European Journal of Medicinal Chemistry 46 (2011) 4980–4991

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Polymeric systems containing dual biologically active ions

Francisco Parra-Ruiz [a,b], Estefanía Toledano [a,b], Mar Fernández-Gutiérrez [a,b], Nina Dinjaski [c], María Auxiliadora Prieto [c], Blanca Vázquez-Lasa [a,b,*], Julio San Román [a,b]

[a] Institute of Polymer Science and Technology, CSIC, C/Juan de la Cierva 3, 28006 Madrid, Spain
[b] CIBER-BBN, Ebro River Campus, Building R&D, Block 5, 1st Floor, Poeta Mariano Esquillor s/n, Zaragoza 50017, Spain
[c] Centre of Biological Research, CSIC, C/Ramiro de Maeztu 9, 28040 Madrid, Spain

ARTICLE INFO

Article history:
Received 1 March 2011
Received in revised form
1 August 2011
Accepted 2 August 2011
Available online 23 August 2011

Keywords:
Quaternary ammonium salts
Bactericide polymers
Non steroidal anti-inflammatory drugs
Dual functional polymers

ABSTRACT

This paper reports the synthesis and characterization of dual functional polymerizable salts containing quaternary ammonium cations ionically linked to non steroidal anti-inflammatory drugs (NSAIDs), and their polymers and copolymeric systems obtained with acrylic monomers of different hydrophilicity, e.g. methyl methacrylate and 2-hydroxyethyl methacrylate. NSAIDs used were meclofenamic acid, ketoprofen and ibuprofen. Sustained release of the NSAID from polymeric and copolymeric samples was observed over a period of 10 days and the hydrophobic/hydrophilic character of both the polymeric system and the drug played a role in the release behaviour. The antimicrobial activity of dual functional monomeric and polymeric derivatives was confirmed against Gram-positive and Gram-negative bacteria and polymeric compounds presented higher bactericidal action than the precursory monomers. The extracts of copolymeric samples had anti-inflammatory activity in a nitric oxide inhibitory assay on RAW 264.7 cells and they produced a NO inhibition around 80% within the first seven days.

1. Introduction

In the latest years, organic salt forms of dual functionality have been developed and attracted much attention in the pharmaceutical industry [1]. Some examples of drugs have appeared in which two biologically active ions were chosen based on the desired physical, chemical and biological properties. Forms of quaternary ammonium cations as antimicrobial agents with active pharmaceutical ingredients such as lidocaine or sodium docusate have been reported as compounds of dual biological function [2]. Other dual functional salt forms of salicylic acid have been prepared using active cations composed of different antibacterial and analgesic drugs [3]. Dual functional salts of 1-methyl-3-butylimidazolium ibuprofenate have been incorporated into porous functionalized silica as a new drug delivery system [4]. Hexafluorophosphate salts of butyl, hexadecyl and octyl-3-methylimidazolium cations have been studied as reservoirs of sucrose and dexametasone with good results [5].

It is well known that quaternary ammonium polymers have antibacterial activity [6–8] and they have found application in the biomedical field, for example in medical devices for the prophylaxis and treatment of implant associated infections [9,10], as coatings of pellets in oral controlled release systems [11,12] or as potential membrane permeation enhancers of drugs [13,14]. Nowadays, antimicrobial polymeric systems with dual functionality are emerging. For example, a bifunctional layer-by-layer construct with permanent microbiocidal activity and capable of releasing different therapeutic agents has been reported [15]. Dual functional polymeric systems having ammonium cations with anions that possess a therapeutic activity are under investigation. It is worth mentioning the work of Jiang et al. [16] that employs polymers bearing quaternary ammonium cations with hydrolysable substitute groups ionically linked to an antimicrobial, antibacterial or antifungal agent, that seem to be adequate for application in wound dressings. The approach of our research team deals with the preparation of acrylic polymeric systems bearing quaternary ammonium cations and non steroidal anti-inflammatory drug (NSAID) as counterions. The ammonium cation will provide permanent antibacterial activity and the counterion, by ionic exchange reaction in the physiological medium, will provide the anti-inflammatory action. Localized delivery of anti-inflammatory agents is still the most effective way to control inflammation and subsequent fibrosis that occurs after implantation of any biomedical device [17].

* Corresponding author. Institute of Polymer Science and Technology, CSIC, C/Juan de la Cierva 3, 28006 Madrid, Spain. Tel.: +34 91 5618806; fax: +34 91 5644853.
E-mail address: bvazquez@ictp.csic.es (B. Vázquez-Lasa).

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doi:10.1016/j.ejmech.2011.08.004

Herein we report the synthesis and characterization of polymerizable salts varying in the ammonium substitute groups and the NSAID counterion as well as their free radical polymerization and copolymerization with acrylic monomers of different hydrophilic character. Thermal behaviour of compounds was studied by Differential Scanning Calorimetry (DSC) and Thermogravimetric Analysis (TGA). Release of NSAID in buffered solution was studied at different pHs. The antimicrobial activity of the obtained derivatives was tested using different Gram-positive and Gram-negative bacteria. The anti-inflammatory action and cytotoxicity was studied using cellular cultures of macrophages and human fibroblasts.

2. Experimental

2.1. Materials

The monomers *N,N*-dimethyl-*N*-benzyl-*N*-(2-methacryloyloxyethylh) ammonium bromide (BBr^-), *N,N*-dimethyl-*N*-(2-methyleylnenaphthyl)-*N*-(2-methacryloyloxyethyl) ammonium bromide (NBr^-) and *N,N*-dimethyl-*N*-(2-hexadecyl)-*N*-(2-methacryloyloxyethyl) ammonium iodide (HI^-) were synthesized as described previously [18]. Methyl methacrylate (MMA) (Aldrich) was used after successive extractions with 5% NaOH solution and distilled water. 2-Hydroxyethyl methacrylate (HEMA) (Aldrich) was purified as reported previously [18]. Sodium meclofenamate (SM) (Park Davis) was used without further purification. Sodium ketoprofenate (SK) and sodium ibuprofenate (SI) were prepared from ketoprofen acid (Aldrich) and ibuprofen acid (Uriach & Cia) respectively, following the procedures described elsewhere [19,20]. The solvents chloroform (SDS), dichloromethane (SDS), ethanol (EtOH) (SDS), dimethylformamide (DMF) (SDS) and dimethylsulfoxide (DMSO) (SDS) were purified by standard procedures. Azobisisobutyronitrile (AIBN) (Merck) was recrystallized from methanol (m.p. 104 °C). Phosphate buffered solutions (PBS) of different pH (Merck) were used as received. The culture medium Dulbecco's modified Eagle's medium enriched with 4500 mg/L of glucose (DMEM) was supplied by Sigma. L-glutamine, penicillin-streptomycin solutions, sodium pyruvate, trypsin-EDTA, 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) and lipopolysaccharide (LPS) (*Escherichia coli* 0127:138) were all purchased from Sigma–Aldrich. Foetal bovine serum (FBS) was supplied by Gibco (Invitrogen) and Greiss reagent (G-7921) by Molecular Probes (Invitrogen).

2.2. Characterization techniques

^1H NMR and ^{13}C NMR Nuclear Magnetic Resonance spectra were recorded in an INOVA-300 spectrophotometer. The spectra of the monomers and polymers were recorded in deuterated chloroform or methanol (10 w/v) at 25 °C. Tetramethylsilane (TMS) was used as internal standard. High resolution mass spectra were performed on an Agilent 6520 Accurate Mass Q-TOF LC/MS. ATR-FTIR spectra were recorded on a Perkin–Elmer Spectrum One spectrophotometer, with an ATR attachment. Thermal analysis was carried out by Differential Scanning Calorimetry (DSC) with a Perkin Elmer DSC-8500. The dry samples (15–20 mg) were placed in aluminium pans and heated from −20 to 150 °C at a constant rate of 20 °C/min. The glass transition temperature (T_g) was taken as the midpoint of the heat capacity transition. Thermal degradation was performed by Thermogravimetric Analysis (TGA) with a TGA Q500 (TA instruments) thermobalance under nitrogen atmosphere at a heating rate of 5 °C/min and a temperature interval between 30 and 600 °C.

2.3. Synthesis of monomers

2.3.1. N,N-dimethyl-N-benzyl-N-(2-methacryloyloxyethyl) ammonium meclofenamate (BM)

To a solution of BBr^- (1.84 mmol) in EtOH (10 mL) at room temperature SM was added (1.84 mmol) and reaction stirred for 2 h. The solvent was evaporated at reduced pressure and the solid dissolved in dichloromethane. The NaBr salt was filtered and the solvent evaporated at reduced pressure. BM was isolated as a white powder. The reaction yield was near to 100%. ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 10.87 (s, 1H, NH), 8.15 (d, 1H, $J = 8.4$ Hz, Ar), 7.42–7.19 (m, 5H, Ar), 7.14 (d, 1H, $J = 7.7$ Hz, Ar), 6.98 (t, 1H, $J = 7.7$ Hz, Ar), 6.93 (d, 1H, $J = 8$ Hz, Ar), 6.50 (t, 1H, $J = 7.7$ Hz, Ar), 6.16 (d, 1H, $J = 8.2$ Hz, Ar), 5.98 (s, 1H, $COCCH_3 = CHaH$), 5.47 (s, 1 H, $OCOCCH_3 = CHbH$), 4.68 (s, 2H, $J = 4.4$ Hz, N^+CH_2), 4.50 (s, 2H, $ArCH_2$), 3.80 (s, 2H, $J = 4.4$ Hz, CO_2CH_2), 3.11 (s, 6H, N^+Me_2), 2.26 (s, 3H, $ArCH_3$), 1.78 (s, 3H, $OCOCCH_3$). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 174.7, 162.2, 146.1, 136.8, 136.0, 134.7, 133.6, 133.1, 132.2, 130.7, 130.5, 129.0, 127.4, 127.3, 127.1, 126.6, 120.2, 117.1, 112.7, 69.0, 62.2, 58.0, 50.3, 20.5, 18.0. IR (ν max/cm^{-1}): 2957 (ν N^+R_4), 1718 (ν C=O), 1492 and 1449 (ν C–C in the aromatic ring and alkyl groups). HRMS (ESI^+) *m/z* [M^+] for $C_{15}H_{22}NO_2$, 248.1645; found, 248.1646. HRMS (ESI^-) *m/z* [$M-H^-$] for $C_{14}H_{10}Cl_2NO_2$, 294.0094, found, 294.0088.

2.3.2. N,N-dimethyl-N-benzyl-N-(2-methacryloyloxyethyl) ammonium ketoprofenate (BK)

To a solution of BBr^- (1.84 mmol) in chloroform (20 mL) SK (1.84 mmol) was added at room temperature and reaction stirred for 2 h. The reaction medium was filtered and the solvent evaporated at reduced pressure. BK was obtained as a white solid. The reaction yield was near to 100%. ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 7.82–7.0 (m, 14H, Ar), 6.12 (s, 1H, $COCCH_3 = CHaH$), 6.63 (s, 1H, $OCOCCH_3 = CHbH$), 5.13 (s, 2H, $ArCH_2$), 4.66 (s, 2H, N^+CH_2), 4.87 (s, 2H, $ArCH_2$), 4.14 (s, 2H, CO_2CH_2), 3.3 (s, 7H, CH, N^+Me_2), 1.91 (s, 3H, $OCOCCH_3$), 1.21 (d, $J = 6.7$ Hz, $CHCH_3$). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 197.4, 181.2, 166.2, 145.4, 137.2, 137.0, 135.0, 133.3, 132.4, 132.0, 130.8, 130.1, 129.2, 128.7, 128.1, 127.7, 127.6, 127.4, 127.0, 68.6, 62.2, 58.0, 50.1, 48.5, 19.3, 18.2. IR (ν max/cm^{-1}): 2968 (ν N^+R_4), 1721 and 1652 (ν C=O), 1478 and 1449 (ν C–C in the aromatic ring and alkyl groups). HRMS (ESI^+) *m/z* [M^+] for $C_{15}H_{22}NO_2$, 248.1645, found 248.1648.

2.3.3. N,N-dimethyl-N-benzyl-N-(2-methacryloyloxyethyl) ammonium ibuprofenate (BI)

To a solution of BBr^- (1.84 mmol) in distilled water (3 mL) SI (1.84 mmol) was added at room temperature and the solution stirred for 15 min. Then, dichloromethane was added and agitation maintained for 2 h. The reaction product was extracted with dichloromethane, dried over $MgSO_4$ and the solvent evaporated at reduced pressure. BI was isolated as transparent oil. The reaction yield was near to 100%. ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 7.53–7.34 (m, 5H, Ar), 7.29 (d, 2H, $J = 6.1$ Hz, Ar), 6.95 (d, 2H, $J = 6.1$ Hz, Ar), 6.01 (s, 1H, $COCCH_3 = CHaH$), 5.65 (s, 1H, $OCOCCH_3 = CHbH$), 4.72 (s, $ArCH_2$), 4.43 (s, 2H, CO_2CH_2), 3.80 (s, 2H, N^+CH_2), 3.62 (c, 1H, $J = 5.7$ Hz, $^-O_2C-CH-(CH_3)Ar$), 2.99 (s, 6H, N^+Me_2), 2.80 (m, 2H, $Ar-CH_2$), 1.94 (s, 3H, $OCOCCCH_3$), 1.77 (nona, 1H, $J = 6.8$ Hz, $CH_2-CH-(CH_3)_2$), 1.42 (d, 2H, $J = 5.7$ Hz, $CH-(CH_3)Ar$), 0.85 (d, 2H, $(CH-(CH_3)_2)$). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 180.6, 166.1, 142.1, 138.6, 135.2, 133.2, 130.5, 129.0, 128.6, 127.6, 127.2, 127.1, 68.6, 62.0, 58.1, 49.8, 48.2, 45.0, 30.1, 22.3, 19.7, 18.2. IR (ν max/cm^{-1}): 2957 (ν N^+R_4), 1722 (ν C]O), 1481 and 1460 (ν C–C in the aromatic ring and alkyl groups). HRMS (ESI^+) *m/z* [M^+] for $C_{15}H_{22}NO_2$, 248.1645, found, 248.1652.

2.3.4. N,N-dimethyl-N-(2-methylenenaphthyl)-N-(2-methacryloyloxyethyl) ammonium meclofenamate (NM)

To a solution of NBr^- (1.32 mmol) in EtOH (10 mL) SM (1.32 mmol) was added at room temperature and solution stirred

for 2 h. The solvent was evaporated at reduced pressure and the solid dissolved in dichloromethane. The NaBr salt was filtered and the reaction product isolated by solvent evaporation at reduced pressure. NM was obtained as a white solid. The reaction yield was 99%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 10.71 (s, 1H, NH), 8.13 (d, 1H, J = 7.3 Hz, Ar), 7.84–7.55 (m, 4H, Ar), 7.55–7.37 (m, 2H, Ar), 7.37–7.20 (m, 1 H, Ar), 7.07 (d, 1H, J = 8.4 Hz, Ar), 6.93 (t, 1H, J = 7.4 Hz, Ar), 6.85 (d, 1H, J = 7.9 Hz, Ar), 6.45 (t, 1H, J = 7.0 Hz, Ar), 6.13 (d, 1H, J = 8.4 Hz, Ar), 5.93 (s, 1H, $COCCH_3$ = CHaH), 5.40 (s, 1H, $OCOCCH_3$ = CHbH), 4.75 (s, 2H, N^+CH_2), 4.52 (s, 2H, $ArCH_2$), 3.76 (s, 2H, CO_2CH_2), 3.08 (s, 6H, N^+Me_2), 2.18 (s, 3H, $ArCH_3$), 1.72 (s, 3H, $OCOCCH_3$). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 175.6, 166.9, 146.7, 137.3, 136.4, 135.1, 134.2, 134.1, 133.1, 132.8, 131.3, 131.1, 129.4, 129.3, 128.9, 127.9, 127.8, 127.6, 127.1, 124.4, 120.4, 117.7, 113.2, 69.9, 62.9, 58.5, 50.8, 20.9, 18.5. IR (ν max/cm^{-1}): 3059 (ν N^+R_4), 1718 (ν C=O), 1496 and 1449 (ν C–C in the aromatic ring and alkyl groups). HRMS (ESI^+) m/z [M^+] for $C_{19}H_{24}NO_2$, 298.1802, found, 298.1806.

2.3.5. N,N-dimethyl-N-(2-methylenenaphthyl)-N-(2-methacryloyloxyethyl) ammonium ketoprofenate (NK)

To a solution of NBr^- (1.32 mmol) in chloroform (20 mL) SK (1.32 mmol) was added at room temperature and the solution stirred for 2 h. The suspension was filtered and the solvent evaporated at reduced pressure giving the monomer NK as a white solid with a reaction yield of 99%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.10 (s, 1H, Ar), 8.01–7.60 (m, 5H, Ar), 7.60–7.28 (m, 6H, Ar), 7.28–7.16 (m, 2H, Ar), 7.12 (d, 1H, J = 7.4 Hz, Ar), 6.96 (t, 1H, J = 7.8 Hz, Ar), 6.02 (s, 1H, $COCCH_3$ = CHaH), 5.51 (s, 1H, $OCOCCH_3$ = CHbH), 5.26 (s, 2H, $ArCH_2$), 4.60 (s, 2H, CO_2CH_2), 4.11 (s, 2H, N^+CH_2), 4.0 (c, 1H, J = 6.8 Hz, CH), 3.31 (s, 6H, N^+Me_2), 1.80 (s, 3H, $OCOCCH_3$), 1.13 (d, J = 6.8 Hz, $CHCH_3$). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 197.3, 181.2, 166.2, 145.4, 137.1, 137.0, 134.9, 133.9, 133.7, 132.7, 132.4, 132.1, 130.1, 129.2, 129.0, 128.7, 128.4, 128.0, 127.7, 127.6, 127.7, 126.9, 124.2, 68.8, 62.2, 58.0, 50.1, 48.5, 19.3, 18.2. IR (ν max/cm^{-1}): 2968 (ν N^+R_4), 1722 (ν C=O), 1481 and 1445 (ν C–C in the aromatic ring and alkyl groups). HRMS (ESI^+) m/z [M^+] for $C_{19}H_{24}NO_2$, 298.1802, found, 248.1806.

2.3.6. N,N-dimethyl-N-(2-hexadecyl)-N-(2-methacryloyloxyethyl) ammonium meclofenamate (HM)

To a solution of HI^- (1.12 mmol) in chloroform (20 mL) SM was added at room temperature (1.12 mmol) and the solution stirred for 2 h. The suspension was filtered and the solvent evaporated at reduced pressure giving the monomer HM as a waxy solid with a reaction yield of 99%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 10.85 (s, 1H, NH), 8.12 (d, 1H, J = 7.6 Hz, Ar), 7.16 (d, 1H, J = 8.0 Hz, Ar), 6.97 (m, 2H, Ar), 6.54 (t, 1H, J = 8.0 Hz, Ar), 6.16 (d, 1H, J = 7.6 Hz, Ar), 5.98 (s, 1H, $COCCH_3$ = CHaH), 5.53 (s, 1H, $OCOCCH_3$ = CHbH), 4.42 (s, 2H, N^+CH_2), 3.78 (s, 2H, CO_2CH_2), 3.25 (s, 8H, N^+Me_2, N^+CH_2–$(CH_2)_{14}$–CH_3), 2.27 (s, 3H, $OCOCCH_3$), 1.82 (s, 3H, $ArCH_3$), 1.60–1.1 (m, 26H, N^+CH_2–$CH_2(CH_2)_{13}$–CH_3), 0.86 (t, J = 6.4 Hz, N^+CH_2–$(CH_2)_{14}$–CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 174.8, 166.7, 146.6, 137.3, 136.6, 135.4, 134.0, 132.6, 131.4, 130.9, 128.0, 127.7, 120.3, 117.7, 113.3, 65.7, 62.6, 58.5, 52.4, 32.3, 30.1, 30.9, 30.0, 29.9, 29.8, 29.6, 26.6, 23.2, 23.1, 21.0, 18.6, 14.6. IR (ν max/cm^{-1}): 3019 (ν N^+R_4), 2917 and 2848 (ν C=H), 1722 (ν C=O), 1500 and 1453 (ν C–C in the aromatic ring and alkyl groups). HRMS (ESI^+) m/z [M^+] for $C_{24}H_{48}NO_2$, 382.3680, found, 382.3681. HRMS (ESI^-) m/z [$M-H^-$] for $C_{16}H_{13}O_3$, 253.0864, found, 253.0870.

2.3.7. N,N-dimethyl-N-(2-hexadecyl)-N-(2-methacryloyloxyethyl) ammonium ketoprofenate (HK)

To a solution of HI^- (1.12 mmol) in chloroform (20 mL) SK (1.12 mmol) was added at room temperature and the solution stirred for 2 h. The suspension was filtered and the solvent evaporated at reduced pressure, giving the monomer HK as a waxy solid with a reaction yield of 99%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 7.70–7.56 (m, 3H, Ar), 7.52–7.39 (m, 2H, Ar), 7.40–7.21 (m, 3H, Ar), 7.11 (t, 1H, J = 7.4 Hz, Ar), 6.10 (s, 1H, $COCH_3$ = CHaH), 5.63 (s, 1H, $OCOCH_3$ = CHbH), 4.59 (s, 2H, N^+CH_2), 4.06 (s, 2H, CO_2CH_2), 3.75–3.32 (m + s, 9H, ArCH CO_2^-, N^+CH_2–CH_2–$(CH_2)_{13}$–CH_3), N^+Me_2), 1.90 (s, 3H, $OCOCCH_3$), 1.70 (m, 2H, N^+CH_2–CH_2–$(CH_2)_{13}$–CH_3), 1.60–1.1 (m, 29H, N^+CH_2–CH_2–$(CH_2)_{13}$–CH_3, $ArCHCH_3CO_2^-$, 0.84 (t, J = 6.6 Hz, N^+CH_2–$(CH_2)_{14}$–CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 197.3, 180.4, 166.2, 145.2, 137.2, 137.1, 135.0, 132.4, 132.1, 130.1, 128.7, 128.1, 127.7, 127.3, 65.3, 62.1, 58.1, 51.8, 48.3, 31.8, 29.6, 29.5, 29.4, 29.3, 29.2, 26.2, 22.8, 22.6, 19.3, 18.2, 14.0. IR (ν max/cm^{-1}): 29557 (ν N^+R_4), 1718 (ν C=O), 1496 and 1449 (ν C–C in the aromatic ring and alkyl groups). HRMS (ESI^+) m/z [M^+] for $C_{24}H_{48}O_2$, 382.3680, found, 382.3694. HRMS (ESI^-) m/z [$M-H^-$] for $C_{14}H_{10}Cl_2NO_2$, 294.0094, found, 294.0088.

2.4. Polymerization reactions

The polymers poly-BM, poly-BK, poly-BI, poly-NK, poly-HK were obtained in DMF solution (1M) at 50 °C, using AIBN (0.5 wt-% with respect to monomer) as a free radical initiator, and reaction time of 24 h to render high conversion polymers. At the end of the reaction, the medium was dialyzed in distilled water; the solid was lyophilized and dried until constant weight under vacuum.

2.4.1. Poly-BM

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.92 (d, 1H, J = 8.0 Hz, Ar), 7.50–7.20 (m, 5H, Ar), 7.18 (d, 1H, J = 7.7 Hz, Ar), 7.0 (d, 2H, J = 8. Hz, Ar), 6.59 (t, 1H, J = 7.7 Hz, Ar), 6.15 (d, 1H, J = 8.2 Hz, Ar), 4.80 (bs, 2H, $ArCH_2$), 4.57 (bs, 2H, N^+CH_2), 3.82 (bs, 2H, CO_2CH_2), 3.04 (bs, 6H, N^+Me_2), 2.27 (s, 3H, $ArCH_3$), 1.16 (bs, 3H, $OCOCCH_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 176.3, 173.4, 145.4, 135.8, 135.7, 132.5, 131.0, 130.8, 130.3, 129.7, 128.6, 128.5, 128.3, 127.1, 126.3, 120.0, 116.8, 116.3, 113.1, 112.6, 68.5, 61.6, 58.5, 49.0, 44.7, 19.6.

2.4.2. Poly-BK

^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 7.78 (s, 1H, Ar), 7. 67 (d, 1H, J = 6.5 Hz, Ar), 7.64–7.26, (m, 12H Ar), 4.67 (bs, 2H, $ArCH_2$), 4.50 (s, 2H, N^+CH_2), 3.88 (s, 2H, CO_2CH_2), 3.62 (c, 1H, J = 6.6 Hz, CH), 3.09 (bs, 6H, N^+Me_2), 1.41 (d, 3H, J = 6.7 Hz, $CHCH_3$), 1.14 (bs, 3H, $OCOCCH_3$). ^{13}C NMR (100 MHz, CD_3OD) δ (ppm): 198.7, 181.5, 178.0, 146.3, 138.7, 138.5, 134.3, 133.8, 133.3, 132.0, 131.0, 130.4, 130.0, 129.5, 129.2, 128.9, 128.6, 69.8, 63.5, 60.1, 50.8, 50.3, 46.5, 20.2.

2.4.3. Poly-BI

^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 7.64–7.34 (bs, 5H, Ar), 7.23 (d, 2H, J = 6.1 Hz, Ar), 6.96 (d, 2H, J = 6.1 Hz, Ar), 4.65 (bs, 2H, $ArCH_2$), 4.49 (bs, 4H, N^+CH_2), 3.87 (bs, 2H, CO_2CH_2), 3.53 (c, 1H, J = 5.7 Hz, ^-O_2C–CH–(CH_3)Ar), 3.07 (bs, 6H, N^+Me_2), 2.32 (d, 2H, J = 7.2 Hz Ar–CH_2), 1.74 (nona, 1H, J = 6.8 Hz, CH_2–CH–$(CH_3)_2$), 1.36 (d, 2H, J = 5.7 Hz, CH–(CH_3)Ar), 1.10 (bs, 3H, $OCOCCH_3$), 0.81 (d, 2H, $(CH-(CH_3))_2$). ^{13}C NMR (100 MHz, CD_3OD) δ (ppm): 182.2, 177.6, 142.4, 140.0, 134.1, 131.8, 130.1, 129.6, 128.0, 69.5, 63.3, 60.1, 50.5, 46.2, 45.8, 31.1, 22.8, 20.2.

2.4.4. Poly-NK

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.02 (s, 1H, Ar), 7.72 (bs, 4H, Ar), 7.61–7.25 (m, 10H, Ar), 7.20 (bs, 1H, Ar), 4.69 (bs, 6H, $ArCH_2$, N^+CH_2, CO_2CH_2), 3.61 (bs, 1H, CH), 3.12 (bs, 6H, N^+Me_2), 1.39 (bs, 3H, $CHCH_3$), 1.18 (bs, 3H, $OCOCCH_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 197.0, 179.6, 176.2, 144.5, 136.7, 136.5, 133.2, 132.2, 132.0, 131.6, 129.2, 128.2, 127.7, 127.4, 127.3, 126.6, 123.9, 68.2, 62.0, 60.0, 49.1, 44.7, 18.8.

2.4.5. Poly-HK

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.78 (s, 1H, Ar), 7.23 (d, 2H, $J = 7.3$ Hz, Ar), 7.57 (m, 1H, Ar), 7.54 (t, 1H, $J = 6.8$ Hz, Ar), 7.49–7.36 (m, 3H, Ar), 7.29 (t, 1H, $J = 7.3$ Hz, Ar), 4.43 (bs, 2H, $N^+CH_2CH_2O$), 3.40 (bs, 2H, $CO_2CH_2CH_2N^+$), 3.54 (bs, 3H, $N^+CH_2CH_2CH_2$, CH), 3.27 (bs, 6H, N^+Me_2), 1.61 (bs, 2H, $N^+CH_2CH_2CH_2$), 1.41 (bs, 3H, $CHCH_3$), 1.31–1.10 (bs, 30H, $N^+CH_2CH_2(CH_2)_{13}CH_3$, $OCOCCH_3$), 0.84 (t, 3H, J = 6.9 Hz, $N^+CH_2(CH_2)_{13}CH_3$).^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 197.3, 177.9, 146.6, 137.4, 137.1, 132.5, 132.2, 130.0, 128.8, 128.3, 127.6, 127.3, 66.3, 61.9, 59.3,50.8, 49.7, 45.4, 31.8, 29.7, 29.6, 29.3, 26.3, 22.6, 20.0, 15.1.

2.5. Copolymerization reactions

BM-co-MMA hydrophobic copolymers with BM:MMA ratios in the feed of 5:95, 10:90, 15:85, 20:80 and 50:50 wt-% were synthesized and characterized under the experimental conditions described in Section 2.4. BM-co-HEMA and BK-co-HEMA hydrophilic copolymers with BM:HEMA and BK:HEMA ratios in the feed of 20:80 wt-% respectively, were obtained by bulk copolymerization using AIBN (0.5 wt-% with respect to monomer) as an initiator at 60 °C for 24 h. At the end of the reaction the copolymeric discs were washed with ethanol to remove residual monomers.

2.5.1. BM:MMA 20:80 copolymer

^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 9.17 (s, 1H, NH), 8.04 (dd, 1H, $J = 7.58$, 1.6 Hz, Ar), 7.30 (m, 5H, Ar), 7.28 (d, 1H, $J = 8.2$, Ar), 7.14 (ddd, 1H, $J = 8.6$, 7.2, 1.3 Hz, Ar), 6.75 (d, 1H, $J = 8.2$ Hz, Ar), 6.29 (d, 1H, J = 8.6 Hz, Ar), 6.27 (s, 1H, Ar), 4.73 (bs, $ArCH_2$), 4.46 (bs, N^+CH_2), 3.91 (bs, CO_2CH_2), 3.60 (bs, CO_2Me), 3.20 (bs, N^+Me_2), 2.38 (s, 3H, $ArCH_3$), 1.84 (bs, $OCOCCH_3$), 1.0 (bs, $OCOCCH_3$), 0.82 (bs, $OCOCCH_3$).

2.5.2. BM:HEMA 20:80 copolymer

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 11.80 (s, 1H, NH), 7.88 (bs, 1H, Ar), 7.82–7.46 (bs, 5H, Ar), 7.42 (bs, 1H, Ar), 7.21 (bs, 1H, Ar), 7.0 (bs, 1H, Ar), 6.59 (bs, 1H, Ar), 6.05 (bs, 1H, Ar), 4.89 (bs, CH_2OH), 4.66 (bs, $ArCH_2$), 3.89 (bs, CO_2CH_2), 3.58 (bs, N^+CH_2, CH_2OH), 3.06 (bs, N^+Me_2), 2.38 (s, 3H, $ArCH_3$), 1.84 (bs, $OCOCCH_3$), 0.94 (bs, $OCOCCH_3$), 0.77 (bs, $OCOCCH_3$).

2.5.3. BK:HEMA 20:80 copolymer

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.88–7.31 (m, 14H, Ar), 4.90 (bs, CH_2OH), 4.66 (bs, $ArCH_2$), 3.90 (bs, CO_2CH_2), 3.58 (bs, N^+CH_2, CH_2OH), 3.04 (bs, N^+Me_2), 1.83 (bs, $OCOCCH_3$), 1.34 (bs, 3H, $CHCH_3$), 0.93 (bs, $OCOCCH_3$), 0.77 (bs, $OCOCCH_3$).

2.6. Release experiments

Discs of 50 mg of poly-BK, poly-BM, poly-BI, BM:MMA 20:80, BM:HEMA 20:80 and BK:HEMA 20:80 samples were used for release experiments. Discs of poly-BK were soaked in 5 mL of PBS at pH 2, 7.4 and 9 and incubated at 37 °C in different experiments. The rest of the samples were soaked in 5 mL of PBS of pH 7.4. In all cases, aliquots were taken at different periods of time and the aliquot was replaced by fresh solution. The aliquot was analyzed by UV spectroscopy in an apparatus Perkin Elmer UV/VIS spectrometer Lambda 16, at 260 nm for ketoprofen, at 278 nm for the meclofenamic acid and at 264 nm for ibuprofen. The corresponding calibration curves ($R^2 = 0.9999$) using free drug (SM, SK and SI) were obtained previously.

2.7. Antimicrobial assays

The bacterial strains *Staphylococcus epidermidis* CECT232, *Staphylococcus aureus subsp. aureus* CECT86, *Salmonella enterica serovar Typhimurium* LT2 and *Pseudomonas aeruginosa* PAO1 were used for determination of antibacterial activity. The minimal inhibitory concentration (MIC) of monomers (BBr^-, BK, NBr^-, NK, HI^- and HK) and polymers (poly-BBr^- and poly-BK) were calculated by microdilution assay according to CLSI (Clinical and Laboratory Standards Institute) standard procedure [21]. Tested compounds were sterilized by UV light exposition. Each compound was dissolved in DMSO at an initial concentration of 0.3 mM to prepare the stock solution. Subsequently, serial dilutions were made in Muller-Hinton broth (MHB, Becton Dickinson) to determine MIC values. Briefly, the sterile 96-well round bottom clear polystyrene plates (Cultek S.L.U., Spain) were prepared by dispensing 100 μL of appropriate dilution of tested compound in culture broth per well. The inocula were prepared with fresh microbial cultures in MHB and added to each well, providing a final concentration of 5×10^5 CFU/mL. A positive control (containing inoculum without tested compound) and negative control (containing tested compound without inoculum) were included in each microplate. Microdilutions of DMSO were used as controls to test the intrinsic antimicrobial activity of the solvent. Multiwell plates were incubated at 37 °C. Each test was performed at least three separate times, each by duplicate. The minimal bactericidal concentration (MBC) was obtained by taking further aliquots of 50 μL from four wells of antibacterial activity assays, starting from the well defining the MIC. MBC was tested in LB (Luria Bertani) agar plates incubated at 37 °C for 24 h [22]. The MBC value was defined as the lowest concentration of tested compounds needed to kill most of the viable microorganisms (≥99.9%) after incubation for a fixed time under a given set of conditions according to standards [21]. Each experiment was conducted in triplicate.

2.8. Cellular assays

The anti-inflammatory activity of the analyzed systems was assessed by a nitric oxide inhibitory assay performed according to the method of Wang et al. [23] and cellular viability was evaluated by a MTT assay [24]. Cells were mouse monocyte macrophages RAW 264.7 (ECACC, Sigma P7) and human skin dermal fibroblasts (HFB, Innoprot, P12). The cell culture medium (DMEM) was modified with HEPES for HFB cells and 110 mg/L of sodium pyruvate for RAW 264.7 cells and supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin and 200 mM L-glutamine (complete medium). Samples analyzed were films of poly-BK (25 mg) and BK:HEMA 20:80 copolymer (115 mg). Each film was immersed in 5 mL of FBS-free DMEM and placed on a roller mixer at 37 °C. The medium was totally removed at different time periods (1, 2, 7, 14 and 25 days) and replaced with fresh medium. All the extracts were obtained under sterile conditions. RAW 264.7 cells were seeded in 96-well plates at a density of 5×10^4 cells/mL. Cells were culture at 37 °C in complete medium in 5% CO_2 incubator for 24 h. Adhered cells were then incubated for 24 h with or without 1 μg/mL of LPS and the corresponding extract which was added at the same time. The nitrite concentration was measured by the Griess reaction [25]. Aliquots of 100 μL of the supernatant from RAW 264.7 cells were reacted with 100 μL of Griess reagent, 1:1 mixture of 0.1% N-(1-naphthyl) ethylenediamine in water and 1% sulphanilamide in 5% phosphoric acid, in a 96 well plate and the absorbance was recorded using a Biotek SYNERGY-HT reader at 548 nm. The nitrite concentration was calculated from a calibration curve previously obtained using known $NaNO_2$ concentrations. Data were expressed as percentage of NO inhibition.

Cellular viability of RAW 264.7 and HFB cells in presence of the extracts of poly-BK and BK:HEMA 20:80 copolymer was evaluated in parallel. Cells were seeded at a density of 5×10^4 cells/mL in complete medium in a sterile 96-well culture plate and incubated

to confluence. After 24 h of incubation the medium was replaced with the corresponding extract and incubated at 37 °C in humidified air with 5% CO_2 for 24 h. A solution of MTT was prepared in warm PBS (0.5 mg/mL) and the plates were incubated at 37 °C for 4 h. Excess medium and MTT was removed and DMSO was added to all wells in order to dissolve the MTT taken up by the cells. This was mixed for 10 min and the absorbance was measured with a Biotek SYNERGY-HT detector using a test wavelength of 570 nm and a reference wavelength of 630 nm. The cellular viability (CV) was calculated from equation:

$$CV = 100 \times (OD_S - OD_B)/(OD_C - OD_B)$$

where OD_S, OD_B and OD_C are the optical density of formazan production for the sample, blank (DMEM without cells) and control respectively.

3. Results and discussion

3.1. Synthesis and characterization of compounds

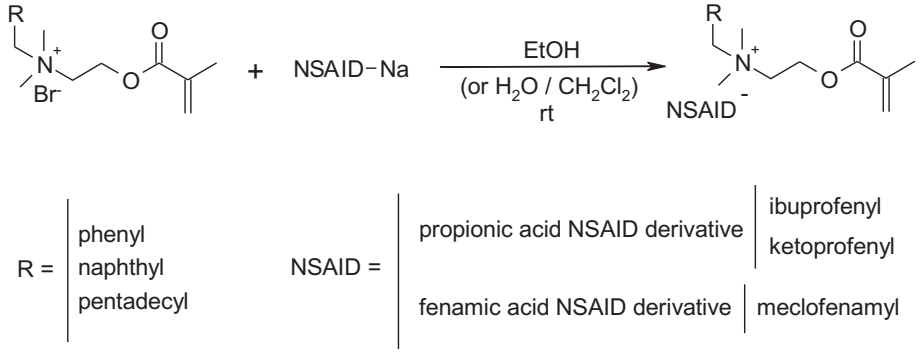
The aim of this work was the preparation of acrylic polymeric systems of dual biological action containing cationic functional groups ionically linked to NSAIDs. Initially, the monomeric compounds were synthesized by one-step ion exchange reaction from the monomeric quaternary ammonium halides and the sodium salts of the drug, following established procedures [26]. In all monomeric synthesis reactions yield was nearly 100%. The chemical structures of monomers shown in Scheme 1 were confirmed by ^1H and ^{13}C NMR, ATR-FTIR and mass spectroscopy (see Experimental section).

The extension of the ion exchange reaction for the BM and BK monomers was studied taking into consideration the resonance signal at about δ 1.9 ppm due to $(CH_3)^{\alpha}$ group of the methacrylate ester and the aromatic signal at δ 6.16 ppm pertaining to the meclofenamate anion and the aliphatic signal at δ 1.4 ppm of the ketoprofenate anion (see Fig. 1). The NSAID content in the monomeric derivatives was equivalent to the ammonium group, indicating that all the halogen anions of the precursory monomers were exchanged by NSAID anions and same result was obtained for the rest of the polymerizable salts. In general, the monomers were white powders not soluble in organic solvents with low solubility parameters but they exhibited solubility in high polar solvents (e.g. methanol, DMSO and chloroform). Solubility in water was poor for all the monomers (0.1–1.6%) and the degree depended of the substituent groups on the cation and the NSAID anion (see Table 1, Supplementary data). The monomers containing ketoprofen, BK and NK, exhibited the higher solubility in water (1.3 and 1.6% respectively). Due to the long alkyl chain of the hexadecyl radical the monomers HM and HK were waxy powders that presented surfactant properties in water. A similar behaviour is reported [26] for other cationic quaternary ammonium monomers which receive the name of surfmers [27].

The polymers poly-BM, poly-BK, poly-BI, poly-NK and poly-HK were obtained by free radical polymerization in DMF solution at high conversion. The polymerization yields were in the range 40–70%, and the polymer poly-HK had the highest yield (Table 1) [27]. Polymerization of the monomers was confirmed by NMR and ATR-FTIR spectroscopy (see Experimental section and Figure 1 of Supplementary data). All the polymers were white powders soluble in polar organic solvents such as chloroform, methanol and DMF but they were practically insoluble in water (Table 1).

Copolymerization of the functional monomers is a way to modulate the physicochemical properties of the polymeric derivatives and the therapeutic doses of the NSAID. Thus, copolymers of dual functional monomers with acrylic monomers of different hydrophilicity were obtained. On the one hand, hydrophobic polymeric systems were attained by copolymerization of BM with MMA. Copolymers were white powders insoluble in water and soluble in methanol, DMF and chloroform. The copolymers were characterized by ^1H NMR spectroscopy (see Experimental section). Assignment of signals confirmed the presence of both monomeric units in the macromolecular chains. Copolymer composition was calculated from the ^1H NMR spectra of copolymeric samples taking into consideration the resonance signals at δ_H, 6.20–6.32, 3.20 and 2.38 ppm corresponding to Ar, $N^+(CH_3)_2$, and $ArCH_3$ groups respectively, of the BM unit and the resonance signal at δ_H, 3.61 ppm pertaining to the CO_2CH_3 group of the MMA unit. Values of copolymer composition are shown in Table 2. BM content in the copolymer was lower than the corresponding feed indicating a lower reactivity of the biologically active monomer versus methyl methacrylate.

On the other hand, BM-co-HEMA and BK-co-HEMA hydrophilic systems were prepared by bulk copolymerization reactions to obtain transparent and hydrophilic discs that can be of interest for applications such as the preparation of intraocular implants [10]. Copolymers were characterized by ^1H NMR spectroscopy (see assignment in Experimental section) and copolymer compositions obtained from their corresponding spectra. The resonance signals at δ_H, 7.88, 7.82–7.46, 7.42, 7.21, 7.0, 6.59 and 6.05 ppm due to Ar protons of the BM unit, the resonance signals at δ_H, 7.88–7.31 ppm due to Ar protons of the BK unit, and the resonance signal at δ_H, 4.90 ppm pertaining to the $\underline{HO}CH_2CH_2OCO$ group of the HEMA unit were considered. Composition data appear in Table 2. Content of BK and BM in the corresponding copolymer was lower than in the feed, as obtained for the BM-co-MMA system, however, reaction yields were high and around 85%. In all cases, the solubility of copolymers in water or physiological fluids was lower than 0.01%.



Scheme 1. Synthesis and chemical structures of dual functional monomers.

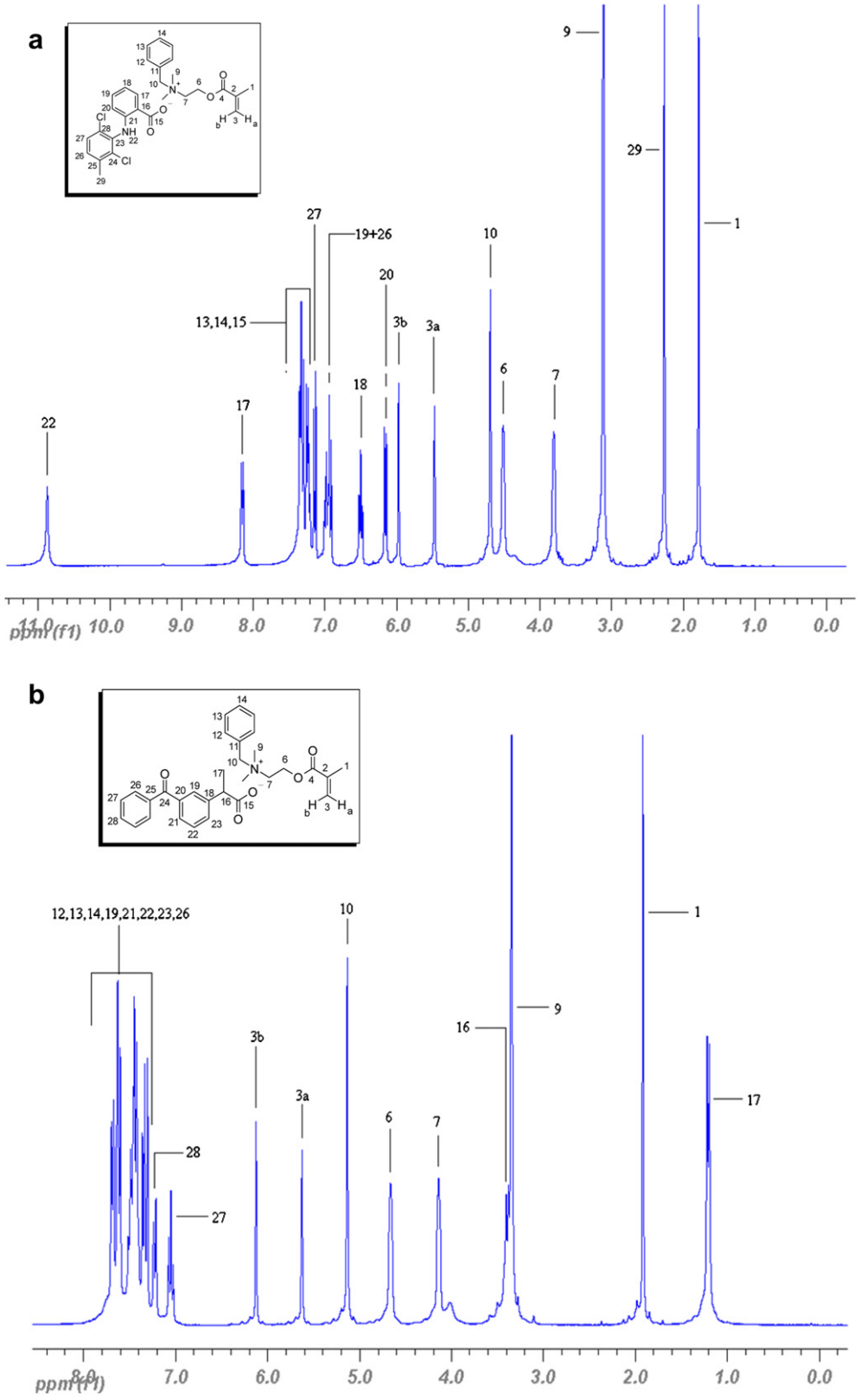


Fig. 1. ^{1}H NMR spectra of the monomers BM (a) and BK (b).

Table 1
Values of polymerization reaction yields and water solubility of the polymers with dual functionality.

Polymer	Poly-BM	Poly-BK	Poly-BI	Poly-NK	Poly-HK	Poly-BBr^-
Yield (%)	52	40	47	46	70	58
Water solubility (%)	0.01	0.07	0.02	0.02	0.01	2.80

3.2. Thermal properties of compounds

DSC and TGA were used for thermal analysis of synthesized monomers and polymers. All thermal results are collected in Table 3. On the part of the NSAIDs, a melting temperature (T_m) between 287 and 291 °C is reported for SM (data sheet of Meclofen®, Mylan Pharmaceuthics Inc.), a T_m of 184 °C is reported for SK [28] and a T_m between 220 and 222 °C is reported for SI [19]. DSC thermograms of BBr^-, NBr^- and HI^- showed characteristic endothermic peaks corresponding to T_m values of 146, 137 and 86 °C respectively. The thermogram of the monomer HI^- also showed a glass transition corresponding to a T_g value of 10 °C. DSC thermograms of all the monomers containing quaternary ammonium-NSAID salts showed a glass transition corresponding to T_g values in the range −3 to 36 °C, depending of the substituent groups on the cation and the NSAID anion. Monomers derived from hexadecyl ammonium meclofenamate and ketoprofenate salts (HM and HK) presented the characteristic endothermic peaks indicative of melting corresponding to T_m values of 69 °C and 80 °C, respectively. These monomers can be considered as ionic liquids which are generally defined as salts with melting points below 100 °C [29]. DSC thermograms of polymers and copolymers showed a glass transition. All the polymers presented T_g values higher than those of the respective monomers as a consequence of the restriction in the mobility due to the macromolecular chains. For BM-co-MMA system, BM:MMA 5:95, 10:90 and 20:80 copolymers had T_g values around that of pure poly-MMA [30] and for the copolymer richest in the bulky dual functional monomer T_g decreased. For the hydrophilic systems, the T_g value of the BM:HEMA 20:80 copolymer was slightly higher than that reported for poly-HEMA [31]. However, value for the BK:HEMA 20:80 copolymer approached that of the hydrophilic homopolymer.

The main parameters of thermal degradation of monomeric and polymeric species are also summarized in Table 3. The thermal stability of the monomers containing benzyl or naphthyl ammonium-NSAID salts ($T_{5\%}$ of 160–171 °C) decreased compared with the respective NSAID salts ($T_{5\%}$ of 309–387 °C) and the monomeric halide derivatives ($T_{5\%}$ of 184–224 °C) (see Figure 2, Supplementary data). However, thermal stability of the monomers containing the hexadecyl ammonium NSAID salts ($T_{5\%}$ of 196–199 °C) was higher than that of the precursory monomer HI^- ($T_{5\%}$ of 184 °C). In general two-step decomposition was observed for the NSAID containing monomers. TGA curves of polymers also showed a thermal decomposition mainly in two degradation stages located between 240 and 293 °C and 427–435 °C, respectively. The T_{max} values of both stages for each polymer were higher than those of the respective monomers. Only for poly-HK degradation occurred in three stages with T_{max} values of 229, 308 and 423 °C respectively. Polymers containing aryl substitute ammonium-NSAID salts had $T_{5\%}$ values lower than that of the respective monomer, except for poly-BM.

The BM-co-MMA copolymeric samples had $T_{5\%}$ values in the range 184–233 °C and they decreased with increasing BM concentration but all of them were higher than that of poly-BM (177 °C). TGA curves of these copolymers showed two main degradation stages. The first one can be associated to decomposition of vinylidene end groups and the most stable step to the random scission of the polymer chain as described for poly-MMA [32]. However, T_{max} values of these steps were higher than those reported for a poly-MMA obtained by a free radical polymerization reaction (270 and 360 °C, respectively) [32,33]. Thermal behaviour of the hydrophilic copolymers showed $T_{5\%}$ values of 233 and 229 °C, also higher than those of the corresponding dual functional polymers. These copolymers degraded in two main degradation steps whereas one main degradation step (T_{max} = 361 °C) has been reported for poly-HEMA [31]. There was no residue at 600 °C in all the copolymers as described for polymethacrylates [34]. Figure 3 (Supplementary data) displays the TGA curves of the three types of copolymers. Then, in general, we can say that copolymers presented a higher thermal stability compared with poly-MMA and poly-HEMA.

Table 2
Copolymerization reaction yields and copolymer composition of systems containing dual functional monomers. Standard deviation values are in brackets.

Sample Code	BM or BK Content in the Feed (wt-%)	BM or BK Content in the Copolymer (wt-%)	Yield (%)
BM:MMA 5:95	5	2.0 [0.10]	57
BM:MMA 10:90	10	4.3 [0.19]	53
BM:MMA 15:85	15	10.6 [0.01]	50
BM:MMA 20:80	20	12.7 [1.20]	51
BM:MMA 50:50	50	29.8 [0.04]	58
BM:HEMA 20:80	20	14.0 [0.01]	86
BK:HEMA 20:80	20	13.0 [0.02]	82

Table 3
DSC and TGA data of NSAID sodium salts, halide precursory monomers and dual functional monomers, polymers and copolymers synthesized in this work.

Sample	T_m[a]/T_g[b] (°C)	$T_{5\%}$ (°C)	T_{max} (main stages)[c] (°C)	Residue at 600 °C (%)
SM	287–291[a,d]	309	313	36.1
SK	184[a,28]	357	387	30.3
SI	220–222[a,19]	387	458	22.2
BBr^-	146[a]	220	273; 428	1.6
NBr^-	137[a]	224	284; 423	4.5
HI^-	86[a]; 10[b]	184	238	0
BM	26[b]	167	176; 274	9.7
BK	29[b]	163	182; 276	16.5
BI	-4[b]	163	187; 229; 431	2.5
NM	36[b]	160	170; 304	14.7
NK	11[b]	171	188; 320	14.9
HM	69[a]; −3[b]	199	249; 322	6.0
HK	80[a]; 9[b]	196	243; 317	6.0
Poly-BM	86[b]	177	293; 433	0.7
Poly-BK	52[b]	96	271; 429	2.2
Poly-BI	49[b]	99	240; 435	1.4
Poly-NK	76[b]	105	282; 427	3.6
Poly-HK	38[b]	160	229; 308; 423	0
BM:MMA 5:95	111[b]	233	297; 398	0
BM:MMA 10:90	95[b]	228	327; 402	0
BM:MMA 20:80	97[b]	196	291; 415	0
BM:MMA 50:50	72[b]	184	260; 416	0
BM:HEMA 20:80	113[b]	233	263; 404	0
BK:HEMA 20:80	106[b]	229	361; 415	0

[a] T_m value.
[b] T_g value.
[c] T_{max} is the temperature of the degradation stage at which velocity of decomposition is maximum.
[d] T_m values reported on the data sheet of Meclofen®.

3.3. Release behaviour

One of the functionalities of the polymeric systems prepared in this work comes from the NSAID captured by ionic force in their

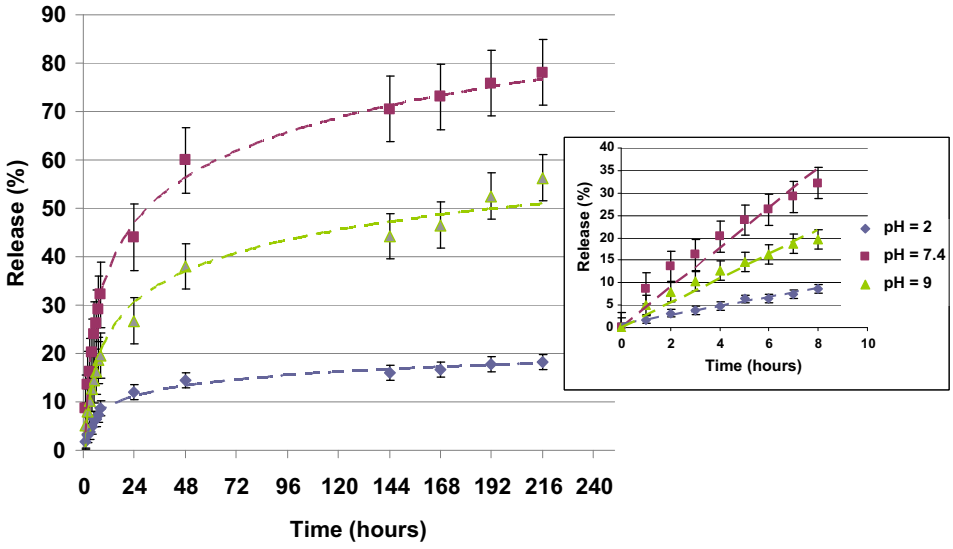


Fig. 2. Ketoprofen release from poly-BK samples soaked in buffered solutions of different pH at 37 °C.

structure. When the polymeric system is soaked in the physiological medium the counterion can be exchanged with another anion and therefore provide the anti-inflammatory action. To investigate this topic, release profiles of poly-BK were obtained at acidic, neutral and basic pH from samples conditioned at 37 °C. This polymer was studied first because of its higher hydrophilic character. Results are shown in Fig. 2. At any pH, release of the NSAID was linear with time during the first 8 h (see inset of Fig. 2). Afterwards, release slowed down approaching equilibrium around 7 days. After 10 days ketoprofen release amounted to 80% of the initial amount at pH 7.4 which means that nearly the majority of the drug anions were exchanged. At basic pH ketoprofenate exchange was close to 50% and it was relatively low at acidic pH, in which only 20% of the ketoprofenate anions were delivered. Fig. 3 shows the release of ketoprofen from the BK-co-HEMA hydrophilic system at neutral pH. An initial rapid release was observed in the first hour (5%), followed by a linear release in the next 8 h when nearly the 14% of the ketoprofenate counterions were exchanged (see inset of Fig. 3). Then, release slowed down and reached the equilibrium after 10 days with 35% ketoprofen released. These results show that the hydrophilic copolymeric system seems to be effective for ketoprofen delivery. In this sense, hydrogels prepared by copolymerization of HEMA and methacrylamide propyltrimethylammonium chloride have been proposed as drug delivery systems in soft contact lenses [35]. Likewise, drug delivery due to ion-exchange is reported for hydrogels that contain phosphate groups in the side chains, in which naphazoline, was incorporated [36].

Ibuprofen release was studied from poly-BI samples at pH 7.4. Release profile (data not shown) indicated a rapid exchange of the drug within the first 24 h (55% ibuprofen release) followed by a more gradual release in the next 7 days. Equilibrium was attained around 10 days with 95% of the drug delivered.

Meclofenamate release profiles were obtained from poly-BM and BM-co-MMA and BM-co-HEMA copolymers at neutral pH

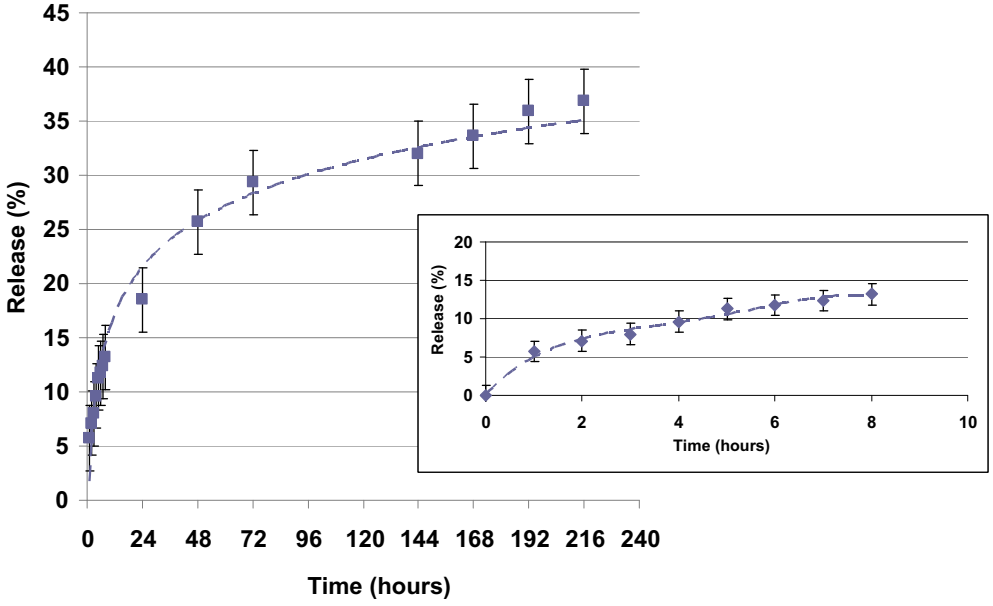


Fig. 3. Ketoprofen release from BK:HEMA 20:80 copolymer in PBS of pH 7.4 at 37 °C.

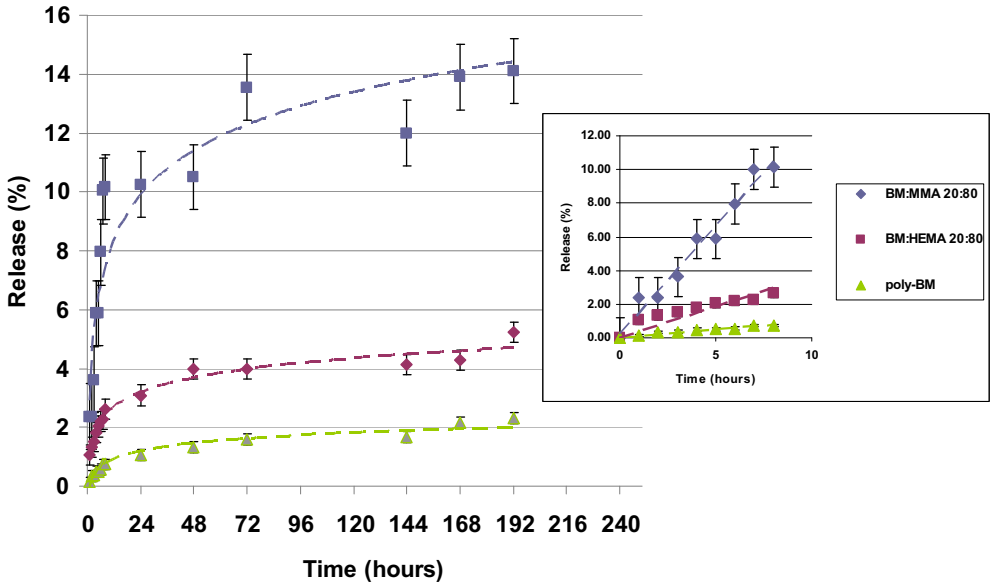


Fig. 4. Meclofenamate release from poly-BM, BM:HEMA 20:80 and BM:MMA 20:80 copolymers in PBS of pH 7.4, all at 37 °C.

and they are represented in Fig. 4. Release of the drug was linear with time during the first 8 h, however, the amount of drug delivered in this period was equal or lower than 10% in all samples (see inset of Fig. 4). At longer times (10 days) meclofenamate release from the homopolymer remained very scarce and only 2% of the anions were exchanged. NSAID release from the hydrophilic copolymer hardly increased, reaching the equilibrium with a 4% of drug exchanged. However, release was higher from the hydrophobic copolymer, and in this case, around 14.5% of initial meclofenamate anions were ion-exchanged. From these results it is deduced that both the hydrophobic/hydrophilic character of the polymeric system and the drug play a role in the release behaviour. Then, both factors should be taken into account for the NSAID delivery when designing a dual functional system for a specific application.

3.4. Antimicrobial activity

Antimicrobial activity was studied against Gram-negative and Gram-positive bacteria. Among Gram-negative strains *P. aeruginosa* PAO1 was selected as a ubiquitous environmental bacterium being one of the top three causes of opportunistic human infections. Furthermore, it is a prototype bacterium with intrinsic resistance to antibiotics and disinfectants. *Staphylococcus enterica* LT2 was also chosen as type Gram-negative pathogen. Gram-positive *S. epidermidis* and *S. aureus* strains were selected as major causes of nosocomial infections. Antimicrobial activity of the dual functional monomers and polymers was analyzed comparatively with those of the corresponding halide precursors. As proceeded previously, ketoprofen derivatives were tested due to their higher hydrophilic character. MIC and MBC values are given in Table 4. In general, the NSAID counterion had little effect on the antimicrobial activity of the monomeric specie, giving MIC and MBC values similar to those of the halide counterion. Only for the naphthyl derivative (NK) the ketoprofenate anion provided a decrease in the bactericidal activity, independent of the strain tested. However, the antimicrobial effect of the monomeric compounds strongly depended of the substitute group of the ammonium cation as reported for 3-alkoxymethyl-1-methylimidazolium salts [37]. The hexadecyl ammonium derivatives (HK, HI^-) showed the lowest MIC and MBC values for all the strains tested, as published in other works [26,27], while the benzyl derivatives (BK, BBr^-) showed the highest values ($>4.0 \times 10^{-2}$ mM). Naphthyl derivatives (NK, NBr^-) presented intermediate values except for the MBC detected in *P. aeruginosa* which was $>4.0 \times 10^{-2}$ mM. In general, MIC values coincided with MBC values for each compound excepting the hexadecyl derivatives monomers for *P. aeruginosa*, which showed MBC values higher than MIC ones. MBC values of the monomers BBr^- and HI^- obtained in this work compared well with those reported by Lu *et al.* [27] and by Callier et al. [26] for the HI^- acrylate (MIC of 275 μM) against *S. aureus*.

For the polymers poly-BBr^- and poly-BK, the incorporation of the ketoprofenate anion improved the bactericidal activity compared with that of the bromide anion. This effect was observed in all bacterial strains being more evident for the Gram-positive strains, in which MIC and MBC values of poly-BK were one order of magnitude lower. Both polymers showed much higher antibacterial activities than their corresponding monomers, e.g. MIC = 1.6×10^{-4} mM for

Table 4
In vitro activity against Gram-negative and Gram-positive strains.

Sample	*P. aeruginosa* PAO1		*S. enterica* LT2		*S. epidermidis* CECT232		*S. aureus* CECT86	
	MIC (mM)	MBC (mM)	MIC (mM)	MBC (mM)	MIC (mM)	MBC (mM)	MIC (mM)	MBC (mM)
HI^-	1.9×10^{-4}	7.5×10^{-4}	4.7×10^{-5}	4.7×10^{-5}	4.7×10^{-6}	4.7×10^{-6}	4.7×10^{-6}	4.7×10^{-6}
HK	1.9×10^{-4}	7.5×10^{-4}	9.4×10^{-5}	9.4×10^{-5}	2.3×10^{-6}	2.3×10^{-6}	4.7×10^{-6}	4.7×10^{-6}
BBr^-	$>5.0 \times 10^{-2}$	$>5.0 \times 10^{-2}$	$>5.0 \times 10^{-2}$	$>5.0 \times 10^{-2}$	5.0×10^{-2}	$>5.0 \times 10^{-2}$	5.0×10^{-2}	$>5.0 \times 10^{-2}$
BK	$>4.0 \times 10^{-2}$	$>4.0 \times 10^{-2}$	$>4.0 \times 10^{-2}$	$>4.0 \times 10^{-2}$	4.0×10^{-2}	$>4.0 \times 10^{-2}$	4.0×10^{-2}	$>4.0 \times 10^{-2}$
NBr^-	2.0×10^{-2}	$>4.0 \times 10^{-2}$	1.0×10^{-2}	1.0×10^{-2}	1.2×10^{-3}	1.2×10^{-3}	1.0×10^{-2}	1.0×10^{-2}
NK	4.0×10^{-2}	$>4.0 \times 10^{-2}$	2.0×10^{-2}	2.0×10^{-2}	2.5×10^{-3}	2.5×10^{-3}	1.0×10^{-2}	2.0×10^{-2}
Poly-BBr^-	1.8×10^{-4}	1.8×10^{-4}	3.7×10^{-4}	3.7×10^{-4}	1.8×10^{-4}	1.8×10^{-4}	1.8×10^{-4}	1.8×10^{-4}
Poly-BK	1.6×10^{-4}	1.6×10^{-4}	1.6×10^{-4}	1.6×10^{-4}	8.0×10^{-5}	8.0×10^{-5}	8.0×10^{-5}	8.0×10^{-5}

poly-BK, what is coherent with bibliography data reported for the polymeric bromide salts containing benzyl and alkyl ammonium substitute groups [27]. This result has been explained by a different mechanism of action of polymers *versus* monomers. It was thought that polymerization increases the positively charged density in polymer coil which enhances the ability to be absorbed onto the negatively charged bacterial surface [6], improving the first step of cationic biocides mode of action [38]. Furthermore, the binding to the cytoplasmatic membrane would be enhanced due to the high density of negatively charged species like acidic phospholipids and membrane proteins [39]. In this work, the polymeric derivatives of the hexadecyl ammonium salt, poly-HI^- and poly-HK, could not be tested due to their reduced solubility [27].

On the other hand, all of the compounds, monomers and polymers, presented lower MIC values against *S. epidermidis* and *S. aureus*, suggesting that the structure of the Gram-positive cell wall is more sensitive to their mode of action [40].

3.5. Anti-inflammatory activity

As stated in previous paragraphs, the anti-inflammatory action of the dual functional polymeric systems will come from the NSAID counterions of the ammonium quaternary salts that are able to ion exchange in the physiological medium. Nitric oxide (NO) inhibitory assay is a recognized test currently used to measure the anti-inflammatory activity. NO is a mediator and regulator in many pathological reactions, especially in acute inflammatory responses [23]. Pro-inflammatory agents, such as LPS, can significantly increase NO production in macrophages through activation of inducible nitric oxide synthase (iNOS) [41]. Then, a LPS-stimulated RAW 264.7 cell assay was employed to evaluate the NO inhibition activity of extracts coming from the polymeric samples. Fig. 5a shows the inhibitory effects of the extracts of poly-BK on LPS treated cells. The extracts of 1 day produced 60% NO inhibition and those collected at longer times produced around 80% NO inhibition. However, cellular viability (CV) of macrophages in presence of extracts was around 20% (data shown in the same figure), what indicates that the concentration of ketoprofen in these samples is high enough to produce a cytotoxic effect on macrophages and therefore, NO inhibition results should not be considered.

A way to control the ketoprofen release is to use copolymers in which the content of the NSAID containing monomer can be adjusted. In this work, different copolymers have been prepared which released ketoprofen in the range of 4–35%. Among them, BK:HEMA 20:80 copolymer was selected and in the first place, the cytotoxic effects of the extracts on macrophages were measured. In this case, CV was around 100% in presence of any extract, indicating absence of cytocytotoxic effect (see Fig. 5b). When the LPS treated

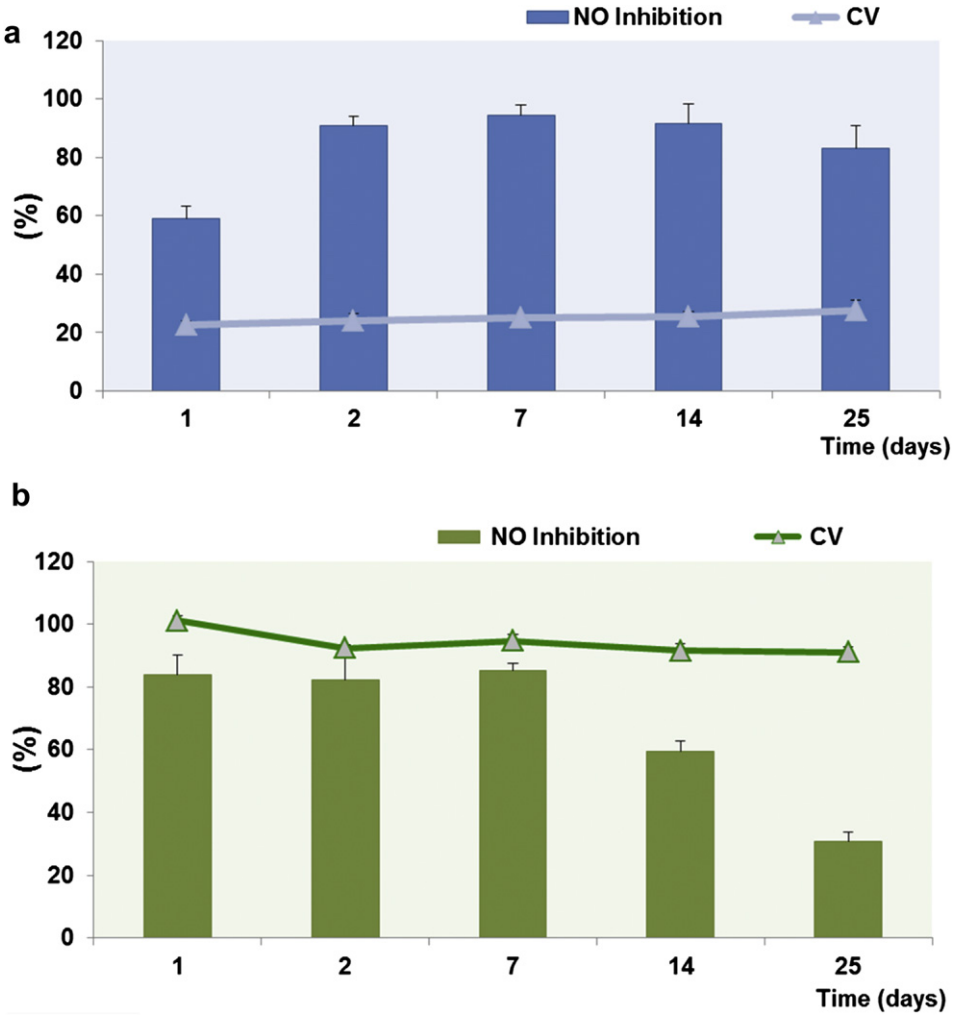


Fig. 5. Inhibitory effects of extracts of poly-BK (a) and BK:HEMA 20:80 (b) samples on nitric oxide production in LPS-stimulated RAW 264.7 cells and cellular viability determined by MTT assay.

macrophages were incubated with the extracts of 1, 2 and 7 days values of NO inhibition oscillated around 80% revealing good anti-inflammatory action. The extracts taken at 14 and 25 days were less effective in their anti-inflammatory activity, producing a NO inhibition of 60 and 40% respectively, what can be related with the lower ketoprofen concentration in the extracts of longer times. Consequently, the copolymer system provides very good anti-inflammatory activity during the first seven days which corresponds with the active release of the anti-inflammatory agent within this period (Fig. 5b).

Cytotoxicity of extracts taken between 1 and 25 days of both poly-BK and BK:HEMA 20:80 copolymer was also evaluated on normal HFB (Figure 4, Supplementary data). For poly-BK, cellular viability in presence of extracts of increasing time was between 50 and 70%, indicating that the biocompatibility is also compromised in this type of cells, mainly for the extracts taken at 1 and 2 days; however, HFB viability in presence of any extract of the copolymer was around 100% with respect to the negative control TMX.

4. Conclusion

Dual functional polymeric and copolymeric systems with different hydrophilicity were obtained based on polymerizable quaternary ammonium salts ionically linked to non steroidal anti-inflammatory drugs. Sustained release of the NSAID depended on the hydrophobic/hydrophilic character of both the polymeric system and the drug. The antimicrobial activity of dual functional polymeric derivatives against Gram-positive and Gram-negative bacteria was higher compared to the bactericidal action of the respective monomers. The extracts of copolymeric samples had anti-inflammatory activity in a nitric oxide inhibitory assay on RAW 264.7 cells and extracts taken within the first seven days produced a NO inhibition around 80%. These results suggest the potential of the copolymeric systems to be used in the fabrication or modification of implant devices, in particular for application in ocular contact lenses and intraocular lenses with functionality and bioactivity.

Acknowledgement

This work was supported by the Ministry of Science and Innovation, (MAT2010-18155 and BIO2010-21049). N. Dinjaski thanks to the *JAE-predoc program* of *CSIC*. F. Parra and M. Fernández-Gutiérrrez also thank to the *JAE-doc program* of *CSIC*. The support of V. Rodríguez in the antimicrobial assays is appreciated.

Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2011.08.004.

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