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Preparation and Evaluation Polyurethane Scaffolds Containing Gelatin Microspheres with Cefotaxime Sodium Delivery for Bone Treatments.

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ABSTRACT

The aim of this study is to prepare polyurethane ways of working are different and evaluation practically as scaffolds for bone tissue engineering .The polymers were synthesized from diethylene glycol (DEOL) and Castrol oil(CO) with 4,4'methylene diphenyl diisocyanate (MDI) with polyelectrolyte used as a chain extender. also prepare in this study, Gelatin microspheres (Gm) as drug delivery system (DDS) ,and loaded microspheres with drug (cefotaxime sodium) has been incorporation with the scaffolds polyurethane to study the extent of delivery in drug from these scaffolds , and also added tri- calcium phosphate(TCP) salts and Hydroxyapatite(HA) for Scaffolds has been studying the impact on those scaffolds.

Keywords: poly urethane, Scaffolds, Castrol Oil, Microspheres, Cefotaxime Sodium

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INTRODUCTION

Bone regeneration is required for healing of open fractures, and healing is often complicated by chronic infection. To reduce the healing time of the patient, it is desirable to promote bone fracture healing and control infection through one surgical procedure. Bioresorbable polyurethanes (PU) have been used extensively in tissue engineering to serve as both a supportive scaffold and drug delivery system due to their biocompatibility and biodegradability [1,2]. Biodegradable polyurethane (PU) scaffolds have been investigated as delivery systems for growth factors [3,4] and antibiotics[5,6] and present several advantages .PU are biocompatible, moderately osteoconductive polymers that biodegrade to non cytotoxic breakdown products in vivo [7-9] .They can be injected into bony defects as a two-component liquid system, which cures in situ to form a solid scaffold with tough mechanical properties and preexisting, interconnected pores [10,11]. Further, the rate of degradation can be controlled by the choice of intermediates used in the synthesis of the scaffold. PUs have also performed well as drug carriers, especially where the goal is to ensure continuous, long-lasting supply of a drug to the body. Such extended-release, or depot, forms are essentially a polymeric base (matrix) with embedded low-molecular bioactive substances.In addition to their extended drug delivery feature, advantages of the depot forms also include reduced side effects. PUs (either linear or network) are a good basis for developing polymeric composites — in fact macromolecular therapeutic systems with controlled physicochemical properties, allowing a developer to vary the drug immobilization level.

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Several carriers have been developed to encapsulate drugs, such as biodegradable polymers (synthetic or natural) and bioactive ceramics, in the form of particulates, membranes, and porous matrix [12]. Among those, hydroxyapatite (HA) has enhanced interest as a drug delivery carrier due to its osteoconductivity and biocompatibility [13]. Practically, the HA porous forms have been used as bone scaffolds to prove an improved bone ingrowth and Osseo integration [14]. However, the brittleness and low strength limited their wider applications in hard tissue implants [13]. To be used effectively in load bearing compartments, the mechanical properties of the HA porous body should be improved. Moreover, as a DDS, the pore structure of the scaffold needs to be controlled in terms of porosity and pore size [15]. More importantly, drugs should be entrapped efficiently to be released for a prolonged period[16]

The use of natural polymers such as gelatin as carriers in controlled drug delivery applications is gaining importance because of their inherent biocompatibility, biodegrade - ability and biosafety .But a common disadvantage of such natural polymers is their structural and thermal labiality which require improvement for controlled and targeted drug release applications. In this context, gelatin a well-known and widely used biopolymer drug carrier has been chosen for improving its drug release characteristics, because the high solubility, and poor mechanical and thermal stability of gelatin under physiological conditions may not facilitate adesirable sustained drug release.

The study also involved the evaluation of the cross-linked gelatin as a drug carrier through in vitro release studies in simulated biological fluids taking Cefotaxime sodium (CS) as a

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model drug. Cefotaxime sodium is a third generation cephalosporin and is widely used in the treatment of microbial diseases. The drug has a relatively short half-life of 0.8- 1.4hr. This antibiotic displays a high antimicrobial potency, a broad antibacterial spectrum, high resistance against the action of β- lactamases, as well as low index of side effects [17]. Fig.(1) shows the structure of Cefotaxime sodium

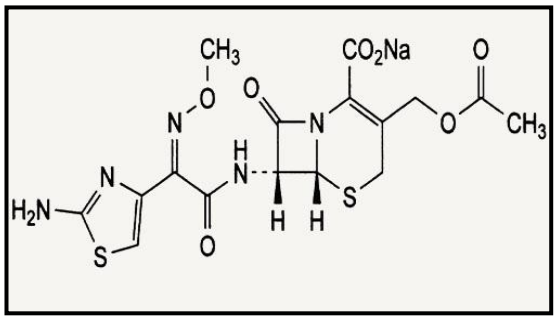

Figure 1: structure of Cefotaxime sodium

The objective of this work was to prepare flexible polyurethane scaffolds that could be employed in soft tissue engineering or as temporary mechanical scaffolds for application. The polymers were synthesized from diethylene glycol (DEOL) and Castrol oil (CO) with 4,4'methylene diphenyl diisocyanate MDI with polyelectrolyte used as a chain extender. MDI was selected as the diisocyanate upon which the hard segment was built since it would be expected to yield putrescine, Mechanical properties of the scaffolds were measured following preparation, and polymer degradation in phosphate buffered saline at 37 °C was characterized over an 11 weeks period.

MATERIALS AND METHODS

Chemicals

Gelatin and glutaraldehyde (50%) was supplied by (B.D.H,England). 4,4'methylene diphenyl diisocyanate was supplied by (Fluka Co., Switzerland). Poly electrolyte was supplied by (Aldrich Co., Germany). Castor oil and Cefotaxime sodium was supplied by (Sigma- Aldrich Co., Germany). All other chemicals were of reagent grade.

Methods

Preparation gelatin microspheres:

Gelatin microspheres (Gm) were prepared using the emulsion/chemical cross-linking method reported in the literature with some modifications [18]. 3 g of gelatin was dissolved in 30 ml distilled water in a water bath at 40° C. Various ratios of drug was dispersed in polymer solution. This solution was added slowly by medical syringe to a beaker containing 300ml of sun flower oil and leave for one hour at a temperature (60 ° C) in a water bath , with constant

stirring .1 ml of glutaraldehyde as a crosslinking agent, while the mixture was stirred at 450 rpm for 15 min. and then cooled the mixture to 0°C with water-ice bath for 60min. Stirring was continued to allow the formation of solid microspheres. The gelatin microspheres then were rinsed in acetone for several times to remove the remaining oil on their surfaces. Finally, Microspheres were filtered, washed 3-4 times of with ethanol, acetone each and dried at room temperature for one day. Repeated the process of preparation of gelatin microspheres using different weights of the drug mg (900,1800,2700) As shown in the table (1)

Table 1: Prepared gelatin microspheres containing CS

Batch code	Drug : Polymer (g)
CS1	0.900 : 3.0
CS2	1.800 : 3.0
CS3	2.700 : 3.0

Preparation hydroxyapatite [19]

130 ml of 1.63(N) ammonical $Ca(NO_3)_2$ solution was added drop wise to a mixture of ammonical $(NH_4)_2HPO_4$ solution with constant stirring with the help of a magnetic stirrer. The pH of the solution was maintained at 10. Hydroxyapatite was formed as per the following reaction:

$$10Ca(NO_3)_2 + 6(NH_4)_2HPO_4 + 2H_2O \rightarrow Ca_{10}(PO4)_6(OH)_2 + 12NH_4NO_3 + 8HNO_3$$

The resulting suspension was boiled for 10 min and cooled in an ice bath overnight to obtain a white gelatinous precipitate. The precipitate was filtered and filtered cake (residue) was dried in an oven at 80◦C. The dried sample of hydroxyapatite was ground to powder.

preparation Polyurethane scaffolds

The synthesis of polyurethane is a condensation type polymerization typically involving the reaction of isocyanate (-NCO) and hydroxyl (-OH) to form the carbamate (-NHCO) linkages .The polymerization is usually a two-step process leading to the formation of segmented polyurethane: (i) Reaction of polyol with diisocyanate to form isocyanate terminated prepolymer and (ii) Chain extension through the reaction of prepolymer and chain extender. Two different polyurethanes were synthesized using diethylene glycol (DEOL) and Castrol oil(CO) as the polyol with 4,4'methylene diphenyl diisocyanate MDI(diisocyanate) and Polyelectrolyte PE (chain extender). The reactions were carried out in a completely Both PEG and Castrol oil were dried under vacuum for 48 hours at 40 °C to remove entrapped water. N,N´-Dimethyl formamide (DMF) used as solvent, was dried over calcium hydride (CaH_2) followed by molecular sieve. Diisocyanate of high (>99%) purity grade was used .The detailed protocol for the synthesis of polyurethane is summarized below:

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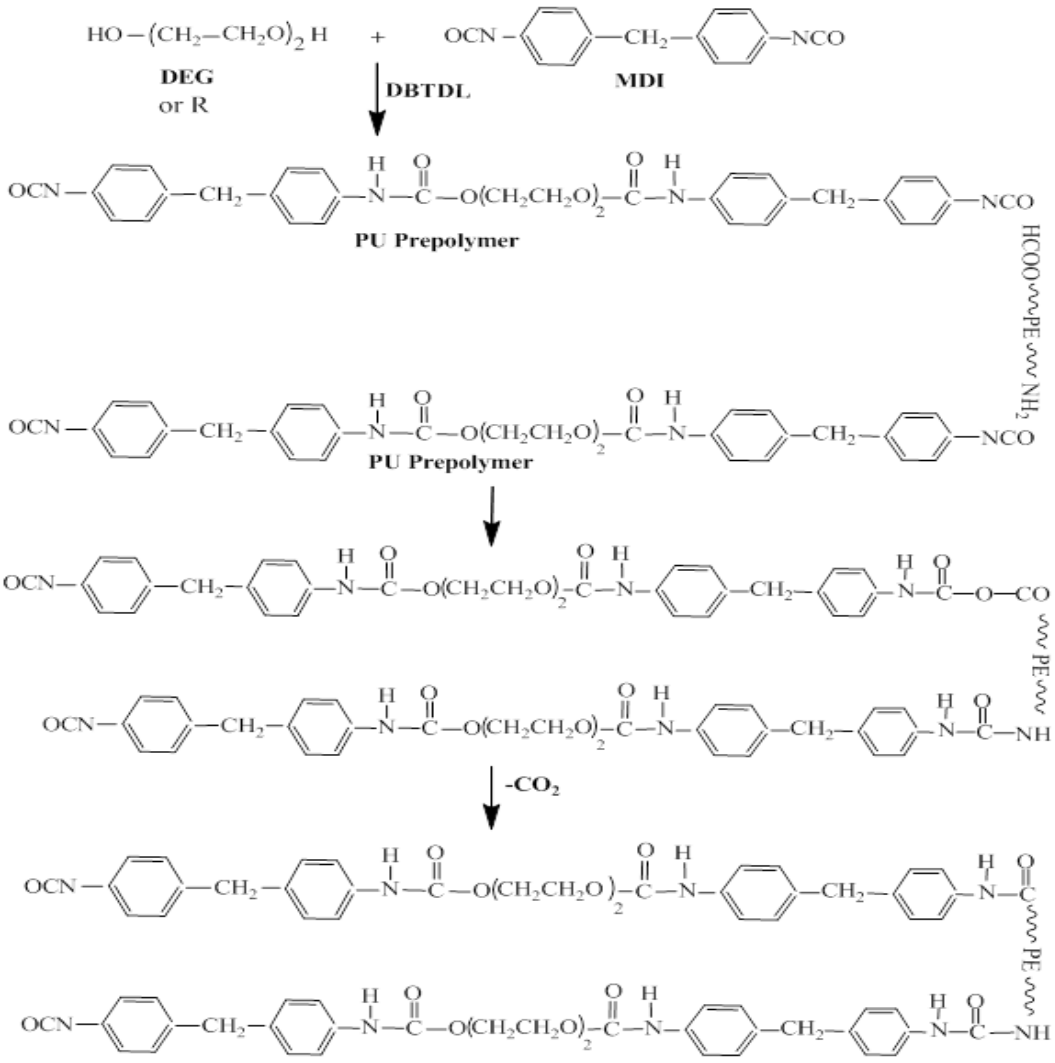

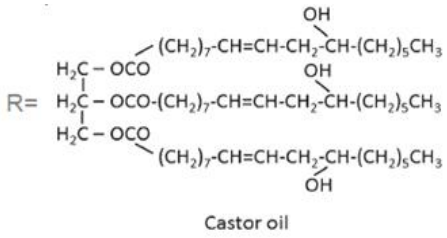

i) The polyol (DEOL or CO) was reacted with MDI at a 1:2 molar ratio in DMF as solvent and 0.1% dibutyltin dilaurate (DBTDL) catalyst to form the prepolymer. Typically, 5 mmol of polyol was added into 40 ml of DMF and 10 mmol of MDI and 2~3 drops of (DBTDL) was added to the reaction mixture with continuous stirring.

ii) PE was added in the second step at a 1:1 molar ratio with the prepolymer. Typically, 5 mmol of PE in 10 mL of water was added.

iii) PU/(HA + TCP)scaffolds and PU / Gm prepared by the same method were set as a control for the succeeding experiments. The polyurethanes synthesized were stored is desiccators for the purpose of characterization and future experiments.

Analytical Methods

Morphological Study

Photomicrographs of microspheres characterized using a digital optical microscope . A small amount of dry microspheres and scaffolds were ultras .Two hundred microspheres were seized by the above mentioned method and the mean diameter as well as size distribution of microspheres were determined.

Fourier Transform Infrared Spectroscopy

FTIR spectrum of the drug, drug-loaded microspheres, blank microspheres , and polyurethane scaffolds were recorded using a FTIR (model 4100 type A,Perkin-Elmer, Norwak, CT, USA) spectrometer using KBr pellets (400–4.000 cm^{-1}) .

Phase analysis by X-ray diffraction (XRD).

X-ray diffraction (XRD) technique (PhilipsX'Pert-MPD system with a CoKa wavelength of 1.5418A°) was used to analyze the structure of the prepared DEOL/MDI and CO/ MDI polyurethane scaffolds. The diffract meter was operated at 40kV and 40mA at a 2ɵ range of 5–69.99°employing a step size of 1.000 /s.

Vitro Release Studies

Briefly,100mg of each prepared microspheres were placed in the synthetic dialysis bags and were immersed into 100mL phosphate buffer solution pH=7.4 after they were fixed in sterilized beakers . Each beaker was accurately covered with glass watch and was fixed on a magnetic stirrer at 100rpm and 37±1^{o}C. 4mL aliquot of the dissolution fluid was withdrawn at regular time interval and was replaced with fresh quantity dissolution fluid. The samples were analyzed spectrophotometrically at 235 nm to determine the dissolved drug concentration (content drug) using UV-spectrophotometer. All the experimental units were analyzed in triplicate (n=3).

Test of Mechanical Properties

The tensile properties of the scaffolds were measured according to ASTM D638-98. Testing was conducted in an Instron testing machine equipped with a 5 lb load cell. A cross-head speed of 10 mm/min was used. The longitudinal direction of the scaffold was cut and tested. Four samples were evaluated for each scaffold.

In vitro degradation of PU scaffolds

Long-term scaffold degradation rates were evaluated by measuring the mass loss for up to 11 weeks. Triplicate 10-mg samples were incubated in 1 ml in each of the Simulated Body Fluid (SBF), and phosphate buffered saline (PBS) , on a shaker at 37 °C [20]. At each time point, scaffolds were removed from the buffer, rinsed in deionized water, dried under vacuum for 48 h, and weighed. The medium was not changed until the targeted time point to minimize phase separation errors resulting from disintegration of the scaffold at longer time points. The oxidative medium comprised 20 wt% hydrogen peroxide (H_2O_2) in 0.1 M cobalt chloride (Co Cl_2) [21]. The cobalt ion and hydrogen peroxide react to form hydroxyl radicals, simulating the oxidative radicals present at the material-macrophage interface. The medium was changed every 3 – 4 days to maintain enzyme activity.

Scaffold porosity

The porosities of the polyurethane foams were studied using a liquid displacement method similar to the procedure reported [22].Ethanol was used as the displacement liquid for this procedure because it penetrated easily into the pores of the polyurethane scaffold. A dry scaffold was placed in a graduated cylinder filled with a predetermined volume (V_1) of ethanol, and this cylinder was then placed in a vacuum for 20 minutes to enable penetration of ethanol into the scaffold pores. The total volume of ethanol containing the sample was recorded as V_2. The scaffold was taken out of the graduated cylinder, and the residual ethanol volume was recorded as V_3. The amount of open pores in the scaffold (P) was calculated according to the following equation :

$$\mathbf{P\ (\%) = (V_1 - V_3)/(V_2 - V_3) \times 100\%}$$

Where (V_2–V_3) = total volume of the scaffold and (V_1–V_3) = volume of ethanol retained in the sample. Three specimens of each sample were used for the porosity measurements and the results were averaged.

Equilibrium swelling Studies of Scaffolds

The % water absorbed by the scaffolds was studied by soaking them overnight in distilled water at room temperature. The% water absorbed was calculated from the following equation:

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$$\% \text{ Equilibrium swelling} = \frac{(W_f - W_i)}{W_i} \times 100,$$

Where Wi is Initial weight of scaffold formulations (mg), and Wf is Weight of scaffold formulations (mg) after soaking the water until saturation (after 24hr)

RESULTS

Morphology and Size Distribution

The release of morphological analysis of SC microspheres Fig.2 , Table (2) showed that smooth and solid microspheres .Increase in drug concentration of CS resulted in increase in mean particle size (MPS) of microspheres. Increase in MPS may be as a result of the release in viscosity of the droplets present in the internal phase caused by the increase in drug concentration **.**

Table 2: Effect of Drug Conc. On MPS

CS Conc. mg	90	180	270
MPS µm	84.5	96.6	128.9

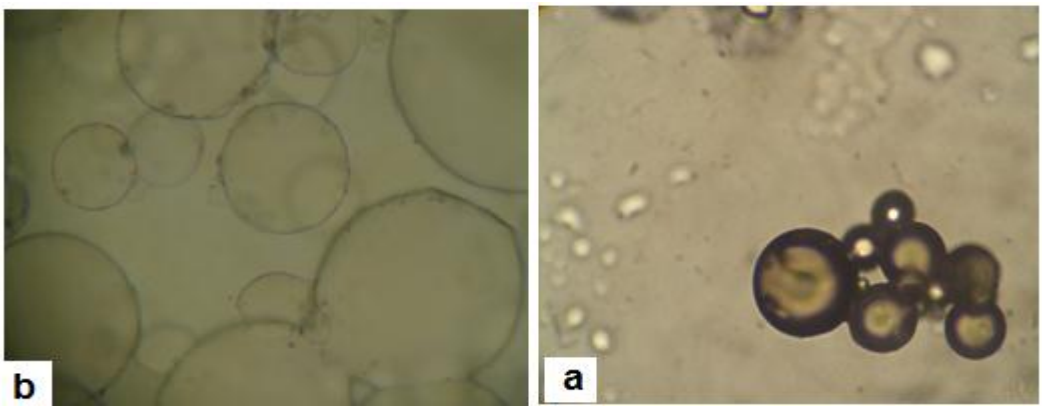

Figure 2: Optical micrograph gelatin microspheres
(a) unloaded microspheres (b) loaded microspheres with drug

The FT- IR spectrum

From the FT-IR spectra of CS, CS-loaded microspheres, blank microspheres , and polyurethane scaffolds, it was observed that all characteristic peaks of CS present in the combination spectrum , thus indicating compatibility of the CS and polymer . IR spectra shown in(Fig. 3 to 7) , data shown in Table 3.

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Table 3: Interpretation of FT-IR spectrums of 1) CS 2) Blank microspheres of gelatin 3) CS microspheres of gelatin 4) DEOL-MDI Scaffolds 5) CO-MDI Scaffold

S.no	IR Spectrum	Peaks(cm^{-1})	Groups	Stretching / Deformation
1	CS	3441&3346	N-H	Stretching
		2825&2946	C- H(asym& sym)	Stretching
		1759	C=O(Lactam)	Stretching
		1730	C=O(Carboxylic ester)	Stretching
		1608	C=C	Stretching
		1541	C=N	Stretching
		1047	C-O	Stretching
2	Blank microspheres of gelatin	3055	N-H	Stretching
		2883&2943	C- H(asym& sym)	Stretching
		1701	C=O Amid	Stretching
		1615	Shiff base	Stretching
		1521	Amid 11	bending
		1319	C-N	Stretching
		1240	C-O	Stretching
3	CS microspheres	3600-3150	CS-polymer (Hydrogen bond)	Stretching
		1674	C=O	Stretching
		1770	C=O(Carboxylic ester)	Stretching
4	DEOL-MDI Scaffold	3441	N-H	Stretching
		2869.&2929	C- H(asym& sym)	Stretching
		1670	C=O Amid	Stretching
		1541	Amid 11	bending
		1325	C-N	Stretching
		1226	C-O	Stretching
5	CO-MDI Scaffold	3302	N-H	Stretching
		2924	C- H (sym)	Stretching
		1708	C=O(Carboxylic group)	Stretching
		1541	N-H	bending
		1598	C=C	Stretching
		1230	C-O	Stretching

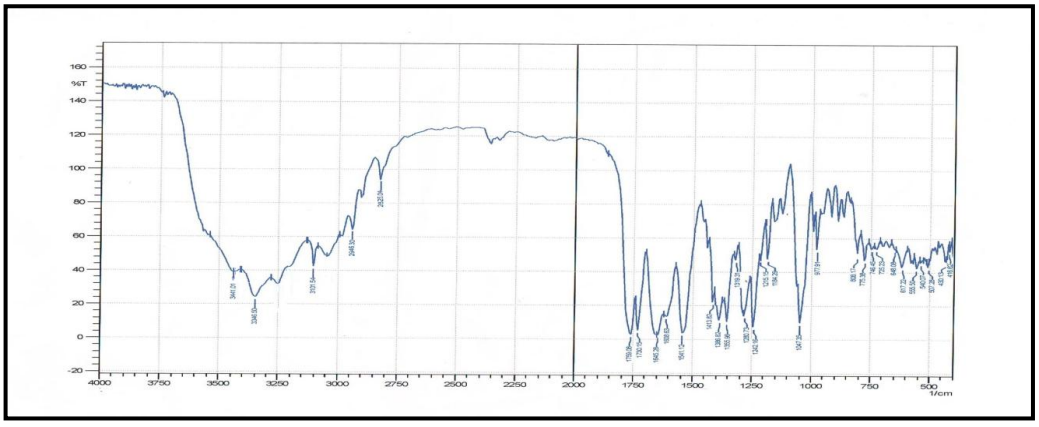

Figure 3: The IR spectrum of CS

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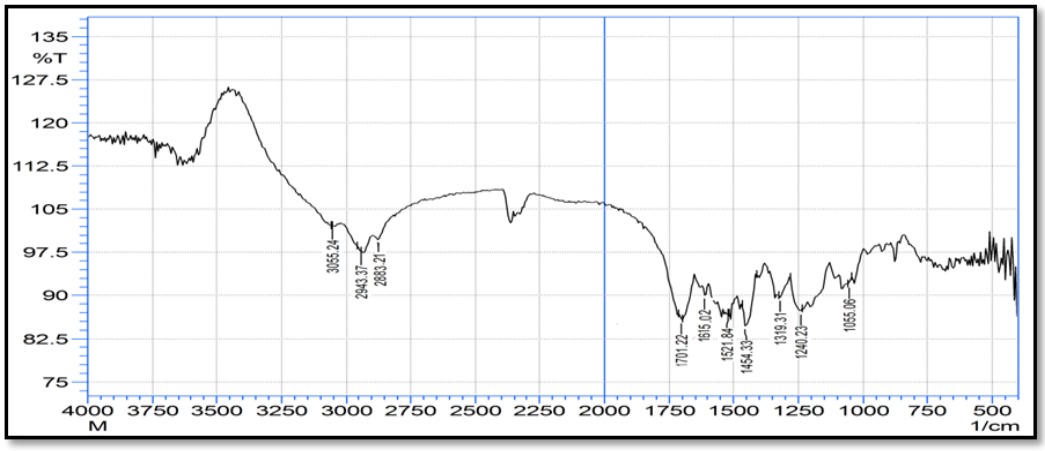

Figure 4: The IR spectrum of Gm

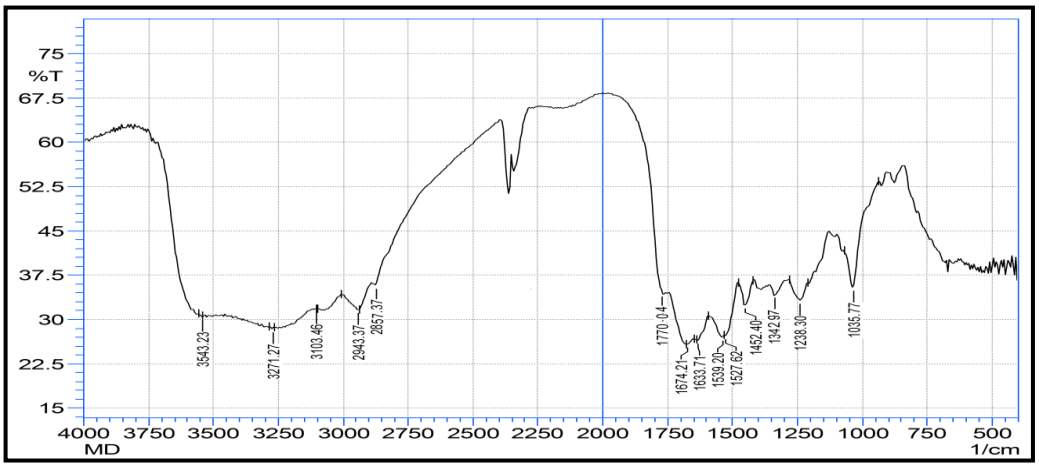

Figure 5: The IR spectrum of Gm+ CS

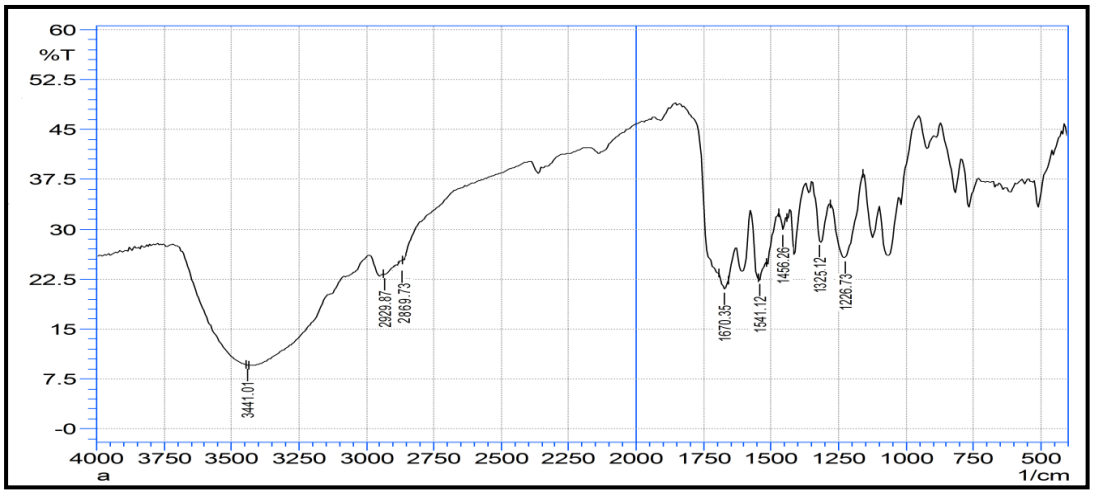

Figure 6: The IR spectrum of DEOL+MDI Scaffold

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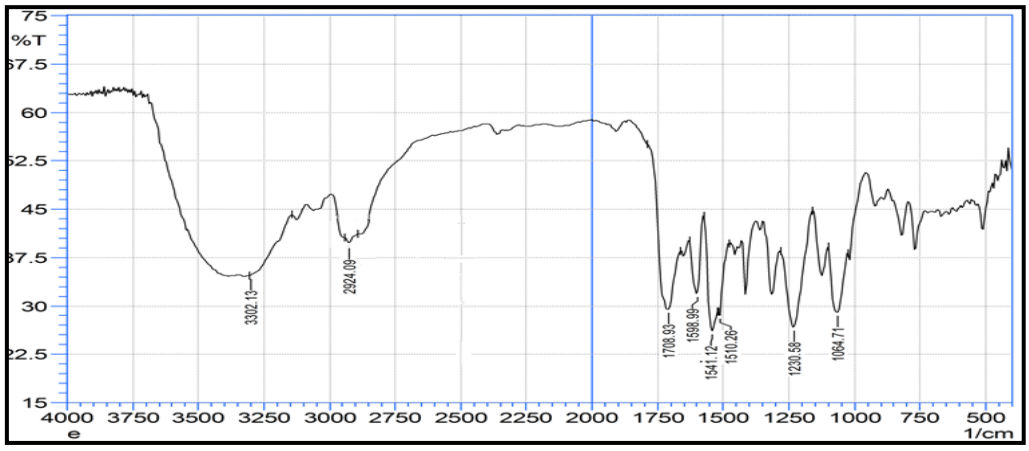

Figure 7: The IR spectrum of CO +MDI Scaffold

X-Ray Diffraction (XRD)

The X-ray diffraction analysis result from the polyurethane scaffolds prepared, with and without the salts (HA+TCP). The polyurethane scaffold with salts has shown some diffraction bands. Hence, it has being identified as crystalline structure due to the superior concentration of salt ,while DEOL/MDI and CO/MDI , have semi-sharp peaks and can observed that as the CO content increases the intensity of the peak at 2θ of 24.7◦ When salts was entrapped into the scaffold matrix, its sharp crystal peaks were overlapped with the noise of the surrounded polymer and disappeared indicating that salts was successfully entrapped into the scaffold matrix system and formation of a new solid phase for salts with low crystallinity [23].

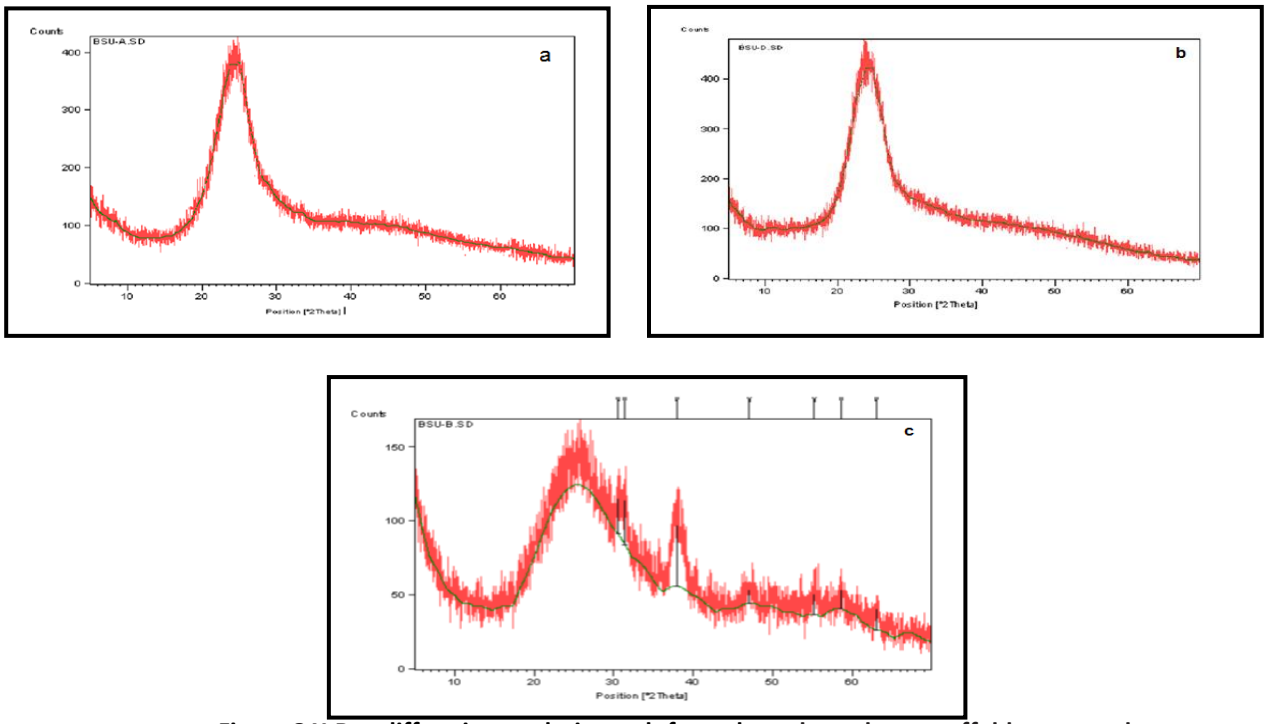

Figure 8 X-Ray diffraction analysis result from the polyurethane scaffolds prepared
a) DEOL/MDI b) CO/MDI c) CO/MDI scaffold with salts

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In Vitro Release Study of CS

100 mg sample of drug-loaded microspheres were placed in the synthetic dialysis bags and were immersed into 100 ml of n-saline phosphate buffer (pH=7.4) after they were fixed in sterilized beakers. The system was placed in the Lap-Shaker at constant temperature 37˚C. Three millilitres of the dispersion medium was withdrawn and filtered through 0.22 µm Millipore filters. The drug concentration was measured at(λ =235 nm)using UV spectrophotometer.

The drug release was evaluated using the following definitions:

$$\text{Drug Release (\%)} = \frac{Amount\ of\ drug\ release\ (mg)}{Total\ amount\ of\ loaded\ (mg)}\ x\ 100$$

The effect of drug loading of microspheres on CS release from microspheres is shown in Fig. 3.It can be seen that by increasing the amount of drug loading from(90 to 270 mg), the rate of drug release from the microspheres increase dramatically .With higher drug loading more drug molecules are available at the surface of microspheres leading to higher initial release .

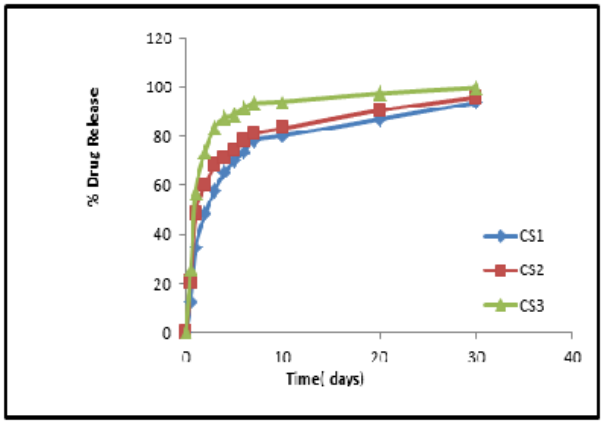

Figure 9: Plot of average percentage CS released from gelatin microspheres

CS3	CS2	CS1	Time(days)
0	0	0	0
25.69	20.27	12.62	0.5
57.4	48.73	34.44	1
73.8	59.89	48.22	2
83.6	68.14	58.06	3
87.5	70.96	65.14	4
88.6	73.87	69.85	5
91.48	78.3	73.47	6
93.5	81.02	78.5	7
94.2	83.23	80.21	10
97.31	90.47	86.93	20
99.56	95.87	93.76	30

Table 4: Evaluation of drug release from microspheres

In Vitro Release Study of CS from scaffolds

Fig. 10 presents the cumulative release of CS from the polyurethane scaffolds in phosphate buffer (pH=7.4) at 37 °C. The release profiles of DEOL-MDI-CS and CO-MDI-CS show an initial “burst” followed by a sustained release of CS from the polyurethane scaffolds. The release mainly depends on the type of polyol structure of the polyurethane: it is significantly higher for the of DEOL-MDI-CS polyurethane than for the DEOL-MDI-CS compared with microspheres loaded drug(CSm).

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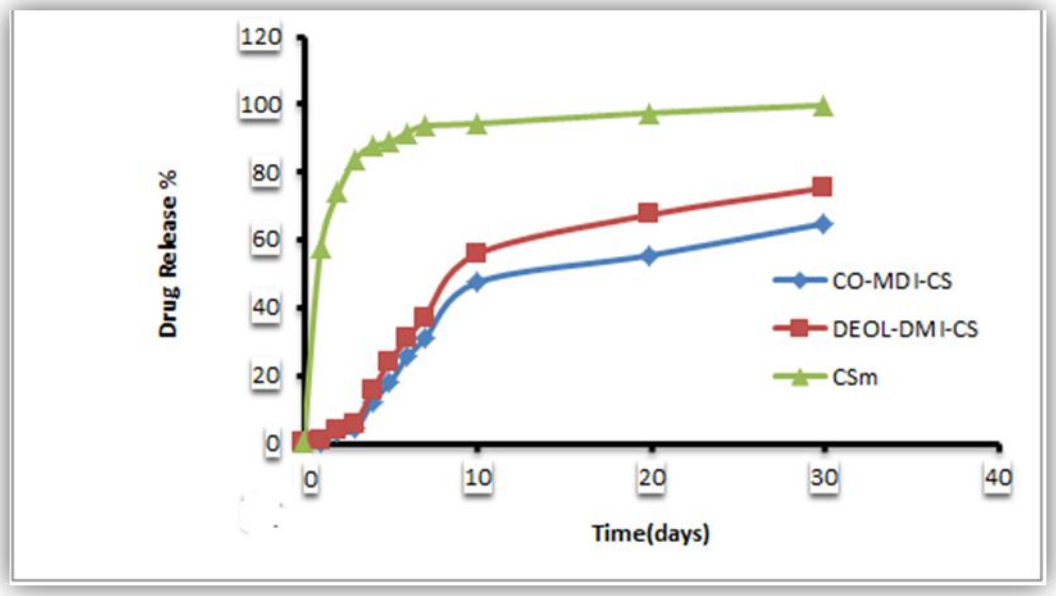

Figure 10: Release curve of Cefotaxime sodium from scaffolds and microspheres

Mechanical properties and porosity

The compressive modulus is an important parameter in tissue formation and thus in the repair of tissue lesions. By changing the type of polyol structure, the content of the soft segment in polyurethane can be controlled. The mechanical properties of the porous polyurethane scaffolds are shown in table 1. The compressive strength and tensile strength of the scaffolding prepared was measured before and after the addition of salts (HA + TCP). The results showed that the scaffold polyurethane without the use of salt, the value of compressive strength it is 3.07 Mpa either the value of the tensile strength was 0.697 Mpa, either the value of the power of compression for models of polyurethane after the addition of salt during preparation was the value of the power of compression it is 4.96 Mpa, either the value of the power tensile strength is 1.82 Mpa.

Table 5: Mechanical properties and porosity of scaffolds

Scaffolds Types	Tensile Strength $\sigma_{(Mpa)}$	Compressive Strength σ_M (Mpa)	Porosity %
DEOL-MDI	0.165	0.358	96
DEOL-MDI + HA +TCP	0.472	1.95	53
CO-MDI	0.697	3.07	92
CO-MDI+HA+TCP	1.82	4.96	48

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The porosity of scaffolds prepared studied using a liquid displacement method, in this way, use of ethanol as a liquid displacement because it penetrated easily into the pores of the polyurethane scaffold. Then calculated as porous polymeric scaffolds in the table 5.

Increasing the crosslinking ratio in polyurethanes improves their mechanical properties by increasing the uniformity and inter connectivity of the pores. The highest percentage of pores (over 96%) was observed for the polyurethane DEOL-MDI scaffold, either the value of the porosity of polyurethane after the addition of salt during preparation was (53%), the porosity decreases and the pore walls becomes thicker.

Equilibrium swelling of Scaffolds

We studied the behavior of the equilibrium swelling Scaffolding prepared as a function of the time of this and that by immersing the samples dry weight information a certain quantity with distilled water at a temperature ° C (30 ± 5). During certain periods of time, raised the models from solution and placed on filter paper to remove water from the surface of the scaffold and weighed for the purpose of calculating the ratio of the Bulge. Calculated the equilibrium swelling ratio of the Scaffolds during 24 hours.

Found that the practical results of all scaffolds compositions prepared up to equilibrium less than seven hours, however experiment lasted for 24 hours. It is noted (Fig. 11) .Scaffolding polymeric Prepared find scaffold DEOL-MDI showed rates of swelling ratio (SR) high, much higher than the CO-MDI because of the presence of proportions more than the totals of hydroxyl and thus a high degree of overlap and thus less ratios bloating, and the results showed rates of swelling ratio of scaffolds without salts much higher than the swelling ratio of scaffolds with salts.

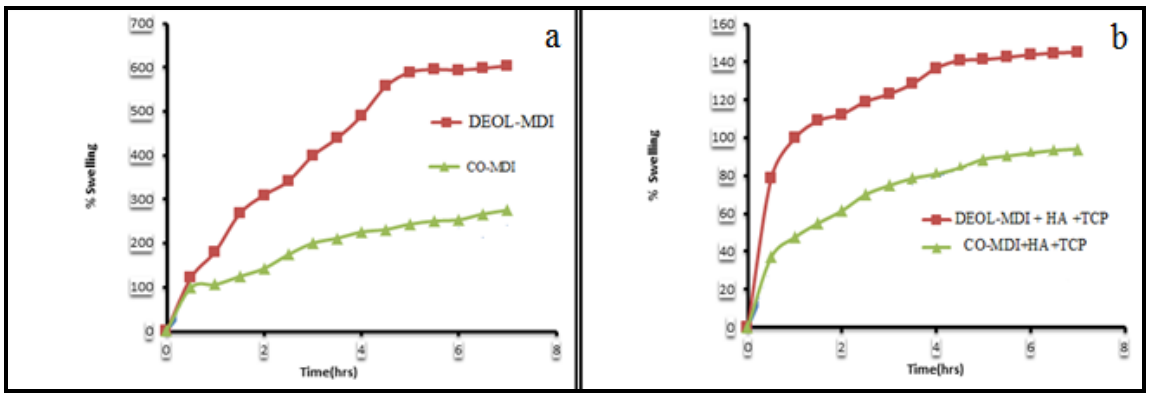

Figure 11: Equilibrium swelling of scaffolds
a) without salts b) with salts

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Degradation properties

Scaffold degradation curves are shown in Fig. 12. Biodegradation rate of the prepared scaffolds was investigated in SBF, and PBS at different time. The results showed a continuous weight loss for each scaffolding models studied, as well as the results show changes in most of the blocks models scaffolds with changes in porosity emerged as a significant increase pore diameter of pores as well as the interdependence of change. And that the rate of decomposition depends on the density of entanglements crosslinking density, where an increase of the density of entanglements resulting decrease in the penetration of water or solutions to the internal surfaces of the polymer and thus lower weight loss scaffolding [24]. The total weight loss of both types of scaffolds increased steadily during the degradation assay. The DEOL/MDI scaffold lost more weight than the CO/MDI scaffold.

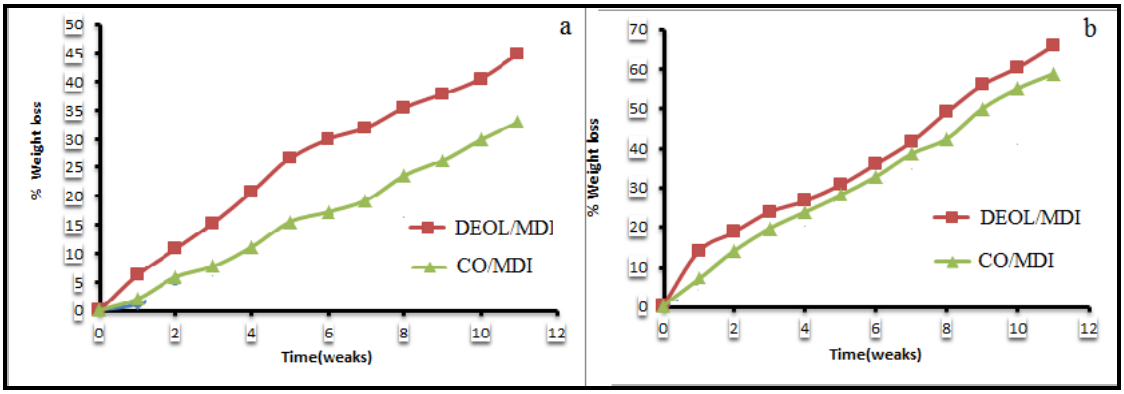

Figure 12: Weight loss of scaffolds
a) in PBS solutions b) SBF solutions

The rate of weight loss for both types of scaffolds in SBF solutions higher than in PBS solutions. Fig. 13 shows optical micrograph of the polyurethane scaffolds hydrolytic degradation over the 6 weeks was done using a light optical microscope.

The polyurethanes scaffolds were cut approximately into 1cm × 1cm squares with thickness of approximately 1 cm. 0.1 M cobalt chloride solution in 20% H_2O_2 were prepared from 30% H_2O_2 solution by proper dilution with distilled water, to understand the effect of oxidative solution on the degradation of the polymers. The polyurethanes scaffolds were added to these solutions at 37±1 °C temperature (physiological body temperature). Samples from each of these solutions were taken out at 3, 7, 14, and 22 days interval and dried in vacuum oven at 40 °C for two days and weighed. The test solutions were changed every 7 days to maintain the ionic concentration relatively constant. The mass loss of the polymers was measured gravimetrically to examine the effect of oxidative degradation. Figs. 14 and 15 shows the loss of mass due to oxidative degradation over the 22 days , about 34% of the mass is lost for DEOL/MDI scaffold compared to only 25% for CO/MDI scaffold.

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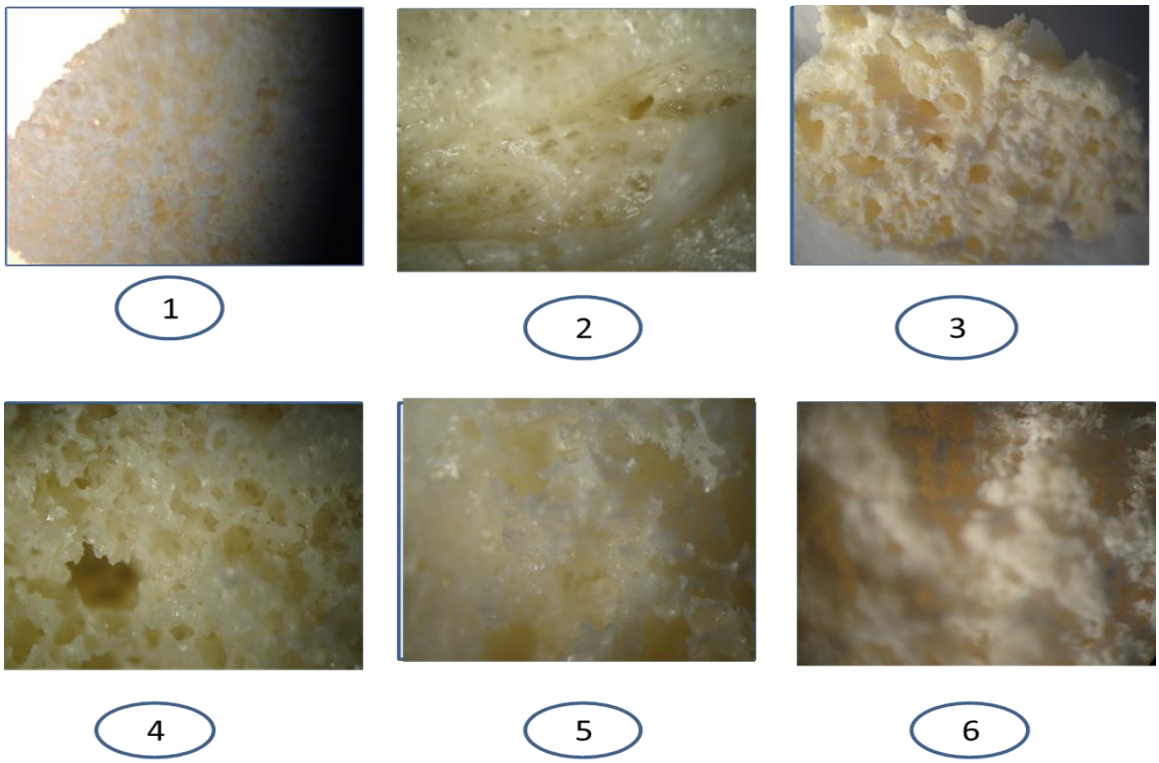

Figure 13: Optical micrograph of the polyurethane scaffolds hydrolytic degradation

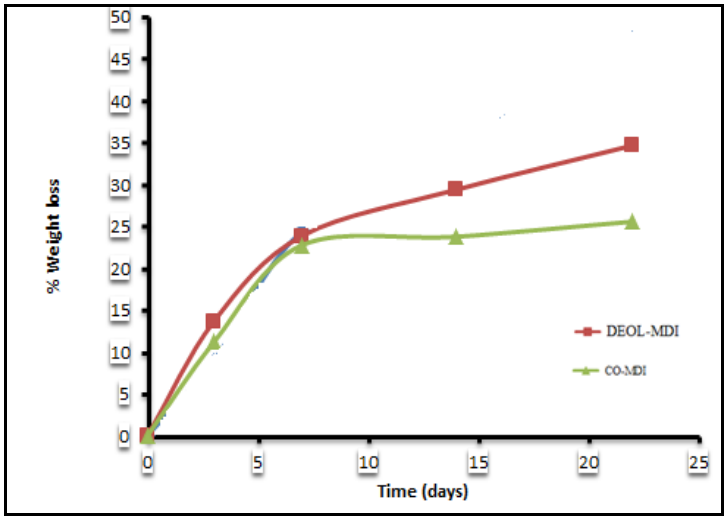

Figure 14: Mass loss of polyurethane scaffolds during oxidative degradation in37 C °

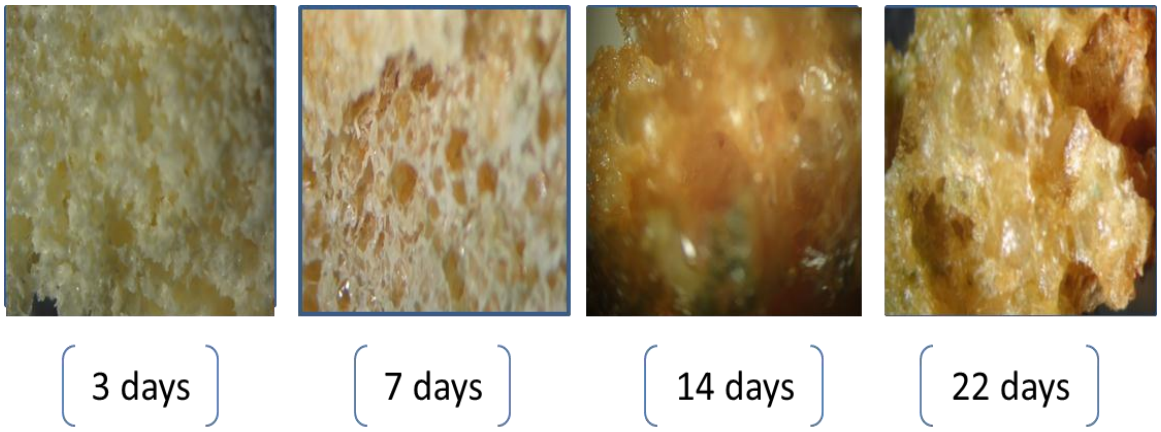

Figure 15: Optical micrograph of polyurethane scaffolds during oxidative degradation

CONCLUSION

In this study, prepare the polyurethane bio-degradable using two different types of polyol diethylene glycol and castor oil, Polyurethane hydrogels are successfully synthesized from CO, which is natural polyol, polyurethanes have density, porosity, degradation rate, and swelling different. Since, CO has more than two hydroxyl groups in each molecule ,the increasing of CO content result in increase in the crosslink density of the polyurethane hydrogels .Increase in CO content not only increase the crosslink density but also increase the water contact angle indicating the hydrophobic. Surfaces and also gave lower values of porosity. the rate of drug release from the microspheres increase dramatically ,when increasing the amount of drug loading microsphere, on the other hand add salt to scaffolding polyurethane was successfully entrapped into the scaffold matrix system ,which lead to improve the mechanical properties of the scaffolds.

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