Synthesis and Characterization of Polymer-Linked Prodrug of Antibacterial Agent for The Targeted Delivery to The Colon by Cold Plasma Technique

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A crystalline acrylic-type polymerizable monomer of Nalidixic acid (NA) was synthesized by linking NA molecule to acrylic acid (AA) via acid-anhydride conjugate. Then, the monomer was polymerized in solid state by vibratory mixing with plasma-irradiated lactose powders as postprocessing-free initiators. The characterization of the resulting polymeric prodrug powders by ESR, XRPD and FT-IR analysis confirmed their synthesis successfully. The in vitro hydrolysis study of the polymeric prodrugs was carried out in three different buffer solutions at 37 °C. The results indicated that the acid-anhydride conjugate of the polymeric prodrugs was relatively stable to acid-catalyzed hydrolysis, and the NA release was in sustained manner with no burst and enhanced in basic media, in which ca. 4%, 35% and 80% of NA was released in 12 h at pH 1.2, pH 6.8 and pH 8.5, respectively. The obtained results suggested that the studied systems could be potential antibacterial polymeric prodrugs to minimize drug-stomach exposure and to use for colonic-targeting drug delivery formulation.

Keywords: Plasma irradiation, Drug delivery system, Antibacterial agent, Polymeric prodrugs

1. Introduction

Drug delivery systems (DDS) are used to transport therapeutic drugs in diseased tissues of the body as needed to safely achieve the desired therapeutic effect. In recent years, the development of a colon targeted DDS, which played a role in treating ulcerative colitis, Crohn’s disease, inflammatory bowel disease and so on, is one of the most active areas in drug research [1]. The goal of any DDS is to provide and maintain therapeutic concentrations of drug at the target biological site, improve the therapeutic index and reduce the adverse side effects.

Among present DDS, polymeric prodrug as carriers, which is a conjugation of a drug with a polymer forms that remains inactive during its delivery to the site of action and is activated by the specific conditions in the targeted site, have shown great potential. The polymeric prodrug exhibits some unique advantages, for example, they can control drug release to maintain drug levels within a desired range, satisfy the need for fewer administrations, optimal use of the drug, and increase patient compliance [2,3].

Over the years, we have been working on the development of DDS by using plasma-assisted method, which was applicable to oral, topical and injectable formulations [4-11]. In continuation of our...
previous work to develop the polymeric prodrugs, herein we report a novel colon-targeted DDS of nalidixic acid (NA), one of antimicrobial agents effective for treatment of urinary tract infections, based on a polymeric prodrug by using plasma technique. If antimicrobial agents are delivered to the colon successfully, it could be applicable for the treatment of intestinal infections and reduced systemic side effects [12].

It was proposed to design an acid–anhydride conjugated prodrug of NA through acrylic based polymer for oral administration. The resultant polymeric prodrug should undergo hydrolysis of its chemical bond to cleave the parent drug in a sustained manner which may result in maintaining the plasma drug level within the therapeutic range. Therefore, the NA release from the system via chemical hydrolysis was also evaluated in buffer solutions mimicking the conditions of upper and lower gastrointestinal tract.

2. Experimental

2.1. Materials

Lactose monohydrate and NA were purchased from Fujifilm Wako Pure Chemical Co. (Tokyo, Japan). The powdered lactose was screened through a 200–235 mesh sieve and dried at 60 °C for over 24 h in vacuo. Acryloyl chloride and triethylamine were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). High-pressure liquid chromatography (HPLC) -grade acetonitrile and dichloromethane were purchased from Kishida Chemicals Co. Ltd. (Osaka, Japan). All reagents and solvents used were of analytical grade and used without further purification.

2.2. Synthetic route for preparation of NA-AA

We designed an acrylate derivative of NA (NA-AA) where NA was linked to acrylic acid via acid-anhydride linkage.

In a two-necked flask, nalidixic acid (696.7 mg, 3.0 mmol) and triethylamine (450 µL, 3.3 mmol) were added in 30 mL of dry CH2Cl2 and stirred for 30 min to obtain a clear solution. The solution was cooled until 0 °C and a solution of acryloyl chloride (543.1 mg, 6 mmol) dissolved in 10 mL of dry CH2Cl2 was added dropwise into the solution. The reaction mixture was stirred at 0 °C for 1 hour and was raised to room temperature with stirring overnight and then washed successively with aqueous solution of NaHCO3 (3 × 30 mL). The processes of the above reactions were monitored by TLC. The organic phase was dried over anhydrous MgSO4, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography using acetone:hexane (30:70) as the eluent and recrystallization from ethyl acetate/hexane. The resulting solid NA-AA monomer was dried in vacuum desiccator overnight to constant weight. Yield: 317.8 mg (37%). The structure and physicochemical properties of the newly synthesized compound were confirmed by elemental analysis, FT-IR, 1H NMR, mass spectral data, and chemical methods. 1H-NMR (400 MHz, CDCl3) δ = 0 ppm (TMS), δ: 1.55 (3H, t, J = 7.3 Hz, Ph-CH2-CH3), 2.68 (3H, s, Ph-CH3), 4.54 (2H, q, J = 7.1 Hz, Ph-CH2-CH3), 6.10 (1H, d, J = 8.4 Hz, -C=CH2 trans), 6.37 (1H, q, J = 4.1 Hz, J = 12 Hz, J = 4.1 Hz, -CH(CO)), 6.68 (1H, d, -C=CH2 cis), 7.28 (1H, d, J = 8.4 Hz, Ar H), 8.61 (1H, d, J = 8.4 Hz, Ar H), 8.76 (1H, s, Ar H). FT-IR (KBr) cm⁻¹: 1753 (C=O acid anhydride), 1642 (C=C). ESI-MS m/z: ([M-H]⁺): 287 (Calcd for C15H14N2O4), found ([M-23]⁺): 309. Anal. Calcd for C15H12N2O4; C, 62.93; H, 4.93; N, 9.79. Found: C, 62.82; H, 4.95; N 9.70.

2.3. Analytical techniques

X-ray powder diffraction (XRPD) was carried out on a Rigaku RINT Ultima III (Rigaku, Tokyo, Japan). The XRPD patterns were recorded with Cu-Kα radiation at 40 kV and 40 mA in the range of 2θ range from 3° to 40° with a step size of 0.02°/min and counting time of 1 s per step.

Measurement of melting point and enthalpy of fusion of the crystalline compounds was performed with a Rigaku Thermo Plus DSC 8230 (RIGAKU, Japan) at a heating rate of 5 °C/min on 2–4 mg samples in aluminum pans with pierced lids under a flow of dry nitrogen. An empty pan was used as reference. Heat of fusion measurements were carried out for NA-AA monomer in triplicate. The temperature axis and the cell constant were calibrated with indium (ca. 3 mg, 99.99%, peak maximum at 156.6 °C and heat of fusion 28.4 J/g).

2.4. Plasma irradiation, plasma-induced polymerization and ESR spectral measurement

Powdered lactose samples (50 mg) were placed in a specially designed Pyrex ampule (30-mm i.d., 100-mm long) connected with a thin-walled capillary tube (2-mm i.d.) at the uppermost part of the ampule. The ampule was filled with argon gas for plasmolysis (40.0 Pa) and sealed. Then the plasma state was sustained for the prescribed period of time with agitation of samples at room temperature by a radio frequency discharge of inductive coupling at a...
frequency of 13.56 MHz with the supplied power of 50 W. The ESR measurements were performed while turning the ampule upside down after plasma irradiation at appropriate intervals, which is fundamentally the same procedure as that reported earlier [13-15].

Polymerization of NA-AA was carried out in accordance with the following procedure. After 120-s plasma irradiation of lactose, the powders were applied to mechanical vibration with NA-AA powder (50% by weight) in a Teflon twin-shell blender (7.8 mm φ, 24 mm long) (Vibrating Mill, Shimadzu Co., Ltd.) containing an agate ball (7.0 mm φ, specific gravity 2.6 g/cm³) for the prescribed period of time at room temperature under strictly anaerobic conditions, and submitted to ESR measurement. All sample manipulations were carried out in a vacuum glove box (Sanplatec Corp., Japan). After the reaction, the residual monomer was analyzed using a HPLC system. ESR spectra were recorded with a JES-FA200 spectrometer (JEOL) with X-band and 100 kHz field modulation. Care was taken to ensure that no saturation occurred and that the line shape was not distorted by excessive modulation amplitude. The square root of the microwave power versus the signal peak height was plotted and a microwave power level of 0.01 mW was chosen. The ESR spectral intensity was determined by double integration. The radical concentration (spin numbers / g) was calculated from the spectral intensities with the aid of calibrated lines obtained from the spectral intensities of poly-methyl methacrylate sample impregnated with DPPH. Measurements of g values were made relative to the fourth signal from the lower magnetic field (g = 1.981) of Mn²⁺ in MgO.

2.5. Nalidixic acid analysis

Quantitative analysis of NA concentrations was performed after sample filtration (PVDF Millipore 0.22 μm) in a high-performance liquid chromatograph (HPLC) system from Waters (Model Alliance 2690, Milford, MA, USA)) using a C18 Inertsil ODS-3 column (150 mm × 3.0 mm i.d., 3 μm, GL Sciences, Inc., Japan), and a photodiode array detector Waters 996 using the Empower software (Waters, Milford, MA, USA). The mobile phase was aqueous ammonium acetate (0.01 M, pH 7.0) and acetonitrile (65:35, v/v). The flow rate was 0.8 mL/min. The column temperature was maintained at 30 °C, and the injection volume was 3 μL. Nalidixic acid was detected by UV at a wavelength of 326 nm, and its typical retention time was 4.0 min.

2.6. In vitro release studies

The monomeric unit of the acrylic polymers synthesized presents a NA pendant group linked by hydrolyzable group. The in vitro hydrolysis behavior of polymeric prodrugs was studied in physiological conditions (aqueous phosphate or hydrochloric acid buffers (900 mL), at 37 °C ± 0.5 °C), using a dissolution test apparatus with an auto sampling apparatus (Toyama Sangyo Co., Osaka, Japan) at 100 rpm.

The release mediums were withdrawn at predetermined time intervals and analyzed for the drug concentrations by the HPLC method described earlier. The bulk medium was supple-mented with the same volume of fresh medium each time. The release studies were conducted in triplicate, and the mean values were plotted versus time. The percent of the drug release was evaluated by using the following definitions:

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\text{Drug Release} \% = \frac{\text{Amount of drug release (mg)}}{\text{Total weight of drug sample (mg)}} \times 100
\]

3. Results and discussion

3.1. Synthesis and characterization of NA-AA

In this study, we designed and synthesized an acrylic anhydride monomer of nalidixic acid, NA-AA, by following the protocol illustrated in Scheme 1. The production was solid at room temperature.

![Scheme 1. The synthesis route of acrylate type derivative of nalidixic acid (NA-AA).](image)

Figure 1 shows the infrared spectra of the NA and NA-AA. Characteristic strong absorption bands in NA were observed at 1715 and 1618 cm⁻¹, corresponding to the stretching vibrations of carbonyl group of carboxylic acid and pyridone, respectively. After acrylation by acryloyl chloride, new absorption bands were observed at 1753 and 1642 cm⁻¹, assigned to the C=O stretching vibration of conjugated anhydride groups and C=C stretching vibration, respectively. These new absorption bands suggest that the acid anhydride groups with acrylate were formed during the acylation process.

Solid-state properties of the NA-AA powder was evaluated using polarized-light microscopy (PLM), XRPD and DSC. The image of the sample (Fig. 2) by direct observation using PLM showed birefringence and column-shaped particles with sizes...
ranging from 50 to 300 µm.

Figure 3 shows the XRPD profiles of the powder preparations. The XRPD pattern of the NA-AA is significantly different from that of the NA because their crystal structures are not identical.

The thermal profiles of NA and NA-AA obtained by DSC experiments are exhibited in Fig. 4. The DSC curve of NA shows a sharp endothermic peak at around 228.7 °C, corresponding to fusion, which is similar to the value reported by Bustamante [16]. By contrast, melting point of NA-AA was approximately 129 °C (∆H = 25.4 kJ/mol), or 100 degrees lower when compared to that of the pure NA, suggested that NA-AA had a decreased thermostability relative to the pure NA.

3.2. Preparation of polymeric prodrug (poly NA-AA) by solid-state polymerization

In the pharmaceutical industry, lactose is one of the most commonly used to help form tablets for more than a century because it has excellent compressibility properties.

In our previous reports, we found that plasma-induced lactose radicals were extremely stable for a long period of time at room temperature and could be utilized as a postprocessing-free radical initiator for solid-state polymerization [17-19].

Figure 5 shows the progressive changes in the ESR spectra when the radical-containing lactose plasma-irradiated for 120 s was submitted to a vibratory mixing using a Teflon vessel and agate ball with NA-AA monomer at room temperature for the prescribed period of time at 60 Hz under anaerobic conditions.

It can be seen from Fig. 5 that ESR spectral patterns of lactose radicals have changed to different one with increasing mixing time, which is similar to that observed in mechanochemical polymerization of several acryloyl vinyl monomeric derivatives of drug substances. According to the earlier work [13,20], it can be speculated that the ESR spectra can be unambiguously assigned to a single end-chain radical (equivalent to a propagating radical) of acrylic polymers.
Fig. 5. Progressive changes of ESR spectra of 120-s plasma-irradiated lactose on vibratory mixing with NA-AA using a Teflon vessel and agate ball under anaerobic conditions.

XRPD analysis was also used to compare the crystal phase of before and after vibratory-mixed powder of plasma-irradiated lactose and NA-AA monomer. Figure 6 shows the XRPD patterns of lactose (intact), physical mixture (PM) of lactose and NA-AA in the ratio of 1:1 (w/w), and mixtures of NA-AA and plasma-irradiated lactose subjected to different mixing times.

As shown in Fig. 6 (b), the XRPD pattern of PM exhibits no peak broadening, indicating a phase separated binary mixture. As shown in Fig. 6 (b), the XRPD pattern of PM contains peaks characteristic of both lactose and pristine crystalline NA-AA. After 10 and 20-min of mixing, only the intensity of the NA-AA peak decreased rapidly and the patterns came to be almost halo patterns, indicating to change the crystalline state of NA-AA into an amorphous state. In contrast, lactose peaks remained even after 60 min mixing.

In addition, FT-IR spectrum of poly NA-AA showed that the characteristic peak at 1754 cm$^{-1}$ of NA-AA spectrum, which is attributed to the stretching vibration of C=C group, has vanished in the poly NA-AA (data not shown).

These results suggested that the solid-state polymerization reaction of NA-AA with plasma-irradiated lactose would be proceeded successfully to synthesize the desired polymeric prodrugs by 60 min mixing of the experimental conditions.

Fig. 6. XRPD patterns of NA-AA monomer vibratory mixed with plasma-irradiated lactose. The spectra are offset for clarity. (a) lactose powder, (b) physical mixture of lactose and NA-AA monomer (1:1), (c) NA-AA vibratory-mixed for 10 min with plasma-irradiated lactose, (d) NA-AA for 20 min and (e) NA-AA for 60 min.

3.3. NA release from poly NA-AA powders

The NA release properties from poly NA-AA were studied in vitro in four different conditions, i.e., pH 1.2, 6.8, 8.5 and 13 (0.1 N NaOH) at 37 °C in order to evaluate the possible time of a prolonged action. As the polymers were not soluble in water, they were dispersed in buffer solution and the hydrolysis was performed in a heterogeneous system.

Figure 7 shows the effect of pH on the release performance of NA from the polymeric prodrugs. The poly NA-AA was chemically unstable at pH 13 and had been rapidly and almost completely released NA within 1 h. On the contrary, the polymeric prodrug was extremely stable in acidic medium and hardly hydrolyzed. Actually, it was observed that less than 2% NA was released at pH 1.2 in 2 h. The amount of NA released at pH 6.8 was also only about 35% after 12 h incubation, which was markedly increased to about 80% at pH 8.5. The results showed that poly NA-AA was pH sensitive and could release drug easily in basic environment.

4. Conclusion

In this study, we have synthesized and successfully crystallized a novel acrylic acid-
anhydride conjugates of NA, from the reaction between acryloyl chloride and NA by the direct acrylation method. Then the crystalline monomeric prodrug of NA was polymerized in solid state by vibratory mixing with plasma-irradiated lactose powders as postprocessing-free initiators. The results of in vitro release test showed that the acid-anhydride conjugate of NA-AA was relatively stable to acid-catalyzed hydrolysis. Moreover, the in vitro release profiles of NA from polymeric prodrugs behavior was in a sustained manner with no burst at pH 1.2–8.5 and the release rate was depended on the pH of the medium. As the pH of the medium is changed from 6.8 to 8.5, the releasing rate was enhanced. The obtained results suggested that this poly NA-AA could be potential anti-infective polymeric prodrugs to manage and minimize drug-stomach exposure and to use for colonic-targeting drug delivery system after oral administration.

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