FUNCTIONALIZATION OF ALGINATE VIA PHOSPHORIZATION: PREPARATION, CHARACTERIZATION.

W. M. Abou-Taleb\textsuperscript{1}, G. D. Roston\textsuperscript{2}, M. S Mohyeldin\textsuperscript{3,4}, A. M Omer\textsuperscript{3}, *T. M. Tamer\textsuperscript{3}, E. F. Shehata\textsuperscript{1} and A. M. Hafez\textsuperscript{1}.

1. Physical Department, Faculty of Education, Alexandria University, Alexandria, Egypt.
2. Physics Department, Faculty of Science, Alexandria University, Alexandria, Egypt.
3. Polymer Materials Research Department, Advanced Technology and New Materials Research Institute, City of Scientific research and advanced technology. New Boarg El-Arab City,21934, Alexandria, Egypt.
4. Chemistry Department, Faculty of Science, University of Jeddah, Jeddah, 21589, Saudi Arabia.

Abstract

In the current study, Phosphorization of Alginate was done via activation of Alginate with Epichlorohydrin then introduces Orthophosphoric as a source of phosphonic groups. Four different molar ratios of Phosphorization were prepared. Phosphorus content was determined using elemental analysis by EDX unit. Physical characterization including moisture content, and ion exchange capacity was examined and confirm phosphorylation step. The structure of the prepared new alginate derivative was characterized using Electronic spectrum (UV-Vis spectrum), Fourier transform infrared FT-IR, Raman Spectroscopy, thermal gravimetric analysis (TGA), Differential scanning calorimetric (DSC) and Scan electron Microscope (SEM).

Introduction:-

Alginate is a member of acidic polysaccharide that has awarded into various applications in environmental, pharmaceutical and biomedical science and engineering due to its helpful properties, including biocompatibility and ease of gelation.

Alginate is an anionic biopolymer commonly obtained from brown seaweed. Alginates are linear polysaccharides. Structurally, it is a copolymer consists of 1→4 linked β-D-mannuronate (M) and its C-5 epimer α-α-L-guluronate (G) residues. The blocks are composed of continuous G residues (GGGGGG), following M residues (MMMMMM), and alternating M and G-residues (GMGMGM). the ratio of guluronate to mannuronate varies depending on the natural source [1].

Alginate is a biomaterial and a polyelectrolyte that are considered to be biocompatible, non-toxic, non-immunogenic and biodegradable [2, 3]. The unique microstructural characteristics of alginate enable it to have been widely used as a type of desired biomaterials in many hot fields such as cell immobilization [4], tissue engineering [5], drug delivery [6-10], and controlled release [11], immobilization of micro-organisms [12], and wound dressing [13] as well as in water treatments.

Corresponding Author:- T. M. Tamer.
Address:- Polymer Materials Research Department, Advanced Technology and New Materials Research Institute, City of Scientific research and advanced technology. New Boarg El-Arab City,21934, Alexandria, Égypt.
Chemical structure of alginate exhibit that it has a number of free hydroxyl and carboxyl groups distributed along its backbone; consequently, it is a fitting model for chemical functionalization. Through derivatization of alginate derivatives by functionalizing available hydroxyl and carboxyl groups, the properties such as solubility, hydrophobicity and physicochemical and biological characteristics may be modified. Several chemical modifications of Alginate were reviewed by authors including; Acylation [14], Sulfation [15], phosphorylation [16], Oxidation [17], Reductive-amination [18], Amidation [19], Copolymerization [20], alkyl sulfonation [21] etc.

In this study, different degrees of phosphorated alginate were prepared via new method by utilizing epichlorohydrin as an activating agent and orthophosphoric acid as a phosphorus source.

Materials and methods:-
Sodium alginate (Alg; medium viscosity ≥2000 cP, 2 % (25°C) (lit)) was purchased from Sigma–Aldrich Chemicals Ltd. (Germany). Epichlorohydrin (purity 99.5%) supplied by Sigma- Aldrich Chemie Gmbh. (USA). Orthophosphoric acid (purity 85% extra pure) supplied by Sigma- Aldrich Chemicals Ltd. (Germany).

2.1. Methods
Prepared 1 gm of Alginate powder (Alg) dissolved in 50 ml distilled water followed by the addition of ECH in different molar ratios; 1: 0.25, 1: 0.5, 1: 1 and 1: 2. The activation process carried at (60°C). After 6 hours, equivalent amounts of orthophosphoric acid were added to the reactor and continuously stirring for additional 6 hr. The obtained mixture of Alg-ph polymer was precipitated using ethanol. Alg-ph was rinsed ten times using absolute ethanol to remove any excess of free reactants. Four different substitutions Alg-ph 1, Alg-ph 2, Alg-ph 3 and Alg-ph 4 were prepared and characterized in comparison with neat Alginate Alg-ph 0.

Characterization:-
3.1. Estimation of Phosphate content
Identification of Phosphate group in new derivative was done using EDX unite of Scanning Electron Microscope (Joel Jsm 6360LA, Japan). Presence of Phosphorus in new derivative simplified identification of it in final product compare to neat alginate.

3.2. Moisture content
Desired weight of dry samples was located in a humidity chamber with 80% humidity ratio, for 24 hr and then reweighed. Moisture content was calculated as follows equation;

\[ \text{Moisture content} \% = \frac{M - M_0}{M_0} \times 100 \]

Where \( M_0 \) is the initial weight of the dry sample and \( M \) is the final weight.

3.3. Infrared spectrophotometric analysis (FTIR)
The structures of Alginate and phosphonated Alginate derivatives were determined by FTIR analysis was carried out using Fourier transform infrared spectrophotometer (Shimadzu FTIR 8400 S), Japan.

3.4. Raman spectroscopy analysis
The micro-Raman scattering spectra were recorded with a Renishaw Raman RM1000 equipped with the 532 nm laser line, an electrically refrigerated CCD camera, and a notch filter to eliminate the elastic scattering. The spectra shown here were obtained by using a 25x microscope objective. The output laser power at the samples was about 0.2 mW. Spectral resolution was 4 cm\(^{-1}\). The spectral scanning conditions were chosen to avoid sample degradation.

3.5. Thermal gravimetric analysis (TGA)
Thermal degradation of Alginate and phosphonated Alginate derivatives was studied using Thermogravimetric analyzer (Shimadzu TGA-50, Japan).

3.6. Scanning electron microscopic analysis (SEM)
Morphological changes of the samples surface were followed using SEM (Joel Jsm 6360LA), Japan.

3.7. UV-Vis Spectroscopic analysis
The electronic absorbance of Alginate and phosphonated Alginate derivatives (2.5 mg/ml) were done using spectrophotometer scanned from 200 -500 nm.
Results and discussion:
The derivatization strategies for alginates depend on reactivity of alginate function groups. Alginate can be modified at the two secondary –OH positions (C-2 and C-3) or the one –COOH (C-6) position. In the current work, phosphorization of alginate was done through activation its hydroxyl groups (at C-2 and C-3) using epichlorohydrin then phosphorization using orthophosphoric acid as shown in Figure 1.

![Figure 1: Schematic preparation of Phosphonated alginate](image)

Physical characterization:

4.1. Moisture content
The moisture content of alginate was estimated and presented in Figure 2. Presence of hydrophilic groups such as hydroxyl and carboxylic groups along alginate backbone enhance it to absorb moisture molecules from surrounding atmosphere it is clear from the figure that the increase of moisture trapped into samples by increasing the content of phosphorization which may be explained by introducing of hydrophilic phosphoric groups.

![Figure 2: Moisture content of alginate and different phosphonate derivatives.](image)

4.2. Electronic spectrum
Alginates have carboxylate as the intrinsic chromophore and hence display circular dichroism around 210 nm matching to the n-π* transition of the carboxyl group. The three types of block sequences present in alginates display very different circular dichroism behavior, the spectrum of poly-L-guluronate being entirely negative, whereas that of poly-D-mannurionate has a strong positive band and mixed sequences show intermediate behavior. Spectra of intact alginates show a peak at ~200 nm, and a trough at ~215 nm (Figure 3), with relative magnitudes varying with composition. The ratio of peak height to trough depth varies almost linearly with the ratio of mannuronate to guluronate residues. A shoulder was observed around 250–290 nm. Absorbance within the 250–290 nm range is commonly attributed to π − π* electron transitions in aromatic and poly-aromatic compounds [22], which could connect with the impurities such as humic acid, phenol, DNA or proteins [23]. The decrease of the
intensity of the band at 270 nm from 1.414 at alginate (Alg-ph 0) to 0.957, 0.682, 0.475 and 0.409 for Alg-ph 1, Alg-ph 2, Alg-ph 3, Alg-ph 4 respectively can be referred to the elimination of attached impurities. This can be explained by repulsion force between immobilized negative phosphonic groups and negative nature of impurities (i.e., humic acid, phenol, DNA or proteins).

![Figure 3: Electronic spectrum of alginate and different phosphonate derivatives.](image)

**4.3. Infrared spectrophotometric analysis (FTIR)**

The FT-IR spectra of alginate and its phosphonate derivatives were done and presented in figure 4. Alginate demonstrates typical characteristic bands related to its polysaccharide structure. A broadband at 3150 cm\(^{-1}\) was attributed to stretching vibration of hydroxyl groups. Bands at 1656 and 1417 cm\(^{-1}\) present in the IR spectrum are assigned to asymmetric and symmetric stretching peaks of carboxylate salt groups. Also, the bands around 1320 cm\(^{-1}\) (C–O stretching), 1130 cm\(^{-1}\) (C–C stretching), 1090 cm\(^{-1}\) (C–O stretching), 1027 cm\(^{-1}\) (C–O–C stretching), and 950 cm\(^{-1}\) (C–O stretching) are attributed to its saccharide structure. On the other hand, a new band appeared at 1736 cm\(^{-1}\) in the spectrum due to the asymmetric and symmetric stretching of –COO– groups. The typical bands of phosphonated derivatives are those that appears at 1204cm\(^{-1}\) d (P=O), d a symmetric 1168 cm\(^{-1}\) (P-O-P), v at 914cm\(^{-1}\) (P-O) and d symmetric at 798 cm\(^{-1}\) (P-O-C) [24].

![Figure 4: FT-IR Spectra of alginate and different phosphonate derivatives.](image)
4.4. Raman Spectroscopy
The Raman spectroscopy analysis of Alginate and different phosphonate derivatives are shown in Figure 5. All the samples exhibit the characteristic asymmetric and symmetric bands of carboxylate ion (COO\(^{-}\)) at 1616–1611 cm\(^{-1}\) and near 1446 cm\(^{-1}\), respectively. In the region 1400–1270 cm\(^{-1}\), three bands assigned to the C–H deformation vibration, and a band around 1200 cm\(^{-1}\) assigned to the C–O stretching vibration are shown [25].

Alginate samples show several bands in the region between 1200–950 cm\(^{-1}\); this region deals with C–OH deformation, C–C–H bending, C–O, and C–C stretching vibrations; every band may be due to contributions of two or more kinds of motions. [26] The region between 950 and 750 cm\(^{-1}\) is called the ‘fingerprint’ or the anomeric region; the spectra of alginate samples present a band near 800 cm\(^{-1}\) assigned to skeletal stretching and deformation modes, and another around 770–730 cm\(^{-1}\) due to ring breathing. Below 700 cm\(^{-1}\), four bands related to the deformation of pyranose rings and C–O–C vibration of glycosidic linkage is shown [25].

![Figure 5: Raman scattering spectra of alginate and different phosphonate derivatives.](image)

4.5. Thermal gravimetric analysis (TGA)
Thermal gravimetric analysis of alginate and its phosphonated derivatives were exanimate at temperature ranged from ambient to 600 °C (figure 6). Alginate demonstrates a general moisture loss band that starts from ambient to around 150 °C, presence of highly hydrophilic bands such hydroxyl and carboxylic groups along backbone simplified trapping of moisture molecules inside chains. Hydrophilicity of polymers was increased significantly by phosphorization process. Moisture content was increased dramatically from 13.26 % on neat polymer alginate (Alg-ph 0) to be 12.62, 20.03, 23.14 and 27.03% for (Alg-ph 1, Alg-ph 2, Alg-ph 3 and Alg-ph 4) respectively.

Alginate exhibits a second weight loss starting from 220 °C that attributed to destructive decomposition of pyranose ring. In the other hand, Phosphonated alginate has a different decomposition behavior. New decomposition rate was observed consequently after elevation of moisture content (i.e., at 150 °C) can be sign to evaporation of physically attached phosphoric acid.
4.6. Differential scanning calorimetry (DSC)
Differential scanning calorimetric analysis of the alginate and its phosphonate derivatives was measured and presented in figure 7. The first endothermic peak observed below 100 °C is attributed to elevation moisture which is attached on internal chains. Alginate exhibits a significant exothermic peak at 245 ºC as a result of cleavage of the glycoside ring. Phosphonate alginate demonstrates a nice endothermic peak with maximum at 140 ºC that can refer to evaporation of physically attached phosphoric acid (boiling point of phosphoric acid 158 ºC).

4.7. Morphological characterization (SEM)
Surface morphological study of alginate and different phosphonate derivatives were performed with Scanning electron microscope. Figure 8 show microstructure of powers surface. From figures, it's clear increase the surface roughness at low phosphorization degree (Alg-ph 1) that can attribute to effect of phosphorization on distortion of crystal structure of alginate.
Identification of immobilized phosphonic groups on polymeric matrix after preparation step was studied by EDX in order to quantitative determine of P % via illustrate elemental analysis. EDX offers use of the X-ray spectrum emitted by a solid sample bombarded with a focused beam of electrons to obtain an elemental chemical analysis. Qualitative analysis involves the identification of the P line (i.e.; P K at 2.013 kev) in the spectrum. Figure 9, illustrate EDX spectrum of alginate and different phosphonate derivatives. It was clear increase P% from 0 in Alg-ph 0 to 0.01, 0.02, 0.03, 0.08 % for Alg-ph 1, Alg-ph 2, Alg-ph 3 and Alg-ph 4 respectively.

**Figure 8:** SEM of alginate and different phosphonate derivatives.

| Alg-ph 0 | Element (keV) | mass% Error% | At% | Cation K |
|---------|---------------|--------------|-----|---------|
| C K 0.277 | 49.38 | 0.40 | 56.51 | 40.9673 |
| O K 0.525 | 50.62 | 1.75 | 43.49 | 46.8986 |
| P K 0 | 0 | 0 | 0 | 0 |
| Total | 100 | 100 |

| Alg-ph 1 | Element (keV) | mass% Error% | At% | Cation K |
|---------|---------------|--------------|-----|---------|
| C K 0.277 | 50.43 | 0.41 | 57.55 | 42.7228 |
| O K 0.525 | 49.56 | 1.89 | 42.45 | 45.1829 |
| P K 2.013 | 0.01 | 0.53 | 0.00 | 0.0162 |
| Total | 100 | 100 |

| Alg-ph 2 | Element (keV) | mass% Error% | At% | Cation K |
|---------|---------------|--------------|-----|---------|
| C K 0.277 | 51.17 | 0.39 | 58.27 | 43.9415 |
| O K 0.525 | 48.80 | 1.85 | 41.72 | 43.9860 |
| P K 2.013 | 0.02 | 0.50 | 0.01 | 0.0337 |
| Total | 100 | 100 |
Figure 9: EDX analysis of alginate and different phosphonate derivatives.

4.8. X-ray diffraction analysis (XRD)

The XRD patterns of sodium alginate and its phosphonated derivatives were shown in Figure 10. Sodium alginate is usually crystalline due to the strong interaction between the alginate chains through intermolecular hydrogen bonding of hydroxyl and carboxylic groups [27]. Three diffraction peaks at 2θ values 13.5°, 22° and 39° were observed for sodium alginate due to the reflection of their (110) plane from poly guluronate unit, (200) plane from poly Mannuronate and the other from amorphous halo [28].

Functionalization of alginate with phosphonic groups via epichlorhydrane exhibit distortion of its crystal form.

Figure 10: XRD of alginate and different phosphonate derivatives.
Conclusion:
In this work new phosphonated derivative of alginate was prepared via activation using epichlorohydrin. Change in chemical structure of alginate was estimated using FT-IR, Raman spectroscopy and UV/Vis spectrum. Thermal behavior of new phosphonated derivatives was investigated using TGA and DSC comparing to neat polymer (Alginate). SEM and XRD give a good view of crystal and morphological structure.

References:
1. A. Haug. Fractionation of alginic acid. Acta Chem Scand. 13, 601–603 (1959).
2. G. Klöck, A. Pfeftermann, C. Ryser, P. Gröhn, B. Kuttler, H. J. Hahn, U. Zimmermann. Biocompatibility of mannnuronic acid-rich alginites. Biomaterials, 18, 707–713 (1997).
3. F.L. Mi, H.W. Sung, S.S. Shyu. Drug release from chitosan–alginate complex beads reinforced by a naturally occurring cross-linking agent. Carbohydrate Polymers, 48 61–72 (2002).
4. G. Orive, S. Ponce, R. M. Hernandez, A. R. Gascon, M. Igartua, J. L. Pedraz. Biocompatibility of microcapsules for cell immobilization elaborated with different type of alginites. Biomaterials, 23, 3825–3831 (2002).
5. J. L. Drury, D. J. Mooney. Hydrogels for tissue engineering: Scaffold design variables and applications. Biomaterials, 24, 4337–4351 (2003).
6. H. Lai, A. AbuKhalil, Q. M. Craig Duncan. The preparation and characterisation of drug-loaded alginate and chitosan sponges. International Journal of Pharmaceutics, 251, 175–181 (2003).
7. A. M. Omer, T. M. Tamer, M. A. Hassan, P. Rychter, M. S. Mohy Eldin, N. Koseva. Development of amphoteric alginate/aminated chitosan coated microbeads for oral protein delivery. International Journal of Biological Macromolecules. 92, 362-370 (2016).
8. M.S. Mohy Eldin, A. M. Omer, M.A. Wassel, T.M. Tamer, M.S. Abd-Elmonem, S.A. Ibrahim. Novel smart pH sensitive chitosan grafted alginate hydrogel microcapsules for oral protein delivery: I. Preparation and characterization. Int J Pharm Sci. 7(10), 320-326 (2015).
9. M.S. Mohy Eldin, A. M. Omer, M.A. Wassel, T.M. Tamer, M.S. Abd-Elmonem, S.A. Ibrahim. Novel smart pH sensitive chitosan grafted alginate hydrogel microcapsules for oral protein delivery: II. Evaluation of the swelling behavior. Int J Pharm Sci. 7 (10), 331-337 (2015).
10. M. S. Mohy Eldin, E. A. Kamoun, M. A. Sofan, S. M. Elbayomi. L-Arginine grafted alginate hydrogel beads: A novel pH-sensitive system for specific protein delivery. Arabian Journal of Chemistry. 8, 3, 355-365 (2015).
11. V. Ramesh Babu, M. Sairam, K. M. Hosamani, T. M. Aminabhavi. Preparation of sodium alginate–methylcellulose blend microspheres for controlled release of nifedipine Carbohydrate Polymers, 69, pp. 241–250 (2007).
12. M. Yakup Arca, A. Çigdem, A. Ergene, B. Gülay, G. Ömer. Ca-alginate as a support for Pb(II) and Zn(II) biosorption with immobilized Phanerochaete chrysosporium Carbohydrate Polymers, 52, 167–174 (2003).
13. E. A. Kamoun, E. S. Kenawy, T. M. Tamer, M.A. El-Meligy, M. S. Mohy Eldin. Poly (vinyl alcohol)-alginate physically crosslinked hydrogel membranes for wound dressing applications: Characterization and bio-evaluation. Arabian Journal of Chemistry. 8, 1, 38-47 (2015).
14. M. J. Franklin, S. A. Douthit, M. A. McClure. Evidence that the algI/algJ gene cassette, required for O acetylation of Pseudomonas aeruginosa alginate, evolved by lateral gene transfer. J Bacteriol, 186, 4759–4773 (2004).
15. R. J. Linhardt, S. Claude Hudson. Award address in carbohydrate chemistry. Heparin: structure and activity. J Med Chem, 46 (2003), pp. 2551–2564.
16. R. J. Coleman, G. Lawrie, L. K. Lambert, M. Whittaker, K.S. Jack, L. Gröndahl. Phosphorylation of alginate: synthesis, characterization, and evaluation of in vitro mineralization capacity. Biomacromolecules, 12, 889–897 (2011).
17. T. Boontheekul, H. Kong, D. Mooney. Controlling alginate gels degradation utilizing partial oxidation and bimodal molecular weight distribution. Biomaterials, 26, 2455–2465 (2005).
18. H. A. Kang, M. S. Shin, J. W. Yang. Preparation and characterization of hydrophobically modified alginate. Polymer Bulletin, 47, 429–435 (2002).
19. C. Galant, A. L. Kjønnsken, G. T. M. Nguyen, K. D. Knudsen, Bo. Nyström Altering associations in aqueous solutions of a hydrophobically modified alginate in the presence of β-cyclodextrin monomers. J Phys Chem B. 110(1):190-5 (2006).
20. M. Z. Liu, L. X. Cao. Preparation of a superabsorbent resistant to saline solution by copolymerization of acrylic acid with sodium polymanunonate Chinese Journal of Applied Chemistry, 19, 455–458 (2002).
21. M. S. Mohy Eldin, A. E. Hashem, A.M. Omer, T. M. Tamer, M. E. Yossuf, M. M. Sabet. Enhancement of solubility and ion exchange capacity of chitosan via N-alkyl sulfonation. Journal de Afrikana, 3(5), 336-349 (2016).
22. L. Trabelsi, N. H. M’sakni, H. B. Quada, H. Bacha, S. Rouesli. Partial characterization of extracellular polysaccharides produced by cyanobacterium Arthospira platensis. Biotechnology and Bioprocess Engineering, 14, 27–31(2009).
23. J. Dusseault, S. K. Tam, M. Ménard, S. Polizu, G. Jourdan, L. Yahia, J. P. Halle. Evaluation of alginate purification methods: effect on polyphenol, endotoxin, and protein contamination Journal of Biomedical Materials Research Part A, 76, 243–251 (2006).
24. R. Silverstein, F. Webster. Spectrometric Identification of Organic Compound, 44 – 293 (1997).
25. W. Rudolph, W. E. Steger, I. Zur Sulfatokomplexbildung in Alumimumsulfat – Lösungen und Hydratschmelzen. Z. Phys. Chem., 172, 31–48 (1991).
26. P. G. Daniele, A. De Robertis, C. De Stefano, A. Gianguzza, S. Sammartano. Salt Effects on the Protonation of Ortho-Phosphate Between 10 and 50 °C in Aqueous Solution. A Complex Formation Model. J. Solution Chem., 20, 495–515 (1991).
27. D. Fang, Y. Liu, S. Jiang, J. Nie, G. Ma. Effect of intermolecular interaction on electrospinning of sodium alginate. Carbohydr. Polym. 85, 276–279 (2011).
28. J. Fabia, C. Z. Slusarczyk, A. Gawlowski. Fibres Supermolecular Structure of Alginate Fibres for Medical Applications Studied by Means of WAXS and SAXS Methods. Text. East Eur. 13(5), 114-117 (2005).