Influence of block versus random monomer distribution on the cellular uptake of hydrophilic copolymers

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ABSTRACT: The use of polymers has revolutionized the field of drug delivery in the past two decades. Properties such as polymer size, charge, hydrophilicity or branching have all been shown to play an important role on the cellular internalization of polymeric systems. In contrast, the fundamental impact of monomers distribution on the resulting biological properties of copolymers remains poorly studied, and is always only investigated for biologically-active self-assembling polymeric systems. Here, we explore the fundamental influence of monomer distribution on the cellular uptake of non-aggregating and biologically passive copolymers. Reversible addition−fragmentation chain-transfer (RAFT) polymerization was used to prepare precisely-defined copolymers of three hydrophilic acrylamide monomers. The cellular internalization of block copolymers was compared with the uptake of a random copolymer where monomers are statistically distributed along the chain. The results demonstrate that monomer distribution in itself has a negligible impact on copolymer uptake.

Over the past few decades, the use of polymers in biomedicine, either in the form of soluble polymers or nanostructures, has revolutionized the field of drug delivery. Pharmacological advantages of using polymer carriers include enhanced solubility, protection against harsh physiological conditions, extension of *in-vivo* lifetime or accumulation in cancer tissues due to EPR effect.[1-3] Polymers are also known to facilitate the transfer of cargos across biological barriers and have been widely used as cell uptake enhancers.[4] The chemical and physical properties of polymeric systems are known to play an important role on their cellular internalization and factors such as polymer size,[5-6] charge,[7] hydrophilicity,[8-9] self-assembling behavior,[10] degree of cross-linking,[11] or branching[12] have already been shown to affect cell uptake.

Less studied is the impact of monomer distribution on the biological properties of copolymers. A few reports demonstrated that monomer distribution can have a significant impact on the ability of polymers to condense DNA or siRNA and carry them inside cells.[13] Copolymers of carbohydrate and cationic monomers have received particular attention as nucleic acid carriers, yet conclusion on the monomer distribution influence seems to differ from one system to another.[14-16] Nonviral gene delivery is a complicated process that combines nucleic acid complexation, cell uptake, cargo release and nucleus membrane crossing. This makes understanding the role of monomer distribution very challenging, and highlights the need for a more fundamental approach to studying the influence of polymer architecture on cell uptake.

The use of modern polymerization techniques such as reversible addition−fragmentation chain transfer (RAFT) polymerization or atom-transfer radical-polymerization (ATRP) has rendered ready accessibility to the preparation of precisely-defined copolymers.[17-18] Copolymers can be arranged in blocks where a particular sequence of (co)monomers can be dictated along the same polymeric chain, or as 'random' copolymers where monomers are statistically distributed along the chain.[19-20] This allows for an easier tuning of polymer physical properties and a better control over the introduction of various functionalities. By mixing together monomers with different hydrophobicity, copolymers are also ideal tools to create nanostructures such as micelles or vesicles.[21-22] Previously, Barz *et al.* reported that statistical copolymers of hydrophobic and hydrophilic methacrylamide monomers internalized better than their block copolymer counterparts due to differences in aggregation behavior.[23] In contrast, the cellular uptake of block copolymers from oxazoline derivatives of various hydrophobicity was shown, using covalently-bound horseradish peroxidase, to be superior to the uptake of their statistical equivalent. These results were associated with the absence of amphiphilicity of the random polymer.[24] Reduction of haemolytic activity was also demonstrated for antibacterial polymers made out of amphiphilic blocks as compared to the randomized version of the same copolymer. Again, differences in monomer hydrophobicity leading to different folding of the

Table 1. Characteristics of copolymers prepared in this study

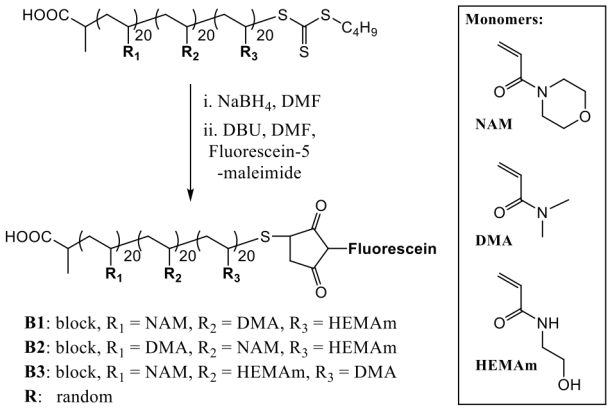
Polymer	Structure	$M_{n,th}$ [a], (g.mol^{-1})	$M_{n,SEC}$ [b], (g.mol^{-1})	$Đ$ [b]	L[c], (%)
A	pNAM-*b*-pDMA-*b*-pHEMAm	7 300	10 900	1.07	98.4
B	pDMA-*b*-pNAM-*b*-pHEMAm	7 300	11 000	1.07	98.5
C	pNAM-*b*-pHEMAm-*b*-pDMA	7 300	10 100	1.09	99.3
R	pNAM-*r*-pDMA-*r*-pHEMAm	7 400	8 500	1.08	99.1

[a] $M_{n,th} = [M]_0 \times \text{conversion} \times M_M / [CTA]_0 + M_{CTA}$

[b] determined by SEC/RI in DMF with pMMA used as molecular weight standards

[c] L = theoretical fraction of living chain

polymers was identified as the main reason for the dissimilarity in cellular behavior.[25] In all, it is becoming clear that cellular internalization is indeed influenced by monomer selection, in different ways for each polymeric system considered, making it difficult to draw general conclusions. This is further complicated by the fact that studies to date focused exclusively on the uptake of amphiphilic copolymers that tend to self-assemble or fold in aqueous solution.[26-28] While these results are pertinent for drug delivery systems that rely on micelles or vesicle formation, an understanding of the fundamental influence of monomer composition on the uptake of single chain copolymers is clearly lacking. In an attempt to fill this knowledge gap, we explored the cellular uptake of various copolymers that do not self-assemble in solution. Various copolymers were prepared using RAFT polymerization, functionalized with a dye and the cellular internalization of block versus randomized copolymers compared.



Scheme 1. Fluorescently-labelled block and random copolymers

Acrylamide monomers were chosen for this study as they are robust, stable towards hydrolysis, and provide high reactivity ratio which makes possible the preparation of well-defined block and random copolymers. (*N*-acryloylmorpholine (NAM), (*N*-(2-hydroxyethyl)acrylamide) (HEAm) and (*N*,*N*-dimethylacrylamide) (DMA) were selected as representative acrylamide monomers. They are neutral monomers that do not induce a lower critical solution temperature (LCST) and, to the best of our knowledge, do not show specific interactions with biological targets that could interfere with our study. They are have been widely used for biomedical applications and were previously shown to be non-toxic to cells.[29-34] While strictly hydrophobic monomers were avoided here to prevent aggregation, this trio of monomers covers an interesting gradient of hydrophilicity (HEAm > NAM >DMA) which is expected to affect the interaction of the resulting copolymers with both extracellular proteins and the lipid bilayer of cells.[35-36] A combination of triblock copolymers (**A**, **B** and **C**), consisting each of 20 units of these three monomers in various sequences, was considered as the most efficient way to study this system (Scheme 1). Additionally, a statistical polymer (p(DMA)-*r*-p(HEAm)-*r*-p(NAM)) (**R**) was also studied as an isomeric analogue to the block copolymers without the sequence defined blocks of monomers. The block copolymers were synthesized via sequential RAFT polymerization, using a recently-described technique that has been demonstrated, both theoretically and experimentally, to yield high end-group fidelity.[37-38] Statistical copolymer **R** was prepared using a one-pot RAFT polymerization of the three monomers. Table 1 summarizes structures and characteristics of the polymers used in the study. Measurement of the polymerization conversion using ^{1}H-NMR shows that all four copolymers are of comparable $M_{n,th}$ values. The fact that the molecular weight reported for copolymer **R** is lower than that of **A**, **B** and **C** is a known artifact of DMF-SEC analysis, and is due to differences in hydrodynamic volume between pHEA and poly(methyl methacrylate) standards used for calibration.[39-40] In the case of **A**, **B** and **C**, grouping of HEAm monomers into blocks enhances this effect, resulting in molecular weight values that appear higher than for random copolymer **R**. In reality, we expect the size for all four copolymers to be comparable, and relatively close to $M_{n,th}$.

Functionalization of the block copolymers with a fluorescent moiety was obtained in a one pot reaction, by reducing the trithioester group of the chain-transfer agent (CTA) into a thiol and reacting it with maleimide-functionalized fluorescein (Scheme 1). Special care was taken in optimizing this step to ensure that subsequent experiments are conducted with uniformly-functionalized polymers. The approach used here is based on previous reports and should ensure maximal functionalization of each polymer chains.[41-43] The reaction was

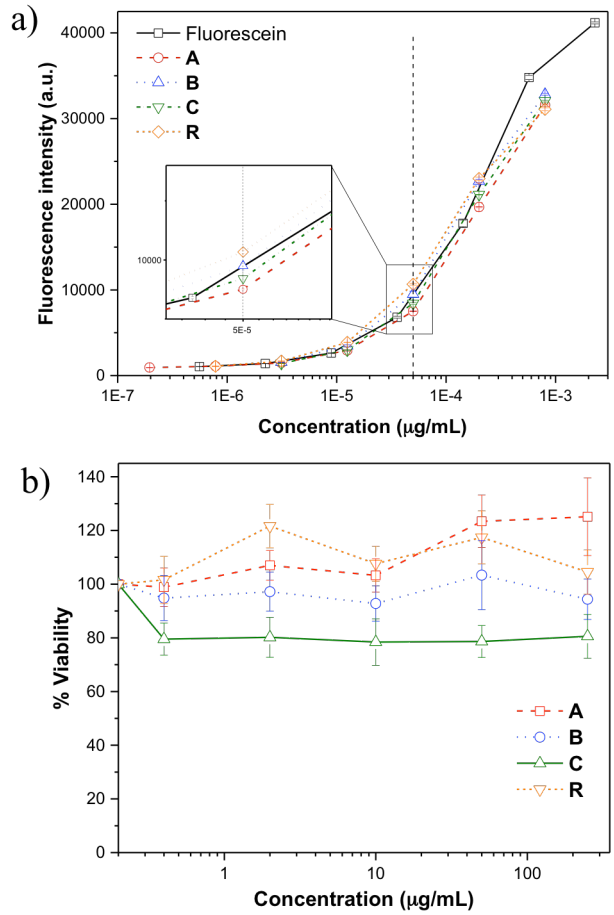


Figure 1. a) Fluorescence profile of the block copolymers and random copolymer. Fluorescence values at 50 µg.mL^{-1} (see insert) were used to calculate the fluorescence correction factor; b) cellular toxicity of copolymers against HeLa cells after 24 hours incubation in the presence of various concentrations of copolymers as measured using MTT assay

allowed to run for six days to drive it towards completion, following which three purification steps (precipitation, filtration and size-exclusion chromatography) were undertaken to ensure complete removal of unconjugated dye molecules. Functionalized polymer chains were characterized via dynamic light scattering (DLS) to confirm the absence of larger self-assembled structures. Measurements in PBS show a hydrodynamic diameter comprised between 4.5 and 7.5 nm for both CTA-polymers and fluorescein-polymers (Figure S1). Due to the hydrophilic nature of these polymers, they are expected to behave as well-solvated single-chains in aqueous solvents. According to the literature, the mean-square end-to-end distance of polyacrylamide chains (7500 Da) in water is approximately 3.2 nm.[44] Using the Stokes-Einstein equation and diffusion coefficients reported by Chamignon *et al.*, the hydrodynamic radius for a linear pNAM of similar molecular weight in PBS (pH = 7.4) can be estimated around 2 nm.[45] Considering these data, the particle hydrodynamic diameters measured by DLS appear to match, or slightly exceed, the values expected for single molecules. Difficulties in measuring light scattering in the presence of large interfering macromolecules prevent us for characterizing the polymers behavior in culture media. As a consequence, it is not possible to definitely conclude on whether polymers are present as single molecules in cell culture media. Yet, for the cell culture experiments described hereafter, we assumed that these hydrophilic polymers are present as single molecules, or at worst aggregates of very few molecules.

Fluorescence spectroscopy measurement of the resulting polymers shows spectra comparable to that of free fluorescein with small differences due to covalent attachment to the polymer (Figure 1, a), but also a trend in fluorescence intensity for the different copolymers (**R** > **B** > **C** > **A**). To determine whether this is due to the presence of free dye molecules or simply to minor differences in RAFT end-group fidelity of the initial polymers, the UV-Vis spectra of the polymers was measured prior to their functionalization. The data, presented in Figure S2, shows a clear correlation between the UV-absorption of the CTA-bearing polymers at 310 nm and fluorescence of the fluorescein-bearing polymers at 490 nm, indicating that differences in the intrinsic fluorescence of the functionalized polymers are likely due to the absence of trithiocarbonate end-groups on dead chains, to different extents depending on the polymer. Indirectly, this result confirms that no free dye molecules remains in our system. To account for these differences, a fluorescence correction factor was calculated using the fluorescence spectra of each polymer and was used to compensate for intrinsic fluorescence discrepancy (Table S1). The validity of this correction was assessed by comparing results obtained when cells were exposed to solutions containing copolymers with equivalent fluorescence intensities against results obtained when cells were incubated with equivalent concentrations of polymers and corrected with the fluorescence correction factor instead (Figure S3).

Next, we turned to characterizing the cellular uptake of these copolymers. In similar studies, Barz and coworkers[23] used concentrations in the range of 0.2 to 1000 µg/mL while Garofalo *et al.*[26] did not exceed 92.5 µg/mL. Preliminary studies with copolymer **R** in HeLa cells (2 hours incubation time) showed that a concentration 50 µg/mL struck a balance of being sufficiently fluorescent while having a fluorescence low enough to avoid saturation-induced screening of potential differences in uptake efficiencies (data not shown). Cytotoxicity assays confirmed that all four copolymers are non-toxic at this concentration (Figure 1,b). The cell uptake of each copolymers in ovarian cancer A2780 and cervical cancer cells HeLa is reported in Figure 2. For each experiment, the fluorescence intensity of 30,000 live cells from each replicate (n = 6) was measured, the median of these data points averaged and corrected with the fluorescence factor to give the values reported for each polymer (Table S1). Incubation of A2780 cells with solutions of copolymers **A**, **B**, **C** and **R** for 2 hours, followed by throughout washes to remove non-internalized polymers, showed a positive uptake of each of the polymer. Block copolymers **A**, **B** and **C** showed negligible uptake differences between each other's, whereas statistical polymer **R** showed a significantly higher uptake ($p < 0.002$). A similar trend was observed for HeLa cells incubated with copolymers for the same amount of time, although the difference between **R** and **A**-**C** was less significant ($p < 0.1$ for **R** and **A**, and $p < 0.01$ for **R** and **B**-**C**). These experiment indicates that monomer distribution has a minor influence on copolymer cell uptake after 2 hours, with statistical distribution of the monomer showing an increase in uptake as compared to block counterparts estimated to 36 % and 18 % for A2780 and HeLa cells, respectively.

In order to gauge whether these results are time-dependent or represent a state of equilibrium, uptake of the polymers by HeLa cells was measured after 24 hours incubation time. The results show an increase of the intracellular fluorescence for block copolymers **A**, **B** and **C** (by 28 %, 32 % and 18 %, respectively) compared to the samples incubated for 2 hours (Figure 2,c). In contrast, the amount of fluorescently-labelled **R** in the cells remained the same than after 2 hours of incubation. As a result, the intracellular fluorescence after overnight incubation showed no significant differences in intensity between each of the block copolymers, and showed no signifi-

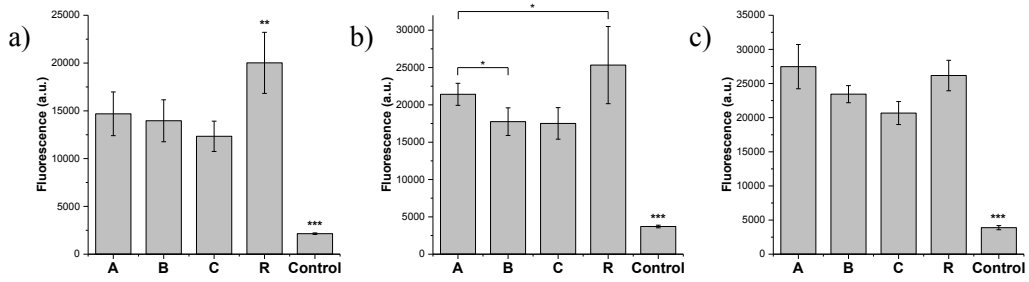


Figure 2. Fluorescence intensity measured in a) A2780 cells incubated for 2 h, b) HeLa cells incubated for 2 h, c) HeLa cells incubated for 24 h at 37 °C, in the presence of block copolymers or random copolymer (50 µg/mL or equivalent). Data represent the mean of fluorescence ± SD for two independent experiments done in triplicates. (*) $p < 0.1$, (**) $p < 0.01$, (***) $p < 0.001$.

cant intensity differences between block polymers and the statistical polymer. Taken together, these data show that monomer distribution does not have an influence on the uptake of copolymers by cells in the long term, but that the internalization of copolymer **R** reaches their maximum intracellular concentration slightly faster than its block counterparts (**A**, **B** and **C**).

The copolymers being neutral and similar in size and aggregation behavior, we hypothesize that changes in cell uptake behavior are possibly related to slight differences in the copolymers hydrophilicities. The consensus in drug delivery is that increased hydrophobicity of macromolecules or nanostructures results in enhanced interaction with the cell membrane and generally facilitate cell uptake.[35, 46] Here, we argue that statistical distribution of the monomers in our system results in a copolymer **R** that is slightly more hydrophobic than its block counterparts. An alternative explanation is that the random arrangement of monomers in **R** results in a greater diversity of consecutive monomer patterns, leading to increased non-specific interactions as compared to the block copolymers (**A**, **B** and **C**). Biologically-inert macromolecules are expected to enter the cells via non-selective endocytosis pathways such as pynocytosis.[47] Both modifying the hydrophilicity of the studied copolymers and increasing their ability to form non-specific interactions with molecules on the cell surface are expected to modify the endocytosis rate of polymers and account for the slightly faster internalization of **R** versus **A**-**C**. Yet, we have demonstrated that these differences become negligible as the incubation proceed for amounts of time superior to two hours.

In conclusion, we have studied the fundamental influence of monomer distribution on the cellular uptake of non-aggregating copolymer chains and demonstrated that monomer distribution in itself, that is for polymeric systems that are biologically passive, has a minor to negligible impact on copolymer uptake overall. Although the random distribution of monomers seemed to favor internalization at first, we showed that it has no significant impact in the longer term.

ASSOCIATED CONTENT

This material is available free of charge via the Internet at http://pubs.acs.org.
Experimental procedures, dynamic light scattering measurement, fluorescence data and additional figures.

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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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