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Synthesis and study of controlled release of ofloxacin from polyester conjugates

Marcin Sobczak[a,*], Grzegorz Nałęcz-Jawecki[b], Wacław L. Kołodziejski[a], Piotr Goś[a], Karolina Żółtowska[a]

[a] Medical University of Warsaw, Faculty of Pharmacy, Department of Inorganic and Analytical Chemistry, ul. Banacha 1, 02-097 Warsaw, Poland
[b] Medical University of Warsaw, Faculty of Pharmacy, Department of Environmental Health Science, ul. Banacha 1, 02-097 Warsaw, Poland

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ABSTRACT

New polymeric conjugates were prepared coupling ofloxacin to two-, three-, four and six-arm, star-shaped poly(ε-caprolactone) and polylactide. The homopolymers were synthesized via ring-opening polymerization of ε-caprolactone, L-lactide and rac-lactide in the presence of glycerol, penthaerythritol, dipentaerythritol and poly(ethylene glycol) as initiators and stannous octoate as a catalyst. The conjugates were characterized by GPC, MALDI-TOF MS, NMR, IR and viscosity methods. Content of Sn has been investigated in polymers by electrothermal atomic absorption spectrometry. Toxicity of monomers, initiators and polymers were evaluated with bacterial luminescence test and two protozoan assays. The in vitro release of ofloxacin from obtained conjugates in pH 7 was investigated.

1. Introduction

Polymers are widely used in biomedical fields as artificial organs, tissue regeneration, macromolecular prodrugs, polymeric drug delivery and therapeutic systems. Controlled drug delivery technology represents one of the most rapidly advancing areas of pharmacy. The polymeric prodrugs, drug delivery and therapeutic systems exhibit unique pharmacokinetics, body distribution and pharmacological efficacy. Drug delivery systems based on macromolecular micelles, coated micro- and nanoparticles, liposomes and various polymer–drug conjugates are extensively studied as novel drugs (Hoste et al., 2004; Järvinen and Järvinen, 1996; Khandare and Minko, 2006; Ouchi and Ohya, 1995; Uhrich et al., 1999).

Bioresorbable and biodegradable polymers like polylactide (PLA), poly(ε-caprolactone) (PCL) and copolymers of lactides (LA) and ε-caprolactone (CL) are very often used as drug delivery systems. Aliphatic polyesters have particularly been developed for implantable pharmaceutical devices, since their use eliminates the step of removing the implant after the drug has been released. Homo- and copolymers of CL and LA are usually prepared by ring opening polymerization (ROP). Cyclic esters are polymerized in the presence of cationic or anionic initiators and coordinative or enzymatic catalysts. Usually the typical polymerization of lactides and lactones is carried out in the presence of tin compounds such stannous octoate ($SnOct_2$). $SnOct_2$ is considered to have a toxicity much lower than other heavy metal compounds, and it is allowed to be used as a food additive in a number of countries (Albertsson and Varma, 2003; Cai et al., 2003; Florjańczyk et al., 2006; Kobayashi et al., 2001; Okada, 2002; Sanda et al., 2002; Sobczak et al., 2008b; Sobczak and Kolodziejski, 2009; Storey and Sherman, 2002).

Recently, the novel biodegradable polyester and polyurethane conjugates of norfloxacin and ciprofloxacin were obtained in our laboratory (Sobczak et al., 2008a, 2010a; Sobczak, 2010b).

In this paper, we describe the synthesis of a series of polyester conjugates of ofloxacin. Ofloxacin is the most prominent member of a group called the fluoroquinolones. It is frequently used as a wide-spectrum antibiotic to treat and prevent infections caused by Gram-positive and Gram-negative bacteria in human bodies.

Chemical structures of the synthesized polymers have been confirmed using ^{1}H- and ^{13}C-solution NMR and FT-IR spectroscopy. Molecular weights of polymers have been determined using gel permeation chromatography (GPC), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and viscosity method.

We describe toxicity test of monomers, initiators and obtained polymers which were evaluated with bacterial luminescence test and two protozoan assays. Although cell culture assays with mammalian cells are currently the most popular in vitro tests for evaluating acute toxicity, other techniques are gaining prominence. They involve the use of bacteria and lower Eucaryotes (Uma et al., 2008). Luminescence assay with *Vibrio fischeri* has become broadly

* Corresponding author. Tel.: +48 22 572 07 55; fax: +48 22 572 07 84.
E-mail addresses: marcin.sobczak@wp.pl, marcin.sobczak@wum.edu.pl (M. Sobczak).

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Table 1
Homo- and copolymerization of CL, DLLA and LLA.

Monomer(s)/initiator/catalyst	M/I/C	Yield (%)	Mn^{GPC} (Da)	PD^{GPC}	Mn^{MS} (Da)	PD^{MS}	Mn^{th}	Mn^{arm}	C_{Sn} (ppm)
CL/PEG/$SnOct_2$	100:2:1	95	6200	1.2	4200	1.1	5615	3100	4
CL/GL/$SnOct_2$	150:2:1	87	8100	1.1	–	–	8642	2700	8
CL/PET/$SnOct_2$	200:2:1	74	7600	1.2	–	–	8572	2500	––
CL/DPET/$SnOct_2$	450:3:1	69	10,700	1.2	–	–	11,935	1800	–
LLA/PEG/$SnOct_2$	100:2:1	74	6000	1.2	3600	1.1	5528	3000	14
LLA/GL/$SnOct_2$	150:2:1	69	8200	1.3	–	–	7544	2700	–
LLA/PET/$SnOct_2$	200:2:1	55	7600	1.2	–	–	8056	1900	12
LLA/DPET/$SnOct_2$	450:3:1	52	10,100	1.3	–	–	11,486	1700	–
DLLA/PEG/$SnOct_2$	100:2:1	79	6100	1.2	3200	1.1	5888	3100	10
DLLA/GL/$SnOct_2$	150:2:1	62	7600	1.2	–	–	6788	2500	–
DLLA/PET/$SnOct_2$	200:2:1	60	8000	1.2	–	–	8776	2000	–
DLLA/DPET/$SnOct_2$	450:3:1	49	10,600	1.3	–	–	10,838	1800	8

Reaction conditions: temp. of 120 °C, time, 24 h. M/I/C, monomer:initiator:catalyst (molar ratio); Mn^{GPC}, number-average molecular weight determined by GPC; PD^{GPC}, polydispersity (Mw/Mn) determined by GPC; Mn^{MS}, number-average molecular weight determined by MALDI-TOF; PD^{MS}, polydispersity (Mw/Mn) determined by MALDI-TOF; Mn^{th}, theoretical number-average molecular weight of polymer: $Mn^{th} = [M]/[I] \times M_{monomer} \times \text{yield} + M_{initiator}$, $M_{PEG} = 200$ Da, $M_{GL} = 92$ Da, $M_{PET} = 136$ Da, $M_{DEPT} = 254$ Da; Mn^{arm}, number-average molecular weight of arm of PCL or PLA, $Mn^{arm} = Mn^{GPC}$/oligomer arm number; C_{Sn}, content of Sn (ppm) determined by ASA.

used as a fast and reliable preliminary test for risk assessment (Novotny et al., 2006). The use of protists, especially protozoa, as non-animal has the potential of reducing, refining and replacing in vivo testing (3R concept). *Tetrahymena* is the most widely used ciliated protozoan (Aptula et al., 2006).

2. Materials and methods

2.1. Materials

ε-Caprolactone (CL, 2-oxepanone, Aldrich 99%) was dried and distilled before use over CaH_2 at reduced pressure. 3,6-Dimethyl-1,4-dioxane-2,5-dione (DLLA and LLA, rac-lactide and L-lactide, Aldrich 98%) was crystallized from a mixture of dry toluene with hexane and dried under vacuum. Poly(ethylene glycol) (PEG) (M_n = 200 Da, Serva Feinbiochemica), glycerol (GL) (Aldrich 99%), pentaerythritol (PET) (Aldrich 99%), dipentaerythritol (DPET) (Aldrich, technical grade), ofloxacin (OFL) (Aldrich 99%) were exhaustively dried under vacuum prior use. Dichloromethane (POCh, Poland) were refluxed over CaH_2 and distilled under argon.

Stannous octoate ($SnOct_2$, tin (II) 2-ethylhexanoate) (Aldrich 95%), dicyclohexylcarbodiimide (DDC) (Aldrich 99%) and dimethylaminopyridine (DMAP) (Aldrich 99%) were used as received.

2.2. Polymerization procedure

Monomers (CL, DLLA, LLA), initiators (PEG, PET, GL, DPET) and the $SnOct_2$ catalyst were placed in a 10 mL glass ampule under argon atmosphere. The reaction vessel was then kept standing in a thermostated oil bath at 120 °C over 24 h (Table 1). When the reaction time was completed, the cold reaction product was dissolved in CH_2Cl_2, precipitated from 5% hydrochloric acid aqueous solution and dried under vacuum for 72 h. The precipitation was repeated three times (Sobczak et al., 2008a; Sobczak, 2010b).

2.3. Macromolecular conjugates synthesis

The conjugates were prepared under argon atmosphere at room temperature immediately before use. The polyesters were dissolved in anhydrous CH_2Cl_2 (1 g/150 mL) and this solution was placed in a 500 mL three-necked flask equipped with a stirrer and addition funnel. DDC (15 mg) and DMAP (10 mg) were added. A solution of OFL in anhydrous CH_2Cl_2 (0.5 g/100 mL) was placed in the funnel and added drop wise into the reactor, while the reaction mixture was vigorously stirred. After the addition procedure was completed, the reaction mixture was left stirring for an additional 24 h. When the reaction time was completed, the reaction product was filtered out and the filtrate was precipitated from 5% hydrochloric acid aqueous solution. The conjugates isolated from the solution (organic phase) were kept under vacuum at room temperature for no more than one week.

2.4. Toxicity assays

Microtox® assay with the luminescent bacteria *V. fischeri* was performed in special glass disposable cuvettes with the lyophilized bacteria purchased from SDI (USA). Samples were incubated at 15 °C for 15 min and the light output of the samples was recorded with a in Microtox® M500 analyzer. As a diluent and a control 2% NaCl was used.

Protoxkit F™ is a multigeneration protozoan growth inhibition bioassay with the ciliate *Tetrahymena termophila*. The test is based on the turnover of the substrate (food suspension) into ciliate biomass. While normal proliferating cell cultures clear the substrate suspension in 24 h, inhibited culture growth is reflected by remaining turbidity (Aptula et al., 2006). The test is based on optical density measurements. The protozoa and the food were obtained from MicroBioTests (Deinze, Belgium). The test was performed in spectrophotometric cuvettes according to the standard operational protocol of the producer. As a diluents and a control deionised water (Milli-Q quality) was used (ProtoxkitF™, 1997).

Spirotox test with the protozoan *Spirostomum ambiguum* was performed according to the standard protocol (Nałęcz-Jawecki, 2005). The test was carried out in disposable, polystyrene multiwell plates (24 wells). Ten organisms were added to each well of the multiwell. The samples were incubated in the darkness at 25 °C for 24 h. Afterwards test responses: different deformations such as shortening, bending of the cell, etc., and lethal response were observed with the use of dissection microscope (magnification of 10). As a diluents and a control Tyrod solution was used.

Stock solutions of monomers (CL, LLA) and initiators (PEG, PET, GL) were prepared with diluents just before the toxicity assays. Four to seven 2-fold serial dilutions of the samples were prepared directly in test containers. Than the test organisms were added to each dilution and samples were incubated according to the test protocols.

Prior to the toxicity test the polymers were pulverized. Two kind of assays were applied a direct contact test and a test of water extracts. In the direct contact test two concentrations of the samples were tested 1 mg l^{-1} and 0.5 mg l^{-1}. The polymer was weighted directly to the test containers, and poured with 1 ml of diluents. The test organisms were incubated with the suspension of the tested polymer. Water extracts were prepared by mixing 10 mg of the polymer with 10 ml of the diluent and incubating for 24 h at 37 °C.

Then the sample was filtered through 0.2 μm filter and the filtrate was assayed in the toxicity tests.

2.5. Measurements

The polymerization products were characterized by means of ^{1}H and ^{13}C NMR (Varian 300 MHz), and FTIR spectroscopy (Spectrum 1000, Perkin Elmer). The NMR spectra were recorded in $CDCl_3$ or DMSO–d_6. The IR spectra were recorded from KBr pellets.

Relative molecular mass and molecular mass distributions were determined by MALDI-TOF MS and GPC techniques. The MALDI-TOF spectra were measured in the linear mode on a Kompact MALDI 4 Kratos analytical spectrometer using a nitrogen gas laser with 2-[(4-hydroxyphenyl)diazenyl] benzoic acid (HABA) as a matrix. GPC measurements were made at 25 °C in the tetrahydrofuran solution using Shimadzu C-R4 Chromatopac apparatus. The molecular weights were calibrated with polystyrene standards.

Macromolecular conjugates viscosity were measured in N,N-dimethylformamide (at 30 °C) using an Ubbelohde viscometer. The concentrations of macromolecular conjugates solutions in DMF were 0.2, 0.4, 0.6, 0.8 and 1 wt.%.

Content of Sn was determined by the method described in (Sobczak et al., 2009).

The amount of released OFL was determined by a UV–vis spectrophotometry (UV-1202 Shimadzu) at the adsorption maximum of the free drug in aqueous buffered solutions (λ = 293 nm) using a 1 cm quartz cell (Cui et al., 2008).

2.6. Biodegradation of polyester conjugates

Dried macromolecular conjugate (1 g) was poured into aqueous buffered solution (100 mL, pH 7) at 37 °C. The mixture was stirred and a 5 mL sample was removed at selected intervals and 5 mL of buffer was replaced. The quantity of released drug was analyzed by means of UV spectrophotometer determined from the calibration curve obtained previously under the same conditions.

2.7. IR and NMR data

OFL: ^{1}H NMR (DMSO–d_6, δ, ppm): 1.55, 2.20, 3.40, 4.25, 4.35, 4.92, 7.43, 8.91; ^{13}C NMR (DMSO–d_6, δ, ppm): 176.7, 166.0, 157.1, 153.8, 146.2, 141.2, 131.9, 124.8, 120.1, 106.6, 103.4, 68.0, 55.2, 50.0, 46.0, 17.9; FTIR (KBr, cm^{-1}): 3470 (υOH), 1710 (υCO), 1619 (υC=C i C=N), 1452 (δCH_2 i ωCH_2), 1194 (δCH, γCH i υC–O), 1102 (rings), 801 (υC–N i δCH_2).

PCL-PEG: ^{1}H NMR ($CDCl_3$, δ, ppm): 1.37 (2H, m, –OCH_2 CH_2C**H$_2$**CH_2CH_2C(O)O–), 1.68 (4H, m, –OCH_2C**H$_2$**CH_2C**H$_2$** CH_2C(O)O–), 2.33 (2H, t, –$OCH_2CH_2CH_2CH_2$C**H$_2$**C(O)O–), 3.39 (2H, t, –OC**H$_2$**C**H$_2$**O–), 3.73 (2H, t, –C**H$_2$**OH, end group), 4.04 (2H, t, –OC**H$_2$**$CH_2CH_2CH_2CH_2$C(O)O–), 4.12 (2H, CL–OC**H$_2$**CH_2O–); ^{13}C NMR ($CDCl_3$, δ, ppm): 24.9 (–OCH_2CH_2C**H**$_2CH_2CH_2$C(O)O–), 25.8 (–$OCH_2CH_2CH_2$C**H**$_2CH_2$C(O)O–), 28.4 (–OCH_2C**H**$_2CH_2CH_2$ CH_2C(O)O–), 33.8 (–$OCH_2CH_2CH_2CH_2$C**H**$_2$C(O)O–), 63.9 (–C**H**$_2$C(O)OH, end group), 64.1 (CL–OC**H**$_2CH_2$O–), 64.5 (–OC**H**$_2CH_2CH_2CH_2CH_2$C(O)O–), 70.1 (–OC**H**$_2$C**H**$_2$O–), 173.8 (–$OCH_2CH_2CH_2CH_2CH_2$C(O)O–); FTIR (KBr, cm^{-1}): 2944 ($\nu_{as}CH_2$), 2867 ($\nu_{as}CH_3$), 1722 (νC=O), 1244 (νC–O).

PCL-GL:^{1}H NMR ($CDCl_3$, δ, ppm): 5.26 (1H, p, =C**H**–O–), 4.29 (2H, d, –C**H$_2$**–O–), 4.06 (2H, t, –CH_2C**H$_2$**OC(O)–), 3.65 (2H, t, –CH_2C**H**$_2$OH, end group), 2.30 (2H, t, –CH_2C**H$_2$**COO–), 1.64 (4H, m, –C**H$_2$**CH_2COO–), 1.37 (2H, m, –CH_2CH_2C**H$_2$**CH_2CH_2–); ^{13}C NMR ($CDCl_3$, δ, ppm): 173.2 (–**C**(O)O–), 63.7 (–CH_2**C**H_2OC(O)–), 62.1 (–**C**H_2–O–), 33.6 (–CH_2**C**H_2COO–), 27.9 (–**C**H_2CH_2OC(O)–), 25.3 (–**C**H_2CH_2COO–), 24.1 (–CH_2CH_2**C**$H_2CH_2CH_2$–); FTIR (KBr, cm^{-1}): 2947 ($\upsilon_{as}CH_2$), 2863 (υ_sCH_2), 1725 (υC=O), 1293 (C–O and C–C), 1241 (υ_{as}COC), 1191 (υOC–O), 1171 (υ_sCOC).

PCL-PET: ^{1}H NMR ($CDCl_3$, δ, ppm): 4.02 (2H, t, –CH_2C**H$_2$**OC(O)–), 3.61 (2H, t, –CH_2C**H$_2$**OH, end group), 2.25 (2H, t, –CH_2C**H$_2$**COO–), 1.59 (4H, m, –C**H$_2$**CH_2COO–), 1.34 (2H, m, –CH_2CH_2C**H$_2$**CH_2CH_2–); ^{13}C NMR ($CDCl_3$, δ, ppm): 173.3 (–**C**(O)O–), 63.9 (–CH_2**C**H_2OC(O)–), 63.6 (C–**C**H_2O–), 33.8 (–CH_2**C**H_2COO–), 33.5 (**C**–CH_2O–), 27.9 (–**C**H_2CH_2OC(O)–), 25.3 (–**C**H_2CH_2COO–), 24.3 (–CH_2CH_2**C**$H_2CH_2CH_2$–); FTIR: 2948 (υ_{as}CH2), 2866 (υ_sCH2), 1728 (υC=O), 1295 (C–O and C–C), 1238 (υ_{as}COC), 1188 (υOC–O), 1171 (υ_sCOC).

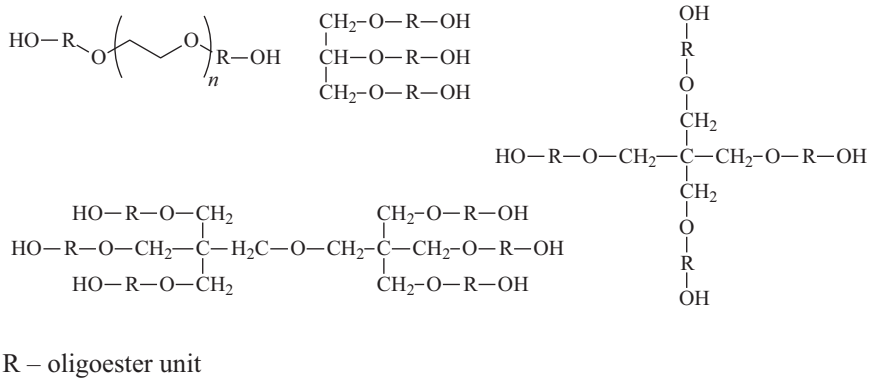
PCL-DPET: ^{1}H NMR ($CDCl_3$, δ, ppm): 1.25 (2H, m, –OCH_2 CH_2C**H$_2$**CH_2CH_2C(O)O–), 1.66 (4H, m, –OCH_2C**H$_2$**CH_2C**H$_2$** CH_2C(O)O–), 2.31 (2H, t, –$OCH_2CH_2CH_2CH_2$C**H$_2$**C(O)O–), 3.65 (2H, t, –C**H$_2$**OH, end group), 4.11 (2H, t, –OC**H$_2$**$CH_2CH_2CH_2CH_2$C(O)O–); ^{13}C NMR ($CDCl_3$, δ, ppm): 24.7 (–OCH_2CH_2C**H**$_2CH_2CH_2$C(O)O–), 25.6 (–$OCH_2CH_2CH_2$C**H**$_2CH_2$C(O)O–), 28.2 (–OCH_2C**H**$_2CH_2$ CH_2 CH_2C(O)O–), 33.1 (**C**–CH_2O–), 33.6 (–$OCH_2CH_2CH_2$ CH_2C**H**$_2$C(O)O–), 63.2 (C–C**H**$_2$O–), 63.7 (–C**H**$_2$C(O)OH, end group), 64.2 (–O**C**$H_2CH_2CH_2CH_2CH_2$C(O)O–), 173.6 (–$OCH_2CH_2CH_2CH_2CH_2$**C**(O)O–); FTIR (KBr, cm^{-1}): 2946 ($\nu_{as}CH_2$), 2869 ($\nu_{as}CH_3$), 1724 (νC=O), 1246 (νC–O).

PLA-PEG: ^{1}H NMR ($CDCl_3$, δ, ppm): 5.17 (1H, q, –C**H**(CH_3)–), 4.36 (1H, q, –C**H**(CH_3)OH, end group), 4.25 (2H, LA–OC**H$_2$**CH_2O–), 3.32 (2H, t, –OC**H$_2$**C**H$_2$**O–), 1.58 (3H, d, –C**H$_3$**); ^{13}C NMR ($CDCl_3$, δ, ppm): 169.9 (–**C**(O)O–), 69.4 (–**C**H(CH_3)–), 16.9 (–**C**H_3); FTIR: 2999 ($\upsilon_{as}CH_3$), 2949 (υ_sCH_3), 2884 (υCH), 1761 (υC=O), 1454 ($\delta_{as}CH_3$), 1346–1389 (δ_sCH_3), 1365–1370 (δ_1CH + δ_sCH_3), 1315–1300 (δ_2CH), 1270 (δCH + υCOC), 1215–1185 (υ_{as}COC + $r_{as}CH_3$), 1131 ($r_{as}CH_3$), 1100–1090 (υ_sCOC), 1045 (υC–CH_3), 960–950 (rCH_3 + υCC), 875–860 (υC–COO), 760–740 (δC=0), 715–695 (γC=O), 515 (δ_1C–CH3 + δCCO), 415 (δCCO), 350 (δ_2C–CH_3 + δCOC), 300–295 (δCOC + δ_2C–CH_3), 240 (τCC).

PLA-GL: ^{1}H NMR ($CDCl_3$, δ, ppm): 5.22 (1H, p, =C**H**–O–), 5.14 (1H, q, –C**H**(CH_3)–), 4.33 (1H, q, –C**H**(CH_3)OH, end group), 4.25 (2H, d,–C**H$_2$**–O–), 1.57 (3H, d, –C**H$_3$**); ^{13}C NMR ($CDCl_3$, δ, ppm): 169.9 (–**C**(O)O–), 69.3 (–**C**H(CH_3)–), 62.4 (–**C**H_2–O–), 16.9 (–**C**H_3); FTIR: 2995 ($\nu_{as}CH_3$), 2945 (υ_sCH_3), 2882 (υCH), 1760 (υC=O), 1452 ($\delta_{as}CH_3$), 1350–1390 (δ_sCH_3), 1360–1365 (δ_1CH + δ_sCH_3), 1315–1300 (δ_2CH), 1270 (δCH + υCOC), 1215–1185 (υ_{as}COC + $r_{as}CH_3$), 1130 ($r_{as}CH_3$), 1100–1090 (υ_sCOC), 1045 (υC–CH_3), 960–950 (rCH_3 + υCC), 875–860 (υC–COO), 760–740 (δC=0), 715–695 (γC=O), 515 (δ_1C–CH_3 + δCCO), 415 (δCCO), 350 (δ_2C–CH_3 + δCOC), 300–295 (δCOC + δ_2C–CH3), 240 (τCC).

PLA-PET: ^{1}H NMR ($CDCl_3$, δ, ppm): 5.19 (1H, q, –C**H**(CH_3)–), 4.37 (1H, q, –C**H**(CH_3)OH, end group), 1.60 (3H, d, –C**H$_3$**); ^{13}C NMR ($CDCl_3$, δ, ppm): 169.4 (–**C**(O)O–), 69.0 (–**C**H(CH_3)–), 63.3 (C–**C**H_2O–), 33.2 (**C**–CH_2O–), 16.5 (–**C**H_3); FTIR: 2996 ($\upsilon_{as}CH_3$), 2945 (υ_sCH_3), 2881 (υCH), 1758 (υC=O), 1451 ($\delta_{as}CH_3$), 1348–1388 (δ_sCH_3), 1355–1360 (δ_1CH + δ_sCH_3), 1315–1300 (δ_2CH), 1270 (δCH + υCOC), 1215–1185 (υ_{as}COC + $r_{as}CH_3$), 1132 ($r_{as}CH_3$), 1100–1090 (υ_sCOC), 1046 (υC–CH_3), 960–950 (rCH_3 + υCC), 875–860 (υC–COO), 760–740 (δC=0), 715–695 (γC=O), 517 (δ_1C–CH_3 + δCCO), 414 (δCCO), 352 (δ_2C–CH3 + δCOC), 300–295 (δCOC + δ_2C–CH_3), 244 (τCC).

PLA-DPET: ^{1}H NMR ($CDCl_3$, δ, ppm): 1.52 (3H, q, –CH(C**H$_3$**)C(O)O–), 4.41 (1H, q, –C**H**(CH_3)OH, end group), 5.19 (1H, q, –OC**H**(CH_3)C(O)O–); ^{13}C NMR ($CDCl_3$, δ, ppm): 17.3 (–OCH(**C**H_3)C(O)O–), 20.9 (–CH(**C**H_3)C(O)OH), 33.6 (**C**–CH_2O–), 63.7 (C–**C**H_2O–), 67.3 (–C**H**(CH_3)OH, end group), 69.2 (–O**C**H(CH_3)C(O)O–), 169.8 (–**C**(O)O–); FTIR (KBr, cm^{-1}): 2995 ($\upsilon_{as}CH_3$), 2945 (υ_sCH_3), 2880 (υCH), 1760 (υC=O), 1450 ($\delta_{as}CH_3$), 1350–1388 (δ_sCH_3), 1355–1360 (δ_1CH + δ_sCH_3), 1315–1300 (δ_2CH), 1270 (δCH + υCOC), 1215–1185 (υ_{as}COC + $r_{as}CH_3$), 1130 ($r_{as}CH_3$), 1100–1090 (υ_sCOC), 1045 (υC–CH_3), 960–950 (rCH_3 + υCC), 875–860 (υC–COO), 760–740 (δC=0), 715–695 (γC=O), 515 (δ_1C–CH_3 + δCCO), 415 (δCCO), 350 (δ_2C–CH_3 + δCOC), 300–295 (δCOC + δ_2C–CH_3), 240 (τCC).



Scheme 1. Structure of polyesters (two-, three-, four- and six-arm, star-shaped).

Table 2
Toxicity of monomers and initiators in mg l^{-1}.

	Microtox®		Protoxkit F™		Spirotox	
	15 min-EC50	15 min-EC20	24 h-EC50	24 h-EC20	24 h-EC50	24 h-EC20
GL	>1000	>1000	>1000	>1000	500 ± 125[a]	375 ± 106
PEG	>1000	>1000	>1000	>1000	>1000	>1000
PET	>100	>100	>100	>100	>100	>1000
CL	7.93 ± 1.86	1.02 ± 0.51	>100	>100	>100	>100
LLA	38.7 ± 10.3	21.8 ± 2.97	35.4 ± 10.3	25.0 ± 7.98	>100	>100

[a] Average ± standard deviation [mg l^{-1}].

3. Results and discussion

3.1. Ring-opening polymerization of ε-caprolactone, L-lactide and rac-lactide: characterization and toxicity tests

The synthesis of polyester and polyurethane conjugate of fluoroquinolones is a continuation of the research initiated by us in 2006. The preliminary results were already published (Sobczak et al., 2008a, 2010a; Sobczak, 2010b). Our experiments have been extended to macromolecular conjugates of ofloxacin.

The purpose of the first part of our research was to obtain a low-molecular weight polyesters which can be subsequently used as polyester conjugates of OFL. The polymerization reactions of CL, DLLA and LLA were carried out like previously (Sobczak et al., 2008a). Poly(ethylene glycol) (PEG), glycerol (GL), penthaerythritol (PET) and dipentaerythritol (DPET) were used as initiators. Process was carried out in the presence stannous octoate ($SnOct_2$) as catalyst. Reaction conditions, yields and average molecular weight of obtained polyesters are shown in Table 1.

The low-molecular weight polyesters with chain-end hydroxyl groups were obtained (Scheme 1). The synthesized PCL and PLA polymers had two-, three-, four- and six-arm star shapes.

The reaction yields were in the 74–95, 62–87, 55–74 and 49–69% range for two-, three-, four- and six-arm star shaped polyesters, respectively.

The number-average molecular weights determined from GPC for CL oligomers lie in the 6200–10 000 Da range, and the polydispersity indexes in the 1.1–1.2 range. For LLA oligomers the Mn values are 6000–10 000 and the Mw/Mn values lie in the 1.2–1.3 range. The number-average molecular weights for DLLA oligomers lie in the 6100–10 000 Da range, and the polydispersity indexes in the 1.2–1.3 range.

The chemical structures of the obtained polymers were confirmed by ^{13}C, ^{1}H NMR and IR studies (Section 2).

The products of polymerization of CL, LLA and DLLA in the presence of PEG were characterized by MALDI-TOF MS method. The MALDI-TOF spectrum of PCL comprises two series of peaks. The most prominent series of peaks is characterized by a mass increment of 114 Da, which is equal to the mass of the repeating unit in the PCL polymer. This series is assigned to PCL terminated with a hydroxyl group and detected as the Na^+ adduct (residual mass: RM = 41 Da). The second series of the peaks is also from PCL terminated with a hydroxyl group, but corresponds to the K^+ adduct (RM = 57 Da). The MALDI-TOF spectrum of PLA comprises two series of peaks, too. The main series comes from PLA terminated with a hydroxyl group and corresponds to the Na^+ adduct (RM = 42 Da), while the second series of smaller peaks is also from PLA terminated with a hydroxyl group, but corresponds to the K^+ adduct (RM = 57 Da).

We have also determined the content of Sn in the polyesters prepared. The concentration of residual tin in final products was in the range of 4–14 ppm (Table 1). The limit of Sn concentration suggested in Pharmacopoeia for some biomedical application is 20 ppm. All of the synthesized polymers contain residual metal at the level below pharmacopoeial standards for materials of blood containers.

The luminescent bacteria *V. fischeri* and two ciliated protozoa *S. ambiguum* and *T. termophila* were used to evaluate the toxicity of monomers, initiators and polymers. Initiators (PEG, PET, GL) were not toxic in the assays (Table 2). CL was toxic only to the bacteria with the threshold value 15 min-EC20 of 1.02 ± 0.51 mg l^{-1}. LLA was 20-fold less toxic to the bacteria and it was the only

Table 3
Toxicity of polymers to Microtox® and Protoxkit F™ tests.

Polymer (mg ml^{-1})	Microtox® (15 min-PE[a])		Protoxkit F™ (24 h-PE)	
	1.0 mg ml^{-1}	0.5 mg ml^{-1}	1.0 mg ml^{-1}	0.5 mg ml^{-1}
P1	35.0 ± 12.1	11.5 ± 2.7	7.3 ± 13.7	0.7 ± 1.1
P2	36.7 ± 7.3	18.1 ± 2.9	10.0 ± 10.2	−1.5 ± 2.3
P3	25.0 ± 6.1	10.0 ± 1.1	3.1 ± 10.5	3.2 ± 1.7
P4	19.2 ± 2.1	5.0 ± 3.7	−12.2 ± 11.4	−22.5 ± 4.9

P1, CL/PEG/$SnOct_2$; P2, CL/GL/$SnOct_2$; P3, CL/PET/$SnOct_2$; P4 LA/PEG/$SnOct_2$.
[a] Percent of toxic effect.

Table 4
Toxicity of polymers in Spirotox test.

Polymer (mg ml^{-1})	Spirotox (24 h-PE[a])		Spirotox (48 h-PE[a])		Spirotox (7 d-PE[a])	
	1.0 mg ml^{-1}	0.5 mg ml^{-1}	1.0 mg ml^{-1}	0.5 mg ml^{-1}	1.0 mg ml^{-1}	0.5 mg ml^{-1}
P1	NT	NT	NT	NT	50	NT
P2	NT	NT	NT	NT	50	NT
P3	NT	NT	NT	NT	NT	NT
P4	NT	NT	NT	NT	NT	NT

NT, not toxic; P1, CL/PEG/$SnOct_2$; P2, CL/GL/$SnOct_2$; P3, CL/PET/$SnOct_2$; P4, LA/PEG/$SnOct_2$.
[a] Percent of toxic effect.

compound toxic to the protozoan *T. termophila* with 24 h-EC20 of 25.0 ± 0.51 mg l^{-1}.

In the direct contact test higher concentration of the P1 (CL/PEG/$SnOct_2$) and P2 (CL/GL/$SnOct_2$) polymers 1 mg ml^{-1} caused the 35–37% inhibition of the luminescence of the bacteria in the Microtox® test and caused 50% deformations in the Spirotox test. Two-fold lower concentration of all tested polymers and water extracts were not toxic in all assays (Tables 3 and 4).

3.2. Synthesis of polyester conjugates of ofloxacin

Biodegradable polymers have been studied extensively over the past few decades to fabricate various novel drug delivery systems such as nanoparticles, microparticles, microspheres, liposomes etc. Preliminary efforts to prepare nanoparticles and implants of polyesters loaded with adsorbed fluoroquinolones has already been reported (Dévaux et al., 2002; Jeon et al., 2000; Jeong et al., 2008; Ramchandani and Robinson, 1998; Sahoo et al., 2010; Silva-Júnior et al., 2008). The first conjugates of fluoroquinolones were obtained by us (Sobczak et al., 2008a, 2010a; Sobczak, 2010b). Macromolecular conjugates technology represents one of the most rapidly advancing areas of science. Polymeric conjugates exhibit unique pharmacokinetics, body distribution and pharmacological efficacy. They characterized more controlled release of drug than nanoparticle matrix or implants loaded with adsorbed fluoroquinolones.

The polyester conjugates were obtained from the reactions of the two-, three-, four, six-arm, star-shaped PCL, PDLA and PLA with ofloxacin (OFL) in the presence of DDC and DMPA (Table 5) (Scheme 2).

The chemical structures of the prepared polyester conjugates were confirmed by ^{1}H, ^{13}C NMR and IR studies. Typical proton NMR spectra of pure OFL and of the reaction products of the two-armed PCL with OFL are shown in Figs. 1–3, respectively. Obviously, the characteristic peaks of ofloxacin can all be found in polyester-OFL, indicating successful preparation of the conjugate.

All these results support the conclusion that the OFL had been conjugated with PCL, PDLA and PLA successfully.

The OFL content in the PCL, PDLA or PLA conjugates calculated by ^{1}H NMR. The signal intensity of the **a** and the signal intensity of the **4** (for PCL) or **6** (for PLA or PDLA) has been compared. The drug content in the conjugates is following: 8.2 (PCL–PEG–OFL), 6.0 (PCL–GL–OFL), 6.5 (PCL–PET–OFL), 8.0 (PCL–DPET–OFL), 6.0 (PLA–PEG–OFL), 6.7 (PLA–GL–OFL), 9.5 (PLA–PET–OFL), 10.6 (PLA–DPET–OFL), 5.8 (PDLA–PEG–OFL), 7.2 (PDLA–GL–OFL), 9.1 (PDLA–PET–OFL), 10.1 (PDLA–PET–OFL) mol.%.

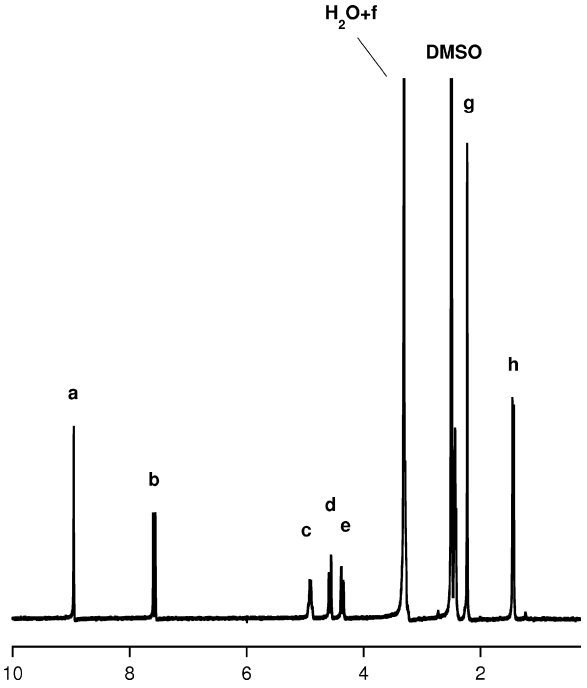
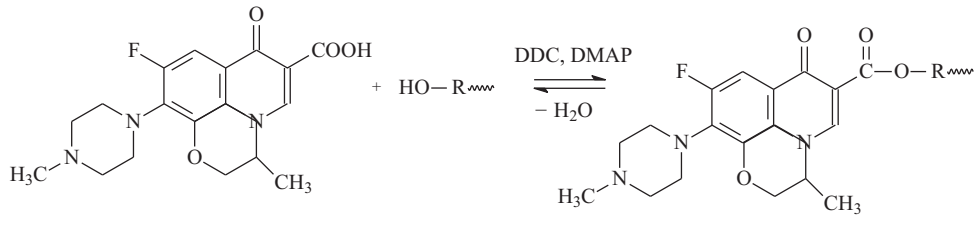


Fig. 1. The ^{1}H NMR spectrum of OFL (in DMSO).

3.3. Ofloxacin release from polyester conjugates

The release of OFL from the macromolecular conjugates was monitored over a 5-week in buffer solution at pH 7 at 37 °C. The in vitro release profiles of OFL from the different polyesters are shown in Table 5. The PLA and PDLA conjugates underwent to faster OFL release as compared to the PCL conjugates. 61% of linked drug was release from PCL–PEG–OFL conjugate within 35 days at pH 7,



Scheme 2. Synthesis of the polyester conjugates of ofloxacin.

Table 5
Ofloxacin release from polyester conjugates.

Polyester conjugates	Time (days)				
	7	21	35	0	35
	% released	% released	% released	η_{inh} (dL g^{-1})[a,b]	η_{inh} (dL g^{-1})[c,b]
PCL–PEG–OFL	26 ± 2	48 ± 3	61 ± 3	0.16	0.15
PCL–GL–OFL	18 ± 2	33 ± 3	43 ± 3	0.21	0.21
PCL–PET–OFL	15 ± 2	31 ± 3	39 ± 3	0.19	0.18
PCL–DPET–OFL	16 ± 2	25 ± 2	41 ± 3	0.27	0.26
PLA–PEG–OFL	37 ± 3	55 ± 3	72 ± 4	0.18	0.16
PLA–GL–OFL	24 ± 3	40 ± 3	59 ± 3	0.24	0.22
PLA–PET–OFL	20 ± 2	30 ± 3	50 ± 3	0.22	0.20
PLA–DPET–OFL	16 ± 2	31 ± 2	54 ± 3	0.28	0.25
PDLA–PEG–OFL	44 ± 2	68 ± 3	84 ± 3	0.18	0.15
PDLA–GL–OFL	31 ± 2	54 ± 3	69 ± 3	0.22	0.19
PDLA–PET–OFL	22 ± 2	40 ± 2	62 ± 2	0.23	0.20
PDLA–DPET–OFL	23 ± 2	43 ± 2	58 ± 3	0.30	0.26

[a] Before incubation.
[b] Measured at 30 °C in DMF.
[c] After 35 h incubation at pH 7.

72% from PLA–PEG–OFL conjugate and 84% from PDLA–PEG–OFL, for example. Similarly, 43% of linked drug was release from PCL-GL-OFL conjugate, 59% from PLA-GL-OFL conjugate and 69% from PDLA-GL-OFL.

We have found that polyester conjugates containing GL, PET and DPET fragments have a higher stability to chemical hydrolysis at pH 7 than macromolecular conjugates obtained in the presence of PEG. The percentage of release OFL after 35 h incubation was about 61% from PCL–PEG–OFL and 43% from PCL–GL–OFL, 39% from PCL–PET–OFL and 41% from PCL–DPET–OFL. Similarly, after 35 h incubation the OFL release was 84% from PDLA–PEG–OFL, 69% from PDLA–GL–OFL, 62% from PDLA–PET–OFL and 58% from PDLA–DPET. Probably, the hydrophilic PEG can influence the drug release. The macromolecular conjugates, which contain PEG fragments, are more hydrophilic and process of their hydrolysis is faster.

The rates of OFL release for the PLA conjugates appear similar but are slower than the PDLA conjugates. The percentage of release OFL after 35 h incubation was about 72% from PLA–PEG–OFL, 59% from PLA–GL–OFL, 50% from PLA–PET–OFL, 54% from PLA–DPET–OFL.

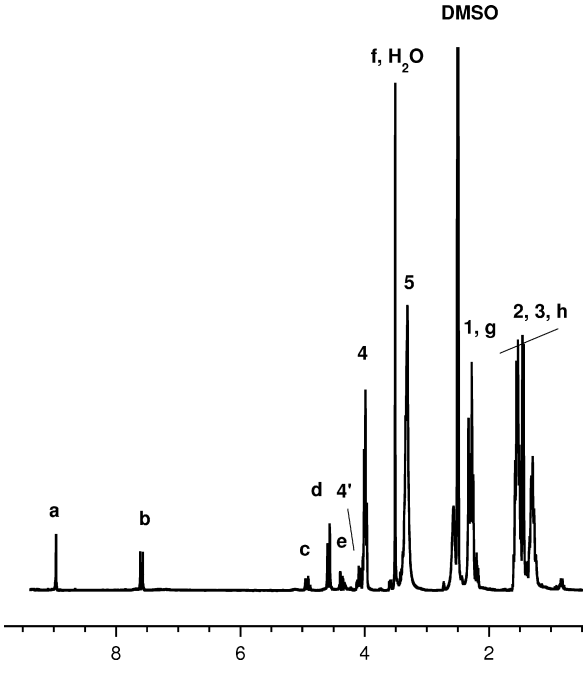


Fig. 2. The ^{1}H NMR spectrum of the conjugate PCL–PEG–OFL (in DMSO).

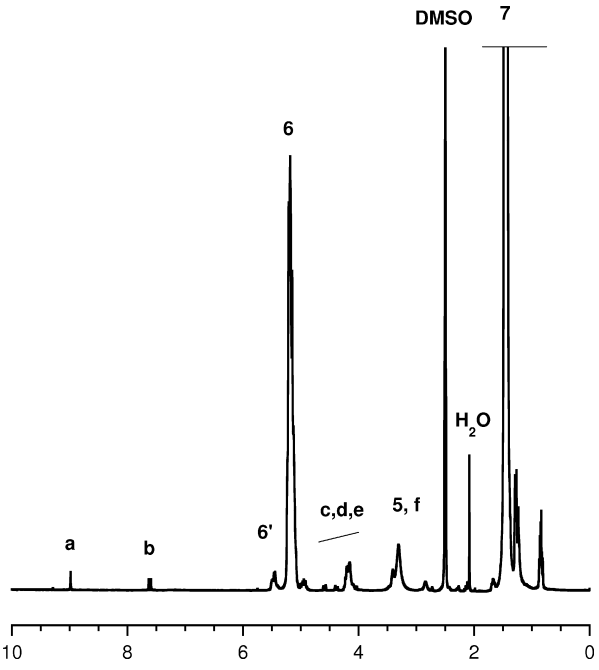


Fig. 3. The ^{1}H NMR spectrum of the conjugate PLA–PEG–OFL (in DMSO).

After 35 h incubation the OFL release was 84% from PDLA–PEG–OFL, 69% from PDLA–GL–OFL, 62% from PDLA–PET–OFL, 58% from PDLA–DPET–OFL. The difference in release rates observed between PLA and PDLA conjugates can be attributed to the difference in their crystallinity (Sobczak, 2010b).

Biodegradation of obtained macromolecular conjugates of OFL was controlled by viscosity method. The intrinsic viscosity of the polymers was determined after 35 days degradation. The results of intrinsic viscosity of original and hydrolytic degraded polyester conjugates are shown in Table 5. Change of the above parameter was relatively small (6–13%). The decrease of intrinsic viscosity, which means decrease of molecular weight of the polyesters, is higher for PLA or PDLA than PCL.

OFL release was faster than decrease of molecular weight of the polyesters, indicating that the hydrolyzed drug diffused through the channels formed in the polymer at advanced stages of degradation. Probably, the release of OFL from PCL and PLA conjugates is a combination of hydrolyzation, diffusion and degradation.

The obtained results demonstrate that the homopolymers of LLA, DLLA and CL are interesting materials for the controlled release of OFL.

4. Conclusions

In our paper the synthesis and characterization of polyester conjugates of OFL has been described. The OFL was covalently connected to the chain end of the two-, three-, four- and six-arm, star-shaped PCL, PDLA and PLA via an ester linkage. The polyesters were obtained by the ring-opening polymerization of CL, LLA and DLLA in the presence of glycol initiators (PEG, GL, PET, DPET) and $SnOct_2$ as a catalyst.

The effects of polyester structure on OFL release were also investigated. It was found that the release rate of the drug depends on the structure of polyesters. The release rate can be extensively modulated by polymerization of LLA, DLA and CL in the presence of PEG, GL, PET and DEPT. The release kinetic of obtained macromolecular conjugates is indeed increased by PEG and decreased by GL, PET, DEPT glycol fragments.

Tested obtained aliphatic polyesters and water extracts are not toxic.

We hope that the obtained polyester conjugates of OFL are good potential candidates for carriers in implant drug delivery systems.

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