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Impact of the polymer side chain chemistry on interactions of amino-acid-derived polyelectrolytes with cells

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Abstract

Polyelectrolytes gained significant attention for their unique, potentially specific association with living cells. Particularly amino-acid-derived polyzwitterions feature specificity to cancer cells, highlighting their potential for biomedical applications. Here, the synthesis of lysine- and phenylaniline-derived polyelectrolytes *via* post-polymerisation modification is presented. The amidation of activated ester prepolymers with protected basic amino acids and their subsequent deprotection yields tailored amino acid-functionalised polyzwitterions comprising side chain chirality. Similarly, the use of achiral neutral amino acids results in polyanionic analogues of these polyzwitterions. *Via* this strategy, a small library of five polymers was created, which enables the careful evaluation of structure-property relationships of polyelectrolytes and their interactions with cells. In particular, we study the influence of side chain motifs: (i) aliphatic vs. aromatic side chains, (ii) permanent vs. non-permanent charge, and (iii) zwitterionic vs. anionic patterns. High-performance liquid chromatography measurements are performed to investigate the influence of both the charge character and aromaticity on the hydrophilicity of the polyelectrolytes. Response to changes in the pH value is studied by dynamic light



scattering (DLS) and electrophoretic light scattering at different pH values. Furthermore, polyelectrolyte stability under biologically relevant conditions, *i.e.*, in the presence of model proteins and more complex protein mixtures from foetal bovine serum is assessed by DLS. Association with cells from different cancer and non-cancer cell lines provides information regarding structural driving forces for association efficiency and specificity. Furthermore, fluorescent imaging shows fast uptake of polyanions into targeted cells making them interesting candidates for drug-delivery applications.

1. Introduction

Limited stability of therapeutic compounds and loss of activity upon modification are major drawbacks of conventional medicine.^{1,2} One strategy to overcome these limitations is the encapsulation into stable carriers perhaps possessing targeting ligands to facilitate the selective delivery to the target site.^{2,3} Besides protecting the drug, these carriers (*e.g.*, composed of synthetic polymers) must possess low-fouling properties and cell-specific moieties (*e.g.* proteins, peptides, aptamers, polysaccharides or small biomolecules).⁴ Building blocks capable of fulfilling these tasks ought to be identified. The engineering of polyelectrolytes has proven to be a powerful strategy to tailor functional molecules for such biomedical application. By systematically varying charge density⁵, backbone architecture⁶, and side chain functionality⁷, polyelectrolytes can be designed to control (i) intermolecular interactions such as polymer-protein interactions (*i.e.* reduce aggregation with proteins)⁸, (ii) self-assembly to form higher-level structures for stable drug transport in the biological environment, and (iii) responsiveness to external stimuli to facilitate cellular uptake.⁹ The latter is important since therapeutic compounds are often cell membrane-impermeable and polyelectrolytes may overcome the membrane barrier efficiently.¹⁰ One way is to harness amino acid transporters – symporters, antiporters (overexpressed in cancer cells), and uniporters – and allow the uptake of anionic, neutral, cationic, or specific amino acid motifs (*e.g.*, leucine, phenylalanine, glycine, proline, and cysteine/glutamate, etc.), respectively.¹¹ Thus, amino acid functionalisation of polymers might improve targeting and internalisation by cancer cells compared to non-cancerous cells.⁶ After membrane engulfment *via* endocytosis, a rapid release of the therapeutics (*i.e.*, endosomal escape) is desired to minimise degradation of the therapeutic compound.¹²



In literature, non-ionic polymers based on polyethylene glycol (PEG) are described as performant tools for aggregation between proteins and polymer nanocarriers.^{13,14} However, tumour cell specificity of PEG itself is comparatively low, thereby calling for decoration with additional targeting ligands (e.g., antibodies), which is both, costly and time-consuming. The targeting efficiency of non-ionic polymers without these ligands is mostly based on the enhanced permeability and retention (EPR) effect¹⁵ – a passive cell targeting method – which is less efficient in larger tumours due to their cellular heterogeneity and thus, intracellular carrier accumulation is limited.¹⁶ Hence, tumour targeting based on specific natural properties of tumour cells is of high interest. Contrarily to PEGylated carrier systems, polyelectrolytes may be designed to tackle the tumour targeting challenge¹⁷ and show superior suitability for the design of next-generation systems: polyelectrolytes combine synthetic precision with selective binding, controlled release properties, tuneable solubility and biocompatibility.^{17,18}

Although classical endocytic pathways such as clathrin-mediated uptake, caveolae-dependent endocytosis and macropinocytosis have been extensively characterised, their applicability to synthetic polyelectrolyte systems remains incompletely understood because polymer–cell interactions are strongly governed by surface charge and interfacial physicochemistry. In contrast to the widely studied cationic delivery vectors, anionic and zwitterionic polymers interact with biological membranes in more subtle ways, often involving weak electrostatic interactions, hydration-layer formation and specific binding to membrane components such as proteins or glycosaminoglycans.^{19–22} These interactions can influence adsorption, membrane wrapping and subsequent internalisation processes, yet the mechanistic details remain considerably less explored than for polycationic systems. Importantly, anionic and zwitterionic polymers are frequently employed to improve biocompatibility, reduce nonspecific protein adsorption and minimise cytotoxicity, owing to their strong hydration and charge-balanced character.^{23–26} Despite these advantages, their comparatively weak electrostatic interactions with cellular membranes often result in lower uptake efficiencies and less predictable intracellular trafficking, highlighting a key limitation of current materials. Moreover, the relationship between polymer architecture, charge distribution and biological response remains insufficiently resolved, particularly for zwitterionic or charge-regulated macromolecules. Consequently, while cationic polymers have historically dominated studies of polymer-mediated delivery,



the development of anionic and zwitterionic polyelectrolytes capable of precisely modulating polymer–membrane interactions represent an important emerging direction for improving cellular compatibility while maintaining effective intracellular transport.

For example, Fuji *et al.* showed that certain polyelectrolyte building blocks, namely zwitterionic polybetaines and amino-acid-derived polyzwitterions can actively accumulate in tumours.^{27,28} They pass the cell membrane by transporter-mediated mechanisms, which offer a new platform for targeted, biocompatible cancer nanomedicine. In addition, amino-acid-derived polyelectrolytes show beneficial features based on their biomimetic properties.^{17,18} Thus, these polymers have *e.g.*, antifouling properties due to their high charge density which strongly binds a hydration layer *via* electrostatic interactions.²⁹ This layer is much more stable than the one associated *via* hydrogen bonding with non-ionic polymers (*e.g.*, poly(*N*-acryloylmorpholine) (PNAM) or PEG)^{30,31} since it forms a significant barrier that biomolecules must overcome before attaching directly to the polymers.³² Moreover, the properties of polyelectrolytes can be tuned by their side chain hydrophobicity,^{33,34} counter ions³⁵ and backbone chemistry⁶ to govern stable polymer performance and transportation in the body at physiological pH values (7.4), while triggering intracellular release mechanisms such as endosomal escape at acidic pH levels (5.0-6.8).³⁶ Hence, the functionality of polyelectrolytes can be used for introduction of targetability and transmembrane transfer efficiency which are important tools to modulate a polymer (complex) for potential medical applications such as drug delivery, gene transfection, and tissue engineering.^{37,38} Synthetically, the design of polyelectrolytes from amino acids is a widely explored field, see, *e.g.*, the recent review on the synthesis and polymerisation of amino acid-derived monomers by our group.³⁹ Studies have furthermore shown that lysine- and ornithine-based brushed polyelectrolytes show low fouling behaviour as they did not show any aggregation towards undiluted serum for 24 h. Furthermore, pH- and temperature responsiveness has been observed for isoleucine- or leucine- and serine-based polymers which are interesting functionalities for various applications as stated above.^{40,41} We previously explored the introduction of permanent positive charges in the polymers to generate zwitterions with a fixed cationic centre. With that, we stabilised the zwitterionic character over a wider pH range.⁴²

Driven by our previous study, which examined impact of the backbone chemistry of amino-acid-derived polyzwitterions on their cellular specificity, we now investigated the effect of



the side chain chemistry as well as alterations to the ionic moiety itself. Similar attempts were previously pursued by Fujii *et al.* where the internalisation of various polymethacrylamides into cancer cells was studied. These authors showed that their cytocompatible polymers were actively taken up by cancer.³⁷ Inspired by these observations, our research aims to enhance the comparability between the developed polymer systems. We intended to achieve this by synthesising polyelectrolytes using activated esters as precursors, thereby mitigating the influence of the degree of polymerisation (X_n) and dispersity (\mathcal{D}) on the final product. We were interested in the influence of the positive charge (presence/absence) and hydrophobicity (aromatic/alkyl-side chain) on protein and cell interactions. Therefore, a small library was created where different amino acid or amino-acid-like structures are substituted in the side chain to obtain zwitterionic or anionic analogue amino acid-derived polyelectrolytes. To summarise, this study aims to elucidate how charge (zwitterionic/anionic), side chain hydrophobicity (aliphatic/aromatic groups) and persistent positive charge influences physicochemical parameters. To obtain this information, the cell association (flow cytometry) and cellular uptake (confocal laser-scanning microscopy) of polyelectrolytes to cancer and non-cancer cells was investigated in a comparative manner. Competitive inhibition assays were applied to study the uptake mechanism further. The systematic variation of side chain building blocks and their influence on cellular interactions provide insights for the development of novel polyelectrolytes for biological applications.

2. Results and Discussion

2.1. Synthesis and characterisation of amino-acid-derived polyelectrolytes

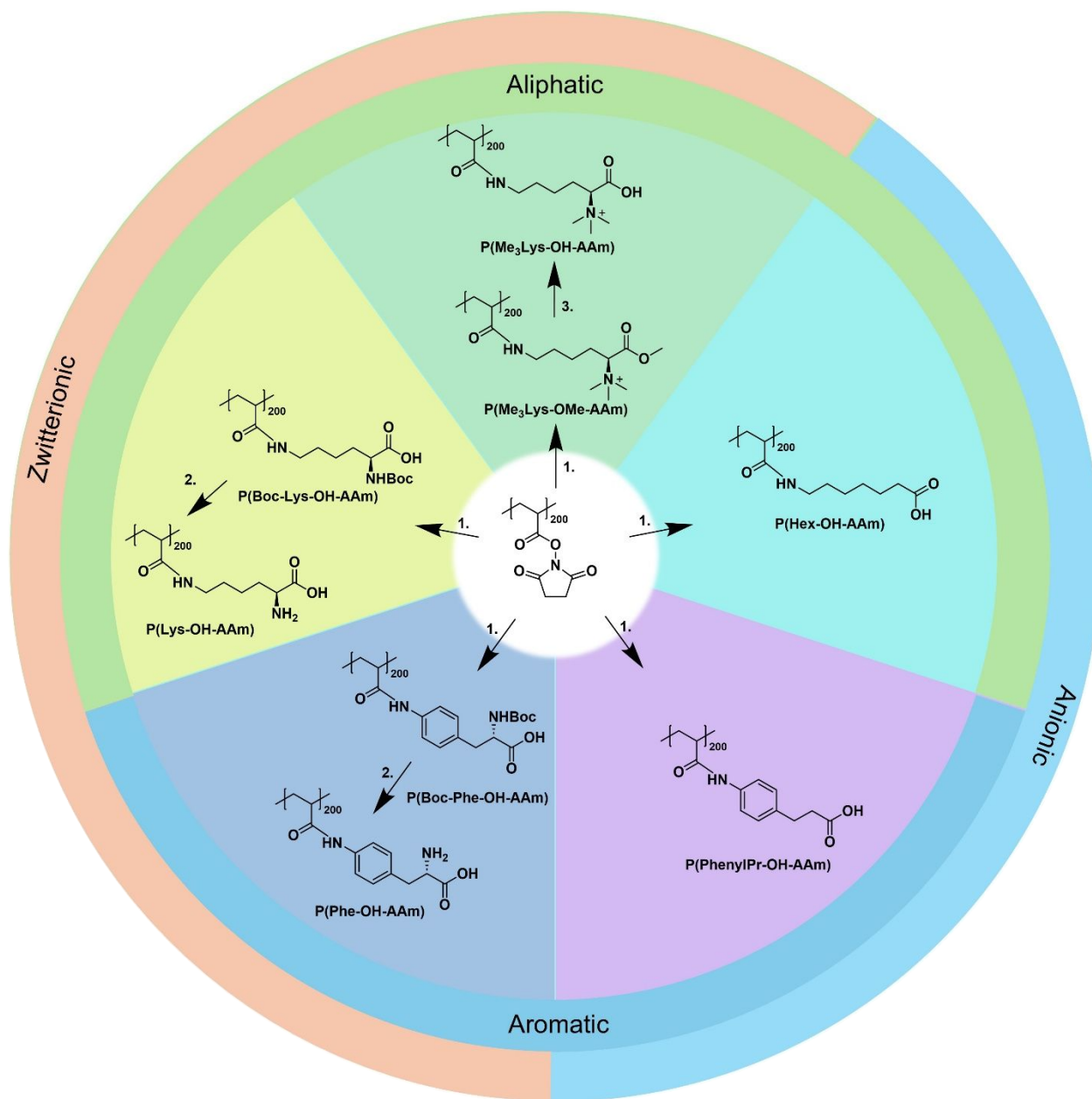
Aiming to investigate the side chain chemistry of amino-acid-derived polyelectrolytes and their interaction with biological matter (*i.e.*, the nature of the zwitterion as well as the spacer between the ionic group and the polymer backbone), we synthesised polyelectrolytes *via* post-polymerisation modification (PPM) of poly(*N*-acryloyl succinimide) (PNAS). Here, PNAS was obtained from reversible addition-fragmentation chain transfer (RAFT) polymerisation and subsequently reacted with different amino acids to design three different polyzwitterions: (i) aliphatic spacer and permanent positive charge, (ii) aliphatic spacer and non-permanent positive charge, and (iii) aromatic spacer



and non-permanent positive charge as well as two corresponding polyanions with (iv) aliphatic and (v) aromatic spacer (**Scheme 1**).

Consequently, the PPM of PNAS with five different reagents was conducted: (i) Me₃Lys-OMe-NH₂,⁴² (ii) *N*-Boc-lysine-OH (Boc-Lys-OH),⁴² (iii) Boc-4-aminophenylalanine (Boc-4-AmPhe-OH), (iv) 6-aminohexanoic acid (6-AmHex-OH), and (v) 3-(4-aminophenyl)propanoic acid (4-AmPhenylPr-OH). The successful PPM was verified via ¹H NMR measurements, showing the disappearance of the characteristic peak of NAS at $\delta = 2.8$ ppm after the reaction, indicating full conversion (**Fig 1A, B and S1**). In case of the aliphatic polymers P(Me₃Lys-OMe-AAm), P(Boc-Lys-OH-AAm), and P(Hex-OH-AAm) a peak shift from $\delta = 2.8$ ppm in the ¹H NMR spectra of the starting compounds (**Fig S2**) to $\delta = 3.0$ to 3.1 ppm in the polymer ¹H NMR spectra (**Fig 1A and S1**) is observed. This indicates a change in environment for the methylene group adjacent to the amine (H3), now positioned adjacent to the amide (**Fig S1 and S2**). The aromatic polymers P(Boc-Phe-OH-AAm) and P(PhenylPr-OH-AAm) are characterised by the broadened aromatic peaks compared to the ¹H NMR spectra (**Fig 1C and S1**). This shows the different chemical environment each aromatic peak has, something typically observed in ¹H NMR spectra of polymers and therefore complementary, to disappearance of the characteristic NAS peak (**Fig S3**), confirming successful PPM.





Scheme 1: Synthesis of P(Boc-Lys-OH-AAm), P(Me₃Lys-OMe-AAm), P(Hex-OH-AAm), P(Boc-Phe-OH-AAm) and P(PhenylPr-OH-AAm) *via* PPM. (1) Et₃N, DMF and Boc-Lys-OH, Me₃Lys-Ome-NH₂, 6-AmHex-OH, Boc-4-AmPhe-OH and 4-AmPhenylPr-OH, respectively. 24 h at room temperature. (2) 50:50 v/v% (TFA/H₂O), overnight at room temperature (3) 0.1 M NaOH, 24 h at room temperature.



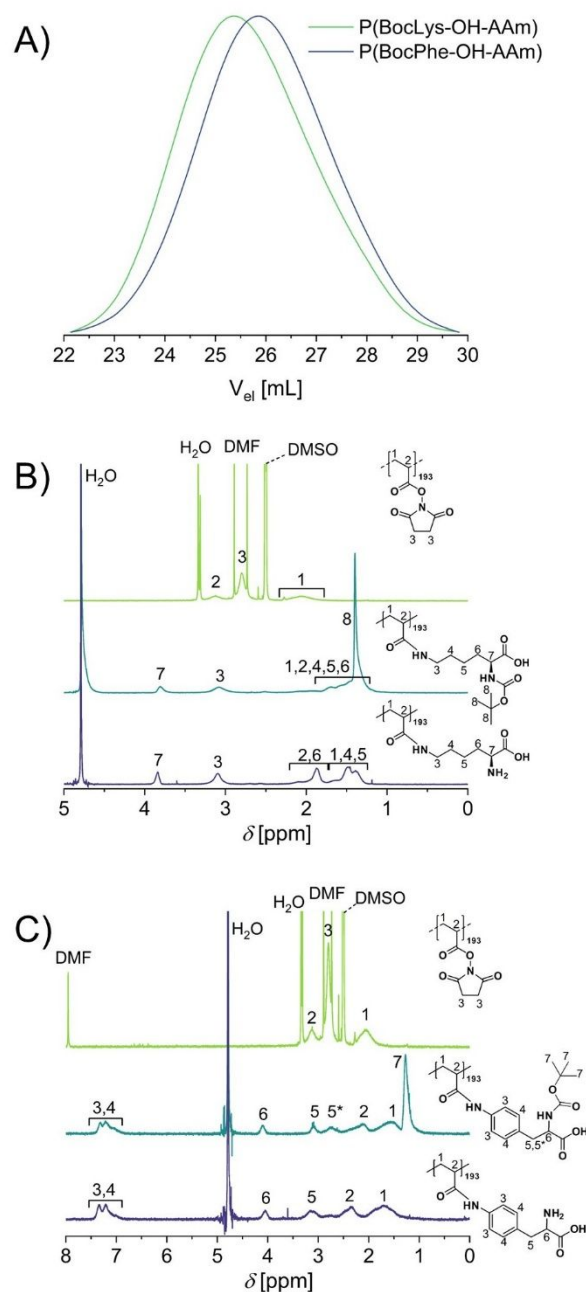


Figure 1: (A) Size-exclusion chromatograms (0.07 M aq. Na_2HPO_4) of P(BocLys-OH-AAm) (green) and P(BocPhe-OH-AAm) (blue). (B) ^1H NMR spectra (300 MHz) of poly(N-acryloyl succinimide) (green, DMSO), P(BocLys-OH-AAm) (cyan, D₂O) and P(Lys-OH-AAm) (blue, D₂O). (C) ^1H NMR spectra (300 MHz) of poly(N-acryloyl succinimide) (green, DMSO), P(BocPhe-OH-AAm) (cyan, D₂O) and P(Phe-OH-AAm) (blue, D₂O).

The zwitterionic polymers P(Lys-OH-AAm) and P(Phe-OH-AAm) were obtained by acidic deprotection of P(Boc-Lys-AAm) and P(Boc-Phe-OH-AAm). Successful deprotection was verified *via* ^1H -NMR as the Boc group signal at $\delta = 1.4$ ppm disappeared (H8 and H7,



respectively, **Fig S4 and S5**). P(Me₃Lys-OH-AAm) was obtained from P(Me₃Lys-OMe-AAm) after basic hydrolysis as previously shown.⁴²

Aqueous size exclusion chromatography (SEC) measurements were conducted to obtain information about the integrity of the polymer chain during the PPM, deprotection and to verify the absence of unwanted chain coupling (*e.g.*, caused by aminolysis of the chain-transfer agent (CTA)). All polymers showed monomodal molar mass distributions (**Fig S6 and S7**). Deprotection changed the dispersity (\bar{D}) of P(Lys-OH-AAm) significantly from 2.25 to 3.27 which was probably caused by the increased charge density and thus, the enhanced interaction with the stationary phase of the column. This also resulted in a significantly later elution time and therefore high decrease in apparent molar mass ($M_{n,app}$) from 17.2 kDa to 2.0 kDa. This effect was not observed for P(Phe-OH-AAm) where \bar{D} was constant (2.47 to 2.14) which can indicate that the aromatic groups prevent interactions with the stationary phase. Both polymers, however, exhibited monomodal molar distributions (**Fig S8**). Generally, all polymers showed increased \bar{D} , with $2.0 < \bar{D} < 3.3$, compared to PNAS ($\bar{D} = 1.9$, **Tab 1**). This broadening may be attributed to the use of a more polar eluent and high density of charged groups in the polymers, which promote hydrogen bonding and ionic interactions with the stationary phase, leading to broadening. Generally, the discrepancies between $M_{n,app}$ and the theoretical molar mass ($M_{n,theo}$) derived from NMR and SEC (**Tab 1**) resulted from the calibration. While $M_{n,theo}$ refers to the mean value derived from the $X_{n,theo}$ which was obtained from conversion of the PNAS precursor, $M_{n,app}$ refers to the calibration standard of the aqueous SEC (*i.e.*, P2VP or PMA).

In addition, the cyanine-5 (Cy5)-labelled polymers (*i.e.*, P(Lys-OH-AAm)-Cy5, P(Me₃Lys-OH-AAm)-Cy5, P(Hex-OH-AAm)-Cy5, P(Phe-OH-AAm)-Cy5 and P(PhenylPr-OH-AAm)-Cy5), which were required for cell association and internalisation experiments, were synthesised *via* sequential PPM with Cy5-amine and the respective amino acids. Those polymers were analysed as described above and the absence of unbound dye was verified by SEC measurements (**Fig S9 and S10**).⁶

Table 1: Polymer characteristics.

Polymer	M^a [Da]	$X_{n,theo}^b$	$M_{n,theo}^b$ [kDa]	$M_{n,app}^c$ [kDa]	\bar{D}^c	t_{ret}^d [min.]



P(Lys-OH-AAm)	200.24	193	38.5	1.9	3.27	11.80
P(Me ₃ Lys-OH-AAm)	243.33	188	46.5	10.7 ^e	1.96 ^e	11.75
P(Hex-OH-AAm)	185.22	193	35.6	16.5	2.30	14.49
P(Phe-OH-AAm)	234.25	193	45.0	13.0	2.14	11.97
P(PhenylPr-OH-AAm)	219.24	193	42.1	11.0	2.71	15.94

^aMolar mass (M) of repeating unit RU after deprotection. ^b $X_{n,theo}$ and $M_{n,theo}$ was calculated from ¹H-NMR. ^c $M_{n,app}$ and \bar{D} was determined by aqueous SEC measurements (0.07M Na₂HPO₄) on a Suprema column set (PMA standard). ^d t_{ret} obtained from HPLC using a reversed phase column with a water/acetonitrile mixture + 0.1 vol% trifluoroacetic acid as eluent. ^edetermined by aqueous SEC measurements (80/20 V% [H₂O/ACN] + 0.1M NaCl + 0.3% TFA) on a Novema column set (P2VP standard).).

2.2. pH-responsiveness and hydrophobicity

The application of polyelectrolytes in biological environments necessitates knowledge about their physicochemical properties, i.e., their hydrophobicity and responsiveness to changes in the pH value, which are important driving forces for interactions with cells and proteins. The Cy5-labelled polymers were subjected to high-performance liquid chromatography (HPLC) measurements to gain information about their relative hydrophobicity (**Fig S11**). At eluent conditions (pH 2)- chosen so the ionic state around the pK_a of the polymers cannot influence the elution time- the anionic polymers P(PhenylPr-OH-AAm)-Cy5 (polymeric retention time (t_{ret}) ~16 min) and P(Hex-OH-AAm)-Cy5 (t_{ret} ~15 min) were the most hydrophobic (**Tab 1, Fig. 2A and S11**). This increased hydrophobicity is explainable by the protonation of the carboxylic acids³⁶ as the pH is below the reported pK_a (pH 4.88 for hexanoic acid and 4.37 for 3-phenylpropanoic acid).⁴³ Furthermore, it was shown that the aromatic side chain influences the hydrophobicity of the polyanions. It is assumed that aromatic spacers feature an increased hydrophobicity due to the higher polarisability of the aromatic rings inducing stronger London dispersion forces with the C18 chains than a flexible alkyl group of similar size on the reversed phase column. In contrast, the zwitterionic polymers, P(Lys-OH-AAm)-Cy5, P(Me₃Lys-OH-AAm)-Cy5 and P(Phe-OH-AAm)-Cy5 showed a similar t_{ret} of ~12 min. Hence, side chain aromaticity or permanent positive charge has no influence on the hydrophobicity of zwitterionic polymers at pH 2.0. Moreover, we calculated the theoretical partition coefficient (LogP) to obtain numerical values for comparison with the experimental data (**Fig S12**). All polymers except P(Lys-OH-AAm) exhibit a positive theoretical logP value



which emphasizes the hydrophobic character of the polymers corroborating the HPLC data. Only P(Lys-OH-AAm) showed a negative theoretical logP value of -0.2319 indicating a slightly hydrophilic character. This could be attributed to the fact that the computational data is based on monomer calculations (one repeating unit per polymer). The algorithm of the trained machine learning model is designed for small molecules;⁴⁴ hence complete polymers were excluded from the calculation of theoretical logP values and future application of algorithms for polymer-based systems could improve accuracy.



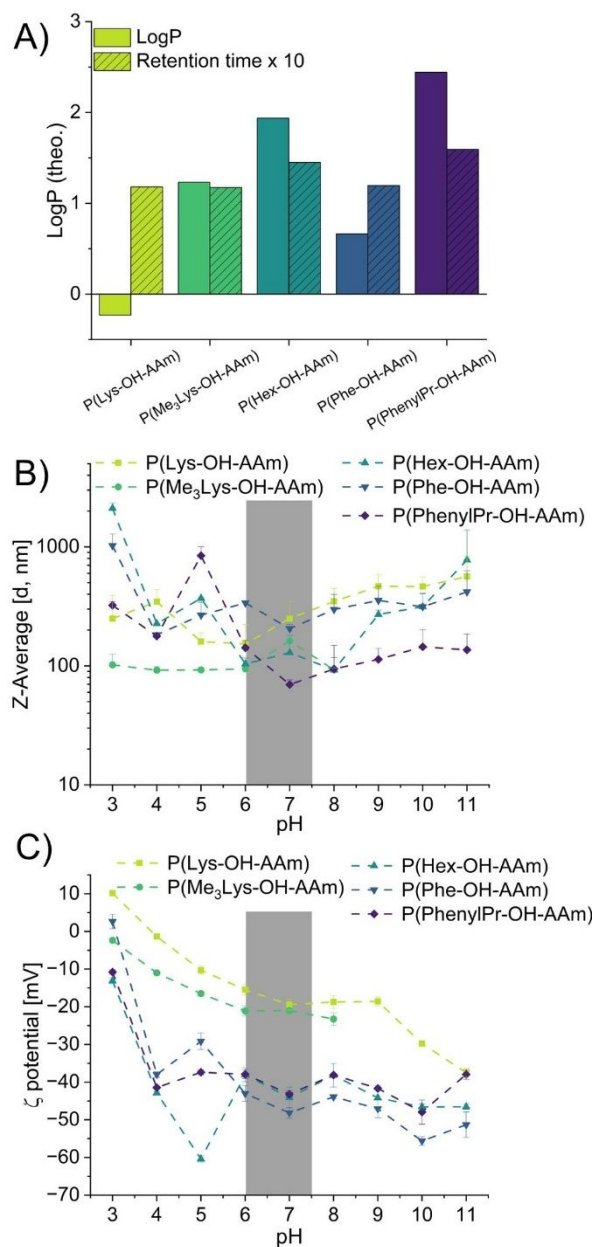


Figure 2: (A) Partition coefficients and retention time (x 10) of the polyelectrolytes. (B) Intensity weighted sizes and (C) ζ -potential of 2 mg mL⁻¹ aqueous solutions at indicated pH values. Grey boxes in (B) and (C) indicate the biologically relevant pH range.

To support these findings, the effect of the pH on self-assembly was studied using dynamic light scattering (DLS) measurements performed at different pH values to get a general overview of its solubility behaviour (**Fig 2B, S13 and S14**). Additionally, changes in particle size serve as an indirect indicator of increased hydrophobicity, as hydrophobic interactions are expected to dominate upon protonation. This behaviour may be particularly important under biologically relevant pH conditions (pH 6.0 – 7.5), even though



the tested pH values exceed this range to give a general overview of physicochemical properties. With DLS measurements, we confirmed the decreased solubility of the polyanions by number size distributions (**Fig S13**). For example, P(Hex-OH-AAm) showed hydrodynamic diameters (D_h) of $> 1\ 000$ nm at pH 3.0 and $D_h \approx 100$ nm at pH 4.0 and 5.0. Moreover, number size distributions of P(PhenylPr-OH-AAm) showed a D_h between 1 and 10 nm at pH values between 6 and 11, as well as $D_h \approx 100$ nm at pH 3.0 and 4.0. At pH 5.0, in comparison, the largest particles were present with diameters larger than 1000 nm. Visually, we could also see aggregation in the polymer solutions at pH values between 3 and 5 for P(Hex-OH-AAm) and P(PhenylPr-OH-AAm) (**Fig S13**). Visual observation of the polymer solutions showed increased turbidity (**Fig S15**) for those polymers at low pH values. Those scenarios could be explained by the pK_a values of hexanoic acid, which is 4.88, and 3-phenylpropanoic acid, namely, 4.37.⁴³ Both values are close to the pK_a values of P(Hex-OH-AAm) and P(PhenylPr-OH-AAm), respectively, since the amide functional groups do not significantly influence the lower pK_a . As the polyanions are partially and fully protonated, around and below their pK_a it is also in agreement with our visual observation of aggregates and particle formation in light scattering measurements. The zwitterionic P(Lys-OH-AAm) showed an average particle diameter of around 10 nm across the whole measured pH range from pH 3.0 to pH 11.0 which was visually observed as a transparent solution. However, for the zwitterionic polymers with an aromatic motif in the side chain and permanent positive charge, aggregation was observed at pH 6.0 to 7.0 (P(Phe-OH-AAm)) and pH 7.0 (P(Me3Lys-OH-AAm)) with a $D_h = 20$ to 100 nm and $D_h \approx 160$ nm, respectively.⁴⁵

Additionally, ζ -potential measurements using electrophoretic light scattering (ELS) provided an insight into the surface charge of the polymers. ELS measurements (**Fig 2C**) showed that the zwitterionic amino acid side chains possessed a positive charge at pH 3.0 due to both, the protonated carboxy group and the amine protonation at low pH values. This character changes to an overall negative net charge when the pH is raised to 4.0 or higher as both functional groups are deprotonated, and thus, the carboxyl group is present as an anion. The measurements for P(Lys-OH-AAm) are also in agreement with previous data of our group. We found that the isoelectric point (pI) is around pH = 4.3 which could be estimated by the expected pK_a . Previously derived amino-acid-derived polyzwitterions from the literature show similar values.^{6,40,42} For P(Phe-OH-AAm), however, the pI



observed by ELS was between pH 3.0 and 4.0 which is considerably lower than the amino acid phenylalanine which is given to be approximately at pH = 5.5.⁴⁶ The lower apparent pI of P(Phe-OH-AAm) observed by ELS compared to free phenylalanine may result from preferential exposure of carboxylate groups at the particle surface, yielding a negative zeta potential at lower pH values. The aggregation detected by DLS near pH 6.0–7.0 further indicates that the polymer attains maximal hydrophobicity around the expected pI. The side chains in both, P(Hex-OH-AAm) and P(PhenylPr-OH-AAm) only possess carboxyl groups that are mostly deprotonated. Even at pH 3.0, the particles were still negatively charged. But the moderate negative ζ -potentials at pH 3.0 (~ -10 mV) for both acid side chains indicated that most of the carboxyl acid groups were protonated and thus neutral. This results in a less negatively charged dispersion with reduced hydrophilic behaviour which can also be observed by the increased particle size of these polymers (from D_h 1 - 10 nm to ≈ 100 nm) during DLS measurement at the respective pH values (pH 3.0-5.0) (**Fig S13C,E**). It has already been shown that aggregation into larger structures under mildly acidic conditions, similar to extracellular pH values at tumour sites (pH ≈ 6.6),⁴⁷ can enhance retention within the tumour tissues as long as they exhibit a small hydrodynamic diameter under physiochemical conditions.

2.3. Interaction of amino-acid-derived polyelectrolytes with proteins

Our previous work demonstrated that both P(Lys-OH-AAm) and P(Me₃Lys-OH-AAm) exhibited minimal tendency for protein fouling when tested with model proteins such as lysozyme and bovine serum albumin (BSA) in Dulbecco's phosphate-buffered saline (DPBS) solution (pH 7.4).⁴² Here, similarly, BSA was chosen as a negatively charged model protein which is mainly present in serum, while lysozyme represented a positively charged model protein and tested in DPBS solution. The effect of foetal bovine serum (FBS) was briefly tested to exclude the formation of sediments.

DLS measurements were performed with the chosen model proteins in DPBS at 37°C for 24 hours (**Fig 3, S16, S17**). Before starting the measurement, the protein solution was mixed with the respective polymers P(Lys-OH-AAm), P(Me₃Lys-OH-AAm), P(Hex-OH-AAm), P(Phe-OH-AAm), and P(PhenylPr-OH-AAm). Based on our previous results, it was expected that there would be strong interaction of the polyanions, P(Hex-OH-AAm) and P(PhenylPr-OH-AAm), with the positively charged lysozyme.⁵ A sudden increase in size



for most of the polymers upon mixing with Lysozyme was observed (**Fig 3 and S16**). P(Hex-OH-AAm), however, remained stable (e.g. no aggregation observed) over the measured period of 24 h while P(PhenylPr-OH-AAm) immediately formed large aggregates resulting in visible sedimentation at the bottom of the cuvette. Consequently, (almost) no particles were measured at the remaining time points as the sediment did not diffuse through the incident laser beam. For the other polymers, a decrease in particle size over time was further observed, indicating the formation of stable complexes, potentially mediated by the ionic charge of the carboxylate group. When looking at BSA, a negatively charged protein with hydrophobic binding pockets (i.e., comprising aromatic amino acid residues), a different image showed. Here, solely the aromatic polymers P(Phe-OH-AAm) and P(PhenylPr-OH-AAm) aggregated with the protein into stable particles with a mean diameter above 100 nm, while none of the aliphatic polymers showed such behaviour (**Fig 3 and S17**). This is in line with previous findings from our group and other studies investigating zwitterionic polymers.^{42,48} However, it is noteworthy that aromatic pi-pi interactions seem to surpass electrostatic interactions. This trend will be investigated in greater detail in the future. The results related to the aliphatic polyelectrolytes polymers confirm the general trend that zwitterionic polymers or surfaces exhibit excellent resistance to biofouling, thereby being able to improve biocompatibility of coupled materials or cargo (i.e. drugs).^{29,49–51}

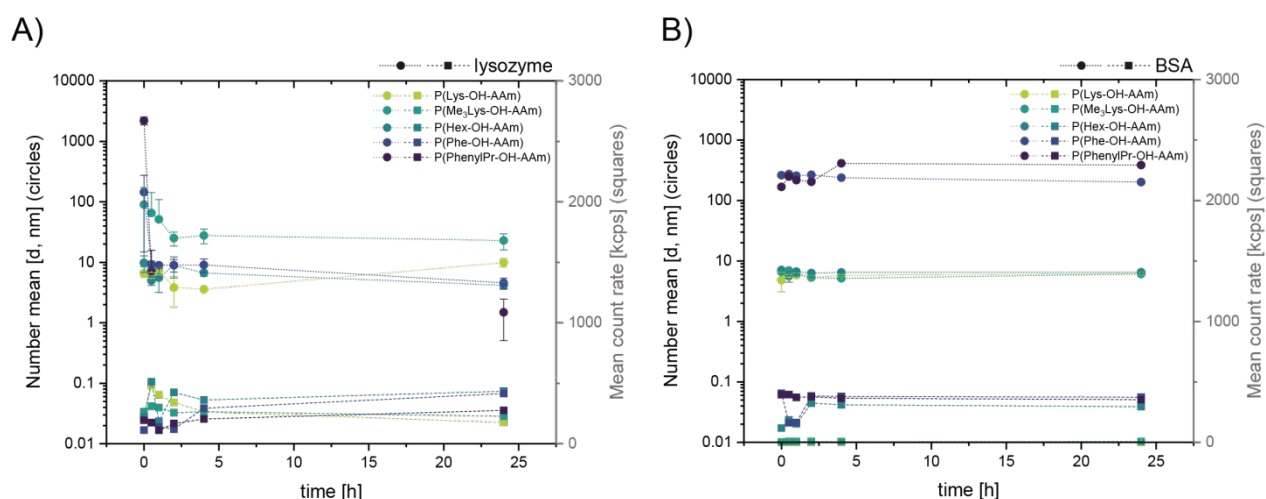


Figure 3: Time-dependent number size distribution of mixtures of (A) lysozyme and (B) BSA with indicated polymers. The concentration of polymers and proteins was 0.5 mg mL^{-1} each in DPBS. Sizes were analysed by DLS measurements at 37°C . Values represent the mean and SD of five measurements with three runs each. Lines are to guide the eye



and do not represent measured values. All measurements of the polymers with and without proteins can be found in the Supporting Information.

The interesting observation of non-aggregating aliphatic polyanions and instantly aggregating aromatic polyanions in DPBS at 37 °C could possibly be explained by their hydrophobicity which is related to the HPLC and pH dependent DLS measurements for these polymers (**Fig S11, S13 and S14**) and compared to the ones shown in our previous work.⁵ There, the more hydrophilic P(Gly-OH-AAm) showed smaller aggregates with lysozyme ($D_h \approx 150$ nm), whereas the more hydrophobic, P(Nva-OH-AAm), showed the formation of larger (although stable) aggregates in combination with lysozyme ($D_h \approx 700$ nm). Comparing t_{ret} values obtained by HPLC, e.g., t_{ret} of P(Hex-OH-AAm) ($t_{ret} = 14.5$ min) and P(PhenylPr-OH-AAm) ($t_{ret} = 15.9$ min) with P(Gly-OH-AAm) ($t_{ret} = 13.2$ min) and P(Nva-OH-AAm) ($t_{ret} = 16.3$ min), we observed a similar retention time for P(PhenylPr-OH-AAm) and P(Nva-OH-AAm) while P(Hex-OH-AAm) has a retention time between P(Nva-OH-AAm) and P(Gly-OH-AAm). Relating the fouling behaviour of the polyanions to their relative hydrophobicity is the most straightforward explanation for the immediate aggregation of the aromatic, more hydrophobic P(PhenylPr-OH-AAm). In comparison, the hydrophobicity of P(Hex-OH-AAm) is insufficient to induce aggregation with lysozyme. Building on these findings, further fouling experiments were conducted in DPBS containing 10 % FBS to evaluate potential polymer aggregation under biologically relevant conditions that are later used during *in vitro* experiments. Here, the absence of an increase in particle size, over a measurement time of 5 h, suggests that no polymer-protein aggregation occurs over the period in which cell association experiments will be performed (data not shown).

2.4. Specificity of amino-acid-derived polyelectrolytes to cancer cells

As the main aim of this study was to evaluate the cellular interactions of amino-acid-derived polyelectrolytes based on their side chain chemistry (i.e., the nature of the side chain spacer and the ionic moiety), comparative cell association studies were conducted. Here, three exemplary cell lines were chosen: (i) MDA-MB-231 and (ii) HCC-78, two cancer cell lines of different origin, as well as (iii) MCF10A and as tissue-matched control cell line for MDA-MB-231. MDA-MB-231, a breast cancer cell line, was studied previously in our group^{6,52,53}, while HCC-78, a lung cancer cell line, served the purpose of expanding



the cancer cell toolbox to exclude that the observed effects were attributed to MDA-MB-231 only.

All polymers were tested towards their cytocompatibility using MDA-MB-231 breast cancer cells by performing a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (**Fig S18**). All synthesised polymers were well tolerated by MDA-MB-231 cells at concentrations up to 1 mg mL^{-1} ($\cong 25 \text{ }\mu\text{M}$) for 24 h. Moreover, these results were in line with our former study on amino-acid-derived polyanions where the cell viability was consistently $\geq 70\%$ for concentrations up to 0.1 mg mL^{-1} ⁵ and demonstrate that cellular tolerance is unaffected by the side chain spacer (aliphatic or aromatic), or the ionic character (permanent) zwitterion or anion) of the polymer.

To analyse the cell association of the polymers, the different cell lines were incubated with the Cy5-labelled polymers. To ensure the absence of cytotoxic effect, a concentration 10-fold lower than the maximum tested concentration in the MTT assay was used (*i.e.*, $0.1 \text{ mg mL}^{-1} \cong 2.5 \text{ }\mu\text{M}$). Based on the concentration-dependent association experiments, ranging from 0.04 to $2.5 \text{ }\mu\text{M}$, with P(Lys-OH-AAm)-Cy5 and MDA-MB-231 (**Fig S19**), a concentration of $2.5 \text{ }\mu\text{M}$ was determined to be suitable for further comparative studies as it resulted in a sufficient fluorescent signal in MDA-MB-231 breast cancer cells.

The cellular association experiments of all polymers were performed on HCC-78 and MDA-MB-231 and MCF10A.⁵⁴ Quantitative evaluation was performed using flow cytometry. PNAM-Cy5 served as internal standard to determine the relative association to different cell types (**Fig S20A**), as it has been reported that there is only unspecific association between cancer cells and non-ionic polymers.^{6,42,53} As we were mainly interested in the targetability of cancer cell lines by these amino-acid-derived polyelectrolytes we further compared the association amongst the different cell lines, *i.e.*, in relation to HCC-78 (**Fig 4**), which will be discussed in the following.



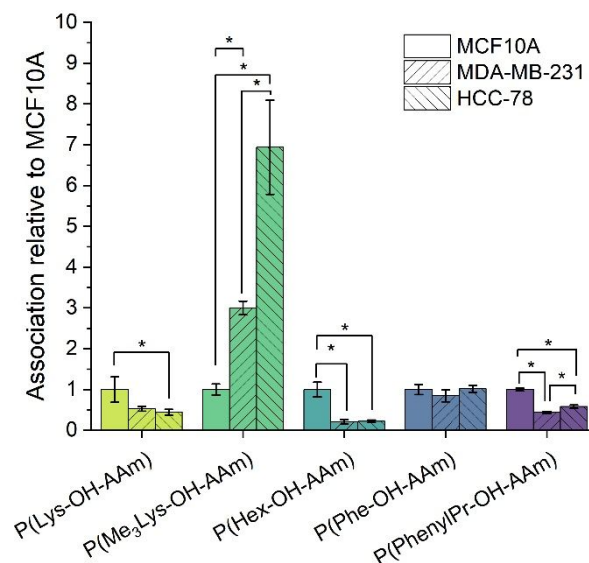


Figure 4: Cellular association of polymers relative to MCF10A determined by flow cytometry. The cells were seeded in 24-well plates (seeding density for each cell line in experimental section) and incubated with 2.5 μ M polymer solutions (500 μ L of FBS-containing cell culture media) at indicated temperatures for 4 h. Values represent mean and SD ($n = 4$). Statistical analysis (unpaired Welch's t-test) * Indicates a significance of $p < 0.05$.

Generally, comparison with HCC-78 and MDA-MB-231 cells showed a statistically significant difference ($p < 0.05$) between association for P(Me₃Lys-OH-AAm)-Cy5 and P(PhenylPr-OH-AAm)-Cy5 while all other polymers showed similar association behaviour to lung and breast cancer cells. Here, the association of P(Me₃Lys-OH-AAm)-Cy5 with MDA-MB-231 was approximately twice as high as with HCC-78. The cell association of P(Lys-OH-AAm)-Cy5, P(Hex-OH-AAm)-Cy5 and P(Phe-OH-AAm)-Cy5 between those two cancer cell lines did not differ significantly. Differences in the cellular association of polyelectrolytes with different cancer cell lines may be attributed to their surface charge^{55,56} or receptor density⁵⁷ and provide valuable information about the importance to investigate various cell types to obtain a broader understanding of the driving forces of cell specificity. Future studies shall investigate these differences in greater detail.

Here, we next compared the interaction of the polyelectrolytes with non-tumorigenic human breast cells (MCF10A). The interaction between the permanently, positively charged P(Me₃Lys-OH-AAm)-Cy5 and MCF10A was determined to be statistically significantly lower compared to the cancer cells ($p < 0.05$) (**Fig 4**). In contrast, the zwitterionic polymer variant P(Lys-OH-AAm)-Cy5 showed a ~2-fold higher association



with MCF10A cells. However, this was not statistically significant compared to cancer cells of the same tissue type (*i.e.*, MDA-MB-231). More interestingly, we observed a ~5-fold stronger association between P(Hex-OH-AAm)-Cy5 and MCF10A. These results strongly indicate that specifically the polymeric anionic charge influences the interaction between human non-cancer cells and polyanions more drastically than to cancer cells. The same trend – however less pronounced – was observed for P(PhenylPr-OH-AAm)-Cy5 and P(Phe-OH-AAm)-Cy5. While zeta-potential measurements showed a comparable charge profile of these three polymers, the cell association and specificity differed. We therefore assume that in addition to the ionic charge, the aromatic side chain hinders the association with non-tumorigenic cell membranes.

2.5. Interaction of amino-acid-derived polyelectrolytes with fibroblasts

Lastly, we compared the cell association of polyelectrolytes with cancer cells to the results with fibroblasts (NIH 3T3, **Fig S20B**), our previous control cell line,⁵³ which provides information about tissue specificity. MCF10A represented a control cell line of non-cancer breast tissue^{54,58}, while NIH 3T3 and L929, both fibroblast cell lines, were chosen to represent other non-cancer cell lines which are regularly investigated in literature and possess a cellularly excreted anionic extracellular matrix composed of hyaluronic acid which is associated to the cells.⁵⁹

It was observed that the overall polymer association to NIH 3T3 fibroblasts was significantly lower - except for P(Me₃Lys-OH-AAm)-Cy5, which showed higher association to the fibroblasts. An explanation for these results may be found in the extracellular matrix of fibroblasts, which is composed of a higher quantity of hyaluronic acid.⁶⁰ This polysaccharide will, as indicated by the results, repel all negatively charged polymers while P(Me₃Lys-OH-AAm)-Cy5, which contains a permanent positive charge, and, thus, an all-over zwitterionic charge at any physiological pH value, is able to associate due to attractive electrostatic interactions. These results highlight that polymeric interactions are strongly dependent on the cell-associated extracellular matrix. In summary, the permanently charged P(Me₃Lys-OH-AAm) exhibited the highest potential for further investigations of specific interaction with cancer cells without affecting healthy human cells of the same origin (*i.e.*, breast tissue) and will be further investigated in the future.



The final part of this study focussed on the clarification about the polymer uptake into cancer cells – represented by MDA-MB-231 breast cancer cells. Clarification was sought by imaging *via* confocal laser scanning microscopy (CLSM) measurements of MDA-MB-231 and L929 fibroblasts after incubation with Cy5-labelled polymers, providing information about the internalisation of the studied polyelectrolytes.

After 1 h of incubation with MDA-MB 231, a strong intracellular fluorescence signal of the polyanions, P(Hex-OH-AAm)-Cy5 and P(PhenylPr-OH-AAm)-Cy5, was observed (**Fig 5**) compared to the untreated cells (**Fig S21**). It should further be noted that some clustering of fluorescence was observed, which may be derived from accumulation within the cellular ultrastructures. The results overall indicated fast and efficient polymer uptake as already suggested by flow cytometry measurements. Previously, a slow but significant accumulation of (chiral) anionic polymers in the cell membrane has been reported.^{5,61,62} This is thought to be due to the combined effects of their anionic charge and hydrophobic properties, which promote membrane association and subsequent internalisation with polyanions.⁵ Here, membrane accumulation appeared to be absent, suggesting that additional motifs (*e.g.*, chirality) contribute to this phenomenon, which should be investigated more deeply in the future. Anionic polymers generally show limited cellular association and internalisation due to the negatively charged nature of the cell membrane surface.³⁶ Moreover, the rapid uptake after 1 h and the intracellular fluorescence signal even after 4 h incubation time (**Fig S21**) observed for both cell lines (MDA-MB 231 and L929) and for all polyanions examined in this study, (P(Hex-OH-AAm)-Cy5 and P(PhenylPr-OH-AAm)-Cy5), highlights the significant role of hydrophobicity in promoting cell interaction and accelerating internalisation.

After 1 h incubation time, the intracellular signal of the zwitterionic polymers P(Me₃Lys-OH-AAm)-Cy5, P(Lys-OH-AAm)-Cy5 and P(Phe-OH-AAm)-Cy5 was low compared to the polyanions, revealing the lowest uptake for P(Me₃Lys-OH-AAm)-Cy5. While this was in line with the flow cytometry results of the two Lys-derived polymers, a deviation for the Phe-derived polyzwitterion was observed. Live cell imaging after 4 h incubation (**Fig S22**), in turn, revealed an intracellular fluorescence signal for this P(Phe-OH-AAm)-Cy5, suggesting a slower internalisation of the aromatic polyzwitterion than for the aliphatic polymers in MDA-MB 231 cells. Thus, it was demonstrated that the polymers were internalised by the cells depending on their characteristics of their side chain.



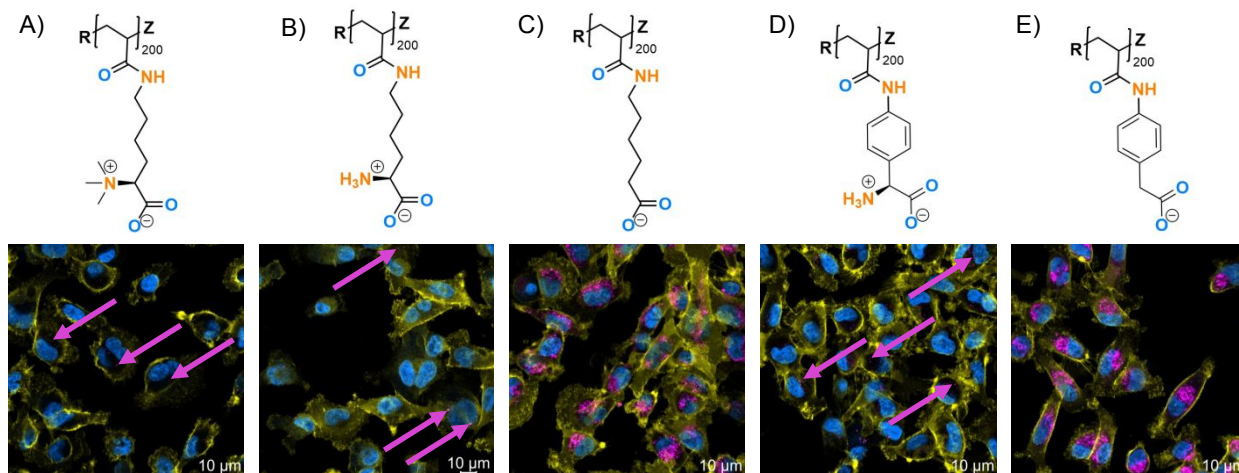


Figure 5. MDA-MB-231 cancer cells visualised by CLSM 1 h after treatment with polymers. Blue: Hoechst 33342/nucleus. Yellow: Cell membrane (CellMask™ Green Plasma Membrane stain). Magenta: Cy5/polymer channel. Cells were treated with Cy5 modified polymers A: P(Lys-OH-AAm)-Cy5. B: P(Me₃Lys-OH-AAm)-Cy5. C: P(Hex-OH-AAm)-Cy5. D: P(Phe-OH-AAm)-Cy5 E: P(PhenylPr-OH-AAm)-Cy5.

2.6. Internalisation pathways of amino-acid-derived polyelectrolytes

Several studies have reported that internalisation of macromolecules mainly follows active pathways.^{63–65} Thus, we quantified the cellular internalisation of the polymers by cell incubation studies at 37 °C and 4 °C for 4 h using MDA-MB-231 breast cancer cells. A reduction in MFI measured by flow cytometry showed signals approximately 60 to 80% lower at 4 °C compared to the respective control group incubated at 37°C (**Fig S23**) indicating reduced polymer-cell interactions at decreased temperatures. At the first glance, the weaker interactions at 4°C combined with the uptake shown in **Fig 5** suggested that uptake mainly takes place *via* active transport pathways, which are often upregulated in cancer cell lines and facilitate enhanced cell growth.^{66–68} However, a more detailed investigation of the complex uptake mechanism of the polymers into cells is needed since the driving force for active transport (e.g., ATP) requires specific inhibition to support the data. In addition, previous research by different groups has further suggested that the cell association of (amino-acid-derived) polyelectrolytes is mainly driven by targeted (active) interactions with components on the cell surface of cancer cells (*i.e.*, receptors or transporters) leading to an enhanced surface interaction and subsequent internalisation *via* endocytic pathways,³⁷ however, previous results were partly controversial, indicating an increased complexity of interactions between polyelectrolytes



and cell surfaces. Based on decreased polymer-cell interaction at lower temperature⁶⁹, the driving forces behind the cellular association of the tested amino-acid-derived polymers were investigated with respect to potential selective interactions with cell-surface transporters. Therefore, competitive inhibitors for different amino acid transporters (AATs), which are overexpressed in MDA-MB-231 (*i.e.*, SNAT1/2, ASCT1/2, LAT1/2, xCT and MCT4)⁵⁷ were applied for a short incubation time (15min), in line with our previous study. Surprisingly, neither of the polymers showed an inhibitor-dependent decrease of uptake, as observed by constantly high MFI values (**Fig S24**). These results suggested an AAT interaction-independent cell association. In general, inhibition of certain transporters can lead to overcompensation by other transporters which could interfere the tests.⁷⁰ However, additional experiments with competitive amino acids (*i.e.*, leucine (Leu, main substrate of LAT transporters)⁷¹, phenylalanine (Phe, main substrate of LAT1)⁷² and glutamic acid (Glu, main substrate of xCT)⁶⁸) for transporter interactions supported these findings (**Fig S25**). Here, only leucine showed a slight trend towards a decrease of polymer cell association. These deviate from previously reported results on CT26 colon adenocarcinoma cells investigating a different kind of polyzwitterions,³⁷ which may be caused by the use of different cell lines.

Based on the reported results, we conclude that the studied polymers do not strongly interact with specific AATs but associate mainly *via* charge interactions with the cell membrane before active internalisation via endocytic pathways. Future studies will aim at examining the cellular association mechanisms with different cell lines in more detail, *e.g.*, by screening a broader range of cell lines and investigating specific uptake mechanisms, aiming for a better understanding of uptake and distribution of polymers inside the cell.

3. Conclusions

The synthesis of amino-acid-derived polyelectrolytes from lysine- and phenylalanine-derived motifs by PPM polymerisation was demonstrated. Defined polyzwitterions were prepared by using Boc-protected compounds and subsequent deprotection. Anionic structural analogues were synthesised likewise. *Via* this approach a library of five polymers was obtained which enabled a study of the effects of side chain alterations on the properties of amino-acid-derived polyelectrolytes. We investigated the influence of a (permanent) zwitterionic character compared to anionic motifs and, additionally, the



influence of aliphatic and aromatic side chain spacers on both the physiochemical and the biological properties/behaviour. HPLC showed that aromaticity in the side chain increased the hydrophobicity, at pH \approx 2, for anionic polymers while no influence was observed for the zwitterionic equivalents. pH-dependent DLS measurements were performed. Here aggregation around and below the pK_a was observed for the anionic polymers. Smaller aggregates were formed around the pI with both zwitterionic polymers, P(Me₃Lys-OH-AAm) and P(Phe-OH-AAm), between pH 6-7 which can enhance retention within tumour tissue as the external pH at tumour sites is slightly lower. Furthermore, DLS measurements in combination with model proteins showed no aggregation with most polymers. Interestingly, the aromatic, anionic polymer, which is more hydrophobic, showed aggregation with the formation of bigger aggregates while the aliphatic less hydrophobic polymer did not show any aggregation over time. No aggregation of the polymers in combination with FBS was observed, allowing further cell experiments. Here, all synthesised polymers were well tolerated by MDA-MB-231 breast cancer cells at concentrations up to 1 mg mL⁻¹ (for 24 h). Cell association with different cell lines showed higher interaction with tumorigenic cells. Further experiments to understand the uptake mechanism were performed by specific amino acid transporter inhibition and competitive uptake assays which revealed no dependence on the studied transporters; however, a strong energy dependent uptake was observed based on the presented results. The fast cellular uptake of the anionic polymers, that was observed by CLSM measurements, make them interesting candidates for further studies towards drug-delivery applications.

4. Experimental Part

4.1. Materials and instrumentation

4.1.1. Materials

Acetonitrile (99.9%), diethyl ether (99.5%), dimethylformamide (99.5%), methanol (99.9%) and sodium hydrogen carbonate (>99.7%) were purchased from FisherScientific. Chloroform (99.8%), dichloromethane (99.8%), isopropanol (99.9%), sodium hydroxide (99.5%), potassium hydroxide (99.5%), hydrochloric acid (32%), Dulbecco's PBS (DPBS), Bovine serum albumin (BSA) and lysozyme, from which the last two obtained as lyophilized powders, were purchased from VWR Chemicals. Triethylamine (99.5%), and potassium carbonate (99.0%) were purchased from Sigma Aldrich. Cyanin5-amine



(95.0%) was purchased from Lumiprobe. 3-(4-aminophenyl)propanoic acid (98%), N-hydroxysuccinimide (98.0%), 2-Amino-2-norbornanecarboxylic acid (BCH, 95%), O-Benzyl-L-serine (BzlSer, 98%), Sulfasalazine (98%), 5-((5-Chloro-2-((5-ethoxyquinoline)-8-sulfonamido)phenyl)ethynyl)-4-methoxypicolinic acid (MSC-4381, 98%) and N-(tert-butoxycarbonyl)-4-amino-L-phenylalanine (98%) were purchased from BLD Pharmatech Ltd. Trifluoroacetic acid (99.0%) was purchased from abcr. Acryloyl chloride (96%) was purchased from ThermoScientific. Boc-Lys-OH (98%) was purchased from Carbolution. 6-Aminohexanoic acid (6-AmHex-OH, >98.5%) was purchased from Fluka. Azobis(isobutyronitril) (AIBN) (Fluka or Sigma Aldrich, 98.0%) was recrystallized from methanol before use. *N*-Acryloxysuccinimide (NAS)⁷³ and 2-propanoic acid (PABTC)⁷⁴ were synthesised and characterised following known procedures in literature.

RPMI 1640 Medium (Sigma Aldrich), Dulbecco's Modified Eagle's Medium low glucose (Sigma Aldrich), Dulbecco's Modified Eagle's Medium (DMEM/F12; Sigma Aldrich), DMEM-LPSTA (Capricorn), Mammary Epithelial Cell Basal Medium (PromoCell) supplemented with BPE-26, hEGF-5, Insulin-2.5, and HC-250, foetal bovine serum (FBS; Bio&Sell), Penicillin/Streptomycin (Bio&Sell), Dulbecco's 1× PBS (Capricorn), cholera toxin (Sigma Aldrich), NIH 3T3 (ACC 59, DSMZ), MDA-MB 231 (HTB-26, ATCC), HCC-78 (ACC 563, DSMZ), MCF10A (#305026, Cytion), L929 (#400260, CLS).

DMEM-LPSTA (Capricorn), MDA-MB-231 ACC-732 (DSMZ), CellMask™ Plasma Membrane Stain Green (Thermo Fisher Scientific), Hoechst33342, trihydrochloride, trihydrate (Thermo Fisher Scientific), 4% Paraformaldehyde solution in PBS (Thermo Fisher Scientific), μ -Plate 24 well (IBIDI), μ -Plate 96 well square (IBIDI), IBIDI Anti-Evaporation Oil (IBIDI).

4.1.2. Proton nuclear resonance (¹H NMR) spectroscopy

¹H- and spectra were recorded on a Bruker Avance 300 (300 MHz) spectrometer equipped with a BACS-120 autosampler and a ¹H, ¹³C, ¹⁹F and ³¹P-BBO probe at room temperature.

4.1.3. Size-exclusion chromatography (SEC)

Aqueous SEC measurement of P(Boc-Lys-OH-AAm), P(Lys-OH-AAm), P(Hex-OH-AAm), P(Boc-Phe-OH-AAm), P(Phe-OH-AAm), P(PhenylPr-OH-AAm), P(Boc-Lys-OH-AAm)-



Cy5, P(Lys-OH-AAm)-Cy5, P(Me₃Lys-OH-AAm)-Cy5, P(Hex-OH-AAm)-Cy5, P(Boc-Phe-OH-AAm)-Cy5, P(Phe-OH-AAm)-Cy5 and P(PhenylPr-OH-AAm)-Cy5 were performed on an instrument consisting of a column set with a Suprema precolumn (particle size = 5 μm) and three Suprema main columns (particle size = 5 μm, 1 × 30 Å; 2 × 1000 Å) with a separation range from 100 to 1 000 000 Da (PSS) together with a variable wavelength detector (1200 Series, Agilent Technologies). As solvent 0.07 M aqueous Na₂HPO₄ was used (for dissolving polymer and as eluting solvent) with a flow rate of 0.8 mL min⁻¹ and the columns were maintained at room temperature. As internal standard ethylene glycol (HPLC grade) was used. The calibration was done with narrowly distributed poly(methacrylic acid) sodium salt homo-polymers (PMA Na salt; PSS calibration kit). An injection volume of 60 μL was used for the measurements. The samples were dissolved with a concentration of 2 mg mL⁻¹ and filtered through a 0.22 μm PTFE Nylon filter before analysis. The UV-detector was set to λ = 600 nm for measurements of Cy5-labeled polymers.

Reported molar masses of polymers in this study are based on the indicated standards and denoted as M_{n,app}.

Aqueous SEC measurements of P(Me₃Lys-OMe-AAm) and P(Me₃Lys-OMe-AAm)-Cy5 were performed on an instrument consisting of a column set with a NOVEMA Max precolumn (particle size = 10 μm) and three NOVEMA Max main columns (particle size = 10 μm, 1 × 100 Å; 2 × 3000 Å) with separation range from 100 to 3 000 000 Da (PSS, Mainz, Germany) together with a variable wavelength detector (1200 Series, Agilent Technologies). As solvent 80:20 Water/ACN mixture + 0.1 M NaCl + 0.1 V% TFA was used (for dissolving polymer and as eluting solvent) with a flow rate of 0.5 mL min⁻¹ and the columns were maintained at room temperature. As internal standard ethylene glycol (HPLC grade) was used. The calibration was done with narrowly distributed Poly(2-vinylpyridine) (narrowly distributed P2VP homopolymers, PSS calibration kit). An injection volume of 60 μL was used for the measurements. The samples were dissolved with a concentration of 2 mg mL⁻¹ and filtered through a 0.22 μm PTFE Nylon filter before analysis. The UV-detector was set to λ = 600 nm for measurements of Cy5-labeled polymers



Size-exclusion chromatography of PNAS and PNAM was performed on an Agilent 1260 Infinity II system equipped with the MDS Refractive Index detector, MWD (UV), MDS Viscometer, PSS SLD 7100 multi angle light scattering detector and PSS GRAM 30 and 1000 column sets with DMAc (+ 5 g L⁻¹ LiBr) as eluent. Measurements were conducted with 100 μ L injection volume at a constant flow rate of 1 mL min⁻¹, with the column oven held at 40°C. All samples were filtered (PTFE 0.22 μ m) before measurements. For calibration, narrowly distributed poly(ethylene oxide) (PEO) standards were used. The UV-detector was set to λ = 600 nm for measurements of Cy5-labeled polymers and λ = 310 nm for end-group removal.

4.1.4. High-performance liquid chromatography (HPLC)

HPLC measurements were conducted with a Jupiter 5 μ m C18 300 Å LC column (250 \times 4.6 mm). The run time was 35 min. A mixture of 0.1 v/v% TFA in water and acetonitrile served as eluent (**Tab 2**). The fluorescence detected was set to λ_{ex} = 640 nm and λ_{em} = 680 nm with 1 \times gain. The ELSD detector was set to λ = 215 nm.

Table 2. Composition of HPLC eluent at indicated time points.

Time [min]	Fraction of 0.1 v/v% aqueous TFA [%]	Fraction of acetonitrile [%]
0	98.0	2.0
5	50.0	50.0
10	0.0	100.0
17	0.0	100.0
25	98.0	2.0
35	98.0	2.0

4.1.5. Data analysis of theoretical logP values

To obtain the theoretical partition coefficients (logP values), the cheminformatics tool RDKit with the descriptor MolLogP (based on Wildman and Crippen)^{44,52} was used. The calculation was done for one repeating unit of each polymer. For this purpose, the following Simplified Molecular Input Line Entry System (SMILE) string were used:

P(PhenylPr-OH-AAm): C(C)CC(=O)Nc1ccc(CCC(=O)O)cc1

P(Phe-OH-AAm): C(C)CC(=O)Nc1ccc(CC([NH3+])C(=O)O)cc1

P(Hex-OH-AAm): C(C)CC(=O)NCCCCC(=O)O

P(Me₃Lys-OH-AAm): C(C)CC(=O)NCCCC(C(=O)O)[N+](C)(C)C

P(Lys-OH-AAm): C(C)CC(=O)NCCCC([NH3+])C(=O)O



4.1.6. Theoretical ionisation state and polymer net charge

The theoretical ionisation state of each pH responsive group in the polymers was calculated via the Henderson Hasselbalch equation (1).

$$\text{Deprotonated fraction (\%)} = \frac{1}{1+10^{pK_a-pH}} \cdot 100\% \quad (1)$$

For calculations the following pK_a values were used as approximation:

P(Lys-OH-AAm): 2.17 (COOH, lysine), 9.13 (α -NH₂, lysine)⁷⁵

P(Me₃Lys-OH-AAm): 1.84 (COOH, betaine)⁷⁶

P(Hex-OH-AAm): 4.88 (COOH, hexanoic acid)⁷⁷

P(Phe-OH-AAm): 1.83 (COOH, phenylalanine), 9.13 (NH₂, phenylalanine)⁷⁸

P(PhenylPr-OH-AAm): 4.73 (COOH, phenylpropionic acid)⁷⁵

The polymer net charge was derived from the proportion of anionic and cationic moieties in charged state, respectively.

4.1.7. Dynamic light scattering (DLS)

DLS was measured on a Malvern Panalytical Zetasizer Ultra device using disposable folded capillary cuvettes. The excitation light source was a He–Ne laser at 633 nm and the intensity of the scattered light was measured at an angle of 173°. The light scattering was measured during an automatic measurement process from minimum 15 runs of 1.68 s run duration or 30 runs of 1.68 s each for each sample. Measurement parameters were set to an equilibrium time and temperature of 60 s and 37 °C. This method measures the rate of intensity fluctuation, and the size of the particles is determined through the Stokes–Einstein equation. The concentration of the polymer solution was 2 mg mL⁻¹. For the pH-responsive measurements, a 2 mg mL⁻¹ solution of the polymer in deionised water was prepared and 0.1 M aq. NaOH or aq. HCl were added until the desired pH-value was reached. Measurements were done with a Mettler Toledo digital pH meter.

4.1.8. Electrophoretic light scattering (ELS)

ELS was used to measure the zeta potential (ζ). The measurement was also performed on a Malvern Panalytical Zetasizer Ultra device using 1.5 mL semi-micro cuvettes or



disposable folded capillary cuvettes by applying laser Doppler velocimetry. For each measurement, automatic measurements including min. 10 runs were carried out using the slow-field reversal and the fast-field reversal mode at 150 V. Each experiment was performed in triplicates at 37 °C after an equilibration time of 60 s. The zeta potential was calculated from the electrophoretic mobility (μ) according to the Henry equation. Henry coefficient $f(\text{ka})$ was calculated according to Ohshima.⁷⁹ The polymer solutions were similarly prepared as for during the DLS measurements.

4.2. Synthesis and characterization

4.2.1. Poly(N-acryloyl succinimide) (PNAS)

2 g of NAS (12 mmol, 200 equiv.) were transferred into a microwave tube (Biotage) equipped with a stirring bar. 0.984 mg (0.006 mmol, 0.1 equiv.) AIBN and 14.28 mg (0.06 mmol, 1 equiv.) PABTC were added, using stock solutions, followed by addition of DMF to reach a final volume of 4 mL. The tube was closed with a septum and vortexed until homogeneous before a sample was taken ($t = 0\text{h}$) for $^1\text{H-NMR}$ spectroscopy in DMSO- d_6 to determine the conversion by comparing the sample with a sample at the end of the reaction. The reaction mixture was deoxygenated by putting the mixture under an argon flow for 30 min. after which it was placed in a preheated oil bath and stirred at 70 °C for 24 h. The reaction was terminated by cooling to RT and purging in air. A sample was taken and analysed *via* $^1\text{H-NMR}$ spectroscopy in DMSO- d_6 . The crude polymer was precipitated in diethyl ether (-80°C) twice. After centrifugation (5000 rpm, 5 min.), the supernatant was discarded, and the polymer was dried under reduced pressure overnight to obtain the product as a yellow viscous oil.

The X_n of the resulting polymer was calculated from $^1\text{H NMR}$ (**Fig S26**) by using the signal of the vinyl CH_2 -group at $\delta = 6.35$ ppm and the reaction solvent at $\delta = 7.96$ ppm both at $t = 0\text{h}$ and 24h . The reaction solvent was used as an internal standard and the decrease of the vinyl signal in relation to the standard was calculated, providing information about the monomer conversion. The conversion was then multiplied with the initial $[\text{M}]/[\text{CTA}]$ ratio to obtain the average degree of polymerisation, *i.e.*, a X_n of 188 for P($\text{Me}_3\text{Lys-OH-AAm}$) and 193 for all others.

Polymers were analysed by DMAc SEC (**Fig S27**, standard: PEG).



PNAS for P(Me₃Lys-OH-AAm): $M_{n,app} = 4\,700\text{ g mol}^{-1}$; $\bar{D} = 1.84$

PNAS for all others: $M_{n,app} = 8\,100\text{ g mol}^{-1}$; $\bar{D} = 1.87$

4.2.2. Post-polymerisation modification of PNAS with Boc-Lys-OH, Me₃Lys-OMe-NH₂, 6-AmHex-OH, Boc-4-AmPhe-OH, 4-AmPhenylPr-OH

The post-polymerisation modification of PNAS is exemplarily described for reactions with Boc-Lys-OH yielding P(Boc-Lys-OH-AAm). Quantities and moles are given relative to the single repeating unit NAS of the polymer and can be found in **Tab 3**.

In a 20 mL reaction vessel (Biotage), PNAS (200 mg, 0.6 mmol, 1.00 equiv.) was dissolved in anhydrous DMF (9.5 mL). 500 μ L triethylamine were added. After that, Boc-Lys-OH (297 mg, 1.2 mmol, 1.03 equiv.) was added, the reaction vial was closed with a septum, and the suspension was stirred at RT for 24 h. Then, the product was purified by dialysis (RC, MWCO 3.5 kDa, SpectraPor) against 1 L brine for one day followed by dialysis against 1 L deionised water for three days with daily water change. Upon lyophilisation, the product was yielded as an off-white powder. Polymers were analysed *via* ¹H NMR and aqueous SEC.

Table 3. Quantities of reagents used for the PPM of PNAS.

Amino acid	n [mmol]	m [mg]
Boc-Lys-OH	1.03	297
Me ₃ Lys-OMe-NH ₂		263
6-AmHex-OH		158
Boc-4-AmPhe-OH		337
4-AmPhenylPr-OH		199

Polymers were analysed by aqueous SEC (0.07 M aq. Na₂HPO₄ (standard: PMA Na salt)). Elugrams can be found in **Fig S6**.

P(Boc-Lys-OH-AAm): $M_{n,app} = 17,200\text{ g mol}^{-1}$; $\bar{D} = 2.25$.

P(Hex-OH-AAm): $M_{n,app} = 16,500\text{ g mol}^{-1}$; $\bar{D} = 2.30$.

P(Boc-Phe-OH-AAm): $M_{n,app} = 12,800\text{ g mol}^{-1}$; $\bar{D} = 2.48$.

P(PhenylPr -OH-AAm): $M_{n,app} = 11,000\text{ g mol}^{-1}$; $\bar{D} = 2.71$.



4.2.3. Post-polymerisation modification of PNAS with Cy5-amine and Boc-Lys-OH, Me₃Lys-OMe-NH₂, 6-AmHex-OH, Boc-4-AmPhe-OH, 4-AmPhenylPr-OH

The post-polymerisation modification of PNAS is exemplarily described for reactions with Boc-Lys-OH yielding P(Boc-Lys-OH-AAm)-Cy5. Quantities and moles are given relative to the single repeating unit NAS of the polymer and can be found in **Tab 4**.

In a 5 mL reaction vessel (Biotage), PNAS (49 mg, 1.4 μmol, 1.00 equiv.) was dissolved in anhydrous DMF (900 μL). 1 mg Cy5-amine (1.5 μmol, 1.1 equiv.) was added using a stock solution. After that, 8 μL of a 1:10 [triethylamine/DMF] mixture were added, and the reaction vessel was closed with a septum. The reaction mixture was stirred in the dark at RT for 24 h. Then, 53 μL triethylamine and Boc-Lys-OH (73 mg, 0.3 mmol, 1.03 equiv.) were added with an additional 940 μL DMF. The reaction vial was closed with a septum, and the suspension was stirred at RT for 24 h. Then, the product was purified by dialysis (RC, MWCO 3.5 kDa, SpectraPor) against 1 L brine for one day followed by dialysis against 1 L deionised water for three days with daily water change. Upon lyophilisation, the product was obtained as a blue powder.

Table 4. Quantities of reagents used for the PPM of PNAS.

Amino acid	n [mmol]	m [mg]
Boc-Lys-OH	0.3	73
Me ₃ Lys-OMe-NH ₂		68
6-AmHex-OH		39
Boc-4-AmPhe-OH		83
4-AmPhenylPr-OH		48.7

Polymers were analysed by aqueous SEC (0.07 M aq. Na₂HPO₄ (standard: PMA Na salt)).

P(Boc-Lys-OH-AAm)-Cy5: $M_{n,app} = 18,600 \text{ g mol}^{-1}$; $\bar{D} = 2.02$.

P(Hex-OH-AAm)-Cy5: $M_{n,app} = 15,900 \text{ g mol}^{-1}$; $\bar{D} = 2.26$.

P(Boc-Phe-OH-AAm)-Cy5: $M_{n,app} = 14,800 \text{ g mol}^{-1}$; $\bar{D} = 2.47$.

P(PhenylPr -OH-AAm)-Cy5: $M_{n,app} = 15,700 \text{ g mol}^{-1}$; $\bar{D} = 2.54$.

4.2.4. Acidic deprotection of P(Boc-Lys-AAm) and P(Boc-Phe-AAm) yielding P(Lys-AAm) and P(Phe-AAm), respectively.



100 mg P(Boc-Lys-AAm) and P(Boc-Phe-AAm) were stirred in a 10 mL 50/50 V% [TFA:water] mixture overnight. \pm 30 mg P(Boc-Lys-AAm)-Cy5 and P(Boc-Phe-AAm)-Cy5 were stirred in the dark in a 4 mL 50/50 V% [TFA:water] mixture overnight. Subsequently, the reaction mixture was purified by dialysis (RC, MWCO 3.5 kDa, SpectraPor) against 1 L deionised water for one day with one water change after five hours followed by lyophilisation to obtain P(Lys-AAm), P(Phe-AAm), P(Lys-AAm)-Cy5 and P(Phe-AAm)-Cy5

Polymers were analysed by aqueous SEC (0.07 M aq. Na₂HPO₄ (standard: PMA Na salt)).

P(Lys-OH-AAm): $M_{n,app} = 2,000 \text{ g mol}^{-1}$; $\bar{D} = 3.27$.

P(Phe-OH-AAm): $M_{n,app} = 13,000 \text{ g mol}^{-1}$; $\bar{D} = 2.14$.

P(Lys-OH-AAm)-Cy5: $M_{n,app} = 4,700 \text{ g mol}^{-1}$; $\bar{D} = 2.08$.

P(Phe-OH-AAm)-Cy5: $M_{n,app} = 12,000 \text{ g mol}^{-1}$; $\bar{D} = 2.59$.

4.2.5. Poly(*N*-acryloylmorpholine) (PNAM)

1.78 mL of NAM (2 g, 14.2 mmol, 200 equiv.) were transferred into a microwave tube (Biotage) equipped with a stirring bar. 1.163 mg (0.0071 mmol, 0.1 equiv.) AIBN and 16.887 mg (0.071 mmol, 1 equiv.) PABTC were added, using stock solutions, followed by addition of DMF to reach a final volume of 4 mL. The tube was closed with a septum and vortexed until homogeneous before a sample was taken ($t = 0 \text{ h}$) for ¹H-NMR spectroscopy in DMSO-d₆ to determine the conversion by comparing the sample with a sample at the end of the reaction. The reaction mixture was deoxygenated by putting the mixture under an argon flow for 30 min. after which it was placed in a preheated oil bath and stirred at 70 °C for 24 h. The reaction was terminated by cooling to RT and purging in air. A sample was taken and analysed *via* ¹H-NMR spectroscopy in DMSO-d₆. The crude polymer was precipitated in diethyl ether (-80 °C). After centrifugation (5000 rpm, 5 min.), the supernatant was discarded, and the polymer was dried under reduced pressure overnight to obtain the product as a yellow viscous oil.

The X_n of the resulting polymer was calculated by using the signal of the vinyl CH₂-group at $\delta = 5.70 \text{ ppm}$ and the reaction solvent at $\delta = 2.90 \text{ ppm}$ both at $t = 0 \text{ h}$ and 24 h, suggesting a X_n of 198.

Polymer was analysed by DMAc SEC (**Fig S28**; standard: PEG).

PNAM-CTA: $M_{n,app} = 8\,300 \text{ g mol}^{-1}$; $\bar{D} = 1.48$



4.2.6. CTA removal of PNAM

The end-group removal of trithiocarbonates was conducted according to a literature procedure.⁸⁰

In a reaction vessel equipped with a stirrer bar, 1.5 g of P(NAM)-CTA (52.5×10^{-6} mol, 1.0 equiv), 173.02 mg of AIBN (1.05 mmol, 20.0 equiv), and 42 mg of Luperox (0.11 mmol, 2.0 equiv) were dissolved in 15 mL of toluene. The vessel was sealed with a septum, and the reaction mixture was deoxygenated with Argon for 30 min. Subsequently, the vial was placed in a preheated oil bath and heated to 80 °C for 2 h under continuous stirring. After cooling to RT, the crude mixture was precipitated in diethyl ether. After centrifugation (5000 rpm, 5 min.), the supernatant was discarded, and the polymer was dried under reduced pressure overnight to obtain the product as a transparent viscous oil. Complete removal of the trithiocarbonate end group was analysed by SEC measurements in DMAc using a UV detector ($\lambda = 310$ nm), showing the disappearance of the polymer UV trace (**Fig S28**).

PNAM-EGR: $M_{n,app} = 1\ 300\ \text{g mol}^{-1}$; $\bar{D} = 1.77$

4.2.7. Cy5-labelling of PNAM-EGR

In a small reaction vessel, 50 mg of PNAM-EGR (16.0×10^{-6} mol, 1.0 equiv.) and 14.47 mg of DMTMM·BF₄ (0.04 mmol, 2.5 equiv.) were dissolved in 20 mL anhydrous DMF and stirred at RT for 30 min. Subsequently, a 10 mg mL⁻¹ solution of Cy5-amine (34.6 mg, 0.05 mol, 3.0 equiv.) in anhydrous DMF was added. The reaction was stirred at RT in the dark overnight. Then, the reaction mixture was precipitated in diethyl ether. After centrifugation (5000 rpm, 5 min.), the supernatant was discarded. The crude product was redissolved in water. Then, the product was purified by dialysis (RC, MWCO 3.5 kDa, SpectraPor) against 1 L brine for one day followed by dialysis against 1 L deionised water for three days with daily water change. Upon lyophilisation, the product was yielded as a blue powder. Dye attachment and purity from unattached dye were confirmed by SEC (**Fig S28**; $\lambda = 612$ nm).

Polymer was analysed by DMAc SEC (standard: PEG).

PNAM-Cy5: $M_{n,app} = 18\ 900\ \text{g mol}^{-1}$; $\bar{D} = 1.31$



4.3. Biological evaluation

4.3.1. Protein fouling

Aggregation of P(Lys-OH-AAm), P(Me₃Lys-OH-AAm), P(Hex-OH-AAm), P(Phe-OH-AAm) and P(PhenylPr-OH-AAm) after incubation with dedicated proteins (BSA and lysozyme) was analysed by DLS measurements. Stock solutions of the polymers and the proteins were prepared separately in DPBS (1 mg mL⁻¹) and mixed in a ratio of 1:1, resulting in final concentrations of $c = 0.5 \text{ mg mL}^{-1}$. The samples were analysed immediately after mixing ($t = 0 \text{ h}$) and at indicated time points (0.5 h, 1 h, 2 h, 4 h, 24 h) thereafter. In between measurements the samples were incubated at 37 °C whilst being shaken at 100 rpm on a thermostated shaker. Measurements were conducted at 37 °C with five measurements and three runs each. After the measurement, samples were placed back into the thermostated shaker immediately.

4.3.2. Polymer stability towards FBS-containing DPBS

Aggregation of P(Lys-OH-AAm), P(Me₃Lys-OH-AAm), P(Hex-OH-AAm), P(Phe-OH-AAm) and P(PhenylPr-OH-AAm) after incubation in DPBS solution containing 10 V% FBS was analysed by DLS measurements. Polymers were weighed in and dissolved into the mixture to obtain a final concentrations of $c = 0.1 \text{ mg mL}^{-1}$. The samples were analysed immediately after mixing ($t = 0 \text{ h}$) and at indicated time points (0 h, 5 min, 10 min, 0.5h, 1 h, 2 h and 5 h) thereafter. In between measurements the samples were incubated at 37 °C without shaking. The light scattering was measured during 30 runs of 1.68 s each for each sample. Measurement parameters were set to an equilibrium time and temperature of 60 s and 37 °C.

4.3.3. Cell culture

MDA-MB-231 cells (HTB-26, ATCC) were maintained in DMEM/F12 cell culture medium, HCC-78 cells were cultured in RPMI 1640 medium, and NIH 3T3 and L929 were cultured in Dulbecco's Modified Eagle's Medium low glucose. All those media were supplemented with 10% foetal bovine serum (FBS), 100 µg mL⁻¹ streptomycin, and 100 U mL⁻¹ penicillin each. MCF10A cells were maintained in Mammary Epithelial Cell Growth Medium with supplements and 100 ng mL⁻¹ cholera toxin. All cells were cultivated at 37 °C in a humidified 5% CO₂ atmosphere.



4.3.4. Cell viability assay

The cell viability assay was slightly modified from our previous procedure.⁴² The cytotoxicity of P(Lys-OH-AAm), P(Me₃Lys-OH-AAm), P(Hex-OH-AAm), P(Phe-OH-AAm) and P(PhenylPr-OH-AAm) were tested using MDA-MB-231 breast cancer cells. The polymers stock solutions were prepared at concentrations ≤ 20 mg mL⁻¹ in demineralised water. The polymers were tested in a concentration range from 0 to 1.0 mg mL⁻¹ MDA-MB-231 cells were cultured as described above. For the cell viability assay, cells (10⁴ per well) were seeded in 96-well plates and allowed to adhere overnight. No cells were seeded in the outer wells. The media was subsequently removed and replaced by fresh polymer containing media. Then, the cells were incubated at 37 °C for an additional 24 h. After that, the media was removed, the cells were washed with 100 μ L DPBS per well, and then fresh media containing thiazolyl blue tetrazolium bromide (MTT) (concentration: 1 mg mL⁻¹) was added (100 μ L per well). Note: MTT (50 mg) was dissolved in 10 mL of sterile DPBS, filtrated (membrane, 0.22 μ m), and 1 to 5 diluted in culture medium prior to use in this assay. After incubation for 3 h at 37 °C, the test medium was discarded, 100 μ L per well of *i*-PrOH were added to each well and the plates were gently shaken in the dark for 15 min to dissolve the formazan crystals. Quantification was done by measuring the absorbance at $\lambda = 580$ nm using a microplate reader (Genios Pro, Tecan). Untreated cells on the same plate served as negative control (100% viability), cells treated with 20% DMSO as positive control (0% viability), and wells without cells as background. Experiments were performed in triplicates on three different plates. Relative cell viability was calculated by using Equation (2).

$$\% \text{ Cell viability} = \frac{\text{Abs. sample} - \text{Abs. background}}{\text{Abs. negative control} - \text{Abs. background}} \cdot 100 \quad (2)$$

4.3.5. Cell association analysis *via* flow cytometry

Cell association assay I: Concentration-dependent polymer association with cells

The MDA-MB-231 cells were cultured as described above. For the experiments, 2×10^5 cells mL⁻¹ were seeded in 2 mL of culture medium in a 6 well plate and cultivated for 24 h prior to treatment. 90 minutes before treatment, the medium was changed to 900 μ L of fresh culture medium. The polymers were diluted in DPBS to a concentration of $c = 0.2$, 1, 5 and 25 μ M and added to the wells (100 μ L). The corresponding final concentrations



of each polymer were $c = 0.04, 0.2, 1.0$ and $2.5 \mu\text{M}$. As negative control (NC), cells were incubated with $100 \mu\text{L}$ DPBS. After 90 minutes of incubation at $37 \text{ }^\circ\text{C}$ in the cell culture incubator, the supernatant was discarded. After a washing step with DPBS cells were harvested by trypsinization, followed by centrifugation and resuspension in DPBS. Flow cytometry measurements were performed using a CytoFLEX S flow cytometer (Beckman Coulter, Krefeld, Germany) equipped with 405 nm, 488 nm and 561 nm lasers. Forward scatter (FSC), side scatter (SSC), Cy5 fluorescence (PC7 filter, 780/760 nm), and propidium iodide (PI) fluorescence (emission 620 nm) were recorded. Negative control samples (mock-treated cells) were used to set measurement parameters and define background fluorescence levels. For each sample, data from at least 10,000 events were collected. Cells were first gated based on FSC and SSC signals (linear scale) to select the non-apoptotic cell population and exclude debris and aggregates (Gate: "Cells"). Subsequently, FSC-A versus FSC-H gating (linear scale) was applied to exclude doublets (Gate: "Single cells"). The relative Cy5 fluorescence of the gated single-cell population was then quantified, enabling statistical analysis of Cy5-labelled polymer–cell interactions. Cy5-positive cells were defined as those exhibiting fluorescence intensities exceeding the autofluorescence of the negative control cells. In parallel, PI fluorescence was used to assess cell viability. Fluorescence intensity histograms (logarithmic scale) were analysed to quantify polymer–cell interactions, expressed as the mean fluorescence intensity (MFI). A detailed gating strategy is provided in **Fig S29**.

The mean fluorescence intensity (MFI) was normalised to the negative control using Equation (3).

$$\text{Normalized MFI [a.u.]} = \frac{\text{MFI}_{\text{sample}}}{\text{MFI}_{\text{NC}}} \times \frac{1}{r_{\text{fluorescence}_{\text{sample}}}} \quad (3)$$

Cell association assay II: Time- and temperature-dependent polymer association with cells

The cell association assay was adapted from Leiske *et al.*⁶ Polymers were investigated for their association with MDA-MB-231, NIH 3T3, HCC 78 and MCF10a. In detail, 4×10^5 MDA-MB-231 cells per well were seeded in a 6-well plate while 2×10^5 HCC 78 cells, 1.8×10^5 NIH 3T3 cells and 4×10^5 MCF10a cells per well were seeded in a 24-well plate and cultivated for 24 h. Then, the media were substituted with $450 \mu\text{L}$ per well fresh medium. After dilution of the polymers in DPBS to a concentration of $c = 25 \mu\text{M}$, $50 \mu\text{L}$ per well of



the respective polymer was added and incubated for 4.0 h at 37 °C and in the cell culture incubator (final c = 2.5 μM). The NC contained only 50 μL DPBS. Moreover, an additional 6-well plate with MDA-MB-231 cells was treated identically, albeit for 4 h at 4 °C (refrigerator). After 4 h of incubation at 37 °C and in the cell culture incubator or 4 °C, the cells were collected for flow cytometer measurements as described above (see Cell association assay I) (n = 4 (two duplicates)). A detailed gating strategy is provided in **Fig S30 to 33**.

The MFI was normalised to MFI of PNAM-treated cells using Equation (4).

$$\text{Normalised MFI [a.u.]} = \left(\frac{\text{MFI}_{\text{sample}}}{\text{MFI}_{\text{NC}}} \times \frac{1}{r_{\text{fluorescence}}^{\text{sample}}} \right) / \left(\frac{\text{MFI}_{\text{PNAM}}}{\text{MFI}_{\text{NC}}} \times \frac{1}{r_{\text{fluorescence}}^{\text{PNAM}}} \right) \quad (4)$$

Association relative to MCF10A using Equation (5)

$$\text{association relative to MCF10A[\%]} = \left(\frac{\text{Normalised MFI}}{\text{Normalised MFI MCF10A}} \right) \times 100 \quad (5)$$

The temperature-dependent reduction of cell association was calculated using Equation (6).

$$\text{Relative cell association} = \frac{\text{MFI}_{4^{\circ}\text{C}} (\text{normalized})}{\text{MFI}_{37^{\circ}\text{C}} (\text{normalized})} \quad (6)$$

Cell association assay III: Polymer-cell association by blocking amino-acid transporters and competitive uptake

2 × 10⁵ MDA-MB-231 cells per well were seeded in a 24-well plate and cultivated for 24 h. Afterward, the medium was replaced by fresh medium without FBS (225 μl per well). For the blocking of transporters, selected amino-acid transporter inhibitors were dissolved in DPBS with final concentrations of 100 mM (BzISer), 100 mM (BCH), 1 mM (Sulfazaline), and 0.5 μM (MSC-4381) and added to the wells (25 μl per well). Inhibitor selectivity profile is provided in **Tab 5**. After 15 min incubation, the medium was discarded and the cells washed with DPBS. Fresh medium was added (450 μl per well) and the prepared polymer solutions in DPBS (c = 25 μM) were added (50 μl per well).



Table 5. Inhibitor selectivity profile across amino acid and monocarboxylate transporters. Alanine/Serine/Cysteine Transporter 1 (ASCT1), Alanine/Serine/Cysteine Transporter 2 (ASCT2), L-type Amino Acid Transporter 1 (LAT1), L-type Amino Acid Transporter 2 (LAT2), Sodium-coupled Neutral Amino Acid Transporter 1 (SNAT1), Sodium-coupled Neutral Amino Acid Transporter 2 (SNAT2), Cystine/Glutamate Antiporter light chain (xCT; system xc-), and Monocarboxylate Transporter 4 (MCT4). “Yes” indicates reported inhibition/interaction in this dataset; “No” indicates no reports on activity. Activities reported according to vendor.

Inhibitor	ASCT1	ASCT2	LAT1	LAT2	SNAT1	SNAT2	xCT	MCT4
BzlSer	Yes	Yes	Yes	No	Yes	Yes	No	No
BCH	No	No	Yes	Yes	No	No	No	No
MSC-4381	No	No	No	No	No	No	No	Yes
Sulfasalazine	No	No	No	No	No	No	Yes	No

For assays with competitive amino acids, a 10× stock of the corresponding compound was prepared in DPBS and then mixed with cell culture media to obtain the 1× concentration. Then, the media were substituted with 450 μL per well fresh medium containing the competing amino acids. After dilution of the polymers in DPBS to a concentration of $c = 25 \mu\text{M}$, 50 μL per well of the respective polymer was added and incubated for 4 h at 37 °C in the cell culture incubator (final $c = 2.5 \mu\text{M}$). The NC contained only 50 μL DPBS. After an incubation time of 4 h at 37°C in the cell culture incubator, the cells were collected for flow cytometer measurements as described above (see Cell association assay I) ($n = 2+2$ in AAT blocking experiment and $n = 2$ in competitive uptake experiment). A detailed gating strategy is provided in **Fig S34**. The normalised MFI was calculated using Equation (3). The inhibitor-dependent reduction of cell association was calculated using Equation (7).

$$\text{Relative cell association} = \frac{MFI_{\text{without inhibitor (normalized)}}}{MFI_{\text{with inhibitor (normalized)}}} \quad (7)$$

4.3.6. Confocal microscopy

For confocal laser scanning microscopy (CLSM), MDA-MB231 cells (DSMZ, ACC 732) were seeded with 2×10^5 cells mL^{-1} and cultured at 37 °C in humidified 5% v/v CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM) Low Glucose (1 g L^{-1}). The medium was supplemented with 10% v/v foetal bovine serum (FBS), 100 U mL^{-1} penicillin and $100 \mu\text{g mL}^{-1}$ streptomycin (culture medium). For the experiments the cells were



seeded in 500 μL medium and incubated for 24 h in an IBIDI 24 well plate. 1 h prior to treatment the medium was changed to 450 μL fresh medium. 50 μL of the polymer solutions were added to the cells which were then incubated for another 1 h. After the incubation the cells were washed twice with warm $1\times$ DPBS, followed by a fixation step with a 2% prediluted paraformaldehyde solution in $1\times$ PBS. The fixation took place at room temperature for 10 min. Afterwards cells were washed twice with warm $1\times$ PBS. Hoechst33342 and CellMask Plasma Membrane Stain green were prediluted in warm cell culture medium and added to each well (0.5 μL of each stain per well). The staining was performed at 37 $^{\circ}\text{C}$ in humidified 5% v/v CO_2 atmosphere for 10 min. Afterwards the cells were washed again twice with $1\times$ PBS and then measured in $1\times$ PBS by CLSM. Imaging was performed using the LSM980 (Zeiss) The lasers had a wavelength of 405 nm (0.2%) (detection wavelength 410 - 481 nm, detection of Hoechst), 488 nm (0.7%) (detection wavelength 492 - 527 nm, membrane) and 639 nm (1.1%) (detection wavelength 644 - 710 nm, detection of Cy5). All images were processed with ZEN software, version 3.7 (ZEN lite) (Zeiss). All measurements were performed at least twice, and the images presented in the paper are representative.

4.3.7 Cell analysis via Cell Discoverer 7

L929 (mouse fibroblast cell line) and MDA-MB-231 (triple-negative human breast adenocarcinoma cell line) were cultured in DMEM (1 g L^{-1} glucose, supplemented with 10% (v/v) FBS, 100 U mL^{-1} penicillin, $100\text{ }\mu\text{g mL}^{-1}$ streptomycin) in an IBIDI 96-well square plate. All cell lines were cultivated at 37 $^{\circ}\text{C}$ in a humidified 5% (v/v) CO_2 incubator. 24 h prior to treatment, cells were seeded at cell densities of 0.1 and 0.2×10^6 cells per mL respectively. 1 h prior to treatment medium has been replaced by 100 μL of fresh medium. 1 h after medium change, medium got exchanged by prewarmed medium supplemented with Hoechst 33342, trihydrochloride, trihydrate to reach a final amount of 0.1 μL of Hoechst solution per well. This solution was then incubated for 10 min in the incubator at 37 $^{\circ}\text{C}$. Afterwards, the solution has been discarded and the cells were washed once with 100 μL prewarmed $1\times$ concentrated DPBS and immediately afterwards, 90 μL of prewarmed medium has been added to the cells, followed by 10 μL of different polymer solutions (P(Lys-OH-AAM), P($\text{Me}_3\text{Lys-OH-AAm}$), P(Phe-OH-AAm), P(Phenyl-OH-AAm), P(Hex-OH-AAm) except for the control well, where 100 μL of medium were added. Final



polymer solutions on cells were 2.5 μM . After adding polymers all treated wells were covered with 100 μL IBIDI anti evaporation oil sterile for cell culture to reduce medium evaporation during experimentation. With finalization of cell preparation, the well has been moved to Carl Zeiss Cell Discoverer 7. Cells were cultivated further at 37 $^{\circ}\text{C}$ at 5% CO_2 and humidified atmosphere. From the first measurement on an image has been made every 1 h for a period of 24 h. For this purpose, the autofocus function of the machine has been used to find cells on basis of nucleus signal. The following laser settings were applied: Hoechst33342 has been measured at 352 nm excitation and 455 nm emission with a laser LED power of 20 % for 2 ms. Cy5 signals have been measured at 650 nm excitation and 673 nm of emission at a laser LED power of 12.1% for 3 ms. Brightfield measurement took place at 10% laser power for 2.5 ms. All samples were measured with plan-apochromat 20 \times /0.95 objective and 1 \times tubulens. The created image settings were identical for each polymer throughout different cell lines and control samples, to obtain comparable results. The measurement has been performed at least twice, and the images are representative.

4.3.8. Statistical analysis

To assess the normal distribution of the data, a Lilliefors test was conducted using Origin2025 (OriginLab, Massachusetts, USA). Afterward a Levene's test was performed to analyse the homogeneity of the variances. In addition, the unequal variances were tested by the unpaired Welch's t-test. Statistical significance was defined as $p \leq 0.05$ for all tests.

5. Associated content

5.1. Supporting information

The supporting information is available free of charge and contains further analysis of the materials in this study, including ^1H spectra, fluorescence spectra, FTIR spectra, SEC data, cell viability data, cell association and uptake data.

5.2. Author contributions

J.D.B. data curation, formal analysis, investigation, methodology, validation, visualisation, writing – original draft; T. M. L. data curation, formal analysis, investigation, methodology,



validation, visualisation, software, supervision, writing – review & editing; C. J. data curation, formal analysis, writing – review & editing; J. P. D. data curation, formal analysis, investigation, methodology, visualisation, writing – review & editing. V. J. methodology, supervision, writing – review & editing; R. F. resources, writing – review & editing; A. T. funding acquisition, resources, supervision, writing – review & editing; M. N. L. conceptualisation, funding acquisition, methodology, project administration, resources, supervision, writing – review & editing.

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6.3. Notes

The authors declare no competing financial interest.

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The primary data are available from the corresponding author upon reasonable request. The supporting information is available free of charge and contains further analysis of the materials in this study, including NMR spectra, SEC data, HPLC data, as well as biological characteristics.

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