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Carboxylated Cy5-labeled Comb Polymers

Passively Diffuse the Cell Membrane and Target

Mitochondria

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KEYWORDS

Spontaneous zwitterionic copolymerization, mitochondria, brush polymer, cyanine dye, 2-oxazoline.

ABSTRACT

A detailed understanding of the cellular uptake and trafficking of nanomaterials is essential for the design of “smart” intracellular drug delivery vehicles. Typically, cellular interactions can be tailored by endowing materials with specific properties, e.g. through the introduction of charges or targeting groups. In this study, water-soluble carboxylated *N*-acylated poly(amino ester) based comb polymers of different degree of polymerization (DP) and side chain modification were synthesized via a combination of spontaneous zwitterionic copolymerization (SZWIP) and redox-initiated reversible addition-fragmentation chain-transfer (RRAFT) polymerization and fully characterized by ^1H NMR spectroscopy and size exclusion chromatography (SEC). The comb polymers showed no cell toxicity against NIH/3T3 and N27 cell lines nor hemolysis. Detailed cellular association and uptake studies by flow cytometry and confocal laser scanning microscopy (CLSM) revealed that the carboxylated polymers were capable of passively diffusing cell membranes and targeting mitochondria. The interplay of pendant carboxylic acids of the comb polymers and the Cy5-label was identified as major driving force for this behavior, which was demonstrated to be applicable in NIH/3T3 and N27 cell lines. Blocking of the carboxylic acids through modification with 2-methoxy ethylamine and poly(2-ethyl-2-oxazoline) or replacement of the dye label with a different dye (e.g. fluorescein) resulted in an alteration of the cellular uptake mechanism towards endocytosis as demonstrated by CLSM. In contrast, partial modification of the carboxylic acid groups allowed to retain the cellular interaction, hence, rendering these comb polymers a highly functional mitochondria targeted carrier platform for future drug delivery applications and imaging purposes.

INTRODUCTION

Nanomedicine is a rapidly evolving research field, which exploits the beneficial properties of nano-engineered materials for biomedical and pharmaceutical applications. Over the last decades, nano-sized organic and inorganic particles have been introduced as excellent candidates for the design of drug delivery, therapeutic and diagnostic systems.¹⁻³ Targeted nanomedicines have received particular attention as they allow to deliver to and act at the site of action, hence, potentially increasing the therapeutic efficacy.⁴⁻⁵ On a cellular level, this means that nanoparticles are tailored to target specific cells and cell organelles, respectively.⁶ The latter can be achieved by the control of the cellular uptake mechanism.⁷⁻⁸ Intracellular trafficking is significantly influenced by the physiochemical properties of the nanocarrier, e.g. its size, shape, charge, and surface modification.⁹⁻¹² Surface charge and modification are two independent factors that mediate intracellular delivery and can be particularly valuable for targeted delivery to specific organelles, such as mitochondria.¹³⁻¹⁴ The major role of mitochondria is in the ATP synthesis by oxidative phosphorylation via the respiratory chain, which causes the highly negative potential of the mitochondrial membrane (about -200 mV).¹⁵ As a consequence, typically positively charged molecules are attracted to mitochondria.¹⁶ Most commonly, mitochondria targeting is achieved through the modification of the nanocarrier surfaces with the lipophilic positively charged triphenyl phosphonium (TPP) group or rhodamine.¹⁷ A second family of dyes, which has received less attention in this regard, are cyanine-based dyes. Active compounds such as drugs have been efficiently transported to mitochondria through the modification of drugs with cyanine dyes,¹⁸ an effect which is mainly associated with the positive charge introduced by the cyanine dye.¹⁹⁻²³ In contrast, in polymer science cyanine dyes have rather found application as labeling agents to track synthetic polymers and nanoparticles in vitro and in vivo. To the best of our knowledge, the cyanine triggered targeting of polymers to mitochondria has not been reported nor is it commonly considered as an option to specifically deliver polymers to these organelles. However, oligonucleotides, which can be considered as natural macromolecules have been demonstrated to target mitochondria when labeled with a cyanine dye despite their overall negative charge induced by the phosphate groups on each nucleotide.²⁴⁻²⁵ Intrigued by this observation we were interested to examine whether it is possible to translate this behavior into synthetic polymers and exploit it for future drug delivery applications. Their high degree of tuneability and tailored properties make synthetic polymers an ideal platform to investigate individual structural aspects and establish

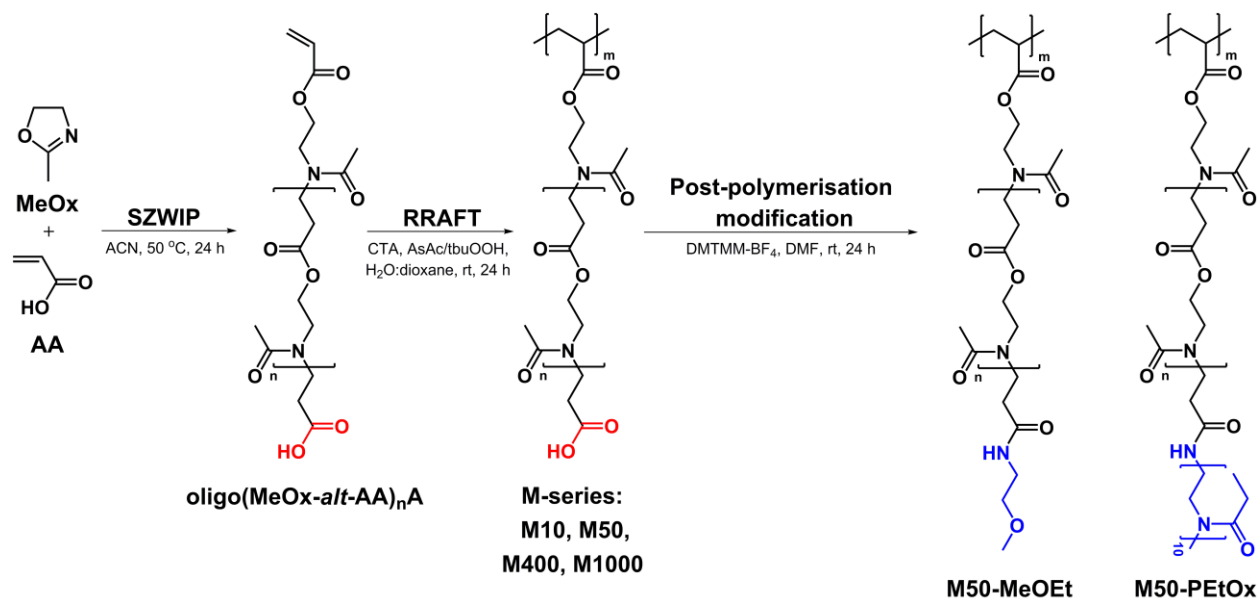
design criteria through structure-property relationship studies. Recently, we introduced the spontaneous zwitterionic copolymerization (SZWIP)²⁶ as a facile method for the synthesis of telechelic α -acrylate, ω -carboxylic acid *N*-acylated poly(amino ester)s.²⁷ These macromonomers provide access to comb polymers bearing side chain carboxylic acids, which render the system negatively charged.²⁸ In contrast to oligonucleotides with their rather chemically inert phosphate groups these comb polymers are readily available for post-polymerization modifications. This allows to alter their physico-chemical properties and introduce other functionalities such as labels, targeting ligands and drugs; making it a highly interesting system for the preparation of targeted drug delivery vehicles.

In this work, we explore the cellular interactions of cyanine 5 (Cy5) labeled negatively charged carboxylated poly(oligo(MeOx-alt-AA)_nA) comb polymers. This particular system was selected since it is water-soluble, does not show a lower critical solution temperature (LCST) behavior as the other members of this comb polymer class, and guarantees a high degree of pendant carboxylic acids.²⁹ We demonstrate that despite their negative charge they efficiently target mitochondria and passively diffuse cell membranes. This behavior was observed in different cell lines and shown to be dependent on the degree of polymerization (DP) and the availability of free carboxylic acid groups. Partial modification of the carboxylic acid groups is shown to not alter the cellular trafficking significantly, hence, rendering it a suitable platform for mitochondria-targeted polymer-drug conjugates.

RESULTS AND DISCUSSION

Comb polymer synthesis and modification. The carboxylated comb polymers used in this study were prepared by the recently reported combination of SZWIP and redox-initiated reversible addition fragmentation chain-transfer (RRAFT) polymerization.^{27, 30} 2-Methyl-2-oxazoline (MeOx) and acrylic acid (AA) were reacted to form the oligo(MeOx-alt-AA)_nA macromonomer. A MeOx-to-AA feed ratio of 1:2 was chosen to enable the quantitative introduction of ω -carboxylic acid end groups as previously reported. Subsequently the heterotelechelic macromonomer was polymerized by RRAFT using 2-(((butylthio)-carbonothioyl)-thio)propanoic acid as chain transfer agent (CTA) to yield the respective comb polymers (M-series) (Scheme 1).

Scheme 1. Schematic representation of the spontaneous zwitterionic copolymerization (SZWIP) of 2-methyl-2-oxazoline (MeOx) and acrylic acid (AA), redox-initiated RAFT polymerization of oligo(MeOx-alt-AA)_nA, and the modification of M50 (ACN: acetonitrile; CTA: 2-(((butylthio)carbonothioyl)thio)propanoic acid; AsAc: ascorbic acid; tbuOOH: tert-butyl hydroperoxide; DMTMM-BF₄: 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium tetrafluoroborate).



By changing the monomer-to-CTA ratio comb polymers of different DP (10, 50, 400, and 1000) were obtained. SEC and DLS investigation of the polymers revealed the preparation of well-defined comb polymers ($\bar{D} < 1.35$; Figure 1a) of different hydrodynamic sizes (Table S1), respectively. To examine the influence of carboxylic acids on the cellular interaction, M50 (Figure

1b) was further modified through amidation with 2-methoxyethyl-amine (MeOEtNH₂) (Figure S1) and amine end-functionalized poly(2-ethyl-2-oxazoline) (PEtOxNH₂).³⁰ By comparing the methyl group signals of the MeOx side chain (H5) to the methyl group signal of the EtOx side chain (H9) in the ¹H NMR spectrum of the M50-PEtOx (Figure 1c) a near quantitative conversion (> 95%) of the carboxylic groups can be assumed. Moreover, a shift towards higher molecular weights in SEC was observed (Figure 1a). Similarly, the hydrodynamic size increased while the zeta potential became less negative after modification (Table S1). All polymers were labeled with Cy5-amine (Figure S2) and extensively purified by dialysis or size exclusion chromatography using NAP-25 columns in order to study their cellular interaction.

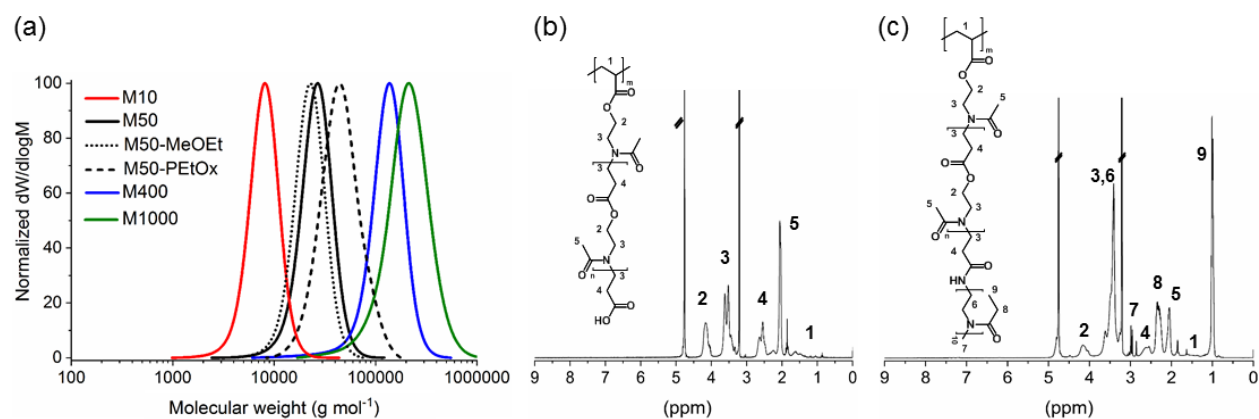


Figure 1. (a) SEC traces of M-series and modified combs, and ¹H NMR (400 MHz, MeOD) of (b) M50 and (c) M50-PEtOx.

In vitro studies. A mouse embryo fibroblast cell line (NIH/3T3) was chosen for the first in vitro studies and the cytotoxicity of all polymers was assessed by an AlamarBlue® assay. The tests indicated that (up to 1.0 mg mL⁻¹, 24 h) the comb polymers do not significantly affect the viability of NIH/3T3 cells (Figure S3a). The oligo(MeOx-alt-AA)_nA macromonomer as well as the comb polymers M50 and M400 were further examined for their hemolytic activity. In all three cases no significant hemolysis was detected, up to 500 µg mL⁻¹ (Figure S4).

Cell association kinetics of Cy5-labeled polymers were studied by flow cytometry. Modified and unmodified comb polymers were incubated with NIH/3T3 cells at 37 °C and the mean fluorescence intensity was measured at different time points (0.25, 0.5, 1, 2, and 4 h). As depicted

in Figure 2a, all polymers showed a time dependent cell association with shorter comb polymers showing the highest values. Moreover, the modification of the carboxylic acids of M50 with MeOEt and PEtOx resulted in a 4-fold decrease of the cell association. In order to further elucidate their cellular interactions, the cell association was studied at 4 °C, and in the presence of pharmacological inhibitors to inhibit potential endocytic pathways. At 4 °C cellular associations showed similar trends but with lower values than at 37 °C (Figure 2b). The cellular association of M10 and M50 was inhibited by 20% followed by M400 with 30% inhibition. In contrast, DP1000 and the modified comb polymers (M50-MeOEt and M50-PEtOx) were inhibited by about 50% and 80-90%, respectively. Similarly, sodium azide/2-deoxyglucose (NaN₃/DOG) treatment had negligible effect on the association of M10 and M50, but remarkably affected the association of M50-MeOEt and M50-PEtOx (Figure S5). Overall, these experiments suggest that the shorter carboxylated comb polymers follow an energy-independent association/uptake while modification of the carboxylic acid groups changes the situation towards an energy-dependent pathway.

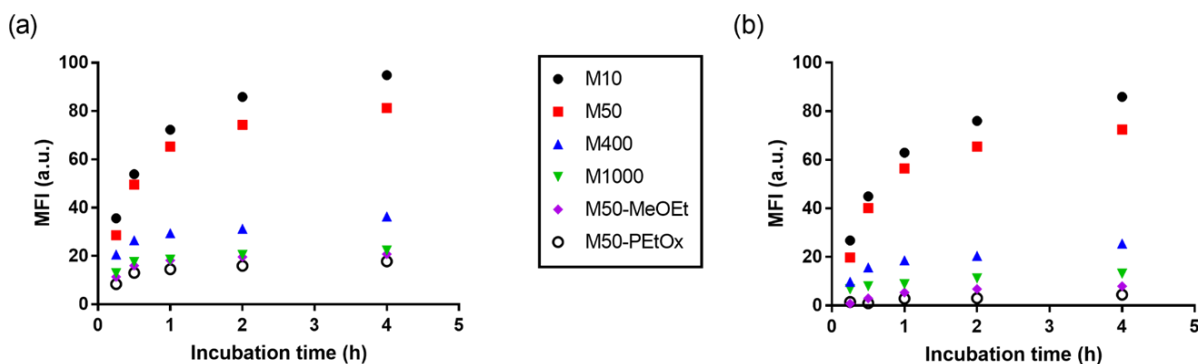


Figure 2. Cell association studies of NIH/3T3 cells with M-series and modified M50, (a) at 37 °C, and (b) at 4 °C. Each polymer was added to NIH/3T3 cells and incubated for different time points (0.25, 0.5, 1, 2, and 4 h). The fluorescence intensity was evaluated by flow cytometry. MFI stands for mean fluorescence intensity.

To visualize and confirm the observations of the substantial effect of the carboxylic group modification made by flow cytometry, more detailed cellular uptake studies using confocal laser scanning microscopy (CLSM) were performed. To study the intracellular localization of the different comb polymers, they were incubated with NIH/3T3 cells expressing SNAP-tag fused to mitochondrial oxidase 8A (Cox8A-SNAPf) and lysosomal-associated membrane protein 1

(LAMP-1), respectively. Figure 3 shows fluorescence images of NIH/3T3 cells incubated with Cy5-labeled M10 and M50 (red channel) and SNAP-Cell TMR-Star to label the mitochondria (green channel). Merged images confirm the colocalization of M10 and M50 with mitochondria. Live cell imaging of the M50 uptake at 37 °C revealed a rapid cellular uptake and cytosol distribution (Video S1, Supporting Information). The cytosol is strongly stained within a few minutes of the injection of the comb polymer solution in serum-containing medium. Typically, such rapid internalization is an indication of a cellular uptake mechanism involving passive translocation through the cellular membrane.³¹ In addition, lysosome colocalization studies showed the lack of colocalization of M10 and M50 with lysosomes in NIH/3T3 cells containing lysosomal-associated membrane protein 1 (LAMP-1) (green channel) (Figure 3b). Increasing the DP of the comb polymers to 400 did not significantly affect the colocalization of the polymer (Figure S6). However, M1000 showed evidence for colocalization with both, mitochondria and lysosomes (Figure S6). In contrast, the modified polymers M50-MeOEt and M50-PEtOx colocalized exclusively with lysosomes (Figure 3b). Noteworthy, similar to the modified polymers, a Cy5-labeled poly(oligo(ethylene glycol) acrylate) (DP50; 24,000 g mol⁻¹) showed no colocalization with mitochondria but with lysosomes highlighting the importance of the pendant carboxylic acid groups (Figure S7). These results were further established when the NIH/3T3 cell line was pre-incubated with NaN₃/DOG to block the cellular ATP synthesis²⁷, and then treated with Cy5-labeled NPAE-based comb polymers. CLSM (Figure 4) confirmed the inhibition of the cellular uptake of the modified polymers, whereas no effect on the cellular uptake of M10 and M50 polymers was observed.

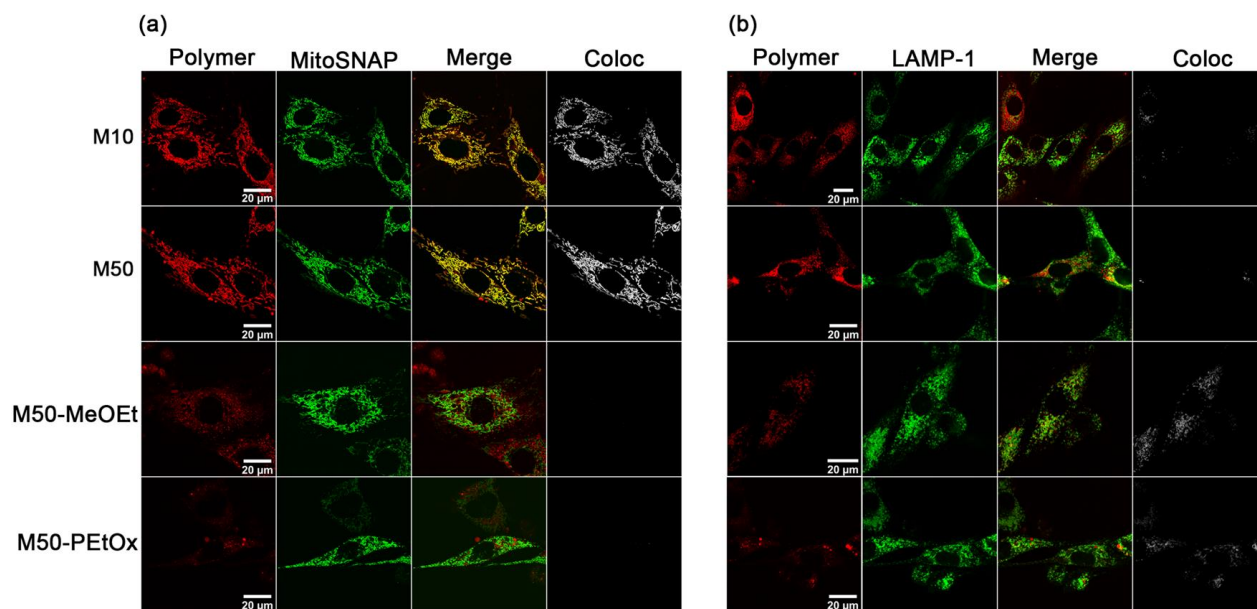


Figure 3. Confocal microscopy images of NIH/3T3 cells treated with M10, M50, M50-MeOEt, and M50-PEtOx (0.2 mg mL^{-1}) incubated for 4 h. (a) Colocalization of M10, M50, M50-MeOEt, and M50-PEtOx (Cy5, red channel) and mitochondria (SNAP, green channel) in NIH/3T3 cells. 1 h before imaging, SNAP was added to cells for 30 min and then cells were washed with media with 20% FBS. (b) Colocalization of M10, M50, M50-MeOEt, and M50-PEtOx (Cy5, red channel) and lysosome (LAMP-1, green channel) in NIH/3T3 LAMP-1 cells.

These observations emphasize the role of the carboxylic acids in the cellular uptake. Moreover, it demonstrates the possibility to switch the cellular uptake of the comb polymers from passive diffusion to endocytosis and modulate their intracellular fate through simple modification of the pendant carboxylic acid moieties.

So as to additionally determine if other organelles are involved in the cytoplasmic distribution of Cy5-labeled M10, M50, and modified comb polymers, endoplasmic reticulum (ER) and trans-Golgi network (TGN) colocalization studies were performed. Selective ER and TGN CellLight® reagents were added 24 h before imaging using CLSM. The comb polymers showed lack of colocalization with ER and TGN, which confirms the selective distribution of Cy5-labeled M10 and M50 polymers in the mitochondria and M50-modified ones in the lysosomes (Figure S8-9).

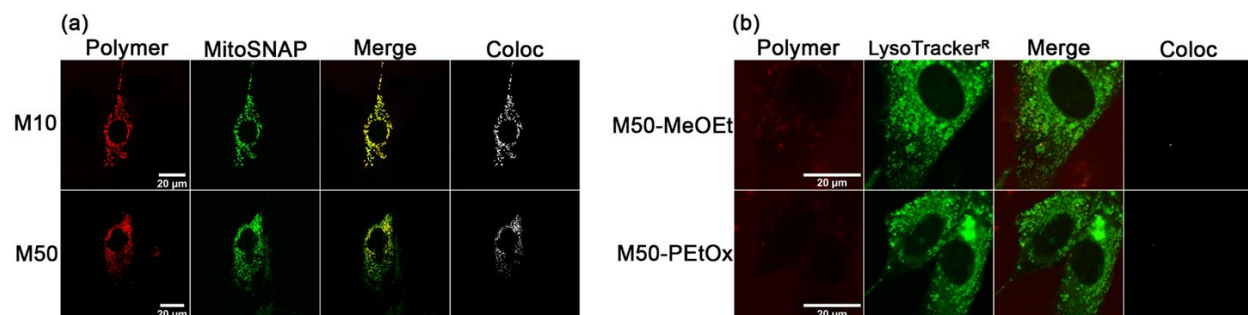


Figure 4. Confocal microscopy images of NIH/3T3 cells demonstrating the effect of endocytosis inhibitor, NaN_3/DOG , on the cellular uptake and colocalization of (a) M10 and M50 (Cy5, red channel) with mitochondria (SNAP, green channel) in NIH/3T3 cells and (b) the M50-MeOEt and M50-PEtOx (red channel) with lysosomes (LysoTracker®, green channel).

Thus far, we established that the ability of Cy5 modified comb polymers to passively diffuse cell membranes and target mitochondria is dependent on the DP and in particular on the presence of free carboxylic acids. To reveal the specific role of Cy5 in the cellular uptake and fate of the comb polymers, M50 was also labeled with fluorescein, a non-cyanine based dye. It is important to note, that although fluorescein is a pH-sensitive fluorophore, the high pH environment in the mitochondria would not affect the signal from fluorescein-labeled polymers.³²⁻³³ Upon incubation with NIH/3T3, CLSM was employed to study the cellular uptake of this differently labeled comb polymer. Similar to altering the polymer charge, changing the Cy5 to fluorescein (Figure S10) resulted in the loss of colocalization with mitochondria, highlighting the synergy of Cy5 and pendant carboxylic groups of the comb polymers for mitochondria targeting.

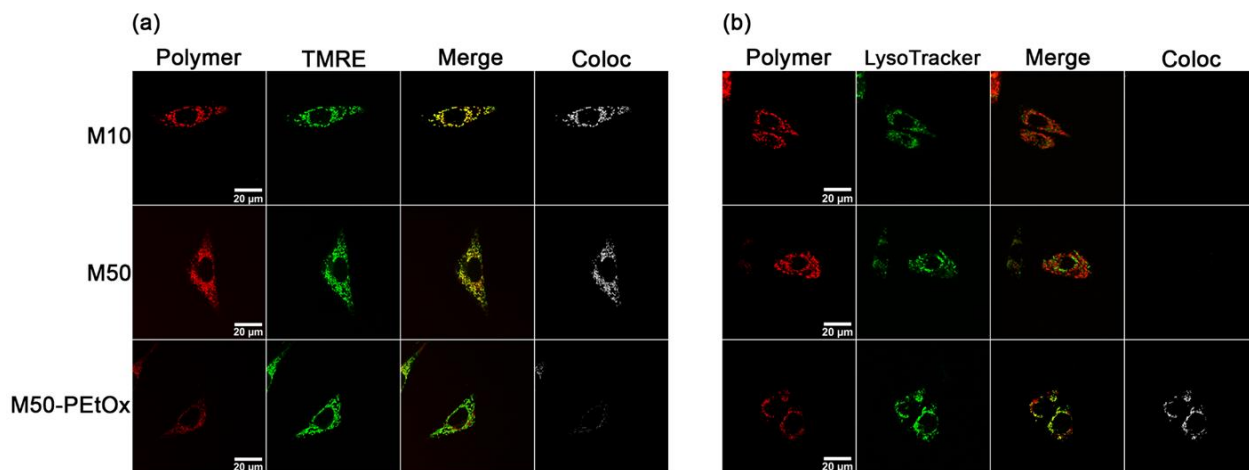


Figure 5. Confocal microscopy images of N27 cells treated with M10, M50, and M50-PEtOx (0.2 mg mL^{-1}) incubated for 4 h. (a) Colocalization of Cy5-labeled comb polymers (Red) and TMRE (Green) in N27 cells. (b) Colocalization of Cy5-labeled M10, M50, and M50-PEtOx (Red) and lysosome (Green) in N27 cells.

To evaluate the broader applicability of the system, a second cell line, namely N27 rat dopaminergic neural cell line, was tested. To this end, we selected the mitochondria targeted comb polymers M10 and M50 as well as lysosome targeted M50-PEtOx. None of the polymers showed toxicity to N27 cells (Figure S3b) with similar values of association to NIH/3T3 in cell association experiment at 37°C and 4°C for M10 and M50, and M50-PEtOx (Figure S11).

CLSM revealed similar colocalization of M10 and M50 with mitochondria, while M50-PEtOx showed lysosome colocalization (Figure 5). To gain more insights in the cellular uptake mechanism responsible for the internalization of the Cy5-labeled M50 and M50-PEtOx as well as the fluorescein-labeled M50 in N27 cells, the effect of temperature and endocytosis inhibitors (Genistein, NaN_3/DOG , and Dyno) was examined (Figure 6). Cy5-labeled M50 cell association was not inhibited by the presence of the inhibitors nor low temperature treatment. In contrast, the fluorescein-labeled M50 and Cy5-labeled M50-PEtOx cell association was significantly affected implying an endocytosis uptake mechanism. Therefore, as observed for NIH/3T3 cells, modification of the pendant carboxylic acid groups or change of the label results in an alteration of the cellular internalization and fate of the polymers.

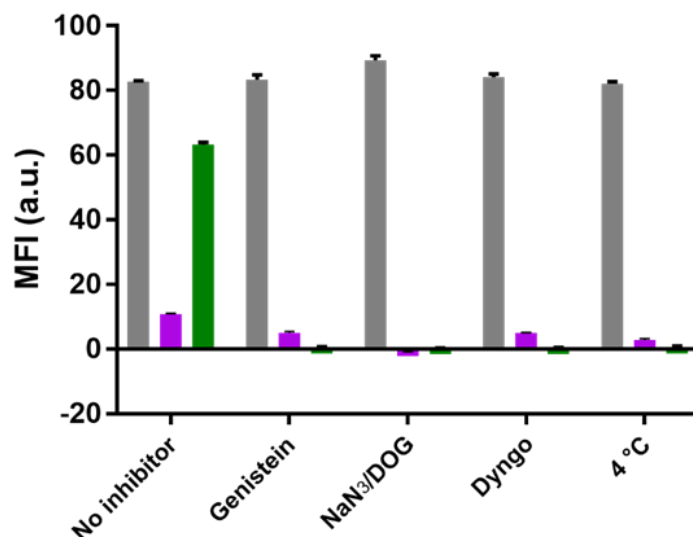


Figure 6. Mean fluorescence intensity measurements representing the effect of endocytosis inhibitors (added 30 min prior to polymer addition to N27 cells): genistein, dyngo, NaN₃/DOG, and cooling to 4 °C on the cell association of Cy5- (grey column) and fluorescein-labeled M50 (green column), as well as Cy5-labeled M50-PEtOx (purple column) in N27 cells. Measurements were performed after 4 h of polymers addition. MFI stands for mean fluorescence intensity.

Since M10, a polymer with as little as 10 carboxylic acids, is sufficient to passively diffuse the cell membrane and target mitochondria, we anticipated that upon partial modification of the carboxylic acids of the longer M50, the cellular interaction behavior will be retained and hence the system can be employed for the future delivery of drugs. To this end, only approximately 20% of the carboxylic side groups were functionalized through amidation with pyridine dithioethylamine (PDS) (Figure S12), a functional group that both enables further post-polymerization modification with various cargo and targeting moieties and is also known to affect the cellular uptake itself.³⁴ CLSM studies after the incubation of N27 cells with M50-PDS revealed an unaltered cellular localization as indicated by the strong colocalization between M50-PDS and mitochondria (Figure 7). This demonstrates that while the quantitative conversion of the carboxylic acids leads to a switch of the cellular processing, partial modification allows to preserve the targeting behavior and thus makes the comb polymers a suitable system for the design of mitochondria targeted polymer-drug conjugates.

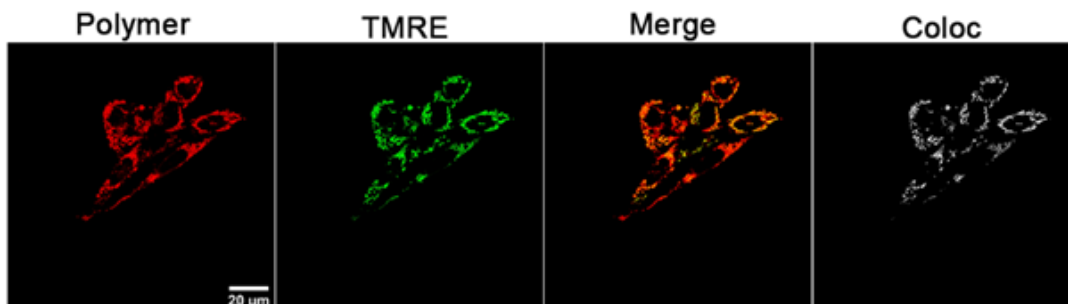


Figure 7. Confocal microscopy images of N27 cells treated with M50-PDS (0.2 mg mL^{-1}) and incubated for 4 h at 37°C and $5\% \text{ CO}_2$. The images demonstrate colocalization of Cy5-labeled M50-PDS (red) with mitochondria TMRE (green) in N27 cells.

CONCLUSIONS

We demonstrate that Cy5-labeled NPAE comb polymers bearing pendant carboxylic acids are cytocompatible, show an energy independent cellular uptake and efficiently target mitochondria despite the overall negative charge of the polymer. The availability of carboxylic groups for amidation reactions allowed for a detailed structure-property relationship study, which identified the interplay of the carboxylic acids, polymer size and the Cy5 label as driving forces for the cellular interaction observed. Passivation of the carboxylic acid functionalities with a small organic compound or short polymer resulted in a change of the cellular uptake and fate towards energy dependent endocytosis process. However, partial modification with a functional entity did not impede mitochondria targeting, hence, demonstrating the potential of the Cy5-labeled NPAE based comb polymers for effective mitochondria bioimaging and drug delivery in the future. Noteworthy, our study highlights the importance of the selection of labels for imaging experiments to avoid misinterpretation of the results.

EXPERIMENTAL SECTION

Materials

Acrylic acid (AA, 99%, anhydrous, Sigma-Aldrich), acetonitrile (ACN, 99.8%, Sigma-Aldrich), 4-methoxyphenol (MEHQ, 99%, Sigma-Aldrich), diethyl ether (DEE, >98%, Sigma-Aldrich), ethanol (>99.8%, Sigma-Aldrich), 1,4-dioxane (>99%, Sigma-Aldrich), Luperox® TBH70X tert-butyl hydroperoxide solution (tBuOOH, 70% wt.% in H₂O, Sigma-Aldrich), L-ascorbic acid (AsAc, Sigma Aldrich), 2-methoxyethylamine (MeOEtNH₂, 99%, Sigma-Aldrich), poly(ethylene glycol) methyl ether acrylate (Sigma-Aldrich), 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM-HCl, >96%, Sigma-Aldrich) and 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium tetrafluoroborate (DMTMM-BF₄, 97%, Sigma Aldrich) were used as purchased without further purification. 2-Methyl-2-oxazoline (MeOx, 98%, Sigma-Aldrich) was distilled to dryness over barium oxide (BaO) and stored in a nitrogen atmosphere. The chain transfer agent (CTA), 2-(((butylthio)-carbonothioyl)thio)-propanoic acid was prepared according to a literature procedure.³⁵ Amine-terminated poly(2-ethyl-2-oxazoline) (PEtOxNH₂) was synthesized following a literature procedure.³⁶ 2-(pyridyldithio)-ethylamine hydrochloride (PDS) was obtained from Speed Chemical, China. Cyanine5 amine was purchased from Lumiprobe. CellLight™ ER-GFP, BacMam 2.0 and CellLight™ Golgi-GFP, BacMam 2.0 were purchased from Thermo Fisher Scientific.

Instruments for polymer characterization

¹H Nuclear Magnetic Resonance (¹H NMR) spectroscopy of all samples was carried out using a Bruker AVANCE III HD 400 MHz spectrometer using deuterated solvents obtained from Sigma-Aldrich. Analyses of polymer solutions were performed using a Shimadzu modular system comprising a DGU-12A degasser, an SIL-20AD automatic injector, a 5.0 µm bead-size guard column (50 x 7.8 mm) followed by three KF-805L columns (300 x 8 mm, bead size: 10 µm, pore size maximum: 5000 Å), a SPD-20A ultraviolet detector, and an RID-10A differential refractive index detector. A CTO-20A oven was used to maintain the columns at 40 °C. *N,N*-dimethylacetamide (DMAc) with 0.03% w/v LiBr was used as the eluent where samples were run isocratically at 1 mL min⁻¹. Polystyrene standards (0.5 to 2000 kg mol⁻¹) were used for calibration. Analyte samples were filtered through 0.45 µm PTFE filters before injection. Molar mass (Mn,

SEC) and dispersity (\bar{D}) values of samples were determined on Shimadzu LabSolutions software. Dynamic light scattering (DLS) analysis was carried out using a Malvern Zetasizer Nano ZS. All measurements were carried out at 25 °C using filtered polymer solutions of 2 mg mL⁻¹ in PBS.

Synthesis of compounds

Synthesis of the macromonomer

In a Schlenk flask under nitrogen, MEHQ (1 mg, 8.06 x 10⁻⁶ mol) was dissolved in anhydrous ACN. Subsequently, 2-methyl-2-oxazoline (2.48 mL, 58.8 mmol) and acrylic acid (4.03 mL, 117.6 mmol) was added. The mixture was stirred for 24 h in an oil-bath at 50 °C. The polymer solution was subsequently cooled, precipitated in DEE and isolated by centrifugation. The polymer was then placed under vacuum to give a colourless-to-yellowish oil product. ¹H NMR and SEC measurements were taken of the final purified sample to determine the degree of polymerization (DP) and molecular weight.

Synthesis of the comb polymers by RRAFT

Four NPAE based comb polymers (M10, M50, M400, and M1000) were prepared by varying the monomer-to-CTA ratio accordingly. Exemplarily, the synthesis of M50 is described: 144 uL of a CTA stock solution (17.13 mg mL⁻¹ in 1, 4-dioxane), macromonomer (0.517 mmol) and 335 uL of an ascorbic acid stock solution (0.68 mg mL⁻¹ in MilliQ) were added to a sample vial. The mixture was deoxygenated for 15 min. In parallel, an aqueous stock solution of tBuOOH (7.4 mg mL⁻¹ in MilliQ) was deoxygenated. 45 uL of the latter was added to the sample vial via a nitrogen-purged syringe. The sample vial was placed in a thermostated water bath set at 25 °C for 24 h. Afterwards, the monomer conversion was determined by ¹H NMR spectroscopy measurements. The comb polymers were purified by dialysis against deionized water (MWCO = 3500 g mol⁻¹) for two days. ¹H NMR and SEC measurements were taken to determine the purity of the final sample.

Preparation of modified M50 systems: M50-MeOEt and M50-PEtOx

12 mg comb polymer was dissolved in 0.3 mL DMF and 15 mg DMTMM-BF₄ (1.5 eq per repeating unit) dissolved in 0.5 mL DMF were added. The solution was stirred for 15 min. Subsequently, 50 mg PEtOxNH₂ (1000 g mol⁻¹) or 3.5 mg MeOEtNH₂ in 0.5 mL DMF were

added. After 24 h the solvent was removed and the crude was redissolved in MilliQ and extensively dialyzed against MilliQ (MWCO = 3500 g mol⁻¹) for four days. ¹H NMR and SEC measurements were conducted to determine the degree of functionalization and the molecular weight of the products.

Labelling of unmodified comb polymers and modified comb polymers with Cy5-NH₂

In a vial, 20 mg of comb polymer was dissolved in 1 mL MilliQ. 0.37 mg DMTMM-HCl (1.3 eq. per polymer; 20 mg mL⁻¹ stock solution) was added and the resulting mixture stirred for 10 min at room temperature. 1.35 mg Cy5-amine (1.5 eq per polymer) were added and the resulting mixture was stirred for 24 h in the absence of light. The product was purified by column chromatography using a NAP-25 column and subsequently dialyzed for 4 days against MilliQ (MWCO = 3500 g mol⁻¹) and freeze-dried to isolate the product.

Synthesis of partially PDS modified M50

25 mg comb polymer was dissolved in 0.5 mL DMF and 7.5 mg DMTMM-BF₄ (0.5 eq per repeating unit) dissolved in 0.5 mL DMF were added. The solution was stirred for 15 min. Subsequently, 6 mg PDS in 0.5 mL DMF were added. After 24 h the solvent was removed and the crude was redissolved in MilliQ and purified by column chromatography using a NAP-25 column and extensively dialyzed against MilliQ (MWCO = 3500 g mol⁻¹) for 4 days. ¹H NMR and SEC measurements were conducted to determine the degree of functionalization and the molecular weight of the products.

Instruments used for in vitro experiments

Spectrofluorophotometer

Fluorescence spectra were obtained using a Shimadzu RF-5301PC spectrofluorophotometer in a 500-μL quartz SUPRASIL cuvette. Fluorescent samples at a concentration of 2.5 μg mL⁻¹ in PBS at pH 7.4 were excited at 600 nm and the emission collected between 620 – 750 nm. The spectrum of PBS alone was obtained before the addition of the polymer and subtracted from all fluorescent spectra.

Flow cytometer

Flow cytometry was performed using a Stratadigm S1000EXI flow cytometer (Stratadigm, California, USA) with a 488 nm excitation and emission collected between 515 – 545 nm and a 642 nm excitation with emission collected between 661 – 691 nm. FCS3.0 files were exported using CellCapTure Analysis Software (Stratadigm, California, USA) and gated by forward and side scatter in FlowJo (version 10, Tree Star, Oregon, USA) before subsequent analysis.

Confocal laser scanning microscope (CLSM)

Confocal imaging was carried out on a Leica SP8 X confocal using a 40x PL APO NA1.3 objective. All images were collected at zoom 3 and 2048x2048 pixel resolution to achieve optimal sampling. Excitation was by 488, 568, and 633 nm laser lines with emission captured at 495-530, 575-610, and 640-700 nm respectively in a sequential order.

In vitro experiments

Cell Culture

NIH/3T3 cells (purchased from ATCC) and N27 rat dopaminergic neural cells (purchased from Merck) used in this study were tested, and cleared for mycoplasma. NIH/3T3 were maintained in Dulbecco's modified Eagles Medium (DMEM) Glutamax™ supplemented 1mM sodium pyruvate 20% v/v fetal bovine serum. N27 cells were kept in RPMI 1640 Medium, GlutaMAX™ Supplemented, HEPES and 5% Penicillin-Streptomycin (10,000 U/mL) obtained from Thermo Frischer and 10% v/v fetal bovine serum. Cells were cultured at 37 °C in a humidified incubator with 5% atmospheric CO₂. Cell counting for passaging was done by adding 0.4% Trypan Blue solution to the cells in medium and using a haemocytometer. Genistein (protein tyrosine kinase inhibitor) and Dyngo® 4a were obtained from Abcam and used without further purification.

Cell viability

Cell viability was tested via AlamarBlue® assay. Cells were seeded the day before on 96 well plates at a density of 10,000 cells per well. The cells were then incubated with polymer samples (0.06 to 1 mg mL⁻¹ in PBS, prepared through serial dilution) and incubated at 37 °C for 24 h in a humidified incubator with 5 % atmospheric CO₂. Samples were run as triplicate. After incubation, the medium was removed and a 10% v/v solution of AlamarBlue® in DMEM is added. The cells

were incubated for a further 4 h at 37 °C. Cell viability is determined by measuring the fluorescence (excitation: 540 nm, emission: 590 nm). Wells incubated without cells were used as blank. Control samples were cells incubated with PBS (without polymer). The viability was calculated using equation below.

$$\text{Cell viability (\%)} = \frac{(\text{FI sample} - \text{FI blank})}{(\text{FI control} - \text{FI blank})} \times 100$$

Where: FI = Fluorescence intensity

Haemolysis studies

The compatibility of the macromonomers, M50, and M400 polymers with erythrocyte membranes was assessed by incubating the polymers (125 or 500 µg mL⁻¹) with red blood cells obtained from a rat. Red blood cells were purified and isolated by repeated centrifugation and washing with PBS. A 0.05 wt. % solution of the red blood cells in PBS buffer was prepared at pH 7.4. A 96 well plate was prepared by transferring 140 µL of the red blood cell solution into a 96 well plate. Polymer solution (10 µL) was added to the wells. The positive control wells contained Triton X-100 and negative controls contained red blood cells without any additions. The polymer samples were incubated for 1 h at 37 °C. Lysed cell contents were isolated and analysed using UV spectrometer at 540 nm. Haemolysis was calculated using equation below.

$$\text{Haemolysis (\%)} = \frac{(P - B)}{(C - B)} \times 100$$

Where:

P = absorbance of polymer sample,

C = absorbance of positive control (100 % haemolysis)

B = absorbance of negative control (blank)

Cell association

Comb polymers were investigated for their association with NIH/3T3 cells and N27 cells via flow cytometry. Cells were seeded at 20,000 cells per 24-well microplates and cultured for 24 h. After incubation with Cy5-labeled polymers (final concentration: 0.2 mg mL⁻¹) for 4 h, the cells were

washed twice with cold PBS and trypsinized. They were harvested and washed twice with cold PBS. Flow cytometry was performed on a Stratadigm S1000EXI flow cytometer. For each sample, 10,000 events were analyzed ($n = 3$ in each experiment). In the analysis of the flow cytometry data, we took into consideration the differences in the level of Cy5 incorporation in the polymers by normalizing the fluorescence intensity accordingly.

Cellular uptake studies with mitochondrial and lysosomal staining

NIH/3T3 COX8A or NIH/3T3 LAMP1 cells were seeded the day before on an 8-chamber plate at a density of 20,000 cells per chamber and incubated at 37 °C in a humidified incubator with 5% atmospheric CO₂. After addition of the polymer solutions, the cells were incubated for 4 h at the 37 °C incubator. After incubation, the cell medium was removed and the cells were washed twice with FluoroBrite DMEM media. 100 µL of a 0.5 % v/v Snap Cell 505 (mitochondrial stain) in Fluoro-Brite was added (in case of mitochondria cellular uptake study) and the cells were incubated for 30 min at 37 °C in a humidified incubator with 5 % atmospheric CO₂. The FluoroBrite DMEM media was removed and the cells were washed twice with FluoroBrite. After 30 min, 100 µL of propidium iodide (PI) in FluoroBrite DMEM media (0.5 µg mL⁻¹) and 100 µL of Hoechst stain in FluoroBrite DMEM media (2.5 µg mL⁻¹) were added. After 5 min, the FluoroBrite DMEM media solutions were removed and a fresh 100 µL of FluoroBrite DMEM media was added. The cellular uptake was studied by confocal laser scanning microscopy. The confocal microscopy images were analyzed using ImageJ software developed by the National Institutes of Health (Bethesda, MD, USA). Cellular uptake study for N27 cells with mitochondrial and lysosomal staining was similar to NIH/3T3 cells. However, instead of using DMEM N27 cells were kept in RPMI 1640 Medium and TMRE was used as mitochondrial stain and LysoTracker® as a lysosomal dye.

Cellular uptake studies with endoplasmic reticulum (ER) and Trans-Golgi network (TGN) staining NIH/3T3 cells were seeded the day before on an 8-chamber plate at a density of 20,000 cells per chamber and incubated at 37 °C in a humidified incubator with 5 % atmospheric CO₂. After addition of the polymer solutions, the cells were incubated for 1 h at 37 °C in a humidified incubator with 5 % atmospheric CO₂. After incubation, the old cell medium was removed and the cells were washed twice with FluoroBrite. 4 µL of CellLight® reagent added directly to the cells

in complete cell medium and mix gently. Return the cells to the culture incubator overnight (≥ 16 h) at 37 °C in a humidified incubator with 5 % atmospheric CO₂. Cells can be imaged the next day. Cells were fixed using 1% PFA solution. The cellular uptake was studied by confocal laser scanning microscopy. The confocal microscopy images were analysed using ImageJ software developed by the National Institutes of Health (Bethesda, MD, USA).

Inhibition of cellular uptake with various inhibitors

N27 cells were plated at 20,000 cells/mL in 8-well microplates and precultured for 24 h. In the case of assays performed at 4 °C³¹, a solution of a Cy5 polymer in cold PBS was added to the cells in amounts such that the final polymer concentration was 0.2 mg mL⁻¹. N27 cells were incubated for 4 h at 4 °C. The inhibitors, sodium azide/2-deoxyglucose (0.1% NaN₃/50 mM DOG),³⁷ Genistein (protein tyrosine kinase inhibitor) (200 µM)³⁷ and Dyngo® 4a (30 µM)³⁸ were added to the cells 30 min prior to polymers addition. Cy5-labeled polymers were added to the inhibitor treated cells to attain a final concentration of 0.2 mg mL⁻¹ and incubated for 4 h at 37 °C. The cells were washed with PBS, trypsinized, harvested, and centrifuged at 5,000 rpm for 4 min. The cells were resuspended in cold PBS and evaluated by flow cytometry.

Supporting Information. Appropriate supporting figures (e.g. confocal microscopy images, cell toxicity), and tables. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Carboxylated Cy5-labeled Comb Polymers Passively Diffuse the Cell Membrane and Target Mitochondria

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