$DMAEMA_{15}$-*star*-MAA_5	5.5, **33.8**	8.1

[a] The experimental hydrodynamic diameter in bold corresponds to the highest peak maximum for each star polymer.

Table 4. Absolute M_ws and Number of Arms of the Star Polymers

no.	polymer formula	M_ws of the star polymers by SLS	M_ws of the linear precursors by GPC	no. of arms[a]
1	$DMAEMA_{15}$-*star*	181 000	2280	84
2	($DMAEMA_{15}$-*b*-MAA_5)-*star*	90 500	2810	32
3	(MAA_5-*b*-$DMAEMA_{15}$)-*star*	245 000	3020	81
4	($DMAEMA_{15}$-*co*-MAA_5)-*star*	100 000	2690	37
5	$DMAEMA_{15}$-*star*-MAA_5	196 000	2260	87

[a] Calculated as the ratios of the M_ws of the star polymers and their linear precursors measured by SLS and GPC, respectively, and shown in the two previous columns.

readily formed due to the presence of the hydrophobic EGDMA core, as observed before with the DMAEMA star homopolymers[17] and the DMAEMA−HEGMA star copolymers.[18] The peaks that corresponded to star polymers ranged from 5.5 to 10.5 nm, lower than the upper theoretical limit because the polymer chains were not fully extended, as expected.

M_ws and Numbers of Arms of the Star Polymers. The M_ws of all the star polymers, as determined by SLS in water, are shown in Table 4. The M_ws ranged from 90 500 to 245 000 g mol^{-1}. The numbers of arms were calculated by dividing these M_ws by the M_ws of the corresponding arms determined by GPC. The numbers of arms ranged from 32 to 87, as expected for star polymers prepared by GTP.[17,30,31] In particular, the lowest M_ws and number of arms corresponded to $DMAEMA_{15}$-*b*-MAA_5-*star* and $DMAEMA_{15}$-*co*-MAA_5-*star*, while for the remaining three star polymers in which the EGDMA cross-linker was added right after addition of DMAEMA, i.e., $DMAEMA_{15}$-*star*, MAA_5-*b*-$DMAEMA_{15}$-*star*, and $DMAEMA_{15}$-*star*-MAA_5, the M_ws and numbers of arms were higher. This may be attributed to a higher cross-reactivity for the DMAEMA−EGDMA pair compared to the THPMA−EGDMA pair.

Transfection Performance. To the best of our knowledge, this is the second report[18] on the use of star copolymers in gene transfection and the first report in which weak base and weak acid monomers were combined to give ampholytic star copolymers for gene transfection. There is another study where ampholytic copolymers were used in transfection. However, in that study the polyampholytes involved were linear, prepared using free-radical polymerization.[37] All star polymers synthesized in the present study were evaluated for their ability to transfect human cervical cancer HeLa cells with pRLSV40-EGFP plasmid DNA by calculating their transfection efficiency, cell viability, and overall transfection efficiency as a function of the amount of polymer and copolymer architecture.

Effect of Amount of Star Polymer. Figure 5 illustrates the effect of the amount of polymer on transfection using each one of the five star polymers: (a) $DMAEMA_{15}$-*star*, (b) $DMAEMA_{15}$-*b*-MAA_5-*star*, (c) MAA_5-*b*-$DMAEMA_{15}$-*star*, (d) $DMAEMA_{15}$-*co*-MAA_5-*star*, and (e) $DMAEMA_{15}$-*star*-MAA_5. The amount of star polymer used was varied between 15 and 120, 180, or 210 μg, while the amount of plasmid DNA used was kept constant at 3 μg. These star polymer/plasmid DNA mass ratios correspond to 7−96 N/P g atom ratios. The results for the commercially available SuperFect at its optimal conditions (12 μg of SuperFect and 1 μg of plasmid DNA) are also shown in Figure 5 in black bands.

The transfection efficiency presented a slight maximum with respect to the polymer amount for polymers $DMAEMA_{15}$-*star*, MAA_5-*b*-$DMAEMA_{15}$-*star*, and $DMAEMA_{15}$-*star*-MAA_5 at 60, 150, and 180 μg, respectively, reflecting optimal physicochemical properties of the complexes at these polymer amounts. The two copolymers, MAA_5-*b*-$DMAEMA_{15}$-*star* and $DMAEMA_{15}$-*star*-MAA_5, presented a maximum at a higher amount of polymer than the DMAEMA star homopolymer due to the presence of the anionic MAA comonomer, necessitating more polymer to achieve the appropriate DMAEMA unit/DNA ratio. Surprisingly, star polymers $DMAEMA_{15}$-*b*-MAA_5-*star* and $DMAEMA_{15}$-*co*-MAA_5-*star* did not present any transfection efficiency at all. For the statistical star polyampholyte, the lack of transfection efficiency can be attributed to the random distribution of the DMAEMA and MAA units which reduced the number of consecutive DMAEMA units and, therefore, the ability of the star copolymer to bind to the negatively charged DNA and destabilize the negatively charged cell membrane. This is in agreement with the previous study that involved a linear statistical DMAEMA-MAA copolymer (35% mol MAA units) that, unlike a linear DMAEMA homopolymer, did not present any transfection efficiency.[37] Another possible reason for the inability of the statistical star polyampholyte to transfer DNA is the relatively low number of arms in this star copolymer, which may further reduce its binding ability. Star polyampholyte $DMAEMA_{15}$-*b*-MAA_5-*star* has an even lower number of arms, which can explain its inability toward transfection.

The heteroarm star polyampholyte $DMAEMA_{15}$-*star*-MAA_5 presented the highest maximum transfection efficiency of all the star polymers, close to 11%, while star polymers $DMAEMA_{15}$-*star* and MAA_5-*b*-$DMAEMA_{15}$-*star* presented maximum transfection efficiencies of 8% and 5%, respectively. The fact that the heteroarm star copolymer and the DMAEMA star homopolymer presented the two highest maximum transfection efficiencies from all the star polymers can be attributed to the distribution of the DMAEMA units at the periphery of the star polymers. The higher transfection efficiency of the former than the latter supports the original hypothesis in this work that the presence of the MAA (at the appropriate location) can facilitate transfection. However, it must be noted that the optimal transfection efficiency of the commercially available transfection reagent SuperFect was 13% and slightly higher than that of all the star polymers in this study.

The cell viability of the four star copolymers was higher than that of the DMAEMA star homopolymer for the same amounts of star polymer tested. The cell viability for all star polymers decreased with the amount of star polymer, reflecting the toxicity of these synthetic polymer vehicles as observed before for linear DMAEMA homopolymers[6,9] as well as DMAEMA star homo- and copolymers.[17,18] The decrease in cell viability was more pronounced for the DMAEMA star homopolymer and the heteroarm star copolymer, indicating the strong interaction between these cationic star polymers and the negatively charged cell membranes which get destabilized, leading to cell death. Given their structure, these two star polymers have only the positively charged DMAEMA units at their exterior and, therefore, interact strongly with oppositely charged entities, in agreement with the cell viability results with the DMAEMA−HEGMA star copolymers.[18]

The overall transfection efficiencies of star polyampholytes $DMAEMA_{15}$-*b*-MAA_5-*star* and $DMAEMA_{15}$-*co*-MAA_5-*star*

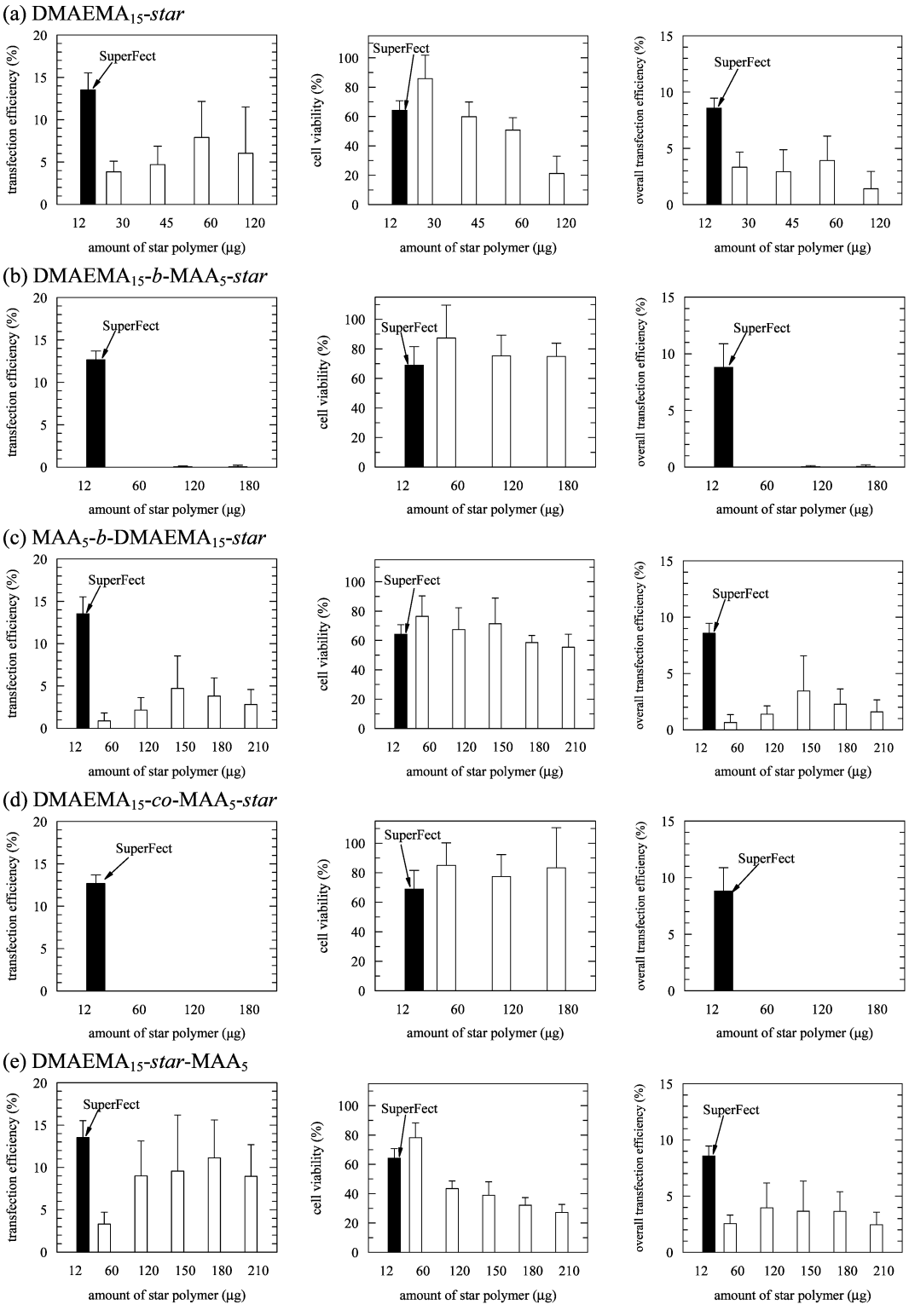

Figure 5. Dependence of the transfection efficiency, cell viability, and overall transfection efficiency on the amount of star polymers (a) $DMAEMA_{15}$-*star*, (b) $DMAEMA_{15}$-*b*-MAA_5-*star*, (c) MAA_5-*b*-$DMAEMA_{15}$-*star*, (d) $DMAEMA_{15}$-*co*-MAA_5-*star*, and (e) $DMAEMA_{15}$-*star*-MAA_5 at a constant amount of plasmid DNA of 3 μg (white bands). The corresponding measurements for SuperFect at the optimum conditions (12 μg of SuperFect and 1 μg of plasmid DNA) are also shown in the figure (black bands).

were zero, while those of the other three star polymers exhibited a slight maximum with the amount of polymer. Both of these trends were due to the calculation of the overall transfection efficiency as the product of transfection efficiency times cell viability: transfection efficiency was zero for the first two star copolymers and presented a maximum with the amount of polymer for the three other polymers, whereas cell viability displayed a gradual decrease with the polymer amount. Thus, the trends in the overall transfection efficiencies resembled those of the transfection efficiencies. The maximum overall transfection efficiencies of star polymers $DMAEMA_{15}$-*star*, MAA_5-*b*-$DMAEMA_{15}$-*star*, and $DMAEMA_{15}$-*star*-MAA_5 were around 4%, lower than the overall transfection efficiency of SuperFect. The results at the maximum overall transfection efficiency for

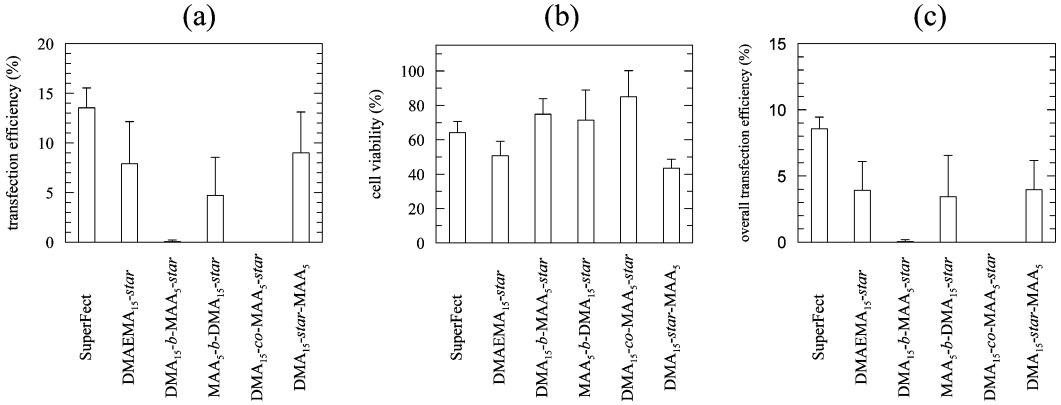

Figure 6. Dependence of (a) transfection efficiency, (b) cell viability, and (c) overall transfection efficiency on star architecture at the optimum conditions for each star polymer plus SuperFect. DMA is a further abbreviation for DMAEMA.

each of the star polymers were extracted from Figure 5 and are presented in Figure 6.

Effect of the Star Architecture. Figure 6 shows (a) the transfection efficiency, (b) the cell viability, and (c) the overall transfection efficiency for the five star polymers plus SuperFect at the maximum of the individual overall transfection efficiency. Examination of parts a and b of the figure can explain the final ranking observed in part c. Although none of the star polymers reached the optimum overall transfection efficiency of the commercially available reagent SuperFect, there was a strong dependence of the transfection results on star architecture. In particular, $DMAEMA_{15}$-*star*, MAA_5-*b*-$DMAEMA_{15}$-*star*, and $DMAEMA_{15}$-*star*-MAA_5 presented the highest overall transfection efficiencies with MAA_5-*b*-$DMAEMA_{15}$-*star* presenting the highest cell viability from the three star polymers. It is noteworthy that the cell viability with MAA_5-*b*-$DMAEMA_{15}$-*star* was even higher than that of SuperFect.

Comparing the transfection performance of these double-hydrophilic ampholytic star copolymers with that of the double-hydrophilic cationic DMAEMA−HEGMA star copolymers, the polyampholyte transfectants were less toxic but less efficient. Thus, the presence of the negatively charged MAA units in the star copolymers may indeed weaken the stability of the polymer−DNA complex, as initially hypothesized. However, this weakening would affect not only the release of the DNA after the complex enters the cell but also the entry of the complex into the cell. In particular, it seems that these two competing effects balance each other, resulting in equal overall transfection efficiencies of the best star polyampholytes and the DMAEMA star homopolymer.

Conclusions

A series of four DMAEMA−MAA ampholytic star copolymers of different architectures were synthesized by sequential group-transfer polymerization (GTP) and subsequent monomer deprotection. The series consisted of two star block copolymers, one statistical star copolymer, and one heteroarm star copolymer with 25% mol MAA units. Moreover, a DMAEMA star homopolymer was also prepared by the same method. After characterization of the polymer structure by GPC and NMR, all star polymers were evaluated for their ability to transfect human cervical HeLa cancer cells. These experiments showed that all four star copolymers displayed a decreased toxicity compared to the DMAEMA star homopolymer for the same amounts of star polymer tested. The overall transfection performance of the star copolymers depended strongly on their architecture. The best transfection reagent was $DMAEMA_{15}$-*star*-MAA_5, which had the highest overall transfection efficiency from the star copolymers, and similar to that of the DMAEMA star homopolymer.

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