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PREPARATION OF NANOCOMPOSITE SILVER-CHITOSAN-ALGINATE FILM AS ANTIBACTERIAL MATERIAL

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Abstract. Preparation of silver-chitosan-alginate (Ag/Kit/Alg) nanocomposite film as an antibacterial material has been done. Nanocomposite film of Ag/Chit/Alg was carried out through casting method of colloidal nanocomposite Ag/Chit/Alg). Colloidal is made through a chemical reduction method of AgNO₃ precursor salts assisted by sunlight with chitosan as a stabilizer agent and reducing as well and NaOH as an accelerator. Furthermore, alginate solution is added to form Ag/Kit/Alg nanocomposite colloidal. The formation of silver nanoparticles was indicated by the appearance of localized surface plasmon resonance (LSPR) phenomenon which was characterized using a UV-Vis spectrophotometer. The shape and size of silver nanoparticles were characterized using TEM. Characterization of Ag/Kit/Alg nanocomposite films includes swelling, mechanical properties, solubility and water vapor permeability (WVP). Testing of antibacterial activity was carried out on film Ag/Chit/Alg nanocomposite using diffusion method. The results showed that the formation of silver nanoparticles was characterized by the appearance of LSPR phenomenon at the peak of 403.50 - 412.50 nm. Silver nanoparticles are spherical shape with a size dominated at 5-9 nm. The physical and mechanical properties of films are influenced by the concentration of silver nanoparticles. The greater concentration of precursor salt AgNO₃ increase swelling and solubility of Ag/Chit/Alg nanocomposite film. The tensile strength of the film tends to decrease due to the presence of Ag nanoparticles. The nanocomposite film of Ag/Chit/Alg has a rough structure with a higher Water Vapor Permeability value compared to Ag/Chit films. The nanocomposite films of Ag/Kit/Alg have high antibacterial activity against *Escherichia coli* (*E. coli*) ATCC 25922 and *Staphylococcus aureus* (*S. Aureus*) ATCC 25923.

1. Introduction

Chitosan can be used as a potential wound dressing material because it has several superior characteristics such as good biocompatibility, antibacterial activity and chemical stability. Previous studies have shown that chitosan can accelerate wound tissue repair and regulate secretion from inflammatory mediators [1]. Chitosan is a hemostat, which helps in blood clotting naturally. Chitosan is gradually depolymerized to release N-acetyl- α -D-glucosamine, which initiates fibroblast proliferation, helps in giving collagen deposition orders and stimulates increased synthesis of natural hyaluronic acid levels at the wound site thereby accelerating wound healing and scar prevention [2].

In making bioactive materials from chitosan film based wound dressing, there is a problem that the antibacterial activity of chitosan films which is relatively weak even for chitosan films have zero zone



of antibacterial activity [3]. To overcome this problem in this research, chitosan-based nanocomposites are made through silver doping (Ag) nanoparticles. Silver metal nanoparticles have been widely studied as having high antibacterial activity [4]. In this case chitosan film can act as drug delivery of silver nanoparticles for antibacterial wound dressing applications.

The manufacture of silver nanoparticles is widely developed using strong reducing agents, for example sodium borohydride which is toxic [5]. Some environmentally friendly weak reducing agents that have been used as reducing agents are trisodium citrate [6], glucose [7] and chitosan [8]. Chitosan as a reductant in making silver nanoparticles is interesting to study because it can also act as a stabilizer agent. The weakness of chitosan as a reducing agent required high temperature (95 °C) and a long time (12 hours). To accelerate the process of reduction is assisted by irradiation of sunlight containing UV light as a trigger for the reduction of silver ions with chitosan as a stabilizer.

Chitosan can play a role in 3 purposes, namely as a stabilizer and a reducing agent as well [9], and forming film [10]. Alginate was added to increase the swelling capacity of chitosan films. Alginate is the family of linear polysaccharides and is composed of guluronic (G) and manuronic (M) units. Chitosan, which is a positively charged polyelectrolyte and alginate, is a negative polyelectrolyte that can help the intra and inter-polymeric bonds through hydrogen bonds. As a result of this bonding complexes chitosan-alginate can form a gel material [11]. Alginate increases the capacity of chitosan-based film swelling [12, 13] which results in having a strong ability to adsorb fluid (exudate) in wounds. Several studies have shown that the chitosan-alginate complex has been applied as for the design of drug-release systems [14], protein separation [15], anticoagulant coatings [16] and wound healing membranes [17].

In this research, silver nanoparticles were made with chitosan as a reducing agent as well as a stabilizer. Natrium hydroxide was use as accelerator with sunlight exposuras as a reducing process trigger. Alginate is added shortly after the formation of these colloidal silver nanoparticles and forms a new composite of silver-chitosan-alginate (Ag/Kit/Alg). This colloidal system is then converted into film by the method of casting and drying at room temperature. In this study also examined the effect of silver nanoparticle concentration on the physical, mechanical and antibacterial activity of the film.

2. Experimental

2.1. Materials

Chitosan with a molecular weight of 1,0173 kD and a degree of deacetylation (DD) 70.5% was synthesized from shrimp shells by Biotech Surindo, Cirebon, Indonesia. Silver nitrate (AgNO_3), acetic acid (CH_3COOH), alginate, sodium hydroxide (NaOH) were purchased from Merck

2.2. Preparation of silver nanoparticles

The Initial step, chitosan of 1% (w/v) solution is made by dissolving chitosan powder in acetic acid 1% (v/v) with strong stirring for 2 hours. Chitosan solution is stored for 1 night to perfect its solubility. AgNO_3 solution (0.012 g/mL) with a volume variation of 0.25; 0.50; 0.75 and 1.00 mL are added in 12.5 mL of 1% chitosan solution (w/v) and stirred for 5 minutes. Then added 1.75 mL of NaOH 2 mL and stirred for 5 minutes again. The formed gel is then exposed to sunlight for 2 hours. A brown gel appears indicating silver nanoparticles have formed. Then added 47.5 mL of chitosan solution was carried to get colloidal silver nanoparticles. The colloidal formed is called the colloidal nanocomposite Ag/Chit. The formation of silver nanoparticles in nanocomposites was characterized using a UV-Vis Spectrophotometer and TEM.

2.3. Preparation of Ag/Chit/Alg nanocomposite film

Ag/Chit/Alg nanocomposite films were made by casting the colloidal Ag/Chit/Alg nanocomposite film. Colloidal Ag/Chit/Alg is made by adding colloidal Ag/Chit to the 1% alginate solution so that the colloidal concentration of alginate is 15% (w / w). In the mixing, Strong stirring was carried out until homogeneous. Then the colloidal Ag/Chit/Alg is dried at room temperature until a dry film is formed. Then the film is peeled, netralized and characterized.

2.4. Characterization silver nanoparticles

Characterization of the formation of Ag nanoparticles colloidal was carried out with ultraviolet-visible (UV-Vis) Shimadzu UV3150 spectroscopy at wavelengths of 300 - 600 nm with 5 times dilution using distilled water. The study of the size, shape and distribution of Ag nanoparticles was carried out using a Transmission electron microscopy (TEM) with a JEM-1400 EX microscope. The sample was thinned and then placed in a copper grid. Then the sample was observed with TEM at a voltage of 120.0 kV. Scion Image software was used to determine particle size and distribution.

2.5. Characterization of Ag/Chit/Alg nanocomposite film

Characterization of Ag/Chit/Alg nanocomposite film samples including Water Vapor Permeability (WVP), swelling, solubility, surface morphology with Scanning Electron Microscopy (SEM). standard anti-bacterial activity test for *Escherichia coli* (*E. coli*) ATCC 25922 and *Staphylococcus aureus* (*S. Aureus*) ATCC 25923 by disk diffusion method.

Water vapor permeability (WVP) is determined gravimetrically according to the ASTM E 96-95 standard method with some modifications. Film thickness is measured and placed on an acrylic cup containing silica gel desiccant. Every 1 hour weighing up to 8 hours.

The water vapor transmission rate (WVTR) is determined by the equation:

$$WVTR = \frac{\left(\frac{G}{t}\right)}{A} (g/hm^2)$$

Water vapor permeability (WVP) is determined by equation:

$$WVP = \frac{WVTR \times d}{\Delta p} (g \text{ mm}/kPa \text{ h } m^2)$$

with G is change in weight (g); t is time (h), A is film area (m²), d is film thickness (mm) and Δp , change in partial pressure passing through the film (kPa).

Scanning electron microscopy (SEM) is used to test the surface of the film. Samples are placed in a carbon-conductive layer and coated with 60% gold and 40% palladium with a sputter coater at a current of 35 mA for 1 minute). Then the sample is observed with a scanning electron microscope (Zeiss DSM 960) with a voltage acceleration of 5, 7 or 10 kV.

Swelling (Sw) test that showed the absorption capacity of the film were determined by wetting the film at pH 7.4 with phosphate buffer (PBS) at room temperature for 30 minutes. Swelling is determined by:

$$Sw = ((W_{30} - W_0) / W_0) \times 100\%$$

where W_{30} = the weight of the film after 30 minutes of absorption, W_0 is the initial weight of the film. The measuring of swelling was carried out on triplicate.

Film solubility is expressed as a percentage of dry film dissolved after being immersed in water for 24 hours at 25°C. The experiments carried out on slow turbulent water. The sample is then dried with an oven to a fixed weight. Solubility is determined by:

$$\%S = ((W_i - W_f) / W_i) \times 100\%$$

Where W_0 =initial weight, W_f =final weight

2.6. Antibacterial activity test

Bacterial colony is taken using an ose needle. Then measure the turbidity level of bacterial suspension with Mc standard. Farland Smear the MHA with bacterial suspension until it is spread evenly. Incubate for 24 hours at 37 °C. Then cut the film with a diameter of 6 mm and placed on the media. Observe the results and measure the inhibition zone (clear zone) using the calipers.

3. Result and Discussion

The study looked at the effect of AgNO₃ concentrations on LSPR silver nanoparticles. Visually the colloidal color and UV-Vis spectra of Ag/Chit nanocomposites are presented in Figure 1. A gradation of colors from light brown which gradually becomes dark brown shows that the amount of silver nanoparticles produced increases with increasing concentration of AgNO₃ used. Figure 1 also shows that the absorbance increases with increasing concentrations of AgNO₃ which indicates that the more

AgNO_3 was added, the more silver nanoparticles that were formed [18]. According to the LSPR position, at increasing of AgNO_3 concentration as precursor for A1-A3, there was a blue-shifted, then followed by red-shifted on A4. It indicate that using precursor in higher concentration cause

The morphology of silver nanoparticles was characterized using TEM on nanocomposite samples with 1.5% AgNO_3 precursor concentration (w/w). Based on the absorbance data in Figure 1, there is a compatibility with TEM images in Figure 2. The appearance of a single LSPR absorption in the range of 400 nm shows that the particles are spherical [19]. The particle size distribution is dominated at 5-9 nm with an average particle size of 1.19 ± 2.9 nm. TEM images show that the particle size is quite uniform and there are only 2 particles with sizes of 19 nm and 29 nm. So we can say that chitosan as a stabilizer is effective in inhibiting the growth of Ag crystals.

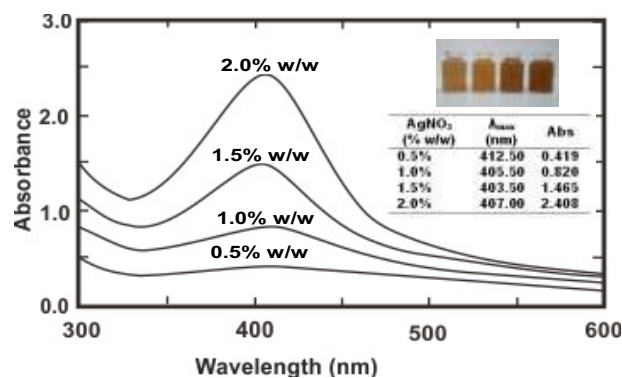


Figure 1. The LSPR Spectra of Colloidal silver nanoparticles in difference of AgNO_3 concentration

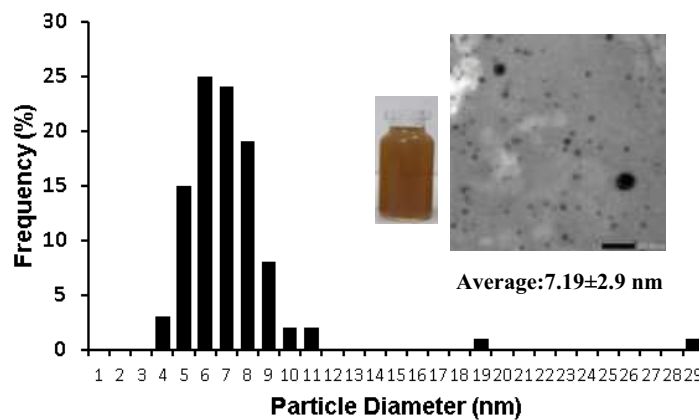


Figure 2. TEM image of silver nanoparticle in colloidal system with precursor AgNO_3 of 1.5% (w/w)

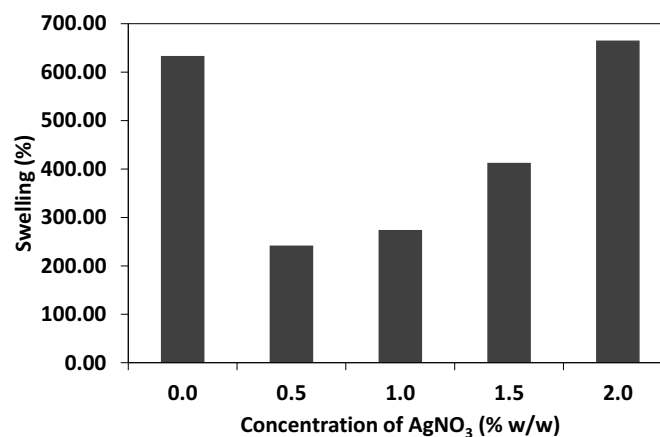


Figure 3. Swelling of Ag/Chit/Alg films at difference of AgNO_3 precursor concentration

Swelling of Ag/Chit/Alg nanocomposite films was observed in a buffer solution for 30 minutes. The swelling test results are shown in Figure 3. Swelling films experience a drastic decrease when silver nanoparticles are added to the film. This decrease in swelling is due to the process of making nanoparticles which involves the use of NaOH as an accelerator. An increase in the amount of silver nanoparticles causes an increase in swelling. This might be due to silver nanoparticles contributing in the binding of water molecules.

The amount of silver nanoparticles in the Ag/Chit/Alg nanocomposite influences the solubility of the film. Without silver nanoparticles, Chit/Alg films can be dissolved in aquademineral for 24 hours to reach 50%. The solubility of this film is due to not all chitosan will coarse with alginate. The acidity of the film causes the remaining chitosan in the film to dissolve in water. Increasing the amount of silver nanoparticles in the use of AgNO_3 precursors to 1% (w / w) decreases solubility, but when the amount of silver nanoparticles increases the solubility of the film increases. This decrease in solubility is probably caused by the process of forming silver nanoparticles which involves the use of NaOH as an accelerator which causes an increase in pH from the film. While the increase in solubility is caused by the presence of silver nanoparticles which causes bonds between chitosan and alginate to get weaker

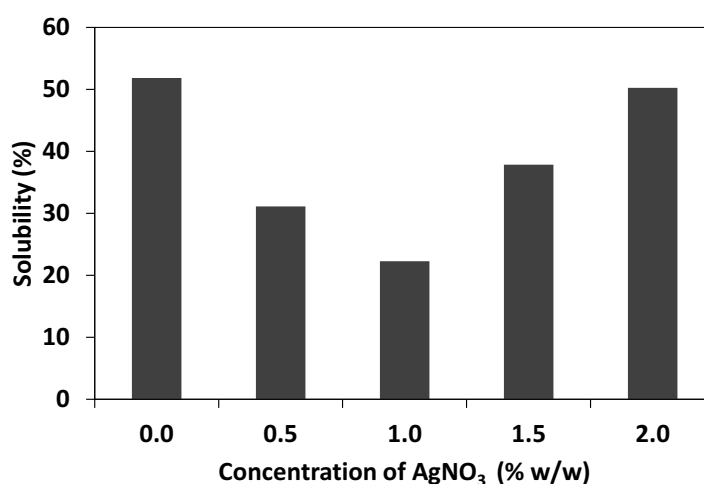
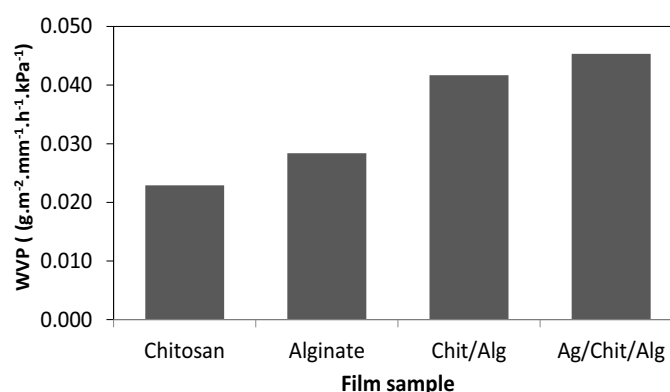
Figure 4. Solubility of of Ag/Chit/Alg film at difference of AgNO_3 precursor concentration

Figure 5. Vater vapour permeability in the various films

Water vapor permeability (WVP) was observed for the chitosan, alginate, Chit/Alg and Ag/Chit/Alg films shown in Figure 5. The WVP film values were increased from chitosan, alginate, Kit/Alg and Ag/Chit/Alg films. This shows that chitosan films have the lowest WVP or are the most resistant to water vapor penetration. When chitosan is added with alginate the Chit/Alg film has a

higher WVP. This is due to the formation of more hollow structures when added due to the formation of bonds between polyelectrolytes. The addition of silver nanoparticles to Chit/Alg films caused an increase in WVP value. This means that the nanoparticles can make water vapor easier to penetrate the film.

The mechanical properties of the films were observed based on the tensile strength and elongation as shown in Figure 6. Chitosan and alginate films have a large tensile strength but low elongation values. Conversely Chit/Alg and Ag/Chit/Alg films have low tensile strength values with high elongation. The formation of bonds between polyelectrolytes causes a decreased tensile strength but the film becomes more stretched. The addition of silver nanoparticles also causes a decrease in the tensile strength of the film. This means that the incorporation of nanoparticles into the chitosan film weakens the bond between the polymers.

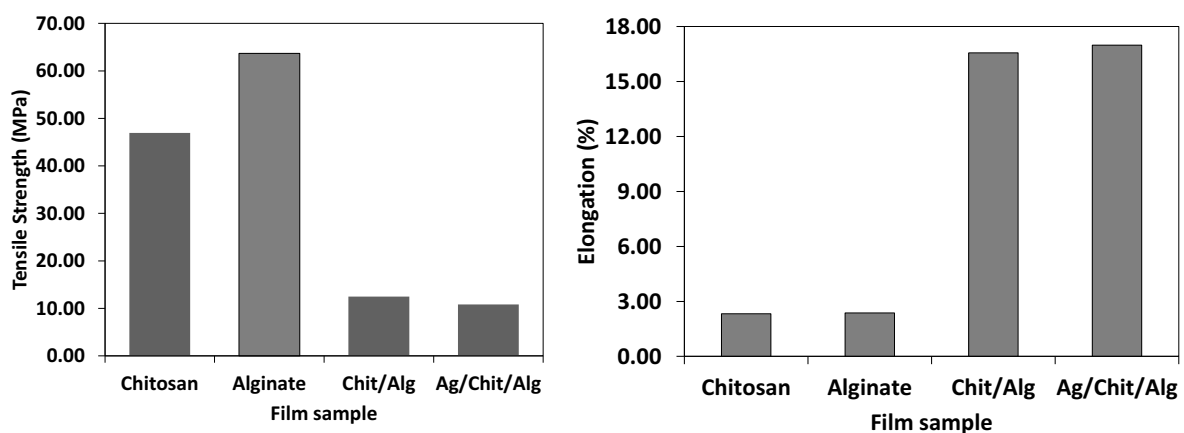


Figure 6. (a) Tensile strength and (b) Elongation in the various films

The antibacterial test results of the bacteria against *E. Coli* and *S. Aureus* can be seen in Figure 7. From Figure 7, it can be seen that the chitosan and alginate films do not have any inhibitory effect on all bacteria tested. The addition of silver nanoparticles to chitosan films or chitosan-alginate films provides good inhibition. An increase in the amount of silver nanoparticles slightly increases the inhibition. The negative chitosan film antibacterial activity test results showed that the antibacterial activity of the nanocomposite film came solely from silver nanoparticles. The more silver nanoparticles in the film show the greater the inhibition.

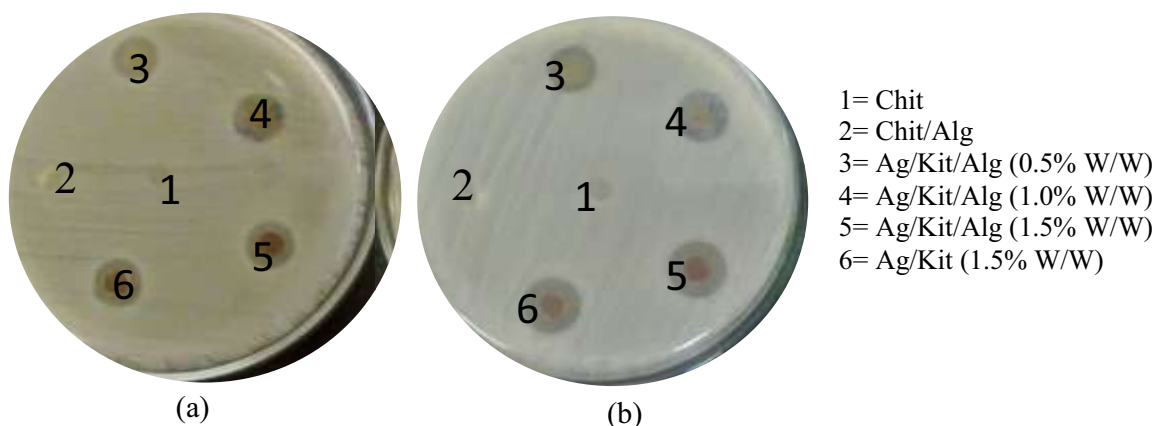


Figure 7. Inhibition zone of film toward (a) *E.coli* ATCC 25922, (b) *S.aureus* ATCC 25923

Silver nanoparticles contained in nanocomposite films can act as a good anti-bacterial material. The nanoparticles have inhibitory zone more than 10 mm either for *E. coli* or *S. aureus* bacterial. There are three possible mechanisms for inhibiting silver nanoparticles, namely (1) Ag^+ ion absorption followed by inhibition of ATP production and DNA replication, (2) Ag nanoparticles and Ag^+ ions form reactive oxygen species (ROS) and (3) Ag nanoparticles directly damage cell membranes [20]. Based on the results of testing with this diffusion method, the mechanism in inhibiting bacterial growth that is most likely for silver nanoparticles in nanocomposite films is the occurrence of silver ion dissolution. This silver ion will diffuse in the bacterial media and enter the bacterial cell which results in the bacteria dying. So that the more silver nanoparticles in the nanocomposite film, the more silver ions are penetrated in bacterial cell which results in wider inhibition zones.

Table 1. Antibacterial activity in terms of inhibition zones

No	Film sample	Inhibition diameter zone (mm)	
		<i>E.Coli</i> ATCC 25922	<i>S.aureus</i> ATCC 25923
1	Chit	0	0
2	Chit/Alg	0	0
3	Ag/Chit/Alg (0.5% w/w)	10.31	11.30
4	Ag/Chit/Alg (1.0% w/w)	11.36	12.72
5	Ag/Chit/Alg (1.5% w/w)	10.85	12.57
6	Ag/Chit (1.5% w/w)	10.74	11.90

4. Conclusion

The results showed that the formation of silver nanoparticles was characterized by the appearance of LSPR phenomenon at the peak of 403.50 - 412.50 nm. Silver nanoparticles are spherical shape with a size dominated at 5-9 nm. The physical and mechanical properties of films are influenced by the concentration of silver nanoparticles. The greater concentration of precursor salt AgNO_3 increase swelling and solubility of Ag/Chit/Alg nanocomposite film. The tensile strength of the film tends to decrease due to the presence of Ag nanoparticles. The nanocomposite film of Ag/Chit/Alg has a rough structure with a higher WVP value compared to Ag/Chit films. The nanocomposite films of Ag/Chit/Alg have high antibacterial activity against *S. aureus* ATCC 25922 and *E. coli* bacteria ATCC 25923.

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