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Postpolymerization Modification Using Less Cytotoxic Activated Ester Polymers for the Synthesis of Biological Active Polymers

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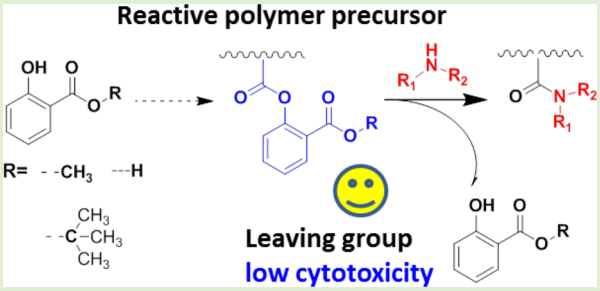
Supporting Information

ABSTRACT: Activated ester polymers, pioneered by Ferruti and Ringsdorf in the 1970s, are attractive polymeric materials because they can easily be converted into functional polymers by reacting with amine nucleophiles. In the present study, methyl salicylate acrylate, salicyl acrylate, and *tert*-butyl salicylate acrylate monomers were polymerized yielding three novel reactive precursors suitable for the postpolymerization modification with primary and secondary amines. The reactivities of poly(pentafluorophenyl acrylate), poly(methyl salicylate acrylic ester), and poly(salicyl acrylate) toward amines were compared by kinetic studies and revealed the practical applicability of salicylic acid based derivatives for efficient postpolymerization modifications. In addition, in vitro cytotoxicity of water-soluble leaving groups, pentafluorophenol and salicylic acid, as well as water-soluble polymers containing the respective activated ester groups were investigated using HeLa cells. In short, compared to the frequently used poly(pentafluorophenyl acrylate), poly(salicyl acrylate) activated ester feature a lower reactivity, but exhibit less cytotoxicity. In this respect, poly(salicyl acrylate) as reactive precursor polymers may become alternative routes for the synthesis of functional polyacrylamides when it comes to advanced applications in vivo.



INTRODUCTION

Postpolymerization modification, also known as polymer analogous reaction, is a versatile synthetic approach in obtaining functional materials.[1−3] The general concept is the chemical transformation of reactive polymer precursors into new functional polymers, thereby enabling the introduction of functional groups, even those that are not compatible with common polymerization conditions or inhibit a precise characterization.[4] Early examples of postpolymerization modification can be traced back to 1840, when Hancock and Ludersdorf independently reported the vulcanization and hydrogenation of natural rubber,[5−8] generating a tough and elastic material. With the general acceptance of the concept of macromolecules, polymer analogous reactions were more and more applied in engineered synthetic polymers.[1] In recent years, the emergence of click type coupling reactions, including azide−alkyne and diene−dienophile cycloadditions, thiol−ene and thiol−yne addition, and substitution reactions based on activated esters laid the foundation for the intensive use of postpolymerization modification reactions.[1,9−12]

Among these, activated ester-amine chemistry has many characteristics of conventional click chemistries, featuring metal free and mild reaction conditions. In 1970s, Ferruti and Ringsdorf proposed activated ester polymers as a novel synthetic tool to prepare pharmacologically active polymer drug conjugates.[13,1] Since then, *N*-hydroxysuccinimide derivatives (NHS), such as poly(*N*-acryloxysuccinimide) (pNAS) and poly(*N*-methacryloxysuccinimide) (pNMAS), have become the most frequently used activated polymer precursors for the preparation of biological relevant functional polymers. However, a drawback of pNAS and pNMAS is their limited solubility in organic solvents, except DMSO and DMF and their hydrolytic instability.[1] Activated ester polymers containing thiazolidine-2-thione are an interesting alternative for pNAS and pNMAS, because polymers featuring thiazolidine-2-thione allow aminolysis in aqueous solution,[14] thereby enabling the application in conjugating with the amine groups located on protein surfaces.[15] Reactive polymers based on azlactone are alternative polymer precursors, with their main advantage being the addition reaction of nucleophiles, that is, no release of a leaving group in this case. Recently, thermoresponsive polymers were derived from postpolymerization modification of poly(2-vinyl-4,4-dimethylazlactone).[8] Later on, Theato et al. introduced activated ester polymers based on pentafluorophenyl (PFP) esters, providing a powerful tool for synthesizing functional polymeric materials.[16−18] Compared with NHS ester polymers, polymers bearing pentafluorophenyl (PFP) ester groups feature a higher reactivity, allowing a practically quantitative conversion with amines under mild conditions, while being analytically advantageous due to the use of ^{19}F

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NMR spectroscopy. Other than that, activated ester polymers based on PFP are (i) easy to synthesize and purify, (ii) soluble in most midrange polar organic solvents, and (iii) allow to monitor the aminolysis process by ^{19}F NMR and FT-IR spectroscopy.[16−18] Due to the wide spectrum of benefits that PFP ester offers, many multifunctional materials, such as reactive coating surfaces and stimuli responsive polymers, have been described in the literature.[19−24] Recently, copolymers containing poly(pentafluorophenyl methacrylate) blocks have been used to prepare well-defined poly(*N*-(2-hydroxypropyl)-methacrylamide) derivatives as drug carriers, enabling precise tracking of the cell uptake behavior as well as the distribution of these highly biocompatible polymers.[25−29] Nevertheless, due to the toxic nature of the pentafluorophenol group that is released during the postmodification step and the possibility of remaining PFP ester groups in the polymer, the utilization of these polymers derived from PFP ester in the biological area is still controversial.

Salicylic acid, derived from plants such as willow bark and latin salix, is a low-cost drug and is classified as nonsteroidal, anti-inflammatory drug (NSAIDS).[30] It is also the active component of acetylsalicylic acid, commonly known as aspirin, one of the most widely used and annually consumed drugs in the world. Aspirin has proved to be effective in modifying proteins by chemical transfer of its acetate group to amino acid side chains.[30] It is demonstrated that the acetylation occurs on the amine of lysine and cysteine residues of cells.[31−33] In the meantime, salicylic acid released during acetylation enabled the pharmacological effects of aspirin in vivo. In light of this evidence for the successful substitution reactions of amines with acetylsalicylic acid, it is reasonable to predict that polymers featuring salicyl esters can act as activated ester polymer precursors. The advantages of activated ester polymer precursors based on salicyl ester would be utilizing a natural product as starting material, which is a cheap (as provided by VWR, the price of salicylic acid is 23.7 € per 500 g, that of pentafluorophenol is 394.6 € per 500g) and enables a green chemistry approach; salicylic acid is an anti-inflammatory, anticancer, antifungal, and antibacterial drug, resulting in less concern about the safety of developing well-defined drug carriers derived from polymer precursors featuring salicyl ester. As a matter of fact, acrylates of salicylic acid have been synthesized and copolymerized with acrylic acid to investigate the release of the drug by hydrolysis.[34] However, to the best of our knowledge, both acrylates of salicylic acid and its derivatives have never been considered as activated ester polymer precursors for postpolymerization modifications.

In this work, we present the preparation of poly(salicyl acrylate) **P2** and its methyl ester and *tert*-butyl ester protected derivatives, poly(methyl-salicylate acrylate) **P1** and poly(*tert*-butyl salicylate acrylate) **P3**. **P1** was developed because of the cheap price (methyl salicylate costs 39.1 € per 500 g, provided by VWR), broad solubility in organic solvents and because it is easy to be prepared, enabling its applicability when manufacturing general functional materials. **P2** is based on salicylic acid, which is a drug itself, can be used in preparing biorelated functional materials. **P3** is based on *tert*-butyl ester protected salicylic acid. The *tert*-butyl ester group is stable toward mild aminolysis and can be cleaved by moderately acidic hydrolysis,[35] allowing **P3** not only to be a reactive polymer precursor itself, but also to be transformed into **P2** via acidic hydrolysis. To the best of our knowledge, it is the first time that these polymers are investigated as successful reactive precursors for the postpolymerization modification of polyacrylates with amines. To demonstrate the practical applicability of salicyl ester based derivatives for efficient postpolymerization modifications, the active ester reactivity of **P1**, **P2**, and the established pentafluorophenyl acrylate (PPFPA) are compared by kinetic studies. In addition, the cell cytotoxicity of salicylic acid, **P2**, PFP, as well as PFP-based active ester polymer precursors, are investigated using HeLa cells.

EXPERIMENTAL SECTION

1. Materials. All chemicals were commercially available and used as received, unless otherwise stated. Dichloromethane (DCM) and dioxane were freshly distilled over calcium hydride and used immediately. Triethylamine (Et_3N) was dried over calcium chloride and distilled previously. Azobis(isobutyronitrile) (AIBN) was recrystallized from methanol prior to use.

2. Structural and Chemical Composition Characterization. ^{1}H NMR spectroscopy was performed on a Bruker 300 MHz. FT-NMR spectrometer with deuterated solvents. The chemical shifts (δ) are given in ppm relative to a standard, tetramethylsilane (TMS). The molecular weight and corresponding molecular weight distribution (M_w/M_n) was determined by gel permeation chromatography (GPC) using polystyrene standards. Unless otherwise stated, the measurements were conducted in THF solution with a flow rate of 1 mL·min^{-1} at 25 °C. Infrared spectroscopy was performed on a Thermo Fisher Scientific Nicolet iS10 using ATR unit. Kinetic IR measurements were conducted on a Reactive IR 45m from Mettler Toledo.

3. Monomer Synthesis. *Methyl-Salicylate Acrylate (M1).* Barbosa et al.[36] have reported the synthesis of **M1** and we synthesized **M1** via a slightly modified synthesis route as follows: A solution of 60 mL of dry DCM containing methyl salicylate (80 mM, 12.16 g) and TEA (84 mM, 8.5 g) was stirred for 10 min in an ice bath. Acryloyl chloride (84 mM, 7 mL) was added dropwise to the mixture. The solution was kept stirring and the formation of product was checked by TLC analysis using ethyl acetate/hexane (1:9 by volume). After 3 h, the mixture was filtrated to remove the precipitated TEA hydrochloride. The filtrate was washed twice with water (30 mL), using a separation funnel and dried over sodium sulfate, and the solvent was removed under reduced pressure. The product was isolated by column chromatography using petrol ether as eluent and was obtained in the form of yellow oil (7 g, yield 77%).

^{1}H NMR (300 MHz, δ, ppm, $CDCl_3$) 8.04 (dd, J = 7.8, 1.7 Hz, 1H), 7.58 (ddd, J = 8.1, 7.5, 1.7 Hz, 1H), 7.41−7.29 (m, 1H), 7.15 (dd, J = 8.1, 1.1 Hz, 1H), 6.71−6.60 (m, 1H), 6.49−6.30 (m, 1H), 6.11−5.99 (m, 1H), 3.84 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.96, 164.60, 150.38, 133.83, 132.76, 131.89, 127.63, 126.08, 123.77, 52.29; IR (ATR mode) 2952.6, 1756.2 (C═O methyl salicylate ester), 1723.6 (C═O methyl ester), 1606.6, 1487.3, 1450.6, 1435.4, 1298.3, 1264.8, 1201.1, 1138.5, 1082.3, 962.2 cm^{-1}; ESI-MS: Calcd for $C_{11}H_{10}O_4$ [M + Na$^+$], 229.0579; found, 229.0493.

Salicyl Acrylate (M2). Boudreaux et al. have reported the synthesis of **M2**.[34] A slightly modified synthesis was conducted as follows: A solution of 175 mL of dry acetonitrile containing salicylic acid (130 mM, 17.94 g) and TEA (390 mM, 54.29 mL) was stirred for 10 min in an ice bath. Acryloyl chloride (140 mM, 11.25 mL) was added dropwise to the mixture. The solution was kept stirring and the formation of product was checked by TLC analysis using ethyl acetate/hexane (1:1 by volume). After 6 h, the mixture was filtrated to remove the precipitated TEA hydrochloride. The solvent was removed under reduced pressure. The compound was dissolved in 100 mL DCM and washed four times with water (50 mL) using a separation funnel. The organic phases were combined and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure. The product was isolated by recrystallization in isopropanol/water (3:1) by dissolving the compound at 60 °C and cooling at −5 °C. The product was obtained in the form of a yellow-tinted solid (11 g, yield 28%).

^{1}H NMR (300 MHz, δ, ppm, $CDCl_3$) 10.66 (d, J = 133.5 Hz, 1H), 8.13 (dd, J = 7.8, 1.7 Hz, 1H), 7.64 (td, J = 7.7, 1.6 Hz, 1H), 7.37 (td, J

= 7.6, 0.9 Hz, 1H), 7.19 (dd, J = 8.1, 0.9 Hz, 1H), 6.77−6.55 (m, 1H), 6.38 (dd, J = 17.3, 10.4 Hz, 1H), 6.04 (dd, J = 10.4, 1.2 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.78 (s), 164.59 (s), 150.98 (s), 134.76 (s), 132.75 (s), 132.46 (s), 127.68 (s), 126.18 (s), 123.97 (s), 122.42 (s); IR (ATR mode) 2972.6, 2635.9, 1756.8 (C═O salicyl ester), 1718.8 (C═O carboxylic acid), 1607.3, 1578.7, 1487.5, 1451.6, 1309.5, 1247.1, 1199.3, 1137.4, 1079.5, 1046.3, 889.6 cm^{-1}; ESI-MS: Calcd for $C_{11}H_8O_4$ [M + Na$^+$], 215.0423; found, 215.0593.

tert-Butyl-salicylate Acrylate (M3). 2-methyl-2-propanyl salicylate (**A**) was synthesized as follows: 1,1′-carbonyldiimidazole (100.3 mM, 16.28 g) and salicylic acid (100.3 mM, 13.7 g) were dissolved in 120 mL of DMF. After stirring at 50 °C for 30 min, the mixture of *tert*-butyl alcohol (200.6 mM, 18.98 mL) and 1,8-diazabicycloundec-7-ene (200.6 mM, 29.83 mL) was added dropwise. The reaction mixture was stirred at 50 °C for 24 h. Then the solution was poured into saturated $NaHCO_3$ (550 mL) and extracted with ethyl acetate (300 mL, 3 times). The organic layers were combined and dried over anhydrous Na_2SO_4, filtered, and concentrated. The compound (colorless oil, 14.4 g) was purified by column chromatography with ethyl acetate/hexane (0.05:1 by volume). Yield: 75%

IR (ATR mode) 3141.5 (O−H), 1667.3 cm^{-1} (*tert*-butyl C═O); ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.07 (s, 1H), 7.80 (dd, J = 8.0, 1.7 Hz, 1H), 7.43 (ddd, J = 8.7, 7.3, 1.7 Hz, 1H), 6.97 (dd, J = 8.4, 0.9 Hz, 1H), 6.92−6.72 (m, 1H), 1.63 (s, 9H).

tert-Butyl salicylate acrylate (M3) was synthesized as follows: A solution of 20 mL dry dichloromethane containing 2-methyl-2-propanyl salicylate (**A**; 10 mM, 1.94 g) and triethylamine (11 mM, 1.53 mL) was kept stirring for 10 min in an ice bath. Acryloyl chloride (10 mM, 0.804 mL) was added dropwise to the mixture. The solution was kept stirring and the formation of product was checked by TLC analysis using ethyl acetate/hexane (0.1:1 by volume). After 6 h, the mixture solution was filtrated to remove the precipitated TEA hydrochloride. The filtrate was washed twice with water (30 mL) using a separation funnel and dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure. The product was isolated by column chromatography using ethyl acetate/hexane (0.1:1 by volume) as eluent and was obtained in the form of colorless oil (1.5 g, yield 60%).

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.94 (dd, J = 7.8, 1.7 Hz, 1H), 7.60−7.45 (m, 1H), 7.37−7.22 (m, 1H), 7.10 (dd, J = 8.1, 1.2 Hz, 1H), 6.70−6.59 (m, 1H), 6.39 (dd, J = 17.3, 10.4 Hz, 1H), 6.05 (dd, J = 10.4, 1.4 Hz, 1H), 1.53 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.33 (s), 164.19 (s), 149.53 (s), 132.93 (s), 132.66 (s), 131.54 (s), 128.02 (s), 125.91 (d, J = 6.4 Hz), 123.38 (s), 81.76 (s), 28.12 (s); IR (ATR mode) 1745 cm^{-1} (*tert*-butyl ester salicylate C═O), 1715 cm^{-1} (*tert*-butyl C═O); Calcd [M + Na$^+$], 271.1049; found, 271.0956.

4. Typical Free Radical Polymerization Procedure. *Poly(methyl-salicylate acrylate) P1.* Free radical polymerization of **M1** was carried out as follows: 1,4-dioxane (1 mL) solution of **M1** (4 g, 100 equiv) and AIBN (1 equiv) were degassed under argon at 25 °C for 20 min. After filling with argon, the flask was immersed in a preheated oil bath of 70 °C for 15 h. The reaction mixture was exposed to air to quench the polymerization. After taking a small portion for checking conversion rate, the solution was diluted with THF and purified by reprecipitation (THF/hexane) to afford a white powder polymer. Yield: 3.2 g (80%).

^{1}H NMR (300 MHz, δ, ppm, $CDCl_3$) 7.73 (d, 1H), 7.10 (m, 3H), 3.78 (m, 3H), 3.30 (s, 1H), 2.30 (t, 2H); IR (ATR mode) 1757.8 cm^{-1} (methyl salicylate ester C═O), 1723.6 cm^{-1} (methyl ester C═O). GPC (PS standard, THF) M_n = 1.35 × 10^4 g/mol, M_w = 4.85 × 10^4 g/mol, M_w/M_n = 3.59.

Poly(salicyl acrylate) P2. **M2** was polymerized as follows: THF solution of **M2** (2 g, 100 equiv) and AIBN (1 equiv) were placed in a Schlenk tube. Three freeze−pump−thaw cycles were performed to degas the solution. The flask was immersed in a preheated oil bath of 65 °C for 20 h. The reaction mixture was exposed to air to quench the polymerization. After taking a small portion to check the conversion rate, the solution was diluted with THF and purified by reprecipitation (THF/diethyl ether) to afford a white powder polymer. Yield: 1.26 g (63%).

^{1}H NMR (300 MHz, δ, ppm, DMSO) 13.08 (S, 1H), 7.81 (m, 1H), 7.21 (m, 3H), 3.11 (s, 1H), 1.97 (ddd, 2H); IR (ATR mode): 1756.8 cm^{-1} (salicyl ester C═O), 1718.8 cm^{-1} (salicylic acid C═O).

Poly(tert-butyl salicylate acrylate) P3. Free radical polymerization of **M3** was carried out as follows: 1,4-dioxane (0.5 mL) solution of **M3** (0.5 g, 100 equiv) and AIBN (1 equiv) were degassed under argon at 25 °C for 20 min. After filling with argon, the flask was immersed in a preheated oil bath of 70 °C for 15 h. Afterward, the reaction mixture was exposed to air to quench the polymerization. After taking a small portion to check the conversion rate, the solution was diluted with THF and purified by reprecipitation (THF/hexane) to afford a white powder polymer. Yield: 200 mg (40%).

^{1}H NMR (300 MHz, δ, ppm, $CDCl_3$) 7.69 (s, 1H), 6.98 (d, J = 34.8 Hz, 3H), 3.16 (d, J = 86.0 Hz, 1H), 2.03 (ddd, J = 82.5, 67.5, 10.1 Hz, 2H), 1.55−0.93 (m, 9H). GPC (PS standard, THF) M_n = 1.86 × 10^4 g/mol, M_w = 3.38 × 10^4 g/mol, M_w/M_n = 1.81. IR (ATR mode): 1758.9 cm^{-1} (*tert*-butyl ester salicylate C═O), 1715 cm^{-1} (*tert* butyl C═O).

5. Typical Procedure for Deprotection of *tert*-Butyl Ester with TFA/Dichloroethane. P3 (0.2 mM, 1 equiv, 50 mg) was dissolved in 1 mL of dry dichloroethane. TFA (2 mM, 10 equiv, 150 μL) was added and the solution was stirred at 25 °C for 6 h. The deprotected polymer was purified by precipiatation (THF/hexane) and obtained in practically quantitative yield.

6. Postpolymerization Modifications with Amines. The postpolymerization modifications of **P1** with amines were performed as follows: after dissolving **P1** (0.2 mM, 1 equiv) in 1 mL of dry 1,4-dioxane, 3 equiv of amine and triethylamine were added to the solution sequentially. The mixture was stirred over 24 h at 50 °C. A small portion was taken for FT-IR measurements.

The postpolymerization modifications of **P2** with amines were performed as follows: after dissolving **P2** (0.2 mM, 1 equiv) in 1 mL of dry 1,4-dioxane and DMSO (1:1), 3 equiv of amine and triethylamine were added to the solution sequentially. The mixture was stirred at 50 °C for 24 h. After removing the solvent under vacuum, the remaining crude produce was redissolved in THF. A small portion was taken for FT-IR measurements.

After dissolving **P3** (0.1 mM, 1 equiv, 25 mg) in 1 mL of dry 1,4-dioxane, 3 equiv of hexylamine and triethylamine were added to the solution sequentially. The mixture was stirred over 24 h at 50 °C. A small portion was taken for FT-IR measurements.

7. Kinetic FT-IR Measurements. A general procedure to investigate the postpolymerization modification kinetically was as follows: the polymer was dissolved in dry solvent (dioxane for **P1** and PPFPA; 50:50 mixture of DMSO and dioxane for **P2**) at a concentration of 0.2 mol/L. The flask was immersed into an oil bath which was preheated to 50 °C. The IR probe was inserted into the solution. Once stable IR spectra could be recorded, 3 equiv of the corresponding amine and TEA were added to the solution. IR spectra were then recorded in time intervals of 1 min during the first hour of the reaction and at 10 min intervals after the first hour. Time-resolved conversion was calculated by the decrease of the carbonyl peak in the IR spectrum. The integration of the peak at 0 min was defined as 0% conversion.

8. Cytotoxicity Tests. Poly((*N*-2-hydroxypropyl)-methacrylamide-*co*-pentafluorophenyl methacrylate) was synthesized by dissolving poly(pentafluorophenyl methacrylate) (0.5 mM, 126 mg, 1 equiv) in 1 mL of dry solvent (50:50 mixture of DMSO and dioxane), hydroxypropylamine (0.8 mM, 60 μL, 1.6 equiv) was added to the solution. The mixture was stirred over 39 h at 20 °C. A small portion was taken to be investigated by ^{19}F NMR spectroscopy. The polymer was purified twice by precipitation (THF/diethyl ether) and then dialyzed against deionized water for 48 h.

8.1. Cell Culture Tests. The cytotoxicity studies of activated ester precursors were carried out using a human HeLa cell line, which was derived from cervix carcinoma cells. The cryopreserved cells were revived and cultured in RPMI 1640 medium, supplemented with 10% fetal calf serum (FCS) plus 1% streptomycin. The cells were grown in a T-25 flask and incubated under 5% CO_2 and 95% humidity at 37 °C. After incubating for 24 h, the cells were harvested using trypsin EDTA

Scheme 1. Synthesis of Poly(methyl-salicylate acrylate) (P1), Poly(salicyl acrylate) (P2), and Poly(*tert*-butyl-salicylate acrylate) (P3) and Their Post-Polymerization Modification with Amines

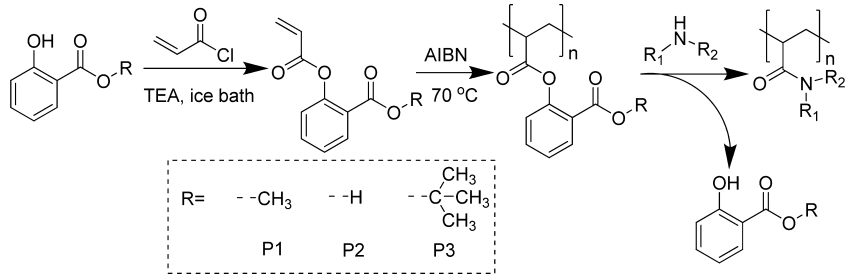

Table 1. Results and Conditions for Polymerization of Different Active Ester Monomers M1 to M3

polymer	monomer	solvent	t (h)	T (°C)	M_n (g/mol)	M_w (g/mol)	M_w/M_n
P1	M1	dioxane	15	70	1.35×10^4	4.85×10^4	3.59
P2	M2	THF	20	65	N	N	N
P3	M3	dioxane	15	70	1.86×10^4	3.38×10^4	1.81

N: not determined.

and were then used for further experiments. The culture medium was changed every 5 days.

8.2. Resazurin Fluorimetric Assay. Resazurin Fluorimetric Cell Viability Assay Kit (Biotium, U.S.A.) was used for cell viability testing according to the company's instructions. Briefly, HeLa cells were seeded at a density of 5000 cells per well in a 96-well plate in 200 μL of culture medium and incubated overnight. The next day, the growth medium was removed and 200 μL of fresh medium containing the desired amounts of compounds and polymers were injected into each well. In the case of testing the cytotoxicity of polymers, the corresponding concentrations of **P2** and poly((*N*-2-hydroxypropyl)-methacrylamide-*co*-pentafluorophenyl methacrylate) were dissolved in 2 μL of DMSO before they were applied to the cells, which means cell culture medium containing 1% DMSO was adopted in this case. Thus, cell viability cultured in medium containing 1% DMSO was tested as blank control. The cells were incubated for 24 h, after which the medium was removed and cells were washed with PBS before adding a mixture of resazurin and RPMI (100 μL) in a volume ratio of 1:10 into each well. After incubating for 4 h, cell viability was monitored by measuring the fluorescence with an excitation wavelength at 550 nm and an emission of 590 nm. Fluorescent intensity generated by the assay was directly related to the number of living cells in the sample. Measurements were carried out using Infinite 200 microplate reader (TECAN, Crailsheim, Germany). Results were presented after subtracting the fluorescence background. The percentage of survival of active ester treated cells was calculated based on the control cells. Correspondingly, cells treated with culture medium containing 1% DMSO were used as blank control (cell viability 95.7%), cell viability was calculated based on this control.

RESULTS AND DISCUSSIONS

1. Monomer Synthesis. Methyl-salicylate acrylate (**M1**), salicyl acrylate (**M2**), and *tert*-butyl-salicylate acrylate (**M3**) were synthesized by alcoholysis of acryloyl chloride in the presence of an auxiliary base, as shown in Scheme 1. **M1** and **M3** were purified by column chromatography and obtained as colorless liquids. **M2** was isolated by recrystallization. The obtained compounds were characterized by ^{1}H NMR spectroscopy (Figures S1−S3) as pure monomers for polymerization.

M1 and **M3** were soluble in many organic solvents such as THF, acetone, dioxane, diethyl ether, *n*-hexane, and DMF. In the case of **M2**, due to the hydrophobic nature of the benzene group and the hydrophilic property of the carboxylic group, it is soluble in organic solvents like THF, DMSO, methanol, dioxane, and DMF, but still insoluble in water. All monomers could be prepared on a large scale and are stable for several months, when stored at 4 °C.

2. Polymerization and Postpolymerization Modification. Polymerization of **M1** to **M3** was conducted via free radical polymerization using AIBN as initiator. The polymerization conditions and the results are summarized in Table 1. All polymers were characterized by ^{1}H NMR and FT-IR spectroscopy (Figures S1−S3). GPC data confirmed that **P1** was obtained with molecular weights of 1.35×10^4 g/mol and molecular weight distributions of 3.59. However, the free carboxylic acid group on the side group of **P2** made it difficult to characterize by GPC. Therefore, **P3** was synthesized because it allows the cleavage of *tert*-butyl ester under moderately acidic conditions, yielding the respective **P2**.[35] Deprotection of the carboxylic acid was conducted in the presence of excessive TFA. ^{1}H NMR spectroscopy documented the complete disappearance of the broad peak around 1.42 ppm (Figure S4), originating from the *tert*-butyl protons of **P3**, and thereby suggesting the successful removal of the *tert*-butyl ester group. ^{1}H NMR and IR (Figure S5) showed that after deprotection of **P3** using TFA, the polymer featured the same characteristic spectrum of **P2** and, consequently, proved the successful indirect way to prepare **P2**. GPC data showed that **P3** was obtained with reasonable molecular weight of 1.86×10^4 g/mol and molecular weight distribution of 1.81 via free radical polymerization.

The polymers were synthesized as activated ester polymer precursors and their postpolymerization modification with amines can be easily monitored by IR spectroscopy. In initial studies, **P1** was selected out of the three polymers because of its good solubility in organic solvents and it is the easiest to prepare. Hexylamine was used to optimize the postpolymerization modification conditions. Aminolysis of **P1** by adding hexylamine several times was conducted at 50 °C. As shown in Figure S6-A, the methyl-salicylate ester peak at 1757.8 cm^{-1} (peak 1) and the methyl ester peak around 1723.6 cm^{-1} (peak 2) decreased simultaneously with the addition of hexylamine. A total of 3 equiv of amine (Figure S6−B) at 50 °C completed the ester modification, while still providing comparable reactivity with the other postpolymerization modification approaches via activated ester polymers.[25,37]

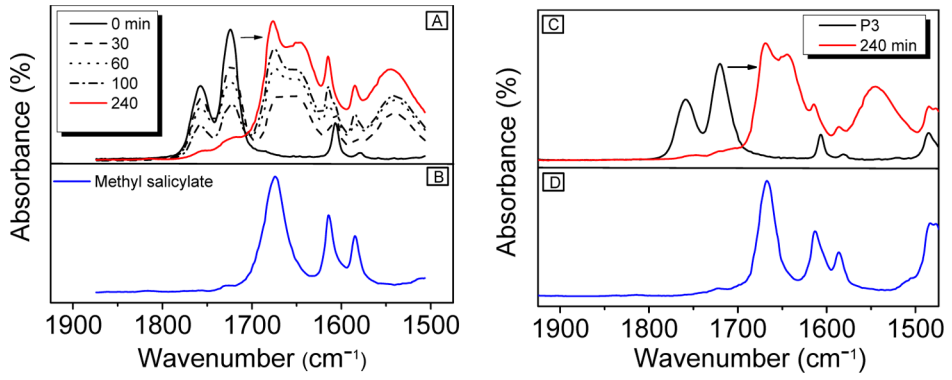

Figure 1. (A) IR (ATR mode) spectra of during postpolymerization modification of **P1** with hexylamine at 50 °C; (B) IR spectra of methyl salicylate; (C) IR (ATR mode) spectra of **P3** before (black solid line) and after postpolymerization modification with hexylamine (red dot line); (D) IR spectra of *tert*-butyl salicylate.

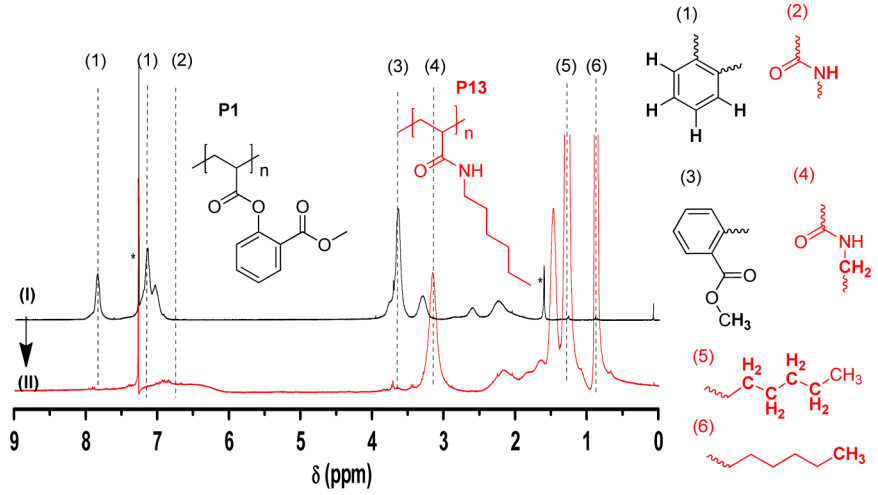

Figure 2. ^{1}H NMR spectra in $CDCl_3$ of **P1** before (I) and after postpolymerization modification with hexyl amine **P13** (II).

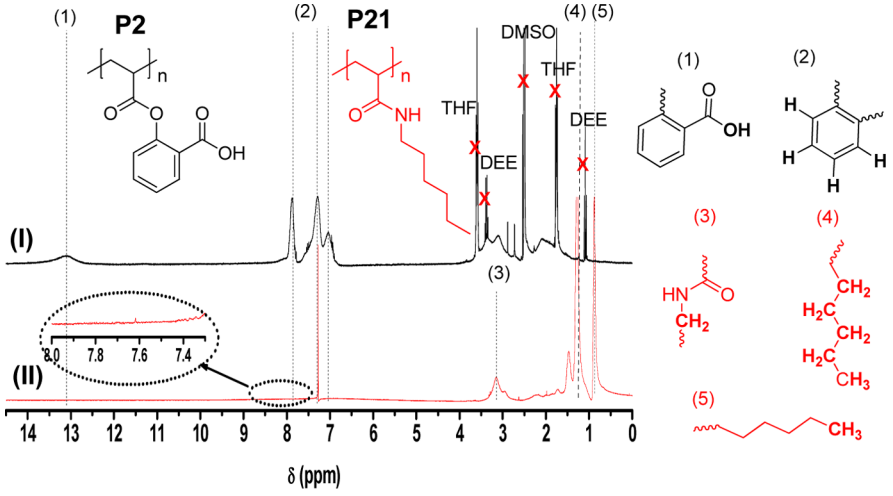

Figure 3. ^{1}H NMR spectra in DMSO of **P2** before (I) and after post modification with hexylamine **P21** in $CDCl_3$ (II).

In terms of **P1** and **P3**, the stability of methyl ester group and *tert*-butyl ester group during aminolysis was investigated. The process of postpolymerization modification with 3 equiv of hexylamine was followed by in situ IR. As shown in Figure 1A, the methyl ester group of **P1** (1723.6 cm^{-1}) gradually shifted to 1674 cm^{-1} as the reaction proceeded, which is the signal of methyl ester group for methyl salicylate (Figure 1B). In the case of **P3**, the in situ IR of **P3** before and after aminolysis also showed that *tert*-butyl ester group (1715 cm^{-1} for **P3**) shifted to 1667.3 cm^{-1} (signal for *tert*-butyl ester of *tert*-butyl salicylate), which means that *tert*-butyl salicylate is the leaving group during the aminolysis process. Thus, it can be concluded that no side reactions occurred during postpolymerization modification of **P1** and **P3** with amines.

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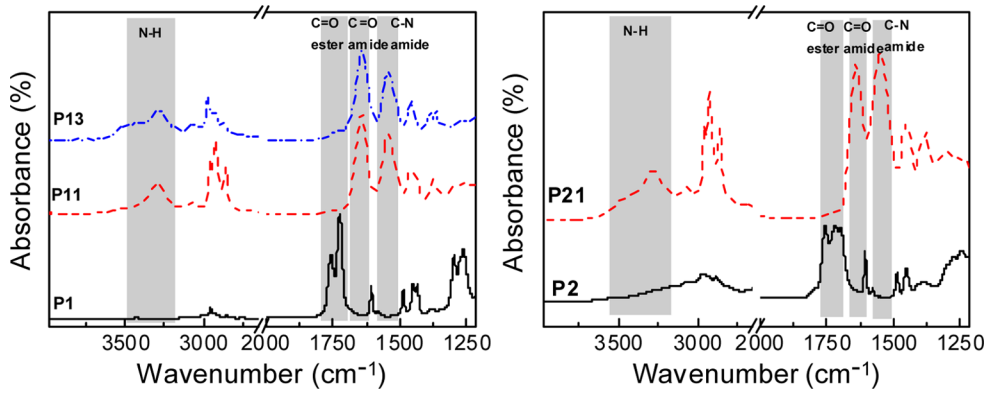
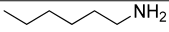
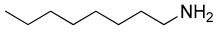
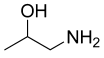
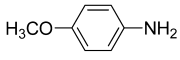
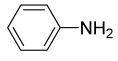
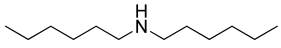


Figure 4. (A) Representative IR of **P1** before (solid black line) and after postpolymerization modification with hexylamine **P11** (red dashed line) and isopropylamine (blue dashed-dotted line) **P13**; (B) Representative IR of **P2** before (solid black line) and after (red dashed line) postpolymerization modification with hexylamine **P21**.

Table 2. Library of Primary and Secondary Amines Used to Modify P1 and P2

Amine	Run	P1[a]	Run	P2[a]
	P11	98%	P21	100%
	P12	98%	P22	100%
	P13	98%	P23	100%
	P14	90%[b]	P24	95%[b]
	P15	50%	P25	60%
	P16	0%	P26	0%
	P17	5%	P27	80%
	P18	8%	P28	60%

[a]Determination by comparison of the intensity of the corresponding characteristic band in the IR spectra of the active ester moiety in the precursor polymer and after modification with amines. [b]The reaction was conducted at 25 °C for 2 days.

In order to provide direct evidence of the successful aminolysis of the obtained activated ester precursors, ^{1}H NMR measurements of the purified polymers before and after postpolymerization modification with hexylamine were carried out and the results for **P1** are shown in Figure 2. The peaks at 7.84, 7.14, and 7.13 ppm owing to the aromatic protons of the salicylate group and the peak at 3.64 ppm originating from the methyl ester group disappeared (Figure 2B), while a broad peak at 6.77 ppm owing to the NH protons of the amide group and a peak at 1.28 ppm as well as a sharp peak at 0.88 ppm originating from the hexyl chain were found. This shows that the methyl salicylate group was fully substituted and that hexylamine was successfully installed via the activated ester postpolymerization modification reaction. In the case of **P2**, as shown in Figure 3I, a broad peak occurred at 13.1 ppm originating from the free acid group of **P2** and three peaks appeared at 7.86, 7.24, and 7.05 ppm owing to the aromatic protons of the salicylic group. After the postpolymerization modification with hexylamine, the signals for aromatic protons disappeared and the product **P21** (Figure 3II) showed the expected signals, which were identical to those found for **P13** (Figure 2II). Postpolymerization modification of **P3** with hexylamine under the same condition was checked as well, ^{1}H NMR (Figure S7) suggests that hexylamine was successfully installed and the product showed that expected signal in the ^{1}H NMR spectrum.

FT-IR spectroscopy is a very useful tool to determine the completeness of the postpolymerization modification via aminolysis. Representative spectra are shown in Figure 4. The absorbance spectrum of the precursor polymer **P1** is plotted in Figure 4 and shows two strong bands at 1757.8 cm^{-1} (methyl salicylate ester C═O stretch) and 1723.6 cm^{-1} (methyl ester C═O stretch) in the carbonyl region as well as a sharp peak with medium intensity at 1602 cm^{-1}, originating from the aromatic stretch of the methyl salicylate group. The FT-IR spectrum of the product of the aminolysis of **P1** with hexylamine is shown in middle plot in Figure 4. **P11** did not show any of the characteristic bands of the precursor polymer, but rather showed a broad peak around 3295 cm^{-1}, owing to the N−H stretching of amide. Compared with the starting material, the absorbance intensity of methylene around 2927 cm^{-1} increased in the C−H stretch region (3000−2800 cm^{-1}),

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revealing the presence of additional $-CH_2-$ groups. Most notably is the carbonyl region, where the strong ester bands (1757.8 and 1723.6 cm^{-1}) completely disappeared, suggesting the complete conversion of the methyl salicylate ester. Instead, the IR spectrum of **P11** showed strong peaks at 1637 and 1545 cm^{-1}, characteristic bands for C═O stretching and C−N bending of amide, respectively. The spectrum of **P1** modified with isopropylamine, **P13**, which is plotted at the top of Figure 3, showed similar bands in the N−H, C═O stretching region and C−N bending region owing to amides. **P13** showed a lesser intensity band in the C−H stretch region, resulting from the fact that isopropylamine features less C−H bonds compared to hexylamine.

In the case of **P2**, the carbonyl region shows two strong broad peaks at 1756.8 cm^{-1} (salicyl ester C═O stretch) and 1718.8 cm^{-1} (carboxylic acid C═O stretch); and it shows a sharp peak with medium intensity at 1602 cm^{-1}, owing to the aromatic stretch of the salicyl ester group. The product of the aminolysis with hexylamine, **P21**, the upper plot in Figure 4B, shows the characteristic bands of N−H stretching, C═O stretching and C−N bending of amide. All in all, IR spectroscopy confirmed the expected structure of the polyacrylamides after aminolysis and demonstrated the complete conversion of the methyl-salicylate ester and salicyl ester, respectively.

In order to show the reactivity of the obtained polymer precursors, different amines were applied for the postpolymerization modification of **P1** and **P2**. As shown in Table 2, for primary amines such as hexylamine, octylamine, and isopropanolamine, both **P1** and **P2** can be converted quantitatively. For isopropylamine, **P1** can reach around 90% of conversion and **P2** can reach almost 100% of conversion when reacting with 3 times of amine at 25 °C for 2 days. For aromatic amines, such as *p*-anisiline, 50% conversion was achieved for **P1** and a slightly higher conversion of **P2** was realized. None of them reacted with aniline. For the above tested amines, **P2** shows a slightly higher reactivity compared with P1 and this reactivity difference seems be more prominent when it comes to secondary amines. For dihexylamine and diethylamine, only a slight aminolysis (less than 10%) happened with **P1**, while **P2** could reach 80 and 60% conversion (Figure S8), respectively. In conclusion, the activated ester precursors have a reasonable reactivity toward amines, making them suitable candidates for the synthesis of functional polyacrylamides via postpolymerization functionalization.

In order to show a direct evidence of the reactivity of the obtained activated ester precursors, postpolymerization modification of poly(pentafluorophenyl acrylate), **P1** and **P2** with hexylamine were compared by kinetic studies. The reactions were tracked by in situ IR measurements. During the postpolymerization modification, the intensity of the carbonyl group of activated ester (1787 cm^{-1} for PFP ester, 1757.8 cm^{-1} for methyl salicylate ester, and 1756.8 cm^{-1} for salicyl ester) decreased. Instead, the intensity of carbonyl group of amide at 1682.4 cm^{-1} increased (Figures S9−13). The time dependence of conversion was determined by the decrease of the area of carbonyl peak. The integration of the peak area at 0 min was defined as 0% conversion. The aminolysis of PPFPA with hexylamine, as shown in Figure 5 (black star curve), was completed within 1 min under the same reaction conditions (polymer concentration 0.2 mM, 3 equiv hexylamine, 3 equiv TEA, 50 °C). **P1** and **P2** reached almost 100% of conversion after 225 min. It is noteworthy that **P2** has a slightly higher reactivity compared with **P1**, which is in agreement with the data shown in Table 2. Obviously, PPFPA has an even higher reactivity, making it the ideal candidate when high reactivity is required. In all other cases, **P1** and **P2** are suitable alternative activated ester precursor polymers.

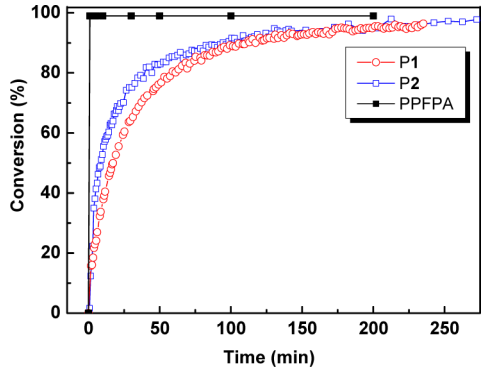

Figure 5. Reaction time dependence of conversion for the aminolysis of PPFPA (black solid squares), **P1** (red circles) and **P2** (blue squares) with hexylamine in the presence of an auxiliary base.

CYTOTOXICITY ASSAY

Due to the high reactivity and quantitative conversion toward primary amines, activated ester polymer precursors, such as poly(pentafluorophenyl methacrylate), have been used for the synthesis of drug carriers in the field of nanomedicine.[24,26−29] However, due to the toxic nature of pentafluorophenol, small amounts of remaining pentafluorophenol after postpolymerization modification is still a controversy when it comes to the application of the drug carrier in vivo. In any case, intensive and time-consuming purification procedures, such as dialysis, must be conducted. In this work, the obtained activated ester precursor **P2** is based on salicylic acid, the active component of aspirin, which is a nonsteroidal anti-inflammtory drug (NSAID).[30] Compared with PFP based polymers, activated ester precursors made of salicylic acid have the potential to solve the safety dispute in terms of biorelated application. Thus, the cytotoxicity of leaving groups and polymers featuring PFP and salicylic acid were investigated using HeLa cell lines. As live cells have enzymes that can reduce resazurin, the resazurin fluorimetric assay is a widely used method in quantifying the cell viability[38,39] and was applied within the present study.

In terms of testing the toxicity of leaving groups, cells were treated with different concentrations of PFP and salicylic acid, ranging from 0 to 1000 μg/mL. As shown in Figure 6, when the concentration was increased from 0 to 1000 μg/mL for samples treated with salicylic acid, the cell viability decreased to 54%, whereas when treated with PFP it decreased to 15%. This shows that PFP exhibited a significantly higher toxicity toward cells. It is reasonable that salicylic acid exhibits toxicity toward cells, especially in the tested high concentration. Noteworthy, after taking Aspirin orally, the normal plasma concentration of salicylate is in the range of 150−300 μg/mL.[40] Higher doses than 350 μg/mL can induce hyperventilation, reversible tinnitus even acidosis.[40] According to our cytotoxicity tests, when treated with 300 μg/mL salicylic acid, the cell viability was around 90%. Whereas when the same amount of PFP was applied, cell viability decreased to around 60%. In summary, PFP showed a higher toxicity than salicylic acid. It should be

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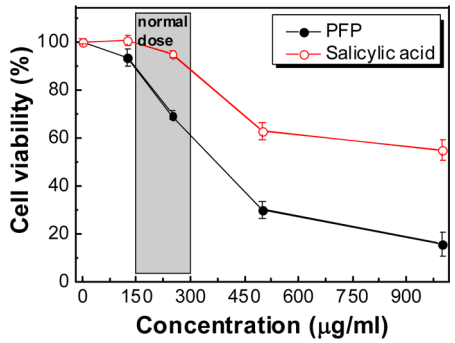

Figure 6. Viability of HeLa cells (human cell line derived from cervix carcinoma cells, adherent) as a function of PFP (solid black circles) and salicylic acid (open red circles) with error bars. Cell viability was determined by resazurin fluorimetric assay.

noted that a suitable concentration of salicylic acid (less than 300 μg/mL) in vivo is acceptable.[40]

Cytotoxicity of polymers containing salicyl and PFP esters were tested using HeLa cell lines as well. In order to improve the solubility of PFP ester polymer in the cell culture medium, hydroxypropylamine was used to partially modify polyPFPMA, yielding poly(*N*-(2-hydroxypropyl)-methacrylamide) (pHPMA), which is known to be a nontoxic drug carrier that has already entered clinical trials.[27,41] Cell toxicity tests also showed that pHPMA did not exhibit any cell toxicity, even up to a dose of 2 mg/mL.[42] Postpolymerization modification of polyPFPMA was conducted using hydroxylpropylamine at 25 °C for 39 h. Retaining PFP ester groups after the postpolymerization modification were proved by ^{19}F NMR and IR spectroscopy (Figure S8), suggesting that the resulting poly((*N*-2-hydroxypropyl)-methacrylamide-*co*-pentafluorophenyl methacrylate) poly(HPMA-*co*-PFPMA) contained 19.5% PFP groups (molar ratio). The amount of the copolymer applied to cell test was calculated based on the neat weight of polyPFPMA (Table S1). As known from pharmaceutical studies, an overdose of aspirin is harmful to the body. Doses higher than 350 μg/mL lead to hyperventilation and result in acidosis at 450 μg/mL.[40] Thus, the cytotoxicity of **P2** below 500 μg/mL, instead of going higher to 1000 μg/mL, was checked in this work. As shown in Figure 7, when the concentration of polymers was increased from 0 to 500 μg/mL, the cell viability treated with **P2** decreased to around 50%, whereas when applying a poly(HPMA-*co*-PFPMA), it decreased

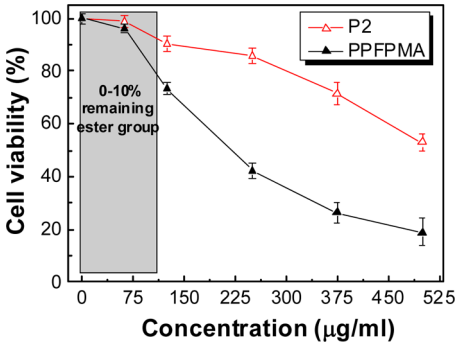

Figure 7. Viability of HeLa cells as a function of the different concentrations of neat polyPFPMA (closed triangles) and **P2** (open triangles); Cell viability was determined by resazurin fluorimetric assay.

to 20%, which is similar to their corresponding leaving groups (Figure 6). It is obvious that polymers containing salicyl ester groups (open triangles in Figure 7) are less toxic than those featuring PFP esters (solid triangles in Figure 7). Noteworthy, during the application of biocompatible polymers or polymer drug conjugates derived from activated ester polymer precursors in vivo, it is the remaining activated ester group in the polymer that is controversial. For example, take the normally applied concentration of polymer (1000 μg/mL) in vivo derived from activated ester polymer precursors. If up to 10% active esters are left after postpolymerization modification, cell viability showed that cells treated with **P2** decreased to 92%, which did not induce serious cytotoxic effect and this toxicity level is still acceptable as biocompatible gene carriers.[43] In contrast, at the same concentration, the cell viability for PPF ester polymer samples decreased to 78% (Figure 7, gray filled pattern region). Thus, functional polymers derived from **P2** featured a lower cytotoxicity, which makes **P2** an ideal activated ester precursor polymer candidate for the preparation of biorelated functional materials.

CONCLUSION

In conclusion, we have successfully synthesized three novel activated ester polymer precursors: Poly(salicyl acrylate) and its methyl ester and *tert*-butyl ester protected derivatives, poly(methyl-salicylate acrylate), and poly(*tert*-butyl-salicylate acrylate). In general, poly(methyl salicylate acrylate) is cheap, has a broad solubility in organic solvents, and can be prepared easily, enabling its applicability in manufacturing general functional materials. Poly(salicyl acrylate) is based on a cheap drug, salicylic acid, the active component of aspirin and can be used in preparing biorelated functional materials. Poly(*tert*-butyl-salicylate acrylate) is not only a reactive polymer precursor itself, but it can also be transformed into poly(salicyl acrylate), allowing an alternative synthesis of poly(salicyl acrylate). With primary amines, all of the three activated esters allowed an almost quantitative functionalization. For secondary amines, poly(salicyl acrylate) reached partial conversion up to 80%. As demonstrated by kinetic IR, poly(salicyl acrylate) and poly(methyl-salicylate acrylate) showed a similar ester reactivity, which was not as high as that of PFP ester. Cytotoxicity tests of leaving groups using HeLa cell lines suggested that salicylate has a lower toxicity than pentafluorophenol. Cell viability tests of polymers containing these activated ester groups also showed that salicyl ester based polymers possess a lower toxicity than PFP based active esters, making these polymers ideal activated ester precursor polymer candidates for the preparation of biorelated functional materials due to their significantly reduced cytotoxicity.

ASSOCIATED CONTENT

Supporting Information

Supplementary spectroscopic data of monomers, poly(*tert*-butyl-salicylate acrylate) and poly((*N*-2-hydroxypropyl)-methacrylamide-*co*-pentafluorophenyl methacrylate), as well as kinetic IR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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