International Journal of Pharmaceutics 408 (2011) 213–222

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Impact of polymer tacticity on the physico-chemical behaviour of polymers proposed as therapeutics

Lorella Izzo[a,*], Peter C. Griffiths[b,**], Renuka Nilmini[b], Stephen M. King[c], Kerri-Lee Wallom[d,1], Elaine L. Ferguson[d,2], Ruth Duncan[b,d]

[a] Dipartimento di Chimica e Biologia, Università degli Studi di Salerno, 84084 Fisciano, Salerno, Italy
[b] School of Chemistry, Cardiff University, Main Building, Park Place, Cardiff CF10 3AT, UK
[c] STFC ISIS Facility, Rutherford Appleton Laboratory, Didcot OX11 0QX, UK
[d] Centre for Polymer Therapeutics, Welsh School of Pharmacy, Redwood Building, King Edward's Avenue, Cardiff CF10 3XF, UK

ARTICLE INFO

Article history:
Received 18 October 2010
Received in revised form 28 January 2011
Accepted 4 February 2011
Available online 20 February 2011

Keywords:
Polymer stereochemistry
Polymer conformation
Polymer therapeutics
Poly(methacrylic acid)

ABSTRACT

Although water-soluble polymers are finding increasing use as polymer therapeutics, there has been little consideration of the effect of polymer stereochemistry on their physico-chemical and biological properties. The aim of this study was to investigate these properties using polymethacrylic acids (PMAs) of similar molecular weights with a difference in syndiotacticity of about 20% of *rr* triad content. Experiments to characterize the solution behaviour were conducted at pHs encountered during the transport, endocytic uptake and intracellular trafficking (7.4–3.0). These showed that with increasing *rr* triads, the polymer become less hydrophobic, a stronger acid, displayed a locally ordered solution conformation at pH 5.5, and interacted more strongly with dodecyl trimethylammonium bromide (DTAB) micelles. Preliminary cytotoxicity experiments using B16F10 melanoma cells showed lower toxicity in the concentration range of 1–100 μg/mL with increased *rr* triads. These observations indicate that the higher content of rr triads could drive a chain organization that minimize the influence of negative charges and so underline the importance of further, systematic studies to investigate the effect of tacticity on the behaviour of polymers in respect of their pharmacokinetics, toxicity and efficacy.

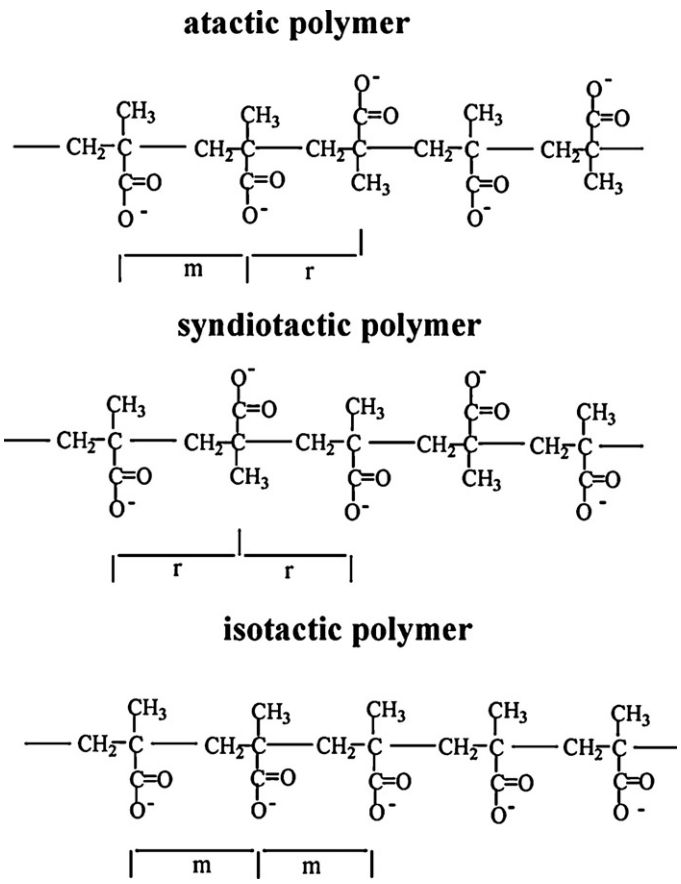
1. Introduction

Over the last three decades functional polymers have been finding increasing use as polymer-therapeutics (reviewed in Duncan, 2003; Vicent and Duncan, 2009). Polymers are being used as therapeutics with intrinsic activity, as platforms for delivery of drugs and proteins, and as non-viral vectors to promote cytosolic delivery of macromolecular drugs. Considering the increasing number of synthetic polymers that have entered clinical development there has been little consideration of the potential effect of polymer stereochemistry on their physico-chemical and biological properties and the implications for the safety/efficacy of the medicines to which they relate. The aim of this study was to investigate the effect of polymer tacticity on physico-chemical and biological properties relevant to the use of polymer therapeutics using poly(methacrylic acid) (PMA) as a model polymer.

Early studies involving the anionic polyelectrolyte PMA showed that, like other polyanions, it displays antiviral activity (De Somer et al., 1968). More recently, PMA has demonstrated the ability to reduce, and even prevent completely, protein thermo-aggregation. This is interesting as this phenomenon is very important for induction of neurodegenerative diseases like Alzheimer's disease, bovine spongiform encephalopathy and Huntington disease (Shalova et al., 2005). From the chemical perspective, the PMA backbone is characterized by the presence of quaternary carbon atoms bearing methyl and carboxylic groups, and thus it can be present with two stereoregular forms, syndiotactic and isotactic, as well as a non-stereoregular atactic form. The simplified Fisher projections (Scheme 1) show that placement of substituent groups in the isotactic structure corresponds to a *meso* or a *m*-placement of two consecutive monomer units (diad). The syndiotactic structure corresponds to a *racemic* or *r*-placement (Frisch et al., 1966). A sequence of three monomer units is called a triad, the isotactic triad is *mm*, the heterotactic triad is *mr*, and the syndiotactic one is *rr*. The prevalence of *mm* or *rr* triads along the polymer

* Corresponding author. Tel.: +39 089 969561; fax: +39 089 969603.
** Corresponding author. Tel.: +44 029 20875858; fax: +44 029 20874030.
E-mail addresses: lizzo@unisa.it (L. Izzo), griffithspc@cf.ac.uk (P.C. Griffiths).
[1] Current address: Department of Pharmacology, University of Oxford, Mansfield Road, Oxford, OX1 3QT, UK.
[2] Current address: Wound Biology Group, School of Dentistry, Cardiff University, Heath Park, Cardiff CF14 4XY, UK.

0378-5173/$ – see front matter © 2011 Published by Elsevier B.V.
doi:10.1016/j.ijpharm.2011.02.003



Scheme 1. Nomenclature of triads relating to atactic, syndiotactic and isotactic PMA.

backbone confers the iso- or syndio-tacticity to the polymer chain respectively. PMA has been already reported to exhibit different solution properties in the isotactic and atactic form. For example, the isotactic PMA is a weaker acid than the atactic, it is insoluble in water below a critical degree of neutralization and shows an irreversible potentiometric behaviour. Furthermore, in the presence of a cationic surfactant such as cetylpyridinium chloride (CPC), the interactions of isotactic chains with CPC starts at a lower surfactant concentration (CAC) than in the case of atactic PMA (van den Bosch et al., 2004; Loebl and O'Neill, 1960; Leyte et al., 1972; Vlachy et al., 2006; Ruiz-Pérez et al., 2008).

The aim of this study was to investigate the physico-chemical behaviour in aqueous solution of atactic and syndiotactic PMAs obtained from hydrolysis of PMMAs of average molecular weight in the range of 43–48 kDa that had slightly increasing amounts (from 60% to 80%) of *rr* triads. In both cases, there were very low amounts of isotactic *mm* triads. Small-angle neutron scattering (SANS) was used to study polymer conformation in solution, whilst surface tension measurement, electron paramagnetic resonance (EPR) and SANS were used to investigate the interfacial interaction with the cationic surfactant DTAB in a unimolecular and micellar form, and consequently to define the effect of increasing stereoregularity on the aggregation behaviour of PMA. These characteristics have been interpreted in terms of the charge on the polymer, quantified by pH titration. Preliminary experiments were also undertaken to investigate the biological properties (cytotoxicity and cell uptake using B16F10 cells) of these PMAs.

2. Materials and methods

2.1. Materials

Syndiotactic polymethylmethacrylate (PMMA) (M_w = 43 kDa, M_w/M_n = 1.02) and N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-Gly-Gly-ONp (4.19 mol% activated groups, M_w = 30 kDa) were from Polymer Laboratories, UK, and used without further purification. Toluene (SigmaAldrich, Steinhem, Germany) was dried over sodium and distilled before use. Methylmethacrylate (MMA) (SigmaAldrich, Steinhem, Germany) was treated with calcium hydride for 4 h and distilled under vacuum. Cu(I)Br (Aldrich, Steinhem, Germany), 2,2′-bipyridine (Aldrich, Steinhem, Germany), 2-bromopropionitrile (BPN) (Aldrich, Steinhem, Germany), methanol (Aldrich, Steinhem, Germany), DTAB (Aldrich, analytical Grade), 16-doxyl stearic methyl ester (16-DSE) (Fluka), optical grade dimethyl sulfoxide (DMSO) (SigmaAldrich, Steinhem, Germany), 1-ethyl-3-(3-dimethylaminopropyl carbodiimide hydrochloride) (EDC) (SigmaAldrich, Steinhem, Germany), 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) (SigmaAldrich, St. Louis, MO), N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) (Fluka), and Oregon Green 488 cadaverine *5-isomer (OG-488-C) (Invitrogen Molecular Probes, Eugene,OR) were all used as received. B16F10 murine melanoma cells were from the American Type Culture Collection (ATCC) (Virginia, USA). Fetal calf serum (FCS), 0.05% (w/v) trypsin–0.53 mM EDTA and RPMI 1640 with GlutaMAX were from Invitrogen Life Technologies (Paisley, UK).

2.2. Synthesis of polymers and their precursors

The stereoregular and atactic samples of PMA were obtained by hydrolysis of the syndiotactic and atactic PMMA respectively. The former was the commercially available product, and the latter was synthesized as described below.

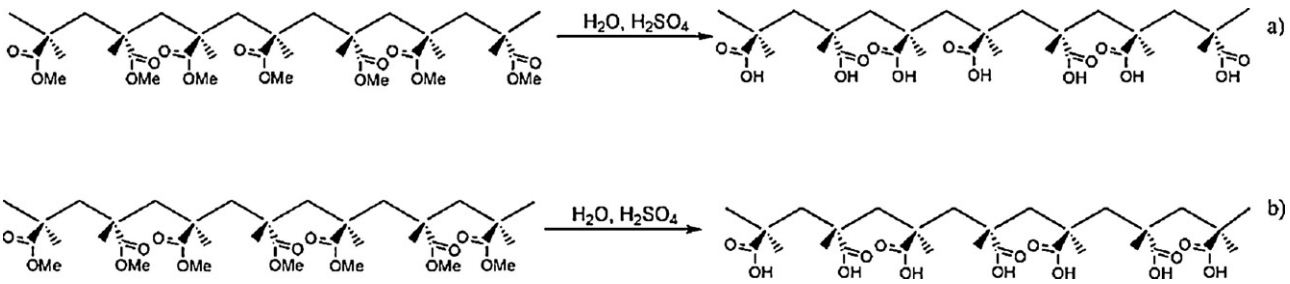
2.2.1. Synthesis of atactic PMMA

Atactic PMMA was synthesized using the atom transfer radical polymerization (ATRP) (Wang and Matyjaszewski, 1995). Polymerization was carried out at 90 °C in a 100 mL thermostated glass-flask equipped with a magnetic stirrer. Toluene (20 mL), Cu(I)Br, 2,2′-bipyridine, BPN and MMA were added sequentially under a nitrogen atmosphere (molar ratio Cu(I)Br:BPN:2,2′-bipyridine:MMA = 1:1:3:100). The reaction was stopped after 4 h by pouring the mixture into methanol. The polymer was recovered by filtration, purified over an aluminium oxide column and dried under vacuum. Both the atactic and syndiotactic PMMA were characterized by ^{1}H NMR and GPC.

^{1}H NMR (400 MHz, $CDCl_3$). Atactic PMMA: δ (ppm) 0.8–1.3 (3H, CH_3), 1.8–2.0 (2H, CH_2), 3.6 (s, 3H, OCH_3). Syndiotactic PMMA: δ (ppm) 0.8–1.0 (3H, CH_3), 1.8 (s, 2H, CH_2), 3.6 (s, 3H, OCH_3).

2.2.2. Preparation of syndiotactic and atactic PMA

Syndiotactic and atactic PMA were obtained by hydrolysis of syndiotactic and atactic PMMA respectively (as described in van den Bosch et al., 2004) (Scheme 2). Typically, PMMA was dissolved in sulphuric acid, the solution was thermostated at 0 °C and water was added dropwise. Anhydride groups were eliminated by refluxing the mixture. The mixture was then dialyzed (molecular weight cut-off 2000 Da) in double-distilled water (ddH_2O) and freeze-dried. The complete transformation of the two PMMA samples into the respective acid form was verified by ^{1}H NMR. In all cases, the degree of hydrolysis was found to be higher than 95%, and within error, the same for both polymers.



Scheme 2. Hydrolysis of PMMAs: (Panel a) atactic and (panel b) syndiotactic polymers.

^{1}H NMR (D_2O). Syndiotactic and atactic PMA: δ (ppm) 0.8–1.2 (3H, CH_3), 1.9–2.0 (2H, CH_2), ~13.0 (1H, COOH).

2.2.3. ^{1}H NMR characterization

^{1}H NMR spectra were recorded on a Bruker AV400 operating at 400 MHz in the Fourier Transform mode and at 293 K. The samples (10 mg) were dissolved in 0.5 mL of $CDCl_3$, or D_2O. Tetramethylsilane (TMS) was used as an internal chemical shift reference.

2.2.4. GPC

Gel permeation chromatography measurements were recorded on the system equipped with a Waters 1525 binary pump, a Waters 2414 RI detector and four Styragel columns (range 10^3–10^6 Å). The measurements were carried out at 25 °C using THF as eluent (1.0 mL min^{-1}) and polystyrene standards as references.

2.3. Interaction with model surfaces

2.3.1. Measurement of surface tension

Surface tension measurements were made using a maximum bubble pressure tensiometer (SITA Online t60) with bubble lifetime of 15 s. The instrument was calibrated using distilled water and checked for linearity using water/ethanol mixtures. In all cases, measurements were made at 25 ± 0.5 °C. Stock surfactant solutions were prepared by dissolving the appropriate mass of surfactant in distilled water to produce a total surfactant concentration of 100 mM. Stock solutions of atactic PMA and syndiotactic PMA 0.4% (w/v) in double distilled water were also prepared, and these solutions mixed in appropriate ratios to obtain solutions of constant polymer concentration with varying surfactant concentration. The surface tension of these polymer/surfactant solutions was then measured after temperature equilibration (10 min). The measurements were conducted at pH 7.4 and 5.5. The data were averaged on three measurements.

2.3.2. Measurement of micelle microviscosity

EPR was used to study the effect of pH and ionic strength on the microviscosity of DTAB micelles in the absence and presence of the polymers. First, spin-probe containing micelles were prepared by addition of 5 mL of DTAB solution (25 mM in distilled water) to glass vials containing an aliquot of a pre-dried spin-probe 16-DSE acetone solution (0.04 mg/mL) such that the concentration ratio of DTAB micelles to spin-probe was ~500:1. The solution was thoroughly mixed and allowed to equilibrate for 1 h. The pH was adjusted by addition of HCl. Polymer/surfactant mixtures were prepared in an analogous fashion by adding DTAB (50 mM; 2.5 mL) and PMA (0.4%, w/v; 2.5 mL) to a glass vial containing pre-dried spin-probe (16-DSE) solution. The pH of the solution was also adjusted by the addition of HCl. For the EPR measurements, an aliquot of the sample was drawn into a capillary tube that was sealed and placed in a quartz EPR tube.

Measurements at room temperature (~22 °C) were recorded on a Bruker EMX using a frequency of 9.29 ± 0.3 GHz. Each spectrum was recorded as the average of five scans. The resultant spectra were transferred to a PC and the line shapes fitted to a Voigt approximation to separate the Gaussian and Lorentzian components of the spectral lines (using LOWFIT) and to locate the resonance field of the three EPR lines to a precision of a few mG. The separation (A^+) of the low and centre lines ($M_I = +1$ and $M_I = 0$) is directly related to the polarity index H (25 °C), defined as the molar ratio of OH groups in a given volume relative to water:

$$A^+ = 14.309 + 1.419H(25\,^\circ\mathrm{C}) \quad (1)$$

For simple surfactant solutions such as DTAB, H (25 °C) corresponds directly to the volume fraction of water in the polar shell and is a sensitive measure of the interaction between a polymer and micelle surface (Griffiths et al., 2004).

2.4. Small-angle neutron scattering (SANS)

Polymer solutions (2%, w/v) were prepared in D_2O and as appropriate, either deuterated or hydrogenous DTAB was added to yield a final surfactant concentration of 5 mM or 100 mM. In all cases, the pH was adjusted to the required value (i.e. pH 7.4, 5.5 or 3.0). The pH values were chosen considering the pHs encountered by polymers therapeutics during the transport, endocytic uptake and intracellular trafficking. Solutions of atactic and syndiotactic PMA (2%, w/v) were also prepared in D_2O at pH 7.4, 5.5 and 3.0. All SANS measurements were performed on the LOQ diffractometer at the ISIS Spallation Neutron Source, Oxfordshire. The scattering data were normalized for the sample transmission and incident wavelength distribution and corrected for the linearity and efficiency of the detector response. LOQ is a fixed geometry, time-of-flight (TOF) instrument and using wavelengths between 2 and 10 Å, spans a Q-range ~0.01–0.3 Å. All the experiments were conducted at 37 °C.

2.5. Evaluation of cytotoxicity

An MTT assay (as described in Ranucci et al., 1991) was used to compare the *in vitro* cytotoxicity of syndiotactic and atactic PMAs towards B16F10 cells, using HPMA copolymer-Gly-Gly-COOH (4.19 mol% carboxylic groups; M_w = 30 kDa) as a reference control. First, to obtain the HPMA copolymer-Gly-Gly-COOH, HPMA copolymer-Gly-Gly-ONp (~100 mg) was dissolved in ddH_2O and 10 molar equivalents of a solution of NaOH (0.1 M) was added and stirred for 4–5 h at room temperature. The resultant product was then dialysed (molecular weight cut-off 2000 Da) in ddH_2O to remove free ONp. The product was isolated and freeze-dried.

For the MTT assay, cells were seeded into sterile 96-well microtitre plates (1×10^4 cells/mL) in 0.1 mL/well of media containing FCS (10%, v/v) and allowed to adhere for 24 h. During culture and throughout the experiment, cells were grown at 37 °C, in humified atmosphere with 5% CO_2. The medium was then removed and polymer-containing media (0.2 μm filter-sterilized, pH 6.8–8.2) was added to the cells. After a further 67 h incubation, MTT (20 μL of a 5 mg/mL solution in PBS) was added to each well and the cells

were incubated for a further 5 h. The medium was then removed and the precipitated crystals solubilized by addition of optical grade DMSO (100 μL) over 30 min. Absorbance was measured at 550 nm using a microtitre plate reader. Cell viability was expressed as a percentage of the viability of untreated control cells. The IC_{50} values were expressed as mean ± SEM.

2.6. Cell association of PMA–OG conjugates by B16F10 cells

The PMA samples were labelled with OG-488-C and purified using the methodology described previously (Ranucci et al., 1991; Richardson et al., 2008). Briefly, PMA (10 mg) was dissolved under stirring in the minimum amount of a phosphate buffer solution (pH 7.4) in a 10 mL round-bottomed flask. EDC (20 molar equiv.) and sulfo-NHS (20 molar equiv.) were added and allowed to dissolve under stirring. Then OG-488-C was added in a 1:1 molar ratio with polymer, and the pH of the solution increased to pH to 9–10 with 0.5 M NaOH. The reaction mixture was stirred in the dark for 5 h, and monitored by thin layer chromatography (TLC). The conjugates were purified by GPC using a disposable PD-10 desalting column containing Sephadex G-25 equilibrated with PBS and characterized prior and after purification by the fluorescence analysis of each SEC fraction. The total OG-488-C content of each probe was determined spectrophotometrically at 485 nm, and the free OG-488-C content measured by using PD-10 re-analysis. Bound OG-488-C was expressed as the percentage of total fluorescence intensity. To study cell association, B16F10 cells were seeded in 6-well plates at a density of 5×10^5 cells/mL in complete clear media and incubated for 24 h to adhere. The medium was then changed for PMA diluted in media (RPMI 1640 with 10% fetal calf serum) and the cells incubated for 1 h at 37 °C. During culture and throughout the experiment, cells were grown in humified atmosphere with 5% CO_2. At each sample time cells were placed on ice, then washed three times with ice-chilled PBS, and PBS (1 mL) was added before the cells were scraped from the plate with a rubber policeman and collected in FACS tubes. After centrifugation at 4 °C ($600 \times g$ for 10 min) the cell pellet was re-suspended in ice-chilled PBS (200 μL), and cell-associated fluorescence determined using a Becton Dickinson FACSCalibur cytometer (CA, USA) equipped with an argon laser (488 nm) with emission filter at 550 nm (10,000 counts per sample). Data were analysed using CELLQuest™ version 3.3 software, the same media – 1640 with 10% fetal calf serum – without PMA added, was used as a control. Cells incubated without polymer were used as background with the fluorescence plot gated for positive (M2) and negative cells (M1). Data are expressed as:

$$\text{cell associated fluorescence} = \frac{\%\,\text{cells that are positive} \times \text{geometric mean of the fluorescence}}{100}$$

3. Results and discussion

Conformational behaviour of the polymers is strongly influenced by the nature and the net charge on the macromolecular chain. Moreover, the tacticity can play an important role in the conformational organization of the chain, such that the physico-chemical and biochemical properties of polymers with different stereochemistry can significantly change with pH (or ionic strength). It is thus surprising that the stereochemistry of polymeric excipients and polymer therapeutics is so rarely discussed.

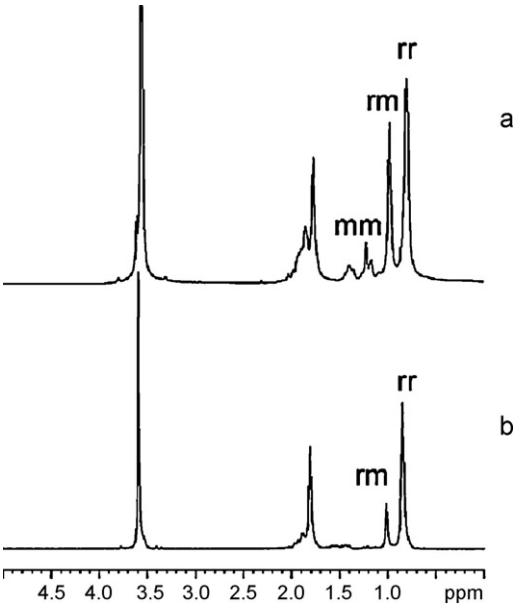


Fig. 1. ^{1}H NMR of PMMAs in $CDCl_3$. (Panel a) Atactic and (panel b) syndiotactic PMMAs. The peaks of the methyl groups are separated in accordance with the three monomeric sequence (triad) distribution along the chain and are indicated with *rr* (syndio), *rm* (hetero) and *mm* (iso).

PMMAs with different degrees of stereoregularity, but similar molecular weight were used here as starting materials. ^{1}H NMR allowed determination of the triad ratio *rr*:*mr*:*mm* (signals seen at 0.85 ppm, 1.02 ppm and 1.26 ppm) and this was 80:17:3 and 59:32:9 for syndiotactic and atactic samples respectively (Fig. 1 and Table 1). The difference of syndiotactic *rr* sequence in the two polymers was not very high, but the atactic PMMA had a typical triad distribution for a PMMA synthesized using ATRP (Kamigaito et al., 2001).

Water-soluble PMAs were then obtained by sulphuric acid hydrolysis of these samples(Scheme 2) (van den Bosch et al., 2004). ^{1}H NMR performed on these PMAs confirmed that the degree of hydrolysis was >95%.

The effect of PMA stereochemistry on solution behaviour has been discussed in relation to local polymer chain conformation which influences the charge density, the hydrophobicity and the chain flexibility (Jerman et al., 2007). It has also been reported that isotactic PMA is a weaker acid than the atactic and syndiotactic forms (Nagasawa et al., 1965). This difference was explained by assuming that the isotactic PMA has a "locally" helical structure in solution, whilst the syndiotactic, similar to atactic, had a more rod-like conformation. Furthermore, the syndiotactic polymer was proposed to have a locally planar zigzag conformation leading to a lower charge density than the isotactic form. However, most studies referring to atactic or stereoregular PMAs do not state the precise stereochemical composition or polymer molecular weight. Here we used two PMAs of similar molecular weight with

Table 1
Characteristics of PMMAs used as starting materials.

PMMA	M_w (kDa)	M_w/M_n	Isotactic % (*mm*)[a]	Heterotactic % (*mr*)[a]	Syndiotactic % (*rr*)[a]
Atactic	48	1.54	9	32	59
Syndiotactic	43	1.02	3	17	80

[a] Triad arrangements (sequence of three MMA monomers in the chain).

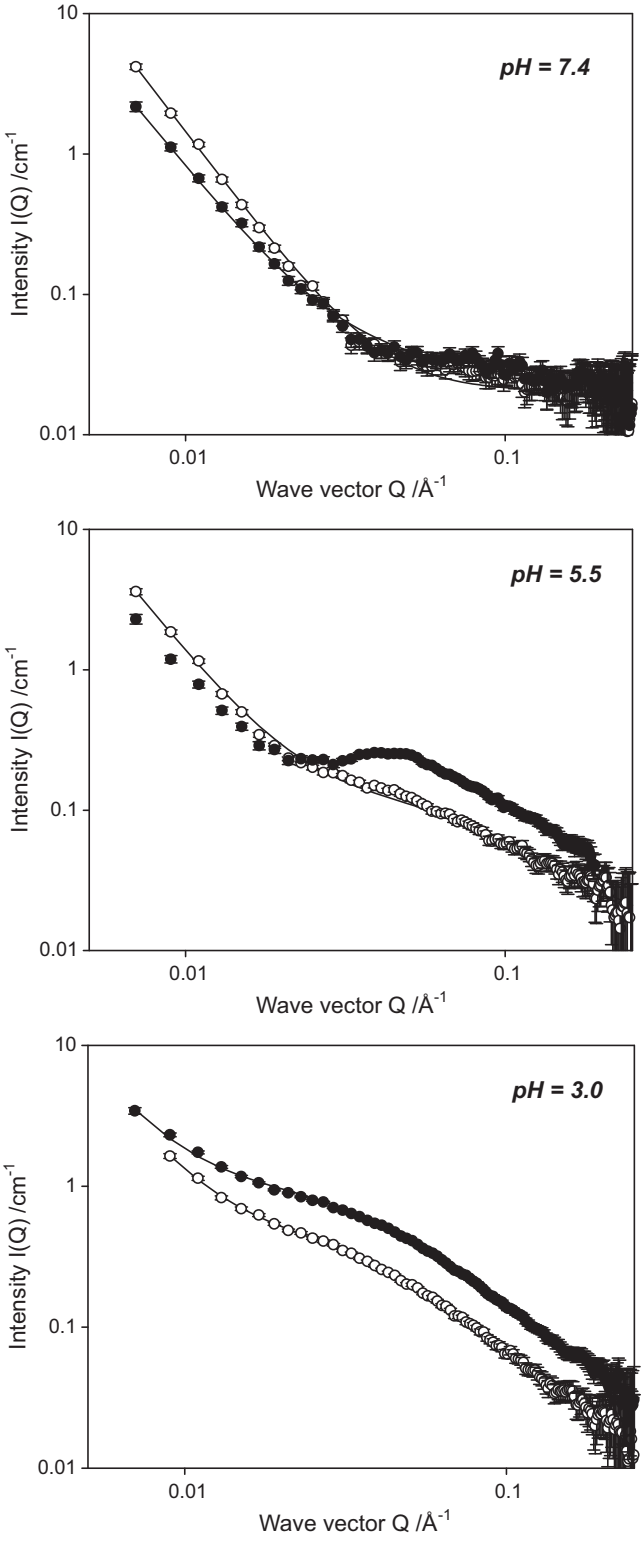


Fig. 2. The effect of pH on the SANS from aqueous 2% (w/v) solutions of atactic (open circles) and syndiotactic PMA (filled circles).

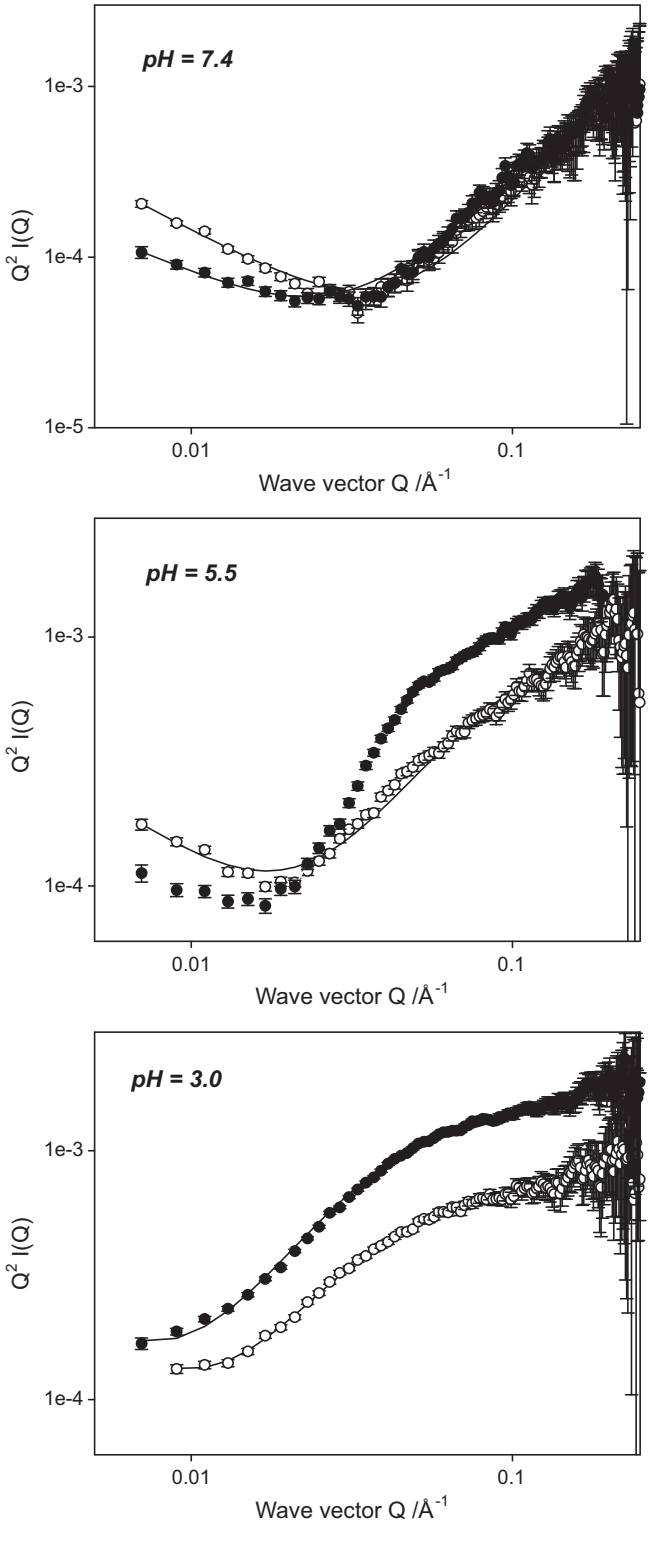


Fig. 3. Kratky representation [$Q^2I(Q)$] of the data shown in Fig. 2. The effect of pH on the scattering from atactic PMA (open circles) and syndiotactic PMA (filled circles).

well-defined stereochemistry to investigate the effect of polymer triad content.

First, physico-chemical properties in aqueous solution were investigated. At a concentration of 1×10^{-5} M both PMAs were weakly acidic as expected, but their pH was slightly different; pH = 5.85 for the atactic form and pH = 5.21 for the syndiotactic form. Titration showed the syndiotactic PMA to be a stronger acid than the atactic one (See Supplementary Material), an observation consistent with the literature (Nagasawa et al., 1965; Vlachy et al., 2006) and the ambient pH of the stock solutions. Given that >95% of the ester pendant groups of starting PMMAs were hydrolyzed, and that the two samples have similar molecular weight, it is reasonable that these variables would not justify the different acidity, suggesting that it is the higher *rr* triad content that promotes a

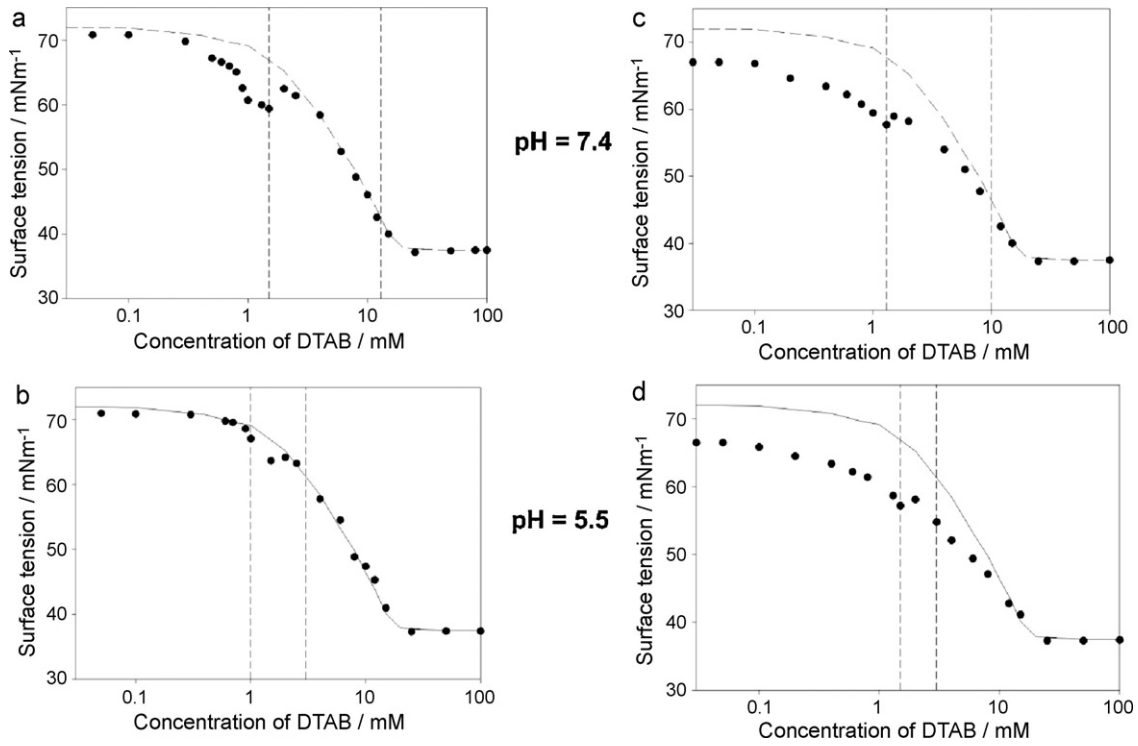


Fig. 4. Concentration dependence of the surface tension of 0.4% (w/v) wt% syndiotactic (panels a and b) and atactic (panels c and d) PMAs in presence of DTAB at pH 7.4 and 5.5. The continuous lines indicate the surface tension of a simple DTAB solution. The vertically bounded regions indicate the "cloudiness zone", as discussed in the text.

greater dissociation of the pedant acidic groups. This could be rationalized by the higher content of *rr* triads inducing the formation of a more extended trans-trans conformation of the polymer chains in solution (Nagasawa et al., 1965), thus minimizing the influence of adjacent negative charges, and stabilizing the arrangement of the carboxylate groups through inter and/or intra-molecular salt bridges.

At high pH, both PMAs showed similar solubility, but with decreasing pH, the atactic PMA become far less soluble than the syndiotactic one. It has been previously reported that isotactic PMA is less soluble than the atactic form, at least at low degrees of neutralization (Loebl and O'Neill, 1960), so it appears that the water solubility of PMA, or its hydrophilicity, increases with increasing *rr* triad content. Obviously the acid-base properties, and the water solubility are both important factors that could significantly influence the biological behaviour of polymeric excipients and/or polymer therapeutics.

The results obtained when the atactic and syndiotactic PMAs (in D_2O) were studied by SANS at different pH (37 °C) are shown in Fig. 2. At pH = 7.4 ($0.8 < \alpha < 0.9$, where α is the degree of neutralization), the scattering obtained from both highly charged PMAs showed a Q^{-n} dependence on Q, albeit with a slight difference in slope, where $n = 2.8$ for the syndiotactic PMA and $n = 3.0$ for the atactic one. This behaviour is normally attributed to a mass fractal morphology. At this pH, a rod-like morphology would be expected, which would show a Q^{-1} dependence. In contrast, at pH = 5.5 ($\alpha < 0.3$) a significant difference was obvious in the two datasets. There remained a weak signature of the initial linear approximate Q^{-3} relationship, but there was a change in the form of the scattering around $Q = 0.04\,Å^{-1}$. For the syndiotactic PMA the discontinuity around $Q = 0.04\,Å^{-1}$ was more pronounced, indicating either the presence of a weak structure peak or the adoption of a different solution conformation. The initial Q dependence was now much weaker. At pH = 3.0, the scattering attained a form that was more akin to that expected for a Gaussian coil, albeit with a weak Q^{-3} upturn at low Q. The fits to the data, using a model comprising the Debye form factor (Gaussian coil) plus a Q^{-3} term, yielded $R_g = 4.0\ (\pm 0.5)$ nm for both the atactic and syndiotactic polymers at this pH, but the relative contribution to the fit from the Debye term in the syndiotactic polymer was roughly double that for the atactic PMA, implying the solution conformation of the syndiotactic PMA had much more random coil character.

The presence of the Q^{-3} dependence was somewhat unexpected. Further insights into PMA structure were gained by recasting the data into a Krakty plot ($Q^2I(Q)$ vs. Q) (Fig. 3). This representation greatly emphasizes the Gaussian coil characteristics (or any deviation from such). At pH = 7.4, the behaviour expected of a

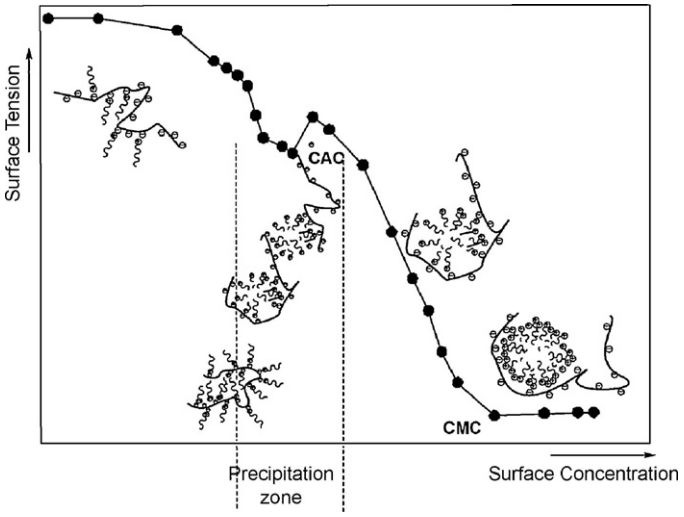


Fig. 5. Schematic representation of a typical surface tension curve and the hypothetical interactions of the PMA with DTAB.

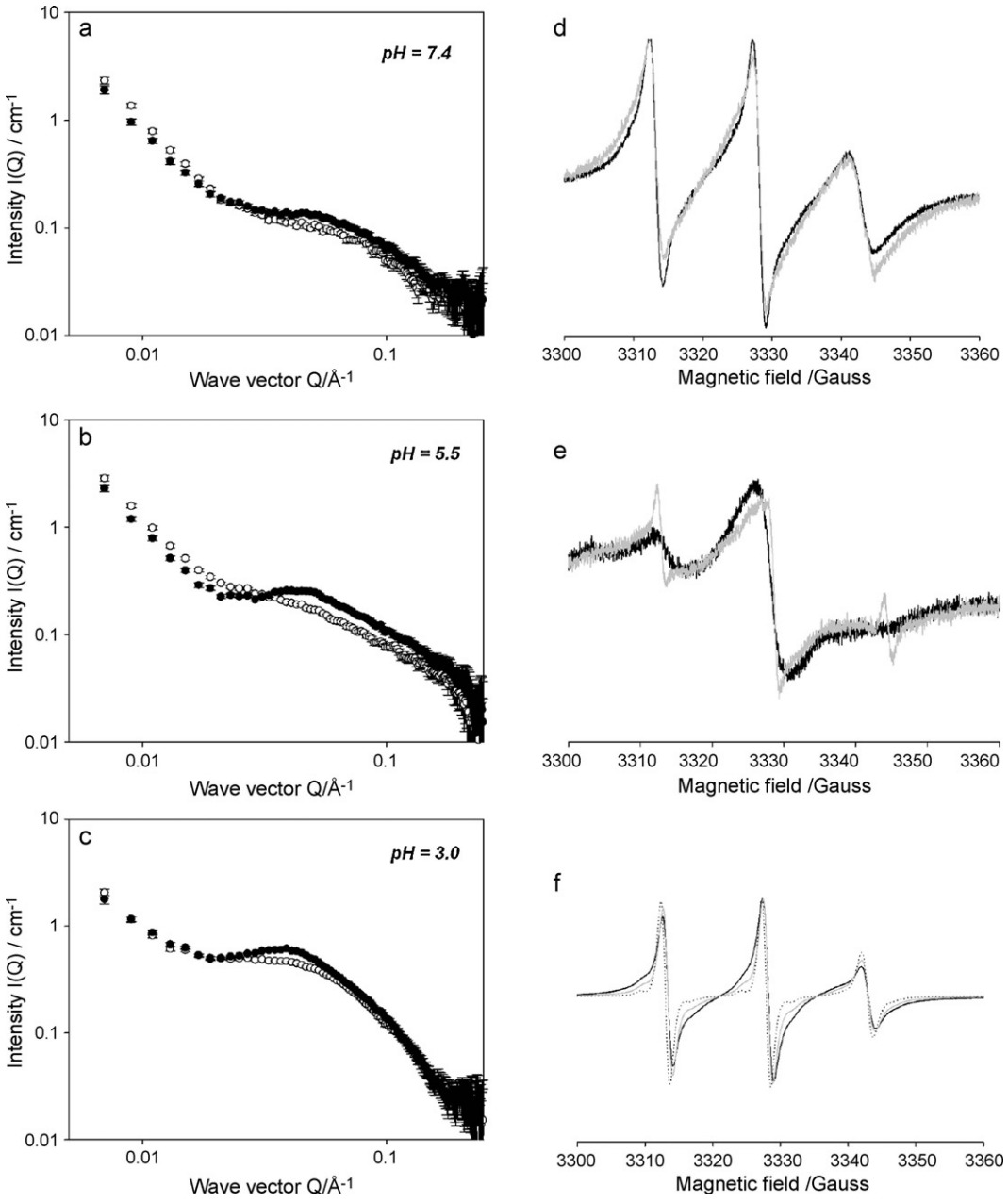


Fig. 6. Small-angle neutron scattering from 2% (w/v) PMA/5 mM d-DTAB at pH 7.4 and 5.5 (panels a and b) and from 2% (w/v) PMA/100 mM d-DTAB at pH 3.0 (panel c) for atactic (open circles) and syndiotactic (filled circles) PMA, and the corresponding EPR spectra for 16-DSE dispersed into these solutions of atactic (grey lines) and syndiotactic (black lines) polymers (panels d–f). Also shown in (f), is the EPR spectrum from 16-DSE solubilized in 100 mM DTAB solution (dashed line).

rod was observed (viz the linearly increasing asymptote at high Q). At pH = 3.0, the horizontal asymptote was almost recovered, i.e. the behaviour expected of a Gaussian coil. At pH = 5.5, the differences in the two datasets are most pronounced, with the atactic form showing a greater rod-like character. In conclusion, the Kratky analysis of the scattering data confirms the presence of rod-like structures for the atactic form, at least over the short length scales (~12.0 nm) consistent with a small number of monomer units.

It is apparent from these SANS data that the local conformation of PMA is partially determined by the electrostatic nature of the coil i.e. a rod-like conformation at pH 7.4 yet a much greater Gaussian coil character at pH 3.0. This would be expected since at pH 7.4 the polymers are more charged and the Coulombic repulsion between the carboxylate groups drives the coil expansion. The change in dimension seen is in accordance with other studies on atactic PMA which showed a pH-induced conformational transition. At pH 4.0 the hypercoiled conformation exists with the methyl groups (due to hydrophobic interactions) preferentially located in the interior, whilst at pH 6.0 the Coulombic repulsions between carboxylate groups induce a chain expansion (Ruiz-Pérez et al., 2008). Processing the scattering data in Fig. 2, yielded R_g ~4.0 nm for both atactic and syndiotactic PMAs at pH 3.0, but at pH 7.4, $R_{g(\text{atactic})} > R_{g(\text{syndiotactic})}$ reflecting a more pronounced coil expansion (to facilitate this comparison, these data have been replotted and are included in the Supplemantary Material). Given that there is increasing appreciation that subtle changes in architecture of nanosized structures can markedly affect their pharmacokinetics (Chen et al., 2009; Geng et al., 2007) in a way that would likely impact on safety and efficacy, these effects of tacticity on structure are important.

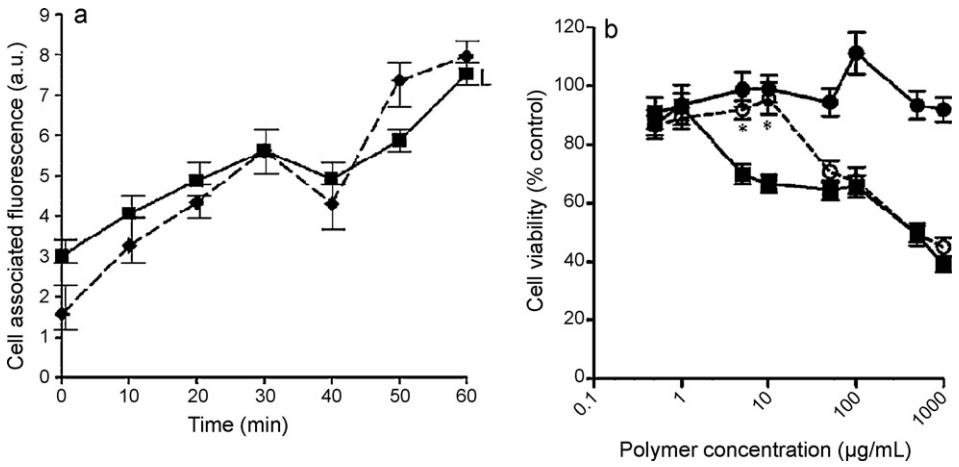


Fig. 7. Cell association and cytotoxicity of PMAs in B16 F10 cells. (Panel a) The cell association of the atactic PMA-OG (broken line) and syndiotactic PMA-OG (solid line). (Panel b) The cytotoxicity after a 72 h incubation of the atactic PMA (filled squares), syndiotactic PMA (open circles) and HPMA copolymer-Gly-Gly-COOH (filled circles). Data represent mean ± SEM, $n = 18$. *Significative differences between syndiotactic and atactic PMA ($p < 0.05$; unpaired students t-test).

To probe the interfacial behaviour of the PMA samples, surface tension of PMA/DTAB solutions was measured as a function of the DTAB surfactant concentration and pH (Fig. 4). The surface tension of the atactic PMA/DTAB solution at pH 7.4 and 5.5 (Fig. 4c and d) was lower than that of the DTAB alone over the surfactant concentration range <20 mM. For the syndiotactic PMA, at both pHs, the differences between the PMA/DTAB mixture and the DTAB-only control were less pronounced (Fig. 4a and b). The lower surface activity of syndiotactic PMA is consistent with the conclusion that the higher *rr* triad content confers greater hydrophilicity to the chain that interacting with the DTAB in solution decreases the surface activity of the surfactant.

Furthermore, both PMAs at dilute surfactant concentrations (both pHs) gave solutions that were transparent indicating that the surfactant–polymer complex was soluble. With increasing surfactant concentration, the solutions became increasingly cloudy and the solubility of the complex decreased as the concentration of charged surfactant approached the polymer charge neutralization point. This cloudiness was accompanied ultimately by phase separation and precipitation within a range of surfactant concentration as indicated in Fig. 4.

The fact that the atactic PMA appeared more surface active than the syndiotactic one, both at pH 7.4 and 5.5, indicates that increasing the *rr* triad content increased the polymer interaction with the cationic surfactant in solution, as reported above and schematically shown in Fig. 5.

The structure of the polymer/surfactant complex was examined in more depth using SANS and EPR experiments conducted at different DTAB concentrations (Fig. 6). As deuterated DTAB in D_2O is invisible to neutrons, changes in the observed scattering seen reflect only perturbations in the conformation of the polymer induced by the surfactant. SANS measurements (Fig. 6a–c) show that at low Q, scattering was similar for both the atactic and syndiotactic PMAs at pH 7.4, 5.5 ([DTAB] = 5 mM) and 3.0 ([DTAB] = 100 mM). It was also similar to that seen for the PMAs alone. However, the peak/shoulder seen around $Q = 0.05$ Å^{-1} at pH 5.5 (Fig. 2) was now enhanced by the addition of DTAB which clearly induced a conformational change at pH 7.4, 5.5 and 3.0 as a consequence of charge reduction along the chain. The peak/shoulder in the scattering around $Q = 0.05$ Å^{-1} was more pronounced for the syndiotactic form, and this feature became more evident with a decrease in pH. Also, the Q^{-n} scattering was more pronounced in the syndiotactic form, reflecting the fact that surfactant addition induced a smaller perturbation in the polymer conformation.

Nitroxide radicals in a fluid environment give a spectrum embodying three sharp lines, with the separation of the low- and mid-field peaks reflecting the polarity sensed by the probe, and the line shape defining by the mobility of the probe (Fig. 6d–f). For the PMA/DTAB mixture ([DTAB] = 5 mM) three broad lines were evident at pH 7.4, indicating that a hydrophobic domain capable of solubilising the spin-probe was present but that the dynamics of the spin-probe were slow and/or anisotropic (Fig. 6d). Some subtle differences were seen between the syndiotactic and atactic PMAs at this pH, but they were at the limits of resolution of the data. However, at pH 5.5 these differences were more pronounced and the emergence of the sharp lines for the atactic PMA indicated the presence of an "immature" hydrophobic environment, but one that was more developed than for the syndiotactic PMA (Fig. 6e). These observations were in agreement with the conclusions drawn above from the surface tension studies.

At higher surfactant concentrations i.e. approaching the saturation point of 100 mM (Fig. 6c), the scattering from the respective polymer/surfactant complex adopted a form that was only weakly dependent on the stereo-conformation, and the only difference shown by the syndiotactic compared to the atactic form was the slightly more pronounced maximum in the range $0.05 < Q < 0.1$ Å^{-1}. The corresponding EPR spectrum (Fig. 6f) showed that the syndiotactic PMA produced a more noticeable decrease in intensity of the high field peak indicating a slower motion of the spin-probe than seen in for the atactic PMA. The differences in these spectra compared to that of the DTAB control suggests that polymer–surfactant complexation led to a micellar structure that was different from the one of a simple DTAB micelle. Preliminary studies designed to investigate whether the physico-chemical changes noted above would impact on the general cytotoxicity and cellular uptake of the atactic and syndiotactic PMAs were therefore undertaken (Fig. 7). The PMA–OG conjugates (~10–50 μg OG/mg conjugate; free OG <0.2% bound OG) showed an increase in cell-associated fluorescence over 1 h at 37 °C. No significant difference was evident between the atactic and syndiotactic PMA except at the first time point and that represents instantaneous binding rather than endocytic internalization (Fig. 7a). HPMA copolymer-Gly-Gly-COOH was used as reference control because it is an atactic copolymer that is commonly used in drug delivery and present a structure similar to PMA, even if the content of COOH groups

are much lower than that of PMAs used in this study (see Section 2).

Whereas the reference control polymer was not toxic (up to 1 mg/mL) after a 72 h incubation with B16F10 murine melanoma cells, both the syndiotactic and atactic PMAs showed concentration-dependant cytotoxicity after the 72 h incubation. Their IC_{50} values (494 μg/mL and 492 μg/mL respectively) were not significantly different (Fig. 7b). However, the atactic PMA reproducibly showed greater toxicity than the syndiotactic PMA over the lower concentration range of 1–100 μg/mL. The difference in amount of COOH groups between control polymer and PMAs can explain the difference in toxicity. Furthermore, it is interesting to speculate that the more compact structure of syndiotactic polymer conferred by its triad composition and the presence of inter and/or intra-molecular salt bridges that minimize the effective negative charges can be responsible for the observed difference in cytotoxicity between PMAs, but further experiments are needed to define the structure–activity relationships of these biological effects more precisely. Cytotoxicity studies using a range of incubation times, and assay methods would bring greater insight, and so would more sophisticated methods to distinguish binding, uptake and intracellular trafficking (Richardson et al., 2010).

Studies examining the effect of tacticity on pharmacological activity are rare. For example, isotactic PMAs, characterized by a larger amount of *mm* triads were found to display greater antiviral activity than atactic PMAs. Furthermore, they inhibited the enzymatic hydrolysis of p-nitrophenyl acetate more strongly than atactic or syndiotactic PMAs (Kuramoto et al., 1984). The precise effects of polymer stereochemistry will undoubtedly depend on the particular polymer concerned, its molecular weight, and the specific biological issue under investigation. However, it is clear that the changes in physico-chemical properties reported here for PMA have broader implications for formulation properties, pharmacokinetics, toxicity and pharmacological activity of other polymers (or batches of the same polymer) that contain stereoregularities. To ensure safety and efficacy of innovative nanomedicines and polymer therapeutics great care should be to document such properties in order to satisfy regulatory authority needs (Gaspar and Duncan, 2009).

4. Conclusions

These studies show that relatively small changes in PMA *rr* triad content cause changes in the solution and interfacial behaviour of the polymer that could impact on biological properties during use as a polymeric excipient or a polymer therapeutic. At pH 5.5 ($\alpha < 0.3$) the syndiotactic PMA showed a pronounced maximum in the SANS over the Q range $0.05 < Q < 0.1$ Å^{-1} not detectable for the atactic form, implying the presence of a possible ordered conformation and/or the presence of aggregates due to intermolecular salt bridges. These differences in solution behaviour (conformation, charge) influenced the association with DTAB micelles. EPR of 16-DSE solubilized in the atactic PMA in the presence of 5 mM DTAB at pH 5.5, showed the emergence of sharp lines characteristic of a mobile environment that was due to a higher hydrophobicity of the atactic form with respect to the syndiotactic PMA, whilst SANS experiments confirmed the presence of local ordered structures or aggregation in the presence of the syndiotactic, but not the atactic PMA. Finally, at DTAB concentrations approaching the saturation limit (100 mM), the two stereo-forms were very similar, with a slightly more pronounced structure peak evident in the syndiotactic form and a less fluid micelle environment (stronger micelle–polymer interaction).

From the biological point of view, the atactic PMA showed greater toxicity than the syndiotactic PMA over the concentration range of 1–100 μg/mL. Probably, the information coming from the physico-chemical investigation such as the more compact structure of syndiotactic polymer and the presence of local ordered structures induced by inter and/or intra-molecular salt bridges that minimize the effective negative charges, can be used to explain the observed difference in cytotoxicity. Undoubtedly, this is just the first step and further experiments in presence of polymers with higher difference in tacticity are needed. It is clear, in fact, that there is a pressing need for more detailed, fundamental studies on the effect of polymer stereochemistry on biological behaviour to enable accurate prediction of the safety and efficacy of novel polymer therapeutics. For statistical polymers the local distribution of side-groups/chains along the polymer backbone as well as overall stereochemistry will be very important.

Acknowledgements

EPSRC is gratefully acknowledged for the provision of a Platform grant (EP/C013220/1). STFC is thanked for provision of neutron beamtime.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2011.02.003.

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