No	ARTIKEL	KOMENTAR PENILAI	TANGGAPAN PENGUSUL	LAMPIRAN
C-01	**KARIL 1** **Judul artikel**: The effect of two different crosslinkers on *in vitro* characteristics of ciprofloxacin-loaded chitosan implants **Penulis**: (1) **Esti Hendradi***, (2) Dewi Melani Hariyadi, (3) Muhammad Faris Adrianto. **Nama Jurnal**: **Research in Pharmaceutical Sciences**, Volume Jurnal: 13, Nomor Jurnal: 1, Tahun Terbit Jurnal: 2018, Halaman: 38-46, ISSN: 1735-5362, Penerbit: Medknow Publications' **Terideks Scopus,Q1** **SJR 2018= 0.57** **Turnitin 19%** **URL:** http://rps.mui.ac.ir/index.php/jrps/article/view/1805/1825 **atau** **URL:** http://rps.mui.ac.ir/index.php/jrps/index	Sebagai pemenuhan syarat utama, beberapa hal perlu diklarifikasi: a. Ket Fig 1 kurang baik karena tidak mendeskripsikan dengan tepat sesuai komposisi gambar tersebut yang terdiri dari gambar 1 A,B,C, D dan E. b. Beberapa gambar yang digunakan di jurnal ini serupa dengan yang **di jurnal IJPPS 2014 (karil no. 17)**. **Mohon klarifikasi dari ybs dengan mengetahui pimpinan PT**	**a.** Pada awal submit gambar dibuat satu-satu. Tetapi karena ada batasan jumlah gambar, tabel dan kata yang ada di naskah maka untuk hasil karakteristik sediaan terdiri dari **porosity (%), density (g/cm^3), hardness (MPa), water uptake (%) dan swelling ratio (%)** dijadikan satu. Permintaan **revisi gambar** ada pada L**ampiran Karil 1.1.** Pada revisi data betul tetapi waktu digabung dalam artikel ada kesalahan satuan yang tertukar yaitu **Density (g/cm^3)** dan **Porosity** (%). Artikel ini sudah dipublikasi tahun 2018, sehingga tidak bisa direvisi. b. **Karil 1** dan **Karil no 17** yang di **publikasi di IJPPS 2016 bukan IJPPS 2014** merupakan penelitian satu payung. Formula pada penelitian Karil 1 berbeda dengan Karil 17. Pada **Karil 1 menggunakan 2 macam crosslinrke**r yaitu **glutaraldehid dan genipin sedangkan p**ada **Karil 17 menggunakan 1 crosslinker** yaitu **glutaraldehid**	**Lampiran Karil 1.1** Permintaan dan revisi gambar **Lampiran Karil 1.2.** Formula **Karil 1** dan **Karil 17**

			dengan konsentrasi yang beda. Formula **Karil** 1(Jurnal **RPS Vol 13(1)**, **2018**) dan **Karil 17 (Jurnal IJPPS Vol 8(1), 2016)** ada pada **Lampiran Karil 1.2.**	
		c. Perlu disertai komunikasi proses review jurnal	c. Korespondensi proses review jurnal RPS ada pada **Lampiran Karil 1.3.**	**Lampiran Karil 1.3** Korespondensi proses review jurnal RPS
		d. Daftar pustaka jumlahnya hanya 20. Sebaiknya lebih banyak lagi untuk level jurnal ilmiah internasional bereputasi	d. Pada artikel yang dipublikasikan di jurnal RPS ada pembatasan kata yang meliputi **kata pada text artikel**, **References, Tabel dan Gambar bukan pembatasan jumlah references (2750 kata)**. Ketentuan pembatasan penulisan author ada pada **Lampiran Karil 1.4.**	**Lampiran Karil 1.4** Ketentuan pembatasan penulisan author
		e. Ada **2 jurnal 2012 dan 2014 yang mirip namun tidak disitasi.**	e. Ada **pembatasan** jumlah kata pada full text artikel termasuk kata pada **References, Tabel dan Gambar yaitu 2750 kata**. Sehingga pemilihan Jurnal yang disitasi menjadi sangat penting. **Jurnal 2012 dan 2014 tidak disitasi karena sudah disitasi pada Karil 17 (Reference no 19 dan 20)**. Sedangkan **Karil 17 disitasi Karil 1** (**Reference no. 10**). **Daftar Pustaka Karil 1 dan Karil 17** terlampir pada **Lampiran Karil 1.5**.	**Lampiran Karil 1.5** Daftar Pustaka Karil 1 dan Karil 17.
		f. Acknowledgment selazimnya lengkap disebutkan nomor kontrak dan tahun hibah.	f. Tidak ada permintaan no kontrak dan tahun hibah pada jurnal.	

		g. Alamat web jurnal seharusnya: http://rps.mui.ac.ir/index.php/jrps/index	g. URL: http://rps.mui.ac.ir/index.php/jrps/article/view/1805/1825 Langsung terbuka artikelnya **Apabila dengan URL:** http://rps.mui.ac.ir/index.php/jrps/index Yang terbuka adalah daftar isi artikel di jurnal. **Jadi keduanya bisa digunakan**	

Karil 1

SURAT PERNYATAAN

Yang bertanda tangan dibawah ini saya,

Nama : Dra. Esti Hendradi,MSi.,Ph.D., Apt.

NIP : 195711141987032001

Departemen : Ilmu Kefarmasian

Menyatakan bahwa mempunyai penelitian dalam bidang penghantaran obat inhalasi yang terdiri dari beberapa proyek penelitian. Untuk itu jika ada kemiripan atau persamaan dalam pendekatan adalah suatu kewajaran. Sementara pada 2 publikasi Karil 1 dan Karil 17 adalah berbeda formula seperti yang ada pada lampiran naskah klarifikasi borang penolakan (lampiran 2 Karil 1).

Demikian saya buat pernyataan ini sebagai klarifikasi Karil 1 untuk digunakan sebagai pengajuan kenaikan jabatan guru besar.

Surabaya, 25 Januari 2021

Mengetahui,

Dekan Fakultas Farmasi Universitas Airlangga

Prof. Junaidi Khotib, SSi., M.Kes., Ph.D., Apt

NIP. 197010221995121001

Yang membuat pernyataan,

Dra. Esti Hendradi,MSi.,Ph.D., Apt

NIP. 195711141987032001

LAMPIRAN I

Gmail **Adrianto Faris <farisadrianto@gmail.com>**

Trs: RPS 16168 editing phase

1 message

Esti Hendradi <estihendradi@yahoo.com> Tue, Sep 5, 2017 at 9:45 AM
Reply-To: Esti Hendradi <estihendradi@yahoo.com>
To: Adrianto Faris <farisadrianto@gmail.com>, MUHAMMAD FARIS <farisadrianto@ff.unair.ac.id>, Dewi Melani <dewiffua96@yahoo.com>

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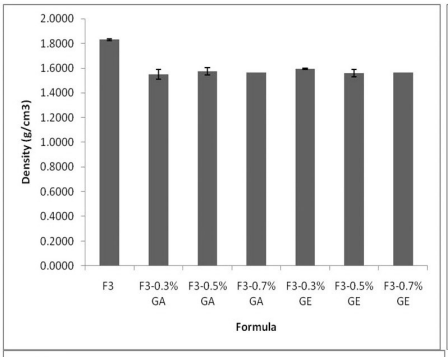

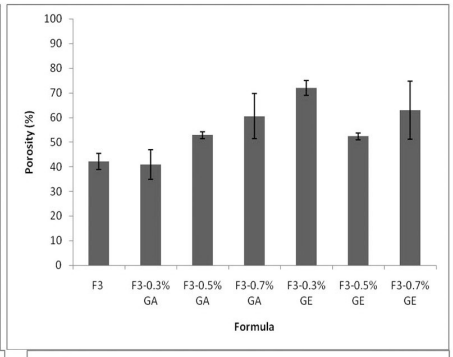

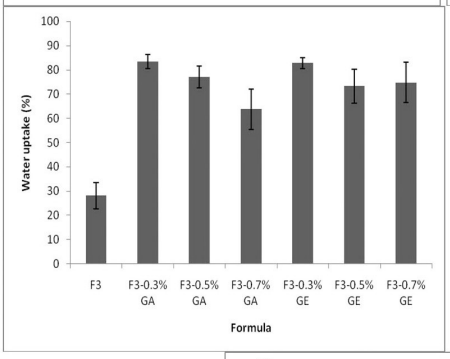

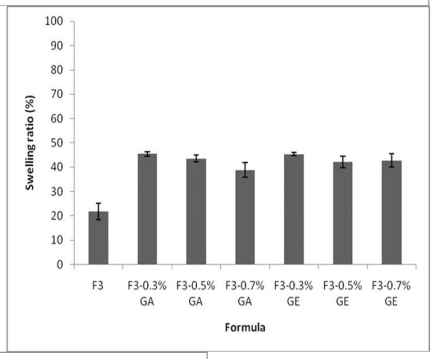

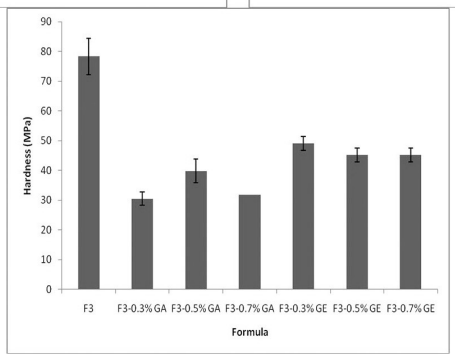

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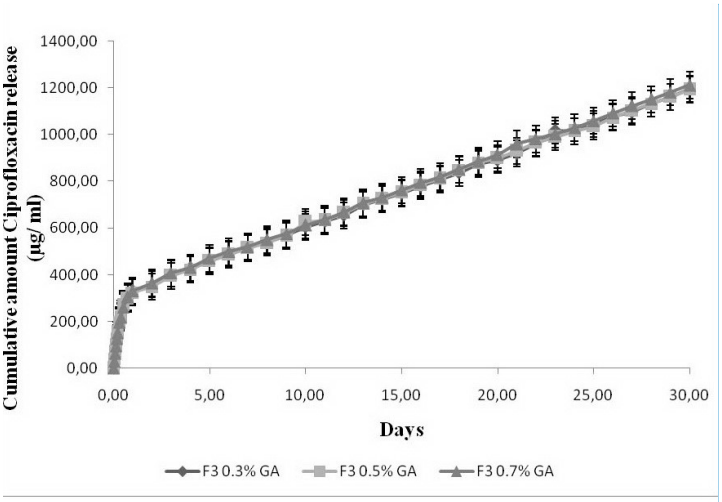
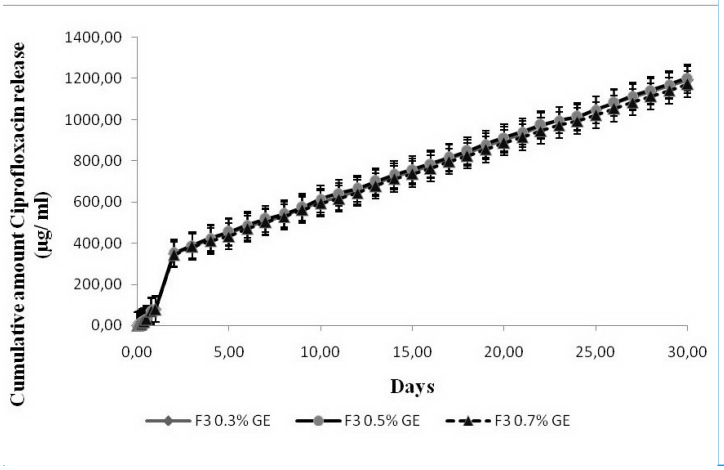
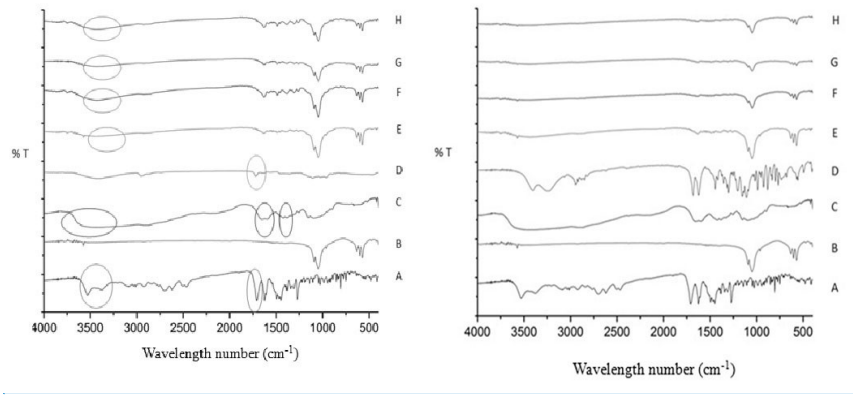




Commented [p2]:
-The cumulative amount of ciprofloxacin released (Y-axis) must be presented as percent cumulative ciprofloxacin released. To do so, the percent released must be calculated with dividing amount released by initial amount of drug in the formulation and then multiplied by hundred.
-Please modify the font of all figures to Times New Roman, un-bolded and legible size (10-12).
-The color of x- and y- axis lines as well as the line for all plots in figures should be black.
-The size of symbols must be 3 and the color of them must be black.
-The thickness of all lines in all figures must be 1 pt and the lines should not be dashed line.
-The symbols description must be replaced in the figures.
Finally please provide the modified excel format.



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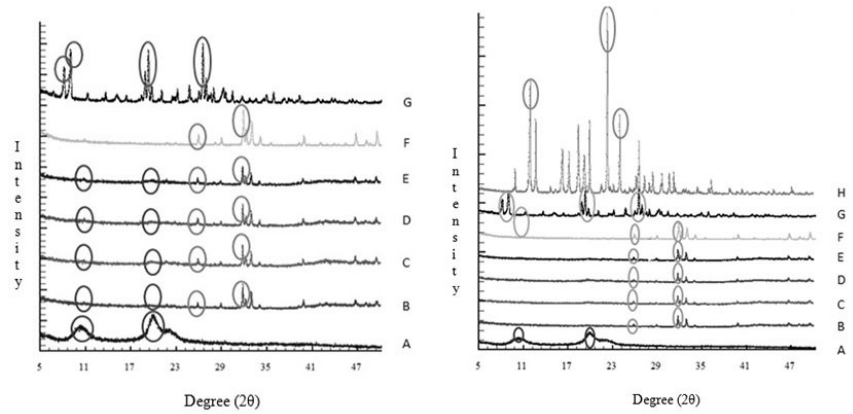

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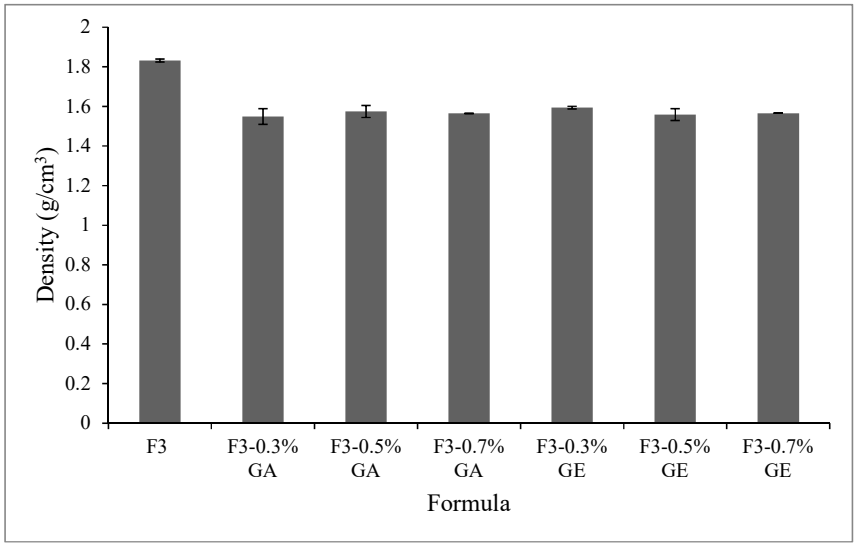
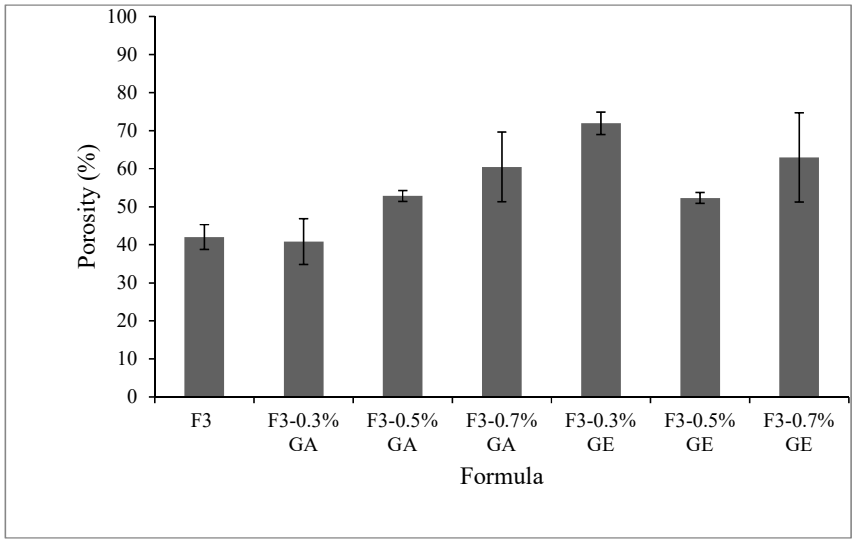
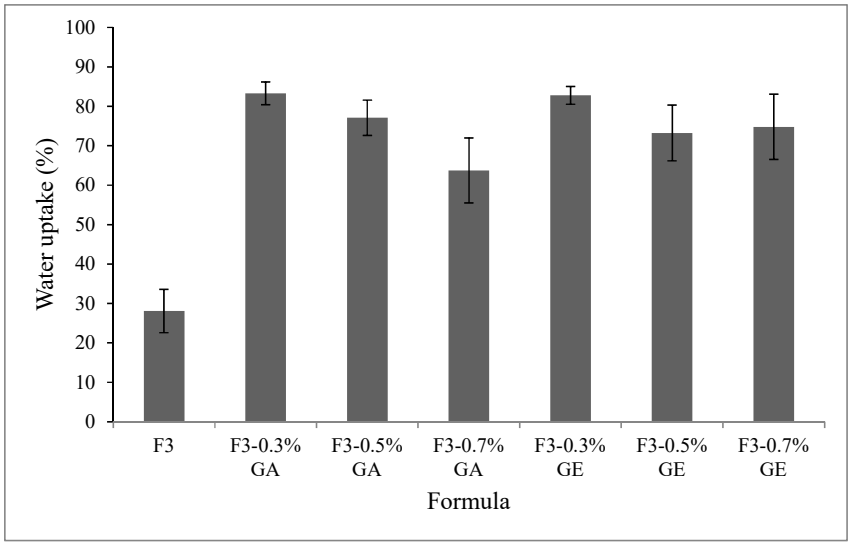
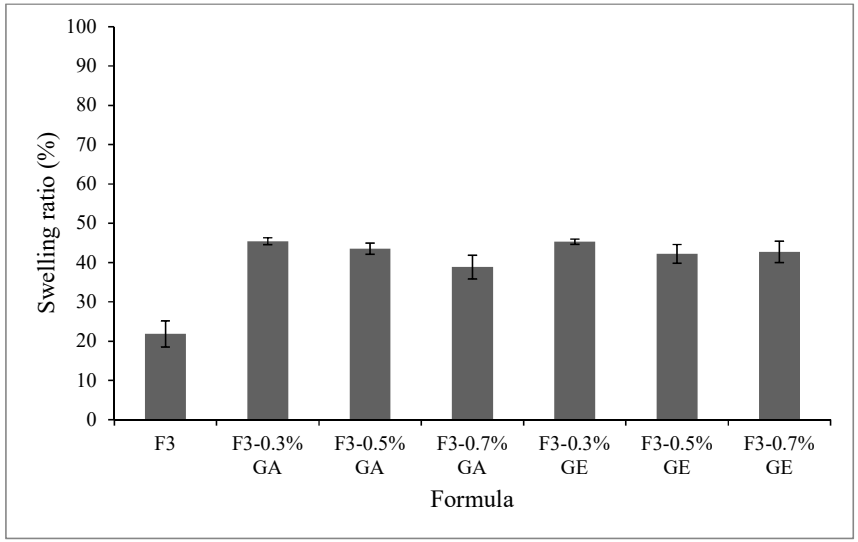
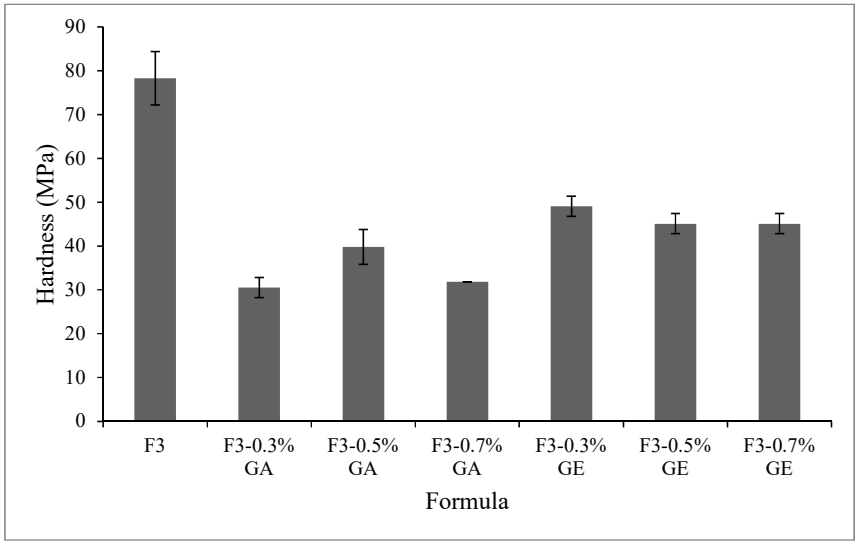

Density (g/cm³)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
F3
F3-0.3% GA
F3-0.5% GA
F3-0.7% GA
F3-0.3% GE
F3-0.5% GE
F3-0.7% GE
Formula


Porosity (%)
100
90
80
70
60
50
40
30
20
10
0
F3
F3-0.3% GA
F3-0.5% GA
F3-0.7% GA
F3-0.3% GE
F3-0.5% GE
F3-0.7% GE
Formula


Water uptake (%)
100
90
80
70
60
50
40
30
20
10
0
F3
F3-0.3% GA
F3-0.5% GA
F3-0.7% GA
F3-0.3% GE
F3-0.5% GE
F3-0.7% GE
Formula


Swelling ratio (%)
100
90
80
70
60
50
40
30
20
10
0
F3
F3-0.3% GA
F3-0.5% GA
F3-0.7% GA
F3-0.3% GE
F3-0.5% GE
F3-0.7% GE
Formula


90
80
70
60
50
40
30
20
10
0
Hardness (MPa)
F3
F3-0.3% GA
F3-0.5% GA
F3-0.7% GA
F3-0.3% GE
F3-0.5% GE
F3-0.7% GE
Formula

LAMPIRAN II

Formula KARIL 1

Hendradi et al. / RPS 2018; 13(1): 38-46

Table 1. The composition of implant formulations.

Formulation	Ciprofloxacin (%)	Composite (%)		Crosslinkers (%)	
		BHA	Chitosan	Glutaraldehyde	Genipin
F3	10	30	60	-	-
F3-0.3% GA	10	30	60	0.3	-
F3-0.5% GA	10	30	60	0.5	-
F3-0.7% GA	10	30	60	0.7	-
F3-0.3% GE	10	30	60	-	0.3
F3-0.5% GE	10	30	60	-	0.5
F3-0.7% GE	10	30	60	-	0.7

(GE), genipine; (GA), glutaraldehyde; (BHA), bovine hydroxyapatite.

Formula KARIL 17

MATERIALS AND METHODS

Materials

Ciprofloxacin was a gift sample from Shangyu Jingxin Pharmaceutical, Shangyu, China CO., LTD. Bovine Hydroxyapatite was obtained from Tissue Bank of Dr. Soetomo Teaching Hospital, Surabaya, Indonesia. Chitosan was obtained from PT. Biotech Indonesia, Cirebon, Indonesia. Glutaraldehyde 25%, glacial acetic acid, Na_2HPO4, KH_2PO4, and NaCl were products of Merck Millipore, Germany. Aquabidestilata was a gift sample from PT. Widatra Bhakti, Pasuruan, Indonesia. All other ingredients used were of analytical grade.

Methods

Preparation of homogeneous chitosan powder

Chitosan flakes were dissolved in acetic acid solution (1%) v/v. The solution was stirred at 400 rpm with a mechanical stirrer for 24 h to produce chitosan solution with 2% w/v concentration. 1 M NaOH solution was added to chitosan solution (2% w/v) until the pH reached neutral (pH =7) to produce chitosan gels. Chitosan gels were dried at 40 °C for 24 h. Dried chitosan gels were sieved using 1 mm sieve to obtain homogeneous chitosan powder.

Formulation of bovine hydroxyapatite-chitosan-ciprofloxacin implants using glutaraldehyde as cross-linking agent

Ciprofloxacin were dissolved in aqua bidest lata, Bovine Hydroxyapatite added gradually and mixed until homogen. Chitosan powder was added to ciprofloxacin-Bovine Hydroxyapatite blend. Aquabidestilata were added gradually with continuous stirring until form wet granules mass. Wet granules mass were sieved using 1 mm sieve and dried overnight (24 h) at 40 °C to obtain dried granules. Dried granules were immersed in glutaraldehyde solution (0.5%, 0.75%, and 1.0% concentration) for 24 h until the colour was changed [14]. The composition of various formulations was made in table 1. Granules were washed three times with aqua bidestilata to remove the residual glutaraldehyde. At the final stage, granules were washed with phosphate buffer saline (PBS) pH 7.40. Granules were dried in an oven 40 °C for 24 h. Dried granules were weighed 100 mg, pressed using tablet press machine with 4.0 mm diameters and the compression pressure was 2 tons.

Table 1: Formulation of implants using glutaraldehyde as cross-link agent

Formulation code	Composite composition (Bovine Hydroxyapatite: chitosan)	Glutaraldehyde concentration (%v/v)
F1	70:30	0.5
F2	70:30	0.75
F3	70:30	1.0

LAMPIRAN III

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A manuscript has been submitted to our journal Research in Pharmaceutical Sciences by Esti Hendradi titled 'The effect of different cross link agent glutaraldehyde and genipin in composites to the physicochemical characteristics and the release of ciprofloxacin implant'. A copy of the acknowledgment mail is attached here with for your reference.

Thanking you
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Research in Pharmaceutical Sciences has received your manuscript entitled "The effect of different cross link agent glutaraldehyde and genipin in composites to the physicochemical characteristics and the release of ciprofloxacin implant" for consideration for publication. The reference number for this manuscript is "RPS_168_16". Kindly quote this in correspondence related to this manuscript.

The manuscript is being reviewed for possible publication with the understanding that it is being submitted to one journal at a time and have not been published, simultaneously submitted, or already accepted for publication elsewhere either as a whole or in part. Online submission of this article implies that the corresponding author has the written consent from all the contributors to act as corresponding author.

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Status of the manuscript titled 'The effect of different cross link agent glutaraldehyde and genipin in composites to the physicochemical characteristics and the release of ciprofloxacin implant' submitted by Dr. Esti Hendradi has been changed and a copy of the mail is as;

Dear Dr. Hendradi

With reference to your manuscript entitled 'The effect of different cross link agent glutaraldehyde and genipin in composites to the physicochemical characteristics and the release of ciprofloxacin implant', please review the comments of the referees from our site http://www.journalonweb.com/jrps. The manuscript would be reconsidered after requisite modifications as per the comments and instructions provided by the journal.

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Manuscript no.: RPS_168_16

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The effect of two different cross-linkers ~~agent glutaraldehyde and genipin in~~on ~~composites to the physicochemical~~in vitro characteristics ~~and the release of~~ of ciprofloxacin-loaded- chitosan implants

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Abstract

The objective of this study was to determine and evaluate a controlled release implant of ciprofloxacin using b~~B~~ovine h~~H~~ydroxyapatite (BHA)-chitosan composite and glutaraldehyde or genipin as cross linking agent. Ciprofloxacin implants were prepared using BHA~~Bovine Hydroxyapatite~~-chitosan-ciprofloxacin composition 30:60:10. This composite was further developed using three different concentrations of glutaraldehyde or genipin (0.3%, 0.5%, and 0.7%). Implants were formed into pellets with 4.0 mm diameter and weighed 100.0 mg using compression method. Further, the prepared ciprofloxacin implant was characterized for porosity, density, water absorption capacity, swelling ratio, disintegration test, compressive strength, compatibility studies (fourier transforms-infrared spectroscopy (FT-IR)), morphology (scanning electron microscope (SEM)), X-ray diffraction (X-RD) study, assay, and *in vitro* drug release. The addition of glutaraldehyde or genipin- as cross-link agent in ciprofloxacin implant showed controlled release profile of ciprofloxacin over a time period of 30 days. This is due to glutaraldehyde or genipin formed compact structure so the porosity, water absorption capacity, and swelling ratio of the implant decreased. SEM photomicrograph revealed low porosity of the implant after cross-link with glutaraldehyde or genipin. The FTIR study confirmed the formation of covalent imine bonds between chitosan and glutaraldehyde. Moreover, the addition of glutaraldehyde or genipin as cross-link agent caused a decrease in the mechanical strength of the implant. Increased concentration of glutaraldehyde or genipin reduced the crystallinity of BHA and chitosan, which were confirmed by X-RD studies. The results obtained from this study indicated that glutaraldehyde or genipin 0.7% had the potential effect to retard ciprofloxacin release from ~~Bovine Hydroxyapatite~~BHA-chitosan-ciprofloxacin implant with diffusion and erotion mechanisms for 30 days in the treatment of osteomyelitis.

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***Keywords**:* Ciprofloxacin i~~I~~mplant ~~ciprofloxacin~~; Cross-linker; Glutaraldehyde; Genipin; Released-

INTRODUCTION

Bones are ~~an~~ essential parts of human body which have an important role in supporting the physiological functions of the body (1). Complications of bone disease and bone disorder caused by traumatic accidents may result in the presence of bone defect. The healing process of bone damage or bone fracture is determined by the level of trauma and soft tissue damage (2). Some cases of bone damage or bone injury cannot be ~~experienced~~ naturally repaired and healed (1). Therefore, clinical rehabilitation to overcome bone defect is expected to rise along

with population growth (3).

Rehabilitation of bone defect ~~cannot be separated from~~are associated with the risk of infection. The number of complications varied from 1% in the case of total joint replacement ~~until~~ to 23% in the case of bone fracture (4). The cause of infection complication is the entry of the bacteria into the bone tissue through the defects. The infection ~~occurred~~ occurs because of a less sterile surgical process, bacteria that adhere to the implant, bacteria in human skin, open wounds, and circulatory patients suffering from infection elsewhere (3,5).

The occurrence of bacterial infections can be ~~corrected~~ treated by administering antibiotics. However, tissue devascularization in the case of bone defect limits the delivery of the~~caused the~~ antibiotic ~~transfer~~ to the target site ~~become stunted~~. This condition ~~caused~~ leads to the lower antibiotic~~the~~ concentration ~~of antibiotic~~ in the target tissues ~~target become low~~ so that and consequently the antibiotic is not able to penetrate into the bacteria biofilm layer ~~of bacteria~~. ~~In consequence,~~This may lead to the bacterial resistance ~~occurs~~ in the target tissue (6). One ~~way~~ approach to ~~overcome~~ limit the risk of infection is to administer ~~giving~~ the antibiotic ~~in~~ through the oral or intravenous route for a long period of time.

To overcome these problems, ~~administering~~ antibiotics can be ~~done~~ delivered locally to the by using specific drug delivery systems. The purpose of ~~this~~ such drug delivery systems is to ~~providing~~ provide adequate drug concentration at a specific location ~~and~~ ensuring drug release profile for a longer period (7). Local drug delivery systems ~~has~~ have several advantages such as ~~: (a) reduce~~minimizing systemic adverse effects, ~~(b)~~ using smaller quantity ~~the number~~ of drugs with greater efficiency ~~are used less and secure~~, avoiding multiple drug therapy, reducing risk of toxicity and ~~(c)~~ ease of the ~~efficacy and efficient to~~ delivery of the drug to target site (8). ~~Administering antibiotic locally can also minimize the side effects and the risk of toxicity than administering antibiotic systemically.~~ In addition, administering antibiotics locally also cause high antibiotic concentration in target tissue (3). The release of antibiotic on the target tissue is expected to take place continuously for a specific period and the concentration is higher than minimum inhibitory concentration (MIC). Controlled release system also can enhance the bioavailability of antibiotic in the target tissue. This system is designed to release the drug in the target tissue with appropriate rate during specific period ~~(~~[3]~~)~~. The release of the drug from implants is affected by the composite. ~~-~~A ~~composite~~ component that regulates the release rate of ~~of t~~the drug from composites is cross-linkers type.

Chitosan as organic material and b~~B~~ovine h~~H~~ydroxyapatite (BHA) as inorganic material were used in this research to increase mechanical strength and bone bioactivity ~~in~~ of the implant~~s~~ (3) and also to control the release rate of ciprofloxacin as the antibiotic. Glutaraldehyde (8) and genipin (9) were used as cross-linkers.

~~The objective of this study was to determine and evaluate a controlled release implant of ciprofloxacin using Bovine Hydroxyapatite~~BHA ~~chitosan composite and glutaraldehyde or genipin as cross-link agent according to drugs characteristic.~~

MATERIALS AND METHODS

Materials

Ciprofloxacin (Shangyu Jingxin Pharmaceutical Co. Ltd.) ~~Bovine Hydroxyapatite (~~BHA~~) from (~~Tissue Bank RSUD DR Soetomo Surabaya, Indonesia); ~~c~~Chitosan with MW 30 -1000 kD (Biotech, Indonesia) ~~mMolecular weight 30-1000 kDalton~~; glutaraldehyde 25% ~~p.a~~ (Merck Millipore, Germany); Genipin ~~was obtained from (~~Challenge Bioproduct Co., Ltd., Taiwan). Glacial a~~A~~cetic acid, ~~glacial p.a (Merck),~~ KBr IR (for s~~S~~pectroscopy), ~~Na_2HPO_4~~ Na_2HPO_4 ~~p.a, (Merck),~~ K_2HPO_4 ~~p.a~~, KH_2PO_4 ~~p.a~~, and NaCl ~~p.a~~ (Merck, German). ~~and~~ Deionized water was used throughout this study~~.Aquabidestilata~~.

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Preparation of homogeneous chitosan powder

Homogenous chitosan powder was obtained by dissolving chitosan flakes in acetic acid solution (1%~~,~~) v/v). The solution was stirred at 400 rpm on a mechanical stirrer for 24 h~~ours~~ to obtain chitosan solution with 2% w/v concentration. 1 M NaOH solution was added into chitosan solution until the pH reached neutral (pH~~,~~ ~~=~~ 7). After the addition of NaOH solution into chitosan solution, chitosan gels could be obtained. Chitosan gels were dried at 40 °C for 24 h~~ours~~.~~.~~ t~~T~~he dried chitosan gels were sieved by 1 mm sieve to produce homogeneous chitosan powder.

Formulation of ciprofloxacin-loaded bovine hydroxyapatite-chitosan-~~ciprofloxacin~~ implant ~~using glutaraldehyde or genipin as cross link agent~~

The implant was ~~produced~~ prepared ~~by~~ using direct compression method. Ciprofloxacin was dissolved in distilled water~~aquabidestilata~~, ~~Bovine Hydroxyapatite~~ BHA was added gradually and mixed until ~~homogen~~homogenized~~ization~~ with ciprofloxacin. Chitosan powder was added to c~~C~~iprofloxacin-~~Bovine Hydroxyapatite~~BHA blend and mixed ~~until homogenization~~well. Distilled water~~Aquabidest~~ was~~ere~~ added gradually with continuous stirring until a~~form~~ wet granules mass formed. Wet granules ~~mass~~ were sieved ~~using~~ through a 1~~-~~-mm sieve~~r~~ and dried overnight (24 h~~ours~~) at 40 °C to obtain dried granules. Dried granules were immersed in glutaraldehyde ~~solution~~ or genipin solution at various concentration of crosslinkers (0.3%, 0.5%, and 0.7%~~ concentration~~) for 24 h~~ours~~ until the color was changed. The composition of various formulations ~~was~~ is ~~mentioned~~ given in Table 1. Granules were washed with distilled water to remove the residual glutaraldehyde ~~and~~ or genipin. At the final stage, granules were washed with phosphate buffer saline (PBS) at pH 7.40. To ensure the absence of glutaraldehyde residues, the rinsed solution tested with Schiff reagents. Granules were dried in an oven at 40 °C for 24 h~~ours~~. Dried granules (100 mg) were weighed out~~ 100 mg~~, pressed using tablet press machine with 4.0 mm diameter and the compression pressure was set to 2 tons.

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Evaluation of implants

Density test

The density of the implant was calculated from the weight of the implant (in the dry state) divided by volume of the implant (10,11) through the equation below. ~~The density of the implant could be calculated using equation 1.~~

$$\text{Density} = \frac{W_i W_i}{V V} \qquad (1)$$

~~where, W_i is the weight of the implant at the initial condition, and V is the volume of the implant.~~

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Porosity test

The implant was weighed in the ~~initial condition (in the~~ dry state and ~~), then the implant was~~ placed in 5 ~~ml~~ mL water~~ for 1 minute~~. The implant was taken out from the water after 1 min~~ute immersion process~~ and placed in filter paper to remove the excess water on the surface of the implant. The implant was weighed again~~ to obtain the wet weight~~ ({10,11}). The porosity of the implant ~~could be~~was calculated using equation 2.

$$\text{Porosity (\%)} = \frac{W_w - W_i}{\text{Implant volume}} \qquad (2)$$

where, Ww is the wet weight of the implant and Wi is the initial weight of the implant. Implant volume was calculated from pellet thickness multiplied ~~with~~ by implant surface area. An implant is a cylindrical form with 4.0 mm diameter and 0.525 mm thickness.

Water absorption capacity and swelling ratio

The implant was weighed in ~~the initial condition (~~dry state and ~~), then implant was~~ immersed in 5 ~~ml~~ mL ~~phosphate buffer saline (~~PBS,~~)~~ pH 7.4 for 1 min~~ute~~ at temperature 37 ~~°C ±.~~± 0.5 °C. The implant was withdrawn and gently blotted with filter paper to remove the excess water and weighed again (10-13). The percentage of water absorption capacity and swelling ratio of the implant was calculated using equations 3 and 4.

$$\text{Water absorption capacity} = \frac{Ww-Wi}{Wi} \times 100 \qquad (3)$$

$$\text{Swelling ratio} = \frac{Ww-Wi}{Ww} \times 100 \qquad (4)$$

where, Wi is the weight of implant in dry state and Ww is the weight of the implant after immersion process in ~~phosphate buffer saline (~~PBS,~~)~~ pH 7.40.

Hardness test

The implant was pressed by load cell compression machine 5 mm/min by autograph E-10 instrument. The hardness of the implant obtained from the force (F, in newton unit) which was displayed at the instrument divided by contact surface area of the implant (in mm unit) (14).

Disintegration Test

Implant was immersed in 5 ~~ml~~ mL ~~phosphate buffer saline~~PBS, pH 7.4 at 37 ~~°C~~ ± 0.5 °C. Visual inspection was done to observe the changing of implant structure which was caused by erosion and degradation (14-16).

Evaluation of implant morphology using scanning electron microscope ~~(SEM)~~

Morphology of the implant was observed using scanning electron microscope (SEM). The samples were fitted to aluminum stubs with conductive paint and were sputter-coated with gold (17). The difference of implant morphology before and after cross-linking process was observed using specific magnification.

Drug content

The implant was placed in a mortar and milled, then transferred into Erlenmeyer flask. 100 ~~ml~~ mL HCL 0~~.~~.1 N was added into Erlenmeyer flask which contained milled implant and stirred for 24 h (400 rpm) until form suspension. The suspension was filtrated, and the filtrate was diluted to determine ciprofloxacin concentration. The absorbance of this solution was observed using spectrophotometer ~~UV~~ultraviolet (UV)-~~Vis~~ visible at three wavelengths (260 nm, 270 nm, and 280 nm). (Δ), a~~A~~bsorbance which was obtained from the observation extrapolated in standard curve equation to obtain ciprofloxacin HCL concentration. Determination of ciprofloxacin content in the implant was done triplicate (18).

In vitro drug release study

The implant was placed in a vial containing 5 ~~ml~~ mL of ~~phosphate buffer saline (~~PBS,~~)~~ pH 7.4. The vial was placed on a shelf and incubated in water bath at 37 ~~°C~~ ± 0.5 °C. Sampling was conducted by pipetting 1 ~~ml~~ mL of elution fluids at predetermined time intervals (1st, 3rd, 5th, 7th, 9th, 12th, 14th, 16th, 18th, 20th, 22th, and 24th h on first day and every 24 h for 30 days) and replaced with fresh buffer to maintain sink condition. The sample solution was filtered with Millipore membrane ()(ø = 0.45 µm). Appropriate dilution was prepared using ~~phosphate buffer saline (~~PBS,~~)~~ pH 7.4. The absorbance of the solution was analyzed using UV spectrophotometer at three wavelengths (260 nm, 270 nm, and 280 nm). Cumulative percent drug release was found at each time interval. The release of ciprofloxacin HCL from the implants was assayed in triplicate (16,19).

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Data analysis

The results of implant evaluation (density, porosity, swelling ratio, water uptake, hardness, and area under the curve (AUC) of *in vitro* release profile) was statistically analyzed using one way aAnalysis of vVariance (ANOVA) with 95% confidences interval.

Characterization of the implant

Fourier transforms infrared ~~(FTIR)~~ spectroscopy

A sample of the implant was combined with KBr and pressed into a pellet. The solid pellet was analyzed using fourier transform-infrared (FT-IR) spectroscopy in the wave number range 4000-400 cm^{-1} (17).

X-ray diffraction study

The X-ray diffraction (X-RD) study was carried out to determine the crystal phases of the implant using monochromatic Cu Kα radiation (40 KV, 30 MA). X-~~ray diffraction~~RD peaks of the implants were compared to the diffraction peaks of pure materials (ciprofloxacin HCL, ~~Bovine Hydroxyapatite~~BHA, and chitosan) in 2θ scan range of 5-50° (17).

RESULTS

Density of implant

The result of density test on the implants that have been cross-linked using glutaraldehyde and genipin can be seen in Fig. 1

Porosity of implant

The porosity of the implants that have been cross-linked using glutaraldehyde and genipin can be seen in Fig. 1.

Water absorption capacity

Water absorption capacity of the implants that have been cross-linked using glutaraldehyde and genipin can be seen in Fig. 1.

Swelling ratio of implant

The swelling ratio of the implants that have been cross-linked using glutaraldehyde and genipin can be seen in Fig. 1.

Hardness of implant

The result of hardness test on the implants that have been cross-linked using glutaraldehyde and genipin can be seen in Fig. 1.

Disintegration test

"~~"~~Disintegration test~~"~~" was done by visual observation after implant have been crosslinked with glutaraldehyde and genipin. The dDisintegration profile of implants with three different concentrations of glutaraldehyde showed that formula with the lowest disintegration was F3-0.7% glutaraldehyde. At the opposite, F3-0.3% glutaraldehyde showed greater disintegration than two others formula. The lower concentration of glutaraldehyde as a cross-linking agent caused hydrolysis process in polymer chains inducing erosion process (~~22~~20). The increase of glutaraldehyde concentration caused an increase in cross-link density. Implants with higher cross-link density had lower hydrophilic groups, so that the structure of the implants became difficult to extend in water (~~23~~21).

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Evaluation of implant morphology using scanning electron microscope ~~(SEM)~~

SEM micrograph of the implant that has been cross-linked using glutaraldehyde and genipin are presented in Fig. 2. Based on the micrograph, it could be seen that there was small pores in the structure. These pores facilitate the release of ciprofloxacin from the implants.

Drug content

The result of ciprofloxacin HCL content in an implant that has been cross-linked using glutaraldehyde and genipin can be seen in Table 2. Drug content of all formulations was determined by UV spectrophotometer using three-wavelength methods.

In vitro drug release study

The cumulative amount of ciprofloxacin that has been released in PBS, pH 7.40, from the implant that has been cross-linked using glutaraldehyde and genipin could be seen in Fig 3. The release profile of ciprofloxacin HCL from the implants showed that ciprofloxacin release was at a therapeutic level of ciprofloxacin for osteomyelitis (2-50 µg/~~ml~~mL) (~~24~~22,~~25~~23). This condition could be kept for 30 days.

Fourirer transform infrared ~~(FTIR)~~ spectroscopy

The infrared spectrum of ciprofloxacin, BHA~~ovine Hydroxyapatite~~, chitosan, implants BHA~~ovine Hydroxyapatite~~-chitosan-ciprofloxacin before the cross-linking process, and implants B~~ovine Hydroxyapatite~~HA-chitosan-ciprofloxacin after the cross-linking process with three different concentrations of glutaraldehyde and genipin can be seen in Fig. 4. FT-IR spectrum of BHA~~ovine Hydroxyapatite~~-chitosan-ciprofloxacin implant after crosslinking process using glutaraldehyde showed a peak shift characteristics of chitosan on wavenumbers 1658,67 cm^{-1} (C=O stretching in amide group) to the lower wavenumbers around~ 1630 cm^{-1}. This band is most probably composed of amide I band of chitosan (appears at 1658.67 cm^{-1}) and the C=N stretching band of Schiff's base that according to the literature appears at wave number 1620-1660 cm^{-1} (8). Moreover, the peak characteristic of aldehyde could not be seen in the FT-IR spectrum of BHA~~ovine Hydroxyapatite~~-chitosan-ciprofloxacin implant after crosslinking process using glutaraldehyde. This condition showed that the implant did not contain free aldehyde group. Based on the results of the FT-IR spectrum, it was known that there was a shift of the N-H stretching vibrations and O-H stretching vibrations from chitosan molecules. In addition, the loss of peak at wave number 1363 cm^{-1} (the vibration bending of CH_3) and 1155 cm^{-1} (the vibration bending of C-O-C) observed in FT-IR spectrum of the implant compared to FT-IR spectrum of pure chitosan. FT-IR spectrum of B~~ovine Hydroxyapatite~~HA-chitosan-ciprofloxacin implant that has been cross-linked using genipin also can be seen in Fig. 4. The spectrum showed a characteristics peak shift of chitosan to the lower wavenumbers. Characteristic peak of chitosan on wavenumbers 1639,55 cm^{-1} (C=O stretching of amides group) shift to the lower wavenumbers 1622.19 cm^{-1} at 0.7% genipin concentration, 1637.62 at 0.5% genipin concentration, and 1622.19 cm^{-1} at 0.3% genipin concentration. Increased of genipin concentration caused an increase of C=C bond intensity of genipin.

X-ray diffraction study

X-~~ray diffraction~~RD of the implants after cross-link using glutaraldehyde can be seen in Fig. 5. Based on the results, it was known that the characteristics peak of ciprofloxacin in 2θ 8,2°, °, 9,0°, °, 19,3°, °, 19,8°, °, and 26,5° did not appear in a diffraction spectrum of ~~Bovine Hydroxyapatite~~BHA-chitosan-ciprofloxacin implant. This condition indicated that ciprofloxacin was molecularly dispersed in the implant. X-ray diffraction of the implant after cross-link using glutaraldehyde showed that the peak intensity of BHA in 2θ ≈ 26° and 2θ ≈

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32° decreased compared to X-ray diffraction of pure BHA and the implants before the cross-linking process. The X-ray pattern of chitosan shows major crystalline peaks at $2\theta \approx 10°$ and $2\theta \approx 20°$. But, the X-ray diffraction of the implants indicated that these peaks became wider and weaker. The decrease crystallinity of chitosan molecules caused by the deformation of hydrogen bond in the molecular structure of chitosan. Substitution of glutaraldehyde molecules destroyed the regular structure of chitosan molecules so that the structure of chitosan molecules became amorph (~~26~~24). A similar case also happened on the B~~ovine Hydroxyapatite~~ HA diffraction. The addition of glutaraldehyde damage regularity on BHA~~ovine Hydroxyapatite~~ crystal lattice. The decreased of BHA~~ovine Hydroxyapatite~~ crystallinity in line with the increased of glutaraldehyde concentration.

DISCUSSION

The result of this research is to obtain bone implant with ciprofloxacin as an active ingredient and chitosan-BHA composite. Hopefully, with the addition of genipin and glutaraldehyde as cross-linker, the implant has good physical characteristics and controlled drug release. In the beginning, implant was characterized. After that, the release of ciprofloxacin from the implant was observed.

Aldehyde groups of glutaraldehyde (C=OH) react with chitosan amine groups ($-NH_2$) produced covalent crosslinking through a Schiff base reaction (8,~~20~~25,~~21~~26).

The crosslinking reaction mechanism between chitosan and genipin occurred in low acidic and neutral conditions. A nucleophilic attack by the amino groups of chitosan on the olefinic carbon atom at C-3 occurred, this condition followed by opening the dihydropyran ring and attacked by the secondary amino group on the newly formed aldehyde group. In other words, genipin act as a dialdehyde (9).

The density of the implants after the cross-linking process with glutaraldehyde was lower than before cross-linking process. Moreover, the result of statistical analysis using one-way ANOVA showed that there was no significant difference in density between the implants which used three different concentration of glutaraldehyde ($P > 0.05$). Based on this result, it could be concluded that the difference of glutaraldehyde concentration did not affect the implant density. On the other hand, statistically, analysis using one-way ANOVA showed that there was a significant difference of implant density before and after cross-link using genipin ($P < 0.05$). The result of statistical analysis using one-way ANOVA showed that there was no significant difference of density between the implants which used three different concentration of genipin ($P > 0.05$). Based on this result, it could be concluded that the difference of genipin concentration did not affect the implant density.

The result of statistical analysis using one-way ANOVA to the formula that using three different concentrations of glutaraldehyde showed that there was a significant difference of porosity between F3-0.3% glutaraldehyde and F3-0.7% glutaraldehyde ($P < 0.05$). Increasing glutaraldehyde concentration of 0.7% caused an increase of porosity than 0.3% glutaraldehyde. The increase of glutaraldehyde concentration caused the structure of the implants became looser. Higher concentration of glutaraldehyde led the structure of the implant became amorph, so that the porosity of the implant increased (27).

Statistically, analysis using one way ANOVA showed that there was a significant difference of porosity before and after cross-link using genipin ($P < 0.05$). The porosity of F3 was significantly different with F3-0.3% genipin and F3-0.7% genipin. In addition, the result of statistical analysis using one-way ANOVA to the formula that using three different concentration of genipin showed that there was a significant difference of porosity between F3-0.3% genipin and F3-0.5% genipin. Increasing genipin concentration of 0.5% caused a decrease in porosity than 0.3% genipin. The increase of genipin concentration caused the

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structure of the implants became more compact. Genipin affected network size which was formed between chitosan ~~chain~~chains. The increase of genipin concentration led to the size of the network became smaller, so that the porosity of the implant decreased.

Water absorption capacity of the implants after the cross-linking process was higher than before cross-linking process. The result of statistical analysis using one way ANOVA to the formula that using three different concentrations of glutaraldehyde showed that there was a significant difference of water absorption capacity between F3-0.3% glutaraldehyde and F3-0.7% glutaraldehyde ($P < 0.05$). The increase of glutaraldehyde concentration to 0.7% caused markedly decrease water absorption capacity. Cross-linking process with glutaraldehyde restricted water molecules to enter into chitosan structure (28). On the other hand, statistically, analysis using one-way ANOVA showed that there was a significant difference of water absorption capacity before and after cross-link using genipin ($P < 0.05$). Water absorption capacity of the implants after the cross-linking process with genipin was higher than before cross-linking process. The result of statistical analysis using one-way ANOVA to the formula that using three different concentrations of genipin showed that there was no significant difference of water absorption capacity between three formulas. Based on ~~this results~~these results, it could be concluded that the difference of genipin concentration did not affect water absorption capacity of the implants.

The result of statistical analysis using one way ANOVA to the formula that using three different concentrations of glutaraldehyde showed that there was no significant difference of swelling ratio among three formulas ($P > 0.05$). On the other hand, there was a significant difference of swelling ratio of the implant, before and after cross-link using genipin ($P < 0.05$). The swelling ratio of the implants after the cross-linking process was higher than before cross-linking process. Statistically, analysis using one way ANOVA showed that there was no significant difference of swelling ratio between three formulas which used three different concentration of genipin ($P > 0.05$).

Cross-linking process using glutaraldehyde caused the characteristic of biomaterial became brittle. Increasing glutaraldehyde as cross-link agent more than 0.2 % ~~decrease~~decreases the mechanical strength of the implants (29). The hardness of the implant after cross-link using genipin was lower than before cross-link. Based on statistical analysis using one- way ANOVA, it could be found that there was a significant difference of implant hardness before and after the cross-linking process. The result of statistical analysis using one- way ANOVA to the formula that using three different concentrations of genipin showed that there was no significant difference of hardness between three formulas.

The disintegration profile of implants with three different concentrations of glutaraldehyde showed that formula with the lowest degradation was F3-0.3% genipin. At the opposite, F3-0.7% genipin showed greater disintegration than two others formula. In the specific case, the increased of cross-link agent concentration caused a decrease of material crystalinity. The cristalinity of F3-0.7% was lower than F3-0.3%, so that the ability of water to penetrate in implants structure became easier and the implants degrade easily.

There was a change of diffraction pattern before and after cross linking process using genipin. This phenomenon indicated a change in the degree of crystallinity. Characteristic peak of chitosan in 2θ 10° and 20° did not appear in the implant diffraction. In addition, characteristic peak of B~~ovine Hydroxyapatite~~ HA in 2θ 26° and 32° decreased compare to the diffraction of pure B~~ovine Hydroxyapatite~~ HA and implant before cross linking process. The increased of genipin concentration in the implant caused a decrease of cristalinity. This can be observed through a decreased of peak intensity in line with the increased of genipin concentration.

There are many factors that influence the drug release profile of the implants. This includes drug concentration in the dosage form, drug solubility, and drug-carrrier interaction. In this research, ciprofloxacin interact with implant~~'~~s' composite (24,30~~-~~,31). Therefore, drug release

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study showed that ciprofloxacin release from bone implant with glutaraldehyde crosslinker with different concentration have no significant difference. In the same way, with difference crosslinker (genipin) also showed no significant difference.

Adding cross-linker to the implant formula could control the drug release either in the initial release to prevent burst effect and in the sustained release process.

The release of ciprofloxacin from BHA-cChitosan-ciprofloxacin implant using glutaraldehyde as cross-linked shows controlled release. The same result happened using genipin as cross-linker.

The release mechanism of ciprofloxacin from BHA-cChitosan-ciprofloxacin using glutaraldehyde and genipin cross-linker starts with the swelling process and followed with drug diffusion. One system involves the diffusion of drugs from a reservoir ~~through a biodegradable matrices~~through biodegradable matrices. Glutaraldehyde and genipin hamper the degradation process so the drug release will be controlled.

Using genipin, the degradation process is faster compared with using glutaraldehyde as crosslinker. Nevertheless, the ciprofloxacin concentration released from the implant using glutaraldehyde and genipin was meet the therapeutic range criteria according to Indonesian Ministry of Health (2-50 ug/mL) for 30 days. Drug release study showed that ciprofloxacin release from bone implant with glutaraldehyde and genipin crosslinker using different concentration have no significant difference. This result happened because using high concentration of cross linker, implant becoming more stiff with less mechanical strength. The decrease of cristalinity forming an amorphous structure contributed with the reduction of mechanical strength (26,27).

To conclude with, bone implants with ciprofloxacin using cChitosan-BHA composite and crosslinker glutaraldehyde and genipin 0.7% showed the best result. Therefore, the release of ciprofloxacin for 30 days meets the standard requirements (2-50 mg). Glutaraldehyde or genipin 0.7% had the potential effect to retard ciprofloxacin release from BHA~~ovine Hydroxyapatite~~-chitosan-ciprofloxacin implant for 30 days in the treatment of osteomyelitis.

CONCLUSION

The results obtained from this study indicated that glutaraldehyde or genipin 0.7% had potential effect to retard and control ciprofloxacin release from BHA~~ovine Hydroxyapatite~~-chitosan-ciprofloxacin implant with diffusion and ~~erotion~~erosion mechanism for 30 days in the treatment of osteomyelitis.

ACKNOWLEDGEMENTS

This work was supported by the grant PUPT from Ministry of Research, Technology and Higher Education of the Republic of Indonesia.

Comments by two anonymous reviewers greatly helped to improve an earlier version of this manuscript.

REFERENCES

1. Porter JR, Ruckh TT, Popat KC. Bone tissue engineering : a review in bone biomimetics and drug delivery strategies. Biotechnol Prog. 2009;25(6):1539-1560.
2. Strobel C, Bormann N, Kadow-Romacker A, Schmidmaier G, Wildemann B. Sequential release kinetics of two (gentamicin and BMP-2) or three (gentamicin, IGF-1 and BMP-2) substances from a one component polymeric coating on implants. J Controlled Release. 2011;156(1):37-45.
3. MouriñoMourino V, Boccaccini AR. Bone tissue engineering therapeutics: controlled drug delivery in three-dimensional scaffolds. J R Soc Interface. 2010;7(43):209-227.
4. Cui, X., Gu, Y., Wang, H., Xie, Z., Luo, S., Zhou, N., Huang, W., and Rahaman, *Met al*. *In vitro* bioactivity,

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cytocompatibility, and antibiotic release profile of gentamicin sulfate-loaded borate bioactive glass/chitosan composites. J Mater Sci Mater Med. 2013;24(10):2391-2403.
5. Gogia JS, Meehan JP, Di Cesare PE, Jamali AA. Local aAntibiotic tTherapy in oOsteomyelitis. Semin Plast Surg 2009;23(2):100-107.
6. Li B, Brown KV, Wenke JC, Guelcher SA. Sustained release of vancomycin from polyurethane scaffolds inhibits infection of bone wounds in a rat femoral segmental defect model. J Controlled Release. 2010;145(3):221-230.
7. Dubnika A, Loca D, Berzina-Cimdina LB. Functionalized hydroxyapatite scaffolds coated with sodium alginate and chitosan for controlled drug delivery. Proc Est Acad Sci. 2012;61(3):193-199.
8. Ostrowska-Czubenko J, PierógPierog M, Gierszweka-Drużyńska M. State of water in non crosslinked and crosslinked hydrogel chitosan membranes. Prog Chem Appl Chitin Its Deriv. 2011;16:147-156.
9. Muzzarelli RAA. Genipin-crosslinked chitosan hydrogel as biomedical and pharmaceutical aids. Carbohydr Polym. 2009:77(1):1-9.
10. Lei L, Li L, Zhang L, Chen D, Tian W. Structure and performance of nano-hydroxyapatite filled biodegradable poly(1,2-propanediol-sebacate)-citrate) elastomer. Polym Degrad Stab 2009;94(9):1494-1502.
11. Liuyun J, Yubao L, Chengdong X. Preparation and biological properties of a novel composite scaffold of nano-hydroxyapatite/chitosan/carboxymethyl cellulose for bone tissue engineering. J Biomed Sci 2009;16(1):651-10.
12. Campos MG, Rawls HR, Innocentini-Mei LH, Satsangi N. *In vitro* gentamicin sustained and controlled release from chitosan cross-linked films. J Mater Sci: Mater Med. 2009;20(2):537-542.
13. Pawar Ashish PY, DerleDeelip V. Development of cost effective biodegradable implants of ciprofloxacin hydrochloride in treatment of osteomyelitis. Asian J Pharm Innov. 2012;2(5):19-37.
14. Budiatin SAS, Zainuddin M, Khotib J. Biocompatible composite as gentamicin delivery system for osteomyelities and bone regeneration. Int J Pharm Pharm Sci. 2014;6(3):223-2261-3.
15. Yang B, Li XY, Shi S, Kong XY, Guo G, Huang M, *et al*. Preparation and characterization of a novel chitosan scaffold. Carbohydr Polym. 2010;80(3):860-865.
16. Teng SH, Lee EJ, Yoon BH, Shin DSWang P, Jun SH, Han CM, Kim HE, Oh JS. Chitosan/nanohydroxyapatite composite membranes via dynamic filtration for guided bone regeneration. J Biomed Mater Res A.Part B 20082009;88(3):569-579580.
17. Tein-Han WW, Misr-a RDK. Biomimetic chitosan-nanohydrixyapatite composite scaffolds for bone tissue engineering. Acta Biomater. 2009;5(4):1182-1197.
18. Ashish YP, Derle DV, Gandhi PP, Nade VN, Patel JS. *In vitro* and *in vivo* characterization of a novel biocompatible implants system for the sustained delivery of ciprofloxacin hydrochloride. J Pharm Res. 2010;3:2122-2126.
19. Stallmann HP, Faber C, Bronckers AL, Amerogen AVN, Wuisman PI. *In vitro* gentamicin release from commercially available calcium-phosphate bone substitute influence of carrier type on duration of the release profile. BMC Musculoskeletal Ddisord,ers 2006;7:1-8.
20. Goncalves VL, Laranjeira MCM, Favere VT, Pedrosa RC. Effect of crosslinking agents on chitosan microspheres in controlled release of diclofenac sodium. Polim Cienc Tecnol. 2005;15:6-12.
Berge J, Reist M, Maayer JM, Felt O, Peppas NA, Gunny R. Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications. Eur J Pharm Biopharm 2004:57:19-34.
21. Prabaharan M, Rodriguez-Perez MA, de Saja JA, Mano JF. Preparation and characterization of poly(L-lactic acid)-chitosan hybrid scaffolds with drug release capability. J Biomed Mater Res B Appl Biomater. 2007;81(2):427-434.Shweta A, and Sonia P. Pharmaceutical and relevance of crosslinked chitosan in microparticulate drug delivery. Int Res J Pharm 2013:4:45-51.
22. Mäkinen TJ, Veiranto M, Lankinen P, Moritz N, Jalava J, Törmälä P, et al. In vitro and in vivo release of ciprofloxacin from osteoconductive bone defect filler. J Antimicrob Chemother. 2005;56(6):1063-1068. Goncalves VL, Laranjeira, MC, Favere VT. Effect of crosslinking agents on chitosan microspheres in controlled release of diclofenac sodium. Polim : Cienc Tecnol 2005;15:6-12
23. Mont MA, Mathur SK, Frondoza CG, Hungerford DS. The effects of ciprofloxacin on human chondrocytes in cell culture. Infection. 1996;24(2):151-155.Prabaharan M, Perez MA, Saja JA, Mano JF. Preparation and characterization of poly(L-lactic acid)-chitosan hybrid scaffolds with drug release capability J Biomed Mater Res Part B 2006;88:427-434.
24. Li B, Shan CL, Zhou Q, Fang Y, Wang YL, Xu F, et al. Synthesis, characterization, and antibacterial activity of cross-linked chitosan glutaraldehyde. Mar Drugs. 2013;11(5):1534-1552.Makinen TJ, Veiranto M, Lankinen P, Moritz N, Jalava J, Tormala P, Aro *HT*. *In vitro* and *in vivo* release of ciprofloxacin from osteoconductive bone defect filler. J of Antimic Chem 2005;56:1063-1068.
25. Berge J, Reist M, Maayer JM, Felt O, Peppas NA, Gurny R. Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications. Eur J Pharm Biopharm. 2004:57(1):19-34.Mont MA, Mathur SK, Carmelita G, Frondoza D, Hungerford DS. The effects of ciprofloxacin on human

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chondrocytes in cell culture. Infection 1996;24:151-155.
26. Shweta A, Sonia P. Pharmaceutical relevance of crosslinked chitosan in microparticulate drug delivery. Int Res J Pharm. 2013:4(2):45-51. Li B, Shan CL, Zhou Q, Fang Y, Wang YL, Xu F, Han LR, Ibrahim M, Guo LB, Xie GL, Sun *GC*. Synthesis, characterization, and antibacterial activity of cross-linked chitosan glutaraldehyde 2013;11:1534-1552.
27. Silva RM, Silva GA, Coutinho OP, Mano JF, Reis RL. Preparation and characterization in simulated body conditions of glutaraldehyde crosslinked chitosan membranes. J Mater Sci Mater Med. 2004;15(10):1105-1112.
28. Sailakshmi G, Mitra T, Chatterjee S., Gnanamani, A. Chemistry behind the elastic nature of the biomaterial prepared usimg oxidized form of glutaraldehyde. Int J of Life Science and Med Res. 2013;4(2):634-745.
29. Mirzaei BE, Ramzani SA, Shaifee M, Dannaei M. Studies on glutaraldehyde crosslinked chitosan hydrogel properties for drug delivery system. Int J Polymr Mater Polymr Biomater. 20152013;62(11):605-611.
30. Stevenson, C.L., Santini, J.T Jr., and Langer, R., 2012. Reservoir-based drug delivery system utilizing microtechnology. Advanced Drug Delivery Reviews, 2012;Vol. 64(14,):p. 1590-1602.
31. Sinko PJ, Singh Y. , (editors). Martin Physical Pharmacy and Pharmaceutical Sciences, Physical Chemical and Biopharmaceutical Principles in the Pharmaceutical Sciences. 6th ed. Wolter Kluwer/Lippincott Williams & Wilkins, Philadelphia, 2011. pp. 300-317.

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Table 1. The composition of implant formulations.

Formulation code	Cyprofloxacin (%)	Composite composition (%)		Cross-linker (%)	
		Bovine hydroxyapatite	Chitosan	Glutaraldehyde	Genipin
F3	10	30	60	-	-
F3-0.3% GA	10	30	60	0.3	-
F3-0.5% GA	10	30	60	0.5	-
F3-0.7% GA	10	30	60	0.7	-
F3-0.3% GE	10	30	60	-	0.3
F3-0.5% GE	10	30	60	-	0.5
F3-0.7% GE	10	30	60	-	0.7

Genipine (GE), glutaraldehyde (GA).

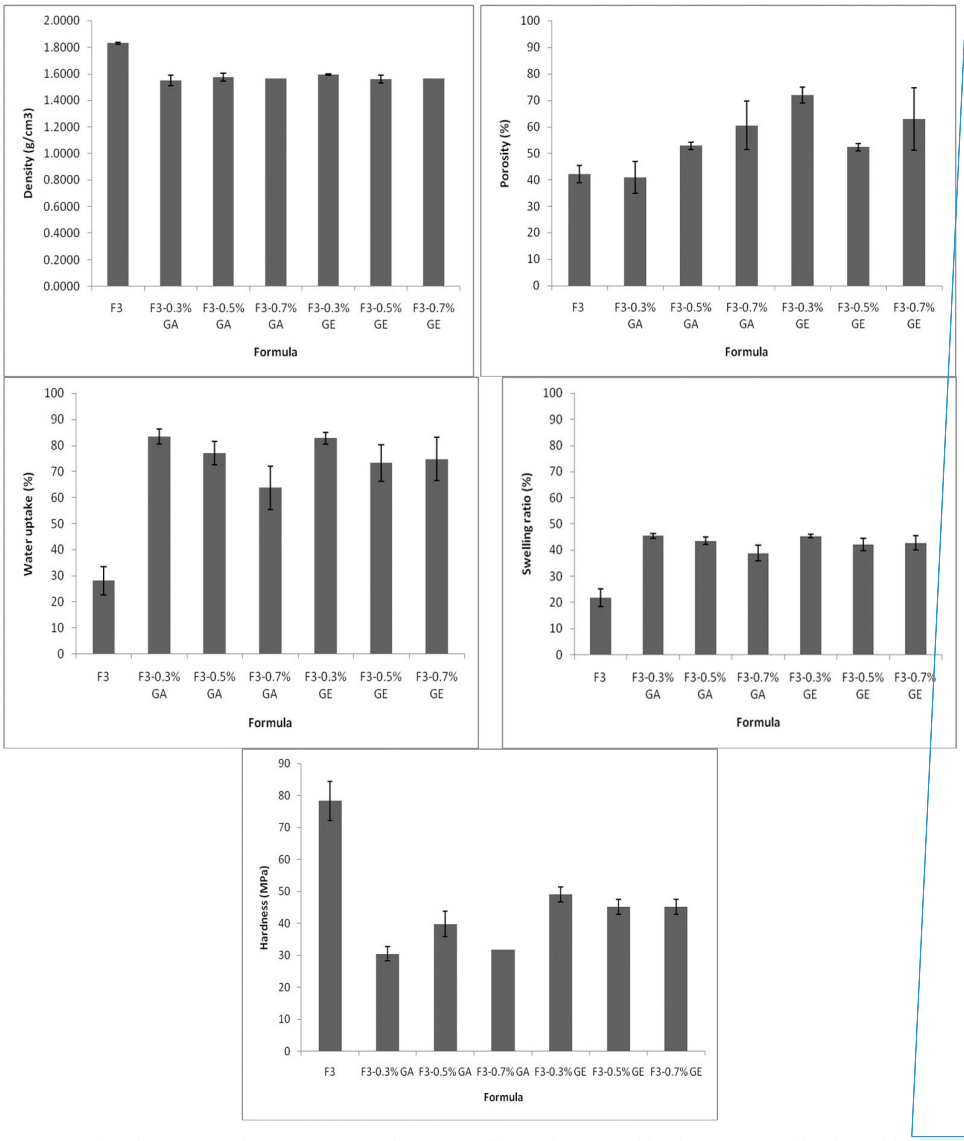

Fig. 1. Density (g/cm3), porosity (%), water uptake (%), swelling ratio (%), and hardness (MPa) of implant with cross-linker glutaraldehyde (GA) and cross-linker genipin (GE). Each column represents the mean ± SD of three determinations.

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-Please modify the font of all figures to Times New Roman, un-bolded and legible size (10-12).
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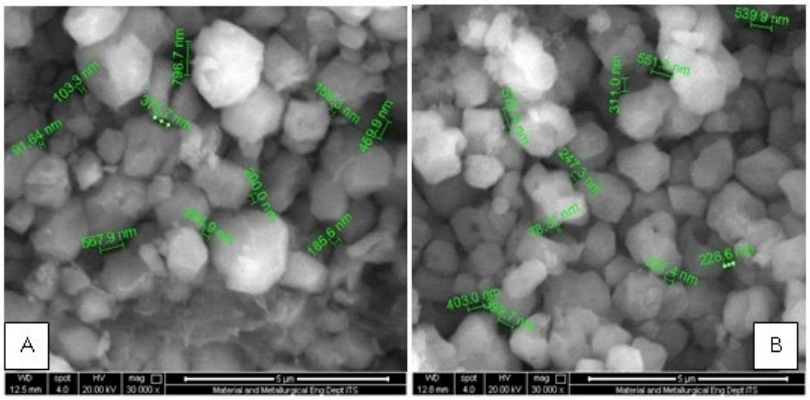

Fig. 2. Scanning electron microscopy~~EM~~ micrograph of ciprofloxacin implant that has been cross linked (with 30.000~~×X~~ magnification): (A) bovine hydroxyapatite-chitosan-ciprofloxacin (30:60:10) with 0.7% glutaraldehyde, (B) bovine hydroxyapatite-chitosan-ciprofloxacin (30:60:10) with 0.7% genipin. The green lines inside in micrograph show the pore size of the implant.

Table 2. Drug content of implant formulations

Formulation code	Drug content (%)
F3-0.3% GA	96.04 ± 7.11
F3-0.5% GA	84.80 ± 10.34
F3-0.7% GA	87.31 ± 3.40
F3-0.3% GE	92.81 ± 7.96
F3-0.5% GE	95.30 ± 1.07
F3-0.7% GE	92.81 ± 2.41

Each data represents the mean ± SD of three determinations. (GA), glutaraldehyde and (GE), genipin.

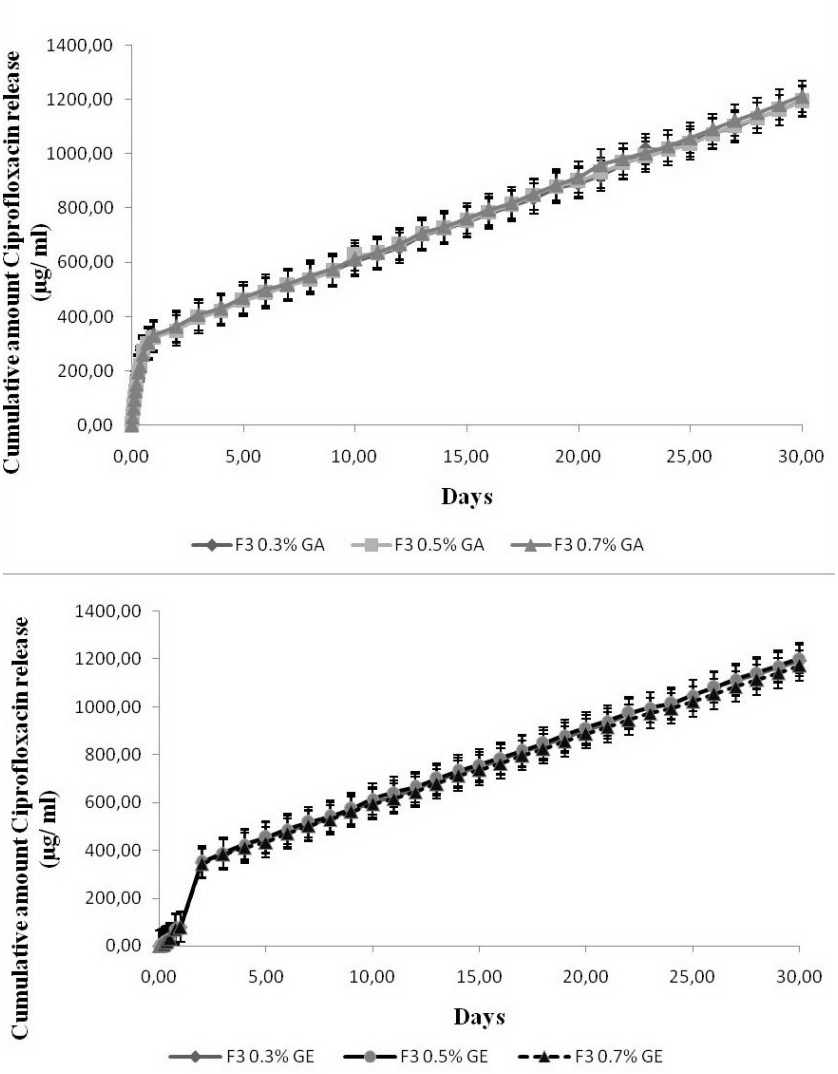

Fig. 3. The profile of cumulative amount of ciprofloxacin release from implant formulation cross-linker glutaraldehyde (GA) and genipin (GE). Each point represents the mean ± SD of three determinations.

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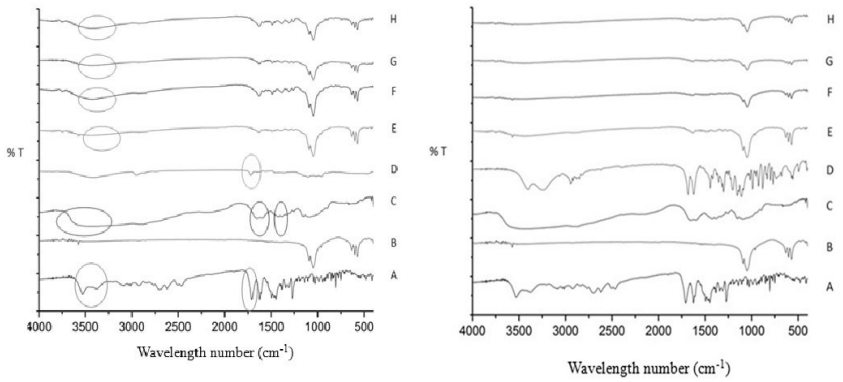

Fig. 4. Fourirer transform infrared~~FT-IR~~ spectrum of (A) ciprofloxacin, (B) bovine hydroxyapatite~~BHA~~, (C) chitosan, (D) glutaraldehyde, (E) formula 3 bovine hydroxyapatite-chitosan-ciprofloxacin implant (30:60:10), (F) formula 3-0.3% glutaraldehyde, (G) formula 3-0.5% glutaraldehyde, and (H) formula 3-0.7% glutaraldehyde (left) and genipin (right).

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Dear DR. Emami, I think this type of FT-IR spectrum is not acceptable and needs to be more qualified and also more details like numbers needs to be included. But it is depend on your valuable decision.

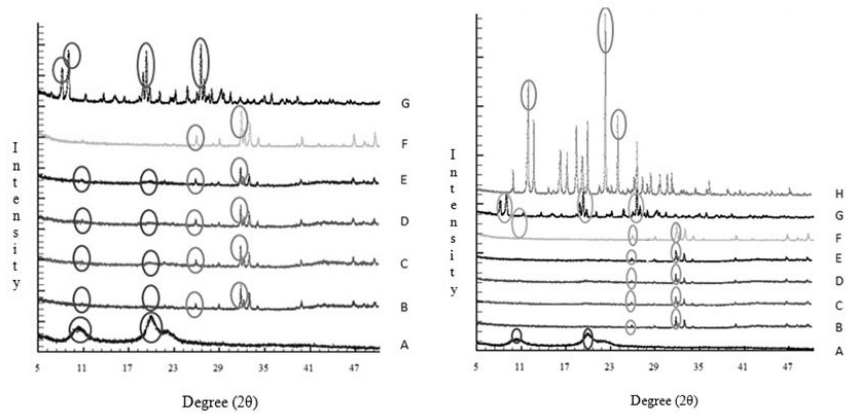

Fig. 5. X-ray diffraction spectrum of (A) chitosan, (B) formula 3 bovine hydroxyapatite(BHA)-chitosan-ciprofloxacin implant (30:60:10), (C) formula 3-0.3% glutaraldehyde, (D) formula 3-0.5% glutaraldehyde (left) and genipin (right), (E) formula 3-0.7% glutaraldehyde (left) and genipin (right), (F) BHA, and (G) ciprofloxacin.

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Gmail **Adrianto Faris <farisadrianto@gmail.com>**

Bls: RPS 16-168 needs major major revision

2 messages

Esti Hendradi <estihendradi@yahoo.com> Mon, Aug 14, 2017 at 5:11 AM
Reply-To: Esti Hendradi <estihendradi@yahoo.com>
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Cc: Adrianto Faris <farisadrianto@gmail.com>, Dewi Melani <dewiffua96@yahoo.com>

Dear Jaber Emami Pharm.D, Ph.D

I am glady inform you that I have received your email regarding revision of my manuscript titled " The effect of different cross link agent glutaraldehyde and genipin in composites to the physicochemical characteristics and the release of ciprofloxacin implant. However, there are few notes we must discuss about this revisio.

1. Regarding your revision, I had already made revision about your point. There are 2 revision from two different reviewers, First revision and second revision (attached to email).
From first revision, we had already answer all the reviewers remarks, including from editor (attached to email).
And then come the second remarks after we made the first revision. We also answer all the remaining question from reviewers (no question from editor on the second revision). (Attached to email).

2. After we made second revision, we received email from RPS stated that our manuscript "The effect of different cross link agent
glutaraldehyde and genipin in composites to the physicochemical characteristics and the release of ciprofloxacin implant" is now acceptable after clearing the dues for publication of the manuscript. (attached to email)
Coming with this email, we must pay 510 USD for the publication and we also already paid the fee (attached to email)

3. After we made the payment, it is stated that we must wait 2-3 weeks for the final check. But after we wait more than 1 month, You sent us an email stated that our manuscript need major revision again. From your remarks in your email, It means our revision from reviewers remarks we made so far are useless. You said the paper does not follow a scientific approach and very poorly written in terms of English language and skills.
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Esti Hendradi, Ph.D

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Thank you submitting your article to RPS journal, your revised article was reevaluated to ensure that all comments have been taking in to account and the
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Journal, reviewd your article and realized that your article needs serious revision
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Editor in Cheif comments
1. The work lacks adequate novelty. So many Micro, macro, and nano carriers have been reported using chitosan and HA to target drugs to the desired tissues. The method also is very old and has no novelty or innovation and tablet or tablet-form composite seems inappropriate for bone damages and fractures.

2. The evaluation and assessment methods are not appropriately and logically selected and described. References given in the method section do not match with the methods described and unrelated to the described method.

3. Observations are all over the places and can not be explained or justified with variables such as the GA or genipin content used in the implants.

4. So many problems and issues are associated with the discussion section. The observations are not correctly discussed and in some occasions are incorrect. Most often the results contradict each other. For instances dissolution profiles are not in accord with density, porosity and water uptake of the formulations. Statistical analysis are not described appropriately in the result and discussion sections. They are non-scientifically and nonstandard are presented in discussion section.

5. Overall, the paper does not follow a scientific approach and very poorly written in terms of English language and skills. Crammer errors and non scientific and incorrect phrases are abundant.

[REVIEWER]1:

In this manuscript, “The effect of different cross link agent glutaraldehyde

and genipin incomposites to the physicochemical" , while I find some interests, I have several criticisms described below.

1. In the " Introduction" the authors should introduce the use of chitosan and hydroxyapatite for bone tissue engineering

2. The genipin and glutaraldehyde crosslinked the chitosan, what's the function of Bovine Hydroxyapatite in the implant? In the FTIR and XRD following BHA in the formulations is not clear and should be discussed.

3. Molecular weight of chitosan should be noted in the materials.

4. "Mechanism of crosslinking" should be transformed to the Result section.

5. For determining the prosity, the authors should display BET test. The implant has a hydrogel structure and adsorbs the water, so using water for prosity test is incorrect.

6. Equation 2 and Equation 4 are the same. This equation is used for determining the swelling ratio and should be omitted from prosity test.

7. For determining the swelling ratio, the duration time of immersion of implants in the buffer should be noted.

8. "Degradation test" should be changed to the "disintegration test".

9. The results of Degradation test were not shown.

10. Analysis of ciprofloxacin was performed at 3 wave lengths, the authors display about this method.

11. In "In vitro drug release study" the volume of release medium is very low (5 ml) in comparison to the sampling volume (1 ml), and 20% of the medium was replaced with fresh buffer at each sampling point time. This can cause error.

12. X and Y axis in Fig 3 should be corrected. Cumulative drug releasedpercent against time should be plotted not cumulative drug releasedconcentration. Indeed, number of days should not have decimal points.

13. The authors didn't discuss why different formula released the drug with the same manner?

14. The SEM images were not uploaded correctly.

15. At the end of discussion the authors should conclude about the study.

16. At the lines number of 161, 238,260, please change "," to "."

17. At the line numbers of 105 please change the red point to black point.

[REVIEWER]2:

The manuscript deals with investigating the effect of type and concentration

of two crosslink agents, glutaraldehyde or genipin, on the physiochemical characteristics of the composite hydrogel of hydroxyapatite and chitosan. Please find the following comments to undertake a major revision:

1- the abstract needs re-writing with respect to the structure and the content. It also contains many typos and grammatical errors.

2- incomplete literature review! the papers on chitosan-hydroxyapatite hydrogel hasn't been addressed.

3- the aim of study has not been well defined at the end of the introduction section.

4- there are some ambiguous parts in the materials and methods section that needs to be revised or explained: homogeneous chitosan powder (homogeneous?), the compression pressure of 2 tons, mechanism of crosslinking section (unrelated to the materials and methods section), the calculation of hydrogel porosity, assay of drug content by extrapolating of the UV-Vis calibration curve constructed at 3 wavelengths, the mechanical strength of the implant which has only been tested in term of hardness in dry state.

5- some parts in the results section are related to the discussion. P values have only been presented in the discussion, so it is suggested to combine the results and the discussion sectons.

6- there are some justifications that have to be re-considered: molecular dispersion of ciprofloxacin in the XRD experiment (or may be only an amorphous drug state), no free glutaraldehyde has been remained (FTIR of free glutarladehyde at the corresponding amount is missing), etc.

7- the discussion section has been started with the non-significant density findings. It is recommended to begin this section with what the study is concerned about and also to discuss only about the most important findings.

8- there is no discussion about the drug release from the implant that should be included.

9- there is no interesting conclusion coming from the effect of type and the concentration of crosslinks on the physicochemical and the drug release properties.

[REVIEWER]3:

The authors review the the effect of different cross link agent glutaraldehyde and genipin in composites to the physicochemical characteristics and the release of

ciprofloxacin implant. This issue is of potential interest and pharmaceutical interest and inputs new insight to the understanding of the topic. However, a few points must be considered prior to publication.

1. There are numerous grammatical points and the manuscript requires a thorough English editing prior to publication.

2. The authors have discussed the availability of retard ciprofloxacin release. The clinical importance has been explained in the introduction. It

would be better to describe the potential clinical benefits in the discussion section.

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Reply to the reviewers' comments

Reviewer Number	Original comments of the reviewer	Reply by the author(s)	Changes done on page number and line number
1	In the " ***Introduction***" the authors should introduce the use of chitosan and hydroxyapatite for bone tissue engineering	Chitosan as organic material and Bovine Hydroxiapatite as inorganic material were used in this research to increase mechanical strength and bone bioactivity in implants [3]. Glutaraldehyde [8] and genipin [9] were used as cross-linker. The objective of this study was to determine and evaluate a controlled release implant of ciprofloxacin using Bovine Hydroxyapatite -chitosan composite and glutaraldehyde or genipin as cross-link agent according to drugs characteristic.	Page 3, 128-133
1	The genipin and glutaraldehyde crosslinked the chitosan, what's the function of Bovine Hydroxyapatite in the implant? In the FTIR and XRD following BHA in the formulations is not clear and should be discussed.	Bovine Hydroxiapatite as inorganic material were used in this research to increase mechanical strength and bone bioactivity in implants [3].	
1	The molecular weight of chitosan should be noted in the materials.	Chitosan Medical Grade (Biotech Surindo). Molecular weight varied from 30-1000 KD according to CoA.	Page 4, 147
1	"***Mechanism of crosslinking***" should be	Already moved to RESULT	Moved to page 6-7,

	transformed to the Result section.		278-288
1	For determining the porosity, the authors should display BET test. The implant has a hydrogel structure and adsorbs the water, so using water for porosity test is incorrect.	There is no spesific equipment available for determining the porosity	
1	Equation 2 and Equation 4 are the same. This equation is used for determining the swelling ratio and should be omitted from porosity test.	**Porosity (%) = Ww-Wi/implant volume** (Eq. 2) Where Ww is the wet weight of implant and Wi is the initial weight of implant. Implant volume calculated from pellet thickness multiplied with implant surface area. Implant are cylindrical form with 4.0 mm diameter and 0.525 mm thickness.	Page 5, 197-202
1	For determining the swelling ratio, the duration time of immersion of implants in the buffer should be noted.	The duration time of immersion of implants in the buffer was 1-minute	Page 5, 207
1	"***Degradation test***" should be changed to the "disintegration test".	Already revised	Page 5, 223
1	The results of ***Degradation test*** were not shown.	"Disintegration test" was done by visual observation after implant have been crosslinked with glutaraldehyde and genipin	Page 7, 310-311
1	Analysis of ciprofloxacin was performed at 3 wavelengths, the authors display about this method.	Multiple wavelengths with three different wavelengths is a spectrophotometric method to eliminate	

		interference absorbance from implant excipients in the determination of ciprofloxacin. The measurement was done for maximum wavelength, 10 nm above, and 10 nm below. (Cazedey and Salgado, 2012).	
1	In "***In vitro drug release study"*** the volume of release medium is very low (5 ml) in comparison to the sampling volume (1 ml), and 20% of the medium was replaced with fresh buffer at each sampling point time. This can cause error.	Implants were meant to be used in bones. Bones contain 31% water, much less than skin and muscles. The addition buffer was homogenized first before sampling. As a result, it didn't interfere with the sampling process.	
1	X and Y axis in Fig 3 should be corrected. Cumulative drug released <u>percent</u> against time should be plotted not cumulative drug released <u>concentration</u>. Indeed, number of days should not have decimal points.	In this research, cumulative drug release isn't calculated in percent against time because concentration are related with daily measurement to calculate the sustain release in the drug itself.	
1	The authors didn't discuss why different formula released the drug with the same manner?	There are many factors that influence the drug release profile of the implants. This includes drug concentration in the dosage form, drug solubility, and drug-carrrier interaction. In this reseach, ciprofloxacin	Page 10, 468-473

		interact with implant's composite [24,30.31]. Therefore, drug release study showed that ciprofloxacin release from bone implant with glutaraldehyde crosslinker with different concentration have no significant difference. In the same way, with difference crosslinker (genipin) aslo showed no significant difference. Added in DISCUSSION	
1	The SEM images were not uploaded correctly.	Reupload SEM image	
1	At the end of discussion the authors should conclude about the study.	To conclude with, bone implants with ciprofloxacin using Chitosan-BHA composite and crosslinker gluaraldehyde and genipin 0.7% showed the best result. Therefore, the release of ciprofloxacin for 30 days meet the standard requirements (2-50 mg). Glutaraldehyde or genipin 0.7% had potential effect to retard ciprofloxacin release from Bovine Hydroxyapatite-chitosan-ciprofloxacin implant for 30 days in the treatment of osteomyelitis.	Page 10, 474-478
1	At the lines number of 161, 238,260, please change "," to "."	Already changed	
1	At the line numbers of 105 please change the red	Already changed	

	point to black point.		
2	the abstract needs re-writing with respect to the structure and the content. It also contains many typos and grammatical errors.	Already revised	
2	incomplete literature review! the papers on chitosan-hydroxyapatite hydrogel hasn't been addressed.	Chitosan-hydroxyapatite hydrogel reviewed in reference [9, 20, 21]	
2	the aim of study has not been well defined at the end of the introduction section.	The objective of this study was to determine and evaluate a controlled release implant of ciprofloxacin using Bovine Hydroxyapatite-chitosan composite and glutaraldehyde or genipin as cross-link agent according to drugs characteristic.	Page 3, 131-133
2	there are some ambiguous parts in the materials and methods section that needs to be revised or explained: homogeneous chitosan powder (homogeneous?), the compression pressure of 2 tons, mechanism of crosslinking section (unrelated to the materials and methods section), the calculation of hydrogel porosity, assay of drug content by extrapolating of the UV-Vis calibration curve constructed at 3 wavelengths, the mechanical strength of the implant which has only been tested in term of hardness in dry state.	homogeneous chitosan powder is grinded chitosan into a powdery form.	
2	some parts in the results section are related to the	According to the RPS journal, RESULT	

	discussion. P values have only been presented in the discussion, so it is suggested to combine the results and the discussion sectons.	and DISCUSSION section must be separated .	
2	there are some justifications that have to be re-considered: molecular dispersion of ciprofloxacin in the XRD experiment (or may be only an amorphous drug state), no free glutaraldehyde has been remained (FTIR of free glutarladehyde at the corresponding amount is missing), etc.	Ciprofloxacin shifting into amorphous is an important factor because ciprofloxacin release depends on the drug solubility.	
2	the discussion section has been started with the non-significant density findings. It is recommended to begin this section with what the study is concerned about and also to discuss only about the most important findings.	Already added The result of this research is to obtain bone implant with ciprofloxacin as an active ingredient and chitosan-BHA composite. Hopefully, with the addition of genipin and glutaraldehyde as cross-linker, the implant has good physical characteristics and controlled drug release. In the beginning, implant was characterized. After that, the release of ciprofloxacin from the implant was observed.	Page 9, 390-394
2	there is no discussion about the drug release from the implant that should be included.	There are many factors that influence the drug release profile of the implants. This includes drug concentration in the dosage form, drug solubility, and drug-carrrier interaction. In this research, ciprofloxacin	Page 10, 468-474

		interact with implant's composite [24,30.31]. Therefore, drug release study showed that ciprofloxacin release from bone implant with glutaraldehyde crosslinker with different concentration have no significant difference. In the same way, with difference crosslinker (genipin) also showed no significant difference. Added to DISCUSSION	
2	there is no interesting conclusion coming from the effect of type and the concentration of crosslinks on the physicochemical and the drug release properties.	This research showed that bone implant with genipin cross-linker with the concentration of 0.3%, 0.5%, and 0.7% has potential in the treatment of osteomyelitis. Genipin also less toxic material compared with other cross-linker available	
3	There are numerous grammatical points and the manuscript requires a thorough English editing prior to publication.	Already revised	
3	The authors have discussed the availability of retard ciprofloxacin release. The clinical importance has been explained in the introduction. It would be better to describe the potential clinical benefits in the discussion section.	Glutaraldehyde or genipin 0.7% had the potential effect to retard ciprofloxacin release from Bovine Hydroxyapatite-chitosan-ciprofloxacin implant for 30 days in the treatment of osteomyelitis.	Page 10, 476-478

		Added in the last section of DISCUSSION	
4	Please provide ACKNOWLEDGEMENT section if applicable.	Added	Page 11, 489-494

The effect of different cross link agent glutaraldehyde and genipin in composites to the physicochemical characteristics and the release of ciprofloxacin implant

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Abstract

The objective of this study was to determine and evaluate a controlled release implant of ciprofloxacin using Bovine Hydroxyapatite-chitosan composite and glutaraldehyde or genipin as cross link agent. Ciprofloxacin implants were prepared using Bovine Hydroxyapatite-chitosan-ciprofloxacin composition 30:60:10. This composite was further developed using three different concentrations of glutaraldehyde or genipin (0.3%, 0.5%, and 0.7%). Implants were formed into pellets with 4.0 mm diameter and weighed 100.0 mg using compression method. Further, the prepared ciprofloxacin implant was characterized for porosity, density, water absorption capacity, swelling ratio, degradation test, compressive strength, compatibility studies (FT-IR), morphology (SEM), X-ray diffraction study, assay, and in vitro drug release.

The addition of glutaraldehyde or genipin as cross-link agent in ciprofloxacin implant showed controlled release profile of ciprofloxacin over a time period 30 days. This is due to glutaraldehyde or genipin formed compact structure so the porosity, water absorption capacity, and swelling ratio of the implant decreased. SEM photomicrograph revealed low porosity of the implant after cross-link with glutaraldehyde or genipin. The FTIR study confirmed the formation of covalent imine bonds between chitosan and glutaraldehyde. Moreover, the addition of glutaraldehyde or genipin as cross-link agent caused a decrease in the mechanical strength of the implant. Increased concentration of glutaraldehyde or genipin reduced the crystallinity of BHA and chitosan, which were confirmed by XRD studies. The results obtained from this study indicated that glutaraldehyde or genipin 0.7% had the potential effect to retard ciprofloxacin release from Bovine Hydroxyapatite-chitosan-ciprofloxacin implant for 30 days in the treatment of osteomyelitis.

Keywords: Implant ciprofloxacin; Cross-linker; Glutaraldehyde; Genipin; Released.

INTRODUCTION

Bones are an essential part of human body which have an important role in supporting the physiological functions of the body [1]. Complications of bone disease and bone disorder caused by traumatic accidents may result in the presence of bone defect. The healing process of bone damage or bone fracture is determined by the level of trauma and soft tissue damage [2]. Some cases of bone damage or bone injury cannot be experienced naturally repair [1]. Therefore, clinical rehabilitation to overcome bone defect is expected to rise along with population growth [3].

Rehabilitation of bone defect cannot be separated from the risk of infection. The number of complication varied from 1% in the case of total joint replacement until 23% in the case of bone fracture [4]. The cause of infection complication is the entry of the bacteria into the bone tissue through the defects. The infection occurred because of a less sterile surgical process, bacteria that adhere to the implant, bacteria in human skin, open wounds, and circulatory patients suffering from infection elsewhere [3,5].

The occurrence of bacterial infection can be corrected by administering antibiotics. However, tissue devascularization in the case of bone defect caused the antibiotic transfer to the target site become stunted. This condition caused the concentration of antibiotic in tissue target become low so that the antibiotic is not able to penetrate into the biofilm layer of bacteria. In consequence, bacterial resistance occurs in the target tissue [6]. One way to overcome the risk of infection is giving the antibiotic in oral or intravenous route for a long period.

To overcome these problems, administering antibiotic can be done locally by using specific drug delivery system. The purpose of this drug delivery system is providing drug concentration at a specific location and ensuring drug release profile for a long period [7]. Local drug delivery system has several advantages (a) reduce systemic effect, (b) the number of drugs are used less and secure, and (c) efficacy and efficient to deliver the drug [8]. Administering antibiotic locally can also minimize the side effects and the risk of toxicity than administering antibiotic systemically. In addition, administering antibiotic locally also cause high antibiotic concentration in target tissue [3]. The release of antibiotic on the target tissue is expected to take place continuously for a specific period and the concentration is higher than *minimum inhibitory concentration* (MIC). Controlled release system also can enhance the bioavailability of antibiotic in the target tissue. This system is designed to release the drug in the target tissue with appropriate rate during specific period [3]. The release of the drug from implants is affected by the composite. A composite component that regulates the release of the drug is cross-linker.

Chitosan as organic material and Bovine Hydroxyapatite as inorganic material were used in this research to increase mechanical strength and bone bioactivity in implants [3]. Glutaraldehyde [8] and genipin [9] were used as cross-linker.

The objective of this study was to determine and evaluate a controlled release implant of ciprofloxacin using Bovine Hydroxyapatite-chitosan composite and glutaraldehyde or genipin as cross-link agent according to drugs characteristic.

MATERIALS AND METHODS

Materials

Ciprofloxacin (Shangyu Jingxin Pharmaceutical Co. Ltd) ; Bovine Hydroxyapatite (BHA) from Tissue Bank RSUD DR Soetomo Surabaya; Chitosan (Biotech Indonesia) Molecular weight 30-1000 kDalton; glutaraldehyde 25% p.a (Merck Millipore-German); Genipin was obtained from Challenge Bioproduct Co., Ltd., Taiwan. Acetic acid glacial p.a (Merck), KBr IR (for Spectroscopy) Na_2HPO_4 p.a (Merck), K_2HPO_4 p.a, KH_2PO_4 p.a, NaCl p.a (Merck-German) and Aquabidestilata

Preparation of homogeneous chitosan powder

Homogenous chitosan powder was obtained by dissolving chitosan flakes in acetic acid solution (1%) v/v. The solution was stirred at 400 rpm on a mechanical stirrer for 24 hours to obtain chitosan solution with 2% w/v concentration. 1 M NaOH solution was added into chitosan solution until the pH reached neutral (pH =7). After the addition of NaOH solution into chitosan solution, chitosan gels could be obtained. Chitosan gels were dried at 40°C for 24 hours. The dried chitosan gels were sieved by 1 mm sieve to produce homogeneous chitosan powder.

Formulation of Bovine Hydroxyapatite-chitosan-ciprofloxacin implant using glutaraldehyde or genipin as cross link agent

The implant produced by compression method. Ciprofloxacin was dissolved in aquabidestilata, Bovine Hydroxyapatite added gradually and mixed until homogen with ciprofloxacin. Chitosan powder was added to Ciprofloxacin-Bovine Hydroxyapatite blend and mixed until homogen. Aquabidest were added gradually with continuous stirring until form wet granules mass. Wet granules mass were sieved using 1 mm siever and dried overnight (24 hours) at 40 °C to obtain dried granules. Dried granules were immersed in glutaraldehyde solution or genipin solution (0.3%, 0.5%, and 0.7% concentration) for 24 hours until the color was changed. The composition of various formulations was mentioned in Table 1. Granules were washed with aquabidest to remove the residual glutaraldehyde and genipin. At the final stage, granules were washed with phosphate buffer saline (PBS) pH 7.40. Granules were dried in an oven at 40 °C for 24 hours. Dried granules were weighed 100 mg, pressed using tablet press machine with 4.0 mm diameter and the compression pressure was 2 tons.

Evaluation of implant

Density test

The density of the implant was calculated from the weight of the implant (in the dry state) divided by volume of the implant [10,11]. The density of the implant could be calculated using Eq. (1).

$$\text{Density} = \frac{Wi}{V} \quad \text{(Eq. 1)}$$

Where Wi is the weight of the implant at the initial condition, V is the volume of the implant.

Porosity test

The implant was weighed in the initial condition (in the dry state), then the implant was placed in 5 ml water for 1 minute. The implant was taken out from the water after 1 minute immersion process and placed in filter paper to remove the excess water on the surface of the implant. The implant was weighed again to obtain the wet weight [10,11]. The porosity of the implant could be calculated using Eq. (2).

Porosity (%) = Ww-Wi/implant volume (Eq. 2)

Where Ww is the wet weight of implant and Wi is the initial weight of the implant. Implant volume calculated from pellet thickness multiplied with implant surface area. An implant is a cylindrical form with 4.0 mm diameter and 0.525 mm thickness.

Water absorption capacity and swelling ratio

The implant was weighed in the initial condition (dry state), then implant was immersed in 5 ml phosphate buffer saline (PBS) pH 7.4 for 1 minute at temperature 37 °C ±.0.5 °C. The implant was withdrawn and gently blotted with filter paper to remove the excess water and weighed again [10-13]. The percentage of water absorption capacity and swelling ratio of the implant was calculated using Eq. (3) and Eq. (4).

$$\text{Water absorption capacity} = \frac{Ww-Wi}{Wi} \times 100 \quad \text{(Eq. 3)}$$

$$\text{Swelling ratio} = \frac{Ww-Wi}{Ww} \times 100 \quad \text{(Eq. 4)}$$

Where Wi is the weight of implant in dry state and Ww is the weight of the implant after immersion process in phosphate buffer saline (PBS) pH 7.40.

Hardness test

The implant was pressed by load cell compression machine 5 mm/min by autograph E-10 instrument. The hardness of the implant obtained from the force (F in newton unit) which was displayed at the instrument divided by contact surface area of the implant (in mm unit) [14].

Disintegration Test

Implant was immersed in 5 ml phosphate buffer saline pH 7.4 at 37 °C ± 0.5 °C. Visual inspection was done to observe the changing of implant structure which was caused by erosion and degradation [14-16].

Evaluation of implant morphology using scanning electron microscope (SEM)

Morphology of the implant was observed using scanning electron microscope (SEM). The samples were fitted to aluminum stubs with conductive paint and were sputter-coated with gold [17]. The difference of implant morphology before and after cross-linking process was observed using specific magnification.

Drug content

The implant was placed in a mortar and milled, then transferred into Erlenmeyer flask. 100 ml HCL 0,1 N was added into Erlenmeyer flask which contained milled implant and stirred for 24 h (400 rpm) until form suspension. The suspension was filtrated, and the filtrate was diluted to determine ciprofloxacin concentration. The absorbance of this solution was observed using spectrophotometer UV-Vis at three wavelengths (260 nm, 270 nm, and 280 nm). Δ Absorbance which was obtained from the observation extrapolated in standard curve equation to obtain ciprofloxacin HCL concentration. Determination of ciprofloxacin content in the implant was done triplicate [18].

In vitro drug release study

The implant was placed in a vial containing 5 ml of phosphate buffer saline (PBS) pH 7.4. The vial was placed on a shelf and incubated in water bath at 37 °C ± 0.5 °C. Sampling was conducted by pipetting 1 ml of elution fluids at predetermined time intervals (1^{st}, 3^{rd}, 5^{th}, 7^{th}, 9^{th} , 12^{th} , 14^{th} , 16^{th} , 18^{th} , 20^{th} , 22^{th} , and 24^{th} h on first day and every 24 h for 30 days) and replaced with fresh buffer to maintain sink condition. The sample solution was filtered with Millipore membrane (ø = 0.45 µm). Appropriate dilution was prepared using phosphate buffer saline (PBS) pH 7.4. The absorbance of the solution was analyzed using UV spectrophotometer at three wavelengths (260 nm, 270 nm, and 280 nm). Cumulative percent drug release was found at each time interval. The release of ciprofloxacin HCL from the implants was assayed in triplicate [16,19].

Data analysis

The results of implant evaluation (density, porosity, swelling ratio, water uptake, hardness, and AUC of in vitro release profile) was statistically analyzed using one way Analysis of Variance (ANOVA) with 95% confidences interval.

Characterization of the implant

Fourier transform infrared (FTIR) spectroscopy

A sample of the implant was combined with KBr and pressed into a pellet. The solid pellet was analyzed using FT-IR spectroscopy in the wave number range 4000-400 cm^{-1} [17].

X-ray diffraction study

The X-ray diffraction study was carried out to determine the crystal phases of the implant using monochromatic Cu Kα radiation (40 KV, 30 MA). X-ray diffraction peaks of the implants were compared to the diffraction peaks of pure materials (ciprofloxacin HCL, Bovine Hydroxyapatite, and chitosan) in 2θ scan range 5-50° [17].

RESULTS

Mechanism of crosslinking

Mechanism of glutaraldehyde crosslinking

Aldehyde groups of glutaraldehyde (C=OH) react with chitosan amine groups ($-NH_2$) produced covalent crosslinking through a Schiff base reaction [8, 20,21].

Mechanism of genipin crosslinking

The crosslinking reaction mechanism between chitosan and genipin occurred in low acidic and neutral conditions. A nucleophilic attack by the amino groups of chitosan on the olefinic carbon atom at C-3 occurred, this condition followed by opening the dihydropyran ring and attacked by the secondary amino group on the newly formed aldehyde group. In other words, genipin act as a dialdehyde [9].

Density of implant

The result of density test on the implants that have been cross-linked using glutaraldehyde and genipin can be seen in Fig. 1

Porosity of implant

The porosity of the implants that have been cross-linked using glutaraldehyde and genipin can be seen in Fig. 1.

Water absorption capacity

Water absorption capacity of the implants that have been cross-linked using glutaraldehyde and genipin can be seen in Fig. 1.

Swelling ratio of implant

The swelling ratio of the implants that have been cross-linked using glutaraldehyde and genipin can be seen in Fig. 1.

Hardness of implant

The result of hardness test on the implants that have been cross-linked using glutaraldehyde and genipin can be seen in Fig. 1.

Disintegration test

"Disintegration test" was done by visual observation after implant have been crosslinked with glutaraldehyde and genipin. The ***Disintegration*** profile of implants with three different concentrations of glutaraldehyde showed that formula with the lowest disintegration was F3-0.7% glutaraldehyde. At the opposite, F3-0.3% glutaraldehyde showed greater disintegration than two others formula. The lower concentration of glutaraldehyde as a cross-linking agent caused hydrolysis process in polymer chains inducing erosion process [22]. The increase of glutaraldehyde concentration caused an increase in cross-link density. Implants with higher cross-link density had lower hydrophilic groups, so that the structure of the implants became difficult to extend in water [23].

Evaluation of implant morphology using scanning electron microscope (SEM)

SEM micrograph of the implant that has been cross-linked using glutaraldehyde and genipin are presented in Fig. 2. Based on the micrograph, it could be seen that there was small pores in the structure. These pores facilitate the release of ciprofloxacin from the implants.

Drug content

The result of ciprofloxacin HCL content in an implant that has been cross-linked using glutaraldehyde and genipin can be seen in Table 2. Drug content of all formulations was

determined by UV spectrophotometer using three-wavelength methods.

In vitro drug release study

The cumulative amount of ciprofloxacin that has been released in PBS pH 7.40 from the implant that has been cross-linked using glutaraldehyde and genipin could be seen in Fig 3. The release profile of ciprofloxacin HCL from the implants showed that ciprofloxacin release was at a therapeutic level of ciprofloxacin for osteomyelitis (2-50 µg/ml) [24, 25]. This condition could be kept for 30 days.

Fourirer transform infrared (FTIR) spectroscopy

The infrared spectrum of ciprofloxacin, Bovine Hydroxyapatite, chitosan, implants Bovine Hydroxyapatite-chitosan-ciprofloxacin before the cross-linking process, and implants Bovine Hydroxyapatite-chitosan-ciprofloxacin after the cross-linking process with three different concentrations of glutaraldehyde and genipin can be seen in Fig. 4. FT-IR spectrum of Bovine Hydroxyapatite-chitosan-ciprofloxacin implant after crosslinking process using glutaraldehyde showed a peak shift characteristics of chitosan on wavenumbers 1658,67 cm^{-1} (C=O stretching in amide group) to the lower wavenumbers ~ 1630 cm^{-1}. This band is most probably composed of amide I band of chitosan (appears at 1658.67 cm^{-1}) and the C=N stretching band of Schiff's base that according to the literature appears at wave number 1620-1660 cm^{-1} [8]. Moreover, the peak characteristic of aldehyde could not be seen in the FT-IR spectrum of Bovine Hydroxyapatite-chitosan-ciprofloxacin implant after crosslinking process using glutaraldehyde. This condition showed that the implant did not contain free aldehyde group. Based on the results of the FT-IR spectrum, it was known that there was a shift of the N-H stretching vibrations and O-H stretching vibrations from chitosan molecules. In addition, the loss of peak at wave number 1363 cm^{-1} (the vibration bending of CH_3) and 1155 cm^{-1} (the vibration bending of C-O-C) observed in FT-IR spectrum of the implant compared to FT-IR spectrum of pure chitosan. FT-IR spectrum of Bovine Hydroxyapatite-chitosan-ciprofloxacin implant that has been cross-linked using genipin also can be seen in Fig. 4. The spectrum showed a characteristics peak shift of chitosan to the lower wavenumbers. Characteristic peak of chitosan on wavenumbers 1639,55 cm^{-1} (C=O stretching of amides group) shift to the lower wavenumbers 1622.19 cm^{-1} at 0.7% genipin concentration, 1637.62 at 0.5% genipin concentration, and 1622.19 cm^{-1} at 0.3% genipin concentration. Increased of genipin concentration caused an increase of C=C bond intensity of genipin.

X-ray diffraction study

X-ray diffraction of the implants after cross-link using glutaraldehyde can be seen in Fig. 5. Based on the results, it was known that the characteristics peak of ciprofloxacin in 2θ 8,2°; 9,0°; 19,3°; 19,8°; and 26,5° did not appear in a diffraction spectrum of Bovine Hydroxyapatite-chitosan-ciprofloxacin implant. This condition indicated that ciprofloxacin was molecularly dispersed in the implant. X-ray diffraction of the implant after cross-link using glutaraldehyde showed that the peak intensity of BHA in $2\theta \approx 26°$ and $2\theta \approx 32°$ decreased compared to X-ray diffraction of pure BHA and the implants before the cross-linking process. The X-ray pattern of chitosan shows major crystalline peaks at $2\theta \approx 10°$ and $2\theta \approx 20°$. But, the X-ray diffraction of the implants indicated that these peaks became wider and weaker. The decrease crystallinity of chitosan molecules caused by the deformation of hydrogen bond in the molecular structure of chitosan. Substitution of glutaraldehyde molecules destroyed the regular structure of chitosan molecules so that the structure of chitosan molecules became amorph [26]. A similar case also happened on the Bovine Hydroxyapatite diffraction. The addition of glutaraldehyde damage regularity on Bovine

Hydroxyapatite crystal lattice. The decreased of Bovine Hydroxyapatite crystallinity in line with the increased of glutaraldehyde concentration.

DISCUSSION

The result of this research is to obtain bone implant with ciprofloxacin as an active ingredient and chitosan-BHA composite. Hopefully, with the addition of genipin and glutaraldehyde as cross-linker, the implant has good physical characteristics and controlled drug release. In the beginning, implant was characterized. After that, the release of ciprofloxacin from the implant was observed.

The density of the implants after the cross-linking process with glutaraldehyde was lower than before cross-linking process. Moreover, the result of statistical analysis using one-way ANOVA showed that there was no significant difference in density between the implants which used three different concentration of glutaraldehyde ($P > 0.05$). Based on this result, it could be concluded that the difference of glutaraldehyde concentration did not affect the implant density. On the other hand, statistically, analysis using one-way ANOVA showed that there was a significant difference of implant density before and after cross-link using genipin ($P < 0.05$). The result of statistical analysis using one-way ANOVA showed that there was no significant difference of density between the implants which used three different concentration of genipin ($P > 0.05$). Based on this result, it could be concluded that the difference of genipin concentration did not affect the implant density

The result of statistical analysis using one-way ANOVA to the formula that using three different concentrations of glutaraldehyde showed that there was a significant difference of porosity between F3-0.3% glutaraldehyde and F3-0.7% glutaraldehyde ($P < 0.05$). Increasing glutaraldehyde concentration of 0.7% caused an increase of porosity than 0.3% glutaraldehyde. The increase of glutaraldehyde concentration caused the structure of the implants became looser. Higher concentration of glutaraldehyde led the structure of the implant became amorph, so that the porosity of the implant increased [27].

Statistically, analysis using one way ANOVA showed that there was a significant difference of porosity before and after cross-link using genipin ($P < 0.05$). The porosity of F3 was significantly different with F3-0.3% genipin and F3-0.7% genipin. In addition, the result of statistical analysis using one-way ANOVA to the formula that using three different concentration of genipin showed that there was a significant difference of porosity between F3-0.3% genipin and F3-0.5% genipin. Increasing genipin concentration of 0.5% caused a decrease in porosity than 0.3% genipin. The increase of genipin concentration caused the structure of the implants became more compact. Genipin affected network size which was formed between chitosan chain. The increase of genipin concentration led to the size of the network became smaller, so that the porosity of the implant decreased.

Water absorption capacity of the implants after the cross-linking process was higher than before cross-linking process. The result of statistical analysis using one way ANOVA to the formula that using three different concentrations of glutaraldehyde showed that there was a significant difference of water absorption capacity between F3-0.3% glutaraldehyde and F3-0.7% glutaraldehyde ($P < 0.05$). The increase of glutaraldehyde concentration to 0.7% caused markedly decrease water absorption capacity. Cross-linking process with

glutaraldehyde restricted water molecules to enter into chitosan structure [28]. On the other hand, statistically, analysis using one-way ANOVA showed that there was a significant difference of water absorption capacity before and after cross-link using genipin ($P < 0.05$). Water absorption capacity of the implants after the cross-linking process with genipin was higher than before cross-linking process. The result of statistical analysis using one-way ANOVA to the formula that using three different concentrations of genipin showed that there was no significant difference of water absorption capacity between three formulas. Based on this results, it could be concluded that the difference of genipin concentration did not affect water absorption capacity of the implants.

The result of statistical analysis using one way ANOVA to the formula that using three different concentrations of glutaraldehyde showed that there was no significant difference of swelling ratio among three formulas ($P > 0.05$). On the other hand, there was a significant difference of swelling ratio of the implant, before and after cross-link using genipin ($P < 0.05$). The swelling ratio of the implants after the cross-linking process was higher than before cross-linking process. Statistically, analysis using one way ANOVA showed that there was no significant difference of swelling ratio between three formulas which used three different concentration of genipin ($P > 0.05$)

Cross-linking process using glutaraldehyde caused the characteristic of biomaterial became brittle. Increasing glutaraldehyde as cross-link agent more than 0.2 % decrease the mechanical strength of the implants [29]. The hardness of the implant after cross-link using genipin was lower than before cross-link. Based on statistical analysis using one-way ANOVA, it could be found that there was a significant difference of implant hardness before and after the cross-linking process. The result of statistical analysis using one-way ANOVA to the formula that using three different concentrations of genipin showed that there was no significant difference of hardness between three formulas.

The disintegration profile of implants with three different concentrations of glutaraldehyde showed that formula with the lowest degradation was F3-0.3% genipin. At the opposite, F3-0.7% genipin showed greater disintegration than two others formula. In the specific case, the increased of cross-link agent concentration caused a decrease of material crystalinity. The cristalinity of F3-0.7% was lower than F3-0.3%, so that the ability of water to penetrate in implants structure became easier and the implants degrade easily.

There was a change of diffraction pattern before and after cross linking process using genipin. This phenomenon indicated a change in the degree of crystallinity. Characteristic peak of chitosan in 2θ 10° and 20° did not appear in the implant diffraction. In addition, characteristic peak of Bovine Hydroxyapatite in 2θ 26° and 32° decreased compare to the diffraction of pure Bovine Hydroxyapatite and implant before cross linking process. The increased of genipin concentration in the implant caused a decrease of cristalinity. This can be observed through a decreased of peak intensity in line with the increased of genipin concentration.

There are many factors that influence the drug release profile of the implants. This includes drug concentration in the dosage form, drug solubility, and drug-carrrier interaction. In this research, ciprofloxacin interact with implant's composite [24,30.31]. Therefore, drug release study showed that ciprofloxacin release from bone implant with glutaraldehyde crosslinker with different concentration have no significant difference. In the same way, with difference crosslinker (genipin) also showed no significant difference.

To conclude with, bone implants with ciprofloxacin using Chitosan-BHA composite and crosslinker glutaraldehyde and genipin 0.7% showed the best result. Therefore, the release of ciprofloxacin for 30 days meet the standard requirements (2-50 mg). Glutaraldehyde or genipin 0.7% had the potential effect to retard ciprofloxacin release from Bovine Hydroxyapatite-chitosan-ciprofloxacin implant for 30 days in the treatment of osteomyelitis.

CONCLUSION

The results obtained from this study indicated that glutaraldehyde or genipin 0.7% had potential effect to retard ciprofloxacin release from Bovine Hydroxyapatite-chitosan-ciprofloxacin implant for 30 days in the treatment of osteomyelitis.

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REFERENCES

1. Porter JR, Ruckh TT, Popat KC, Bone tissue engineering : A review in bone biomimetics and drug delivery strategies. Biotechnol. Prog 2009;25:1539-1553.
2. Strobel C, Bormann N, Romacker A, Schmidmaier, G, Wildemann B. Sequential release kinetics of two (gentamicin and BMP-2) or three (gentamicin, IGF-1 and BMP-2) substances from a one component polymeric coating on implants. J Controlled Release 2011;156:37-45.
3. Mourino V, Boccaccini AR. Bone tissue engineering therapeutics : controlled drug delivery in three-dimensional scaffolds. J R Soc Interface 2010;7:209-27.
4. Cui, X., Gu, Y., Wang, H., Xie, Z., Luo, S., Zhou, N., Huang, W., and Rahaman, M. In vitro bioactivity, cytocompatibility, and antibiotic release profile of gentamicin sulfate-loaded borate bioactive glass/chitosan composites. J Mater Sci 2013;24,2391-2403.
5. Gogia JS, Meehan JP, Cesare PE, Jamali AA. Local Antibiotic Therapy in Osteomyelitis. Semin Plast Surg 2009;23:100-107.
6. Li B, Brown KV, Wenke JC, Guelcher SA. Sustained release of vancomycin from polyurethane scaffolds inhibits infection of bone wounds in a rat femoral segmental defect model. J Controlled Release 2010;145:221-230.
7. Dubnika A, Loca D, Cimdina LB. Functionalized hydroxyapatite scaffolds coated with sodium alginate and chitosan for controlled drug delivery. Proc Est Acad Sci 2012;61:193-199.
8. Ostrowska J, Pierog M, Gierszweka M. State of water in non crosslinked and crosslinked hydrogel chitosan membranes. Prog Chem Appl Chitin Its Deriv 2011;16:147-156.
9. Muzzarelli RAA. Genipin-crosslinked chitosan hydrogel as biomedical and pharmaceutical aids. Carbohyd Polym 2009:77:1-9.
10. Lei L, Li L, Zang L. Structure and performance of nano-hydroxyapatite filled biodegradable poly(1,2-propanediol-sebacate)-citrate) elastomer. Polym Degrad Stab 2009;94:1494-1502.
11. Liuyun J, Yubao L, Chengdong X. Preparation and biological properties of a novel composite scaffold of nano-hydroxyapatite/chitosan/carboxymethyl cellulose for bone

tissue engineering. J Biomed Sci 2009;16:1-10.
12. Campos MG, Rawls HR, Innocentini-Mei LH, Satsangi N. In vitro gentamicin sustained and controlled release from chitosan cross-linked films. J Mater Sci : Mater Med 2009;20:537-542.
13. Ashish P, DerleDeelip V. Development of cost effective biodegradable implants of ciprofloxacin hydrochloride in treatment of osteomyelitis. Asian J Pharm 2012;2:19-37.
14. Budiatin SA, Zainuddin M, Khotib J. Biocompatible composite as gentamicin delivery system for osteomyelities and bone regeneration. Int J Pharm Sci 2014;6:1-3.
15. Yang B, Li XY, Shi S, Kong X, Guo G, Huang M. Preparation and characterization of a novel chitosan scaffold. Carbohydr Polym 2010;80:860-865.
16. Teng SH, Lee EJ, Wang P, Jun SH, Han CM, Kim HE. Chitosan/nanohydroxyapatite composite membranes via dynamic filtration for guided bone regeneration. J Biomed Mater Res Part B 2008;88:569-579.
17. Han WW, Misr a RD. Biomimetic chitosan-nanohydrixyapatite composite scaffolds for bone tissue engineering. Acta Biomater 2009;5:1182-1197.
18. Ashish YP, Derle DV, Gandhi PP, Nade VN, Patel JS. In vitro and in vivo characterization of a novel biocompatible implants system for the sustained delivery of ciprofloxacin hydrochloride. J Pharm Res 2010;3:2122-2126.
19. Stallmann HP, Faber C, Bronckers AL, Amerogen AV, Wuisman PI. In vitro gentamicin release from commercially available calcium-phosphate bone substitute influence of carrier type on duration of the release profile. BMC Musculoskeletal disorders 2006;7:1-8.
20. Berge J, Reist M, Maayer JM, Felt O, Peppas NA, Gunny R. Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications. Eur J Pharm Biopharm 2004:57:19-34.
21. Shweta A, and Sonia P. Pharmaceutical and relevance of crosslinked chitosan in microparticulate drug delivery. Int Res J Pharm 2013:4:45-51.
22. Goncalves VL, Laranjeira, MC, Favere VT. Effect of crosslinking agents on chitosan microspheres in controlled release of diclofenac sodium. Polim : Cienc Tecnol 2005;15:6-12
23. Prabaharan M, Perez MA, Saja JA, Mano JF. Preparation and characterization of poly(L-lactic acid)-chitosan hybrid scaffolds with drug release capability J Biomed Mater Res Part B 2006;88:427-434.
24. Makinen TJ, Veiranto M, Lankinen P, Moritz N, Jalava J, Tormala P, Aro HT. In vitro and in vivo release of ciprofloxacin from osteoconductive bone defect filler. J of Antimic Chem 2005;56:1063-1068.
25. Mont MA, Mathur SK, Carmelita G, Frondoza D, Hungerford DS. The effects of ciprofloxacin on human chondrocytes in cell culture. Infection 1996;24:151-155.
26. Li B, Shan CL, Zhou Q, Fang Y, Wang YL, Xu F, Han LR, Ibrahim M, Guo LB, Xie GL, Sun GC. Synthesis, characterization, and antibacterial activity of cross-linked chitosan glutaraldehyde 2013;11:1534-1552.
27. Silva RM, Silva GA, Coutinho OP, Mano JF, Reis RL. Preparation and characterization in simulated body conditions of glutaraldehyde crosslinked chitosan membranes. J.Mater Sci Mater Med 2004;1105-1112.
28. Sailakshmi G, Mitra T, Chatterjee S., Gnanamani, A. Chemistry behind the elastic nature of the biomaterial prepared usimg oxidized form of glutaraldehyde. Int J of Life Science and Med Res 2013;4:64-75.
29. Mirzaei BE, Ramzani SA, Shaifee M, Dannaei M. Studies on glutaraldehyde crosslinked chitosan hydrogel properties for drug delivery system. Int J Polym Mater

579 Polym 2015;62:605-611.
580 30. Stevenson, C.L., Santini, J.T., and Langer, R., 2012. Reservoir-based drug delivery
581 system utilizing microtechnology. **Advanced Drug Delivery Reviews**, Vol. 64, p.
582 1590-1602.
583 31. Sinko PJ, Singh Y. (editor). Martin Physical Pharmacy and Pharmaceutical Sciences.
584 6th Ed. Wolter Kluwer/Lippincott Williams & Wilkins, Philadelphia 2011: 300-317.

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Original Article

The effect of two different crosslinkers on *in vitro* characteristics of ciprofloxacin-loaded chitosan implants

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Abstract

The objective of this study was to determine and evaluate a controlled release implant of ciprofloxacin using bovine hydroxyapatite (BHA)-chitosan composite and glutaraldehyde or genipin as crosslinking agents. Ciprofloxacin implants were prepared using BHA, chitosan, ciprofloxacin at 30:60:10 and using three different concentrations of glutaraldehyde or genipin (0.3, 0.5, or 0.7%) as crosslinkers. Implants were formed as mini-tablet with 4.0 mm diameter weighing 100 mg using compression method. Further, the prepared ciprofloxacin implants were characterized for porosity, density, water absorption capacity, swelling, degradation, compressive strength, compatibility (Fourier transforms-infrared spectroscopy (FT-IR)), morphology (scanning electron microscope (SEM)), X-ray diffraction (X-RD), and *in vitro* drug release. The addition of glutaraldehyde or genipin as crosslinkers in ciprofloxacin implant showed controlled release profile of ciprofloxacin over a time period of 30 days. SEM photomicrograph revealed low porosity of the implant after crosslinking with glutaraldehyde or genipin. The FTIR study confirmed the formation of covalent imine bonds between chitosan and glutaraldehyde. Moreover, the addition of glutaraldehyde or genipin as crosslinkers caused a decrease in the mechanical strength of the implant. Increased concentration of glutaraldehyde or genipin reduced the crystallinity of BHA and chitosan, which were confirmed by X-RD studies. The results obtained from this study indicated that glutaraldehyde or genipin had the potential effect to retard ciprofloxacin release from BHA-chitosan-ciprofloxacin implant for 30 days.

Keywords: Ciprofloxacin implant; Crosslinker; Glutaraldehyde; Genipin

INTRODUCTION

Bones are essential parts of human body which have an important role in supporting the physiological functions of the body (1). Complications of bone disease and bone disorder caused by traumatic accidents may result in the presence of bone defect. The healing process of bone damage or bone fracture is determined by the level of trauma and soft tissue damage (2). Some cases of bone damage or bone injury cannot be naturally repaired and healed (1). Therefore, clinical rehabilitation to overcome bone defect is expected to rise along with population growth (3).

Rehabilitation of bone defects is associated with the risk of infection. The number of complications varied from 1% in the case of total joint replacement to 23% in the case of bone fracture (4). The cause of infection complication is the entry of the bacteria into the bone tissue through the defects. The infection occurs because of a less sterile surgical process, bacteria adhering to the implant, bacteria in human skin, open wounds, and circulatory patients suffering from infection elsewhere (3,5). The occurrence of bacterial infections can be treated by administering antibiotics. However, tissue devascularization in the case of bone defect limits the delivery of the antibiotic to the target site. This condition leads to the lower antibiotic concentration in the target tissues and consequently the antibiotic is not able to penetrate into the bacteria biofilm layer. This may lead to the bacterial resistance in the target tissue (6).

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To overcome these problems, antibiotics can be delivered locally by using specific drug delivery systems.

The purpose of such drug delivery systems is to provide adequate drug concentration at a specific location ensuring drug release profile for a longer period (7). Local drug delivery systems have several advantages such as minimizing systemic adverse effects, using smaller quantity of drugs with greater efficiency, avoiding multiple drug therapy, reducing risk of toxicity and ease of the delivery of the drug to target site (8). In addition, administering antibiotics locally also cause high antibiotic concentration in target tissue (3).

The release of antibiotic on the target tissue is expected to take place continuously for a specific period and the concentration is higher than minimum inhibitory concentration (MIC). Controlled release system also can enhance the bioavailability of antibiotic in the target tissue. This system is designed to release the drug in the target tissue with appropriate rate during specific period (3).

The combination of bovine hydroxyapatite (BHA) as inorganic material and chitosan as organic material could construct implants with porous structure and adequate mechanical strength to support bone formation. But, previous study revealed that drug release from hydroxyapatite-chitosan composite was so fast.

In this study, chitosan as organic material and BHA as inorganic material were used to increase mechanical strength and bone bioactivity of the implant (3) and also to control the release rate of ciprofloxacin as the antibiotic. Glutaraldehyde (8) and genipin (9) were used as crosslinkers.

MATERIALS AND METHODS

Materials

Following materials were used in the present study. Ciprofloxacin (Shangyu Jingxin Pharmaceutical Co. Ltd,); BHA (Tissue Bank RSUD DR Soetomo Surabaya, Indonesia); chitosan (Biotech, Indonesia); glutaraldehyde 25% (Merck Millipore, Germany); Genipin (Challenge Bioproduct Co. Ltd., Taiwan). Glacial acetic acid, KBr IR (for spectroscopy), Na_2HPO_4, K_2HPO_4, KH_2PO_4, and NaCl (Merck, Germany). Deionized water was used throughout the study.

Preparation of homogeneous chitosan powder

Homogenous chitosan powder was obtained by dissolving chitosan flakes in acetic acid solution (1%, v/v). The solution was stirred at 400 rpm on a mechanical stirrer for 24 h to obtain chitosan solution with 2% w/v concentration. 1 M NaOH solution was added into chitosan solution until the pH reached neutral (pH, 7). After the addition of NaOH solution into chitosan solution, a chitosan gel was obtained. Chitosan gel was dried at 40 °C for 24 h. the dried chitosan gel was sieved by 1 mm sieve to produce homogeneous chitosan powder.

Formulation of ciprofloxacin-loaded bovine hydroxyapatite-chitosan implant

The implant was prepared using direct compression method. Ciprofloxacin was dissolved in distilled water; BHA was added gradually and mixed until homogenized with ciprofloxacin. Chitosan powder was added to ciprofloxacin-BHA blend and mixed well. Distilled water was added gradually with continuous stirring until a wet mass formed. Wet mass were sieved through a 1-mm sieve and dried overnight (24 h) at 40 °C to obtain dried granules. Dried granules were immersed in glutaraldehyde or genipin solution at various concentrations (0.3, 0.5, or 0.7%) for 24 h until the color was changed. The composition of various formulations is given in Table 1.

Granules were washed with distilled water to remove the residual glutaraldehyde or genipin. At the final stage, granules were washed with phosphate buffer saline (PBS) at pH 7.40. To ensure the absence of glutaraldehyde residues, the rinsed solution tested with Schiff reagents. Granules were dried in an oven at 40 °C for 24 h. Dried granules (100 mg) were weighed out, pressed using tablet press machine with 4.0 mm diameter and the compression pressure set to 2 tons.

Table 1. The composition of implant formulations.

Formulation	Cyprofloxacin (%)	Composite (%)		Crosslinkers (%)	
		BHA	Chitosan	Glutaraldehyde	Genipin
F3	10	30	60	-	-
F3-0.3% GA	10	30	60	0.3	-
F3-0.5% GA	10	30	60	0.5	-
F3-0.7% GA	10	30	60	0.7	-
F3-0.3% GE	10	30	60	-	0.3
F3-0.5% GE	10	30	60	-	0.5
F3-0.7% GE	10	30	60	-	0.7

(GE), genipine; (GA), glutaraldehyde; (BHA), bovine hydroxyapatite.

Evaluation of implants

Density test

The density of the implant was calculated from the weight of the implant (in the dry state) divided by volume of the implant through the equation below.

$$\text{Density} = \frac{\text{W}}{\text{Implant volume}} \quad (1)$$

where, W is the weight of the implant. Implant volume was calculated by multiplying the implant thickness by implant surface area. An implant was cylindrical with 4.0 mm diameter and 0.525 mm thickness.

Porosity test

The implant was weighed in the dry state and placed in 5 mL water. The implant was taken out from the water after 1 min and placed on a filter paper to remove the excess water on the surface of the implant. The implant was weighed again (10). The porosity of the implant was calculated using equation 2.

$$\text{Porosity (\%)} = \frac{\text{W}_\text{w} - \text{W}_\text{i}}{\text{Implant volume}} \quad (2)$$

where, W_w is the wet weight of the implant and W_i is the initial weight of the implant.

Water absorption capacity and swelling ratio

The implant was weighed in dry state and immersed in 5 mL PBS, pH 7.4 for 1 min at 37 ± 0.5 °C.

The implant was withdrawn and gently blotted with filter paper to remove the excess water and weighed again (11). The percentage of water absorption capacity and swelling ratio of the implant was calculated using equations 3 and 4.

$$\text{Swelling ratio} = \frac{\text{W}_w - \text{W}_i}{W_i} \times 100 \quad (3)$$

$$\text{Water absorption capacity} = \frac{\text{W}_w - \text{W}_i}{W_w} \times 100 \quad (4)$$

where, W_i is the weight of implant in the dry state and W_w is the weight of the implant after immersion process in PBS, pH 7.40.

Hardness test

The implant was pressed by load cell compression machine 5 mm/min by autograph E-10 instrument. The hardness of the implant obtained from the force (F, in newton unit) which was displayed at the instrument was divided by contact surface area of the implant (in mm unit) (10).

Degredation test

Implant was immersed in 5 mL PBS, pH 7.4 at 37 ± 0.5 °C. Visual inspection was done to observe the changing of implant structure which was caused by erosion and degradation (12).

Evaluation of implant morphology

Morphology of the implants was observed using a scanning electron microscope (SEM). The samples were fitted to aluminum stubs with conductive paint and were sputter-coated with gold. The differences between implant morphology before and after crosslinking process were observed using specific magnification.

Drug content

The implant was placed in a mortar and milled, then transferred into an Erlenmeyer flask. 100 mL HCL 0.1 N was added into Erlenmeyer flask contained milled implant and stirred for 24 h at 400 rpm until a suspension was obtained. The suspension was filtrated and the filtrate was diluted to determine ciprofloxacin concentration. The absorbance of this solution was observed using a spectrophotometer at three wavelengths of 260, 270, or 280 nm. (Δ), absorbance which was obtained from the observation extrapolated in

standard curve equation to obtain ciprofloxacin HCL concentration. Determination of ciprofloxacin content in the implant was done in triplicate (10)

In vitro drug release study

The implant was placed in a vial containing 5 mL of PBS, pH 7.4. The vial was placed on a shelf and incubated in water bath at 37 ± 0.5 °C. Sampling was conducted by pipetting of 1 mL of elution fluids at 1, 3, 5, 7, 9, 12, 14, 16, 18, 20, 22, and 24 h on first day and every 24 h for 30 days and replaced with fresh buffer to maintain sink condition. The sample solution was filtered with Millipore membrane (ø = 0.45 μm). Appropriate dilution was prepared using PBS, pH 7.4. The absorbance of the solution was analyzed using UV spectrophotometer at three wavelengths of 260, 270, and 280 nm. Cumulative percent of drug release was found at each time interval. The release of ciprofloxacin HCL from the implants was assayed in triplicate (10).

Characterization of the implant

Fourier transforms infrared spectroscopy

A sample of the implant was combined with KBr and pressed into a tablet. The solid tablet was analyzed using Fourier transform-infrared (FT-IR) spectroscopy in the wave number range 4000-400 cm^{-1}.

X-ray diffraction study

The X-ray diffraction (X-RD) study was carried out to determine the crystal phases of the implant using monochromatic CuKα radiation (40 KV, 30 MA). X-RD peaks of the implants were compared to the diffraction peaks of pure materials (ciprofloxacin HCL, BHA, and chitosan) in 2θ scan range of 5-50 °.

Data analysis

The results of implant evaluation including density, porosity, swelling ratio, water uptake, hardness were statistically analyzed using one way analysis of variance (ANOVA) with 95% confidences interval.

RESULTS

Physical characteristics of the implants

Physical characteristics including density, porosity, water absorption capacity, swelling ratios, and hardness of the glutaraldehyde or genipin crosslinked implants are shown in Fig. 1. The content of ciprofloxacin HCL in implants is also shown in Table 2.

Degradation test

Degradation test of implants crosslinked with glutaraldehyde or genipin was done by visual observation. The result showed formulations containing 0.7% of crosslinkers had lowest degradation time. In contrast, formulations containing 0.3% of crosslinker showed longest degradation time.

Implant morphology

SEM micrograph of the implants crosslinked with glutaraldehyde or genipin is presented in Fig. 2. Based on the micrograph, it could be seen that there was small pores in the structure. These pores facilitate the release of ciprofloxacin from the implants.

In vitro drug release study

The *in vitro* release profiles of ciprofloxacin from prepared implants in PBS, pH 7.40 are illustrated in Fig 3. The release profile of ciprofloxacin HCL from the implants showed that ciprofloxacin release was at a therapeutic level of ciprofloxacin for osteomyelitis (2-50 μg/mL) (13). This condition could be kept for 30 days.

Fourirer transform infrared spectroscopy

The infrared spectrum of ciprofloxacin, BHA, chitosan, implants BHA-chitosan-ciprofloxacin before the crosslinking process and implants BHA-chitosan-ciprofloxacin after crosslinking process with three different concentrations of glutaraldehyde and genipin can be seen in Fig. 4.

Table 2. Drug content of implant formulations

Formulations	Drug content (%)
F3, 0.3% GA	96.04 ± 7.11
F3, 0.5% GA	84.80 ± 10.3
F3, 0.7% GA	87.31 ± 3.40
F3, 0.3% GE	92.81 ± 7.96
F3, 0.5% GE	95.30 ± 1.07
F3, 0.7% GE	92.81 ± 2.41

Each data represents the mean ± SD of three determinations. (GA), glutaraldehyde and (GP), genipin.

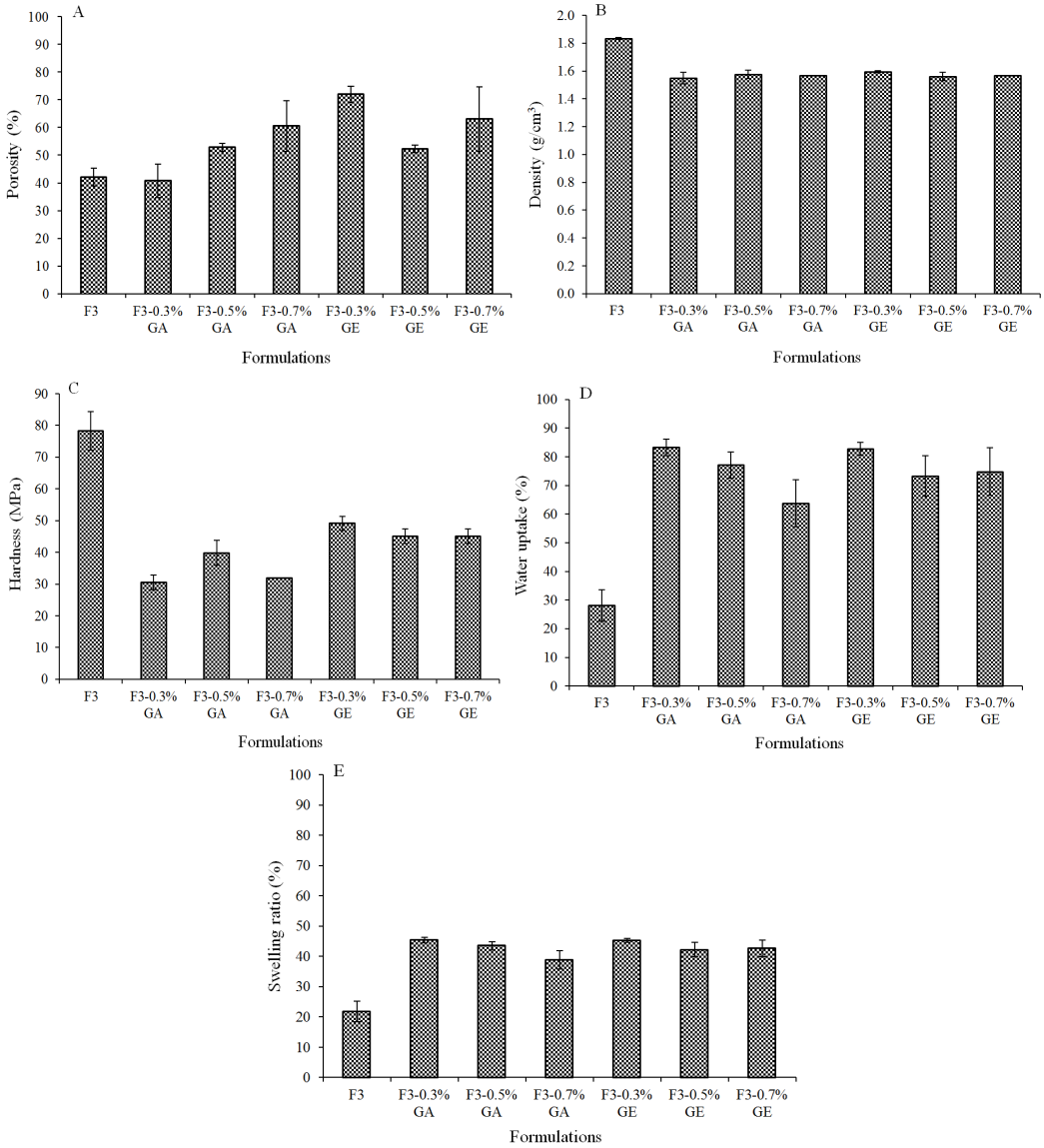

Fig. 1. Porosity (g/cm3), density (%), hardness (MPa), water uptake (%), and swelling ratios (%) of implants with crosslinker glutaraldehyde (GA) and crosslinker genipin (GE). Each column represents the mean ± SD of three determinations.

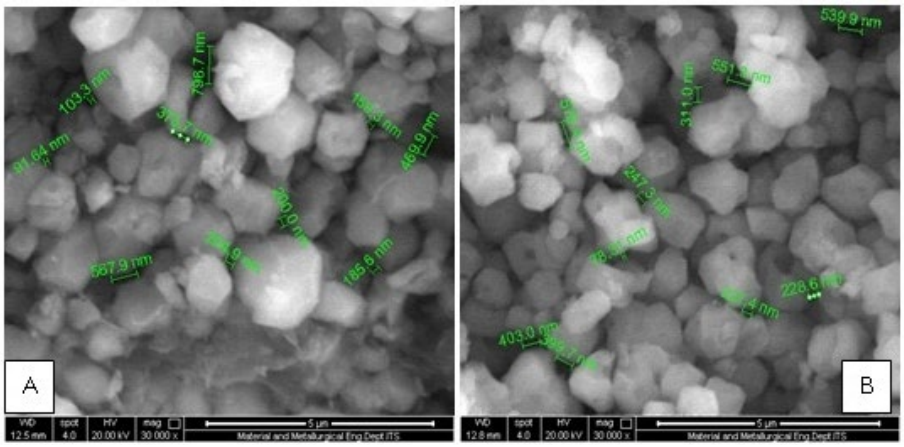

Fig. 2. Scanning electron microscopic micrographs of crosslinked ciprofloxacin implants (with 30.000 × magnification). (A) bovine hydroxyapatite-chitosan-ciprofloxacin (30:60:10) with 0.7% glutaraldehyde, (B) bovine hydroxyapatite-chitosan-ciprofloxacin (30:60:10) with 0.7% genipin. The green lines inside the images show the pore size of the implants.

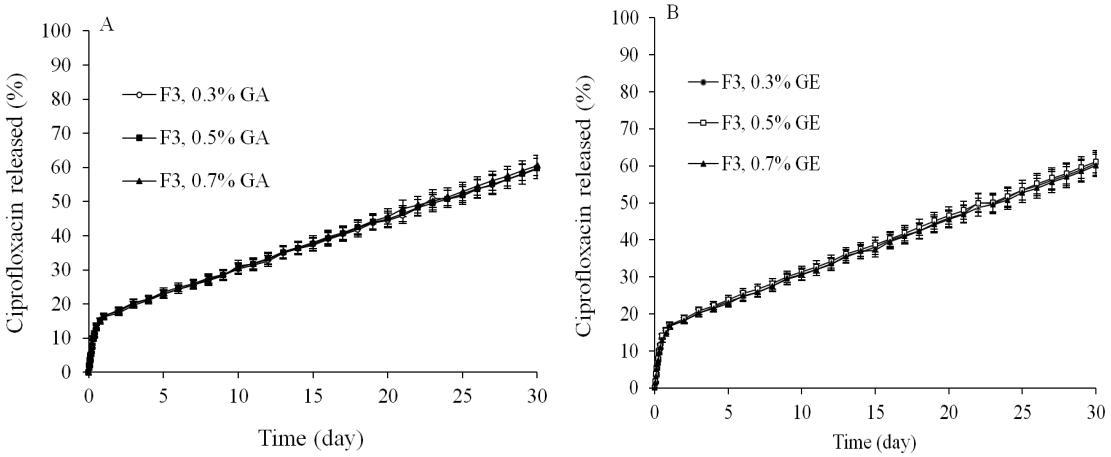

Fig. 3. The profile of cumulative ciprofloxacin released from implants crosslinked with glutaraldehyde (GA) or genipin (GE). Each point represents the mean ± SD of three determinations.

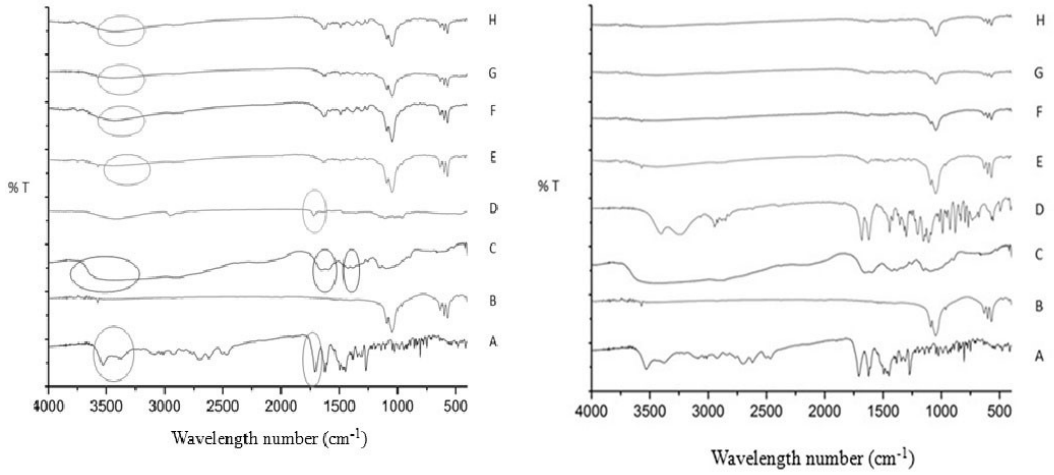

Fig. 4. Fourirer transform infrared spectra of (A) ciprofloxacin; (B) bovine hydroxyapatite; (C) chitosan; (D) glutaraldehyde; (E) formulatin 3, bovine hydroxyapatite-chitosan-ciprofloxacin implant (30:60:10); (F) formulation 3, 0.3% glutaraldehyde (left) and genipin (right); (G) formulation 3, 0.5% glutaraldehyde (left) and genipin (right); and (H) formulaion 3, 0.7% glutaraldehyde (left) and genipin (right).

FT-IR spectrum of BHA-chitosan-ciprofloxacin implant after crosslinking process using glutaraldehyde showed a peak shift characteristics of chitosan on wavenumbers 1658,67 cm^{-1} (C=O stretching in amide group) to the lower wavenumbers around 1630 cm^{-1}. This band (1630 cm^{-1}) is most probably composed of amide I band of chitosan (appears at 1658.67 cm^{-1}) and the C=N stretching band of Schiff's base that according to the literature appears at wave number 1620-1660 cm^{-1} (8). Moreover, the peak characteristic of aldehyde could not be seen in the FT-IR spectrum of BHA-chitosan-ciprofloxacin implant after crosslinking process using glutaraldehyde. This condition showed that the implant did not contain free aldehyde group. Based on the results of the FT-IR spectra, it was known that there was a shift of the N-H stretching vibrations and O-H stretching vibrations from chitosan molecules. In addition, the loss of peak at wave number 1363 cm^{-1} (the vibration bending of CH_3) and 1155 cm^{-1} (the vibration bending of C-O-C) observed in FT-IR spectrum of the implant compared to FT-IR spectrum of pure chitosan. FT-IR spectrum of BHA-chitosan-ciprofloxacin implant that has been crosslinked with genipin also can be seen in Fig. 4. The spectrum showed a characteristic peak of chitosan (C=O stretching of amides group) shift to the lower wavenumbers. In addition, increasing genipin concentration caused an increase of C=C bond intensity of genipin. The obtained data from FTIR study evidenced intermolecular interaction between components in the system.

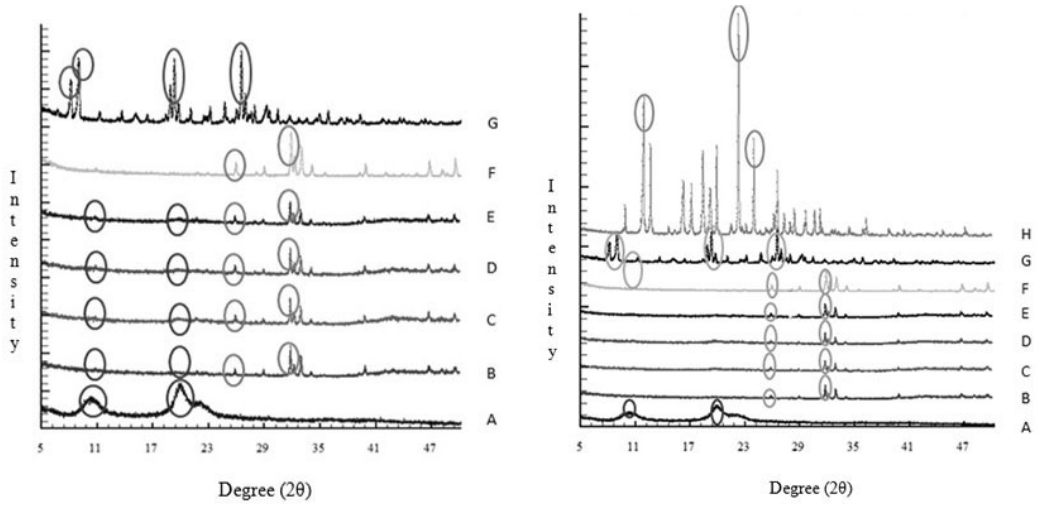

Fig. 5. X-ray diffraction spectra of (A) chitosan; (B) formulatin 3 bovine hydroxyapatite (BHA)-chitosan-ciprofloxacin implant (30:60:10); (C) formula 3, 0.3% glutaraldehyde; (D) formulation 3, 0.5% glutaraldehyde (left) and genipin (right); (E) formulation 3, 0.7% glutaraldehyde (left) and genipin (right); (F) BHA, (G) ciprofloxacin; and (H)

X-ray diffraction study

X-RD of the implants after crosslinking with glutaraldehyde and genipin are demonstrated in Fig. 5. Based on the results, it was known that the characteristic peak of ciprofloxacin in 2θ 8.2 °, 9.0 °, 19.3 °, 19.8 °, and 26.5 ° did not appear in a diffraction spectrum of BHA-chitosan-ciprofloxacin implant. This condition indicates that ciprofloxacin was molecularly dispersed in the implant. X-ray diffraction of the implant after crosslinking with glutaraldehyde or genipin showed that the peak intensity of BHA in 2θ ≈ 26 ° and 2θ ≈ 32 ° decreased compared to X-ray diffraction of pure BHA and the implants before the crosslinking process. The decreased of BHA crystallinity was in line with the increased of glutaraldehyde and genipin concentrations. This condition indicated the addition of glutaraldehyde and genipin damage regularity on BHA crystal lattice.

The X-ray pattern of chitosan shows major crystalline peaks at 2θ ≈ 10 ° and 2θ ≈ 20 °. But, these peaks became wider and weaker in X-ray diffraction of the implants. This finding could be due to decreased crystallinity of chitosan molecule caused by the deformation of hydrogen bond in the molecular structure of chitosan. Substitution of glutaraldehyde and genipin molecules destroyed the regular structure of chitosan molecules so that the structure of chitosan molecules became amorph (14)

DISCUSSION

The purpose of this research was to obtain chitosan-BHA composite containing ciprofloxacin as a bone implant. The obtained data showed that with the addition of genipin or glutaraldehyde as crosslinkers the implant has good physical characteristics and controlled drug release.

Fig. 1A shows the porosity of implants crosslinked with different concentrations of glutaraldehyde or genipin. Interestingly, as glutaraldehyde or genipin concentrations increased, the porosity of implant was also increased though not significant. In most studies, the porosity decreased with increasing crosslinking concentration. In agreement with our study, such phenomenon observed by Bie, *et al,* (15) when chitosan-collagen scaffold was crosslinked with genipin (0.1-2%). Gorczyca, *et al.* (16) also reported similar results when crosslinked porous chitosan–collagen–gelatin scaffolds were prepared using genipin with concentrations between 0.5-2%. These findings explained more favorable condition for ring-opening polymerization of genipin and its long-range crosslinking effect on polymeric blends.

The density of the implants after the crosslinking process with glutaraldehyde or genipin was lower than that before crosslinking process. This is probably due to the increased porosity of the implants after crosslinking. However, as shown in Fig. 1B, there were no significant differences between densities of the

implants prepared with three different concentrations of genipin or glutaraldehyde ($P > 0.05$). Based on these results, it could be concluded that the different concentrations of genipin or glutaraldehyde did not affect the implant density. This may be due to the outer layers of implants being crosslinked, thereby limiting crosslinking of inner layers (17).

As shown in Fig. 1C hardness of the implants was decreased greatly when the implants crosslinked with genipin and glutaraldehyde possibly due to increased porosity. Xu, *et al.* (18), also showed the mechanical strength of a calcium phosphate-chitosan scaffold depends mainly on porosity and lower porosity is helpful to enhance the biomechanical strength of the engineered constructs.

In agreement with our findings, Schiffman, *et al.* (19) showed that increasing glutaraldehyde concentration as the crosslinker more than 0.2 % decreased the mechanical strength of the implants. In another study by Mi, *et al.* (20), crosslinking of chitosan membrane with glutaraldehyde or genipin up to a certain concentration (0.5 mM) increased its ultimate strength. However, with further increasing the concentration of crosslinkers, the mechanical strength of membrane decreased. This finding was explained with disruption of hydrogen bond interaction between chitosan molecules and reduction of its crystallinity.

As it is shown in Figs. 1D and 1E, water absorption capacity and swelling ratio of the implants after crosslinking process with glutaraldehyde or genipin was higher than that before crosslinking process.

The crosslinks in implant formed by the glutaraldehyde and genipin increased the porosity and reduced the crystallinity as well as mechanical strength of the implants which increased the swelling ratio and water content of implants. However, we observed that increasing genipin or glutaraldehyde concentrations does not have a significant effect on water absorption capacity and swelling ratios of the implants.

The degradation test showed formulations containing 0.7% crosslinker has lowest degradation time.

In contrast, formulations containing 0.3% crosslinker indicated longest degradation time. This could be due to the lower mechanical strengh of formulations containing 0.7% crosslinker, thus the penetration of water to the implant structure became easier and the implants degraded faster. The degradation process is faster once genipin was used as crosslinking agent compared to glutaraldehyde.

There are many factors influencing the drug release rate from implants. This includes drug concentration in the formulation, drug solubility, and drug-carrier interaction. In addition, addition of crosslinker to the implant could control the drug release. Implants prepared with different concentrations of glutaraldehyde and genipin showed almost the same release profiles possibly due to the interaction of ciprofloxacin with implants' composite. Ciprofloxacin concentration released from the implants will meet the therapeutic range of ciprofloxacin according to Indonesian Ministry of Health (2-50 ug/mL) for 30 days. As shown in Fig. 3, the suitable drug release can be obtained with the lowest glutaraldehyde and genipin content in order to limit the toxicological effects of the cross linking agents.

CONCLUSION

To conclude with, bone implants containing ciprofloxacin using chitosan-BHA composite and crosslinkers glutaraldehyde or genipin 0.3% showed the best results. Therefore, the release of ciprofloxacin for 30 days meets the standard requirements (2-50 mg). Glutaraldehyde or genipin 0.3% had the potential effect to retard ciprofloxacin release from BHA-chitosan-ciprofloxacin implant for 30 days in the treatment of osteomyelitis.

ACKNOWLEDGEMENTS

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REFERENCES

1. Porter JR, Ruckh TT, Popat KC. Bone tissue engineering : a review in bone biomimetics and drug

delivery strategies. Biotechnol Prog. 2009;25(6):1539-1560.
2. Strobel C, Bormann N, Kadow-Romacker A, Schmidmaier G, Wildemann B. Sequential release kinetics of two (gentamicin and BMP-2) or three (gentamicin, IGF-1 and BMP-2) substances from a one component polymeric coating on implants. J Control Release. 2011;156(1):37-45.
3. Mouriño V, Boccaccini AR. Bone tissue engineering therapeutics: controlled drug delivery in three-dimensional scaffolds. J R Soc Interface. 2010;7(43):209-227.
4. Cui X, Gu Y, Wang H, Xie Z, Luo S, Zhou N, *et al*. *In vitro* bioactivity, cytocompatibility, and antibiotic release profile of gentamicin sulfate-loaded borate bioactive glass/chitosan composites. J Mater Sci Mater Med. 2013;24(10):2391-2403.
5. Gogia JS, Meehan JP, Di Cesare PE, Jamali AA. Local antibiotic therapy in osteomyelitis. Semin Plast Surg 2009;23(2):100-107.
6. Li B, Brown KV, Wenke JC, Guelcher SA. Sustained release of vancomycin from polyurethane scaffolds inhibits infection of bone wounds in a rat femoral segmental defect model. J Control Release. 2010;145(3):221-230.
7. Dubnika A, Loca D, Berzina-Cimdina L. Functionalized hydroxyapatite scaffolds coated with sodium alginate and chitosan for controlled drug delivery. Proc Est Acad Sci. 2012;61(3):193-199.
8. Ostrowska-Czubenko J, Pieróg M, Gierszweka-Drużyńska M. State of water in non crosslinked and crosslinked hydrogel chitosan membranes. Prog Chem Appl Chitin Its Deriv. 2011;16:147-156.
9. Muzzarelli RAA. Genipin-crosslinked chitosan hydrogel as biomedical and pharmaceutical aids. Carbohydr Polym. 2009:77(1):1-9.
10. Rani K, Primaharinastiti R, Hendradi E. Preparation and evaluation of ciprofloxacin implants using bovine hydroxyapatite-chitosan composite and glutaraldehyde for osteomyelitis. Int J Pharm Pharm Sci. 2016;8(1):45-51.
11. Liu Y, An M, Wang L, Qiu H. Preparation and characterization of chitosan-gelatin/glutaraldehyde scaffolds. J Macromolecul Sci, Part B. 2014;53(2):309-325.
12. Teng SH, Lee EJ, Yoon BH, Shin DS, Kim HE, Oh JS. Chitosan/nanohydroxyapatite composite membranes via dynamic filtration for guided bone regeneration. J Biomed Mater Res A. 2009;88(3):569-580.
13. Mäkinen TJ, Veiranto M, Lankinen P, Moritz N, Jalava J, Törmälä P, *et al*. *In vitro* and *in vivo* release of ciprofloxacin from osteoconductive bone defect filler. J Antimicrob Chemother. 2005;56(6):1063-1068.
14. Li B, Shan CL, Zhou Q, Fang Y, Wang YL, Xu F, *et al*. Synthesis, characterization, and antibacterial activity of cross-linked chitosan glutaraldehyde. Mar Drugs. 2013;11(5):1534-1552.
15. Bi L, Cao Z, Hu Y, Song Y, Yu L, Yang B, *et al*. Effects of different cross-linking conditions on the properties of genipin-cross-linked chitosan/collagen scaffolds for cartilage tissue engineering. J Mater Sci Mater Med. 2011;22(1):51-62.
16. Gorczyca G, Tylingo R, Szweda P, Augustin E, Sadowska M, Milewski S. Preparation and characterization of genipin cross-linked porous chitosan-collagen-gelatin scaffolds using chitosan-CO_2 solution. Carbohydr polym. 2014;102:901-911.
17. Yuan Y, Chesnutt BM, Utturkar G, Haggard WO, Yang Y, Ong JL, *et al*. The effect of cross-linking of chitosan microspheres with genipin on protein release. Carbohydr Polym. 2007;68(3):561-567.
18. Xu HH, Simon CG Jr. Fast setting calcium phosphate-chitosan scaffold: mechanical properties and biocompatibility. Biomaterials. 2005;26(12):1337-1348.
19. Schiffman JD, Schauer CL. Cross-linking chitosan nanofibers. Biomacromolecules. 2007;8(2):594-601.
20. Mi FL, Tan YC, Liang HC, Huang RN, Sung HW. *In vitro* evaluation of a chitosan membrane cross-linked with genipin. J Biomater Sci Polym Ed. 2001;12(8):835-850.

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REFERENCES

1. Porter JR, Ruckh TT, Popat KC. Bone tissue engineering : a review in bone biomimetics and drug delivery strategies. Biotechnol Prog. 2009;25(6):1539-1560.
2. Strobel C, Bormann N, Kadow-Romacker A, Schmidmaier G, Wildemann B. Sequential release kinetics of two (gentamicin and BMP-2) or three (gentamicin, IGF-1 and BMP-2) substances from a one component polymeric coating on implants. J Control Release. 2011;156(1):37-45.
3. Mouriño V, Boccaccini AR. Bone tissue engineering therapeutics: controlled drug delivery in three-dimensional scaffolds. J R Soc Interface. 2010;7(43):209-227.
4. Cui X, Gu Y, Wang H, Xie Z, Luo S, Zhou N, *et al. In vitro* bioactivity, cytocompatibility, and antibiotic release profile of gentamicin sulfate-loaded borate bioactive glass/chitosan composites. J Mater Sci Mater Med. 2013;24(10):2391-2403.
5. Gogia JS, Meehan JP, Di Cesare PE, Jamali AA. Local antibiotic therapy in osteomyelitis. Semin Plast Surg 2009;23(2):100-107.
6. Li B, Brown KV, Wenke JC, Guelcher SA. Sustained release of vancomycin from polyurethane scaffolds inhibits infection of bone wounds in a rat femoral segmental defect model. J Control Release. 2010;145(3):221-230.
7. Dubnika A, Loca D, Berzina-Cimdina L. Functionalized hydroxyapatite scaffolds coated with sodium alginate and chitosan for controlled drug delivery. Proc Est Acad Sci. 2012;61(3):193-199.
8. Ostrowska-Czubenko J, Pieróg M, Gierszweka-Drużyńska M. State of water in non crosslinked and crosslinked hydrogel chitosan membranes. Prog Chem Appl Chitin Its Deriv. 2011;16:147-156.
9. Muzzarelli RAA. Genipin-crosslinked chitosan hydrogel as biomedical and pharmaceutical aids. Carbohydr Polym. 2009;77(1):1-9.
10. Rani K, Primaharinastiti R, Hendradi E. Preparation and evaluation of ciprofloxacin implants using bovine hydroxyapatite-chitosan composite and glutaraldehyde for osteomyelitis. Int J Pharm Pharm Sci. 2016;8(1):45-51.
11. Liu Y, An M, Wang L, Qiu H. Preparation and characterization of chitosan-gelatin/glutaraldehyde scaffolds. J Macromolecul Sci, Part B. 2014;53(2):309-325.
12. Teng SH, Lee EJ, Yoon BH, Shin DS, Kim HE, Oh JS. Chitosan nanohydroxyapatite composite membranes via dynamic filtration for guided bone regeneration. J Biomed Mater Res A. 2009;88(3):569-580.
13. Mäkinen TJ, Veiranto M, Lankinen P, Moritz N, Jalava J, Törmälä P, *et al. In vitro* and *in vivo* release of ciprofloxacin from osteoconductive bone defect filler. J Antimicrob Chemother. 2005;56(6):1063-1068.
14. Li B, Shan CL, Zhou Q, Fang Y, Wang YL, Xu F, *et al.* Synthesis, characterization, and antibacterial activity of cross-linked chitosan glutaraldehyde. Mar Drugs. 2013;11(5):1534-1552.
15. Bi L, Cao Z, Hu Y, Song Y, Yu L, Yang B, *et al.* Effects of different cross-linking conditions on the properties of genipin-cross-linked chitosan/collagen scaffolds for cartilage tissue engineering. J Mater Sci Mater Med. 2011;22(1):51-62.
16. Gorczyca G, Tylingo R, Szweda P, Augustin E, Sadowska M, Milewski S. Preparation and characterization of genipin cross-linked porous chitosan-collagen-gelatin scaffolds using chitosan-CO_2 solution. Carbohydr polym. 2014;102:901-911.
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18. Xu HH, Simon CG Jr. Fast setting calcium phosphate-chitosan scaffold: mechanical properties and biocompatibility. Biomaterials. 2005;26(12):1337-1348.
19. Schiffman JD, Schauer CL. Cross-linking chitosan nanofibers. Biomacromolecules. 2007;8(2):594-601.
20. Mi FL, Tan YC, Liang HC, Huang RN, Sung HW. *In vitro* evaluation of a chitosan membrane cross-linked with genipin. J Biomater Sci Polym Ed. 2001;12(8):835-850.

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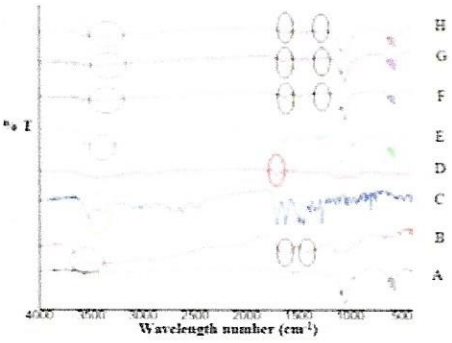

Fig. 10: FTIR spectrum of, (A): Bovine hydroxyapatite; (B): Chitosan; (C) Ciprofloxacin; (D) Glutaraldehyde; (E) Implants before cross-linking process; (F): Formula 1; (G): Formula 2; (H): Formula 3

Moreover, the characteristic peaks intensity of Bovine Hydroxyapatite in $2\theta \approx 26^\circ$ and $2\theta \approx 32^\circ$ decreased in X-ray diffraction of the implants after cross-linking process compared to X-ray diffraction of pure BHA and the implants before the cross-linking process. Based on this fact, it could be concluded that increasing glutaraldehyde concentration caused the decrease of implants crystallinity [14]. Characteristic peaks of ciprofloxacin also did not observe in X-ray diffraction of the implants after the cross-linking process. This condition indicated that ciprofloxacin was molecularly dispersed in the structure of the implants.

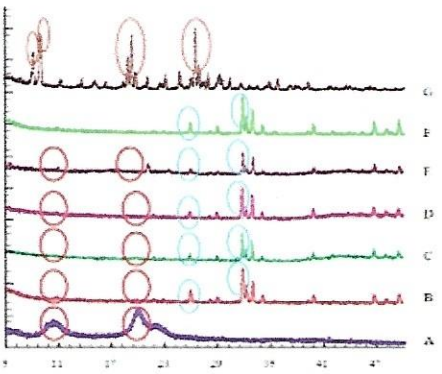

Fig. 11: X-ray diffraction spectrum of (A): Chitosan; (B): Implant before cross-linking process; (C) Formula 1; (D) Formula 2; (E) Formula 3; (F): Bovine Hydroxyapatite; (G): Ciprofloxacin

CONCLUSION

Bovine Hydroxyapatite-chitosan-ciprofloxacin implants with glutaraldehyde as cross-link agents are characterized by low porosity, low water uptake capacity, and minimal swelling ratio. But, glutaraldehyde decreased the mechanical strength of the implants due to the decrease of material crystallinity. The addition of glutaraldehyde as cross-link agent in Bovine Hydroxyapatite-chitosan-ciprofloxacin implants produced controlled release profile of ciprofloxacin. Glutaraldehyde inhibited burst release of ciprofloxacin from the implants. The release of ciprofloxacin from the implants ranged from *in vitro* therapeutic level of ciprofloxacin for osteomyelitis. Therefore from this study it is proved that Bovine Hydroxyapatite-chitosan-ciprofloxacin implants with glutaraldehyde as a cross-link agent has a potential to control ciprofloxacin release for thirty days in the treatment of osteomyelitis.

CONFLICT OF INTERESTS

Declared none

REFERENCES

1. Porter JR, Ruckh TT, Popat KC. Bone tissue engineering: a review in bone biomimetics and drug delivery strategies. Biotechnol Prog 2009;25:1539-53.
2. Strobel C, Bormann N, Romacker A, Schmidmaier G, Wildemann B. Sequential release kinetics of two (gentamicin and BMP-2) or three (gentamicin, IGF-1 and BMP-2) substances from a one component polymeric coating on implants. J Controlled Release 2011;156:37-45.
3. Mourino V, Boccaccini AR. Bone tissue engineering therapeutics: controlled drug delivery in three-dimensional scaffolds. J R Soc Interface 2010;7:209-27.
4. Gogia JS, Meehan JP, Cesare PE, Jamali AA. Local antibiotic therapy in osteomyelitis. Seminars in Plastic Surgery 2009;23:100-7.
5. Aviv M, Berdicevsky I, Zilberman M. Gentamicin-loaded bioresorbable films for prevention of bacterial infections associated with orthopedic implants. J Biomed Mater Res A 2007;83:10-8.
6. Li B, Brown KV, Wenke JC, Guelcher SA. Sustained release of vancomycin from polyurethane scaffolds inhibits infection of bone wounds in a rat femoral segmental defect model. J Controlled Release 2010;145:221-30.
7. Dubnika A, Loca D, Cimdina LB. Functionalized hydroxyapatite scaffolds coated with sodium alginate and chitosan for controlled drug delivery. Proc Est Acad Sci 2012;61:193-9.
8. Iqbal MM, Gupta S, Sagar S, Ibrahim M. Design and evaluation of subcutaneous implantable drug delivery system of tramadol using natural biodegradable polymer. Ann Phytomed 2012;1:30-8.
9. Lambert HP, O'Grady FW. Quinolones. In: Lambert HP, Grady O. editors. Antibiotic and Chemotherapy. 6th ed. Edinburgh: Churchill Livingstone; 1992. p. 245-62.
10. Armstrong EP, Elsberry VA. Bone and joint infections. In: Dipiro JT, Talbert RL, Yee GC, Matzke GR, Wells BG, Posey LM. editors. Pharmacoteraphy. A Patophysiologic Approach. 3rd ed. Stamford: Appleton and Lange; 1997. p. 2221-35.
11. Jin HH, Kim DH, Kim TW, Shin KK, Jung JS, Park HC, *et al. In vivo* evaluation of porous hydroxyapatite/chitosan-alginate composite scaffold for bone tissue engineering. Int J Biol Macromol 2012;51:1079-85.
12. Usha KM, Nandeesh BN. Physiology of bone formation, remodeling, and metabolism. In: Radionuclide and Hybrid Bone Imaging. Berlin: Springer Berlin Heidelberg; 2012. p. 29-57.
13. Teng SH, Lee EJ, Wang P, Jun SH, Han CM, Kim HE. Functionally gradient chitosan/hydroxyapatite composite scaffolds for controlled drug release. J Biomed Mater Res Part B 2009;90:275-82.
14. Monteiro OA, Airoldi JC. Some studies of crosslinking chitosan-glutaraldehyde interaction in a homogeneous system. Int J Biol Macromol 1999;7:209-27.
15. Ostrowska J, Pierog M, Gierszweka M. State of water in non-crosslinked and crosslinked hydrogel chitosan membranes. Prog Chem Appl Chitin Its Deriv 2011;16:147-56.
16. Lei L, Li L, Zang L. Structure and performance of nano-hydroxyapatite filled biodegradable poly(1,2-propanediol-sebacate)-citrate) elastomer. Polym Degrad Stab 2009;94:1494-502.
17. Campos MG, Rawls HR, Innocentini-Mei LH, Satsangi N. *In vitro* gentamicin sustained and controlled release from chitosan cross-linked films. J Mater Sci: Mater Med 2009;20:537-42.
18. Liuyun J, Yubao L, Chengdong X. Preparation and biological properties of a novel composite scaffold of nano-hydroxyapatite/chitosan/carboxymethyl cellulose for bone tissue engineering. J Biomed Sci 2009;16:1-10.
19. Ashish P, Derle Deelip V. Development of cost effective biodegradable implants of ciprofloxacin hydrochloride in the treatment of osteomyelitis. Asian J Pharm 2012;2:19-37.
20. Budiatin SA, Zainuddin M, Khotib J. Biocompatible composite as gentamicin delivery system for osteomyelitis and bone regeneration. Int J Pharm Sci 2014;6:1-3.
21. Yang B, Li XY, Shi S, Kong X, Guo G, Huang M. Preparation and characterization of a novel chitosan scaffold. Carbohydr Polym 2010;80:868-5.

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22. Teng SH, Lee EJ, Wang P, Jun SH, Han CM, Kim HE. Chitosan/nanohydroxyapatite composite membranes via dynamic filtration for guided bone regeneration. J Biomed Mater Res Part B 2008;88:569-79.
23. Han WW, Misra RD. Biomimetic chitosan-nanohydroxyapatite composite scaffolds for bone tissue engineering. Acta Biomater 2009;5:1182-97.
24. Ashish YP, Derle DV, Gandhi PP, Nade VN, Patel JS. *In vitro* and *in vivo* characterization of a novel biocompatible implants system for the sustained delivery of ciprofloxacin hydrochloride. J Pharm Res 2010;3:2122-6.
25. Stallmann HP, Faber C, Bronckers AL, Amerogen AV, Wuisman PI. *In vitro* gentamicin release from commercially available calcium phosphate bone substitute influence of carrier type on the duration of the release profile. BMC Musculoskeletal Disord 2006;7:1-8.
26. Berger J, Reist M, Maayer JM, Felt O, Peppas NA, Gunny R. Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications. Eur J Pharm Biopharm 2004;57:19-34.
27. Mirzaei BE, Ramzani SA, Shaifee M, Dannaei M. Studies on glutaraldehyde crosslinked chitosan hydrogel properties for drug delivery system. Int J Polym Mater Polym 2015;62:605-11.
28. Goncalves VL, Laranjeira MC, Favere VT. Effect of crosslinking agents on chitosan microspheres in controlled release of diclofenac sodium. Polim Cienc Tecnol 2005;15:6-12.
29. Prabaharan M, Perez MA, Saja JA, Mano JF. Preparation and characterization of poly(L-lactic acid)-chitosan hybrid scaffolds with drug release capability. J Biomed Mater Res Part B 2006;88:427-34.
30. Silva RM, Silva GA, Coutinho OP, Mano JF, Reis RL. Preparation and characterization in simulated body conditions of glutaraldehyde crosslinked chitosan membranes. J. Mater Sci Mater Med 2004;1105-12.
31. Sailakshmi G, Mitra T, Chatterjee S, Gnanamani A. Chemistry behind the elastic nature of the biomaterial prepared using oxidized form of glutaraldehyde. Int J Life Sci Med Res 2013;4:64-75.
32. Makinen TJ, Veiranto M, Lankinen P, Moritz N, Jalava J, Tormala P, *et al. In vitro* and *in vivo* release of ciprofloxacin from osteoconductive bone defect filler. J Antimicrob Chemother 2005;56:1063-8.
33. Mont MA, Mathur SK, Carmelita G, Frondoza D, Hungerford DS. The effects of ciprofloxacin on human chondrocytes in cell culture. Infection 1996;24:151-5.
34. Li B, Shan CL, Zhou Q, Fang Y, Wang YL, Xu F, *et al.* Synthesis, characterization, and antibacterial activity of cross-linked chitosan-glutaraldehyde. Mar Drugs 2013;11:1534-52.
35. Monteiro OA, Airoldi JC. Some studies of crosslinking chitosan-glutaraldehyde interaction in a homogeneous system. Int J Biol Macromol 1999;26:119-28.