Possibility of Wound Dressing Using Poly(L-leucine)/poly(ethylene glycol)/poly(L-leucine) Triblock Copolymer

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<Abstract>

ABA-type block copolymers composed of poly(L-leucine)(PLL) as the A component and poly(ethylene glycol)(PEG) as the B component were synthesized by ring-opening polymerization of L-leucine N-carboxyanhydride initiated by primary amino group located at both ends of PEG chain. A silver sulfadiazine(AgSD)-impregnated wound dressing of sponge-type was prepared by the lyophilization method. Morphological structure of this wound dressing obtained by scanning electron microscopy(SEM) was composed of a dense skin layer and a macroporous inner sponge layer. Equilibrium water content(EWC) of wound dressing was above 10%. It increased with an increased of PEO content in the block copolymer due to the hydrophilicity of PEO. AgSD release from AgSD-impregnated wound dressing in PBS buffer(pH=7.4) was dependent on PEG composition in the block copolymer. Therefore, EWC and release of AgSD can be control by PEG composition. Antibacterial capacity of AgSD-impregnated wound dressing was examined in agar plate against *Pseudmonas aeruginosa* and *Stapplococus aruous*. Cytotoxicity of the wound dressing was evaluated by studing mouse skin fibroblast(L929). From the behavior of antimicrobial releasing and the investigation of the suppression of bacterial proliferation, it was supposed that the wound dressing containing antibiotics could protect the wound surfaces from bacterial invasion to suppress the bacterial proliferation effectively. In cytotoxicity observation, cellular damage was reduced by the controlled released of AgSD from the LEL sponge matrix of AgSD-medicated wound dressing. In vivo test, granulous tisue formation and wound contraction for the AgSD and DHEA impregnated wound dressing were faster than any other groups.

Key words : Wound dressing, Lyophilization method, Antibacterial capacity

Introduction

Tissue repair and regeneration of full-thickness skin wounds, particularly when infected tissue are present, is achieved by covering a non-medicated or medicated wound dressing[1-2]. A non-medicated wound dressing such as Biobrane[R3] by Woodroof, biosynthetic wound dressing composed of silicon membrane and a collagen and glycosaminoglycon sponge matrix by Yannas[4], living skin by Bell[5], non-woven fabric type of Meipec[R] manufactured by Utsuo[6], and many type polyurethane wound dressing[7] were used as conjunction with antimicrobial cream and ointment. In these cases, it is difficult for wound dressing to adhere to the wound surface due to the presence of cream or ointment beneath the wound dressing and it delayed to promote wound healing, because cream or ointment causes damage to tissue and a patient feels a pain due to the frequent replacement of the wound dressing. Therefore, the concept of drug delivery system(DDS) has been accepted to full-thickness skin wound care, and antimicrobial drug-impregnated wound dressing are proven effectively in controlling bacterial invasion even through a porous matrix[8]. Among the wound dressing, a silver sulfadiazine(AgSD)-impregnated poly(L-leucine)(PLL) sponge(trade name; Epicul)[9-10] developed by Kuroyanagi has several advantage : 1)excellent biocompatibility, 2)decomposition into nontoxic compound, 3)sponge structure by lyophilization, 4)antibacterial drugs that can be incorporated without impairment. But PLL sponge matrix has several disadvantages : 1)low solubility in organic solvent due to distinctly hydrophobic property, 3)low bioadhesion, and 4) slow biodegradation.

In this study, we wish to report possibility of AgSD as antimicrobial agent[11] and dihydroepiandrosterone(DHEA) as immune enhancer[12] impregnated wound dressing using poly(L-leucine)/poly(ethylene glycol)/poly(L-leucine) (abbreviated as LEL) copolymer which may have several advantages than PLL homopolymer wound

dressing, such as 1)release of antibacterial drugs that can be controlled in a sustained fashion, 2)increase of flexibility, 3)control of biodegradation, 4)absorption of body fluid, 5)non-immunogenic effect. The ultimate goal of this study is to evaluate antibacterial capacity and wound healing effect of these wound dressing in vitro and in vivo.

Experimental

Material

Amine-terminated poly(ethylene glycol)PEG (H_2N-PEG-NA_2, M. W. ca. ,2000) was kindly provided by Japan Oil and Fat Co.. Benzene, methylene dichloride, formaldehyde, hematoxylin-eosin ,DHEA and diethyl ether were purchased from Sigma Chemical Co. and used without purification. Silver sulfadiazine(AgSD) was obtained from Dong Wha Pharm. Ind. Co..

Synthesis of LEL triblock copolymers

L-leucine N-carboxy-anhydride(LL-NCA) was prepared by a similar method described in the literature[13]. PLL/PEG/PLL(abbreviated as LEL) triblock copolymers were prepared by ring-opening polymerization of LL-NCA initiated with amine-terminated PEG(NH_2-PEG-NH_2 or ATPEG) in a methylene dichloride, at a total concentration of LL-NCA and Me-PEG-NH_2 of 3%(w/v), at room temperature for 72 hrs as a similar method reported previously[14-15]. The reaction mixture was poured into a large excess of dimethyl ether to precipitate the PLL/PEG/PLL triblock copolymers. The resulting copolymer was washed with diethyl ether and then dried in vaccum oven at room temperature for one day. The unreacted monomer and ATPEG do not precipitate from a mixture of methylene dichloride and diethyl ether.

Measurement of ^1H NMR spectroscopy

^1H NMR spectroscopy of the LEL triblock copolymers was measured to estimate the composition and molecular weight of the block copolymers, using a JEOL FX 90Q NMR spectrometer in deuterated trifluoroacetic acid(CF_3COOD). As the number-average molecular weight(2,000) of PEG is known, the number-average molecular weight of the PLL block of the block copolymer can be estimated from the copolymer composition via the peak intensities assigned to both polymer block.

Measurement of Infrared(IR) spectroscopy

IR spectra of samples prepared by KBr method were measured with Bruker IFS-66 FTIR spectrometer between 4,000 and 400 cm^{-1}.

Preparation of silver sulfadiazine-impregnated wound dressing

The PLL/PEG/PLL block copolymer was dissolved in benzene (1.5 wt/wt%) at 70°C, then became a gel at room temperature. The AgSD impregnated PLL/PEO/PLL wound dressing was prepared from a benzene solution of PLL/PEG/PLL by adding AgSD during this stage. A fine nylon mesh with mechanical supporter is incorporated in the middle of the medicated wound dressing to form two layers, an outer thin membrane and an inner sponge structure. Finally, they were lyophilized for 1 day.

Scanning electron microscope(SEM) observation

The morphological of the wound dressing was observed using a SEM(JEOL, JSM 5400, JAPAN). Prepared wound dressing were fixed on an adhesive tape and coated with gold/palladium. Observation was performed at 25kV.

Measurement of water content

To measure the water content, preweighed dry samples($2cm^2$) were immersed in distilled water at 37℃. After the excess surface water was removed and tapped with filter paper, the weight of swollen samples was measured at specific time intervals. Water content was determined according to the following equation :

$$\text{Water content}(\%) = [(W_s - W_d)/W_s] \times 100$$

where W_s and W_d are the weight of swollen and dry samples, respectively.

In Vitro Release Test

The release test of AgSD from LEL wound dressing was carried out in 1 ml phosphate buffered saline(PBS, pH=7.4) in a shaking incubator at 37℃. 1 ml aliquot was taken and replaced with fresh PBS at specific time points. The concentration of the samples were determined by UV spectrophotometer(Shimadzu, the model of UV-1,201) at 256 nm and expressed by the total release amount of AgSD(wt.-%).

Bactericidal test of AgSD-impregnated wound dressing

In this study, *Pseudomonas aeruginosa* with gram-negative bacteria and *Staphylococcus aruous* with gram-positive bacteria were used. These are important bacteria in burn disease. Bactericidal capability of AgSD-impregnated wound dressing was evaluated on agar plate seeded with bacteria. A AgSD-impregnated wound dressing(2× 2cm) was then placed on a bacteria-seeded agar plate and cultured in an incubator at 37℃ for 2 days. After a period of 2 days, the AgSD-impregnated wound dressing was excluded and $2cm^2$ of the agar immediately beneath the wound dressing was cut out by pora and homogenized in 10㎖ of a sterile phosphate buffer solution(PBS, pH=7.4) after diluted repeatedly to a 1/10 concentration. The diluted solution was inoculated on a nutrient agar plate. After 2~3 days of culture at 37℃, the bacterial colony was counted, and the number of bacteria beneath the wound dressing was calculated and expressed in the number of bacteria per cm^2.

Cytotoxicity test of wound dressing against cultured L929 cells

The cellular response of the cultured L929(mouse fibroblasts) cells were evaluated with MTT(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide; thizoyl blue) assay. The RPMI 1640 500㎕ was added to wound dressing sterilized by ethylene oxide gas, then the cell was incubated 37℃ at for 2 days. The cell density in the wound dressing was $5\times10^3/100\mu\ell$. After incubation for 2 days, the well was treated with 25㎕ of MTT at 37℃ for 4 hrs. And then OD(optical density) at 570~630nm was measured by MTT method.

Preparation of burn wound

The full-thicness burn wound of the dorsum of BALB/C mouse(6~8 weeks) was prepared on by 20 seconds exposure to the heat block at 90℃, after then burn wound was covered with sterile wound dressing sterilized by ethylene oxide gas and fixed with paper tepe. 3 days after coverage, the burn wound was surgically incised, and then excised wound was covered with new wound dressing. This wound dressing was changed at every week with new wound dressing.

Histological observation

The mouse were killed at 4 weeks after drug-impregnated wound dressing application, and biopsy specimens of the underlying wound dressing were taken and fixed in 10% formaldehyde. Biopsy specimens of these wound tissue were then stained with hematoxylin-erosin and evaluated histologically.

Results and Discussion

LEL triblock copolymers were prepared by ring-opening polymerization of LL-NCA initiated with Me-PEG-NH_2 in a methylene dichloride solution as scheme shown in Fig 1. It is assumed that the polymerization mechanism is the primary-amine mechanism in which the initiator amine undergoes a nucleophilic addition to the C-5 carboxy group of the NCA, as suggested by Goodman et al.[16].

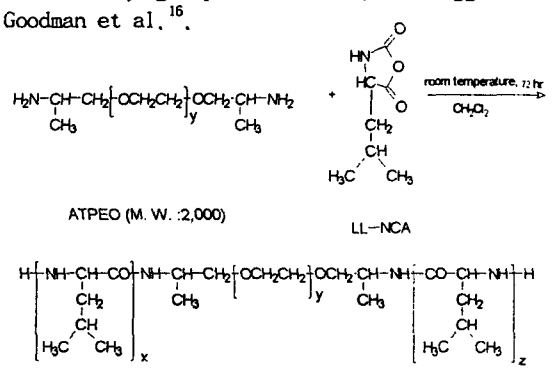


Fig 1. Synthetic scheme of PLL/PEG/PLL triblock copolymer

Fig. 2 shows representative ^1H NMR spectrum of the LEL-1 block copolymer. In Tab. 1 are listed the amount of PEG and the molecular weight of copolymers obtained from ^1H NMR spectra. The block copolymer composition and number-average molecular weight were estimated from peak intensities of methylene proton signal(3.9 ppm) of the PEG block and methyl proton signal(0.9 ppm) of PLL block in the spectrum.

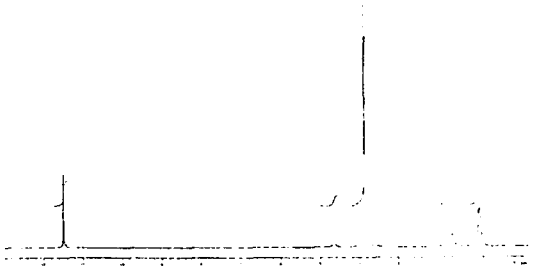


Fig 2. ^1H NMR spectrum of LEL-1 triblock copolymer in trifluoroacetic acid-d_1(CF_3COOD)

Fig. 3 shows FT-IR spectra of LEL triblock copolymers and PLL homopolymer in the region of 1,800-500cm^{-1}. The amide I, II and V bands of these LEL block copolymers appear at 1,650 cm^{-1}, 1,550 cm^{-1}, 615 cm^{-1}, respectively, at the same wavenumbers as for the PLL homopolymer. It was found that the PLL block exists in the α-helical conformation, as in PLL homopolymer.

Table 1. Characterization of PLL homopolymer and LEL triblock copolymers prepared.

Polymer	Content of monomeric units in mol-%[a)]		$\overline{M_n}$
	PEO	PLL	
PLL	0	100	300,000
LEL-1	17.4	82.6	25,900
LEL-2	28.6	71.4	14,500

a) content of monomeric units in the block copolymers estimated by ^{1}H NMR measurement.

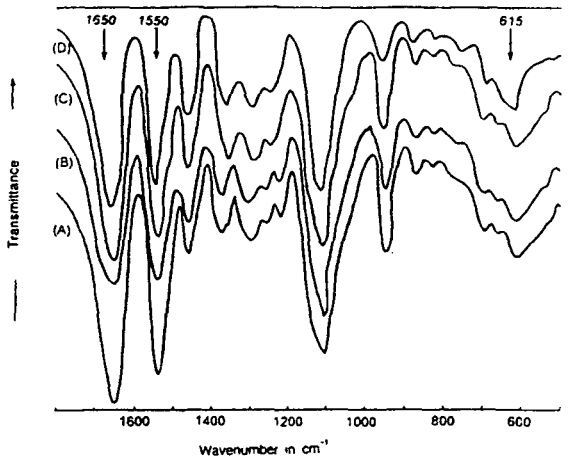
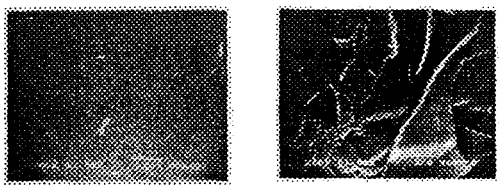


Fig 3. FT-IR spectra of LEL triblock copolymers and PLL homopolymer .

Scanning electron micrographs of outer surface and inner surface of the wound dressing are shown in Fig 4. The morphological structure of this wound dressing was composed of a dense skin layer and macroporous inner sponge layer. The dense skin layer provide several function such as control of water loss through evaporation, inhibition of body fluid loss and protection from external contamination. Also, macroporouse inner sponge layer have a function such as promotion of drainage, prevention of exudate buildup and preparation of an optimum wound bed for autografting. Therefore, this wound dressing have a optium structure to promote wound healing.



(a) Outer surface (b) Inner surface

Fig 4. SEM of LEL-1 wound dressing

Water content of wound dressing against an incubation time in distilled water at 37℃ is shown in Fig 5. These results showed that water contents were dependent on the mole fraction of PEG and the water content increased with increasing PEG mole fraction due to the hydrophilicity of PEG. Wound dressing composed of PLL and PEG are expected to produce different degree of matrix hydration depending on the amount of PEG. Water content composed of only PLL homopolymer was very low as below 5%, therefore this wound dressing is not sufficient to absorb exudate.

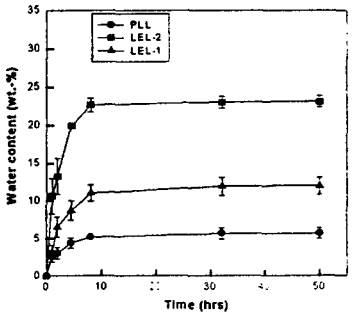


Fig 5. Water content of LEL wound dressing against PEG content.

Fig 6. shows total amount of AgSD released from AgSD-impregnated wound dressing against fraction of PEG. These results indicated that the release of AgSD from wound dressing rapidly increased with increasing mole fraction of PEG in the wound dressing. These observed phenomena could be explained in relation to the water content of the wound dressing as shown in Fig 5. The penetration of water molecules within the sponge-type wound dressing become easier with the increase of hydrophilicity of PEG in the wound dressing. From these results, release of AgSD from LEL block copolymer wound dressing can Bacteria-seeded agar plate were used to determine the bactericidal efficacy of the AgSD-impregnated(51μg/cm^2) wound dressing.

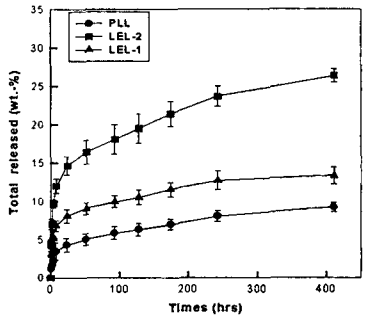


Fig 6. Release of AgSD from AgSD-loaded synthetic wound dressing against mole fraction of PEG(Drug loading : 20wt%)

The results of bactericidal test against *Pseudomonas aeruginosa* are shown in Fig 7. A strain of *Pseudomonas aeruginosa* from a patient with septicemia was used for these studies. Initial seeding density of *Pseudomonas aeruginosa* were 1×10^3 organisms/cm^2 . As a control, vaseline gauze utilized commonly in burn therapy was used. Anti-bactericidal capacity of vaseline gauze is very low. In the case of AgSD-impregnated wound dressing, number of residual bacteria decreased with an increased of PEG content due to the fast release of AgSD in the AgSD-impregnated wound dressing as shown in Fig 6. Bacteria were not detected in LEL-2(PEG : 29 mol%) wound dressing. Therefore, bactericidal capacity can be control by PEG content and drug loading content.

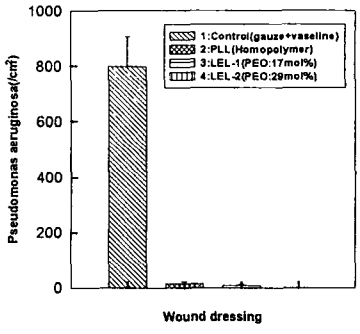


Fig 7. Number of residual *pseudomonas aeruginosa* in the agar plate covered with AgSD-impregnated wound dressing.

Fig 8. shows results of bactericidal test against *Staphylococcus aureous*. Initial seeding density of *Staphylococcus aureous* were 1×10^5 organisms/cm^2. It was found that the anti-bactericidal capacity for *Staphylococcus aureous* was almost similar to the *Pseudomonas aeruginosa* one. These results could be explained in relation to the drug release test and bacterial test against *Pseudomonas aeruginosa*. From the behavior of antimicrobial agent releasing and the antibacterial test of antimicrobial agent-impregnated wound dressing, it can be supposed that the wound dressing containing antibiotics could protect the wound surface from bacterial invasion to suppress the bacterial proliferation effectively.

Fig 9. shows cytotoxicity of drug loading(50 μ g/cm^2) wound dressing against culture L929 cells(mouse fibroblast). In Vitro cellular response of mouse fibroblast(L929 cells) was examined by adding some pieces of AgSD non-impregnated or AgSD-impregnated wound

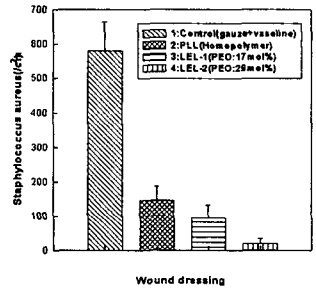


Fig 8. Number of residual Staphylococcus aureus in the agar plate covered with AgSD-impregnated wound dressing.

dressing, it was found that all wound dressings were not much different in cytotoxicity.

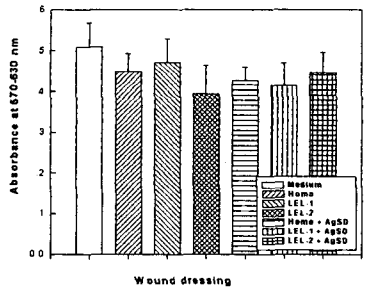


Fig 9. Cytotoxicity of LEL wound dressing against cultured L929 cells.

In vivo test, the derided wound surface was covered with each wound dressing for 4 weeks. Four weeks after application, the progress of

granulous tissue formation and wound contraction of AgSD and DHEA impregnated LEL wound dressing been faster than other four groups(vaseline gauze, HA, LA and HAD)

Conclusion

ABA-type block copolymers composed of poly(L-leucine)(PLL) as the A component and poly(ethylene oxide)(PEG) as the A component were synthesized by ring-opening polymerization of L-leucine N-carboxtanhydride initiated by primary amino group located at both ends of PEG chain and characterized. A silver sulfadiazine(AgSD)-impregnated wound dressing of sponge-type was prepared by the lyophilization method. Morphological structure of this wound dressing obtained by scanning electron microscopy(SEM) was composed of a dense skin layer and a macroporous inner sponge layer. Equilibrium water content(EWC) of wound dressing was above 10%. AgSD release from AgSD release from impregnating wound dressing in vitro was dependent on PEG composition. Therefore, EWC and release of AgSD can be control by PEG composition. From the behavior of antimicrobial releasing and the investigation of the suppression of bacterial proliferation, it was supposed that the wound dressing containing antibiotics could protect the wound surfaces from bacterial invasion to suppress the bacterial proliferation effectively. In cytotoxicity observation, cellular damage was reduced by controlled released of AgSD from the LEL sponge matrix of AgSD-medicated wound dressing . All the properties of wound dressing was found to be enough for the desired properties of an ideal artificial skin. The result of animal test showed that the formation of scar on wound surface was markedly minimized.

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