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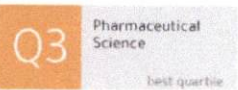
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PREPARATION AND EVALUATION OF CIPROFLOXACIN IMPLANTS USING BOVINE HYDROXYAPATITE-CHITOSAN COMPOSITE AND GLUTARALDEHYDE FOR OSTEOMYELITIS

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ABSTRACT

Objective: The objective of this study was to develop and evaluate a controlled release implant of ciprofloxacin using Bovine Hydroxyapatite-Chitosan composite and glutaraldehyde as cross-link agent.

Methods: Ciprofloxacin implants were prepared using Bovine Hydroxyapatite-Chitosan composite composition 70:30. This composite was further developed using three different concentrations of glutaraldehyde (0.5%, 0.75%, and 1.0%). Implants were formed into pellets with 4.0 mm diameters and weighed 100.0 mg using compression method. Further, the prepared ciprofloxacin implants were 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.

Results: The addition of glutaraldehyde as cross-link agent in ciprofloxacin implants showed controlled release profile of ciprofloxacin over a time period 30 d. This is caused by glutaraldehyde formed compact structure, so the porosity, water absorption capacity, and swelling ratio of the implants decreased. Scanning Electron Microscope photomicrograph revealed low porosity of the implants after cross-linking with glutaraldehyde. The FTIR study confirmed the formation of covalent imine bonds between Chitosan and glutaraldehyde. However, the addition of glutaraldehyde as a cross-link agent caused a decrease in the mechanical strength of the implants. Increased concentration of glutaraldehyde reduced the crystallinity of BHA and Chitosan, which were confirmed by XRD studies. In consequence, the mechanical strength of the implants decreases.

Conclusion: The results obtained from this study indicated that glutaraldehyde has the potential effect to retard ciprofloxacin release from Bovine Hydroxyapatite-Chitosan-ciprofloxacin implants for 30 d in the treatment of osteomyelitis.

Keywords: Ciprofloxacin, Bovine Hydroxyapatite, Chitosan, Glutaraldehyde, Cross-link, Implants, Osteomyelitis.

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INTRODUCTION

The research about controlled drug delivery systems has been developed to fulfill therapeutic requirements in specific diseases. One of the medical fields which required controlled drug delivery systems is orthopedics. Complication of bone disease or bone disturbance which is due to an injury-induced bone defect [1]. Hence, the provision of restorative implants is needed to reconstruct damaged bone tissue.

Reconstruction of bone linked to the risk of infection [2]. This is caused by the entry of bacteria into bone tissue [3]. Bacteria attached on the surface of bone tissue or implants, and then made a biofilm layer. Biofilm layer which is produced by bacteria could protect themselves from antibiotics and human immune systems [3, 4]. Administration of long period, intravenous antibiotics and oral antibiotics had several limitations in bone infection (osteomyelitis) [5]. Tissue devascularization in a bone defect caused obstruction of antibiotic delivery systems in target tissue. Moreover, giving high doses of antibiotics in a long period caused systemic toxicity and side effects [5,6]. To overcome this problem, antibiotics could be given locally using controlled drug delivery systems. Controlled drug delivery systems produced drug release in specific site for a specific time period [7]. Therefore, drug delivery systems were developed to optimize the therapeutic properties of drug products and render them more safe, effective, and reliable. Implantable drug delivery systems are an example of such systems available for therapeutic use [8].

The release of antibiotics in bone tissue was expected to last continuously for a definite period with concentration more than MIC (Minimum Inhibitory Concentration). One of the antibiotics, which had bactericidal properties of the bacteria in the case of bone infection (osteomyelitis) is ciprofloxacin. Ciprofloxacin has been most widely used fluoroquinolone for bacterial bone infection since

the minimal inhibitory concentration (MIC) of ciprofloxacin is low (0.25-2 µg/ml) for most of the pathogens that cause osteomyelitis such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, and *Proteus mirabilis* [9, 10]. Biomaterial implants which act as the ciprofloxacin controlled delivery system can be developed as an alternative therapy in osteomyelitis. Biomaterial which is used to design implants must be biocompatible, biodegradable, osteoconductive, angiogenic, and the mechanical strength could support the structure of bone tissue [11].

The composite can be designed as a biomaterial to get adequate physical capability and controlled the release of ciprofloxacin. The combination of Bovine Hydroxyapatite as inorganic material and chitosan as organic material could construct implants with porous structure and adequate mechanical strength to support bone formation. Calcified bone contains about 25% organic matrix, 5% water, and 70% inorganic mineral (hydroxyapatite) [12]. The composite can be designed using similar composition with the bone component to obtain good physical characteristics of the implants. The composition of Bovine Hydroxyapatite and chitosan in the composite can be formed in 70:30 ratio. The previous study revealed that drug release from hydroxyapatite-chitosan composite was so fast. Approximately 30% of tetracycline hydrochloride was released from the scaffolds during the initial 2 hour. An almost complete drug release of 93% was reached for the composite within 120 hour [13]. However, hydroxyapatite-chitosan composites assessed inadequate for the delivery of antibiotics during 4-6 w in osteomyelitis. The addition of glutaraldehyde as a cross-linking agent was purposed to enhance physical characteristics of the implants and obtained controlled release profile of ciprofloxacin. Optimization of glutaraldehyde concentration was made in three different concentrations. The concentration of glutaraldehyde was 0.5%; 0.75%; and 1.0%.

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, Na₂HPO₄, KH₂PO₄, 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 bidestilata, 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

Mechanism of crosslinking

Aldehyde groups of glutaraldehyde (C=O) react with chitosan amino groups (-NH₂) produced covalent crosslinking through a Schiff base reaction [15].

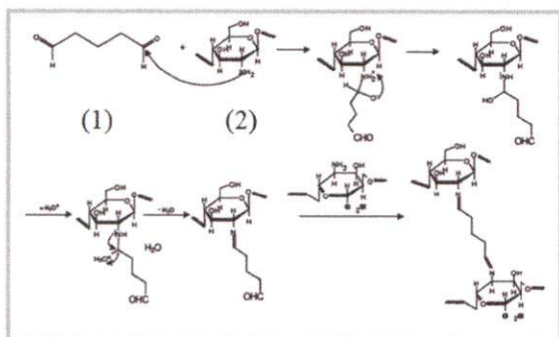


Fig. 1: Cross-linking reaction of glutaraldehyde (1) and chitosan (2) [15]

Evaluation of implants

Density and porosity test

The density of the implant was obtained by weighing the implant and calculating the volume of the implant. Density was calculated from the weight of the implant divided by the volume of the implant [16-18].

$$\text{Density} = \frac{W_i}{V}$$

Where, W_i is the weight of the implant at initial condition

V is volume of the implant

Porosity test was conducted by weighing the implant in the initial condition (at time = 0). The implant was placed in 5 ml water for 1 minute. The implant was taken out from the water at an appropriate interval and blotting away the excess water using filter paper. The implant was weighed again to obtain the wet weight.

$$\text{Porosity (\%)} = \frac{W_w - W_i}{W_i} \times 100$$

Where, W_w is the wet weight, W_i is initial weight

Swelling and water uptake study

Dry implant was weighed and immersed in 5 ml phosphate buffer saline (PBS) pH 7.4 at temperature 37 °C ± 0.5 °C. The implant was withdrawn at appropriate intervals, and then the implant was gently blotted with filter paper to remove the excess water and weighed again. The percentage of swelling ratio and water uptake of the implant was calculated using following equation [16-19].

$$\text{Swelling ratio} = \frac{W_w - W_i}{W_w} \times 100$$

$$\text{Water absorption capacity} = \frac{W_w - W_i}{W_i} \times 100$$

Where, W_i is the weight of implant in dry state

W_w is the weight of the implant after immersion process in phosphate buffer saline (PBS) pH 7.40.

Degradation test

Degradation test was done by immersing the implant at 5 ml phosphate buffer saline pH 7.4 at 37 °C ± 0.5 °C. Visually inspection was done to observe the changing of implant structure which was caused by erosion and degradation [20-22].

Hardness test

The hardness of the implant was tested by the autograph E-10 instrument. The implant was pressed by load cell compression machine 5 mm/min. 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) [20].

Evaluation of implant morphology using scanning electron microscope (SEM)

The morphology of the implant was examined in scanning electron microscope (SEM). The samples were fitted to aluminum stubs with conductive paint and were sputter-coated with gold. Pore diameter was calculated from SEM micrograph. The pore size was calculated using a minimum 40 pores from different places of the cross-section of the implant [23].

Drug content

One milled implant was placed in 100 ml HCL 0.1 N and stirred for 24 h (400 rpm) until form suspension. The suspension was sonicated for 30 min. After sonication process, the filtrate of the suspension was filtered using millipore membrane with 0.45 mm diameter. The filtrate was pipetted 1 ml, transferred in a 25 ml volumetric flasks, and diluted using phosphate buffer saline (PBS) pH 7.4. The absorbance of this solution was observed using spectrophotometer UV-Vis at three wavelengths (260 nm, 270 nm, and 280 nm). Absorbance which was obtained from the observation extrapolated in standard curve equation to obtain ciprofloxacin HCL concentration [24].

In vitro drug release study

Drug release from the implant was studied by vial method. The drug release study was performed by immersing the implant in a vial containing 5 ml of phosphate buffer saline (PBS) pH 7.4. Vial was placed in a shelf and incubated in water bath at 37 ± 0.5 °C. Sampling was conducted by pipetting 1 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 d) and replaced with fresh buffer to maintain sink condition. The sample solution was filtered with millipore membrane ($\phi = 0.45$ μ m (micrometer)). 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). Drug concentration in the sample was determined using standard calibration curve. Cumulative percent drug release was found at each time interval. The release of ciprofloxacin HCL from the implants was assayed in triplicate, mean and S. D was also determined [22, 25].

Data analysis

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

Characterization of implants

Fourier transforms infrared (FTIR) spectroscopy

Fourier transform of infrared (FTIR) spectroscopy was conducted in the wave number range 4000-400 cm^{-1} . The sample was combined with KBr and pressed into a pellet. The solid pellet was analyzed using FT-IR spectroscopy [23].

X-ray diffraction study

The X-ray diffraction study was carried out to determine the crystal phases of the implant using monochromatic Cu K α radiation (40 KV, 30 MA). The 2θ scan range was 5-50 °. X-ray diffraction peaks of the implants were compared to the diffraction peaks of pure materials (ciprofloxacin HCL, Bovine Hydroxyapatite, and chitosan) [23].

RESULTS AND DISCUSSION

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

Formulation of the implants was begun by producing dry granules. Dry granules were brown, spherical in shape, and the diameters were approximately 1 mm. Dry granules then pressed using tablet machine to produce cylindrical pellet with 4 mm diameters.

Evaluation of implants

Density and porosity

The density of the implant with three different concentration of glutaraldehyde can be seen in fig. 3. Statistically analysis using one-way anova showed that there was no significant difference of density between the implants which used three different concentration of glutaraldehyde ($P > 0.05$). Based on this results, could be concluded that the difference of glutaraldehyde concentration did not affect the implant density.

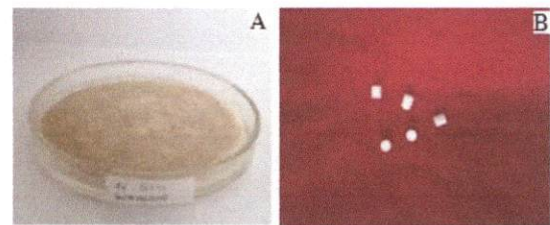


Fig. 2: Formulation process of bovine hydroxyapatite-chitosan-ciprofloxacin implants using glutaraldehyde, A) dry granules, B) implants (cylindrical pellets)

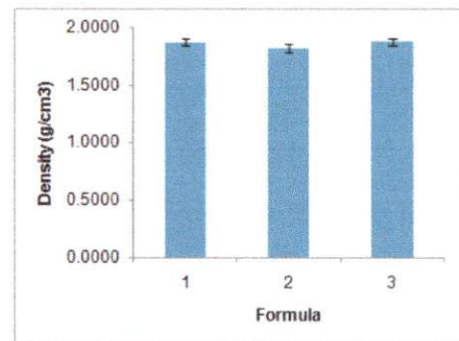


Fig. 3: Density of F1 to F3 formulations

The porosity of the implant with three different concentrations of glutaraldehyde is shown in Fig.4. Porosity of F3 (1.0% glutaraldehyde) was significantly different with F1 (0.5% glutaraldehyde) ($*P < 0.05$). Increasing glutaraldehyde concentration of 1% caused a decrease in porosity than 0.5% glutaraldehyde. The increase of glutaraldehyde concentration caused the structure of the implants became more compact. Glutaraldehyde affected network size which was formed between chitosan chains. The increase of glutaraldehyde concentration led to the size of the network became smaller, so that the pores diameter also decreased [26].

Swelling and water uptake study

The swelling ratio of F1 to F3 formulation is indicated in fig. 5. The swelling mechanism depends on protonation of amino groups in chitosan molecules. Protonation caused repulsion of chitosan chains and dissociation of interactions like intramolecular hydrogen bonding [26, 27]. Cross-linking process with glutaraldehyde could decrease protonation of amino groups in chitosan molecules, reduce the relaxation of chitosan chain, and lower repulsion mechanism of chitosan chains. Based on this condition, F1 to F3 showed low swelling ratio of the implants.

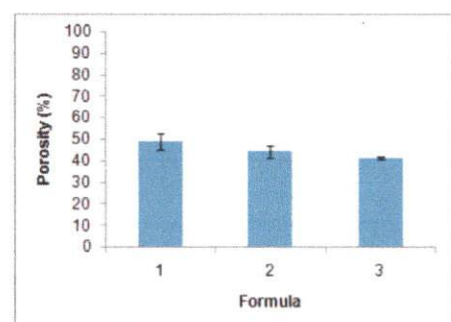


Fig. 4: Porosity of F1 to F3 formulations

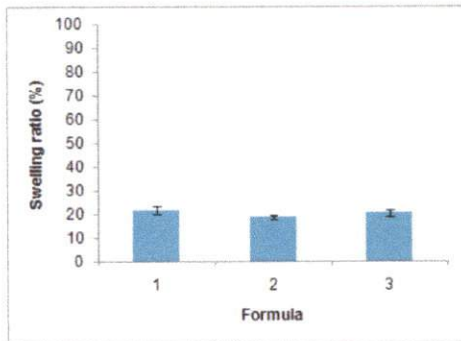


Fig. 5: Swelling ratio of F1 to F3 formulations

Water uptake of formula 1 (0.5% glutaraldehyde) was significantly different from formula 3 (1.0% glutaraldehyde) (** $P < 0.01$). The increase of glutaraldehyde concentration to 1.0% caused a markedly decrease in implant water uptake capacity. Cross-linking process with glutaraldehyde restricted water molecules to enter into chitosan structure [27]. Therefore, the highest concentration of glutaraldehyde (1.0%) produced an implant with the lowest water uptake capacity. Percent water uptake of F1 to F3 formulation can be seen in fig. 6.

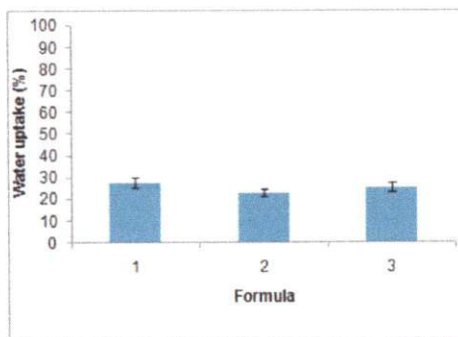


Fig. 6: Water uptake of F1 to F3 formulations

Degradation test

The degradation profile of implants with three different concentrations of glutaraldehyde showed that formula with the lowest degradation was F2 (glutaraldehyde 0.75%). At the opposite, F1 (glutaraldehyde 0.5%) showed greater degradation than two others formula. The lower concentration of glutaraldehyde as a cross-link agent caused hydrolysis process in polymer chains inducing erosion process [28]. Because of this phenomenon, F1 easier to degrade compare to two others formula. 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 [29]. Limitation of expansion ability was caused by the compact structure of the implants after cross-link process. But, increasing glutaraldehyde concentration of 1.0% in F3 produced implants with higher degradation ability compares to F2 (glutaraldehyde 0.75%). In general, degradation of the implants decrease as the cross-linking degree increased because of the more dense covalently linked network. For the specific case, a more complex relationship is found between cross-linking degree with glutaraldehyde and degradation rate, because the crystalline content in the material is also changing [29]. This condition was demonstrated by F3 which easier to degrade compare to F2. The crystallinity of F3 was lower than F2, so that the ability of water to

penetrate in implants structure became easier and the implants degrade easily [30].

Hardness

Hardness of the implants (F1 to F3) is as shown in fig. 7. The increase of glutaraldehyde concentration to 1.0% in F3 caused a significant decrease of implants hardness compare to F1 and F2. Using high concentration of glutaraldehyde as cross link agent caused modification of implants structure became amorph [30]. Cross-linking process using glutaraldehyde caused the characteristic of biomaterial became brittle. Increasing glutaraldehyde as cross-link agent more than 0.2% decreases the mechanical strength of the implants [31].

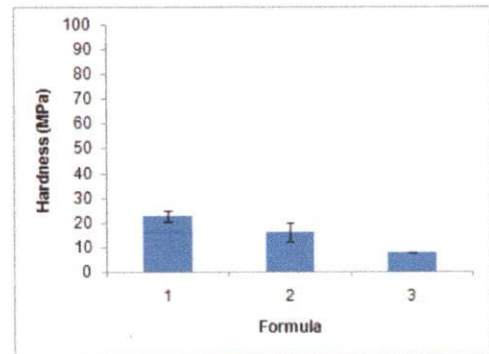


Fig. 7: Hardness of F1 to F3 formulations

Evaluation of implants morphology using scanning electron microscope (SEM)

SEM micrograph of the implant is presented in fig. 8. Surface morphology of the implant after the cross-linking process was dense, rough, and porous. The structure of the implant was porous and composed of irregular pores. In this structure, chitosan covered Bovine Hydroxyapatite particles which seemed as hexagonal particles. This condition led to the structure of the implant became compact, the pores became smaller, and the number of pores decreases [29]. F3 showed the smallest pore size among three formulas and the number of pores became lower. Increasing glutaraldehyde concentration caused a decrease of pore size.

Drug content

All the implants had a uniform distribution of ciprofloxacin HCL in all the formulations. Drug content of all formulations was determined by UV spectrophotometer using three-wavelength methods and reported in table 2.

Table 2: Drug content of F1 to F3 formulations

Formulation code	Drug content (%)
F1	91.32±1.99
F2	95.04±4.52
F3	94.56±1.82

In vitro drug release study

The cumulative percent release from three formulations (F1 to F3) was determined and shown fig. 9. 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) [32-33]. This condition could be kept for 30 d. Take into account this profile, it could be concluded that glutaraldehyde inhibited the burst release of ciprofloxacin from the implants. Area under curve (AUC) of F1 to F3 was calculated and analyzed being used one way ANOVA to compare the release of ciprofloxacin from three formulas. In general, an increase of glutaraldehyde concentration decreased the

drug release from the implants [28]. But, increasing glutaraldehyde concentration to 1.0 % in F3 showed no significant difference of ciprofloxacin release compared to F2. The decrease of material

crystallinity after cross-linking process caused implants structure became amorph, so that ciprofloxacin was easier to dissolve and release from the implants [30].

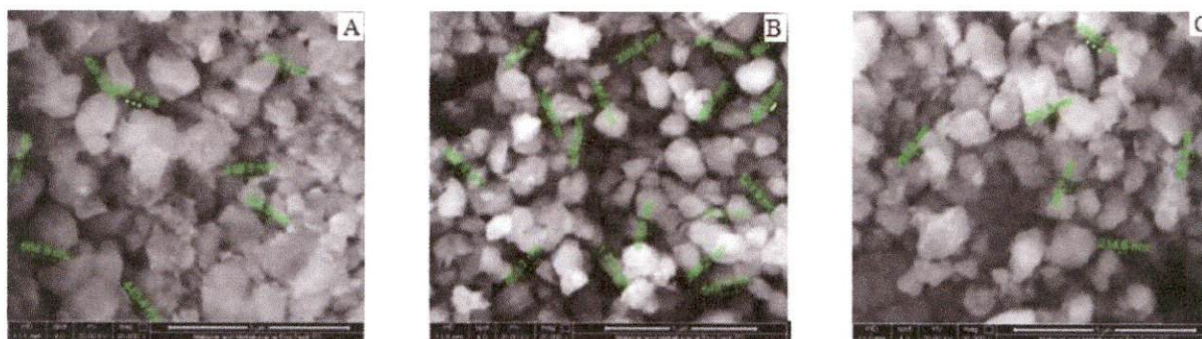


Fig. 8: SEM micrograph of the implants, A): F1 (0.5% glutaraldehyde), B): F2 (0.75% glutaraldehyde), C): F3 (1.0% glutaraldehyde)

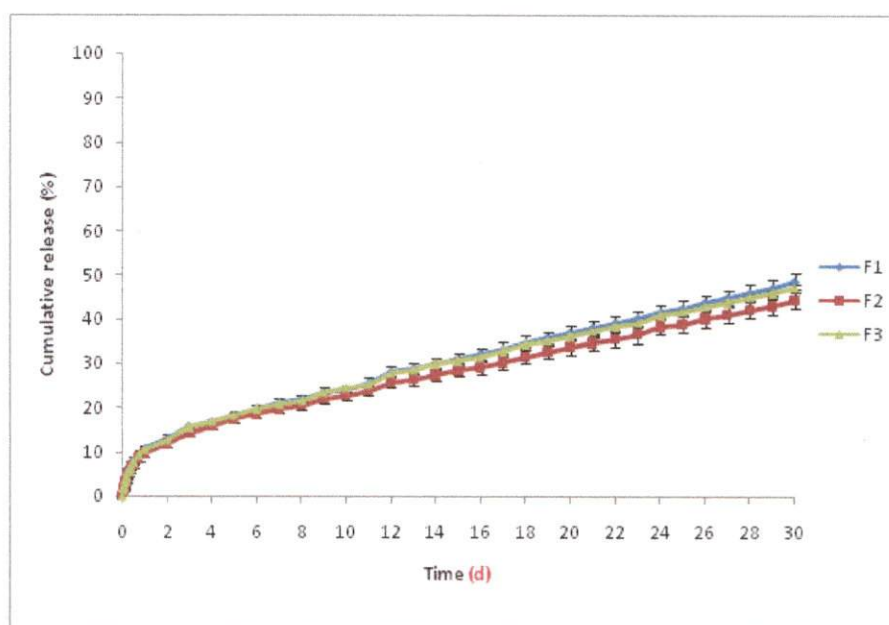


Fig. 9: Ciprofloxacin release profile of F1 to F3 formulations

Characterization of implants

Fourier transformed infrared spectroscopy (FT-IR)

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 (F1 to F3) is shown in fig. 10. In the spectrum of the implants before cross-linking with glutaraldehyde, chitosan absorption bands situated at 1658.67 cm^{-1} (C=O stretching in amide group). Some changes can be noted after the cross-linking process with glutaraldehyde. The peak at 1658.67 cm^{-1} shifts to lower wave numbers $\sim 1630\text{ cm}^{-1}$. 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 $1620\text{--}1660\text{ cm}^{-1}$ [15]. Moreover, it is not observed any band at $\sim 1715\text{ cm}^{-1}$, connected with free aldehyde group. The increase of glutaraldehyde concentration to 1.0% caused a successive increase in the intensity of the ethylene bond (C=C) at 1583 cm^{-1} [27]. This fact can be

attributed to the increase of glutaraldehyde molecule contribution in the cross-linking reaction so that this condition increases the crosslinking chain [27]. Infrared spectrum of the implants after cross-linking process also showed a shift of N-H and O-H stretching vibration of chitosan molecules (3475.49 cm^{-1}) to 3420 cm^{-1} in implants. This condition indicated interactions between the materials which composed the implants.

X-ray diffraction study

X-ray diffraction of the implants after cross-linking process are shown in fig. 11. X-ray diffractogram of the implants are compared with X-ray diffraction of Bovine Hydroxyapatite, chitosan, ciprofloxacin, and the implants before the cross-linking process. The X-ray pattern of chitosan shows major crystalline peaks at $2\theta \approx 10^\circ$ and $2\theta \approx 20^\circ$. But, thX-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 [34].

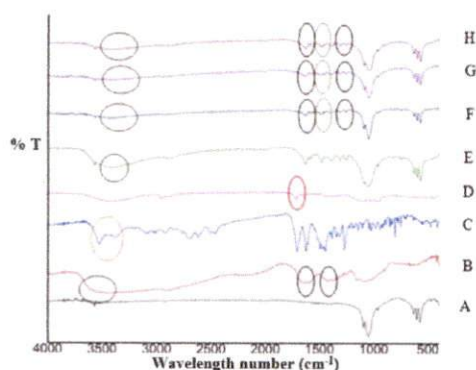


Fig. 10: FTIR spectrum of, (A): Bovine hydroxyapatite; (B): Chitosan; (C) Ciprofloxacin; (D) Glutaraldehyde; (E) Implants before cross-linking process; (F): Formula 1; (G): Formula 2; (H): Formula 3

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 can 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.

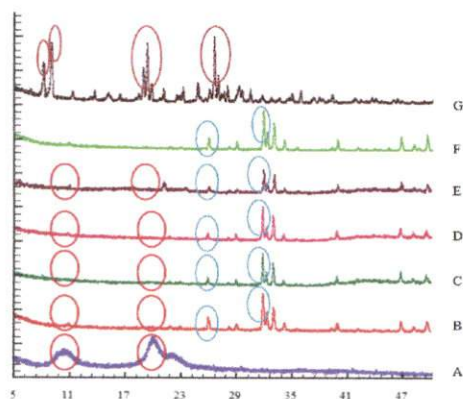


Fig. 11: X-ray diffraction spectrum of (A): Chitosan; (B): Implant before cross-linking process; (C) Formula 1; (D) Formula 2; (E) Formula 3; (F): Bovine Hydroxyapatite; (G): Ciprofloxacin

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

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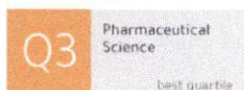
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