Development and testing of a simple, economical, and reliable lab-scale static model to study bacterial biofilm using *Escherichia coli*

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Abstract:

Bacteria can develop resistance to antibiotics through several mechanisms. In this study, we focus on one of the mechanisms based on biofilm growth. Biofilms are organized communities of micro-organisms buried firmly on extra polymeric matrix attached to the living surface. The matrix which is formed surrounding the bacteria makes them tolerant to harsh environment conditions as well as resistant to antibacterial treatment. The development of biofilms in clinical or hospital setting is the major reason for several infections. They have negative impact when they are formed on medical devices as it is difficult to eradicate it using disinfectants or antibiotics available. Several *in vitro* models have been implemented in the last decade to evaluate antimicrobial activity on bacterial biofilms. There is lack of simple, economical and reliable static biofilm models to study biofilms. In this scenario, considering the importance of this research area, we planned to design laboratory scale working models to study biofilms. We used *Escherichia Coli* as a model organism. E. coli biofilms are made up of a bacterial colony enclosed in an extracellular polymeric substance (EPS) matrix that protects the bacteria from the environment and contributes to infection. The emergence of antibiotic resistance exacerbates the variety of structural components of biofilm, making its eradication more difficult. This new model will contribute to deeper knowledge about the physiology, structure, formation and composition of biofilms. This method can be used as an appropriate and up-to-date technique to study biofilms at laboratory settings.

Keywords: Biofilms, Antibiotic resistance, Infection, *Escherichia coli*, static model

INTRODUCTION:

Bacteria can develop resistance to antibiotics through several mechanisms. In this study, we focus on one of the mechanisms based on biofilm growth. Bacterial biofilms have been linked to several infections, ranging from infections caused by exogenous devices like catheters or prosthetic joints to chronic tissue infections like those found in cystic fibrosis patients' lungs. Multiple tolerance mechanisms in biofilms make them resistant to antibiotic treatment (phenotypic resistance). This contributes to the persistence of biofilm infections despite antibiotic use, which raises the risk of antibiotic resistance (genetic resistance). To maximize antibiotic treatment of biofilm infections, researchers must first understand the interplay between phenotypic and genetic resistance mechanisms acting on biofilms, as well as the variety of environmental factors that affect the effect of antibiotics [1].

A biofilm is a community or group of microorganisms that has become irreversibly bound to a surface and is encased in an Extracellular polymetric Substance (EPS), increasing their resistance to host cellular and chemical responses [2] These pathogens pose an added threat to humans due to their increased tolerance to widely used antibiotics. Biofilm-bound microorganisms are difficult to extract because biofilm is more resistant to biocides than microorganisms that are not bound in biofilm. Surface attachment, structural heterogeneity, genetic diversity, dynamic population interaction, and an extracellular matrix of polymeric material are all characteristics of biofilms. In most cases, a single Celled organism can behave in one of two ways. The first is the well-known free floating or planktonic method, in which single cells float or swim in a liquid medium independently. The second stage is attached, in which cells are tightly packed and strongly attached to one another, forming a solid surface [3].

In the gastrointestinal tract, E. coli is the most common type of facultative anaerobic bacteria. Several main factors, including various extracellular appendages, are implicated in E. coli surface colonization, and their expression and activity are finally regulated, both spatially and temporally, to ensure efficient events leading to matured biofilm formation [3]. cc [4]**.** The National Institutes of

Health (NIH) reported that about 65% of microbial infections are associated with Biofilm formation [4]**.** Biofilm infections are estimated to be responsible for up to 80% of all infections in humans and animals, posing a major health challenge [1]**.** Planktonic bacteria are individual micro-organisms, whereas biofilms are well-connected organizations of millions of such microorganisms. Bacterial biofilms are highly resistant to antibiotics when they are formed inside the matrix of a biofilm [4, 5]. The National Institutes of Health (NIH) reported that about 65% of microbial infections are associated with Biofilm formation [6, 7]**.** At research level, development of biofilm working models will help to mimic real environmental conditions [8, 9]. Considering the importance of this research area, we planned to design a simple, economical, and reliable laboratory scale working model to study biofilms [10]. We used silicon tube catheters for biofilm formation and testing. We also tested the ability of selected antibiotics to prevent the formation of biofilm.

MATERIALS AND METHODS

Materials and reagents

Medical / clinical grade silicone tube urinary catheters were used in this study. Biofilm forming bacterium *Escherichia coli* (ATCC 25922) was used as test organism. Dehydrated media like Nutrient broth, Tryptic soy broth (TSB), Glucose, Brain heart infusion broth, Blood base agar, Mueller Hinton Agar were procured from HI media, India. Crystal violet (0.1%), Congo red (0.8 g/L), and Acridine orange (1%) dyes, all chemicals consumables used were research grade procured from Sigma Aldrich

Experiments to confirm the formation of biofilms, quantification, and structural assessment:

Tube method

Tube method is a qualitative test for detection of biofilm producing micro-organism which forms visible lining surrounding the walls of the tubes [11]. 10mL of Tryptic soy broth (TSB) was served with 1% glucose and the test organism was inoculated and incubated for 37°C for 24 hours and the

tubes were decanted and washed with phosphate buffer saline solution (pH7.2) and stained with 1% Crystal violet for 15-20 minutes and excess stain was removed with deionized water and dried. The test was carried in triplicate. T1-single loop of culture, T2-double loop of culture, T3-triple loop of culture C-control-without any organism. The tubes which showed visible lining around the tubes were recorded [12].

Congo red agar test (CRT)

It is a qualitative test to detect biofilm forming organisms, which produces color change in the colonies when they are inoculated on the Congo red agar (CRA) medium [13, 14]. It is a chromatic evaluation for detection of biofilms as Black colonies are formed by biofilm producing organism and non-biofilm produces reddish pink colored colonies on the CRA plates. The plates were poured with the composed media allowed to solidify and then streaked with concerned organism to check the biofilm producing organism.

Biofilm formation using 96 well plate method

This is a quantitative method which is used for quantitative method for detection of biofilm where the polystyrene plate was inoculated with the bacterial suspension present in Tryptic soy broth and incubated for 48h at 37°C [15]. After incubation the biofilm formation takes place as the wells around the walls and the plates were decanted and gently washed with Phosphate buffer saline and after which the fixation is done using sodium acetate. The plate was stained with 0.1% crystal violet and allowed to stand for 15-20 minutes at room temperature, excess stain was washed with deionized water. The plates were dried and again resolubilized with methanol/glacial acetic acid and read spectrophotometrically using ELISA microplate reader at 570 nm. The TSB with 1% glucose serves as blank which is used to identify whether biofilm is formed or not. The OD value greater than blank are determined as biofilm producer [12]. Plate 1: The culture was loaded as 100µl, 200µl, 300µl of test organisms and Control with no organism & incubated at 37°C; Plate 2: The broths containing organism with different concentration of antibiotics such as 100µg,200µg,300µg,400µg and the broth containing only the organism without adding any antibiotic was loaded and incubated. When, (i) mean

OD value is <0.120, then, no adherence and weak biofilm formation; (ii) mean OD value is 0.120-0.240, then, moderate adherence and biofilm formation; (iii) mean OD value is >0.240, then, strong adherence and high biofilm formation.

Docking of Protein extracted from E.coli with antibiotic ciprofloxacin

The protein 1JW2 (HEMOLYSIN EXPRESSION MODULATING PROTEIN Hha FROM *Escherichia coli*) is an important protein which is related to the regulation of biofilm formation [16]. Hence, docking this protein with antibiotics such as ciprofloxacin will provide an insight into the interaction that occurs between them. Before docking, it is important to measure the flexibility of the residues in the protein, which can computed using B factor. Docking studies was performed for the protein extracted from *E. coli* with ciprofloxacin antibiotic.

RESULTS AND DISCUSSION

The tube method, CRT, 96 well plate method were performed in static model in order to confirm the biofilm formation.

Tube method

Tube method is a qualitative assay used for the detection of biofilm producing organisms because of occurrence of visible biofilm. The crystal violet used here stains the biofilm formed by the organism so that the results can be easily visualized. The third polystyrene test tubes indicate biofilm formation as compared with the first two tubes (Figure 1).

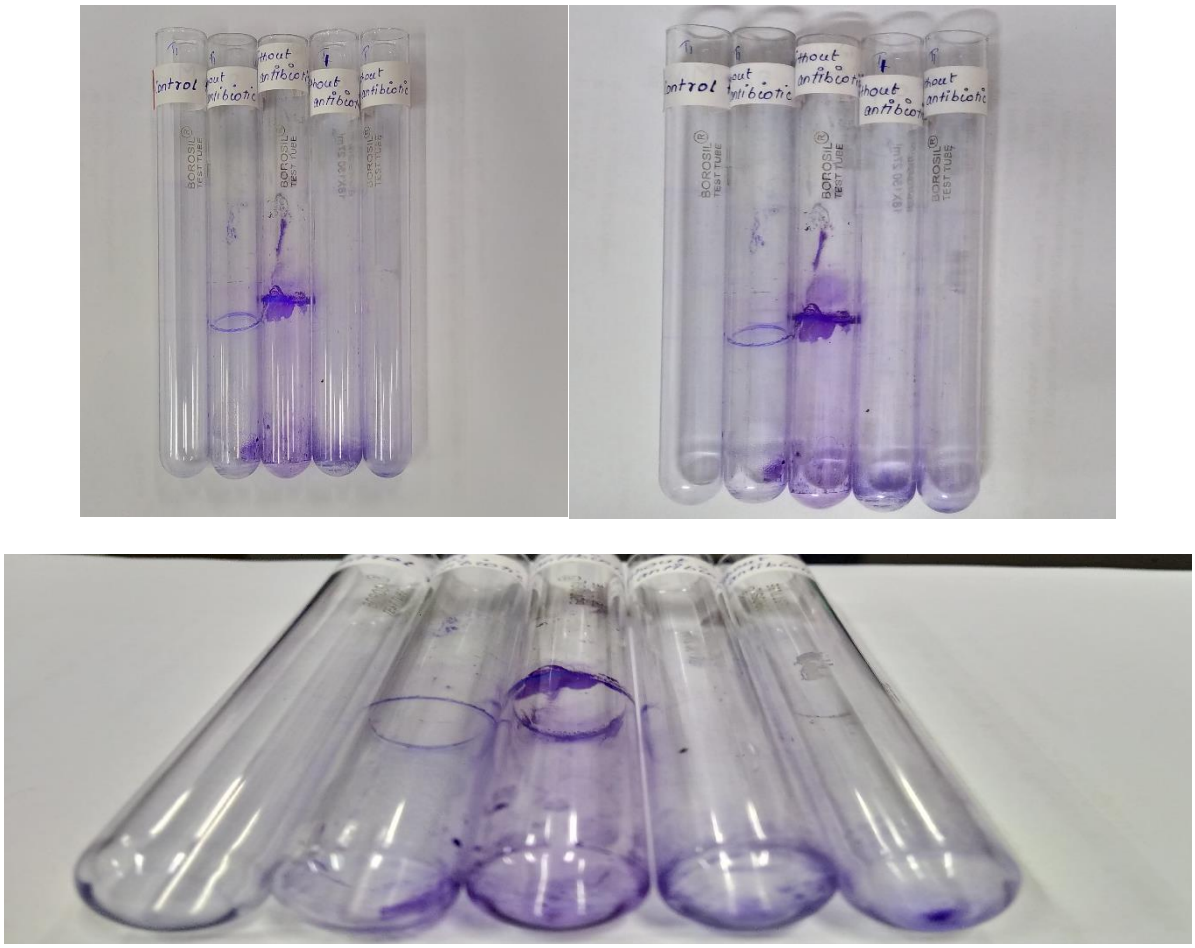


Figure 1: Tube method qualitative assay used for the detection of biofilm producing organisms because of occurrence of visible biofilm. The crystal violet used here stains the biofilm formed by the organism so that the results can be easily visualized.

Congo red agar test (CRT)

The CRA plate test uses a solid medium namely Congo red agar. This method allows for direct analysis of the colonies and the identification of slime-forming strains (which appears as black colonies on the congo red agar) and non-slime forming strains (red-colored colonies). Black colonies denote that the organism has the ability to form biofilms (Figure 2).

Figure 2: Congo red agar test (CRT). Black colonies denote that the organism has the ability to form biofilms

96 well plate method

The 96 well Microtitre plate is a quantitative analysis of biofilm formation. After incubation for 2 days at 37°C the plates were decanted, washed with Phosphate buffer saline and stained for 15-20 minutes using 1% crystal violet, decolorized using acetic acid and read spectrophotometrically at 570 nm (Figure 3).

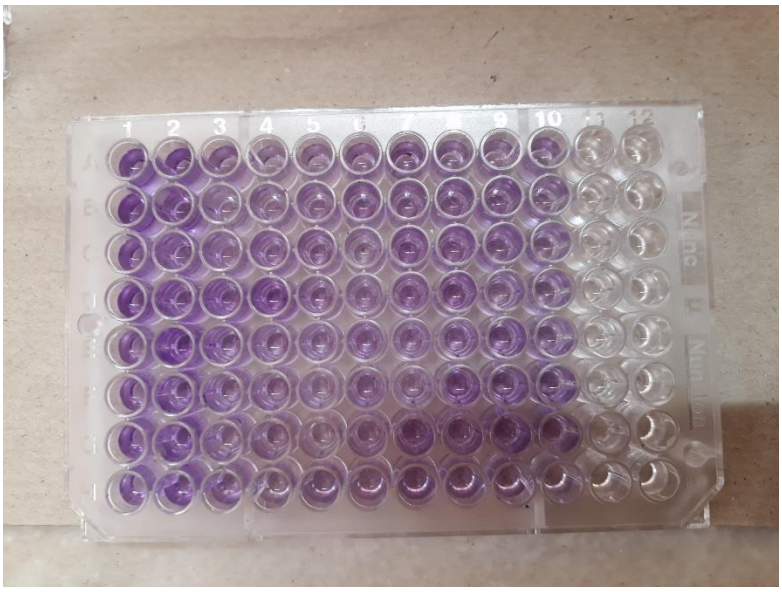


Figure 3: The 96 well Microtitre plate is a quantitative analysis of biofilm formation. 1% crystal violet staining used to confirm the formation of biofilm.

Docking of Protein extracted from E.coli with antibiotic ciprofloxacin

The protein 1JW2 (HEMOLYSIN EXPRESSION MODULATING PROTEIN Hha FROM *Escherichia coli*) has no beta strands which will be displayed in red colour. This Hha protein is a regulator of biofilm formation [16]. Hence, docking this protein with antibiotics will provide an insight into the interaction that occurs between them. Before docking, it is important to measure the flexibility of the residues in the protein, which can computed using B factor. B factor in a protein structure indicates true static or dynamic mobility of an atom in a protein. The blue colour regions in the B factor plot presented in Figure 1 corresponds to Helix and green colour regions relates to turns and random coils.

Low values of B factor indicate well-ordered regions in protein and high values are specific to more flexible residues or protein regions. The B factor and deformity values are well within defined boundary, and hence the residues in the protein are stable that are less prone for mobility. The Eigen value of above 1.0 indicated stability and hence the protein can be used for rigid body docking with the selected antibiotics (Figure 4 & Figure 5).

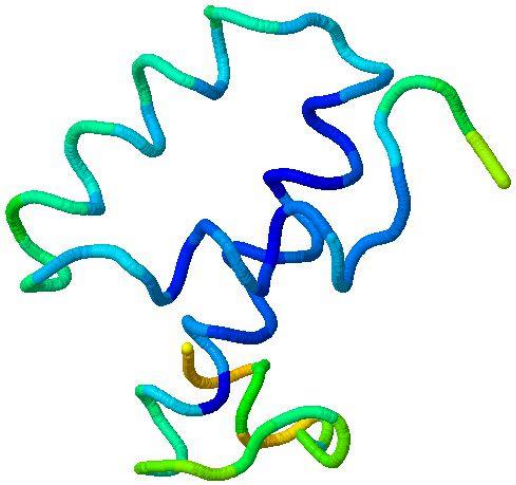
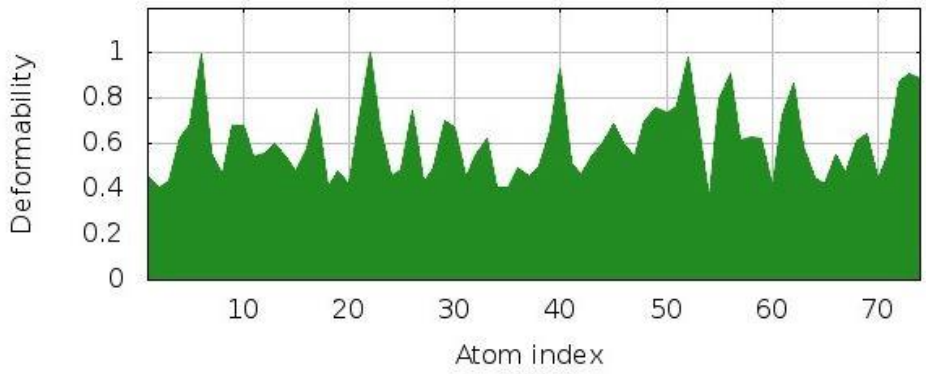


Figure 4: 1JW2 (HEMOLYSIN EXPRESSION MODULATING PROTEIN Hha FROM *Escherichia coli*.

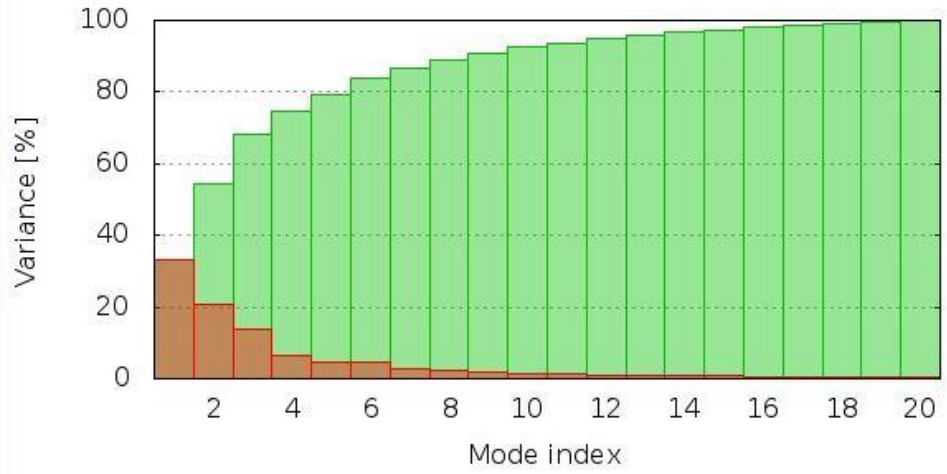
B-factor/Mobility

The main-chain deformability is a measure of the capability of a given molecule to deform at each of its residues. The location of the chain 'hinges' can be derived from high deformability regions.



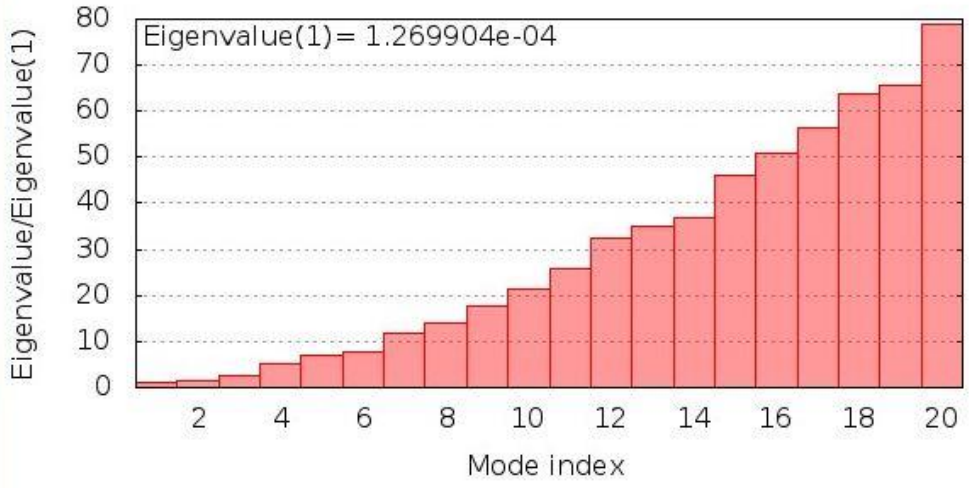
Variance

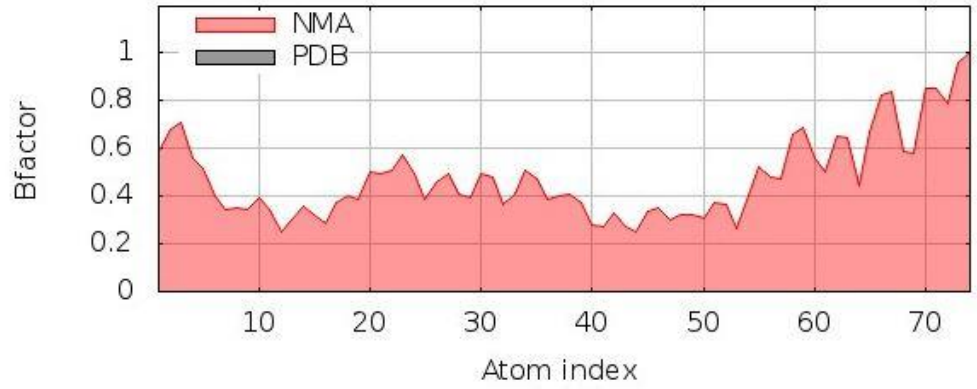
The variance associated to each normal mode is inversely related to the eigenvalue. Colored bars show the individual (red) and cummulative (green) variances.



Eigenvalues

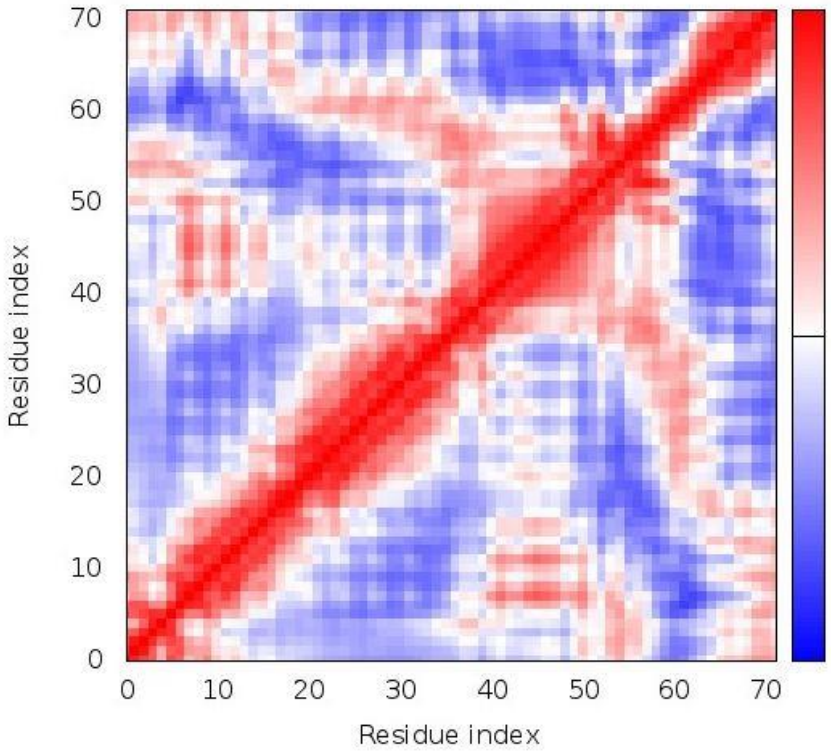
The eigenvalue associated to each normal mode represents the motion stiffness. Its value is directly related to the energy required to deform the structure. The lower the eigenvalue, the easier the deformation.





Covariance map

Covariance matrix indicates coupling between pairs of residues, i.e. whether they experience correlated (red), uncorrelated (white) or anti-correlated (blue) motions.



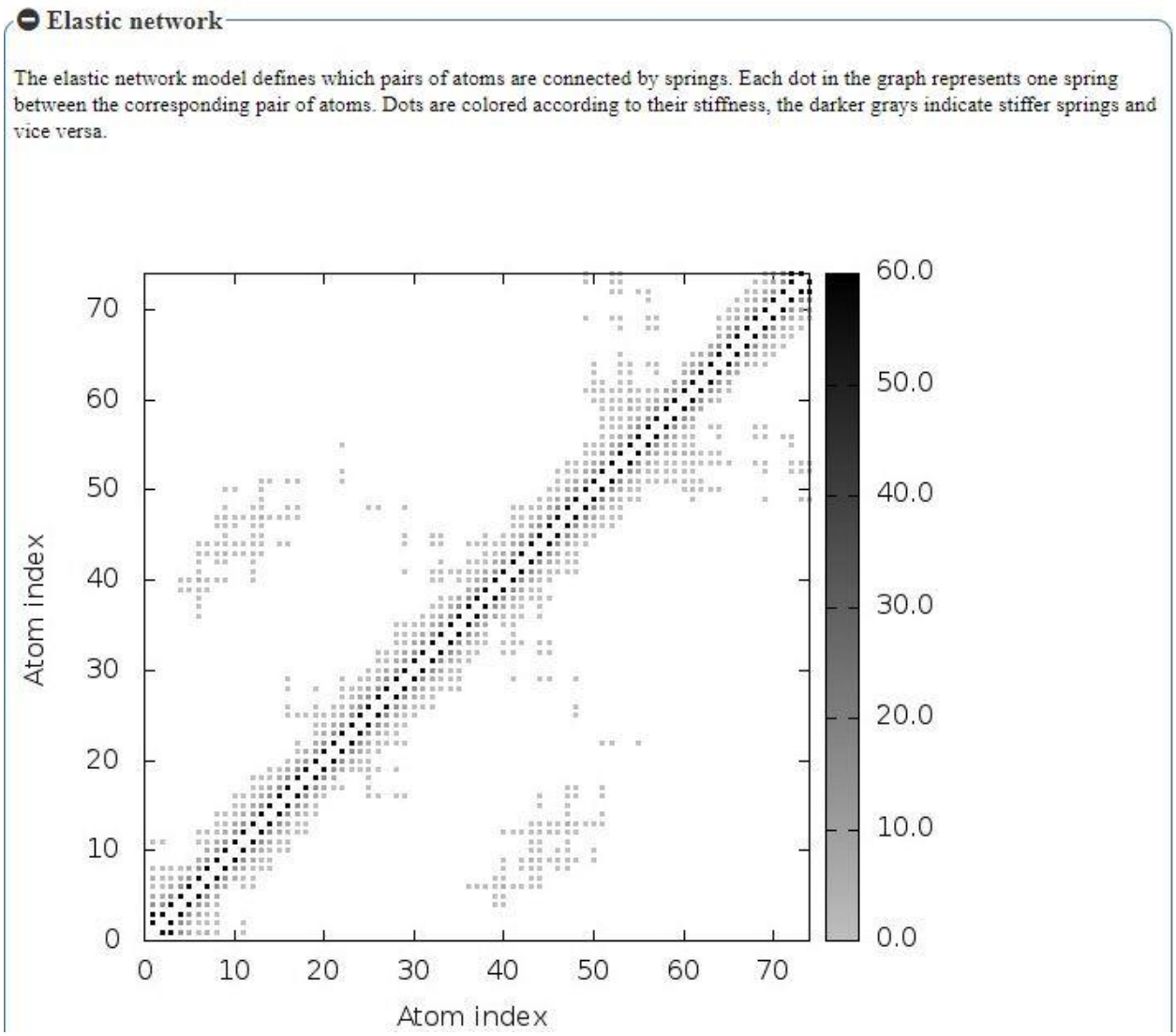


Figure 5: Docking of Protein extracted from E.coli with antibiotic ciprofloxacin

CONCLUSION

Treatment of biofilm related infections using antibiotics is difficult, due to the protective layer build by the cells in the biofilms. Antibiotics have very minimum or limited penetration into biofilm matrix. From the results we obtained, antibiotics can be effective in preventing the formation of biofilms formed by *Escherichia coli* however, the dosage or concentration of antibiotics may vary to treat the samples which are obtained from the hospitals or clinics directly. In this study, we used static model to study biofilm formation by *Escherichia coli* We successfully developed and tested a simple, economical and reliable lab scale static model to study biofilm. Similar biofilm testing models have

been reported in few earlier publications [17], but the present proposed model is much more economical and simpler to design, validate and replicate. Thus, this model will be of more advantage with minimum requirements. Molecular mechanism behind the formation of biofilm can be explored further.

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CONFLICT OF INTEREST

All authors confirm there is NO conflict of Interest.

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