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OPTIMIZED FORMULATION OF VANCOMYCIN LOADED THERMOREVERSIBLE HYDROGEL FOR TREATMENT OF ORTHOPEDIC INFECTIONS

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ABSTRACT: The aim of the present study was to develop suitable controlled release injectable vancomycin thermoreversible chitosan/ β-glycerophosphate hydrogel system for treatment of orthopedic infections. The effect of different formulation variables on the gelation temperature was studied. The prepared vancomycin loaded Chitosan/β-glycerophosphate thermoreversible hydrogels were characterized regarding gelation temperature, *in-vitro* vancomycin release and enzymatic degradation rate in presence of lysozyme. Antimicrobial activity of the released vancomycin was tested against the gram-positive methicillin resistant *Staphylococcus aureus* (MRSA) by agar diffusion method. The results showed that chitosan/ β-glycerophosphate solution were able to form gels at body temperature depending on chitosan degree of deacetylation, concentration, pH and β-GP concentration. The prepared thermoreversible hydrogels sustained vancomycin release for 21 days. Released vancomycin concentrations ranged from 3 to 95 folds the reported minimal inhibition concentration (MIC 90) for MRSA at all release study time points. Chitosan/β-glycerophosphate thermoreversible hydrogels were enzymatically degradable and 17.57% of the gel weight remained after 21 days. The preserved antimicrobial activity found after release of the antibiotic from the hydrogel elected this delivery system for further *in-vivo* and toxicological investigations.

INTRODUCTION: Bone and joint infections, including osteomyelitis and septic arthritis, are often difficult to treat and require prolonged courses of antimicrobial therapy in association with surgical drainage or debridement.

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Delayed or ineffective treatment of these orthopedic infections, due to their poor vascularity, causes significant morbidity in terms of pain, loss of function with need for further surgery and antibiotics. Most of these infections are caused by Gram-positive cocci including *Staphylococcus* spp. and enterococci, increasingly resistant to commonly used antibiotics such as methicillin and other β-lactam agents [1].

Vancomycin is an effective therapy empirically used for treating serious orthopedic infections with less toxic effects on osteoblasts and skeletal cells

compared to other antibiotics [2, 3]. It should be administered intravenously over a minimum of 60 minutes to avoid infusion-related reactions for a period reaching 6 weeks or longer [4]. This is usually associated with serious adverse effects including hypotension, urticaria, pruritis, phlebitis, nephrotoxicity, and ototoxicity [5]. Vancomycin nephrotoxicity, observed in 5–25% of patients, usually limits the antibiotic dose and duration of administration resulting in incomplete bacterial eradication and development of bacterial resistance [6].

For this reason, the delivery of local antibiotics for the treatment of musculoskeletal infection has become increasingly popular. High local antibiotics levels facilitate diffusion to the poor vascular areas reaching the resistant organisms. Among these systems are the implants, micro and nanoparticles delivery systems [7-9]. Despite the advantages offered by these antibiotic delivery carriers, most of them lack applicability due to concerns about the delivery or implantation of the systems at infection sites, degradadability and tolerability of the systems in addition to the difficulty of their industrial fabrication [7,9,10].

Gels, in particular the thermoreversible ones, are swollen networks possessing both the cohesive properties of solids and the diffusive transport characteristics of liquids. Their reversible nature confers the capacity to make, break and modify the bonds responsible for holding the network [11]. In this respect, Chitosan/ β-glycerophosphate (Ch/β-GP) hydrogel is liquid at room temperature and can be easily administered and pack any target shape upon injection and turns into biodegradable and biocompatible gel as response to physiological temperature [12]. Both Ch and β-GP have osteogenic activity and have been able to support *in-vitro* and *in-vivo* accumulation of cartilage matrix by primary chondrocytes, while persisting in osteochondral defects at least one week *in- vivo* [13].

The aim of this study was to develop vancomycin injectable thermoreversible Ch/ β-GP hydrogel system for treatment of orthopedic infections. The effect of Ch degree of deacetylation (DD), concentration and pH as well as β-GP concentration on the properties of the prepared thermosensitive gels were studied. Release of vancomycin from the prepared gel and gel degradation were studied *in-vitro.* The antibacterial activity of the released antibiotic was tested against MRSA. Using the prepared gel, we expected to control the rate of antibiotic release over a prolonged period of time eliminating the need for multiple dosing. This will in turn, increase the efficacy of drugs and enhance the patients compliance.

MATERIALS AND METHODS:

MATERIALS: Vancomycin: supplied from Sigma pharmaceutical company Egypt. Ch high molecular weight, β-GP disodium salt, N-acetyl-D-glucosamine, glucosamine, lysozyme (from chicken egg white 58.1 U/mg protein), phosphoric acid and glacial acetic acid: from Sigma–Aldrich Company, St. Louis USA. Potassium dihydrogen ortho phosphate, sodium hydroxide, sodium chloride, potassium chloride, sodium dibasic hydrogen ortho phosphate and hydrochloric acid: from Fluka, Switzerland. All other reagents and chemicals were of analytical grade.

METHODS:

Determination of chitosan degree of deacetylation (DD): Ch DD was determined by the previously described first derivative UV spectrophotometric method [14]. Briefly, 0.5% w/v solution of Ch in 85% w/v phosphoric acid was heated at 60^{o}C for 40 minutes after which the solution was filtered, diluted 1:100 with deionized water and then incubated at 60^{o}C for 2 h. This solution was scanned in the range of 190 to 400 nm using 0.85% v/v phosphoric acid as blank using a UV spectrophotometer (Schimadzu 240, Japan). The first derivative value at 203nm (wavelength of minimum interference with glucosamine) was recorded and the DD was calculated according to the following equation[14]:

$$DD\% = 100 - \frac{\left(\frac{m1}{203.21}\right)*100}{\left(\frac{m1}{203.21}\right)+\left(\frac{m2}{161.17}\right)} \quad \textbf{Equation 1}$$

Where: *m1* is the mass of acetyl-glucosamine in 1mL Ch solution, calculated from a previously constructed calibration curve of acetyl-glucosamine; *m2* is the mass of glucosamine in 1mL Ch solution, calculated as m2=M-m1. The mass of Ch (M) in the 1 mL solution (step2) was calculated from: M= (M1*M3)/(M1+M2), where M1 is mass of solid Ch sample taken for analysis (100 mg); M2 is mass of 20 mL 85% w/v phosphoric acid (step 1); M3 is mass of 1mL Ch solution in concentrated phosphoric acid, 203.21 and 161.17 are the molecular weights of acetyl-glucosamine and glucosamine respectively.

Preparation of highly deacetylated Chitosan: An alkaline hydrolysis procedure was adopted to increase Ch DD as follow[15]: A 10 % w/v of Ch in 50% w/v sodium hydroxide solution was heated at 100^{o}C for 2 h. The obtained precipitate was washed consecutively and repeatedly with deionized water, methanol and acetone till neutrality and then dried in an oven at 60^{o}C till complete dryness [15]. Dried Ch samples were stored in a desiccator for further characterization. DD for the modified Ch sample was determined as previously explained.

FT-IR spectroscopy of Chitosan: The infrared spectra of Ch with and without alkaline deacetylation were recorded on a FT-IR Spectrometer (JASCO 4000, USA). A well mixed mixture of Ch and KBr (2 mg sample in 100 mg of KBr) was made in a disc and the spectra were recorded in the range 4000-400 cm^{-1}.

Preparation of thermoreversible chitosan /β-glycerophosphate hydrogel: Aqueous acetic acid solution (0.75%v/v) containing 1 and 2% w/v of Ch, with different DD were prepared, autoclaved (Thermo Scientific autoclave, Varioklav laboratory, Germany) at 121°C for 20 minutes, cooled down to room temperature and then stored at 4°C. Serial concentrations of β- glycerophosphate were prepared in deionized water, sterilized using syringe filters (Durapore®, 0.22 µm pore size, Millipore, USA) then stored at 4°C. Sterilized, cold β-GP solution (0.1-1 g/mL) was added drop wise to the Ch solution under stirring using mechanical stirrer (FALC instruments Trevi Glio (BG), Italy) in an ice bath (volume ratio of Ch: β-GP, 9:1) and the final concentration of β-GP in Ch solutions ranged from 1-10% w/v [16].

Mixing was continued for 15 minutes and the prepared solutions were kept at 4˚C. The procedure was repeated on selected Ch solutions after dialysis against 1 liter of distilled water for 6 days with daily changes of water (till constant Ch solution pH). The prepared formulae are shown in **Table 1**.

For the preparation of medicated hydrogel, vancomycin (1% w/v) was first dissolved in Ch solution and the thermoreversible hydrogels were prepared as previously explained.

Characterization of thermoreversible chitosan /β-glycerophosphate hydrogel:

Determination of hydrogel pH: Five mL of each formula were transferred to a glass beaker dipped in an ice bath and the pH of was measured using a pH meter (Jenway Ltd, UK).

Sol-to-gel temperature of thermoreversible chitosan /β-glycerophosphate hydrogel: Sol-to-gel transition temperature was measured using a simple test tube inverting method. Briefly, an aliquot of 2mL of each refrigerated formula was transferred to a test tube maintained in a thermostatically controlled water bath (Poly science 9006, USA) at 25^{o}C. The water bath temperature was increased gradually by 1^{o}C, then by 0.1^{o}C in the region of gelation and the test tube was left to equilibrate for 5 minutes at each temperature [17].

Determination of gelation time: Two mL of each refrigerated thermosensitive solution was transferred to a test tube incubated in a water bath at 37^{o} C. The flowability of the sample was observed every 30 seconds by tilting the tubes. The time after which flowing of the samples stopped was taken as the gelation time [18].

Scanning electron microscopy (SEM): The shape and surface morphology of selected plain and vancomycin loaded thermoreversible Ch/β-GP hydrogel were observed by SEM (JXA-840A, Japan). A volume of 5 mL of thermoreversible Ch/ β-GP hydrogel was incubated in a water bath at 37^{o}C. When the sample was transformed into gel, it was freeze dried using BenchTop Manifold Freeze Dryer (Millrock Technology, Inc, USA) for 48 h. The sample was finally vacuum coated with gold and the surface was examined by SEM.

***In-vitro* vancomycin release from thermo-reversible chitosan /β-glycerophosphate hydrogel:** A volume of 1 mL of vancomycin loaded thermosensitive Ch solution was placed in a dialysis membrane (Spectra/Por® CE, MWCO 1000Da, Flat, width 8mm and diameter 5mm, China) and was allowed to gel in an incubator (Julabo Biother 37, Germany) at 37°C. Each dialysis membrane was placed in 50 mL phosphate buffer saline pH 7.4 in a stoppered conical flask placed on thermostatically controlled shaker (D3006 Brug Wedal, Germany) at 50 ± 1 rpm for 21 days. At predetermined time intervals, aliquots of 1mL of the release medium were sampled and replaced with the same amount of fresh dissolution medium to maintain a constant volume. Drug concentration was determined Spectrophoto-metrically at the predetermined λ_{max} (280 nm).

Release kinetics: Release data were analyzed using the following equation [19]:

$$\frac{M_t}{M} = Kt^n \qquad \textbf{Equation 2}$$

Where *Mt*/*M* is the fraction of drug that has been released at time t, *k* is a kinetic constant and *n* is the exponent related to the release mechanism .The constant *n* is termed the diffusional exponent and equal to 0.5 for diffusional (Fickian) release, 1 for zero-order kinetics and $0.5 < n < 1$ for anomalous (non-Fickian) release [19].

***In-vitro* enzymatic degradation:** Degradation of vancomycin thermoreversible Ch/β-GP hydrogel was examined with respect to weight loss under aqueous conditions in the presence of lysozyme. A volume of 1mL of the prepared formula was allowed to gel in a beaker placed in an incubator at 37°C then was suspended in 10 mL of phosphate buffered saline (pH 7.4) containing 4mg/mL lysozyme at 37°C [20]. The sample was placed in a thermostatically controlled shaker at 50 ± 1 rpm for 21 days. At predetermined time intervals of time, remaining autogel was removed from the medium, frozen at -80°C then lyophilized at -50°C for 48 h. The degradation was assessed by determining the weight loss % using the following equation:

$$\textbf{Weight loss}\% = \frac{W0-Wt}{W0} * 100 \qquad \textbf{Equation 3}$$

Where W_0 and W_t were the weights of the dry lyophilized gel before and after degradation at time t, respectively.

***In-vitro* microbiological activity:** The antibiotic activity of vancomycin in the release eluents withdrawn at different time intervals was tested against standard strain of MRSA as described by Julia *et al*., (2011) [21]. The bacteria were subcultured in 10 mL broth overnight at 37 °C. The broth culture was then diluted with sterile phosphate buffer, so that the density of the inoculum was $l0^9$ CFU/mL and was then added to 20 mL of the medium (agar Oxoid Inc., at pH 7.3). Three holes, each of 5 mm in diameter, were punched on every plate, and filled with 10 µl of vancomycin eluents. After incubation at 37°C for 24 h, the diameter of the clear zone surrounding the wells was measured. The activity was calculated after subtracting the diameter of the central well (5 mm) for each sample using a linear calibration curve prepared for vancomycin concentrations in the range 20 to 200 µg/mL.

Statistical analysis: All experiments were done in triplicate. Blank thermoreversible gels were always prepared and tested along with their medicated counterparts. Data are presented as mean of the three replicates for each experiment ± SD. Unpaired student-t test was used for comparing between two variables and probability values *P* value <0.05 was considered significant.

RESULTS AND DISCUSSION: As Ch DD of deacetylation greatly affects its physicochemical properties, it deemed necessary to start by the determination of this parameter [22].

Determination of chitosan degree of deacetylation: Ch DD (i.e. the mole fraction of N-glucosamine expressed as a percentage of total Ch sample) was determined using an indirect spectrophotometric method. The assay depends on measuring the absorbance intensity of Ch acetyl group using a first derivative spectrophotometric method **(Figure 1a)** [14]. Ch was firstly heated in phosphoric acid at 60°C for 40 min to allow for its complete dissolution and avoid its reaggregation. Higher temperature or longer heating time can lead to the formation of 5-hydroxymethylfurfural (HMF), a dehydration product of hexoses,

including glucosamine and acetyl-glucosamine [23]. HMF can significantly reduce the number of acetyl-glucosamine units increasing the calculated DD. Therefore, UV scanning of Ch was followed in the broad range of 190-400nm to ensure the absence of HMF peak, which has a characteristic absorption peak at 285 nm [23]. Diluting Ch solution with water before heating for 2h at 60^{o}C is an important step to avoid overestimation of DD. In presence of concentrated acids and heat, polysaccharides undergo complex reactions forming some intermediates which can block acetyl group. Hydrolyzing these possible complexes in aqueous media by dilution avoid overestimation of DD [14]. After applying these conditions, Ch was scanned **(Figure 1b),** the absorption intensity of N-acetyl GLc at 203nm was obtained and its concentration was found from the corresponding calibration curve, The DD value was calculated using equation 1 and was found to be 78.05%±1.38.

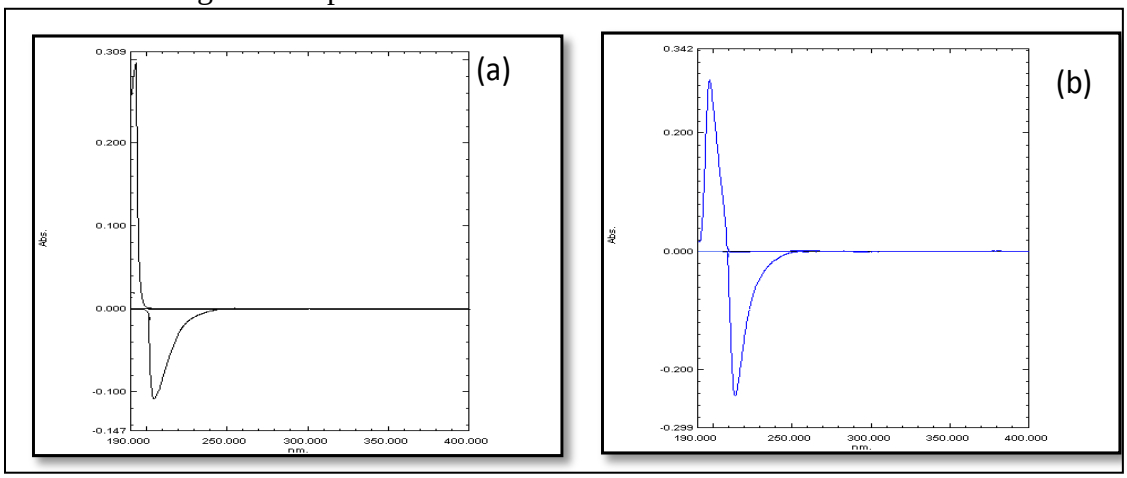

FIGURE 1: ZERO AND FIRST DERIVATIVE SPECTRUM OF (A) 5 MG/ML N-ACETYL GLC AND (B) HMW CHIN 0.85% V/V PHOSPHORIC ACID.

Preparation of highly deacetylated chitosan: The process of deacetylation involves the removal of acetyl groups from the molecular chain of Ch leaving behind a high proportion of chemically reactive amino group (-NH2). This helps to ensure high positive charge density, with higher mucoadhesion [22]. Deacetylation also affects the biodegradability and immunological activity [24]. In this work, the alkaline deacetylation process was used to increase Ch DD by heating Ch sample at 100˚C for 2 h [25]. The obtained deacetylated product was washed using deionized water, methanol and acetone consecutively as suggested by previous investigators [14]. Using this sequence, the drying process lasted only 12 h due to the hygroscopic nature of the organic solvents used which extracted water in Ch sample and then rapidly evaporated [24]. Ch, after alkaline hydrolysis for 2 h, was scanned as previously described in the range (190-400 nm) and the first derivative was manipulated. The results show that alkaline treatment resulted in a significant increase (*P<0.05*) in DD from 78.05%±1.38 to 94.08% ± 1.48 as calculated using equation 1.

It has been shown that, although the DD is directly proportional to alkali concentration and heating time yet, the complete deacetylation can never be achieved by this heterogeneous deacetylation process without modification [25].

Characterization for chitosan by FT-IR spectroscopy: FT-IR can efficiently be used for the detection of chemical functional groups in a sample, investigation of intermolecular interaction as well as the change in their intensity. It was therefore employed to characterize the effect of alkaline treatment during the deacetylation process. **Figure 2a** shows the FT-IR spectrum of unmodified Ch where the following absorption bands could be observed: N-H stretching band at 3437 cm^{-1}, a broad absorption band in the range 3000- 3500 cm^{-1} attributed to an overlap between O–H and N-H bands and peaks around 2885, 1424, 1380 and 1080 cm^{-1} resulting from the stretching vibrations of aliphatic C–H, amide II, amide III and C–O–C bonds respectively and more importantly, the band around 1650 cm^{-1} attributed to amide I (-NH) deformation of –NHCOCH3 [26].

An obvious decrease in intensity of the later band could be seen in **Figure 2b**, for sodium hydroxide treated Ch, corresponding to untreated Ch. This decrease in the intensity of amide I band was due to Ch deacetylation confirming the preparation of Ch with a higher degree of deacetylation as explained in previous studies [24].

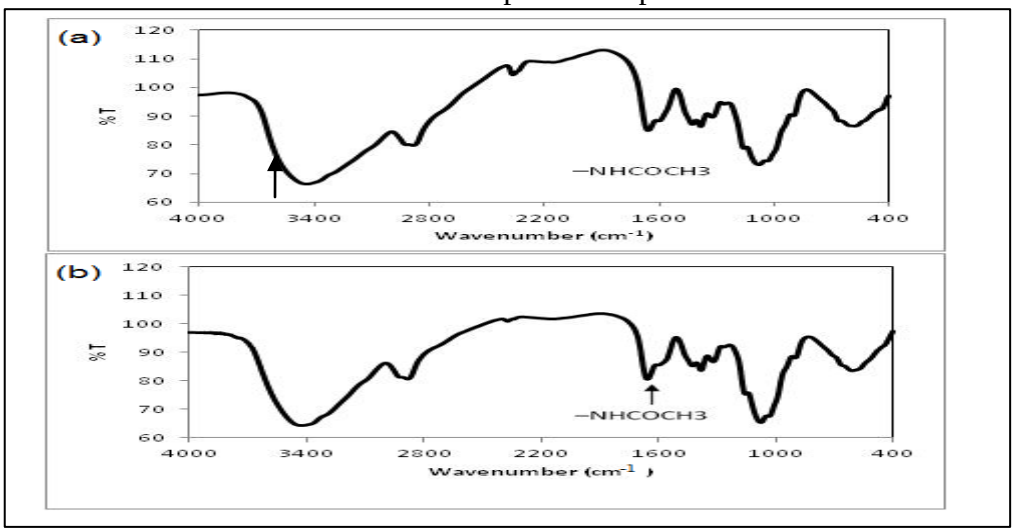

FIGURE 2: FT-IR OF (A) UNMODIFIED CH AND (B) CH AFTER 2 HR ALKALINE DEACETYLATION

Preparation of plain thermoreversible chitosan /β-glycerophosphate hydrogel: The gelation of a Ch/β-GP solution is thermally induced via several types of interactions including electrostatic attractions/repulsions, hydrogen bonding and hydrophobic effects [27, 28]. When a weak base, as disodium β-GP solution, is added to an acidic Ch solution, the pH increases and electrostatic attractions between positively charged Ch (NH_3^+) and negatively charged phosphate molecules (-HPO_4^{-1} or -PO_4^{-2}) of β-GP can occur. Moreover, the charge neutralization caused by β-GP anion causes a reduction in electrostatic repulsion between the Ch chains leading to an increase in hydrogen bonding interactions along Ch interchains [27]. Furthermore, glycerol molecules of β-GP form a shield of water around Ch molecules inhibiting their aggregation at low temperature. By raising the temperature, Ch-Ch interactions become dominant resulting in a phase transition from a liquid to a gel while hydrogen-bonding interactions are reduced. Phosphate groups function as a sink for the protons released as a result of breaking of Ch hydrogen bonding [29]. Ch chains are subsequently brought close to precipitation resulting in the gelation of the Ch/β-GP formulation [27, 29].

In this study, Ch with different degree of deacetylation (LDD and HDD) was used to prepare thermoreversible autogels by mixing with β-GP in concentrations ranging from 1 to 10% w/v and the gelation process was observed at different temperatures up to 50°C. The gelation temperatures of the different studied systems are shown in **Table 1**.

Effect of chitosan degree of deacetylation: The results in Table 1 show that 1% w/v Ch with low DD (LDD) solutions failed to form thermoreversible hydrogel with the tested concentration range of β-GP and 10% of the gelator was needed to give gels with physiologically acceptable gelation temperature (32-34°C) at 2% polymer concentration. At this concentration, appropriate physiological gelation temperature (32-34°C) was also achieved using 6% w/v β-GP for high Ch DD respectively. At this β-GP concentration, an obvious significant decrease in gelation temperature from > 50°C to 32.63°C ± 0.73 ($P<0.05$) can be seen using HDD instead of LDD. The decrease in gelation temperature with increasing Ch DD can be explained as follow: during the gelling course of Ch/ β-GP, the hydrated Ch molecular chains are gradually dehydrated; they interacted with each other and then rearranged themselves to form crystalline regions with gel formation. Conformation and chain flexibility in the gelling process play an important role. Ch chain flexibility increased as DD increased [30]. This effect facilitated rotation of the glycosidic bond,

rendering the gelation process easier which resulted in decrease in the gelation temperature. Furthermore, Ch with higher DD had more amine groups and therefore could form more cross-links with the phosphate group of glycerophosphate. This also led to an increase in the gelation rate and consequently decreased the gelation temperature [31]. Similarly, previous studies had proved that increasing Ch DD reduced significantly the gelation temperature [32].

TABLE 1: GELATION TEMPERATURE OF DIFFERENT CH/B-GP HYDROGELS.

Chitosan characteristics		Gelation temperature (^oC) at different β-GP concentration (%w/v)									
DD%	**Conc. (%w/v)**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
78.05	**1**	← No gelation →									
	2	←			>50			→	41.40 ± 0.25	39.10 ± 0.15	34.10 ± 0.10
94.08	**1**	>50		44.20 ± 0.10	33.90 ± 0.10	←		NA*		→	
	2		>50		46.10 ± 0.10	36.50 ± 0.15	32.60 ± 0.73	←	NA	→	

*NA: not applied.

Effect of chitosan concentration: Table 1 shows that doubling Ch concentration increased the gelation temperature significantly from 33.9°C ±0.1 to 46.1°C ±0.1 ($P<0.05$) in presence of 4% w/v β-GP. This can be justified by the greater viscosity of Ch, with higher density of hydrogen bonding requiring higher energy (temperature) to breakdown these bonds and allow for the formation of Ch/ β-GP thermoreversible hydrogel.

Effect of β-glycerophosphate concentration: A significant decrease in gelation temperature could be noticed by increasing β-GP concentration using the same Ch DD or concentration. It could be noticed from Table 1 that, high gelation temperatures exceeding 50^{o}C were seen with LDD-Ch at β-GP concentration up to 7% w/v. Furthermore, high concentration of β-GP amounting of 10% w/v was necessary to obtain solution which can gel at body temperature. On the other hand, high DD-Ch succeeded to gel at less than 37^{o}C using only 6% w/v β-GP. Obviously, there is an inverse relation between the gelation temperature and the concentration of β-GP used. The phosphate group of β-GP is the counter-ion for Ch, when its concentration increases, enhancement of Ch ammonium groups ($-NH_3^+$) charge neutralization occurs [30]. Therefore, reducing both Ch chain charge density and electrostatic repulsion between the NH_3^+ groups and increasing the chain flexibility [30]. Ch chains are then easier to close, entangle, interact and turn into gel.

Several strategies have been previously reported to increase the biocompatibility of Ch/ β-GP systems. This includes the use of inorganic phosphate salts or crosslinkers such as glyoxal and hydroxyethyl cellulose [28, 33]. In this study, an alternative approach based on the dialysis of the acidic Ch solution before the addition of β-GP was tried in this study with 2% w/v HDD- Ch as shown in **Table 2**.

Effect of initial chitosan solution pH: Another important factor related to Ch ionization is the effect of pH. Therefore, the pH value of Ch solution was measured before and after dialysis and the gelation temperatures of the compared autogels were determined as shown in Table 2. It is clear that the pH of Ch solution was significantly increased from 5.32 ± 0.01 to 6.16 ± 0.05 ($P<0.05$) due to dialysis. Furthermore, this process led to a significant decrease in the gelation temperature from 36.5^{o}C±0.73 to 31.4^{o}C±0.2 using 5% w/v β-GP (Table 2). This may be attributed to a lower degree of Ch protonation occurring at higher pH

value. The decrease in degree of Ch protonation leads to a probable decrease in its solubility. A possible increase in hydrophobic interactions between Ch molecules occurred in an endothermic process. Protons are easily released and hence hydrating water is more easily removed from the Ch [34]. Furthermore, and as previously discussed, lower Ch molecular charge density leads to reduced charge repulsion between the –NH3+ groups of Ch results in a more flexible chain [30]. These results are similar to those reported by previous studies [34].

TABLE 2: EFFECT OF CH DIALYSIS ON GELATION TEMPERATURE OF CH/ B-GP THERMOREVERSIBLE GELS PREPARED WITH 2% W/V CH WITH 94.08 % DD

Ch characteristics		Gelation temperature (^oC) at different β-GP concentration (%w/v)					
DD%	**pH**	1	2	3	4	5	6
94.08	**5.32**	←	>50	→	46.1± 0.1	36.5± 0.15	32.6± 0.73
	6.16	←	>50	→	43.6± 0.11	31.4± 0.2	NA*

* NA: not applied.

Based on the criteria of attaining an optimum gelation temperature below body temperature at a low β-GP concentration to maintain biocompatibility, vancomycin was loaded in the gel composed of 2% w/v dialyzed-Ch (with DD 94.08%) and 5% w/v β-GP (F1).

Preparation and characterization of vancomycin loaded thermoreversible chitosan /β-glycerophosphate hydrogel: As seen in **Table 3**, the gelation temperature increased significantly (*P<0.05*) from 31.4^{o}C±0.2 to 35.5^{o}C ±0.3 after vancomycin incorporation. This was actually accompanied by a significant decrease (*P<0.05*) in the pH of Ch/ β-GP solution from 6.99±0.02 to 6.70±0.10 after vancomycin addition. This pH decrease was probably the result of the ionization of vancomycin carboxylic acid group at pH 6.7 i.e. below one of its isoelectric points (pH =7.2) [35]. The pH decrease enhanced the charge density along the Ch chain which subsequently required more thermal energy to initiate gelation. Similarly, the gelation time was also significantly increased from 2.63 min ±0.15 to 3 min ±0.1.

TABLE 3: PHYSICAL CHARACTERIZATION OF CH/B-GP THERMOREVERSIBLE HYDROGELS.

Parameter	Plain autogel	Vancomycin loaded autogel
pH	6.99 ±0.02	6.70±0.10
Gelation Temperature (^oC)	31.40 ±0.20	35.50±0.30
Gelation Time (min)	2.63±0.15	3.00 ±0.10

Morphology study of thermoreversible chitosan /β-glycerophosphate hydrogels: The morphological architectures of the prepared plain and vancomycin loaded Ch/ β-GP thermoreversible hydrogel are displayed in **Figure 3**. The micrographs show clearly irregular deeply porous structure with rough surface. The addition of vancomycin obviously increased surface roughness with a decrease in the noticed porosity in the plain formula. No drug crystals could be seen on vancomycin loaded thermosensitive gel indicating the distribution of vancomycin in the interior of the gel [17].

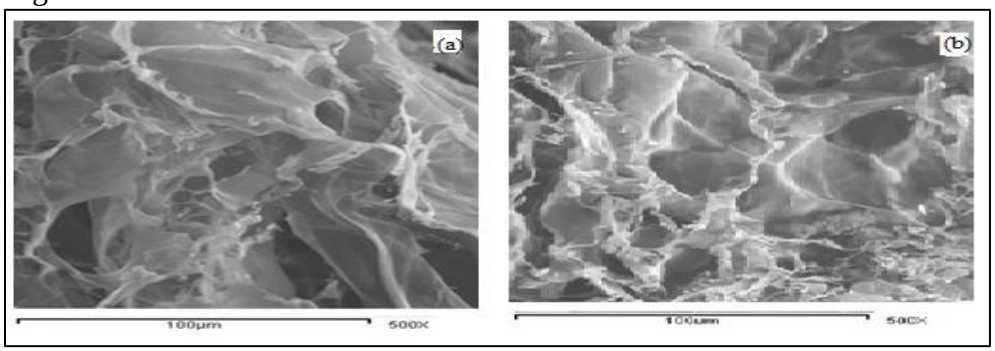

FIGURE 3: SEM OF PLAIN (A) AND VANCOMYCIN (B) CH/ B-GP THERMOREVERSIBLE HYDROGEL

***In-vitro* release of vancomycin from thermoreversible chitosan /β-glycerophosphate hydrogel:** The release of vancomycin from Ch/ β-GP autogel formula F1 is illustrated in **Figure 4a**. Vancomycin powder, as received, was dissolved rapidly achieving 100% of vancomycin release in 45min. It is obvious that the inclusion of vancomycin into Ch/β-GP autogel had sustained the drug release where 95.66% ±3.78 of vancomycin was released in 21 days. No burst release was noticed indicating the proper incorporation of the drug in the gel matrix. The release kinetic data of vancomycin release from the formulated Ch /β-GP gel revealed an n value of ≈0.8 denoting an anomalous release mechanism (coupled erosion-diffusion mechanism).

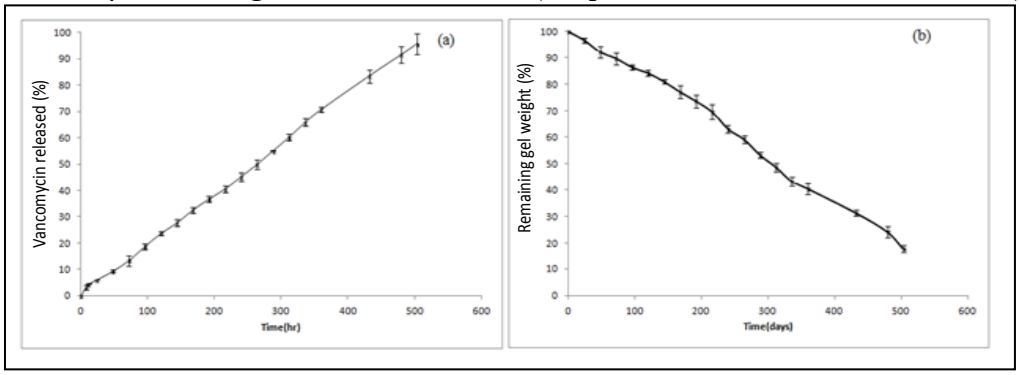

FIGURE 4: *IN-VITRO* (A) RELEASE PROFILE OF VANCOMYCIN FROM CH/B-GP THERMOREVERSIBLE GEL FORMULA F1 AND (B) GEL DEGRADATION

***In- vitro* enzymatic degradation:** Polymers biodegradation and accumulation remain the biggest challenges for their clinical application in drug delivery. In order to predict the biodegradation behavior of F1, the weight loss method was used and vancomycin loaded Ch/β-GP was incubated in PBS pH 7.4 in presence of lysozyme, the weight remaining at different time intervals for a period up to 3 weeks is illustrated in **figure (4B)**. There was a gradual decrease in the remaining gel amounts and only 17.57% ±1.43 of the prepared gel was retained for 21 days.

Lysozyme, present in many body fluids was used in this study to evaluate the degradation of the gel. Because Ch was the main constituent of the gel [36, 37], we assume that the weight loss of the hydrogel in lysozyme solution resulted mainly from breakage of the glycosidic bonds of Ch molecules. Relating these data with those obtained with the release of vancomycin revealed that no accumulation of the polymers used will be expected after drug release. The drug was released *in-vitro*, in a synchronized manner with the delivery system degradation, confirming the erosion-coupled diffusion drug release mechanism. This prolonged vancomycin delivery will also eliminate the need for multiple dosing.

***In-vitro* microbiological activity:** Released vancomycin concentrations were found to be above the reported minimal inhibition concentration (MIC 90) for MRSA (=2 µg/mL) [38] at all-time points of the release study. The lowest vancomycin concentration released was 6.28 µg/mL, equivalent to 3 folds that of the MIC90 while, the highest concentration (191.32µg/mL) was equivalent to 95-fold of the MIC90.

Figure 5 illustrates the antibiotic activity of the released vancomycin at different time intervals. The calculated bactericidal activity of the 48 h sample was 93.48% ± 1.61 after 48 hr. This antibiotic activity showed slight decrease at each successive time point where it significantly decreased after 21 days of release study reaching 74.62% ± 3.54. This slight decrease is probably due to the partial hydrolysis of vancomycin in aqueous dissolution medium [21]. We assume that this partial hydrolysis does not exist in the body. Further confirmation could be withdrawn for future *in-vivo* experiments.

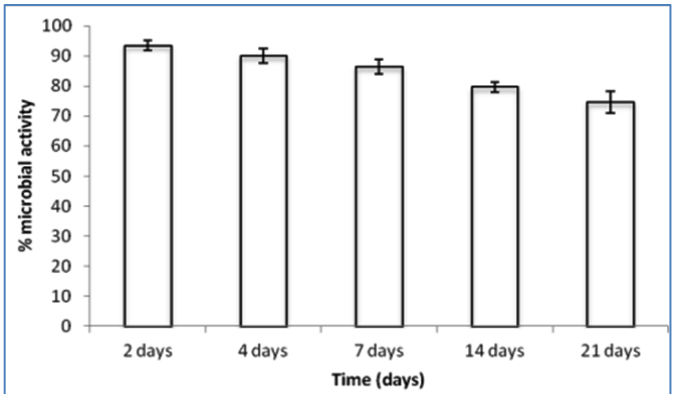

FIGURE 5: *IN-VITRO* ANTIBACTERIAL ACTIVITY OF THE PREPARED VANCOMYCIN CH/B-GP THERMOREVERSIBLE HYDROGEL

CONCLUSION: In this study, developed an injectable vancomycin Ch/β-GP thermoreversible gel for MRSA treatment with sustained delivery of vancomycin was developed. The Ch/β-GP solution underwent thermoreversible gelation around body temperature. Ch DD, concentration, pH and β-GP concentration effects on thermogelation process were evaluated. Manipulating the degree of deacetylation, concentration, pH of chitosan and the concentration of β-GP could lead to a gelation temperature for the Ch/β-GP thermoreversible hydrogel close to body temperature. The use of dialyzed highly deacetylated Ch solution (DD 94.08%, pH 6.16) reduced the concentration of β-GP required for gelation at physiological concentration which is essential to improve gel biocompatibility.

The prepared system was able to sustain vancomycin release for 21 days with no burst effect. The gel maintained its structural integrity in presence of lysozymes which is essential to avoid burst release of drug *in-vivo*. The released vancomycin concentration range was equivalent to 3 to 95 folds that of the MIC90 of vancomycin solution. The bactericidal activity of vancomycin was calculated as 93.48% ± 1.61 after 48 h and 74.62% ± 3.54 in 21 days. This study provides rationale for further *in-vivo* and toxicological investigations.

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REFERENCES:

1 Haidar R, Boghossian AD and Atiyeh B: Duration of post-surgical antibiotics in chronic osteomyelitis: Empiric or evidence-based? Int J Infect Dis. 2010; 14(9):e752-e758.
2 Vandecasteele SJ and De Vriese AS: Recent changes in vancomycin use in renal failure. Kidney Int. 2010; 77:760-764.
3 Ketonis C, Barr S, Adams CS, Shapiro IM, Parvizi J and Hickok NJ: Vancomycin Bonded to Bone Grafts Prevents Bacterial Colonization. Antimicrobial agents and chemotherapy 2011; 55(2):487-494.
4 Hicks RH and Hernandez J: Perioperative Pharmacology: A Focus on Vancomycin. AORN J 2011; 93(5):593-599.
5 Gutierrez K: Pharmacotherapeutics: Clinical Reasoning in Primary Care. St Louis, MO, Saunders, 2008.
6 Fowler VG, Boucher HW, Corey GR, Elias A, Karchmer AW, Rupp ME, et al: Daptomycin versus standard therapy for bacteremia and endocarditis caused by *Staphylococcus aureus*. N Engl J Med 2006; 355(7):653-665.
7 Li B, Brown KV, Wenke JC and 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: 221-230.
8 Zongping X, Xin L, Weitao J, Changqing Z, Wenhai H and Jianqiang W: Treatment of osteomyelitis and repair of bone defect by degradable bioactive borate glass releasing vancomycin. J. Control. Release 2009; 139:118-126.
9 Honary S, Ebrahimi P and Hadianamrei R: Optimization of particle size and encapsulation efficiency of vancomycin nanoparticles by response surface methodology. Pharm. Dev. Technol. 2014; 19(8):987-998.
10 Samit K N, Prasenjit M, Subhasis R, Biswanath K, Dipak KD and Debabrata B: Local antibiotic delivery systems for the treatment of osteomyelitis – A review. Materials Science and Engineering C 2009; 29:2478-2485.
11 Ramteke KH, Dhole SN and Patil SV: Transdermal Drug Delivery System: A Review. Journal of Advanced Scientific Research 2012; 3(1):22-35.
12 Zhi-peng C, Wen L, Dan L, Yan-yu X, Hong-xuan C, Jun C, Weidong L, et al: Development of brucine-loaded microsphere/thermally responsive hydrogel combination system for intra-articular administration. J. Control. Release 2012; 162:628-635.
13 Limin W and Jan PS: Thermogelling chitosan and collagen composite hydrogels initiated with b-glycerophosphate for bone tissue engineering. Biomaterials 2010; 31:3976-3985.
14 Wu T and Zivanovic S: Determination of the degree of acetylation DA of chitin and chitosan by an improved first derivative UV method. Carbohyd. Polym. 2008; 73 (2):248-253.
15 Ying W, Katherine AMC, Brant PV and Tam B: Synthesis, characterization and ionic conductive properties of phosphorylated chitosan membranes. Macromol. Chem. Phys. 2003; 204:850-858.
16 Diao Zy, Fu HL, Nie CL, Hao LJ, Yang DP and Chen WH: Controlled release of transforming growth factor-beta receptor kinase inhibitor from thermosensitive Chitosan-based hydrogel: Application for prevention of capsular contracture. Chin. Med. J. 2011; 124(2):284-290.
17 Hui YZ, Xi GC, Ming K, Cheng SL, Dong SC and John FK: Effect of molecular weight and degree of chitosan deacetylation on the preparation and characteristics of chitosan thermosensitive hydrogel as a delivery system. Carbohyd. Polym. 2008; 73:265-273.

18 Hang TT, Han H, Ian L, Crispin RD and Dave ED: Chitosan-dibasic orthophosphate hydrogel: A potential drug delivery system. Int. J. Pharm. 2009; 371:134-141.

19 Peppas NA: Analysis of fickian and non-fickian drug release from polymers. Pharm. Acta Helv. 2009; 60:110-111.

20 Ganji F, Abdekhodaie MJ and Ramazani ASA: Gelation time and degradation rate of chitosan-based injectable hydrogel. Journal of Sol-Gel Science and Technology 2007; 42:47-53.

21 Julia S, Uwe G, Elke V, Michael S and Thomas K: The effect of porosity on drug release kinetics from vancomycin microsphere/calcium phosphate cement composites. Journal of Biomedical Materials Research Part B: Applied Biomaterials 2011; 99B (2):391-398.

22 Yuan Y, Chesnutt BM, Haggard WO and Bumgardner JD: Deacetylation of Chitosan: Material Characterization and in vitro Evaluation via Albumin Adsorption and Pre-Osteoblastic Cell Cultures. Materials 2011; 4:1399-1416.

23 Jun M, Shao Y, Ho CT, Koetter U and Lech S: Structural identification of nonvolatile dimerization products of glucosamine by gas chromatography-mass spectrometry, liquid chromatography-mass spectrometry, and nuclear magnetic resonance analysis, J. Agric. Food Chem. 2003; 51(21):6340-6346.

24 Tolaimate A, Desbrieres J, Rhazi M and Alagui A: Contribution to the preparation of chitins and chitosans with controlled physicochemical properties. Polymer 2003; 44 (26):7939-7952.

25 Inmaculada A, Marian M, Ruth H, Inés P, Beatriz M, Niuris A, et al: Functional characterization of chitin and chitosan. Current Chemical Biology 2009; 3:203-230.

26 Tanya SG, Kulanthai SSG, Narasinganallur SV, Kilavan PK: Extraction purification and characterization of chitosan from endophytic fungi isolated from medicinal plants. World Journal of Science and Technology 2011; 1(4): 43-48.

27 Cho J, Heuzey MC, Begin A and Carreau PJ: Physical gelation of chitosan in the Presence of β-glycerophosphate: The effect of temperature. Biomacromolecules 2005; 6:3267-3275.

28 Hoemann CD, Chenite A, Sun J, Hurtig M, Serreqi A, Buschmann MD, et al: Cytocompatible gel formation of chitosan-glycerol phosphate solutions supplemented with hydroxyl ethyl cellulose is due to the presence of glyoxal. J. Biomed. Mater. Res., Part A 2007; 83 A (2):521-529.

29 Ta HT, Dass CR and Dunstan DE: Injectable chitosan hydrogels for localised cancer therapy. J. Control. Release 2008; 126:205-216.

30 Chen RH, Tsaih ML and Lin WC: Effects of chain flexibility of chitosan molecules on the preparation, physical, and release characteristics of the prepared capsule. Carbohyd. Polym. 1996; 31:141-148.

31 Min-Lang T, Hsiang-Wei C, Hui-Chuan Y, Ya-Shen L and Ying-Die T: Effect of chitosan characteristics and solution conditions on gelation temperatures of chitosan/2 glycerophosphate/nanosilver hydrogels. Carbohyd. Polym. 2011; 84:1337-1343.

32 Hsiang-Wei C, Ya-Shen L, Ying-Die T and Min-Lang T: Effects of chitosan characteristics on the physicochemical properties, antibacterial activity, and cytotoxicity of chitosan/2-glycerophosphate/nanosilver hydrogels. Journal of Applied Polymer Science 2013; 127(1):169-176.

33 Crompton KE, Goud JD, Bellamkond RV, Gengenbach TR, Finkelstein DI, Forsythe JS, et al.,: Polylysine-functionalised thermoreversible chitosan hydrogel for neural tissue engineering. Biomaterials 2007; 28:441-449.

34 Kim S, Nishimoto SK, Bumgardner JD, Haggard WO, Gaber MW and Yang, Y: A chitosan/ glycerophosphate thermo-sensitive gel for the delivery of ellagic acid for the treatment of brain cancer. Biomaterials 2010; 31: 4157-4166.

35 Salvatore F, Marcello C, Francesco DA and Carla P: Enantio separation of amino acid derivatives by capillary zone electrophoresis using vancomycin as chiral selector. Electrophoresis 2002; 23:3035-3040.

36 Jatuporn N, Atchariya F, Sukkid Y and Jarupa V: Potential of an injectable chitosan/starch/β-glycerol phosphate hydrogel for sustaining normal chondrocyte function. Int. J. Pharm. 2010; 391:115-124.

37 Xingyi L, Xiangye K, Juan Z, Yingjing W, Yujun W, Shuai S, et al: A novel composite hydrogel based on chitosan and inorganic phosphate for local drug delivery of camptothecin nanocolloids. J. Pharm. Sci. 2011; 100(1):232-241.

38 Sancak B, Yagci S, Gür D, Gülay Z, Ogunc D, Söyletir G, et al: Vancomycin and daptomycin minimum inhibitory concentration distribution and occurrence of heteroresistance among methicillin-resistant Staphylococcus aureus blood isolates in Turkey. BMC Infect. Dis. 2013;13(583).

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