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Pseudomonas aeruginosa Cell Wall Polysaccharide-Iron Oxide Nanoparticle Augments the Formation of Biofilm by the Bacterium

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Abstract
Pseudomonas aeruginosa is very ubiquitous microorganism which is a member of the normal microbial flora in human. The bacterium forms biofilm that is responsible for many persistent and chronic infections. *P. aeruginosa* biofilm formation is mediated by two soluble lectins, LecA (PA-IL) and LecB (PAIIL) that are essential extracellular virulence factor of *P. aeruginosa.* In this study bacteria isolated from direct biopsy samples of colorectal cancer (CRC) were used. Iron oxide nanoparticles (IONP) were prepared by coprecipitation method and subsequently coated with *Pseudomonas aeruginosa* polysaccharide using the principles of electrostatic interaction. The Fourier transform infrared spectroscopy (FTIR) and scanning probe microscope (SPM) analyses confirmed the successful coating of polysaccharide on the surface of iron oxide nanoparticles. The preparation appeared to have 1649 cm^{-1} ($CONH_2$) and 1543 cm^{-1} (N-H) band. Particle size for polysaccharide coated iron oxide increased from 85.09 nm for naked iron oxide nanoparticles to 107.20 nm. Bacterial polysaccharide coated iron oxide nanoparticles was used to inhibite *Pseudomoas aeruginosa* biofilm on saliva coated 96-well polystyrene.It was seen that exposure of cells to polysaccharide coated iron oxide showed increased biofilm formation on optimally coated saliva on polystyrene plates.The highest augmentation was recorded in a concentration of 100mg/ml and in saliva of Lewis group Lea^+ and approaching (75%) $p>0.05$ and the lowest augmentation was achieved in a concentration of 1mg/ml and approached (5%) $p=1.4\times10^{-3,}$ Lea-b-secretor gave (5%) augmentation $p=2.6\times10^{-3}$ while Lea-b- secretor saliva coat showed (15%) augmentation $p=0.05$ compared with naked iron oxide nanoparticles.Percentage augmentation of polysaccharide coated iron oxide nanoparticles in concentration of 100mg/ml, 10mg/ml and 0.1mg/ml was non significant $p>0.05$ except for Leb saliva used in concentration of 0.1mg/ml that was highly significant (44.5%) augmentation $p=3.3\times10^{-3}$in compared with iron oxide nanoparticles. In 0.01 mg/ml polysaccharide-iron oxide nanoparticle concentration augmentation reached (5.9%) $p=0.03$ for Lea-b-non secretor while Leb saliva (46%) augmentation $p=0.03$ in addition Lea gave (15%) augmentation and Lea-b- secretor showed (26.7%) augmentation $p>0.05$. This imply Lewis molecules determinants and bacterial lectin interaction in biofilm formation. Lectin are involved in biofilm formation.
Keywords: key words, polysaccharide-iron oxide, nanoparticles, *Pseudomonas aeruginosa* biofilm.

1. Introduction
Pseudomonas aeruginosa, is a Gram negative bacterium, important opportunistic pathogen (Goossens 2003; Easten 2005). It is often a long-term bowel tenant, colonizing the intestine of about three percent of healthy people. This germ is usually harmless in the intestine, but it can turn deadly (Easten, 2005), via its ability to adhere to and disrupt the intestinal epithelial barrier (Alverdy *etal*., 2000). *P. aeruginosa* carbohydrates play a main role in virulence. They are major components of the Gram-negative bacterial envelope lipopolysaccharides (LPSs) and alginate, the mucoid exopolysaccharide which is essential for the development of *P. aeruginosa* monospecies biofilms (Allison and Sutherland. 1987 and Scharfman *etal.*, 2001). The bacterium's host selectivity is based on the carbohydrate specificity of its lectins and adhesins, the host carbohydrates, including mucins of the lumen lining mucosa and cell surface glycoconjugates act as specific targets for the pathogen binding and are involved in establishment of infection (Scharfman *etal.*, 2001). The soluble lectins PA-IL and PA-IIL are specific for galactose and fucose, respectively (Gilboa-Garber, 1982). These Lectin are involved in biofilm formation.

2. Materials and Methods
2.1 Bacterial strains
Two isolates of *Pseudomonas aeruginosa* from direct biopsy samples of patients with colorectal cancer (CRC) were obtain from Kurdistan center for Gastroenterology and Hepatology (KCGH), Sulaimani/Iraq. VITEK2 system was used for characterization of the isolates.

2.2 Isolation of *P. aeruginosa* polysaccharide
Overnight *P. aeruginosa* culture grown in Tryptose soy broth, at 37°C for 24h, was heavily streaked onto ten

tryptose soya agar Petri dishes and incubated for 18 h at 37°C.After, 4 ml of saline was added to each plate, the growth was harvested with the aid of a Pasteur pipettes, and bacteria were collected by centrifugation at 4000 rpm for 15 minutes. The pellet was washed three times with saline using centrifugation, and treated with 5ml of 1% acetic acid, incubated at 100°C in water bath for 1 hour, cooled and centrifuged at 4000rpm for 20 minutes. The supernatant was taken and polysaccharide content was estimated.

2.3 Determination of polysaccharide concentration of *P. aeruginosa*

Phenol-sulfuric acid method was used (Dubios *etal.*, 1956). Two and a half milliliter of concentrated sulfuric acid, 0.5 ml of 5% phenol and 0.5ml of polysaccharide extracted were mixed. The mixture was incubated in water bath at 100℃ for 15 minutes and cooled. The absorbance (Optical density) of the characteristic yellow orange color was measured by a spectrophotometer at490 nm. Polysaccharide concentration was estimated according to curve using glucose as the standard sugar.

2.4 Preparation of polysaccharide-coated iron oxide nanoparticles.

100mg of naked iron oxide nanoparticles was prepared (Kedar *etal*., 1997) using electrostatic attraction principle the naked particles were coated with 10ml of 400µg/ml *P. aeruginosa* polysaccharide in a total volume of 100ml distilled water. The mixture was stirred for six hour at room temperature. Finally, the particles were recovered by using a strong magnet, washed 6 times with distilled water and dried at 37℃ to obtain the final product.

2.5 Characterization of polysaccharide−iron oxide nanoparticles

The size and morphology of nanoparticles preparation were determined by Scanning probe microscope (SPM). (Department of chemistry, Faculty of Science, University of Baghdad. Baghdad− Iraq) and Fourier transform infrared spectroscopy (FTIR) of bare and conjugated nanoparticles (Department of chemistry, Faculty of science, university of AL-Mustansiryiah. Baghdad− Iraq).

All samples were scanned over a range of 600–4000 cm^{-1} on a 2400S spectrophotometer.

2.6 Nanoparticles inhibition of Biofilm formation

This was pursued according to the protocol described by O'Toole and Kolter (1998). Saliva was diluted 1:16 in sterile saline and used to coat polystyrene 96 wells using 200 µl saliva. 100 mg/ml nanoparticle samples were used as standared stock solution. 100 µl of the standard stock solution was added into the tube containing 900µl tryptose soy broth with 1% (w/v) glucose to get (10mg/ml), followed by doubling dilution to get 1, 0.5, 0.25 mg/ml nanoparticles. 180 µl of the particle concentration was added to each wells followed by adding 20 µl of overnight *Pseudomonas aeuginosa* culture grown in tryptose soy broth at 37°C to each 96 wells saliva coated well and incubated at 37°C for 24 hours. The medium was discarded and the wells were gently washed twice with 200 µl of distilled water and dried. The wells were stained with 200 µl of 0.1% crystal violet and incubated for 10 minutes at room temperature and washed three times with 200 µl distilled water and allowed to dry completely. To each dried well, 200µl of 97% ethanol was added. Finally, the optical density (O.D) for each well was determined at 595 nm using a microplate ELISA reader (Human, Germany). Controls included cultures without nanoparticles. (O'Toole and Kolter, 1998).

2.7 Statistical analysis

Statistical analysis was done using a computer program for Epidemiological statistics. t-test was used to compare means of tests and controls. Values were regarded significant if $p \leq 0.05$.

3. Results and discussions

3.1 Characterization of bacterial polysaccharide – Iron oxide nanoparticles.

Successful polysaccharide iron Oxide nanoparticles formation was confirmed by FTIR. The appearance of characteristic bands such as C-O-C, C-O of the carbohydrates at 964, 989, 1022, and 1037 cm^{-1} was depicted (Figure 1) (Davis and Mauer 2010). The band at 1616 cm^{-1} is due to amide I group (C–O) of Iron oxide nanoparticles (Figure 1).

Appearance of 1649 cm^{-1} peak (amide I) and 1543 cm^{-1} peak (amide II) indicated successful coating in polysaccharide- iron oxide nanoparticles. The peak 1649 cm^{-1} in CH spectra refer to the amide linkage ($CONH_2$),1246cm^{-1} refer to P=O and C-O-C stretching (amide III) of polysaccharide.

Disappearance of carboxylate ester having a C=O that absorbs in the range of 1,780 to 1,730 cm^{-1} and absorbance at 1246 cm^{-1} (COO stretching of the O-acetyl group in alginate) was reported (Nivens *etal*., 2001).

Particle sizes determined by SPM indicated that the nanoparticles are close to spherical. The particle size distribution and histogram seen in figure (3) has an average diameters of 107.20 nm and ranging from (80-140) nm Figure (2).

3.2 Effect of *Pseudomonas aeruginosa* polysaccharide coated iron oxide nanoparticle on *Pseudomonas aeruginosa* biofilm formation

The effect of *Pseudomonas aeruginosa* polysaccharide coated iron oxide nanoparticles on biofilm formation by *P. aeruginosa* on mucin saliva is depicted in table 1.

The highest augmentation was recorded at a concentration of 100mg/ml and in saliva of Lewis group Lea^+ coat augmentation reached (75%). The lowest augmentation was achieved in a concentration of 1mg/ml on Leb coat (5±2.9) which was highly significant $p=1.4\times10^{-3}$. However, Lea-b- secretor coated saliva gave (5±1.4) $p=2.6\times10^{-3}$ while Lea-b- secretor saliva coat gave (15±6) augmentation p=0.05. Augmentation percentage of polysaccharide coated iron oxide nanoparticles was non significant for the concentration of 100mg/ml, 10mg/ml and 0.1mg/ml p>0.05 (table 4-17) except Leb saliva used in concentration of 0.1mg/ml that was highly significant (44.5±3.8) $p=3.3\times10^{-3}$ in comparsion with iron oxide nanoparticles. In 0.01 mg/ml polysaccharide-iron oxide nanoparticle concentration and on Lea-b-non secretor coat augmentation (5.9-54.1) p=0.03 while Leb saliva coat gave (46±22.5) augmentation p=0.03 non significant augmentation was seen in Lea (15±12) and Lea-b- secretor coat (26.7±25.9) as well p>0.05.

Pseudomonas aeruginosa produced three exopolysaccharides pel, psl and alginate each of them involved in biofilm formation (Ryder *etal*., 2007).

While, Psl is composed from mannose and galactose produce through planktonic growth, arranging attachment to surfaces and contributing to microcolony formation. Psl is also important for adherence to mucin-coated surfaces (Ma *etal*., 2006). Pel is a glucose-rich, cellulose-like polymer (Friedman and Kolter 2004). It has been shown to plays a role in cell-to-cell interactions in *P. aeruginosa* PA14 biofilms, Psl functions as a scaffold, holding biofilm cells together in the matrix at early stages (Colvin *etal*. 2011).

The augmentation of biofilm on Iron oxide nanoparticles coated with homologous bacterial polysaccharide might be attributed to using by the bacterial its polysaccharide as ligand in establishing its biofilm. It was reported that bacterial lectins and its polysaccharide interaction are involved in biofilm formation (Ryder *etal*., 2007).

The phenomena of the augmentable biofilm formation on *Pseudomonas aeruginosa* polysaccharide-Iron oxide nanoparticles could be mediated by the interaction of bacterial lectins (PAIL and PAIIL) with sugar components of the polysaccharide as well as the saliva ligands.

In this instance, the system reported here in, provide a model to study this interacting components, aiming to dissect factors that contribute to biofilm formation by this bacterium, aiming in inhibition of this structure.

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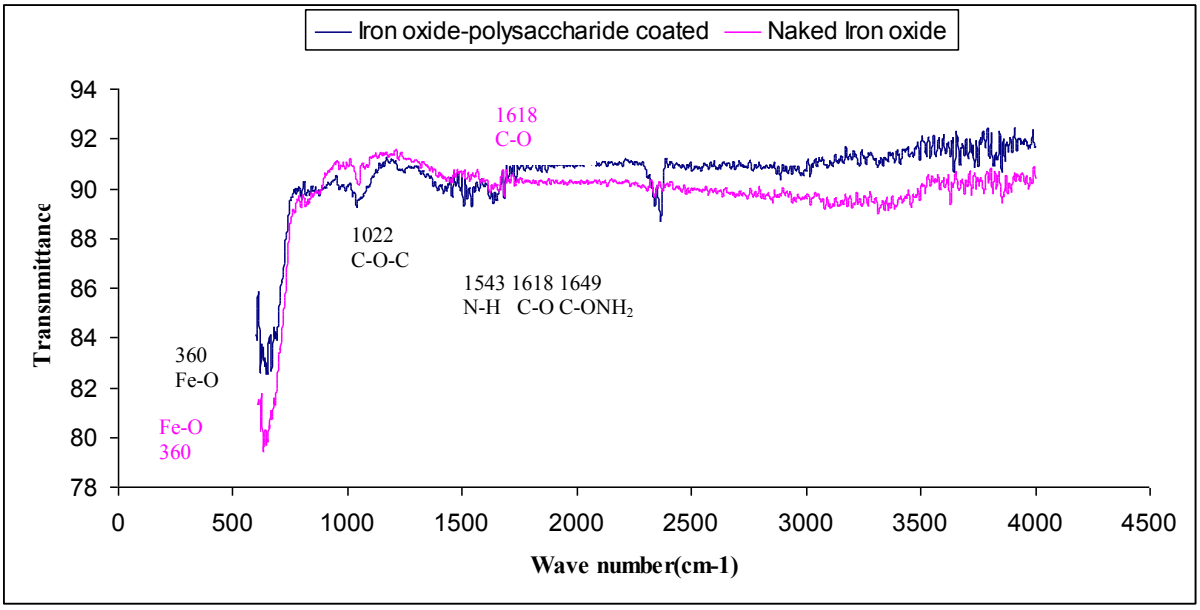

Figure 1. FTIR spectra for, naked iron oxide, and polysacchride-iron oxide particles.

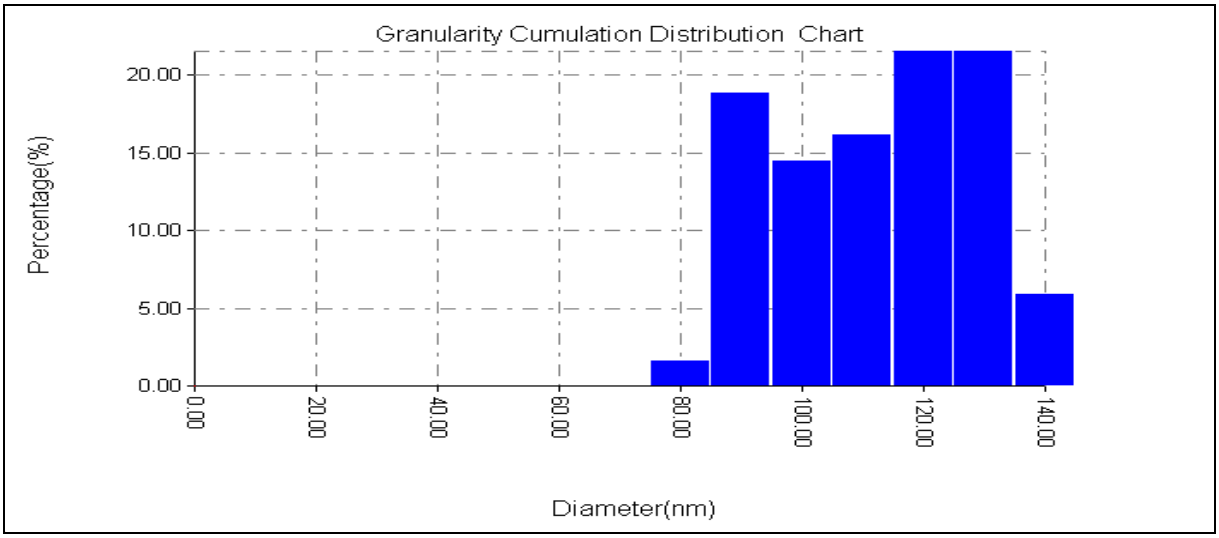

Figure 2. SPM particle size distribution histogram of polysaccharide coated iron oxide nanoparticle

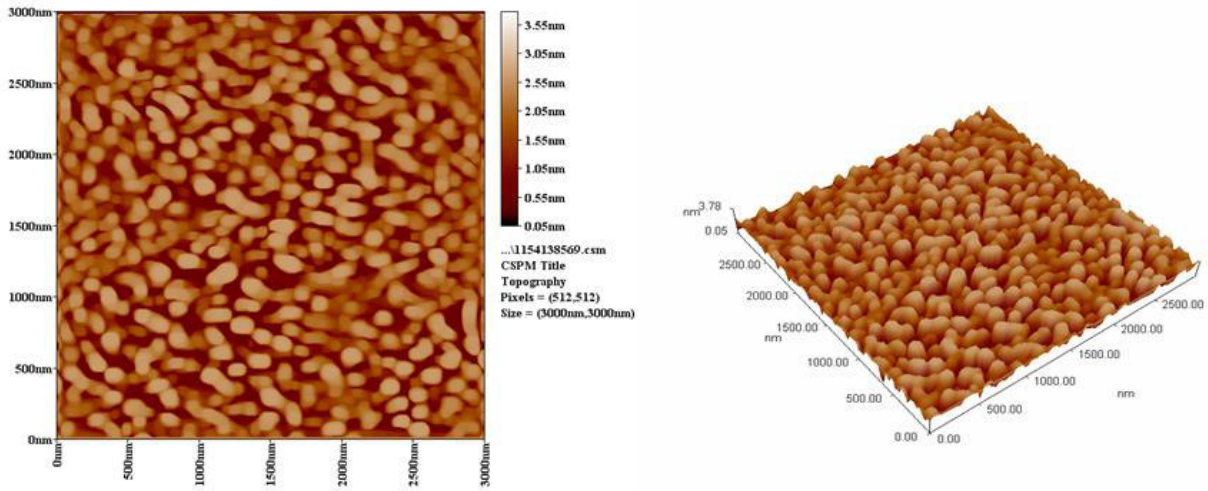

Figure 3. SPM polysaccharide coated iron oxide nanoparticle

Table 1. The effect of *Pseudomonas aeruginosa* polysaccharide coated iron oxide nanoparticle on *P. aeruginosa* biofilm formation on mucin saliva Lewis types.

Polysaccharide Iron oxide nanoparticle mg/ml	% Inhibition (Mean ±SD) Saliva Lewis type							
	Lea^+	p-value	Leb^+	p-value	Lea^-b^-Non secretor	p-value	Lea^-b^- secretor	p-value
100	75±31.7	0.3	31±15.2	0.9	46.3±8.9	0.4	70.3±26	0.3
10	40±29	0.4	36±12.7	0.8	31±6.5	0.3	52.5±17.6	0.4
1	4.3±1.6	0.09	5±2.9	1.4×10^{-3}**	5±1.4	2.6×10^{-3}**	15±6	0.05*
0.1	9±4.3	0.8	44.5±3.8	3.3×10^{-3}**	24±13.9	0.9	36.7±13.7	0.4
0.01	15±12	0.7	46±22.5	0.03*	41±8	0.03*	26.7±25.9	0.4

*$p \leq 0.05$ significant
** $p \leq 0.01$ highly significant
*Augmentation was calculated using the formula :

$$\% \text{ Augmentation} = \frac{\text{Test O.D} - \text{Control O.D}}{\text{Control O.D}} \times 100$$

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