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Degradable Polymers and Nanoparticles Built from Salicylic Acid[a]

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Abstract: As more evidence emerges supporting the possibility that non-steroidal anti-inflammatory drugs, especially aspirin (acetyl salicylic acid), might have a role in the prevention and management in certain types of cancer, there have been several attempts to fabricate salicylic acid-based polymers that can be employed in the targeted therapy of tumours. The primary disadvantage so far has been in use of non-therapeutic polymeric backbones that constitute the majority of the therapeutic particle's size. The focus of this research is the creation of a biodegradable polymer consisting only of salicylic acid, and its use as the main building block in targeted nanotherapeutics that would consequently provide both high local dose and sustained release of the active moiety. In this work, we demonstrate the synthesis and degradation of

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polysalicylates, and modulation of their size and hydrolytic stability through formation of nanostructures.

Introduction

There is a growing body of evidence indicating that non-steroidal anti-inflammatory drugs (NSAIDs), particularly aspirin, may have a chemopreventive role in some types of tumours.[1,2] Results from meta-analyses of observational studies and randomised controlled trials suggest that the regular use of aspirin in cancer patients, specifically those with breast,[3] colorectal,[4] or prostate[5,6] cancer inversely correlates with cancer incidence, as well as being linked to a decrease in relapse and mortality rates and a reduction in metastases.[7] However, the mechanism by which aspirin exhibits its anti-tumour effects is still not fully understood, and both COX-dependent and -independent pathways have been proposed.[8]

Since the prolonged exposure of aspirin carries certain risks such as gastrointestinal bleeding and cerebral haemorrhage even at low doses,[9,10] there is an ongoing debate on the risk-benefit ratio of prescribing aspirin in cancer for chemoprevention, and hence there are currently no guidelines advocating such practice.[11,12]

Because the anti-inflammatory effects of aspirin could be attributed to its hydrolysis by-product, salicylic acid (SA),[13] there have been several studies focusing on the creation of SA-based polymers for the controlled release of the this molecule in high local doses.[14–18] However, since the synthesis of these polymers involves conjugating SA to a poly(anhydride-esters) or poly(xylitol-adipate) backbone, the loading capacity of the active moiety is limited, and the effect of other components arising from degradation must also be considered. In our group, we are actively investigating high local dose drug delivery through the creation of polymers comprised primarily of drug molecules.

The goal of this research is to develop a biodegradable polymer that comprises solely of SA to eventually create a particle with a high-loading capacity and sustained-release of the drug while taking advantage of the characteristics that can make nanoparticles more targeted towards cancer tissue and less likely to exert action on healthy tissues.[19] This could enhance the therapeutic effects of salicylic acid in cancer patients while minimizing side effects. Herein, we discuss a method for synthesis of polysalicylate (PSA), and further conjugation with polyethylene glycol (PEG) to both improve biocompatibility, and lead to the formation of self-assembled nanostructures as possible targeted therapeutic particles.

Results and Discussion

1. Polysalicylates (PSA)

1.1. Synthesis and Characterization

Polysalicylate was prepared using a condensation polymerisation method proposed by White et al.[20] in which salicylic acid is heated with acetic anhydride at high temperatures under a nitrogen atmosphere (in that report, the PSA was used as capping agents for polyphenylene ethers). This method gives a linear polymer of 15-50 repeating units of SA with an acetyl group on one end and a carboxyl group on the other.

Based on this method, polysalicylates in our experiments were prepared by a two-stage process. The first stage involved heating salicylic acid with 1.1 equivalents of acetic anhydride under nitrogen while refluxing at temperatures ranging between 150-200˚C for varying durations of time (6 to 24 hours). In the second stage, temperature was raised to 250-300˚C, nitrogen was disconnected, and the acetic acid by-product was distilled away with vacuum assistance over

periods of time ranging from 6 to 24 hours. Upon cooling, the polymer formed as a glassy solid that could easily be extracted from the reaction vessel.

The resulting polysalicylates were longer given more elevated temperatures and longer reactions times, and were soluble in dichloromethane, tetrahydrofuran, dimethyl sulfoxide, and chloroform. Long chains were partially soluble in acetone while short chains showed good solubility in that solvent. The polymer was practically insoluble in water, ethanol, methanol, hexane, and diethyl ether.

For the purpose of this study, we created a short polymer, with 9 repeating units of SA, by heating the monomer with acetic anhydride in the first stage at 150 ˚C for 20 hours, and at 250 ˚C for 16 hours in the second stage. A lower molecular weight was targeted to facilitate degradation studies. The polymer was further purified by dissolving it in dichloromethane (DCM) and precipitating it with methanol. This helped in washing out the monomer along with the short chains, thus enhancing the polydispersity profile of the polymer. To create a block-copolymer, an acyl chloride of the polysalicylate was made by reacting the polymer with thionyl chloride. The acyl chloride was then esterified under basic conditions with monomethyl polyethylene glycol (mPEG, $M_n=$ 1900) to create PSA-b-mPEG.
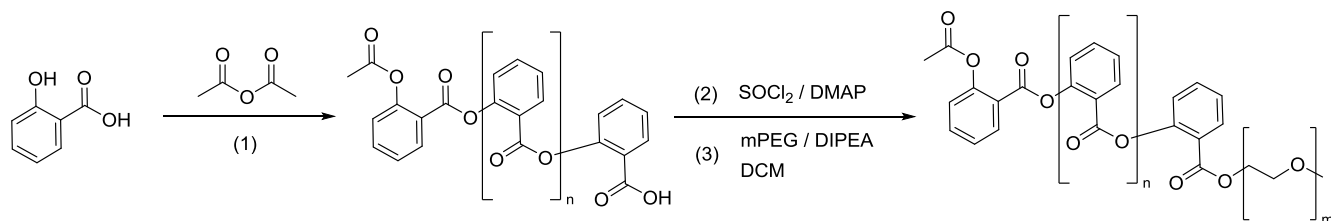

Figure 1 - Reaction scheme for synthesising polysalicylates (PSA) and polysalicylates-polyethylene glycol (PSA-mPEG) co-polymer. (1) Salicylic acid is heated with acetic anhydride under nitrogen and then vacuum is used to remove the resulting acetic acid leaving PSA as a glassy solid. (2) Polysalicyloyl chloride is prepared by heating PSA with thionyl chloride and DMAP as a

catalyst. (3) The resulting polysalicyloyl chloride is then reacted with mPEG in the presence of DIPEA to give PSA-b-mPEG co-polymer.

^{1}H NMR (400 MHz, CDCl$_3$) and gel permeation chromatography (GPC) were used to characterise the resulting polymers. As illustrated in **Figure 2**, the NMR spectrum exhibits broadening of the peaks of the aromatic region (6.9 – 8.3 ppm) typical for a polymer. By integrating the peak at 8.13 ppm that belongs to one of the benzene ring hydrogen with the peak at 2.17 that belongs to the hydrogens of the acetyl end group the number of the salicylate repeating units in the polymer can be determined.
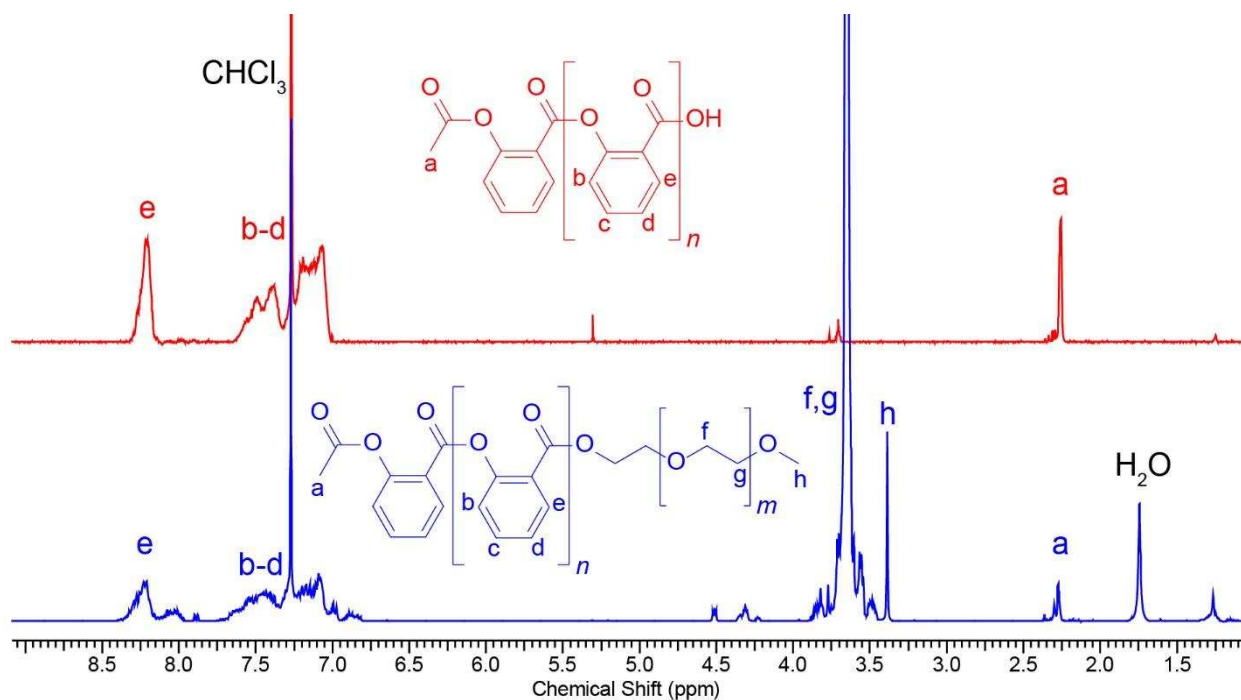

Figure 2- ^{1}H-NMR spectrum of PSA (above) and PSA-mPEG (below) in CDCl$_3$.

GPC with a refractive index detector was used to evaluate the average molecular weights and the polydispersity of the polymer. The number average molecular weight (M$_n$) was 688, the weight average molecular weight (M$_w$) was 1075, and the dispersity (Đ) was 1.56. The response vs retention time chromatograms as well as the differential molecular weight distributions (DMWD) are shown in **Figure S1** and **S2**, respectively.

1.2. Degradation

In order to assess the biodegradability of the synthesised polymer, an accelerated degradation test was conducted in several media representing different semblances of biological conditions. Accurately weighed amounts of the polymer were separately added to water (pH ≈ 7), sodium hydroxide (2 mol dm^{-3}, pH ≈ 14), phosphate-buffered saline (PBS, pH ≈ 7.4), 10% foetal bovine serum (FBS) in PBS (pH ≈ 7.4), and 36 units mL^{-1} solution of porcine liver esterase (PLE) in PBS (pH ≈ 7.4), giving suspensions in each case. Analysis was carried out using UV spectroscopy in the range of 200-360 nm and the concentration of SA released in each medium was compared to a solution of SA in the respective medium that corresponds to 100% degradation of the polymer (**Figure 3**). Quantitative comparison was conducted at a wavelength of 296 nm. Initial degradation studies at 37 °C showed negligible release of SA, thus subsequent studies were conducted at a higher temperature to accelerate degradation. The polymer-containing media were hence incubated at 90°C, and samples from the supernatants were taken at different time points (2, 4, 8, 24, and 96 hours) to measure the amount of SA released in each respective medium.

The maximum release of SA observed with water was only about 5%, and a modest 11% release in enzyme-free PBS was also noted. High pH levels appeared to have the greatest effect on the polymer's ester bonds since an almost complete degradation happened with the NaOH solution after only 2 hours. The polymer showed a promising SA release of 60% and 49% in FBS and PLE respectively after 96 hours, which could indicate that a more sustained release might be achieved in the biology where a similar enzymatic composition exists at a lower temperature.
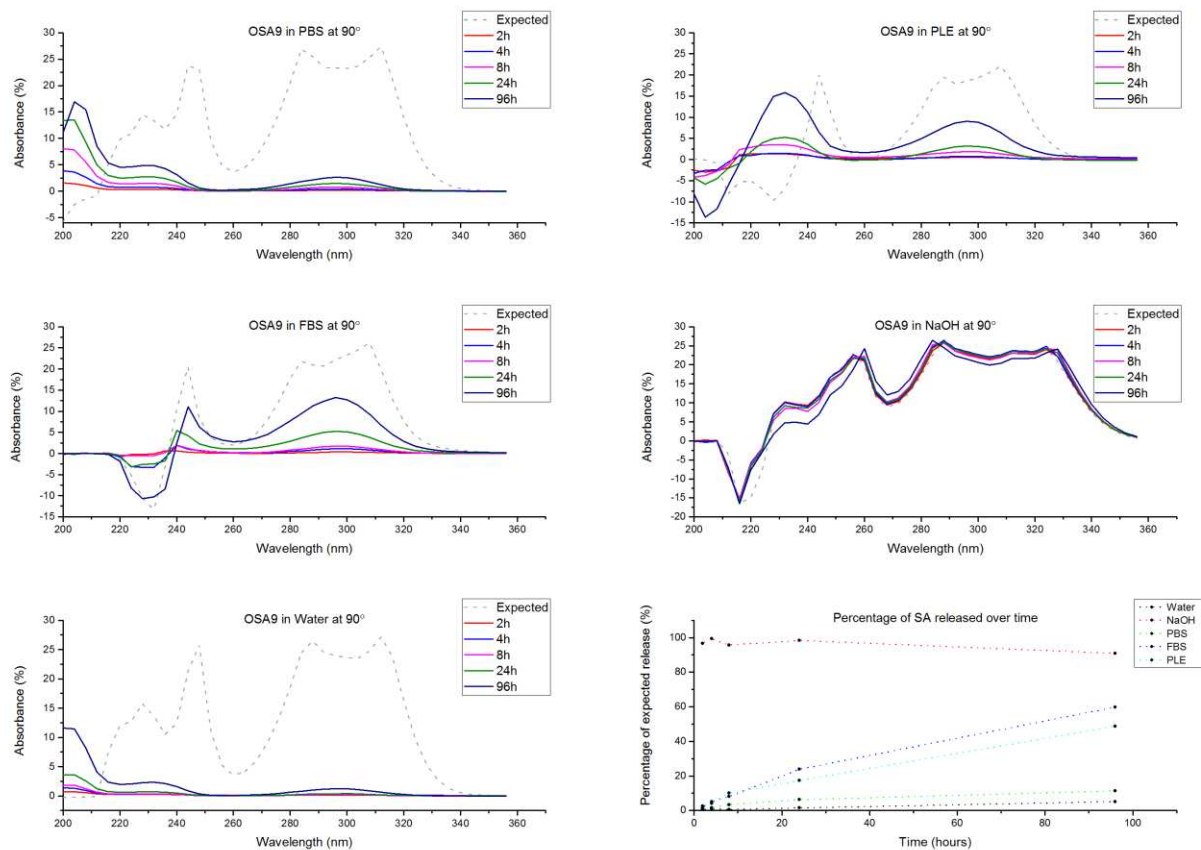
Figure 3 - Degradation of PSA in PBS, NaOH, Water, PLE, and FBS over a period of 96 h at 90 ℃. *Differences in the 'Expected'* curves are due to their being measured in the different media. The graph in the right lower corner shows the release percentage as measured at the wavelength of 296 nm.

It is worth noting that, even though high temperatures most likely lead to the denaturing of enzymes in FBS and PLE, Weingand-Ziade et al. demonstrated that denaturing might have a more complicated kinetic that is also pressure-dependent and that moderate pressure might actually protect enzymes from inactivation[21]. We speculate that the enzymatic activity of the media in our study might have been preserved in a related manner.

2. Polysalicylate-block-mPEG Co-polymer

2.1. Synthesis and Characterisation

Conjugating nanoparticles with PEG, also known as PEGylation, can enhance the pharmacokinetic profile of chemical entities. PEG enhances the half-life of nanoparticles by increasing their hydrophilicity, as well as "stealthing" them against the reticuloendothelial system (RES) which reduces the chances of their scavenging[22,23]. Since PEG is hydrophilic, its conjugation to PSA will create an amphiphilic polymer which would be expected to self-assemble into nanoscale micellar structures. This kind of polymeric micelles is a promising form of drug delivery systems in terms of stability, release profile, and tumour targeting.[24,25] Increasing the size of the constructs through self-assembly to the range of 100-200 nm can provide a level of passive tissue targeting and accumulation by taking advantage of the physiochemical changes in solid tumours' microenvironment.[26–28] Creating the delivery system within this size range also have an important role in decreasing renal clearance and splenic filtration and thus further enhancing bioavailability.[29] In our study, we synthesised a co-polymer of PSA and mPEG for the purpose of creating micelles as nanocarriers for targeted therapy that have the hydrophobic core consisting of the drug intended for delivery itself, i.e. the PSA, and a hydrophilic PEG shell that gives the micelles all the advantages mentioned above.

We created a PEG conjugate of our polysalicylate polymer by the method mentioned above. The co-polymer was characterised using ^{1}H NMR (400 MHz, CDCl$_3$) and GPC (Fig. 2, S1, S2). Integrating the peak at 8.13 ppm that belongs to one of the hydrogens of the benzene ring with the 3.55 ppm peak of the polyethylene glycol hydrogens showed a PSA:PEG ratio of 1:1.27 indicating a successful coupling, but with a residual amount of PEG which defied extraction. GPC analysis ($M_n = 2998$, $M_w = 3315$, $Đ = 1.11$) was in line with expected values. DMWD of the co-polymer is

shown in **Figure S2** along those of the polymer and the PEG. The shift of the co-polymer to the right indicates the successful coupling between the polymer and the PEG.

Although ^{1}H NMR and GPC data suggests the successful PEGylation of PSA, it also indicates that the purification process is not complete. This is due to the difficulty in removing the mPEG from the end product, and since this impurity could possibly cause some inaccuracy in the future degradation studies of the co-polymer, this issue will be examined carefully to avoid any inconsistencies.

2.2. PSA-b-mPEG Micelles

We took advantage of the fact that our co-polymer has a hydrophilic and a hydrophobic end to create simple micelles as a means for the delivery of polysalicylates to target tissues.

Two solutions of polysalicylates in THF (1 and 10 mg/mL) were prepared. As they were being independently stirred in round-bottom flasks, de-ionised water was added dropwise to the solutions to form micelles until a 10-fold dilution was achieved. The resulting micelles were analysed using dynamic light scattering (DLS) immediately after preparation and after 24 and 48 hours.

DLS analysis showed a uniform distribution of size with the mean of about 250-300 nm for both samples (**Figure S3, S4**). The analysis of the samples over the following 48 hours showed minor changes in size distribution and correlograms suggesting that the nanostructure were stable for at least that period of time. However, given the fact that the theoretical total length of two co-polymer molecules head-to-head would not exceed 30 nm the larger size indicates that the particles formed by the micellisation process are vesicular rather than star micelles. This was corroborated using the transmission electron microscopy (TEM) after staining with 2% solution of uranyl acetate

(**Figure 4**). The images indicated that unilamellar and multilamellar spheres of sizes consistent with the DLS results were formed.

Degradation studies on the micelles was complicated by a difficult deconvolution of the spectrum of free PSA from that of the nanostructures in solution, hampering our attempts to accurately examine the kinetics of the system under different conditions at this stage.
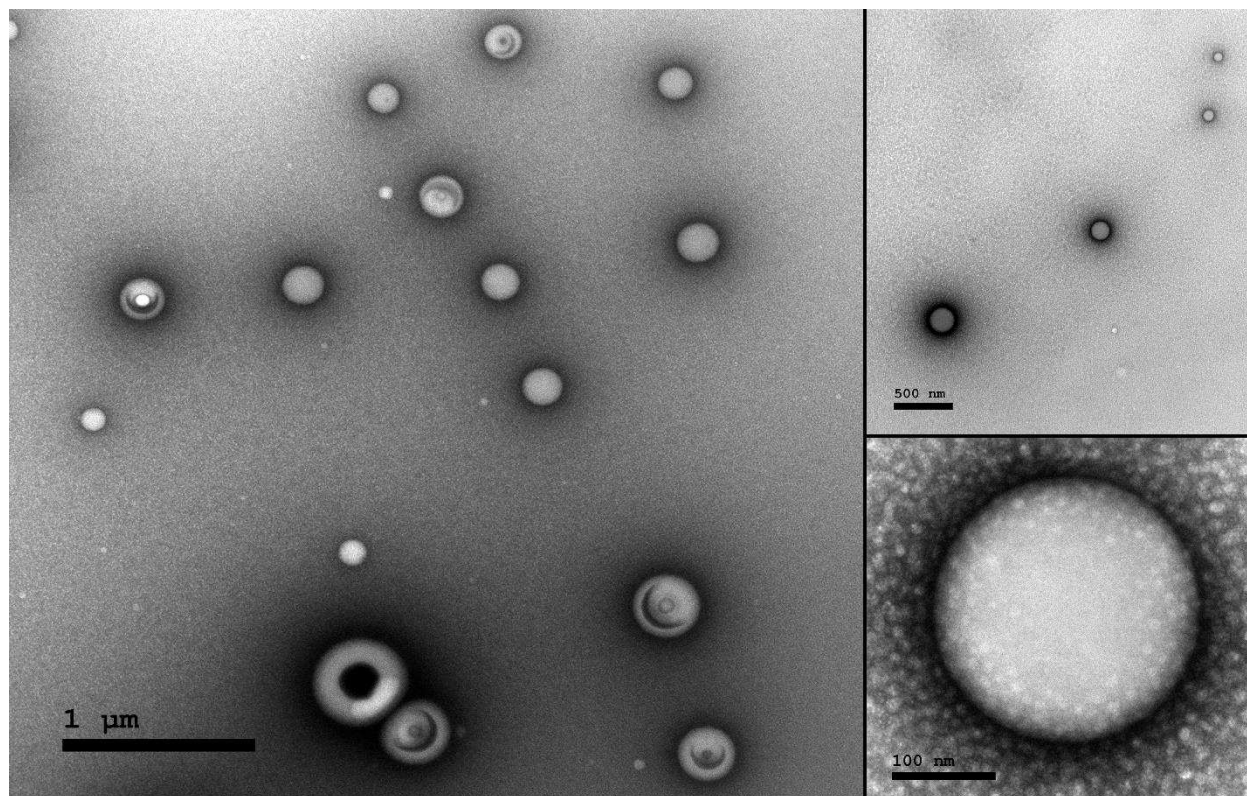

Figure 4 - PSA-mPEG particles as observed with TEM after staining with uranyl acetate.

Conclusion

We have shown that polymers comprised entirely of pharmaceutically active molecules can be synthesised, and moreover show sustained release of the active compound under biologically relevant conditions. By creating block copolymers, we can access prospectively biocompatible forms of the system which show modulated release profiles. Polymeric micelles that have a high

loading of salicylic acid in the form of a biodegradable, sustained-release polymer and that are both compatible with the organism and shielded from its clearance mechanisms due to its PEG shell might have a promising role in chemoprevention and increasing cancer survival especially when they fall in the preferable size range of approximately 100-200 nm that increases their ability to passively target and accumulate in cancer tumours. We are currently exploring the self-assembly phase diagram of the copolymers with respect to chain length and block ratio, which will we correlate with release profiles and pharmacological activity in cancer cell lines.

Acknowledgement

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