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Cockle Shell nanoparticles and Chlorhexidine Loaded Alginate/Cockle Shell Nanobiocomposite Bone Scaffold: Preparation and Drug Release Evaluation

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Abstract: Objective: This study was designated for preparation of cockle shell nanoparticles and evaluation of drug release from alginate/cockle shell nano bio composite bone scaffold. Materials & Methods: In this study, nano cockle shell powder was prepared by mechano-chemical method utilizing ball milling machine and tween 80. The prepared nano cockle shell powder was characterized by scanning electron microscope (SEM) and transmission electron microscope (TEM) to investigate the morphology and size of the prepared nano cockle shell particles. After that, sodium alginate (SA)/cockle shell scaffold was fabricated by freeze-drying technique and then loaded with 1% and 2% chlorhexidine by a stirring process. The fabricated scaffold was characterized by SEM, TEM, FTIR and EDX. Finally, drug encapsulation and drug release were evaluated by using UV-spectrophotometer. Results: The scanning electron microscope (SEM) results showed that the prepared cockle shell powder was present in the form of nano particles with particle size range from(63nm-145nm). Moreover, it revealed that the prepared scaffold had a porous structure and a homogenous distribution of calcium ions that crosslinked to alginate polymer chains. The FTIR spectra of the prepared alginate/calcium carbonate scaffold showed that CHX drug was successfully loaded into alginate/cockleshell scaffold. As regard to the drug release, the 2% loaded scaffold recorded higher significant percentage of drug release than the 1% CHX loaded scaffold at different time intervals. Conclusion: Based on the overall results, the CHX loaded alginate/ nano calcium carbonate scaffold has proved to be a suitable nanocarrier that exhibits a sustained release of CHX drug for many hours. Thus, it could be considered as an effective bone scaffold in treatment of infectious bone diseases.
Keywords: Nano cockle shell powder, alginate/cockle shell scaffold, scanning electron microscope, EDX, FTIR Analysis

Introduction: Progress in the field of biomaterials as materials for medical use has brought a great expansion in the field of tissue engineering. Focus on fields such as bone tissue engineering has further narrowed down to help in accelerating tissue healing and performance of the developed materials [(1)]. Recently, cockle shell is being exploited as a material for biocomposite scaffold in bone tissue engineering, which demonstrates its ability as efficient biomaterial of calcium carbonate aragonite polymorph. In fact, the abilities of calcium carbonate aragonite polymorph nanoparticles derived from cockle shells as a delivery agent have been successfully corroborated for antibacterial drug carrier therapy [(2)].

Using bone tissue engineering technique, a bone scaffold carrier can be designed to carry seeded cells and biological factors, and then implanted in the bone defect, to promote bone repair. The progress of this recent technology eliminates the drawbacks of autogenous and allogeneic bone grafts addition, by adding many biological factors, the process of bone repair can be accelerated and the treatment time can be shortened [(3)].

Bone defects are usually caused by infection, trauma, surgery, and diseases, such as osteoporosis and arthritis, and bone tissue replacement is required to restore function. As the human body itself cannot repair large bone defects, tissue healing must be promoted by surgical intervention. At present, allogeneic and autologous bone transplantations are the main clinical treatment strategies. However, the hazard of immune rejection secondary infection, donor disease, and less blood supply required to be circulated through artificial replacement using bone tissue engineering [(4)]. Therefore, the progress of appropriate tissue engineering techniques may help bypass the above drawbacks and accelerate tissue healing. Bone repair is a long-term approach, and thus tissue engineering can be used to enhance bone repair. The standard bone scaffold should have structure, compositions, and biomechanical properties matching those of natural bone. It acts as a template for bone deposition and provide mechanical support, regulate cell adhesion, proliferation, and differentiation, and supply cells with a microenvironment for osteoblast differentiation [(5)].

Alginate is a natural polymer which is purified from brown seaweeds. It represents an organic component of the scaffold. The polymer is known to be non-toxic, non-immunogenic, biocompatible and biodegradable that has been considered as safe by the Food and Drug Administration. It is composed of linear unbranched chains of randomly arranged α-l-guluronate (G block) and β-d-mannonate (M block) units. The presence of free hydroxyl and carboxyl functional groups enables alginate to form intramolecular and intermolecular hydrogen bonds[(6)].Nanotechnology is very important science that concentrates on the continuous designing and manipulation of materials (atoms and molecules) to produce structures at the nano meter scale size ranging from (1-100 nm) with the same initial unique features of the material used, with broad nanoscale schemes in clinical applications for diagnostic, protective as well as therapeutic purposes . The whole application of

nanotechnology from the synthesis processes, control release profiling, monitoring of biological processes and diagnosis is named a "Nano-medicine" [7].

Chlorhexidine Gluconate is a bis-biguanide which has been used as an antibacterial drug for the past 60 years since it was introduced by the Imperial Chemical Industries Limited in the early 1960's as a surface and topical antimicrobial and disinfectant agent. Then it has found its way into medical catheters, as self-releasing gels in various devices used in medicine [8]. Moreover, it is effective against both Gram-positive and Gram-negative strains as well as fungi. It has both bacteriostatic and bactericidal actions. Chlorhexidine has excellent anti-plaque activity and unique property of binding to hard and soft tissues. Chlorhexidine is now regularly used by operators when they treat patients with fixed appliances in orthodontic and maxillofacial surgeries [9]. This study aimed to prepare nano cockle shell powder and alginate/nano cockle shell scaffold for chlorhexidine encapsulation and drug release evaluation.

Materials and Methods: The materials used in this study, their specification, composition, manufacturer and batch number are summarized in table (1).

Table (1): Chemical compositions, manufacturer, batch numbers of materials used in the study.

Material name	Presentation	Manufacturer	Batch number
Sodium alginate	Powder	Ningbo Inno pharma chem co. Ldt, China	9005-38-3
Polysorbate 80 (Tween 80).	Solution	Refine chemical co., Ltd	9005-65-6
Nano cockle shell	Powder	Nano Gate Lab, Egypt	-
Phosphate Buffer solution (PBS).	solution	SERANA CO. for chemicals, Germany	01071721BD1
Chlorhexidine digluconate.	Solution	Sigma Aldrich, Germany	41385.AC

II- Methods:

Ethical approval was obtained from Research Ethics Committee (REC) of the faculty of Dental Medicine for girls, Al-Azhar University, code REC-MA-24-02.

Sample size calculation [10]

Based on Yaun et al. (2021), Using G power statistical power Analysis program (version 3.1.9.4) for sample size determination, A sample size (n=5 in each group), was sufficient to detect a small effect size (d) = 2.12, with an actual power (1-β error) of 0.8 (80%) and a significance level (α error) 0.05 (5%) for two-sided hypothesis test.

a. Preparation of micron-sized cockle shells powder [8]

The cockle shells were obtained from a local beach, red sea. The collected cockle shells were scrubbed under tap water with a brush to remove dirt and rinsed with double-distilled water (DDW), then the cleaned shells were boiled for 10 min and cooled to room temperature. The shells were thoroughly washed with distilled water and then oven-dried in Memmert UM500 oven (GmbH Co, Schwalbach, Germany) at 50°C for 7 days. Then the cockle shells were finely crushed using a mortar and pestle, and then blended using a blender machine (Premier Super-g, India) into a micron cockle shell powder. finely ground with a stainless-steel blender (Blender®; HCB 550, Stamford, CT, USA), and sieved using a 75 μm aperture-sized stainless-steel laboratory test sieve (Endecott Ltd., London, UK). The produced micron-sized cockle shell powder was further desiccated in an oven at 50°C for 7 days' duration for complete dry up.

b. Preparation of Nano cockle shell powder[8]

Preparation of nano cockle shell powder was carried out using ball milling technique. 2 g of the 75-μm of cockle shell powder was added 20 mL of deionized water. The mixture was sonicated for fifteen minutes, then 1 mL of Tween 80 (Wuhan, Hubei, China) was added and the suspension was stirred at 1100 rpm for 2 hours at room temperature using hot plate-magnetic stirrer (JOAN lap Co, China). The slurry obtained from this process was filtered and rinsed with 18.0-cm-sized double ring filter papers (Filter CF, China). The filtrate was centrifuged for 10 min at 14,000 rpm, and the supernatant was discarded. The pellet was dispersed upon the addition of 20 mL deionized water. The collected nanoparticles were dried in an oven at 50 ºC for 48 h. Upon drying, the nanoparticles were added to the dry milling container (8 × 11 cm). Milling was carried out using a programmable ball miller with a 10:1 ball to powder ratio, with ceramic balls (eight balls of 1.6 cm diameter and two balls of 2.5 cm diameter). This jar was placed on a programmable ball milling machine (R Etch co, Japan) and operated at 120 rpm for 120 h. The resultant cockle shell nano powder (Figure 4) was then stored in an oven at 50 ºC.

I-Characterization of nano cockle shell powder:

1- Scanning Electron Microscopy:

The surface morphology of nano cockle shell powder was examined by scanning electron microscope (SEM) (FESEM, Quattro S, Thermo Scientific). 1g of prepared powder was spread onto adhesive tape and sputtered coated with conductive gold layer under high vacuum. Once the powder was sputtered, the images were acquired at accelerating voltage 30KV, with resolution 3nm and at low and high magnification 60,000 X ,120.000X, respectively [11].

2- Transmission electron microscopy (TEM) analysis:

A small trace of the powder 0.2g in weight was added to 10ml distilled water in a plastic tube and was then transferred to ultrasonic mixture for 1hour to form a suspension. A drop of the sample solution was placed on a 400-mesh carbon film copper grid at magnification 20000X [12].

3- Energy Dispersion X-ray (EDX) Spectroscopy:

Energy dispersion X-ray (EDX) spectroscopy (Hitachi, Japan, S-3000H electron microscope with accelerating voltage of 15 kV) was used to study the elemental composition of the fabricated nano cockle shell powder [13].

4-Fourier-Transform Infrared (FTIR)Spectroscopy analysis:

A Fourier transform infrared spectrometer (Vertex 70 RAM II IR (Bruker, Germany) was used to determine the functional groups of nano cockle shell powder. Analysis was performed by means of a thermo electron corporation FTIR spectrometer. The powder 0.05g in weight was mixed with 0.3g of potassium bromide (KBr) in agate mortar and pressed into 0.1 mm thickness pellet. The infrared spectrum was recorded within the ranges of 4000 to 400 cm−1 at a 2 cm-1 resolution [14].

II. Nano bio composite alginate/ calcium carbonate scaffold fabrication: -

Alginate/Calcium carbonate bone scaffold was fabricated by Freeze-Drying technique [15]**.** The alginate/calcium carbonate scaffold was prepared by dissolving 0.3 g sodium alginate (MW = 10,000–600,000 g/mol) in 6 ml of deionized water at room temperature and stirred until dissolved. 0.7 g of Nano cockleshell powder (Nano gate, Egypt) was gradually added to previous mix at room temperature and stirred until a homogenous suspension was formed.

50% citric acid solution was then added at different volumes until a desirable moldable consistency was obtained. The mixed materials were then molded into custom made cylindrical Teflon mold with dimension (1cm diameter x 1cm thickness) [16] and allowed to harden. After that, Chlorhexidine digluconate solution was then added to the mixture with two different concentrations 1wt% and 2 wt.% [17].

All components in the mold were freezed to -80^{o}c for 6 hours followed by freeze -drying in (-80^{o}c for 8hours, vacuum degree is 8pa) using freeze dryer (Christ, Germany). Scaffolds were then removed from the circular molds and soaked in10% calcium chloride ($CaCl_2$) that was purchased from China (Ningbo Inno pharma chem co. Ltd., China.) for 1hour, then the prepared scaffold was washed three times in distilled water to remove any $cacl_2$ ruminants [9].

III-Characterization of alginate/Calcium carbonate scaffold.

All previous characterization tests were performed for the prepared alginate/cockle shell scaffold.

IV-Drug encapsulation efficiency (DE):

Firstly, a calibration curve was prepared for CHX ranging from a concentration of 2% to 0.0625%. The amount of CHX Encapsulated in the scaffold was determined using a UV-Vis spectrophotometer (Cary series UV-Vis- NIR, Australia) by direct method [18].

Scaffolds fabricated with chlorhexidine were soaked in 10ml phosphate buffer solution (PBS), then they were centrifuged at 8000rpm for 15minute.Presence of chlorhexidine in the supernatant was evaluated using spectrophotometer at 245 nm and the concentration of total CHX encapsulated was estimated based on the standard curve of CHX. Then drug encapsulation (DE) was calculated according the following relationships:

Drug encapsulation= $\frac{\text{Initial Concentration-Free concentration}}{\text{Initial concentration}}$ (18).

V- Drug release study:

Typically, the Scaffold was dispersed into 15 mL phosphate buffer saline pH 7.4. Then the suspension was put into dialysis bag (SERVA, Germany, MWCO 10:12 KDa) which was directly immersed into 60 mL of the corresponding solution at 37 ^{o}C. The UV– absorbance of the released scaffold was recorded at 245 nm to determine the concentration of CHX at time t (Ct). The cumulative release percentage of CHX was calculated according to the following equation:

$$\text{Cumulative Drug release \%} = \frac{V_o C_o + Vsam\,(C_t - C_o)}{m} * 100$$

Where (m) is the initially loading amount of CHX in the scaffold (g), Vo represents the total volume of the release solution (Vo = 75.0 mL), (Vsam) is the volume per sample, (Ct) is the concentration at time (t)and (Co) is the initial concentration (19).

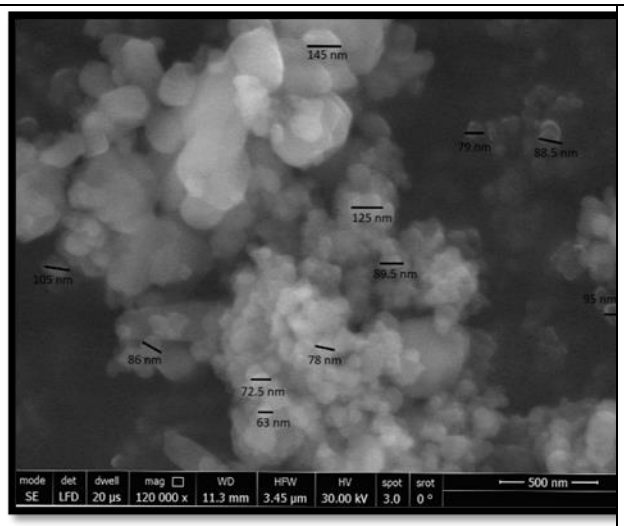
Statistical analysis:

Data management and statistical analysis were performed using the Statistical Package for Social Sciences (SPSS) version 20. Numerical data were summarized using mean, standard deviation, confidence intervals and range. Data were explored for normality by checking the data distribution and using Kolmogorov-Smirnov and Shapiro-Wilk tests. Based on their normal distribution groups were compared using independent t test; while repeated measures ANOVA was used for intra (within) group comparisons. Two ways ANOVA test was used to study the effect of time and group variables and their interaction. All p-values are two-sided. P-values ≤0.05 were considered significant.

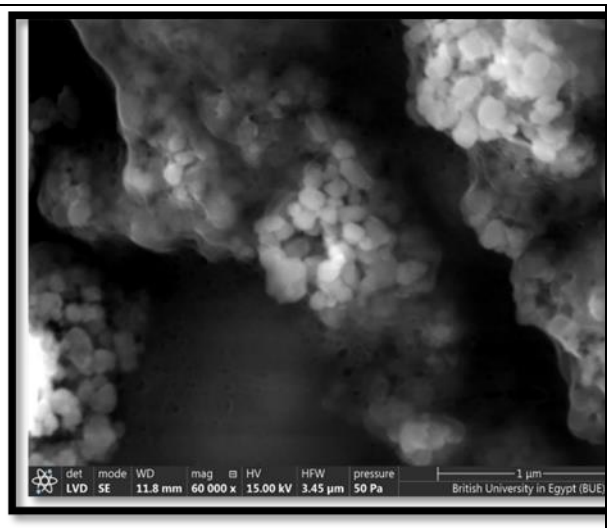
Results and Discussions:

1-Scanning electron microscope analysis:

Results of SEM revealed that the cockle shell $CaCO_3$ powder was successfully grinded into fine powder particles. The surface morphology of nano cockle shell powder sample was observed by SEM at 120000X magnification was shown in (Figure.1). Moreover, the prepared cockle shell $CaCO_3$ powder had orthorhombic shape as shown in (Figure1). In addition, the size range of most nano particles was (63-145nm) in diameter as shown in (Figure.1) which was confirmed by transmission electron microscope as shown in (Figure 3).Scanning electron micrograph of alginate/nano cockle shell scaffold at 60000X magnification demonstrated the homogenous distribution of calcium ions that crosslinked the alginate polymer chains producing typical egg-box pattern as shown in (Figure 2).Additionally, the alginate/nano cockle shell micrograph showed the successful entrapment of CHX into the prepared scaffold and its diffusion into porosities as shown (Figure.2)



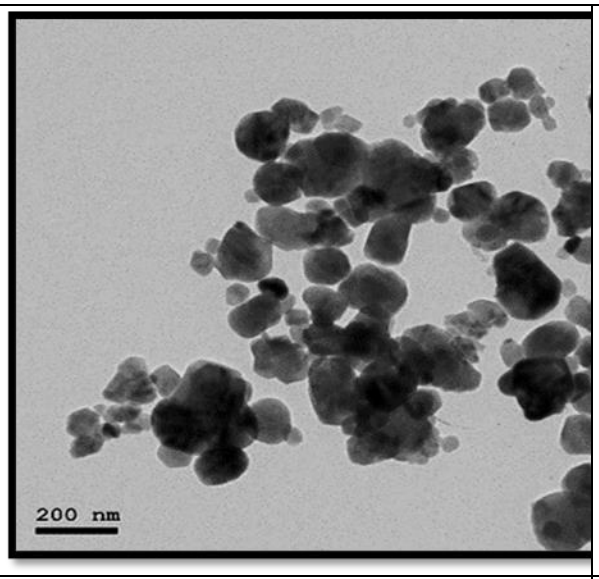
(figure.1): SE Micrograph of prepared Nano cockle shell powder at magnification120000 X



(**figure.2):**SE Micrograph of alginate/cockle shell Scaffold at magnification60000X

2-Transmission electron microscope analysis (TEM):-

Micrograph of the prepared nanocockle shell powder revealed uniform orthorhombic shape particles with diameter (63-154nm) at magnification 20000X.(Figure.3). Selected area electron diffraction (SAED) investigation showed spotted sharp rings indicating a polycrystalline nature of nano cockle shell powder particles as shown in (Figure.4).



(figure.3): TE micrograph of cockle shell powder cockle shell at 20000X

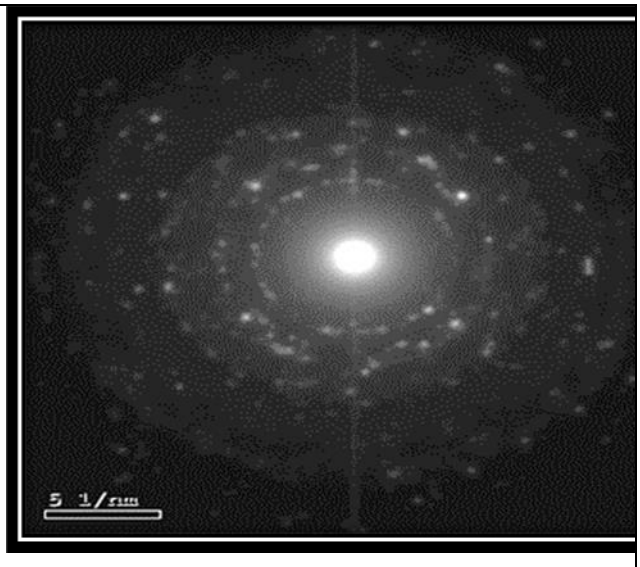
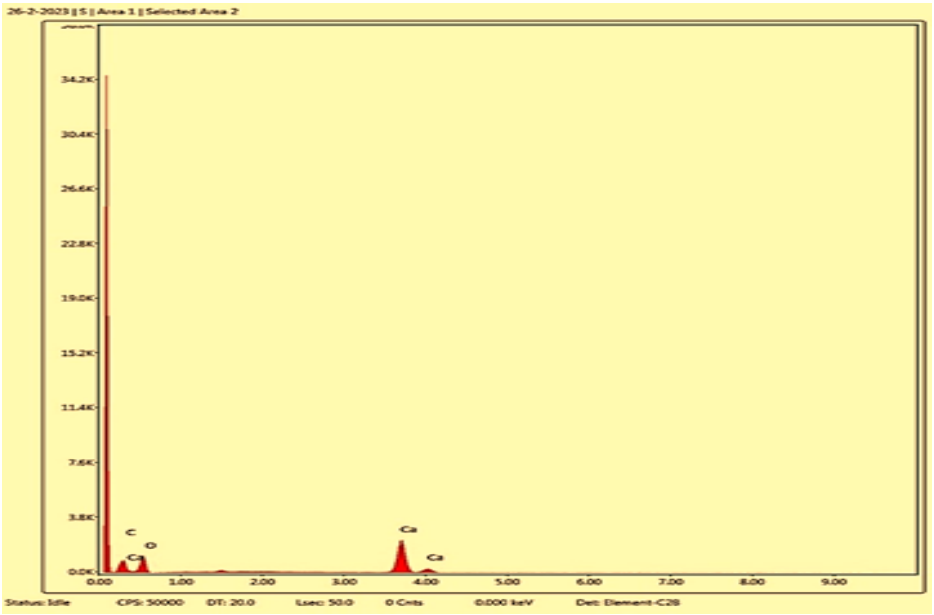


figure.4): Selected area electron diffraction for the powder

3-Energy Dispersive X-ray analysis (EDX):

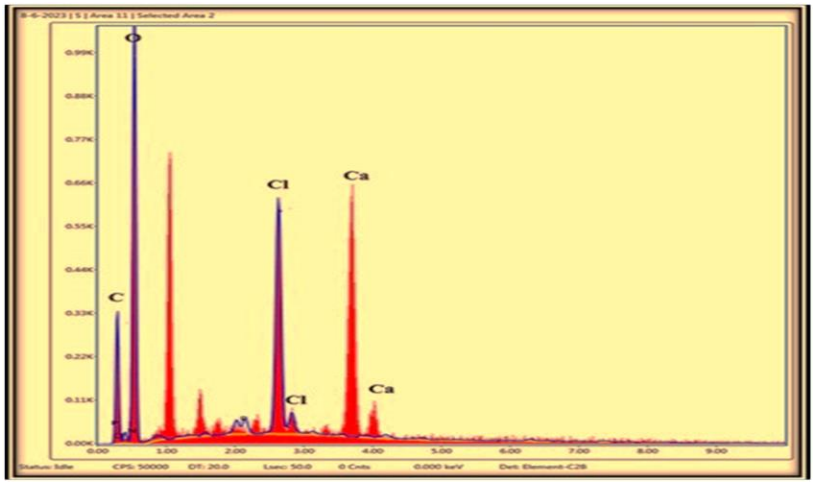
The elemental analysis was carried out at three different spectrums of the sample. The elemental constituents of the prepared cockle shell $CaCO_3$ powder were investigated at each spectrum. Table (2) and (Figure.5) showed that (Ca), (C) and (O) are the major components of the nano cockle shell powder. Presence of chloride (Cl), nitrogen (N) in the spectra of 2%CHX loaded alginate/cockle shell scaffold proved the successful encapsulation of Chlorhexidine into the prepared scaffold as shown in table (3) &(Figure.6).



(Figure.5): EDX of nano cockle shell powder.

Table (2): Percentage of elemental contents of nano cockle shell powder

Element	Weight %	Atomic %	Error%
C	9.8	18.02	7.58
O	38.96	53.76	10.83
Ca	51.32	28.22	2.38



(Figure.6): EDX of 2% chlorhexidine alginate/cockle shell scaffold.

Table (3): Percentage of elemental contents in 2% CHX loaded scaffold

Element	Weight%	Atomic%	Error%
Ca	28.05	38.61	12.36
C	2.32	2.74	24.56
O	46.07	47.61	9.79
N	0.85	0.45	16.29
Cl	22.72	10.6	3.33

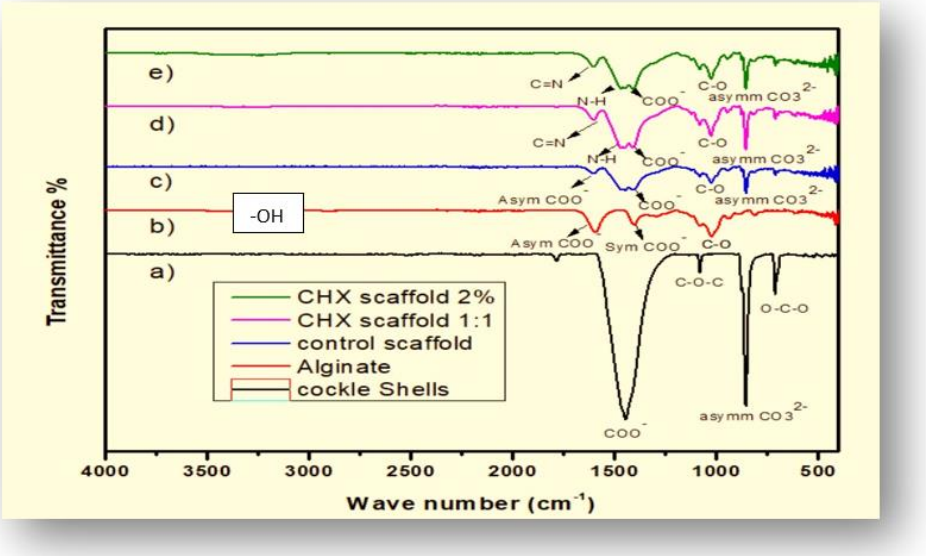
4-FTIR analysis:

Regarding the prepared nano cockle shell powder, FTIR spectra confined characteristic peaks attributed to carbonate group $(CO_3)^{-2}$. The very strong and large band assigned to presence asymmetric and vibrational bands of carbonate group $(CO_3)^{-2}$ of calcium carbonate aragonite at 1445$cm^{-1}$and bending vibration of carbonate group. $(CO_3)^{-2}$ that appeared strong and sharp at 857cm^{-1}. Bending vibration of (C-O) bond was observed in 857cm^{-1}. Moreover, the weak asymmetric stretching vibration band was attributed to $(CO_3)^{-2}$ that appeared at 1085cm^{-1}. The peak at 712cm-1 was attributed to internal plane bending vibration of (O-C-O) as shown in (Figure.7a).

FTIR of alginate spectrum revealed broad band at 3460cm-1 indicating the presence of stretching vibration of (-OH). The band appeared at 1406cm-1 in alginate spectrum indicating presence of symmetric stretching vibration of carboxylic group coo- as shown in (Figure.7b). which shifted to longer wave length in the alginate /calcium carbonate scaffold

as shown in (Figure.7c) this shift was explained by chemical reaction of carboxylic group of alginates with calcium of nano cockle shell powder.

FTIR of 1%, 2%Chlorhexidine scaffold showed new characteristic groups which were attributed to the incorporation of chlorhexidine drug into the prepared scaffold. The presence of stretching band at wave length 1470cm-1was related to amine group (N-H) in the prepared scaffold, also appearance of stretching vibrational band at 1612cm-1 was attributed to imine group of 1,2% chlorhexidine containing scaffold as shown in (Figure.7d,e). The band at 3480cm-1was attributed to the reaction of amide group of CHX with (-OH) group of sodium alginate and the formation of hydrogen bond which increased in intensity with increasing the CHX concentration as shown in (Figure.7d,e). These groups confirmed the incorporation of chlorhexidine into the scaffold.



(Figure.7): FTIR spectra of a)Nanocockle shell powder ,b) FTIR of alginte powder, c)FTIR of alginate/calcium carbonate scaffold , d)FTIR of scaffold loaded with 1%chlorhexidine , E) FTIR of scaffold loaded with 2%chlorhexidine.

5- Drug encapsulation: Table (4) shows the mean and standard deviation (SD) values of the amount of encapsulated 1%,2%Chlorhexidine concentrations to alginate/calcium carbonate scaffold. There was a statistically significant difference between the two tested groups (P value=0.000). Results revealed that 2%CHX loaded scaffold group showed statistically higher drug encapsulation value (98.24±1.09) than 1% Chlorhexidine loaded scaffold group (97.89±0.77).

Table (4): The mean and standard (SD) values of the amount of encapsulated 1%, 2%drug concentrations to alginate/calcium carbonate scaffold.

Groups	**Mean**	**SD**	**P. value**
1% Chlorhexidine loaded scaffold	97.89%	0.77	**0.000***
2% Chlorhexidine loaded scaffold	98.24%	1.09	**0.000***

6-Drug release:

Table (5) shows the mean and standard deviation (SD) values of the percentages of drug release from 1%CHX loaded scaffold and 2% CHX loaded scaffold at different time intervals. The highest % of released CHX was observed in 2%CHX loaded scaffold after 10hours that recorded (90 ±1.33) and the lowest % of released CHX was observed in 1%CHX loaded scaffold after 1hour that recorded (41.72±0.31) as shown in (Figure.8)

Table (5): Comparison between cumulative drug release results (mean values ± SD) between 1%CHX loaded scaffold and 2% CHX loaded scaffold at different time intervals.

Groups / **Time**	**1 hour**	**2 hours**	**3 hours**	**4 hours**	**5 hours**	**8 hours**	**10 hours**	**P- value**
	Mean± SD %	Mean± SD%	Mean± SD%	Mean± SD%	Mean± SD%	Mean± SD%	Mean± SD%	
1%CHX loaded Scaffold	41.72± 0.31	47.91± 0.65	56.49± 1.0	59.36± 0.74	68.97± 0.82	71.37± 1.23	77.67± 0.71	<0.001
2%CHX loaded Scaffold	50.52± 0.51	52.02± 0.51	62.87± 0.71	68.72± 0.93	74.98± 1.05	80.76± 1.19	90± 1.33	<0.001
P- value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	

Significance level p≤0.05, *significant, ns=non-significant.

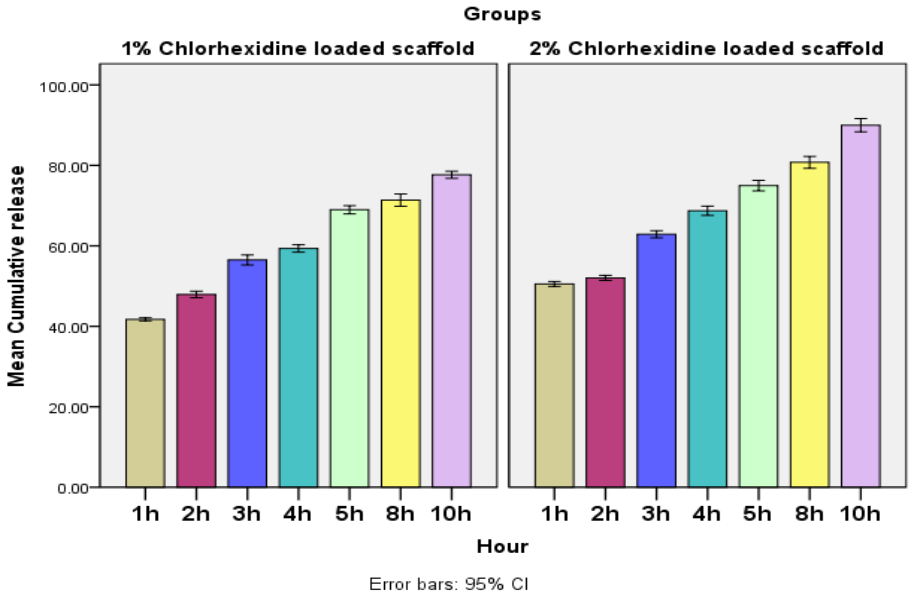


Figure (8): Bar chart illustrating mean value of cumulative drug release in both groups at different time intervals.

Discussion:

In biomedical engineering, excellent regeneration of tissue engineering bone defects demands a three-dimensional biocompatible scaffold, which can ensure cell attachment and proliferation and provide sufficient mechanical support for bone tissue healing [20]. In recent years, although the area of bone tissue engineering has developed rapidly, the repair of infected bone defects is still a challenging goal [21]. The purpose of this work was to develop nano cockle shell powder and alginate/cockle shell scaffold loaded with chlorhexidine drug delivery system, which has favorable bacteriostatic properties, sustained drug release efficacy, and biocompatibility. The scaffold can continuously deliver antiseptic agent to the bone defect during bone healing and better repair of the infected bone defects [22].

Calcium carbonate nanoparticles could be prepared by both top-down [23] and bottom-up methods [24]. An important consideration is that the formulation process must be strong enough to ensure high reproducibility and be organized to allow for the ease of scale-up production. This study involves the usage of both the chemical and the mechanical processes to synthesize the cockle shell $CaCO_3$ nanoparticles. In the initial stages of the nanoparticle synthesis, Tween 80 was used as the nonionic surfactant to prevent the nanoparticles from growing and aggregating during the vigorous stirring step [25].

Cockle shell nano particles were prepared by top-down mechanical method where micron sized particles were broken down into smaller nanometer-sized particles. The mechanical dry milling is an acceptable fine particle synthesis method where mechanical energy is applied to the solid material to break the bonding between the atoms or molecules [26]. The

use of ceramic balls and their constant hitting with the container helps break the particles further. Besides, this method is more effective and less expensive than a high-pressure homogenizer technique [27].

Calcium carbonate (CaCO3) is one of the most abundant minerals that has attracted widespread attention in the biomedicine field due to its biocompatibility, biodegradability, pH-sensitivity, low cost, also increased mechanical properties and stability of the prepared scaffold [28]. Hence, the $CaCO_3$ nanoparticles were used as drug/gene delivery vehicles [29]. Regarding the organic scaffold material, the alginate was used in fabrication of alginate /cockle shell scaffold as it is hydrophilic and water-soluble and forms a hydrogel in the presence of polyvalent cations [30]. As a natural polysaccharide, alginate shows a pH-dependent anionic character and interacts with cationic polyelectrolytes, making it suitable for the loading of cells [31]. It also possesses specific physicochemical properties (hydrophilicity, porosity, morphology, mechanical properties, biodegradability, biocompatibility) to mimic the cell environment in vivo as closely as possible [32]. Scaffolds are designed as drug delivery platforms for the controlled release of therapeutic bioactive agents (growth factors, vitamins, hormones, antibiotics) that support tissue regeneration and promote the healing process [33]. The combination of nano cockle shell and Sodium alginate can significantly improve the mechanical properties of scaffolds while simulating the composition of natural bone [34].

In the current study, freeze-drying technique was chosen for the fabrication of cockle shell/alginate scaffold as it has a significant effect on the formation of pores before lyophilization. The hydrogel samples presented here were initially frozen at − 80 ºC for a period of 8h, followed by freeze drying in − 80 ºC until lyophilization. The slow freezing process allows for the formation of ice crystals from the water component of the hydrogels within the hydrogel, leaving behind pores within the architecture when freeze-dried [18]. $CaCl_2$ solution was added during fabrication of scaffold because it acts as chemical crosslinking agent which has the ability to form hydrogels with a three-dimensional network structure. This characteristic permit preparing of drug loaded alginate/cockle shell scaffold [35]. Furthermore, 2% CHX was chosen because it is commonly used in dentistry, showing an effective antimicrobial effect mainly through electrostatic attraction, binding negatively charged receptors of the bacterial cell wall, and interfering with microbial metabolism leading to their death [36].

Characterization of cockle shell calcium carbonate powder was done using scanning electron microscope to show the crystal size and surface morphology of the cockle shell CaCO3 powder. The cockle shell CaCO3 powder is a non-conducting surface therefore the SEM images were taken at high vacuum mode by using gold conductive layer to obtain sharp images. As shown in (Figure1), the SE micrograph proved that the cockle shell CaCO3 powder was successfully grinded into fine powder grains. It revealed that the prepared cockle shell CaCO3 powder had rhombohedral shape. These rhombohedral like structures were typical aragonite phase of cockle shell [37].

The particle size of nano cockle shell powder ranged from 65nm-145nm which was confirmed by transmission electron microscope image as shown in (Figure.3). The uniquely obtained nano-size could be attributed to the controlled synthetic conditions employed. Another possible explanation for the nanoparticle dispersity could be due to the negatively charged layer of tween80 which aided in the repulsions of nanoparticles from each other and also, due to electrostatic repulsion and the conjugate hydration surface layer preventing aggregation and increasing conjugate stability as similarly reported by Jazayeri et al. (2021) [38].Scanning electron microscopy (SEM)of alginate/cockle shell scaffold image at 60000X magnification revealed homogeneous distribution of calcium ions contributed by nano cockle shell powder which is an important factor that form cross-linking of alginate molecules in order to produce typical egg-box pattern with well -organized pore structure of alginate /calcium carbonate scaffold as shown in (Figure.2). The alginate/$caco_3$ scaffold showed the successful entrapment of chlorhexidine into the prepared scaffold and diffusion into porosities as shown in (Figure.2) [39].

EDX test was carried out to investigate the chemical composition of the prepared nano cockle shell powder and the fabricated scaffold. Results showed that cockle shell CaCO3 only contain Calcium (Ca), Carbon(C), and oxygen(O) without any impurities as shown in (Figure.5) &(table.2) indicating the success of mechanical-chemical method in synthesis of nano cockle shell powder. These findings were in accordance with Ismail et al. (2021) [40]. Kiranda et al. (2018) [41] who stated that the chemical composition of cockle shell $CaCO_3$ consists of only Ca, C, and O. The EDX spectra of chlorhexidine loaded scaffold (Figure.6) &(table.3) exhibited the characteristic peaks for Ca, C, O of cockle shell powder and also chloride (Cl) of and nitrogen(N) of encapsulated chlorhexidine drug which ensure the successful entrapment of drug into the fabricated scaffold [42].

As regard to Chlorhexidine encapsulation, UV- spectrophotometry was chosen for measuring drug encapsulation because it is sensitive, less time-consuming, and cost-effective [43]. 2%CHX scaffold showed the greater % of drug encapsulation with (98.24±1.09%) than 1% CHX scaffold that recorded drug encapsulation (97.89±0.77%) as shown in (table 4). These results could be explained by the probable formation of hydrogen bonding between the carboxylic groups of alginates and the CHX–NH groups, resulting in an increase in CHX- loading efficiency [20,44].

Drug release from scaffold was studied as a function of time as shown in (Figure.8). It's clearly represented that the Chlorhexidine (CHX) released from all the porous alginate/nano cockle shell scaffolds showed a sustained release profile. In the first release (1 hours) about (50.52±0.51%) of encapsulated CHX was released from 2%CHX loaded scaffold which was significantly higher than CHX released from 1%CHX loaded scaffold that recorded (41.72±0.31). These results could be explained by several mechanisms simultaneously, such as diffusion of phosphate buffer solution into the polymeric matrix, the swelling and dissolution processes leading to polymer relaxation. The higher percentage of drug release from 2%Chlorhexidine scaffold than 1%Chlorhexidine scaffold was due to higher drug

encapsulation to the 2%CHX scaffold than 1%CHX scaffold [45] .Thereby, increasing the diffusion coefficient of the CHX molecules within the scaffold matrix and a slight improve in the release profile took place accordingly [20]. A higher concentration of CHX was maintained during early bone defect regeneration, inhibiting the growth of bacteria in the bone defect, and providing a bacteria-free environment during the early stages of bone regeneration. The prolongation of the release time up to 10h reveals that the structure of the scaffold has been conserved, owing to the existence of cross linking between alginate and cockle shell $caco_3$ which partially delayed the dissolution of alginate resulting in a delay in the CHX release rate. Moreover, the interactions between CHX's amine groups, which were gradually deprotonated at high pH and could bind with the available positively charged calcium of cockle shell, resulted in sustained release behavior [46].

Conclusions:

Based on the overall results, the CHX loaded alginate/ nano calcium carbonate scaffold has proved to be a suitable nanocarrier that exhibits a sustained release of CHX drug for many hours. Thus, it could be considered as an effective bone scaffold in treatment of infectious bone diseases.

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