View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Warwick Research Archives Portal Repository

Manuscript version: Author's Accepted Manuscript
The version presented in WRAP is the author's accepted manuscript and may differ from the published version or Version of Record.

Persistent WRAP URL:
http://wrap.warwick.ac.uk/114334

How to cite:
Please refer to published version for the most recent bibliographic citation information. If a published version is known of, the repository item page linked to above, will contain details on accessing it.

Copyright and reuse:
The Warwick Research Archive Portal (WRAP) makes this work by researchers of the University of Warwick available open access under the following conditions.

Copyright © and all moral rights to the version of the paper presented here belong to the individual author(s) and/or other copyright owners. To the extent reasonable and practicable the material made available in WRAP has been checked for eligibility before being made available.

Copies of full items can be used for personal research or study, educational, or not-for-profit purposes without prior permission or charge. Provided that the authors, title and full bibliographic details are credited, a hyperlink and/or URL is given for the original metadata page and the content is not changed in any way.

Publisher's statement:
Please refer to the repository item page, publisher's statement section, for further information.

For more information, please contact the WRAP Team at: wrap@warwick.ac.uk.

warwick.ac.uk/lib-publications

Please do not adjust margins

Journal Name

ARTICLE

Reverse-phase high performance liquid chromatography (RP-HPLC) as a powerful tool to characterise complex water-soluble copolymers architectures

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Raoul Peltier,[a] Agnieszka Bialek,[a] Agnès Kuroki,[a] Caroline Bray,[a] Liam Martin,[a] Sébastien Perrier[a,b,c]*

Recent progress in modern polymer synthesis techniques have led to the design of complex functional materials, which can be difficult to analyse accurately. While size-exclusion chromatography (SEC) or mass spectrometry (MS) are typically used to gain information about molecular weight distribution, chemical structure and molecular architecture, there is a lack of available method for characterising compositional heterogeneity (*i.e.* monomer distribution). In contrast with SEC in which separation occurs by hydrodynamic volume, interaction-based chromatography (IC) separates compounds according to their affinity for a stationary phase, which has proven useful on gaining information about the general chemical structure of copolymers in the past. Here, we explore the potential of reverse-phase high performance liquid chromatography (RP-HPLC) as a tool for the characterisation of monomer segmentation in charged water-soluble copolymers. A library of acrylamide copolymeric systems, prepared via reversible addition-fragmentation chain transfer (RAFT) polymerisation, is used to demonstrate the influence of monomer distribution (diblock, multiblock and statistical) on the elution time. The robustness of the method is tested by studying a range of copolymers with varying charge, charge content and hydrophobicity, as well as by using various solvent systems or column lengths. Results highlight the efficiency of RP-HPLC to separate copolymers with varying segmentation, with a limitation observed for branched architecture.

Introduction

Water-soluble copolymers are important materials associated with a wide range of applications from food additives to rheological modifiers, as well as in the biomedical field where they are often used to enhance drug solubility or stability, increase drug cellular uptake, or even direct the drug to tumour areas.[1] By incorporating monomers with different chemical functions, copolymers can be further tailored to exhibit specific properties. For example, amphiphilic block copolymers, which tend to self-assemble in morphologies such as vesicles, micelles, cylinders,[2] are commonly used for practical applications such as antibacterial or antifouling coatings,[3] as structural support for the growth of encapsulated cells,[4] or as vectors for enhanced drug delivery.[5] Until recently, control over the copolymer sequence was limited to either statistical or diblock copolymers. However, novel polymerisation methods such as reversible addition-fragmentation chain transfer (RAFT) polymerisation or atom-transfer radical polymerisation (ATRP) and single electron transfer living radical polymerisation (SET-LRP) have granted access to more complex architectures with a higher number of segmentations in the form of multiblock copolymers.[6-9] To a similar degree as molecular weight and chemical composition, the segmentation of copolymers was shown to have a major impact on the physical properties of the resulting materials, including their stability, solvation or self-assembly behaviour.[10-13] For example, polymer sequence was demonstrated to affect the glass transition temperature of copolymers of ethylene glycol methyl ether acrylate (EGMEA) and tert-butyl acrylate (tBA).[14] In other example, segmentation was also shown to have a dramatic influence on the interaction of copolymers with lipid membranes.[15,16] By varying the monomer distribution along the backbone, Kuroda *et al.* were able to design copolymers that selectively interacted with the membrane of bacteria while presenting relatively low haemolytic profiles.[17]

Despite these developments, the characterisation of copolymers remains non-trivial, mostly because of the numerous parameters to be considered, including molecular weight distribution, chemical structure (*i.e.* choice of monomers, end groups), molecular architecture (*i.e.* linear versus branched), as well as chemical heterogeneity (*i.e.* monomer distribution) of the copolymers.[18] Size-exclusion chromatography (SEC) is the method of choice for polymer analysis, yet its separation based on differences in the

[a] *Department of Chemistry, University of Warwick, Coventry, CV4 7AL, UK.*
[b] *Warwick Medical School, University of Warwick, Coventry, CV4 7AL, UK*
[c] *Faculty of Pharmacy and Pharmaceutical Sciences, Monash University, 381 Royal Parade, Parkville, Victoria 3052, Australia*
Electronic Supplementary Information (ESI) available: [additional figures]. See DOI: 10.1039/x0xx00000x

Please do not adjust margins

Please do not adjust margins

hydrodynamic volumes of the polymeric chains only provides information about copolymer size and eventually its architecture.[19] Light scattering or viscometry detection in SEC can help elucidate accurate molecular mass average and copolymer architectures.[20] However, characterisation of water-soluble polymer via SEC in aqueous environment remains challenging due to the necessity to use salts which can interfere with the separation process.[21] This is especially true for highly charged polymers whose separation is prone to a variety of electrostatic interferences.[22,23] Gradient Polymer Elution Chromatography (GPEC), a separation method based on difference in the solubility of copolymers with varying chemical composition, is useful to determine the chemical composition distribution (CCD) of copolymers.[24-26] Methods such as MALDI-TOF mass spectrometry, IR or NMR are also used to gain information about the chemical composition of the copolymer chains.[27] Despite these advancements, the range of methods currently available for characterising compositional heterogeneity in water-soluble copolymers remains limited to nonexistent. Currently, the preferred method involves determination of the reactivity ratio of each of the individual monomers to estimate the tendency of one of the monomer to self-propagate and create a gradient within a given statistical copolymer.

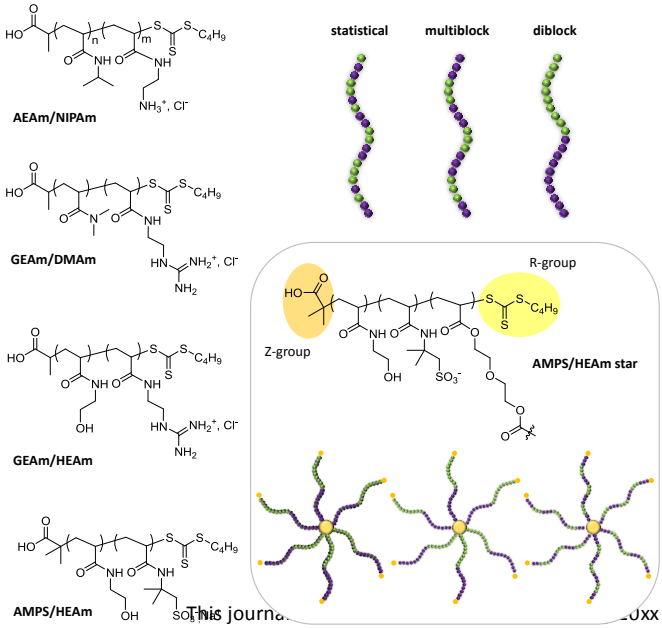
Almost 30 years ago, Glockner *et al.* demonstrated the use of interaction-based chromatography (IC) to differentiate between statistical and block copolymers of styrene and *t*-butyl methacrylate.[28] Despite the convenience of the method, reports describing the characterisation of copolymers using IC remain unusual.[29-31] In contrast with SEC, in which separation occurs by size, IC separates compounds according to their affinity for a stationary phase (*i.e.* chromatographic column) chosen accordingly. While bare silica column is the preferred choice for hydrophobic copolymers,[32,33] interaction with silica columns functionalised with hydrophobic chains, commonly referred to as reverse-phase high performance liquid chromatography (RP-HPLC), appears as a more versatile approach which allows characterisation of a large variety of copolymers. Using a phenyl or C18-functionalised column, hydrophobic copolymers have been separated using a mixture of organic solvents such as tetrahydrofuran (THF) and methanol (MeOH).[34] The composition of hydrophilic copolymers, for example resulting from the hydrolysis of poly(vinyl alcohol), can be characterised via RP-HPLC using a gradient of water and organic solvent.[35,36] While simple IC-based characterisation of copolymers typically gives information about the general chemical structure of copolymers, more in-depth characterisation of block copolymers, telechelic polymers or polymer blends composition can be obtained using liquid chromatography at the so-called "critical point of adsorption" of one of the homopolymer block.[37-39] At this critical point, namely a set of temperature and solvent conditions at which enthalpic and entropic factors are balanced for one polymer constituent, retention time is independent of molar mass of one of the homopolymer and therefore the retention time for copolymers is only reliant of chain length of the other block component.[40,41] This was successfully used to characterise a large number of water-soluble[42-44] and water-insoluble copolymers,[45,46] but requires time-consuming optimisation to determine the critical point of the studied system. Alternatively, two-dimensional approaches in which liquid chromatography techniques are coupled to another characterisation device such as gel permeation chromatography (GPC)[47] or mass spectrometry (MS)[48,49] have also been used in the past to generate rich maps of copolymers structure, yet these requires complex equipment which might not be accessible to most laboratories.

While these techniques have made possible the separation of copolymers according to the number and structure of their functional groups, the characterisation of monomer distribution within copolymers remains a major challenge.[50] In this study, we demonstrate the potential of RP-HPLC as a tool for the characterisation of monomer distribution in charged water-soluble copolymers. We demonstrate the influence of monomer distribution (block, multiblock and statistical) on the elution time of charged acrylamide copolymeric systems prepared via RAFT polymerisation (Scheme 1). The robustness of the method is explored by studying copolymer systems with varying molecular weight, charge, composition, hydrophilicity and architecture. To our knowledge, this is the first report of a chromatographic tool to characterise segmentation of water-soluble copolymers.

Experimental

Materials

Water (H_2O; Fischer Scientific, HPLC gradient grade), acetonitrile (ACN; Fischer Scientific, HPLC gradient grade), methanol (MeOH; Fischer Scientific, HPLC grade), trifluoroacetic acid (CF_3CO_2H; Sigma-Aldrich, 99%), tetrahydrofuran (THF; Honeywell, 99.9%), 2,6-di-tert-butyl-4-methylphenol ($C_{15}H_{24}O$; BHT; Sigma-Aldrich, 99%). Homopolymers and copolymers were synthesised via RAFT polymerisation using previously reported protocols. Compound synthesis and their characterisation via SEC and ^{1}H-NMR was reported previously.[51-53]



Please do not adjust margins

Please do not adjust margins

Scheme 1. Chemical structure of copolymer systems used in this study.

Table 1. Structure and molecular weight of homopolymers and copolymers

Instrumentation

	Name	Structure	Distribution	$M_{n,th}$[a] (g.mol^{-1})	$M_{n,exp}$ (g.mol^{-1})	$Đ_{exp}$
AEAm/NIPAm	H^{AEAm}_{98}	$p(AEAm)_{98}$	-	15000	21000[b]	1.1[b]
	H^{NIPAm}_{104}	$p(NIPAm)_{104}$	-	12000	14400[b]	1.11[b]
	$S^{AEAm/NIPAm}_{32/73}$	$p(AEAm_{32}$-*s*-$NIPAm_{73})$	Statistical	13300	17900[b]	1.09[b]
	$S^{AEAm/NIPAm}_{52/53}$	$p(AEAm_{52}$-*s*-$NIPAm_{53})$	Statistical	14000	18800[b]	1.09[b]
	$S^{AEAm/NIPAm}_{73/32}$	$p(AEAm_{73}$-*s*-$NIPAm_{32})$	Statistical	14900	21600[b]	1.12[b]
	$D^{AEAm/NIPAm}_{31/72}$	$p(AEAm_{31}$-*b*-$NIPAm_{72})$	Diblock	12900	16000[b]	1.1[b]
	$D^{AEAm/NIPAm}_{44/46}$	$p(AEAm_{44}$-*b*-$NIPAm_{46})$	Diblock	13400	18000[b]	1.17[b]
	$D^{AEAm/NIPAm}_{70/29}$	$p(AEAm_{70}$-*b*-$NIPAm_{29})$	Diblock	14800	19000[b]	1.2[b]
	$M^{AEAm/NIPAm}_{30/72}$	$p(NIPAm_{18}$-*b*-$AEAm_{10}$-*b*-$NIPAm_{18}$-*b*-$AEAm_{10}$-*b*-$NIPAm_{18}$-*b*-$AEAm_{10}$-*b*-$NIPAm_{18})$	Heptablock	13100	15800[b]	1.29[b]
	$M^{AEAm/NIPAm}_{50/50}$	$p(AEAm_{10}$-b-$NIPAm_{10})_5$	Decablock	12100	17000[b]	1.38[b]
	$M^{AEAm/NIPAm}_{72/33}$	$p(AEAm_{18}$-b-$NIPAm_{11}$-b-$AEAm_{18}$-b-$NIPAm_{11}$-b-$AEAm_{18}$-b-$NIPAm_{11}$-b-$AEAm_{18})$	Heptablock	14000	17800[b]	1.31[b]
	H^{AEAm}_{23}	$p(AEAm)_{23}$	-	3700	7200[b]	1.07[b]
	H^{NIPAm}_{25}	$p(NIPAm)_{27}$	-	3300	4200[b]	1.12[b]
	$S^{AEAm/NIPAm}_{8/19}$	$p(AEAm_7$-*s*-$NIPAm_{18})$	Statistical	3600	5600[b]	1.1b
	$S^{AEAm/NIPAm}_{12/12}$	$p(AEAm_{12}$-*s*-$NIPAm_{12})$	Statistical	3400	5900[b]	1.08[b]
	$S^{AEAm/NIPAm}_{17/6}$	$p(AEAm_{17}$-*s*-$NIPAm_7)$	Statistical	3500	6300[b]	1.08[b]
	$D^{AEAm/NIPAm}_{7/13}$	$p(AEAm_5$-*b*-$NIPAm_{16})$	Diblock	2800	5600[b]	1.1[b]
	$D^{AEAm/NIPAm}_{10/12}$	$p(AEAm_{12}$-*b*-$NIPAm_{11})$	Diblock	3200	6200[b]	1.08[b]
	$D^{AEAm/NIPAm}_{14/7}$	$p(AEAm_{16}$-*b*-$NIPAm_5)$	Diblock	3200	6500[b]	1.07[b]
GEAm/DMAm	H^{GEAm}_{41}	$p(GEAm)_{41}$	-	6600	9750[b]	1.14[b]
	H^{DMAm}_{40}	$p(DMAm)_{40}$	-	4200	5900[b]	1.11[b]
	$S^{GEAm/DMAm}_{20/20}$	$p(GEAm_{20}$-*s*-$DMAm_{20})$	Statistical	5400	8600[b]	1.1[b]
	$D^{GEAm/DMAm}_{20/20}$	$p(GEAm_{20}$-*b*-$DMAm_{20})$	Diblock	5400	8050[b]	1.11[b]
	$T^{GEAm/DMAm}_{20/20}$	$p(GEAm_{10}$-*b*-$DMAm_{10}$-*b*-$GEAm_{10}$-*b*-$DMAm_{10})$	Tetrablock	5400	9400[b]	1.08[b]
GEAm/HEAm	H^{HEAm}_{40}	$p(HEAm)_{40}$	-	4800	8100[b]	1.12[b]
	$S^{GEAm/HEAm}_{20/20}$	$p(GEAm_{20}$-*s*-$HEAm_{20})$	Statistical	5700	9200[b]	1.12[b]
	$D^{GEAm/HEAm}_{20/20}$	$p(GEAm_{20}$-*b*-$HEAm_{20})$	Diblock	5700	9700[b]	1.13[b]
	$T^{GEAm/HEAm}_{20/20}$	$p(GEAm_{10}$-*b*-$HEAm_{10}$-*b*-$GEAm_{10}$-*b*-$HEAm_{10})$	Tetrablock	5700	9950[b]	1.17[b]
AMPS/HEAm (linear)	H^{AMPS}_{10}	$p(AMPS)_{10}$	-	2500	5500[c]	1.09[c]
	H^{AMPS}_{20}	$p(AMPS)_{20}$	-	4800	8100[c]	1.10[c]
	H^{AMPS}_{50}	$p(AMPS)_{50}$	-	11600	13000[c]	1.11[c]
	H^{AMPS}_{79}	$p(AMPS)_{79}$	-	18400	19000[c]	1.18[c]
	H^{AMPS}_{99}	$p(AMPS)_{99}$	-	23000	17600[c]	1.16[c]
	H^{AMPS}_{198}	$p(AMPS)_{198}$	-	45600	29900[c]	1.25[c]
	H^{AMPS}_{396}	$p(AMPS)_{396}$	-	91000	41300[c]	1.51[c]
	H^{HEAm}_{79}	$p(HEAm)_{79}$	-	9300	4700[c]	1.51[c]
	$S^{AMPS/HEAm}_{56/23}$	$p(AMPS_{56}$-*s*-$HEAm_{23})$	Statistical	15700	14600[c]	1.21[c]
	$S^{AMPS/HEAm}_{40/39}$	$p(AMPS_{40}$-*s*-$HEAm_{39})$	Statistical	13900	13900[c]	1.13[c]
	$S^{AMPS/HEAm}_{24/55}$	$p(AMPS_{24}$-*s*-$HEAm_{55})$	Statistical	12100	11200[c]	1.20[c]
	$D^{AMPS/HEAm}_{56/24}$	$p(AMPS_{56}$-*b*-$HEAm_{24})$	Diblock	15000	11100[c]	1.29[c]
	$D^{AMPS/HEAm}_{39/39}$	$p(AMPS_{39}$-*b*-$HEAm_{39})$	Diblock	13600	8300[c]	1.35[c]
	$D^{AMPS/HEAm}_{23/55}$	$p(AMPS_{23}$-*b*-$HEAm_{55})$	Diblock	11800	7500[c]	1.35[c]
	$T^{AMPS/HEAm}_{40/40}$	$p(AMPS_{20}$-*b*-$HEAm_{20})_2$	Tetrablock	14000	12400[c]	1.23[c]
	$O^{AMPS/HEAm}_{40/40}$	$p(AMPS_{10}$-*b*-$HEAm_{10})_4$	Octablock	13900	16700[c]	1.48[c]
AMPS/HEAm (star)	Star-H^{AMPS}_{50}	star-$p(AMPS)_{50}$	-	-	67000[c]	1.15[c]
	Star-H^{AMPS}_{99}	star-$p(AMPS)_{99}$	-	-	126000[c]	1.17[c]
	Star-H^{AMPS}_{198}	star-$p(AMPS)_{198}$	-	-	199000[c]	1.16[c]
	Star-$S^{AMPS/HEAm}$	star-$p(AMPS_{40}$-*s*-$HEAm_{39})$	Statistical	-	101000[c]	1.22[c]
	Star-$T^{AMPS/HEAm}$	star-$p(AMPS_{20}$-*b*-$HEAm_{20})_2$	Tetrablock	-	162000[c]	1.18[c]
	Star-$O^{AMPS/HEAm}$	star-$p(AMPS_{10}$-*b*-$HEAm_{10})_4$	Octablock	-	180000[c]	1.27[c]
	Star-$D^{AMPS/HEAm}$	star-$p(AMPS_{39}$-*b*-$HEAm_{39})$	Diblock	-	111000[c]	1.17[c]

[a] Theoretical molecular weight calculated using monomer conversion as determined by ^{1}H NMR

[b] Determined for the Boc-protected polymers by SEC/RI in DMF using PMMA as molecular weight standards.

[c] Determined by aqueous-SEC with PEG standard

Please do not adjust margins

Please do not adjust margins

RP-HPLC chromatograms of water-soluble (co)polymers were recorded on either an Agilent 1260 Infinity HPLC instrument equipped with photodiode array (PDA) detector, or on a Shimadzu Prominence HPLC equipped with photodiode array (PDA) detector. HPLC systems were equipped with either an Agilent eclipse XDB C18 column (150 mm x 4.6 mm, 5 µm diameter particle size, 8 nm pore size) or a Phenomenex Luna – C18, (250 mm x 4.6 mm, 5 µm diameter particle size, 10 nm pore size). Water was used as solvent A, acetonitrile or methanol was used as solvent B. All solvents were complemented with 0.04% of trifluoroacetic acid (TFA).

Linear homopolymers and copolymers were dissolved in water (1 mg/ml). Star-shaped homopolymers and copolymers were dissolved in water (10 mg/ml). Injection volumes were 100 µL for all samples. Flow rate was fixed at 1.000 mL/min. Unless otherwise noted, temperature was set at 37°C. Signal was recorded by UV lamp within the range of the wavelength between 200 nm and 600 nm. Chromatograms are reported at 309 nm, which corresponds to the absorbance of the trithiocarbonate of the RAFT agent. Data were extracted and subsequently plotted and analysed using OriginPro 9.1®.

Results and discussion

Copolymer selection

Water-soluble copolymers of acrylate or acrylamide derivatives have gained popularity with the development of aqueous living-polymerisation techniques.[54] In this study, we chose to use acrylamide-based polymers due to their enhanced stability towards hydrolysis as compared to acrylates.[55] Copolymers and homopolymers prepared for this study are reported in Table 1, along with their experimental molecular weight and dispersity. The synthesis of these compounds via RAFT polymerisation and their characterisation was reported previously.[51-53] RAFT polymerisation was chosen as it offers good control over the monomer distribution, and it allows the preparation of well-defined multiblock copolymers. In addition, RAFT polymerisation introduces a trithiocarbonate group as an end group, which conveniently absorbs at 309 nm, allowing the tracking of the elution of the polymers using a simple UV detector for HPLC.

The polymeric systems investigated here, described in Scheme 1, were chosen for their relevance in the medicinal and pharmaceutical field. Copolymers of *N*-(2-aminoethyl)acrylamide (AEAm) and *N*-isopropylacrylamide (NIPAm) were recently shown to have promising antibacterial activity, as they could selectively disrupt bacterial membrane while remaining relatively non-toxic to red blood cells.[51] Copolymers containing guanidine-ethyl acrylamide (GEAm), an acrylamide mimic of Arginine, were shown to have a useful cell-penetrating activity as they can interact with lipid membrane of mammalian cells and to help macromolecules cross into the cytosol.[52] Two systems, containing either *N,N*-dimethylacrylamide (DMAm) or *N*-(2-hydroxyethyl)acrylamide (HEAm) as co-monomers, were studied to investigate the impact of co-monomer hydrophilicity on the separation method. Finally, copolymers containing 2-acrylamido-2-methyl-1-propane sulfonic acid (AMPS), a monomer commonly used in applications such as rheological modifiers, scaffold for cell culture or as a heparin-mimic, were studied as an example of anionic polymers.[53,56,57] Star copolymers AMPS and HEAm were also used to investigate the influence of polymer architecture on the separation method.

AEAm/NIPAm copolymeric system

A small library of copolymers with a targeted degree of polymerisation (DP) of 100, with varying segmentation (diblock, multiblock and statistical) and cationic content (30, 50 and 70% of AEAm) were prepared (Table 1) and systematically characterised via RP-HPLC using a C18 column (4.6 × 250 mm) and UV detection (309 nm). Initially, a gradient of water and acetonitrile (ACN) was used as eluting solvent and homopolymers of p(NIPAm)$_{100}$ (H^{NIPAm}_{100}) and p(AEAm)$_{100}$ (H^{AEAm}_{100}) were used to optimise the solvent gradient. All

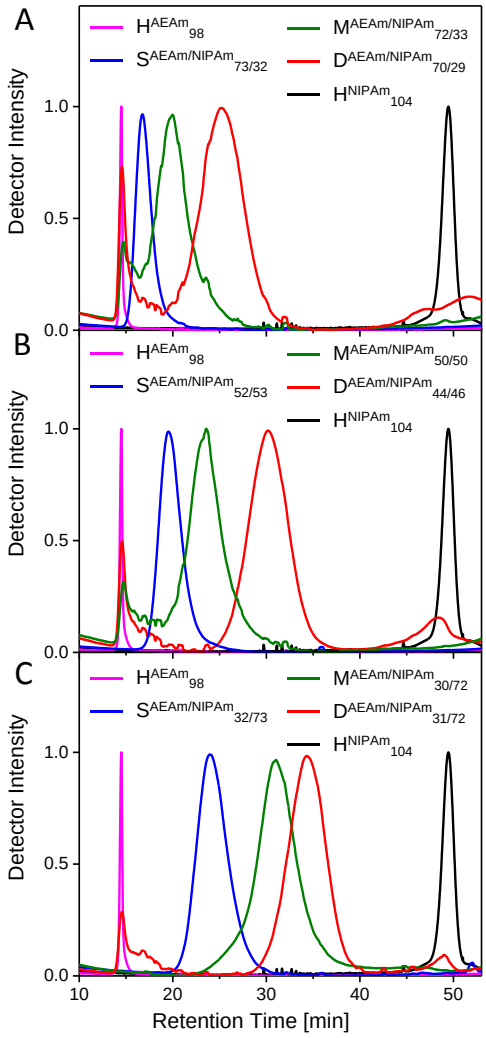


Figure 1. AEAm/NIPAm (DP = 100) with varying segmentation. HPLC chromatograms of copolymers with various monomer distribution for a ratio of AEAm/NIPAm of approximately A) 70/30, B) 50/50, C) 30/70. Homopolymers are included for references. Solvent: water/ACN. Gradient: 1 to 95% ACN in 50 minutes at 37 ºC. Column: C18 (4.6 mm × 250 mm).

This journal is © The Royal Society of Chemistry 20xx

Please do not adjust margins

measurements were carried out at 37°C. Despite the thermo-responsive nature of pNIPAm, the influence of temperature on the retention time of H^{NIPAm}_{100} was found to be minor, which was attributed to the presence of organic solvent in the system (Figure S1).

Chromatograms sorted by segmentation and cationic content are represented Figure 1 and Figure S2, respectively. As expected, increasing the percentage of charged monomer decreases the retention time of the overall polymer, as the overall hydrophilicity of the polymeric chains is increased. This is in agreement with the lower retention time observed for the cationic homopolymer $p(AEAm)_{100}$ in comparison with the comparatively more hydrophobic $p(NIPAm)_{100}$. The influence of monomer distribution follows a trend in which the statistical copolymer elutes before the multiblock counterpart, which in turn elutes before the diblock copolymer. This difference can be attributed to a better distribution of the positive charges of the primary amine group of AEAm in the statistical copolymers, which maximises their interaction with the mobile phase and minimises interactions with the hydrophobic column. In contrast, the segregation of the charged pendant groups in the diblock potentially shields some of the charges, thus increasing the relative hydrophobicity of the copolymer.

Next, the influence of increasing the stationary phase area on the separation of copolymers with different segmentations was investigated. Figure 2 represents the elution conditions

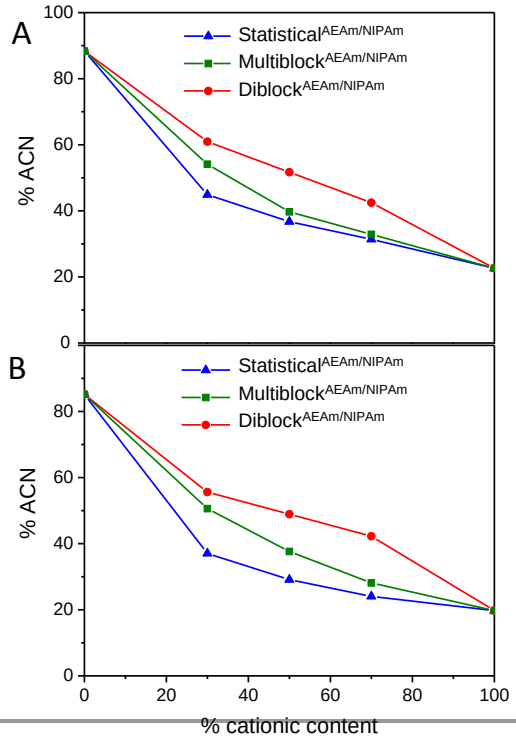


Figure 2. Influence of column length. Chromatographic separation of copolymers with varying cationic content and segmentation using A) column 1: 4.6 mm × 250 mm, 400 m^2/g of C18 stationary phase, B) column 2: 4.6 mm × 150 mm, 180 m^2/g of C18 stationary phase. %ACN values (y-axis) corresponds to the concentration of ACN at which the peaks elute. %cationic content values (x-axis) corresponds to the percentage of charged monomer (AEAm) present in each copolymer. Homopolymers are included for references. Solvent: water/ACN. Gradient: 1 to 95% ACN in 50 minutes at 37 ºC.

obtained for copolymers of $p(NIPAm\text{-}co\text{-}AEAm)_{100}$ using two C18 columns with similar diameter and particle size, as well as with relatively close pore size (10 and 8 nm), but with a length of 250 mm and a surface area of 400 m^2/g (17.5% carbon loading) (column 1) versus a length of 150 mm and a surface area of 180 m^2/g (10% carbon loading) (column 2), respectively. The separation efficiency, defined by the discrepancy between the percentages of ACN required to elute the respective copolymers, is illustrated in Figure 2 and reported in Table S1 and Table S2. Interestingly, a better separation of copolymers with identical composition but different monomer distributions is observed in the case where a shorter column with less surface area is used. Upon initial desorption of the polymer chains from the stationary phase at a given percentage of acetonitrile, eluting copolymers are forced to interact with more stationary phase as they flow through the column. The present results suggest that the initial desorption from the stationary phase results in better separation of copolymers with varying distribution as compared to the subsequent eluting phase, which appear to mitigate the initial desorption-based separation instead.

The influence of the eluting solvent system was then investigated by replacing the mobile phase from water/ACN to water/MeOH, also commonly used in RP-HPLC. The increased retention times illustrate the reduction of the eluting power of methanol in comparison to acetonitrile (Figure S3). This is consistent with previous reports in the literature.[58] Consequently, a better separation of the various segmentations in the copolymer with high cationic content was observed. However, the use of a water/MeOH solvent system presents a limitation in the nature of the compounds which can be characterised, as the less polar diblocks $p(AEAm_{50}\text{-}b\text{-}NIPAm_{50})$ and $p(AEAm_{70}\text{-}b\text{-}NIPAm_{30})$, and homopolymer $p(NIPAm)_{100}$, did not elute from the column even upon reaching 95 % of MeOH as the mobile phase.

Shorter diblock and statistical copolymers (DP = 25) were prepared in order to evaluate if chain length has an influence on the separation of copolymers with various segmentation. Using a similar water/ACN gradient, the homopolymers of DP = 25 eluted at approximately the same time as the homopolymers with DP = 100. However, significant differences were observed in the case of copolymers separation (Figure 3). While statistical copolymers showed a similar retention time regardless of the DP, the elution time of diblock copolymers decreased significantly with decreasing DP, resulting in a decreased separation of the statistical and diblock copolymers. Again, this phenomenon can be attributed to the partial screening of charges in the cationic block. With the number of repeating units increasing within the block, the screening phenomenon is amplified, in turn reducing the hydrophilicity of the overall molecules further than in the case of shorter chains.

GEAm/DMAm and GEAm/HEAm copolymeric system

A different cationic system, comprising an Arginine-mimicking acrylamide monomer (GEAm), was studied next. In particular, the influence of the hydrophilicity of the co-monomer was

Please do not adjust margins

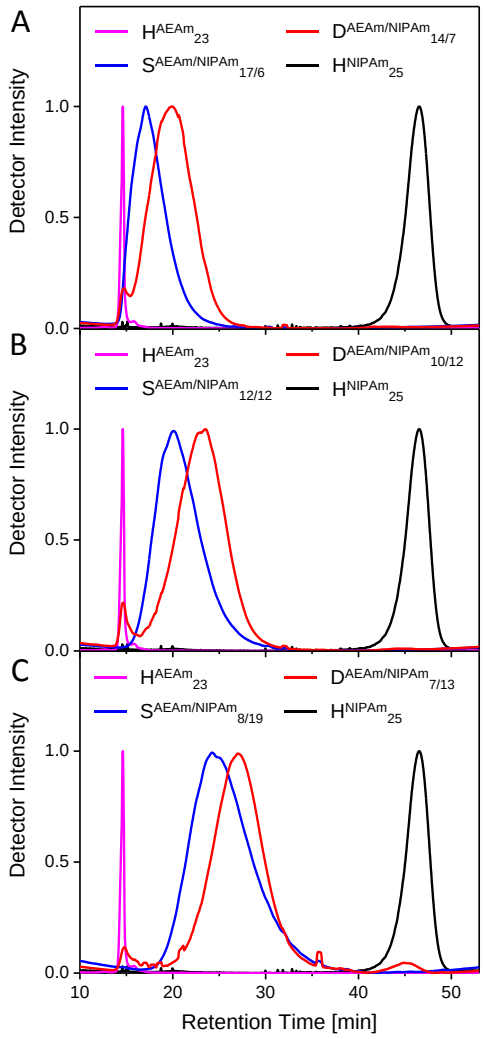


Figure 3. AEAm/NIPAm (DP = 25) with varying segmentation. HPLC chromatograms of copolymers (DP = 25) with various architecture for a ratio of AEAm/NIPAm of approximately A) 18/7, B) 12/13, C) 7/18. Homopolymers are included for references. Solvent: water/ACN. Gradient: 1 to 95 % ACN in 50 minutes at 37 ºC. Column: C18 (4.6 mm × 250 mm).

investigated by comparing GEAm/DMAm against the more hydrophilic GEAm/HEAm copolymeric system. All the copolymers studied were previously shown not to assemble in aqueous environment,[59] which should ensure that aggregation of the copolymers does not interfere with the separation process.

Statistical, tetrablock and diblock copolymers (DP = 40) were characterised using a gradient of either water/ACN (Figure 4) or water/MeOH (Figure S4). Homopolymers of p(GEAm)$_{40}$ ($H^{GEAm}{}_{40}$), p(DMAm)$_{40}$ ($H^{DMAm}{}_{40}$) and p(HEAm)$_{40}$ ($H^{HEAm}{}_{40}$) were used to optimise the solvent gradients. As expected, both p(GEAm-*co*-DMAm) and p(GEAm-*co*-HEAm) polymers show an elution pattern similar to that of the p(AEAm-*co*-NIPAm) system, in which the statistical polymer elutes first, followed by the multiblock and diblock copolymers. A better separation was obtained in the case of GEAm/DMAm copolymers compared to the GEAm/HEAm system, suggesting that decreasing the hydrophilicity of the co-monomer (exemplified by the respective homopolymer retention time) results in a better separation of the various copolymer segmentations. This is in accordance with the dramatically better separation obtained for the AEAm/NIPAm system, in which NIPAm is significantly less hydrophilic than DMAm and HEAm (Scheme 1). Interestingly, homopolymer p(GEAm)$_{40}$ ($rt_{water/ACN}$ equal to 23.59 ± 0.04 min) eluted significantly later than statistical copolymer p(GEAm$_{20}$-s-HEAm$_{20}$) ($rt_{water/ACN}$ equal to 21.72 min ± 0.02 min) in both water/ACN and water/MeOH systems. The errors associated with these results were calculated using the standard deviation of three separate repeat of the same measurement (Table S3). DLS study of p(GEAm$_{20}$-s-HEAm$_{20}$) previously showed an absence of large scale self-assembly for this copolymer in aqueous solvent.[52] This difference in retention time could then be explained by a difference in the overall polarity of the two polymers in solution. While the homopolymer p(GEAm$_{40}$) is charged along the entire chain, the presence of both charged and non-charged monomers in p(GEAm$_{20}$-s-HEAm$_{20}$) potentially results in an unimolecular conformation in solution where the two monomer are segregated to some extent. While this is expected to be minimal due to electrostatic repulsion, it might result in an increased polarity of the solvated polymeric chains.

AMPS/HEAm copolymeric system

The robustness of the method was tested using a copolymeric system consisting of an anionic monomer, 2-acrylamido-2-methyl-1-propane sulfonic acid (AMPS), and *N*-(2-hydroxyethyl)acrylamide (HEAm). Chromatograms of linear copolymers with various segmentations and various anionic

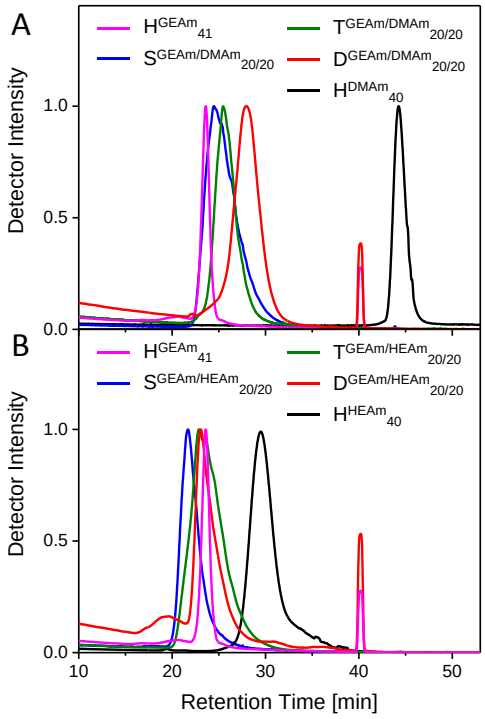
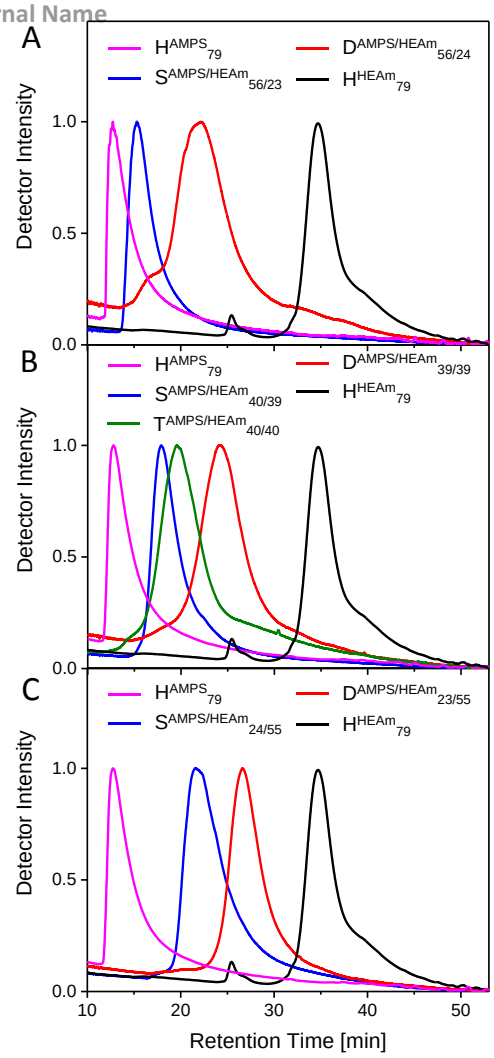


Figure 4. Influence of co-monomer hydrophobicity. HPLC chromatograms of copolymers (DP = 40) with various architecture for A) GEAm/DMAm copolymers, B) GEAm/HEAm copolymers. Homopolymers are included for references. Sharp peak at 40 min corresponds to residual CTA. Solvent: water/ACN. Gradient: 1 to 50 % ACN in 50 minutes at 37 ºC. Column: C18 (4.6 mm × 250 mm).

This journal is © The Royal Society of Chemistry 20xx

Please do not adjust margins

Please do not adjust margins



contents were recorded in both water/acetonitrile (Figure 5) and water/methanol (Figure S5). Homopolymers of $p(AMPS)_{80}$ (H^{AMPS}_{80}), $p(HEAm)_{80}$ (H^{HEAm}_{80}) were used to optimise the solvent gradients. For both mobile phase systems, the elution order for the various copolymers (statistical, octablock, tetrablock and diblock) is in accordance to what was observed for cationic copolymers. Overall, this demonstrates that the use of RP-HPLC for the characterisation of monomer distribution in copolymer is robust to dramatic structural changes in the polymeric chemical structure.

Figure 5. AMPS/HEAm (DP = 80) with varying segmentation. HPLC chromatograms of copolymers (DP = 80) with various architecture for a ratio of AMPS/HEAm of approximately A) 56/24, B) 40/40, C) 24/56. Homopolymers are included for references. Small peak at 25 min corresponds to an impurity in the monomer. Solvent: water/ACN. Gradient: 1 to 35 % ACN in 50 minutes at 37 ºC. Column: C18 (4.6 mm × 250 mm).

Finally, the influence of copolymer architecture, and whether the present method could also be used to characterise segmentation in more complex structure, such as highly branched polymers, was investigated. Star-shaped homopolymers[60] of AMPS and star-shaped copolymers of AMPS/HEAm prepared via an "arm-first approach", in which a previously-synthesised arm is chain extended in the presence of a multifunctional monomer that behaves as a cross-linker, were selected as they should allow direct comparison between the linear and star polymers.[53] It is noteworthy that these star homopolymers were not purified and therefore contain some unreacted linear homopolymers and copolymers which elute at 10 min and 13 min, respectively. Comparison of the linear homopolymers with their star-shaped equivalents show a significant increase in elution time for the star-shaped polymers (Figure S6). These results suggest that differences in architecture, which typically translates into differences in the ratio of hydrodynamic radius to molecular weight for a given molecule, have a significant effect on the retention time of the compound. While the small discrepancy between the column pore size (10 nm) and the size of the star polymers (1-2 nm) is expected to impact the interaction with the stationary phase, a decreased retention time would be expected from polymeric particles being too large to enter particle pores.[61] A better explanation lies in the availability of functional groups in the star polymer to interact with the column. The star polymers are crosslinked via the Z- end of the polymeric chains, thus presenting the R- group extremity at the star surface (Scheme 1). The mobile phase being acidic due to the addition of TFA, the carboxylic acid at the R- group of the chain transfer agent is protonated and, alongside with the two methyl groups, forms a less hydrophilic moiety than the rest of the charged polymeric chain. Hence, the results suggest that the close proximity of the arms in the star polymers creates steric hindrances that limit interaction of the stationary phase with the entire polymeric chain, favouring interactions with the functional group at the surface of the star instead. To confirm

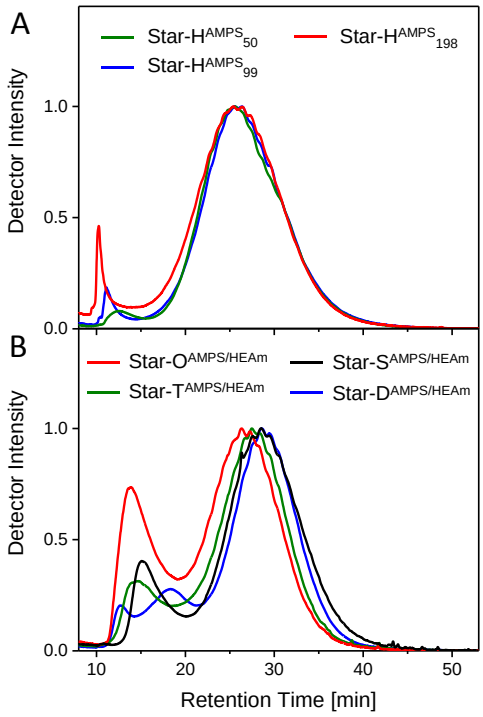


Figure 6. Star shaped anionic copolymers. HPLC chromatograms of a) star-shaped homopolymers of AMPS, b) star-shaped copolymers of AMPS/HEAm with various branch segmentation. Solvent: water/ACN. Gradient: 1 to 50 % ACN in 50 minutes at 37 °C. Column: C18 (4.6 mm × 250 mm).

Please do not adjust margins

Please do not adjust margins

this, cross-linked star homopolymers of AMPS with varying size (DP 50, 100, and 200) were compared. As expected, results showed a negligible difference in elution times (Figure 6), in contrast with data obtained for a library of linear homopolymers of AMPS with DP varying from 10 to 400 (Figure S7). For the later, differences in size for the lower DP homopolymers resulted in a significant shift in the elution time, which can be attributed to the increasing influence of the hydrophobic RAFT end group on the interaction with the stationary phase with decreasing size of the hydrophilic polymeric chain. In contrast, no clear difference in retention time was observed for H^{AMPS}_{100}, H^{AMPS}_{200}, H^{AMPS}_{400}, indicating that this effect becomes negligible above a certain molecular weight.

Star shaped copolymer with varying segmentation were also investigated (Figure 6, B). As expected, no clear separation could be obtained between the star polymers and a seemingly incoherent order of elution was observed instead. This confirms that above a certain branching threshold, interaction with the column are mostly driven by the functional group at the stars surface. Additionally, the broad nature of the elution peaks, associated with differences in the degree of cross-linking and the number of arms incorporated, is also expected to mask the potential differences in elution times otherwise observed for narrower peaks. Taken together, these results highlight a major limitation of the use of RP-HPLC for monomer dispersion characterisation in copolymers with larger branched architecture. While this steric-effect is expected to have a negative effect on the separation between various star polymers, it however highlights the potential utility of RP-HPLC as a technique to separate and potentially purify polymers with varying architectures.

Conclusions

RP-HPLC using a C18 column was successfully used to separate water-soluble linear polymers with varying monomer distribution. The study demonstrates that the elution pattern, statistical < multiblock < diblock, is consistent across a variety of copolymers, anionic or cationic. The separation of these copolymers is assumed to be due to a better repartition of the charges in the statistical copolymers as compared to the more segregated ones, thus reducing the affinity of the statistical for the hydrophobic C18 chains of the stationary phase. For a given mobile phase gradient, the separation of copolymers with varying segmentation was shown to increase with increasing molecular weight and decreasing comonomer hydrophilicity. The improved separation observed for AMPS/HEAm system in comparison with GEAm/HEAm systems demonstrates that the separation efficiency is however highly dependent on the choice of monomers, underlying that additional work is required to make the present technique quantitative. However, this study demonstrates that RP-HPLC can reliably be used as a qualitative tool to analyse copolymers with unknown distribution. For example, comparison of the retention time of an unknown copolymer with a known sequentially-synthesized diblock equivalent would give valuable information on the monomer distribution. In contrast, the method did not allow for separation of star-shaped copolymers with varying segmentation, possibly due to the close proximity of the chains impairing interaction with the column.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

We thank Lubrizol (CB), CSIRO (AK), the Royal Society Wolfson Merit Award (WM130055; SP) and the Monash-Warwick Alliance (LM, RP, SP) for financial support.

Notes and references

1. V. G. Kadajji and G. V. Betageri, *Polymers*, 2011, **3**, 1972-2009.
2. Y. Mai and A. Eisenberg, *Chem. Soc. Rev.*, 2012, **41**, 5969-5985.
3. X. Ding, C. Yang, T. P. Lim, L. Y. Hsu, A. C. Engler, J. L. Hedrick and Y. Y. Yang, *Biomaterials*, 2012, **33**, 6593-6603.
4. X. M. Mo, C. Y. Xu, M. Kotaki and S. Ramakrishna, *Biomaterials*, 2004, **25**, 1883-1890.
5. E. V. Batrakova and A. V. Kabanov, *J. Controlled Release*, 2008, **130**, 98-106.
6. G. Gody, R. Barbey, M. Danial and S. Perrier, *Polym. Chem.*, 2015, **6**, 1502-1511.
7. L. Martin, G. Gody and S. Perrier, *Polym. Chem.*, 2015, **6**, 4875-4886.
8. A. Anastasaki, C. Waldron, P. Wilson, C. Boyer, P. B. Zetterlund, M. R. Whittaker and D. Haddleton, *ACS Macro Lett.*, 2013, **2**, 896-900.
9. E. Baeten, J. J. Haven and T. Junkers, *Polym. Chem.*, 2017, **8**, 3815-3824.
10. G. Hattori, Y. Hirai, M. Sawamoto and T. Terashima, *Polym. Chem.*, 2017, **8**, 7248-7259.
11. M. S. Kent, M. Tirrell and T. P. Lodge, *Journal of Polymer Science Part B-Polymer Physics*, 1994, **32**, 1927-1941.
12. E. N. Govorun, A. R. Khokhlov and A. N. Semenov, *European Physical Journal E*, 2003, **12**, 255-264.
13. M. W. M. Fijten, J. M. Kranenburg, H. M. L. Thijs, R. M. Paulus, B. M. van Lankvelt, J. de Hullu, M. Springintveld, D. J. G. Thielen, C. A. Tweedie, R. Hoogenboom, K. J. Van Vliet and U. S. Schubert, *Macromolecules*, 2007, **40**, 5879-5886.
14. J. Zhang, R. Deubler, M. Hartlieb, L. Martin, J. Tanaka, E. Patyukova, P. D. Topham, F. H. Schacher and S. Perrier, *Macromolecules*, 2017, **50**, 7380-7387.
15. J. Moraes, R. Peltier, G. Gody, M. Blum, S. Recalcati, H.-A. Klok and S. Perrier, *ACS Macro Lett.*, 2016, **5**, 1416-1420.
16. K. Koschek, M. Dathe and J. Rademann, *ChemBioChem*, 2013, **14**, 1982-1990.
17. Y. Oda, S. Kanaoka, T. Sato, S. Aoshima and K. Kuroda, *Biomacromolecules*, 2011, **12**, 3581-3591.
18. D. Held and P. Kilz, *Macromolecular Symposia*, 2005, **231**, 145-165.
19. H. G. Barth, *Adv. Chem. Ser.*, 1986, 31-55.

 This journal is © The Royal Society of Chemistry 20xx

Please do not adjust margins

Please do not adjust margins

20. A. M. Striegel, in *Multiple Detection in Size-Exclusion Chromatography*, American Chemical Society, 2004, vol. 893, ch. 1, pp. 2-12.
21. R. W. Lewis, R. A. Evans, N. Malic, K. Saito and N. R. Cameron, *Polym. Chem.*, 2017, **8**, 3702-3711.
22. S. Kadokura, T. Miyamoto and H. Inagaki, *Die Makromolekulare Chemie*, 1983, **184**, 2593-2601.
23. I. V. Blagodatskikh, M. V. Sutkevich, N. L. Sitnikova, N. A. Churochkina, T. A. Pryakhina, O. E. Philippova and A. R. Khokhlov, *J. Chromatogr. A*, 2002, **976**, 155-164.
24. P. J. C. H. Cools, F. Maesen, B. Klumperman, A. M. van Herk and A. L. German, *J. Chromatogr. A*, 1996, **736**, 125-130.
25. G. Glockner, D. Wolf and H. Engelhardt, *Chromatographia*, 1994, **38**, 749-755.
26. A. Snijder, B. Klumperman and R. Van der Linde, *Journal of Polymer Science Part a-Polymer Chemistry*, 2002, **40**, 2350-2359.
27. N. Fandrich, J. Falkenhagen, S. M. Weidner, B. Staal, A. F. Thuenemann and A. Laschewsky, *Macromol. Chem. Phys.*, 2010, **211**, 1678-1688.
28. G. Glöckner and A. H. E. Müller, *J. Appl. Polym. Sci.*, 1989, **38**, 1761-1774.
29. H. Pasch, E. Esser, C. Kloninger, H. Iatrou and N. Hadjichristidis, *Macromol. Chem. Phys.*, 2001, **202**, 1424-1429.
30. W. Lee, H. Lee, J. Cha, T. Chang, K. J. Hanley and T. P. Lodge, *Macromolecules*, 2000, **33**, 5111-5115.
31. K. Baran, S. Laugier and H. Cramail, *Journal of Chromatography B: Biomedical Sciences and Applications*, 2001, **753**, 139-149.
32. C. Y. Ryu, J. Han and W. S. Lyoo, *J. Polym. Sci., Part B: Polym. Phys.*, 2010, **48**, 2561-2565.
33. S. Teramachi, A. Hasegawa, Y. Shima, M. Akatsuka and M. Nakajima, *Macromolecules*, 1979, **12**, 992-996.
34. G. Glöckner and D. Wolf, *Chromatographia*, 1992, **34**, 363-368.
35. J. V. Dawkins, T. A. Nicholson, A. J. Handley, E. Meehan, A. Nevin and P. L. Shaw, *Polymer*, 1999, **40**, 7331-7339.
36. S. P. Reid, E. Meehan and J. V. Dawkins, *J. Liq. Chromatogr.*, 1994, **17**, 3043-3053.
37. H. Pasch and K. Rode, *Macromol. Chem. Phys.*, 1996, **197**, 2691-2701.
38. W. Lee, S. Park and T. Chang, *Anal. Chem.*, 2001, **73**, 3884-3889.
39. S. Phillips and S. V. Olesik, *Anal. Chem.*, 2002, **74**, 799-808.
40. S. L. Phillips, L. Ding, M. Stegemiller and S. V. Olesik, *Anal. Chem.*, 2003, **75**, 5539-5543.
41. D. Berek, *Macromolecular Symposia*, 1996, **110**, 33-56.
42. H. Pasch, C. Brinkmann, H. Much and U. Just, *J. Chromatogr. A*, 1992, **623**, 315-322.
43. H. Pasch, M. Augenstein and B. Trathnigg, *Macromol. Chem. Phys.*, 1994, **195**, 743-750.
44. Y. Z. Wei, Y. F. Chu, E. Uliyanchenko, P. J. Schoenmakers, R. X. Zhuo and X. L. Jiang, *Polym. Chem.*, 2016, **7**, 7506-7513.
45. H. Pasch, Y. Gallot and B. Trathnigg, *Polymer*, 1993, **34**, 4986-4989.
46. H. Pasch, *Polymer*, 1993, **34**, 4095-4099.
47. H. Pasch, M. Adler, D. Knecht, F. Rittig and R. Lange, *Macromolecular Symposia*, 2005, **231**, 166-177.
48. H. Lee, W. Lee, T. Chang, S. Choi, D. Lee, H. Ji, W. K. Nonidez and J. W. Mays, *Macromolecules*, 1999, **32**, 4143-4146.
49. G. Jia and Q.-H. Wan, *J. Chromatogr. A*, 2015, **1395**, 129-135.
50. R. Epping, U. Panne and J. Falkenhagen, *Anal. Chem.*, 2017, **89**, 1778-1786.
51. A. Kuroki, P. Sangwan, Y. Qu, R. Peltier, C. Sanchez-Cano, J. Moat, C. G. Dowson, E. G. L. Williams, K. E. S. Locock, M. Hartlieb and S. Perrier, *ACS Appl. Mater. Interfaces*, 2017, **9**, 40117-40126.
52. L. Martin, R. Peltier, A. Kuroki, J. Town and S. Perrier, *Biomacromolecules*, 2017, DOI: 10.1021/acs.biomac.1028b00146.
53. C. Bray, R. Peltier, H. Kim, A. Mastrangelo and S. Perrier, *Polym. Chem.*, 2017, **8**, 5513-5524.
54. M. F. Cunningham, *Progress in Polymer Science*, 2008, **33**, 365-398.
55. G. Gody, T. Maschmeyer, P. B. Zetterlund and S. Perrier, *Macromolecules*, 2014, **47**, 639-649.
56. X. Hu, Y. Wang, L. Zhang, M. Xu, W. Dong and J. Zhang, *Carbohydr. Polym.*, 2017, **174**, 171-181.
57. P. Gurnani, C. Bray, R. A. E. Richardson, R. Peltier and S. Perrier, *Macromolecular Rapid Communications*, 2018.
58. M. Gilar, A. Jaworski and T. S. McDonald, *J. Chromatogr. A*, 2014, **1337**, 140-146.
59. E. F. Banwell, E. S. Abelardo, D. J. Adams, M. A. Birchall, A. Corrigan, A. M. Donald, M. Kirkland, L. C. Serpell, M. F. Butler and D. N. Woolfson, *Nat. Mater.*, 2009, **8**, 596-600.
60. J. M. Ren, T. G. McKenzie, Q. Fu, E. H. H. Wong, J. Xu, Z. An, S. Shanmugam, T. P. Davis, C. Boyer and G. G. Qiao, *Chem. Rev.*, 2016, **116**, 6743-6836.
61. J. D. Pearson, N. T. Lin and F. E. Regnier, *Anal. Biochem.*, 1982, **124**, 217-230.

Please do not adjust margins