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Title: Designing dapsone polymer conjugates for controlled drug delivery

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Corresponding Author: Dr. Luis Rojo,

Corresponding Author's Institution: King's College London

First Author: Luis Rojo

Order of Authors: Luis Rojo; Mar Fernandez-Gutierrez; Sanjukta Deb; Molly Stevens; Julio San Roman

Abstract: Polymer-drug conjugates have significantly influenced polymer therapeutics over the last decade via controlled pharmacokinetics. Dapsone (4,4'-diamino diphenylsulphone) is not only widely used in the treatment of leprosy but forms an essential component in the treatment of autoimmune inflammatory diseases and malaria. However, its low bioavailability and non-specific distribution in the body leads to absorption throughout organs including skin, liver, and kidneys that can cause serious side effects. Thus, in this study we report the synthesis of polymer-drug conjugates of dapsone covalently bonded to macromolecular chains towards the development of new bioactive polymeric formulations with anti-inflammatory properties. Dapsone was functionalised with an acrylic moiety in which the acrylamide residue was directly bonded to one of the aromatic rings of dapsone. This functionalization yielded an unsymmetrical dapsone methacrylamide (DapMA) structure, which on free radical polymerisation and co-polymerisation with HEMA yielded polymers of hydrocarbon macromolecules with pendant dapsone units. Thermal and size-exclusion chromatographic analysis revealed an increase in thermal stabilisation of the homopolymer (p(DapMA)) in comparison to the copolymer (p(Dap-co-HEMA)) with relatively high average molecular weight. The polymer conjugates exhibited high stability with low dapsone release from the polymeric backbone due to hydrolysis. However, a significant anti-inflammatory activity in a nitric oxide inhibition assay confirmed that this property was the consequence of only the macromolecular composition and not related to the release of low molecular weight compounds. Thus, the conjugation of dapsone to macromolecular systems provides a synthetic route to incorporate this drug into polymeric systems, facilitating their development into new anti-inflammatory therapies

Statement of Significance

The dapson-conjugated methacrylic monomer and polymer derivatives with anti-inflammatory properties described are previously unreported. The scientific impact of this work lies in its potential to expand the clinical applications of dapson toward the development of advanced anti-inflammatory therapies based on polymer-therapeutic approaches. These approaches facilitate the treatment of existing rare auto-immune and other inflammatory related diseases.

Designing dapsone polymer conjugates for controlled drug delivery

**Luis Rojo^{*1,2,3}, Mar Fernandez-Gutierrez¹, Sanjukta Deb², Molly M. Stevens^{3,4,5}
and Julio San Roman¹**

¹Institute of Polymer Science & Technology, CSIC and CIBER-BBN.

Juan de la Cierva, 3, 28006, Madrid, Spain

²Division of Tissue Engineering and Biophotonics, King's College London Dental Institute, Guy's Hospital, London, United Kingdom

³Department of Materials, Imperial College London.

⁴Institute for Biomedical Engineering, Imperial College London.

⁵Department of Bioengineering, Imperial College London.

*** Corresponding author: luis.rojo_del_olmo@kcl.ac.uk**

Abstract

Polymer-drug conjugates have significantly influenced polymer therapeutics over the last decade via controlled pharmacokinetics. Dapsone (4,4'-diamino diphenylsulphone) is not only widely used in the treatment of leprosy but forms an essential component in the treatment of autoimmune inflammatory diseases and malaria. However, its low bioavailability and non-specific distribution in the body leads to absorption throughout organs including skin, liver, and kidneys that can cause serious side effects. Thus, in this study we report the synthesis of polymer-drug conjugates of dapsone covalently bonded to macromolecular chains towards the development of new bioactive polymeric formulations with anti-inflammatory properties. Dapsone was functionalised with an acrylic moiety in which the

acrylamide residue was directly bonded to one of the aromatic rings of dapsone. This functionalization yielded an unsymmetrical dapsone methacrylamide (DapMA) structure, which on free radical polymerisation and co-polymerisation with HEMA yielded polymers of hydrocarbon macromolecules with pendant dapsone units. Thermal and size-exclusion chromatographic analysis revealed an increase in thermal stabilisation of the homopolymer (p(DapMA)) in comparison to the copolymer (p(Dap-co-HEMA)) with relatively high average molecular weight. The polymer conjugates exhibited high stability with low dapsone release from the polymeric backbone due to hydrolysis. However, a significant anti-inflammatory activity in a nitric oxide inhibition assay confirmed that this property was the consequence of only the macromolecular composition and not related to the release of low molecular weight compounds. Thus, the conjugation of dapsone to macromolecular systems provides a synthetic route to incorporate this drug into polymeric systems, facilitating their development into new anti-inflammatory therapies

Keywords

Dapsone, HEMA, polymer-drug conjugate, anti-inflammatory polymers, nitric oxide inhibition assay

Graphical abstract

1. Introduction

During the last century, sulfone derivatives have been extensively used in medicine because of their versatile bioactive, biocidal and anti-inflammatory properties. Dapsone is structurally one of the simplest sulfones but also recognised as an active therapeutic agent from this family of compounds. As an antibiotic, dapsone acts against bacteria and protozoa inhibiting the synthesis of dihydrofolic acid through competition with para-aminobenzoate for the active site of dihydropteroate synthase [1]. In addition, dapsone has been successfully used as an essential component for the treatment and prophylaxis of leprosy, actinomycetoma, *Pneumocystis pneumonia*, and malaria. The anti-inflammatory action of the drug is not related with its antibacterial action and is still not fully understood. Dapsone has been reported to interfere with the activation or function of a large number of polymorphonuclear leukocytes, predominantly neutrophils and eosinophils, into the affected tissue and shows a noticeable activity in reducing or suppressing the symptoms of many autoimmune [2] and skin [3] related diseases such as acne fulminans, actinomycetoma, brown recluse spider bites, celiac disease, cicatricial pemphigoid, dermatitis herpetiformis, bullous pemphigoid, Behcet's disease, and lupus erythematosus.

One of the main disadvantages of oral administration of dapsone stems from its low solubility and non-specific distribution in the body, once absorbed, it is distributed throughout the organs in the body including skin, liver, and kidneys [4]. Dapsone has also been reported to cross the blood-brain barrier and the placenta and is found in breast milk [5, 6]. Hence, dapsone therapies are still associated with drawbacks of hemolysis, methemoglobinemia and patient non-compliance [7]. To render the treatment more effective and reduce these side effects, a better control over pharmacokinetics and site specific delivery are needed. Thus far, the design of

polymer formulations for controlled delivery of dapsone has been limited to encapsulation within polymeric systems, for which mainly polysaccharides have been used [8, 9]. Although these methodologies have improved the pharmacokinetics, the therapeutic effects of these polymer formulations were found to be modest and a reduction of the complications associated with dapsone therapies were not sufficiently achieved.

Recent developments in the field of polymer-conjugates have provided very promising examples of FDA approved and marketed pharmaceutical products [10]. Anti-inflammatory therapies involving the incorporation of low molecular weight drugs into macromolecular systems such as ibuprofen and aspirin polymer-drug conjugates have shown promising advances that enhance the bioavailability and compatibility within the body [11, 12]. Nonetheless, biomedical approaches based on dapsone polymer-drug conjugates have not yet been fully exploited. Chemically incorporating 4, 4'-diamino diphenylsulphone (dapsone) into a polymer system has only been reported in elastomeric formulations for engineering applications based on its capacity to act as a curing agent in epoxy resins. This results in a high performance elastomeric material with enhanced thermal and mechanical properties, composite materials, and structural adhesives that are unrelated to the current work [13].

In view of the limitations of current dapsone treatment, a polymer system that intrinsically contains the active dapsone molecule anchored to the macromolecular polymer structure will be extremely beneficial and efficacious for therapeutic action. This would lead to enhanced control of pharmacokinetics whilst providing a novel approach for safe use of this promising drug. The covalent linking of dapsone into macromolecular chains is advantageous as it will reduce the migration of dapsone into the surrounding tissues and improve hydrolytic and biological stability.

This study reports for the first time the synthesis and characterisation of a polymerisable derivative of dapsone, dapsone methacrylamide (DapMA) (Figure 1) and the polymers thereof. These systems have the potential for the development of bioactive polymeric formulations with anti-inflammatory properties by virtue of the dapsone residues anchored to the macromolecular chains, which have not yet been reported.

2. *Materials and Methods*

2.1. *Chemicals*

Dapsone (97 %) was purchased from Aldrich and recrystallised from methanol. Methacryloyl chloride (97 %, Aldrich) and triethylamine (98 %, Scharlau) were purified by distillation under reduced pressure. 2-Hydroxyethyl methacrylate (HEMA) (97 %, Sigma–Aldrich) was purified according to the literature by water-hexane selective extraction and washing [14]. 2,2-Azobisisobutyronitrile (AIBN) (98 %, Merck) was recrystallised using methanol. Ethylene glycol dimethacrylate (EGDMA) (98%, Aldrich) was used as received. Solvents used were high performance liquid chromatography (HPLC) grade and all other reagents were analytical grade purchased from Sigma-Aldrich.

Phosphate-buffered solution (PBS) of pH 7.4 (Sigma) was used as received. Thermanox (TMX) control disks were supplied by Labclinics S. L., and aqueous solutions of Triton X-100 were supplied by Aldrich. Tissue culture media, additives, trypsin, and 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) were purchased from Sigma.

2.2. *Synthesis of Dapsone Methacrylamide monomer*

Dapsone (4g, 16 mmol) was dissolved in 150 mL of ethyl acetate containing triethylamine (2.8 mL, 20 mmol). A stoichiometric amount of methacryloyl chloride (1.2 mL, 16 mmol) dissolved in 10 mL of ethyl acetate was added dropwise with constant stirring at room temperature under nitrogen atmosphere, followed by stirring the reaction mixture for a further 24 h at room temperature. The reaction medium was filtered to remove the triethylamine hydrochloride and any unreacted reagents were removed by successive extraction with 0.5M HCl and saturated solutions of HNaCO_3 and NaCl. After drying over anhydrous Na_2SO_4 , the solvent was removed by distillation under reduced pressure and the crude product was purified by a triple recrystallisation in methanol, ethyl acetate, followed by diethyl ether and dried under vacuum until constant weight.

2.3. Synthesis of poly(dapsone methacrylate) homopolymer and poly(hydroxyethyl methacrylate-co-dapsone methacrylate) co-polymer.

Homopolymer p(DapMA) and co-polymer p(DapMA-co-HEMA) (50% feed molar ratio) were synthesized by free radical polymerisation and named P100 and P50, respectively. The appropriate co-monomer mixtures were dissolved in DMF ($[\text{M}] = 0.25 \text{ mol} \cdot \text{L}^{-1}$) containing AIBN ($1.5 \cdot 10^{-2} \text{ mol} \cdot \text{L}^{-1}$), and the reaction mixtures were deoxygenated with N_2 for 15 min and the reaction containers were then transferred to an oven at 50 °C for 24 h. After this period, the reaction mixture was precipitated in ethanol and centrifuged for 5 min at $6 \cdot 10^3$ rpm. The product obtained was re-suspended in fresh ethanol and centrifuged again. The final product was filtered, washed with fresh ethanol and dried under vacuum to constant weight.

Cross-linked polymers and co-polymers were additionally prepared by dissolving the corresponding monomer mixture in 0.4 mL of DMF ($[\text{M}] = 1 \text{ mol} \cdot \text{L}^{-1}$) containing AIBN ($1.5 \cdot 10^{-2} \text{ mol} \cdot \text{L}^{-1}$) and EGDMA (1 mol%) as crosslinking agent.

The reaction mixtures were deoxygenated with N₂ for 15 min, sealed in polypropylene cylindrical moulds (12 mm in diameter) and transferred to an oven at 50 °C for 24 h. After this period the gels were demoulded rinsed with DMF in order to remove all unreacted products and extensively washed with methanol in an orbital shaker by replacing the medium every 30 minutes for 4 hours. After this cycle the samples were dried under vacuum at 30 °C to constant weight.

2.4. Characterisation techniques

¹H and ¹³C NMR spectra were recorded on an INOVA-300 spectrophotometer, operating at 300 and 75.5 MHz, respectively. The spectra were recorded by dissolving the corresponding sample (10% w/v) in deuterated dimethylsulfoxide (DMSO-d₆) and using tetramethylsilane (TMS) as internal standard at 25 °C. An indirect detection experiment, heteronuclear multiple quantum coherence (HMQC), was performed to observe correlation between carbon-13 nuclei while detecting high-sensitivity protons. A pulse delay of 3 s between 90° pulses (13.5 μs), 32 scans, and 1024 data points for t₁ increment, of 256 increments, sweep width of 2500 Hz in the F₂ dimension and 13,000 Hz in the F₁ dimension were used in the experiment with gap decoupling of carbon-13.

ATR-FTIR fingerprint region spectra were recorded on a Perkin–Elmer Spectrum One spectrophotometer with an ATR attachment from 550 to 2,000 cm⁻¹ with a resolution of 4 cm⁻¹ and transmittance scales were normalized for all recorded spectra.

Mass spectra were obtained with an electrospray ionization mass spectrometer (Agilent Series 1100) using 1mg/ml solutions in H₂O/methanol/acetonitrile (49.5/49.5/1 vol%) as mobile phase.

Number (M_n) and weight (M_w) average molecular-weight distribution and polydispersity index ($PI=M_w/M_n$) were determined by size exclusion chromatography (SEC) (Perkin–Elmer) using polymer solutions in DMF ($3\text{ mg} \cdot \text{mL}^{-1}$) with LiBr (0.1% w/v) at a flow rate of $0.3\text{ mL} \cdot \text{min}^{-1}$, using two ResiPore columns (250mm - 4.6mm, Varian) thermostated at $70\text{ }^{\circ}\text{C}$ and a differential refractometer as detector. M_n and M_w were determined based on the calibration curve obtained from monodisperse poly(methylmethacrylate) (PMMA) (Perkin) standard samples.

Glass transition temperatures (T_g) were measured by differential scanning calorimetry (DSC, Perkin–Elmer DSC 8500). Samples (15 mg) were placed in aluminium pans and heated under nitrogen flow ($20\text{ mL} \cdot \text{min}^{-1}$) from -20 to $190\text{ }^{\circ}\text{C}$ at constant rate of $10\text{ }^{\circ}\text{C} \cdot \text{min}^{-1}$. T_g was taken as the midpoint of the heat capacity transition observed. Thermogravimetric diagrams were obtained using a thermogravimetric analyser using a TGA Q500 (TA instruments) apparatus, under a stream of nitrogen at a heating rate of $5\text{ }^{\circ}\text{C} \cdot \text{min}^{-1}$ from 40 - $600\text{ }^{\circ}\text{C}$.

2.5. In vitro drug release, degradation, cell culture, cytotoxicity and anti-inflammatory activity of dapsone derivatives.

In vitro drug release and degradation studies were carried out using crosslinked P100 and P50 disk shaped samples of 12mm in diameter. *In vitro* dapsone release from each composition was studied by soaking three different crosslinked samples in 2 mL of three different release media at pH 3, 7 and 10 buffered solutions and at $37\text{ }^{\circ}\text{C}$ under continuous agitation using an orbital shaker. At desired time points, the medium was refreshed under sterile conditions. The extracts were mixed with methanol (1:1 v/v) for dapsone release and quantification was done by UV spectroscopy detection at 295 nm using a Perking Elmer Lambda 16 instrument. Known concentration solutions of dapsone ($0 - 10\text{ }\mu\text{g} \cdot \text{mL}^{-1}$) were used for

calibration purposes ($R^2 > 0.99$). Degradation of each composition was studied by gravimetric measurements before and after soaking three different crosslinked samples in 1 mL of PBS buffered solutions maintained at 37 °C under continuous agitation using an orbital shaker. At each desired time point, three different samples were used and consequently dried and weighed. All the experiments were carried out in triplicate.

Cell culture studies were performed using monocyte macrophages RAW 264.7 (ECACC, Sigma, P8) cells. The culture medium was Dulbecco's modified Eagle's medium enriched with 110 mg · mL⁻¹ of sodium pyruvate (DMEM, Sigma) and supplemented with 10 vol% of fetal bovine serum (FBS, Gibco), 200mM L-glutamine, 100 units · mL⁻¹ penicillin, and 100 mg · mL⁻¹ streptomycin. Cultures were maintained at 37 °C in humidified air with 5 vol% CO₂, and the culture medium was carefully replaced at selected time intervals under sterile conditions.

The cytotoxicity of the compounds were assessed by MTT assay. 0.5 mg · mL⁻¹ solutions of linear p(DapMA) and p(DapMA-co-HEMA) copolymer prepared in water and were successively diluted to a concentration range of 0.5 - 2 · 10⁻⁴ mg · mL⁻¹. Cells were seeded in a sterile 96-well culture plate at a density of 5 · 10⁴ cells · mL⁻¹ in complete medium and incubated to confluence for 24 h. Following this 50 mL of each sample dilution were added (n = 8) and incubated for 24, 48, and 72 h. After each time point, the medium was removed and 100 mL MTT solution prepared in warm PBS (0.5 mg · mL⁻¹) was added to each well and plates incubated at 37 °C for 4 h. The excess medium and MTT were then removed and 100 mL of DMSO was added to each well in order to dissolve the formazan produced by viable cells. The absorbance was measured using a test wavelength of 570 nm (Biotek ELX808 IU detector).

Samples with a cell viability over 70% were selected for the determination of the anti-inflammatory activity by the nitric oxide inhibitory method reported by Wang et al [15]. Briefly, raw cells were seeded at a density of $5 \cdot 10^4$ cells $\cdot$ mL⁻¹ in complete medium in a sterile 96-well culture plate and incubated for 24 h. Adhered cells were then incubated with or without 1 mg $\cdot$ mL⁻¹ of lipopolysaccharide (LPS) (*Escherichia coli* 0127:138, Sigma-Aldrich) for 30 min when 50 mL of the respective polymer solutions were added and incubated at 37 °C for other 24 h. Nitrite concentration was measured by the Griess reaction [16] using 150 mL of the cell supernatants with 150 mL of an aqueous solution (13.33 vol%) of Griess reagent [1:1 mixture of 0.1 wt% N-(1-naphtyl) ethylenediamine in H₂O and 1wt% sulphanilamide in 5 wt% phosphoric acid] in a 96-well plate and the absorbance was recorded after 30 min of reaction at 540 nm. A NaNO₂ curve was used as an external calibration ($R^2 > 0.99$). An additional MTT assay was performed as described above for the calculation of the corresponding dose-response curves of relative cell viability of the treated cells with the LPS to delineate the concentrations of the sample that depressed MTT-formazan production by 50% (IC₅₀ value). The results were normalised respect to the corresponding blanks, and statistically tested with ANOVA ($p < 0.05$).

3. Results

3.1. Dapsone Methacrylamide monomer

Dapsone was functionalised with an acrylic moiety, in which the methacrylamide residue was directly bonded to one of the aromatic rings of dapsone leading to unsymmetrical structure of DapMA forming a white powder with a yield 52% after the recrystallization procedure. The reaction scheme for the acylation of dapsone is illustrated in Figure 2 and the DapMA monomer was characterized by ¹H

and ^{13}C NMR spectroscopy and both and HMQC spectra are respectively illustrated in Figures 3 and 4 showing the following peak assignment:

^1H -NMR spectrum *DapMA* ($\text{DMSO}-d_6$): δ_{H} 10.13 (1H, $\text{CO}-\text{NH}-\text{C}_{\text{Ar}}-\text{SO}_2$), 7.83 , $J = 7.9$ Hz (2H_{Ar} , $\text{CO}-\text{NH}-\text{C}-\text{CH}-\text{CH}-\text{C}-\text{SO}_2$), 7.76 (2H_{Ar} , $\text{NH}_2-\text{C}-\text{CH}-\text{CH}-\text{C}-\text{SO}_2$), 7.51, $J = 8.7$ Hz (2H_{Ar} , $\text{CO}-\text{NH}-\text{C}-\text{CH}-\text{CH}-\text{C}-\text{SO}_2$), 6.62, $J = 8.7$ Hz (2H_{Ar} , $\text{NH}_2-\text{C}-\text{CH}-\text{CH}-\text{C}-\text{SO}_2$), 6.11 (2H, $\text{NH}_2-\text{C}_{\text{Ar}}$), 5.80 and 5.54 (2H, $\text{CH}_2=\text{C}(\text{CH}_3)\text{CO}$), 1.93 (3H, $\text{CH}_2=\text{C}(\text{CH}_3)\text{CO}$).

^{13}C -NMR spectrum *DapMA* ($\text{DMSO}-d_6$): δ_{C} 167.2 ($\text{CO}-\text{NH}$), 153.5 ($\text{NH}_2-\text{C}-\text{CH}-\text{CH}-\text{C}-\text{SO}_2$), 143.0 ($\text{CO}-\text{NH}-\text{C}-\text{CH}-\text{CH}-\text{C}-\text{SO}_2$), 139.9 ($=\text{C}(\text{CH}_3)-\text{CON}$), 137.2 ($\text{CO}-\text{NH}-\text{C}-\text{CH}-\text{CH}-\text{C}-\text{SO}_2$), 129.18 ($\text{NH}_2-\text{C}-\text{CH}-\text{CH}-\text{C}-\text{SO}_2$) 128.5 ($\text{CO}-\text{NH}-\text{C}-\text{CH}-\text{CH}-\text{C}-\text{SO}_2$) 127.4 ($\text{NH}_2-\text{C}-\text{CH}-\text{CH}-\text{C}-\text{SO}_2$), 120.8 ($\text{C}^{\beta}\text{H}_2$), 119.9 ($\text{CO}-\text{NH}-\text{C}-\text{CH}-\text{CH}-\text{C}-\text{SO}_2$), 113.0 ($\text{NH}_2-\text{C}-\text{CH}-\text{CH}-\text{C}-\text{SO}_2$), 18.5 ($\text{C}^{\alpha}\text{H}_3$)

3.2. *Dapone Methacrylamide homopolymer and co-polymer*

The dapson methacrylamide was used for both homo and copolymerisation with HEMA. Linear p(*DapMA*) (P100) and p(*DapMA-co-HEMA*) (P50) copolymers of HEMA with dapson (Figure 2) were synthesized by free radical polymerisation using AIBN in dimethylformamide as solvent at 50 °C and both obtained in the form of white powders and soluble in organic solvents such as DMSO and DMF and N,N-dimethylacetamide. Solubility in water is limited but confirmed at concentrations of $0.5 \text{ mg} \cdot \text{mL}^{-1}$ or less. P100 and P50 linear polymers were characterised by ^1H and ^{13}C NMR spectroscopy showing the following peak assignment:

^1H -NMR spectrum p(*DapMA*) ($\text{DMSO}-d_6$): δ_{H} 9.3 (bs, $\text{CO}-\text{NH}-\text{C}_{\text{Ar}}-\text{SO}_2$), 8.6 – 6.4 (bm, $\text{CO}-\text{NH}-\text{C}-\text{CH}-\text{CH}-\text{C}-\text{SO}_2$, $\text{NH}_2-\text{C}-\text{CH}-\text{CH}-\text{C}-\text{SO}_2$, $\text{CO}-\text{NH}-\text{C}-\text{CH}-\text{CH}-\text{C}-\text{SO}_2$, $\text{NH}_2-\text{C}-\text{CH}-\text{CH}-\text{C}-\text{SO}_2$), 6.11 (bs, $\text{NH}_2-\text{C}_{\text{Ar}}$), 2.90 - 1.7 (m, CH_2^{β}), 0.83 (bs CH_3^{α}).

^{13}C -NMR spectrum p(*DapMA*) (DMSO- d_6): δ_{C} 175.9 ($\underline{\text{C}}\text{O-NH}$), 153.5 ($\text{NH}_2\text{-}\underline{\text{C}}\text{-CH-CH-C-SO}_2$), 142.5 ($\text{CO-NH-}\underline{\text{C}}\text{-CH-CH-C-SO}_2$), 137.5 ($\text{CO-NH-C-CH-CH-}\underline{\text{C}}\text{-SO}_2$), 129.5 ($\text{CO-C-CH-CH-}\underline{\text{C}}\text{-SO}_2$) 127.4 ($\text{CO-NH}_2\text{-C-CH-}\underline{\text{C}}\text{-CH-C-SO}_2$) 126.1 ($\text{NH}_2\text{-C-}\underline{\text{C}}\text{-CH-CH-C-SO}_2$), 120.8 ($\text{CO-NH-C-}\underline{\text{C}}\text{-CH-CH-C-SO}_2$), 113.1 ($\text{NH}_2\text{-C-}\underline{\text{C}}\text{-CH-CH-C-SO}_2$), 66.5 ($\underline{\text{C}}^{\beta}\text{H}_2$), 46.2 ($\underline{\text{C}}^{\alpha}\text{H}_3$)

^1H -NMR spectrum p(*DapMA-co-HEMA*) (DMSO- d_6): δ_{H} 9.3 (bs 1H, $\text{CO-NH-}\underline{\text{C}}_{\text{Ar}}\text{-SO}_2$), 8.3 – 6.2 (bm, $\text{CO-NH-C-CH-CH-}\underline{\text{C}}\text{-SO}_2$, $\text{NH}_2\text{-C-CH-CH-}\underline{\text{C}}\text{-SO}_2$, $\text{CO-NH-C-CH-CH-C-SO}_2$, $\text{NH}_2\text{-C-CH-CH-C-SO}_2$), 6.10 (bs, $\text{NH}_2\text{-}\underline{\text{C}}_{\text{Ar}}$), 4.8 (bs $\text{O}(\text{CH}_2)_2\text{OH}$), 4.35 - 3.1 (bm $\text{O}(\text{CH}_2)_2\text{OH}$), 2.90 - 1.5 (bm CH_2^{β}), 0.1 - 1.4 (bm CH_3^{α}).

^{13}C -NMR spectrum p(*DapMA-co-HEMA*) (DMSO- d_6):

δ_{C} 179.8 - 175.5 ($\underline{\text{C}}\text{O-NH}$, COO), 154.3 ($\text{NH}_2\text{-}\underline{\text{C}}\text{-CH-CH-C-SO}_2$), 143.3 ($\text{CO-NH-}\underline{\text{C}}\text{-CH-CH-C-SO}_2$), 138.2 ($\text{CO-NH-C-CH-CH-}\underline{\text{C}}\text{-SO}_2$), 130.2 ($\text{CO-C-CH-CH-}\underline{\text{C}}\text{-SO}_2$) 128.1 ($\text{CO-NH}_2\text{-C-CH-}\underline{\text{C}}\text{-CH-C-SO}_2$) 126.5 ($\text{NH}_2\text{-C-}\underline{\text{C}}\text{-CH-CH-C-SO}_2$), 121.8 ($\text{CO-NH-C-}\underline{\text{C}}\text{-CH-CH-C-SO}_2$), 113.4 ($\text{NH}_2\text{-C-}\underline{\text{C}}\text{-CH-CH-C-SO}_2$), 66.8 ($\text{O}(\text{CH}_2\text{-CH}_2\text{OH})$), 59.3 ($\text{O}(\text{CH}_2\text{-CH}_2\text{OH})$), 54.2 – 43.2 ($\underline{\text{C}}^{\beta}\text{H}_2$), 21.3 – 13.9 ($\underline{\text{C}}^{\alpha}\text{H}_3$)

3.3. Characterisation techniques

Figure 5 shows the ^1H -NMR spectra of the homopolymer and co-polymer system in which the corresponding signal of each monomeric units could be observed. Co-polymer composition was determined from ^1H NMR spectra of P50 co-polymer sample using the integral of the hydroxyl protons signals ($\text{CH}_2\text{-OH}$, 4.8 ppm) and oxyethylene protons signals at ($-\text{O}(\text{CH}_2)_2\text{O}-$, 4.3 - 3.1 ppm) and those of the aromatic ring protons at 6.5 - 8.4 ppm. In addition Figure 6 shows the FTIR fingerprint region spectra of Dapsone, DapMA monomer and linear dapsone-conjugated polymers p(DapMA) (P100) and p(DapMA-co-HEMA) (P50) where the

characteristic vibrational bands to can be observed (most representative bands: $\delta_{\text{asym}, \text{C=O}}$ 1,720 cm^{-1} and 1,770 cm^{-1} ; $\nu_{\text{N-H}}$ 1,619 cm^{-1} ; $\nu_{\text{C=CH}_2}$ 1,639 cm^{-1} ; $\nu_{\text{ArC=C}}$ 1,589 cm^{-1} ; ν_{SO_2} 1,290 cm^{-1} ; $\nu_{\text{C-N}}$ 1,280 cm^{-1}).

Table 1. Composition, Glass transition Temperature (T_g), Number Average Molecular Weight (M_n) Polydispersity Index (P.I.) and Temperatures of maximum degradation speed (T_{max}) and half weight loss (T_{50%}) of p(DapMA) (P100) and p(DapMA-co-HEMA) (P50)

Sample	DapMA in the feed (mol %)	DapMA in the copolymer (mol %)	T _g (°C)	M _n (Kg/mol)	P.I.	T _{max} (°C)	T _{50%} (°C)
P100	100	100	129	106	2.2	359	420
P50	50	48	114	62	1.96	470	454

Molecular weight and polydispersity determined by SEC and thermal characteristic of P100 and P50 polymers are summarized in Table 1. The glass transition temperature of the p(DapMA) homopolymer was 129 °C, which decreased to 114 °C with the addition of HEMA units to form the co-polymer. The thermal stabilities of P50 and P100 were investigated with TGA in a nitrogen stream, and the thermogravimetric curves are compared in Figure 7. The p(DapMA-co-HEMA) copolymer thermogram showed four degradation stages, and the derivative of the weight loss versus temperature showed four corresponding peaks centred at 250, 301, 350, and 470 °C. In contrast, the p(DapMA) homopolymer thermogram showed only two main degradation stages, corresponding to peaks centred at 358 and 435 °C.

3.4. *In vitro* dapsone release, degradation, cytotoxicity and anti-inflammatory activity.

Weight loss determined by gravimetric measurements showed that the polymers were stable when immersed in PBS solutions at 37 °C with a weight loss lower than 0.1 % after 21 days. The release of dapsone at three different pHs

(acidic, neutral, basic) is shown in Figure 8. The sustained release profiles of dapsones were comparable at different pH for all polymer conjugates with a slight increase in dapsones release for the P50 copolymers at pH 10.

Table 2. In vitro parameters determined for p(DapMA) (P100) and p(DapMA-co-HEMA) (P50)

Sample	Weight loss in SBF (%)	Maximum dapsones released at pH7 based on copolymer composition ($\times 10^{-2}$ %)	IC50 ($\mu\text{g/ml}$)
P100	0.2	3	32.5
P50	1.9	5	42.9

The nitric oxide (NO) inhibitory effects of the dapsones polymers on LPS treated cells after 24 h of incubation is shown in Figure 9 revealing significant inhibition on NO production compared to those of LPS-stimulated control cultures with no polymer drug added. In addition, the incorporation of HEMA into the copolymers reduced their cytotoxicity as observed in (Figure 10) for the dose-dependent effect with IC50 values ranging between 32 and 43 $\mu\text{g}\cdot\text{ml}^{-1}$ (Table 2). This revealed the absence of cytotoxic effects (CV > 99%) associated to the polymer extracts used for the NO inhibition studies.

4. Discussion

A typical acylation reaction was used for the synthesis of the DapMA by reaction with methacryloyl chloride using triethylamine as a catalyst under mild conditions [17]. Since dapsones contains two reactive aromatic amine functional groups it can lead to the formation of an undesirable side product namely bis-acrylamide as shown in Figure 1. However, the yield of this double acylation product

could be kept to a minimum by using stoichiometric amounts of the acyl compounds. The mono functional derivative of the symmetric sulfamides could be easily isolated and both unreacted dapsone and bis-acrylated derivatives were removed using recrystallization techniques [18]. Although a modest yield is obtained, this simple and easy synthetic process of obtaining DapMa did not warrant further optimisation in order to increase the yield since sufficient amounts of the desired monomer could be easily obtained [17]. The ^1H and ^{13}C NMR peak assignment is presented in *Section 3.1* and were further supported by the two-dimensional HMQC spectra (Figure 4) and by previous works reporting NMR studies on (4-substituted aryl) sulfones which shown peak assignments in concordance with the reported here [19]. The amidation reaction was confirmed by the appearance of the bands corresponding to the methacrylic residue (5.8 and 5.5 ppm) and amide proton (10.1 ppm).

HEMA monomer is known to impart a certain degree of ductility and hydrophilicity to polymeric systems and is beneficial for tuning mechanical and biological properties thus makes it a candidate of choice for biocompatible polymer drug conjugates preparation. Both P100 and P50 linear polymers were characterised by FTIR and ^1H and ^{13}C NMR spectroscopies. The lack of unconverted residual monomers within the isolated polymers was confirmed by the absence of the characteristic ^1H -NMR resonance signals assigned to methacrylic protons (5.80 and 5.54 ppm) and IR $-\text{C}=\text{CH}_2$ band ($1,640\text{ cm}^{-1}$) as observed in the corresponding monomer ^1H -NMR and FTIR spectra (Figure 5 and Figure 6 respectively) indicating that the polymerisation reaction proceeded via the methacrylic double bonds to yield hydrocarbon macromolecular chains with pendant dapsone and hydroxyethanol moieties. Solubility is a very important property of all polymeric biomaterials in general and of these dapsone-conjugates in particular as it determines the synthetic

methodologies and plays a key role in the bioactivity and degradability of these polymers. Linear polymers of dapson P100 and P50 are fully soluble in organic polar solvents and poorly soluble in water. However, when the reaction was carried out in the presence of a crosslinking agent, insoluble polymers were obtained, indicating the formation of a cross-linked network with dimensional stability permitting their use for *in vitro* drug release and degradation studies while keeping the dapson sequence distribution along the macromolecular microstructure as determined for the linear copolymers.

The DapMA homo and copolymers were soluble in DMF, and the number average molecular weight values determined by SEC were relatively high ($M_n > 50\text{kDa}$) (Table 1) with polydispersity indices near to 2.0 as expected for conventional free-radical reaction processes and therefore confirming this polymerization mechanism proposed.

The four degradation stages of p(DapMA-co-HEMA) copolymer suggested a degradation mechanism similar to the described for analogue acrylic polymers. According to well established theories [20, 21], the first stage of degradation originates from sterically hindered linkages that result from head-to-head coupling during polymerisation. The second stage of degradation is attributed to the decomposition of the vinylidene chain end due to disproportionation, with the rest of the stages being attributed to random scission of the main chain leading to depolymerisation of the acrylic polymer. In contrast, the p(DapMA) homopolymer thermogram suggesting the absence of head-to-head coupling reactions during the polymerisation and absence of vinylidene chain ends. The higher stability of p(DapMA) can be associated with the stabilization of the free radicals produced in the thermal degradation due to the presence of dapson moiety that provide with

antioxidant properties associate to its capacity for stabilize free radicals and radical scavenger action [22]. This observation is akin to anti-oxidant properties previously described for dapson and thus beneficial when the polymer interacts with cells under physiological conditions [23].

EGDMA constitutes a common reagent for preparing biocompatible crosslinked polymers due to its effectiveness and desired polymerization kinetics [24]. The aim of crosslinking the linear P50 and P100 polymers was to prepare insoluble matrices with a desired size and shape to support the in vitro drug release and degradation experiments. Release of dapson was studied using a UV detector, which is a simple, fast and practical method with a detection limit of $2 \text{ mg} \cdot \text{mL}^{-1}$ [25]. The results indicated a sustained release from both homopolymer and co-polymer as expected for amide linkages (Figure 8) and were comparable at three different pHs (acidic, neutral, basic) for all polymer conjugates with a slight increase in dapson release for the P50 copolymers at pH 10. This mechanism indicates that the hydrolysis of the amide groups occurs only along the hydrophobic dapson sequences and water accessibility is facilitated with the presence of HEMA sequences. It is important to highlight that the time points selected in this study were chosen from an experimental point of view and were not necessarily biologically relevant. However, from a biomedical perspective the hydrolysis mechanism by which the drug can be delivered is highly dependent on many other factors relative to both physical-chemical properties of the polymer drug conjugate, place of action and biological and enzymatic activity of the degradation milieu. Amide linkage constitute a widespread approach of drug conjugation towards the greater control over pharmacokinetics and targeted delivery where the main release mechanism is accepted to be enzymatically mediated (peptidases, matrix metalloproteinase,

collagenase, prostate specific membrane antigen, plasmin and others) [26]. The biodegradation rate of vinyl copolymers depends on a number of parameters such as hydrophilicity/hydrophobicity balance, composition, monomer sequence distribution, molecular weight or micro and macrostructure among other biological characteristics of the host tissues which drive the degradation mechanism of the macromolecules within body environments. It is well known that the degradative process of relatively low to medium molecular weight (<100 KDa) (meth)acrylic based copolymers occurs first through the hydrolysis of lateral residues followed by a random excision of the main backbone leading to a reduction of molecular weight that permits further bio-elimination and excretion of degradative products. The copolymers described in this work comprise of well-known biocompatible and bioresorbable hydrocarbon backbones analogous similar to established systems such as poly(hydroxymethyl methacrylate) and poly(methacrylic acid) that permit us to hypothesise an analogous biodegradation mechanism in the body [27]. Meanwhile, gravimetric degradation studies of samples in buffered fluid showed a negligible loss of weight during the course of the experiment, confirming the stability of these materials in aqueous conditions. As a result, the *in vitro* behaviour of the polymers is a consequence of the macromolecular composition and not only due to the release of low molecular weight compounds. The conjugation of dapsone to macromolecular systems thus provides a synthetic route to incorporate this drug into polymeric systems, with the potential to synthesise polymers with inherent anti-inflammatory action.

The nitric oxide inhibition assay constitutes a well-established method to determine the macrophage mediated *in vitro* activity of anti-inflammatory polymer drug conjugates. This assay consists in the quantification of NO production in

macrophage cultures through activation of inducible nitric oxide synthase by a pro-inflammatory LPS before the evaluation of NO inhibition activity from extracts of the samples [11, 28]. The inhibitory effects of the dapson polymers on LPS treated cells indicate good anti-inflammatory response for all samples with NO inhibition (mean ranges P100: 67-83 % and P50 67-90 %) with a maximum NO inhibition capacity between the two copolymers significantly higher for P50 after 24 hours of incubation ($p > 0.05$), corresponding to a qualitative anti-inflammatory activity order of P50 > P100. This activity sequence indicates that despite HEMA monomer not possessing inflammatory effects by itself, its presence in the macromolecule contributes to a synergistic effect in improving the overall anti-inflammatory capacity of dapson mainly related to the dapson release mechanism. In addition, the incorporation of HEMA into the co-polymers reduced their cytotoxicity as resulted from their higher IC50 values.

5. Conclusions

The synthesis of dapson-conjugated methacrylic monomer and polymers provides a new approach of obtaining high molecular weight macromolecular systems with *in vitro* anti-inflammatory properties. This approach will enable exploiting the clinical applications of dapson further and has great potential in the development of advanced anti-inflammatory therapies.

Figures:

Dapsone

DapMA

DapBMA

Figure 1. Chemical structures of dapsone and its derivative monomers dapsone methacrylamide (DapMA) and bis-methacrylamide (DapBMA).

Figure 2. Synthetic route for the preparation of dapsone conjugated monomer DapMA, and polymers p(DapMA) and p(DapMA-co-HEMA).

Figure 3. Assignment and ¹H and ¹³C NMR spectra of the dapsonsone-conjugated monomer DapMA in DMSO-d₆ at 25 °C.

Figure 4. ^1H - ^{13}C HMQC spectra of the dapsone-conjugated monomer DapMA in DMSO- d_6 at 25 °C.

Figure 5. ^1H NMR spectra of the linear dapson-conjugated polymers p(DapMA) (upper) and p(DapMA-co-HEMA) (lower) in DMSO- d_6 at 25 °C.

Figure 6. Normalized ATR-FTIR spectra of Dapsone, DapMA monomer and linear dapsone-conjugated polymers p(DapMA) (P100) and p(DapMA-co-HEMA) (P50).

Figure 7. TGA (upper) and DTG (lower) curves of dapson-conjugated polymers p(DapMA) (P100) and p(DapMA-co-HEMA) (P50)

Figure 8. Cumulative released dapson from conjugated polymers p(DapMA) (P100) and p(DapMA-co-HEMA) (P50) at pH 10 (squares), pH 7 (triangles) and pH 3 (spheres) in the time frame of 21 days.

Figure 9. Inhibitory effects of p(DapMA) (P100) and p(DapMA-co-HEMA) (P50) polymers on nitric oxide production in LPS-stimulated RAW 264.7 cells (upper) and relative cell viability (lower) of the treated cells with the LPS and polymers. The results were normalized respect to the corresponding blanks and significant differences calculated with respect to LPS control (* $p > 0.05$) and between samples (§ $p > 0.05$).

Figure 10. Comparison of dose–response curves of dapsones-conjugated polymers p(DapMA) (P100) and p(DapMA-co-HEMA) (P50) normalised against control cultures with no polymers added. From these diagrams the IC₅₀ values were determined.

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Statement of Significance

The dapson-conjugated methacrylic monomer and polymer derivatives with anti-inflammatory properties described are previously unreported. The scientific impact of this work lies in its potential to expand the clinical applications of dapson toward the development of advanced anti-inflammatory therapies based on polymer-therapeutic approaches. These approaches facilitate the treatment of existing rare auto-immune and other inflammatory related diseases.

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