Malolactonate Polymers and Copolymers for Biomedical Applications

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Summary: A new class of malolactonate polymers and copolymers with a wide range of lateral chain structures were synthesized by anionic ring opening polymerization of alkyl malolactonate monomers. The monomers were prepared in good yields according to established procedures. Final macromolecular and thermal characteristics were in agreement with the designed monomer structures. Molecular weights in the range 4-20 kD were attained, as a result of chain transfer reactions. Representative polymeric compounds displayed stability up to 200 °C. Many of the obtained poly(alkyl malolactonate)s were able to sustain and promote with 3T3 murine fibroblasts adhesion and proliferation onto their surface.

Keywords: alkyl malolactonates; anionic polymerization; biocompatibility; biodegradable polymers; tissue engineering

Introduction

Bioerodible and biodegradable polymers represent a class of extremely useful materials for a number of biomedical and pharmaceutical applications. One good example is provided by drug delivery techniques, which in recent years have taken advantage of degradable polymeric matrices for improving and modulating excretion pathways of the releasing device after overall drug depletion.[1] In tissue engineering, a rapidly developing branch of biomedicine that attempts to solve the dramatic problem of tissue loss or organ failure, degradable materials are studied to provide polymer scaffolds where the transplanted cells can remodel their intrinsic tissue organization and hence ultimately lead to the desirable 3D structure and physiological functionality of a regenerated organ.[2] However, before being selected for any biomedical

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application, biodegradable polymers need careful investigation of their interactions and compatibility with the human body, in order to avoid tissue damage and immunogenic responses that could arise against the whole polymeric matrix and its low molecular weight degradation products.[3] Actually, this requirement greatly lowers the number of possible polymeric candidates. Up to date, natural or artificial poly(ester)s constitute the most developed class of degradable biomaterials, because of their excellent mechanical properties and good biocompatibility.[4] Nevertheless, the strongly hydrophobic nature and the lack of available reactive sites of most poly(ester)s used for bio-applications [poly(lactic acid), poly(glycolic acid), poly(lactic acid-*co*-glycolic acid), poly(ε-caprolactone)] often ask for special synthetic methods to realize true biologically activated materials.[5]

Poly(malic acid) represents a very interesting material for biomedical use, since it was shown to be biocompatible[6] and to degrade under physiological condition to non-toxic malic acid.[7] In addition, the side-chain carboxylic groups can be functionalized to obtain a large set of polymers and copolymers with different physical-chemical characteristics, which already proved useful for realizing biocompatible devices.[8] It is also worth noting that the stereogenic centers in the poly(malolactonate) chain may be exploited for further tuning the polymer characteristics and bioactivity. Straightforward procedures for the preparation of both malolactone monomers and the corresponding polymers were developed over the last twenty years starting from commercially available natural products.[9]

Following our continuous interest in the production of bioerodible and biodegradable functional polymers for biomedical applications,[10-13] we undertook the preparation and characterization of a series of poly(ester)s and copolyesters using new malolactonate monomers. The side chains of these malic residues were functionalized by esterification with readily available alcohols; they were selected either to adjust the polymer hydrophilic-hydrophobic balance (aiming at specific recognition sites of natural terpene structures), or to provide a reactive site for further functionalization. The interactions of these new materials with complex biological environments were eventually tested in suitable cell culture experiments.

Experimental

Synthesis of Monomers

The monomers were synthesized by modifying the original procedures for malolactonate preparation developed by Guerin *et al.*[14] A detailed description of the synthetic procedures has been reported.[15]

Synthesis of Poly(ester)s

For liquid monomers, the polymerization reactions were carried out in bulk, whereas solid monomers were dissolved in the minimum amount of anhydrous THF to obtain a homogeneous mixture. The monomers or comonomer mixtures were placed in a schlenck tube. The bottom of the tube was previously coated with tetraethylammonium benzoate by evaporation under vacuum of an ethanol solution (0.14-0.16 M) of the quaternary ammonium salt. Monomer/initiator ratio was set to 1000/1 m/m. The mixture was stirred under nitrogen atmosphere for 4-31 days at 38-42 °C (Table 1). The prepared polymers were purified by double precipitation in absolute ethanol from concentrated dichloromethane solutions (1/10 dichloromethane/ethanol volume ratio), and dried under high vacuum for 12 h prior to characterization.

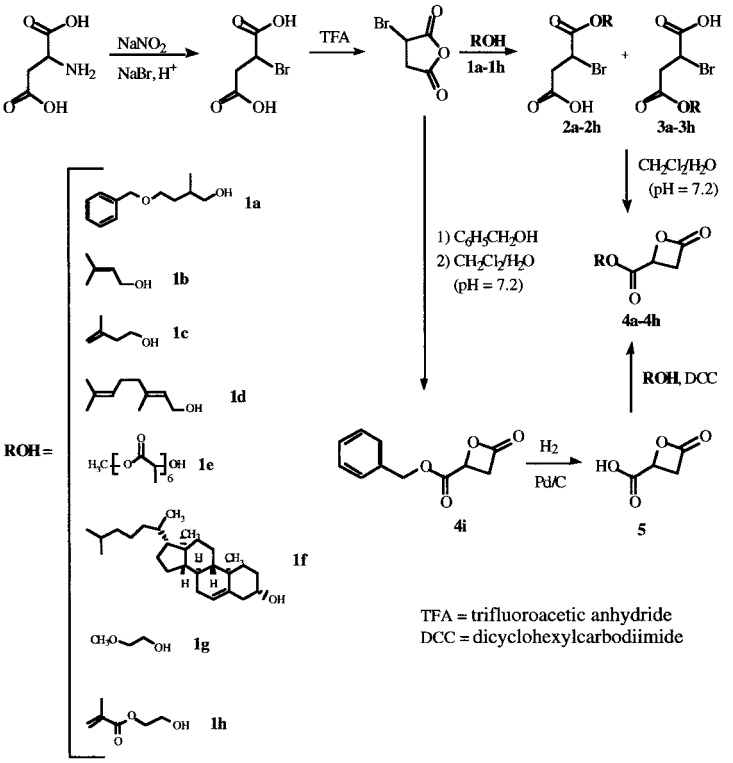
Biological Evaluation of the Poly(ester)s

Experiments were carried out on thin polymer films obtained by slow evaporation of 5 wt % polymer in chloroform solutions. The cell line used in all the experiments was 3T3 balb/c murine fibroblast. Before cell seeding, the polymeric films were sterilized by exposure to UV light for 10-20 min. In each run, the cells were seeded in culture medium at $3 \cdot 10^4$ cell/cm^2 density, and then incubated at 37 °C in a 5 % CO_2 atmosphere. After two hours, the culture medium was replaced with a fresh one, thus removing the cells that did not adhere to the polymeric film. The attached cells were allowed to proliferate for up to 72 hours, which represents the typical time needed by the 3T3 cells to grow and to reach confluence on tissue-culture poly(styrene) plates (TCP) (the reference polymeric materials). WST-1 cell proliferation reagent was employed for a quantitative evaluation of cell proliferation on the polymeric films.[16]

Results and Discussion

Synthesis of monomers

β-Malolactonate monomers were prepared according to two different synthetic routes (Scheme 1).



Scheme 1. Synthesis of β-Malolactonate monomers.

Compounds **4(a,b,c,d)** and benzyl malolactonate were synthesized in three steps from racemic aspartic acid.[14,15] Initially, the α-amino group of the amino acid was replaced by a bromine atom. Then, a linear monoterpene side chain was introduced by reaction of the corresponding monoterpenol **1(a,b,c,d)** with activated bromosuccinic anhydride, that was previously obtained by dehydration of bromosuccinic acid with trifluoroacetic anhydride. The preferential formation

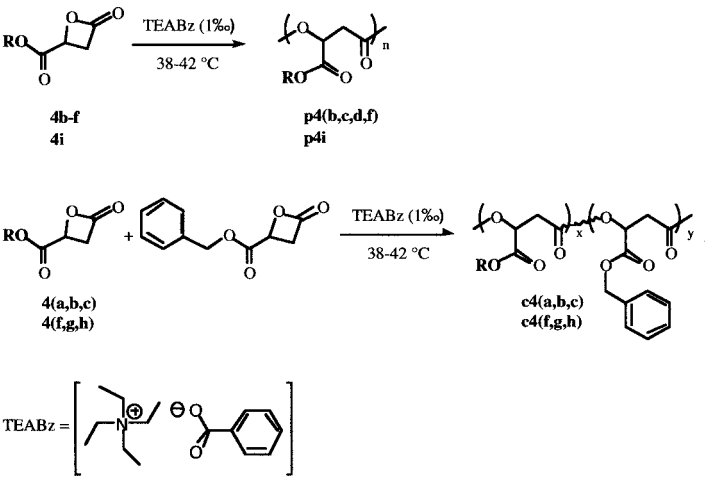
(71-74%) of lactonizable alkyl monoesters **2(a,b,c,d)** was confirmed by ^{1}H-NMR analysis of the product mixtures.[17]

Eventually, intramolecular S_N2_i reaction of the β-halocarboxylic acids led to the formation of lactones **4(a,b,c,d)**. Selected linear monoterpene alcohols **1(b,c,d)** were commercially available, whereas **1a** was synthesized from 3-methyl-3-buten-1-ol by a two-step procedure involving benzylation of the hydroxyl group followed by hydroboration/oxidation of the alkene double bond.

The second series of monomers was synthesized by direct esterification of malolactone, previously prepared by hydrogenolysis of benzyl malolactonate. This method appears preferable when bulky or hydrophilic alkyl groups are to be introduced in the malolactonate structure.[9] The spectroscopic characteristics of the synthesized compounds resulted in agreement with the proposed structures. Overall yields (referred to the precursor alcohol) were comprised in the 12-45 % range. No significant dependence of the yield on the nature of the ester groups was detected, except for **4c**, whose surfactant properties negatively influenced the reaction work up, and **4h**, which contains an acrylic group reactive towards free-radical polymerization process.

Synthesis of polymers

Polymerization of the synthesized lactones was investigated by anionic ring-opening polymerization (Scheme 2) either in bulk or in anhydrous THF at 38-42 °C, by using tetraethylammonium benzoate (1‰ in mol) as initiator (Table 1).



Scheme 2. Synthesis of β-Malolactonate polymers.

In order to obtain strongly hydrophobic materials, only lactones **4(b,c,d,e,f)** were submitted to homopolymerization experiments. Monomer **4i** was homopolymerized as a reference. In the copolymerization of **4(a,b,c)** and **4(f,g,h)** with **4i**, comonomer ratios were selected to give materials with variable degree of hydrophobicity, or to introduce reactive groups in the poly(benzyl malolactonate) matrix, as in the case of **4h**.

Table 1. Anionic polymerization of β-malolactonate monomers

Sample	Feed **4i** (mol %)	Time (days)	Yield (%)	Polymer **4i** (mol %)	M_w	M_w/M_n	Tg [a] (°C)
p4b	0	31 [b]	37	0	11200	1.19	2.1
p4c	0	24 [b]	28	0	9900	1.15	-13.6
p4d	0	16 [b]	33	0	12800	1.23	-33.7
p4e	0	21 [b]	31	0	6650	1.25	36.3
p4f	0	4 [c]	91	0	24800	1.70	-
p4i	0	8 [b]	91	0	20900	1.23	33.7
c4a	55	24 [b]	41	60	3100	1.15	1.2
c4b	39	24 [b]	71	42	10600	1.43	13.6
c4c	49	24 [b]	74	53	8800	1.47	8.8
c4f	79	4 [c]	85	76	6500	1.73	42.2
c4g1	83	4 [c]	62	85	12150	1.80 [d]	28.3
c4g2	39	4 [c]	68	43	16700	1.69 [d]	14.1
c4h1	94	8 [b]	71	96	5350	1.30	27.6
c4h2	91	8 [b]	73	94	5500	1.26	28.0

[a] 2nd heating cycle. [b] In bulk. [c] In THF solution. [d] Bimodal distribution.

Polymerization experiments were allowed to proceed until disappearance of the lactone band at 1848 cm^{-1} in the FT-IR spectrum of the polymerization mixture. Reaction times ranged from 4 to 30 days (Table 1). Faster polymerization rates were recorded in THF rather than in bulk, thus suggesting a marked dependence of the reaction kinetics upon the diffusivity of the monomers in the polymerization mixture. No clear correlation of the polymerization rate with the structural characteristics of the monomers was observed. The polymer samples were obtained as white amorphous materials, soluble at room temperature in polar aprotic solvent such as DMSO, DMF, acetone, and chloroform.

Polymer Characterization

For all polymeric samples, typical absorption bands for the ester linkage (1750-1740, 1190-1160, and 1100-1050 cm^{-1}) were recognized in the respective FT-IR spectrum. The $^{1}H/^{13}C$-NMR spectra of the poly(ester)s were consistent with the predicted macromolecular structure. Interestingly, weak signals due to terminal vinyl protons were detected in the proton NMR spectra of all the polymers. This finding indicates that the polymerization reactions were perturbed by a "deprotonation-rearrangment" chain transfer process which is known to affect anionic ring-opening polymerization of α-unsubstituted β-lactones.[18] Accordingly, GPC values resulted one order of magnitude lower (Table 1), despite the monomer/initiator ratio was set to obtain final molecular weights of about 10 KDa. However, no significant dependence of polymer molecular weight on the nature of side ester groups was detected.

The polydispersity indices were found to be between 1 and 2; these are in agreement with a not-controlled living anionic polymerization. Higher polydispersity values were recorded for polymers prepared in THF, where chain-transfer mechanisms are likely to be more active. In most cases, the poly(ester)s displayed a monomodal distribution of molecular weights. In the case of copolymers, this suggested a statistical distribution of the two residues along the chain. This was also supported by the copolymer composition determined by ^{1}H-NMR, which resulted very close to that of the feed mixtures (Table 1). Only **c4g1** and **c4g2** samples exhibited a bimodal

distribution, more marked at the largest **4g** content, thus indicating the preferential tendency of this monomer to homopropagation.

DSC analysis of the poly(ester)s did not evidence the occurrence of endothermal transitions, thus ruling out the presence of a crystalline phase. This result must be attributed to the formation of atactic polymer structures due to the lack of stereoelectivity in the polymerization process of the racemic monomers. Indeed, isotactic poly(alkyl malolactonate)s obtained from enantiopure monomers often show semi crystalline characteristics.[19] All poly(ester)s displayed well-defined glass transitions (Table 1), with the only exception of **p4f**. The recorded Tg values spanned about 80 °C, depending upon the length as well as the nature of the side groups. The Tg values computed for **c4b** (15.4 °C) and **c4c** (10.2 °C) by the Couchman-Fox equation[20] were in good agreement with experimental data, thus substantiating the random distribution of monomeric units. Interestingly, only one Tg was detected for **c4g1-c4g2**; this indicated that the different components that constitute the copolyesters, gave rise to a homogeneous phase in the solid state.

Thermogravimetric analyses of representative polyesters and copolyesters were carried out under nitrogen atmosphere to determine if the side group structure could affect the stability of the materials (Table 2). In the case of homopolymers, slightly lower onset degradation temperatures were found for materials with linear lateral chains. Weight loss values suggested that the first and second degradation steps of **p4d** and **p4e** correspond to the disruption of the side chains and polymer backbone, respectively. Instead, the weak first degradation step of **p4f** may be attributed to the loss of two methane and one hydrogen molecules (weight loss $\approx$ 7.4%), which would generate a stable conjugated structure in the cholesteryl side chain. It is worth noting that the thermal behavior of copolyesters **c4b** and **c4g1** was different from that of **p4i**, the parent homopolymer. Indeed, introduction of a second hydrophobic side chain (**c4b**) led to a 30 °C decrease in the degradation onset temperature. A two-step degradation mechanism, with initial loss of the terpenic lateral chain, was also observed. In the case of the more hydrophilic **c4g1**, complete degradation occurred at higher temperatures in a single step. These observations suggest that the chain stability is related to the polarity of monomeric units and their ability to establish effective inter- and intra-chain interactions.

Table 2. Thermogravimetric analysis of the prepared poly(ester)s.

Sample	T_1 [a] (°C)	Δw_1 [b] (%)	Td_2 [a] (°C)	Δw_2 [b] (%)
p4d	187	48	217	50
p4e	195	57	280	40
p4f	225	8	250	80
p4i	237	99	-	-
c4b	194	18	238	81
c4g2	258	100	-	-

[a] Degradation onset. [b] Weight loss, values in parentheses were calculated by the Couchman-Fox equation.

Biological Characterization

Thin films of the synthesized poly(β-lactone)s were used in cell culture experiments to obtain a preliminary evaluation of their cytocompatibility. The films were prepared by slow evaporation of 5 % by weight chloroform solutions. Only pBzML1, pBzML2, cChML20, and cMEML20 provided cytocompatible polymeric surfaces that allowed for cell adhesion and growth. The absence of cell proliferation observed for the other poly(β-lactone)s may be ascribed to the physical-chemical characteristics of their surface that resulted unfavorable to serum protein-mediated cell adhesion. However, the residual presence of low molecular weight moieties that have some degree of cytotoxicity cannot be completely ruled out. Interestingly, the WST-1 assay indicated that cell proliferation on pBzML1, pBzML2, cChML20, and cMeML20 was linearly related to the static contact angle for water on the polymer film samples, which increased on the more hydrophobic polymeric surfaces (Figure 1).

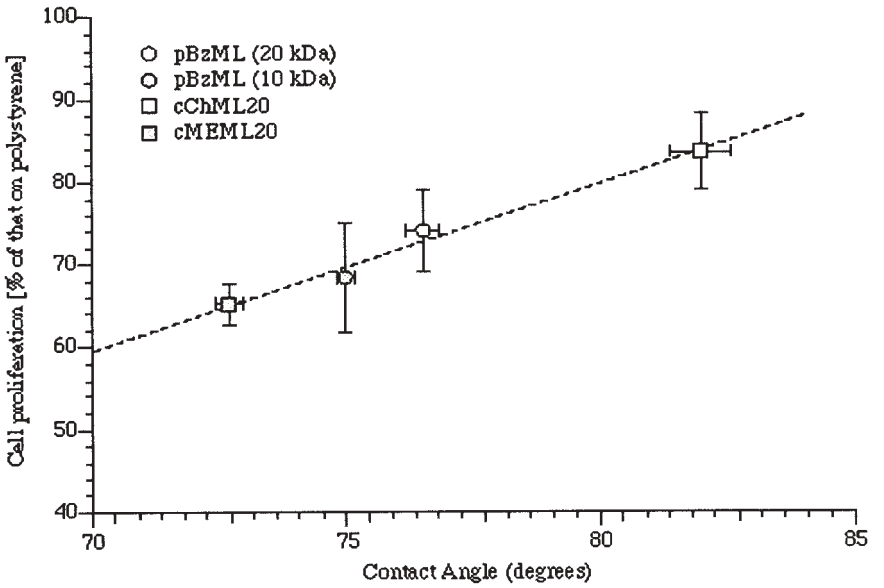


Figure 1. Dependence of 3T3 cell proliferation on the water contact angle of poly(β-lactone) substrates.

This behavior seems to indicate a strong dependence of serum protein absorption on the hydrophilic-hydrophobic balance of the poly(β-lactone) surface, although the different side chains can play some specific biological effects. The reported contact angles were measured on poly(β-lactone) films preliminarily soaked in phosphate buffer (PBS), in order to test polymeric surfaces under conditions similar to those used in cell culture experiments. Indeed, the contact angle of the same poly(β-lactone)s were found to decrease by 15-20° upon incubation in PBS, thus implying that hydrophilic and hydrophobic groups move outward and inward, respectively, on the polymer surface following contact with the buffer water solution. Optical microscopy observation showed that the morphology of 3T3 cells grown on pBzML1, pBzML2, and cChML20 was very close to that of control cells on TCP (Figure 2). On the contrary, 3T3 cells on cMEML20 showed rather peculiar characteristics, such as very small rounded shapes and aggregation in elongated islets.

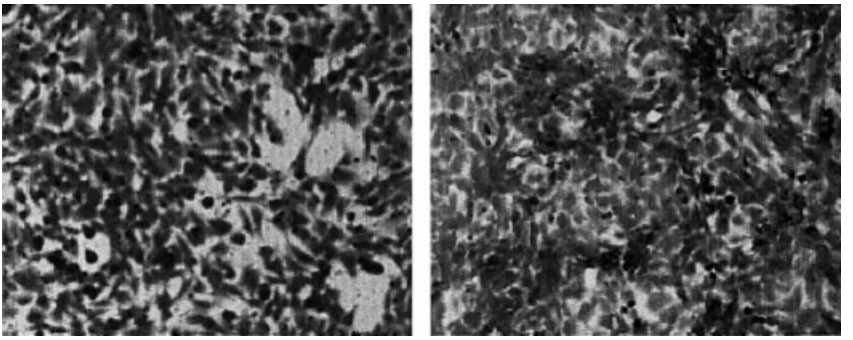


Figure 2. Optical micrograph (20 X) of 3T3 cells on cChML20 film (left), and tissue culture poly(styrene) (right).

Conclusions

Anionic ring opening polymerization of alkyl malolactonates bearing side groups of different structure was successfully performed in THF at 37-42 °C by using tetraethylammonium benzoate as initiator. The lower molecular weights of the obtained poly(alkyl malolactonate)s compared to the theoretic ones were attributed to the presence of chain transfer reactions affecting the polymerization process. Cell proliferation onto the polymer surface was related to the hydrophobic characteristics of the samples, although some effects due to the specific chemical nature of the side groups could not be completely ruled out.

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