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Biodegradation of Poly (aspartic acid-citric acid) Copolymers by Natural Bacteria from the Receiving Water System

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ABSTRACT

Poly (aspartic acid-citric acid) copolymer (PAC) is a novel high efficient water-treatment agent, and whether it is biodegradable determines its application prospect in actual circulating cooling system. In this paper, the biodegradability of PAC was researched by natural bacterial from the receiving water of domestic sewage in circulating cooling system, and investigated by carbon dioxide evolution tests. The copolymer (PAC) was characterized by FTIR, ¹H NMR and ¹³C NMR. The results showed that the degradation ratio of PAC on 10 days and 28 days respectively reached 28.05% and 79.73%, which indicated that PAC belonged to the biodegradable water treatment agent. With the increase in the addition ratio of citric acid in copolymerization process and the decrease in the molecular weight of copolymer, the biodegradability of PAC was significantly enhanced. Cu²⁺ could decrease the degradation rate through inhibiting enzymatic activities. In addition, compared with other commercial scale inhibitors, biodegradable property of PAC was excellent.

INTRODUCTION

Basing on the concept of "green chemistry", the current trend for inhibitors use is towards more environmental-friendly agents that are undoubtedly the trend of development^[1]. Long-term accumulation of water-treatment agents in the environmental medium will lead to uncontrollable environmental pollution. It is an important process for reducing the exposure concentration of these chemicals in the environment and preventing their dispersion and accumulating in specific environmental compartments^[2]. A biodegradable substance is expected to cause less ecological problems in the long term than a persistent one^[3-4]. Therefore, biodegradable performance has become a new important parameter for the evaluation of water treatment agents.

Currently available "green" water treatment agents mainly include polyaspartic acid (PASP), polyepoxysuccinic acid, and natural polymers. Poly-aspartic acid (PASP) is a promising biopolymer. As the water treatment agent, PASP showed a superior performance in inhibiting the scale, especially for CaCO₃ and CaSO₄, but its scale inhibition rate for Ca₃(PO₄)₂ and corrosion inhibition efficiency were not outstanding, thus limiting its large-scale commercial applications^[4-6]. Modified polyaspartic acid was used as the substitute of PASP.

The biodegradable performance evaluation methods of chemicals are divided into two types: aerobic and anaerobic methods. The former includes aerobic rate method, matrix removal method, microbial index method, and carbon dioxide evolution test. The latter includes gas production and volatile acid assays, volatile suspended solid assay, and radio labeled compound assay^[7-8]. The Organization for Economic Cooperation and Development (OECD) is leading international efforts to standardize biodegradation test methods. The well-established OECD carbon dioxide (CO₂) method^[9] based on Sturm's original test^[10] is usually

used for assessing biodegradability of more or less soluble organic chemicals. Evolution of carbon dioxide is considered by the OECD standard to be the only unequivocal proof of microbial activity.

In the article, Poly (aspartic acid-citric acid) copolymer (PAC) was synthesized by the two kinds of monomers (aspartic acid and citric acid). PAC was characterized by Infrared spectroscopy (IR) and Nuclear Magnetic Resonance spectroscopy (^1H NMR and ^{13}C NMR). Its biodegradability was investigated by the carbon dioxide gas method under different conditions (citric acid content, molecular weight, Cu^{2+} content and commercial scale inhibitors).

EXPERIMENTAL

Materials and equipment

Poly(aspartic acid-citric acid) copolymer (PAC) was made in the laboratory. Amino trimethylene phosphonic acid (ATMP) is industrial product from Shandong leonardo chemical Co., Ltd. Polyaspartic acid (PASP) was purchased from Shanghai source biological technology Co., Ltd. Other reagents used in the experiment were supplied by Tianjin Fine Chemical Co., Ltd.

Glass instrument was soaked in lotions (50 g $\text{K}_2\text{Cr}_2\text{O}_7$ and 1000 mL H_2SO_4) for 30 min, washed with tap water, soaked in lotions for another 60 min, washed with distilled water, and dried at 105°C to remove all the organic or toxic substances.

Characterization of PAC

FTIR: A small amount of purified sample PAC was by vacuum drying at 60°C to constant weight, which were mixed with KBr and pressed onto disk. FTIR spectra in the range $400\text{-}4000\text{ cm}^{-1}$ of the copolymers were recorded by a Perkin Elmer Spectrum 100 spectrometer made in USA.

^1H NMR and ^{13}C NMR: ^{13}C NMR and ^1H NMR spectra of PAC were measured with a Bruker Advance AV 500 MHz NMR spectrometer made in Switzerland. 5 mg sample and 1 mL D_2O was used for the measurement.

Methods for biodegradability of PAC

The test device: As shown in **Figure 1**, the test device is composed of a gas supply system (1, 2, 3, 9, 10), and a system of detecting gas (4), and a CO_2 capture system (5, 6, 7, 8). The air without CO_2 was supplied by the gas supply system. The CO_2 capture system absorbed CO_2 produced from the detected mixed liquid in three levels. The water seal was provided to prevent outside air into 7, which could affect experiment results at the end of the test device.

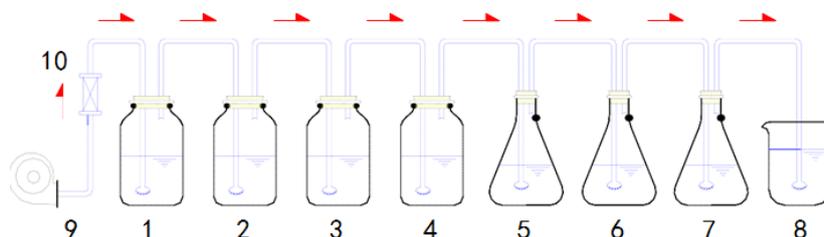


Figure 1. Schematic diagram of test device

Preparation of the detected mixed solution

Solution A was prepared with 8.5 g KH_2PO_4 , 21 mL 75 g K_2HPO_4 , 33.4 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, and 0.5 g NH_4Cl in a 1000 mL volumetric flask. Solution B was prepared with 22.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in a 1000 mL volumetric flask. Solution C was prepared with 36.4 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in a 1000 mL volumetric flask. Solution D was prepared with 0.25 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and a drop of HCl in a 1000 mL volumetric flask.

The detected mixed solution was prepared with 800 mL H_2O , 10 mL Solution A, 1 mL Solution B, C, and D, and 40 mg tested carbon source substance (Conversion PAC: 111.4827 mg; PASP: 95.8 mg; glucose: 100 mg; phenylamine: 51.7 mg) in a 1000 mL volumetric flask, and was adjusted to about 7.4.

Preparation of the inoculum solution

Before the test, the impurities in raw water (containing bacterial) were removed by using coarse filters. Filtered water was cultivated on the cultivation plate in a light incubator for 24 h according to the plate counting method. Finally, the amount of bacterial was estimated in streak plate method to determine the dilution ratio of inoculation. The experimental result was 10^2 (the amount of bacteria in the bacterial solution was $10^4\text{-}10^5$ CFU/mL, $\text{TOC} < 4\text{ mg/L}$).

Carbon dioxide evolution test

In this paper, biodegradability of PAC was investigated by carbon dioxide evolution tests. 1 mL bacterial solution was added into the detected mixed solution (the 4th jar in **Figure 1**). The air flow without CO_2 was pumped into the detected mixed solution at a flow rate of about 80-120 mL/min. One test cycle was 28 days and the testing temperature was maintained at $23 \pm 1^\circ\text{C}$. The experiment was carried out in the dark. When experiment began, the content of CO_2 generated for 5th sample was

determined according to the titration every other day. At the same time, 6th and 7th samples were moved forward as 5th and 6th samples (**Figure 1**). Then water sealing was added at the third level. Containers were replaced until the test was completed [11].

In the blank control group, 1 mL 10 g/L HgCl₂ was added into the detected mixed solution to inhibit microbial activity. After 15 days, the same dose of HgCl₂ was added again.

The results were expressed by the content of CO₂ generated. CO₂ generated from the detected mixed solution was absorbed by Ba(OH)₂ solution in acid-base titration. Phenolphthale acted as the indicator, and the residual Ba(OH)₂ was titrated with 0.05 M HCL until the end titration point of the pink disappeared. The degradation percentages (DM%) was calculated according to Eq. (1):

RESULTS AND DISCUSSION

Characterization of PAC

FTIR analysis: As shown in **Figure 2**, Fourier Transform Infrared Spectroscopy (FTIR) was used to characterize the functional groups that were responsible for the biodegradability of copolymer. It is seen from the curve that the characteristic absorption peaks of N-H bond and C=O bond stretching vibration appear at the wave numbers of 3392.55 cm⁻¹ and 1604.66 cm⁻¹, respectively. The reason is the stretching vibration absorption peaks of -C=O at around 1850-1600 cm⁻¹. The bond connected with C, O and N absorption peaks of C=O is 1715 cm⁻¹, 1735 cm⁻¹ and 1680 cm⁻¹, respectively. Moreover, because of the conjugate effect of C=O and nitrogen atom, the electron clouds of C=O bond moved to C-N single bond, resulting in a decrease of its electron cloud density and a reduction in the force constant. Finally, the absorption peak of C=O was 1650 cm⁻¹. The peaks at around 911.75 cm⁻¹ and 1298.22 cm⁻¹ represent C-O of carboxyl stretching vibration and -OH out of plane deformation vibration respectively. The band at 1074.28 cm⁻¹ in **Figure 2** was caused by the bending vibration of -CH₂- and -CH-. The absorption peaks at 1635.52 cm⁻¹ and 526.53 cm⁻¹ indicated the β structure of aspartic acid monomer polymerization. These peaks indicated that the copolymerization of aspartic acid and citric acid did occur. The FTIR analysis results showed that the function groups of copolymer had similar characteristics to those of PASP synthesized by thermal polymerization of aspartic acid [12-14], confirming that the copolymer used in this study was PAC.

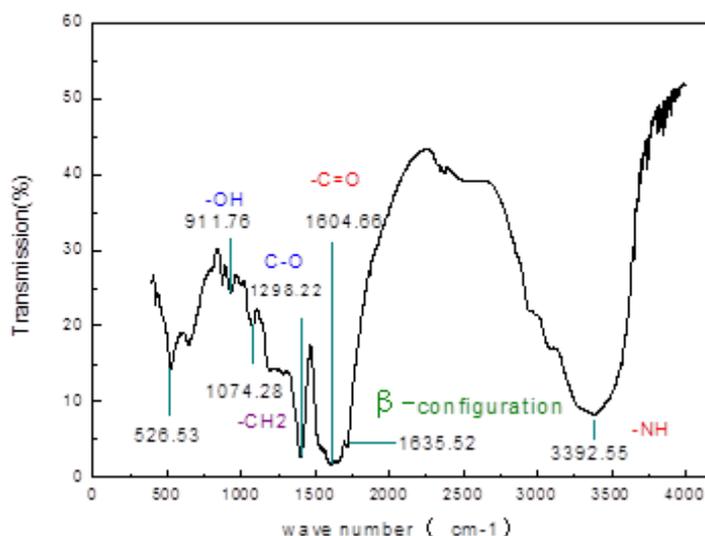


Figure 2. FTIR spectra of the copolymer PAC.

¹H NMR and ¹³C NMR analysis

Figure 3 shows the ¹³C spectra of PAC copolymer. In contrast with related ¹³C NMR spectrum chemical shift values, chemical shifts at 173.98 ppm, 172.46 ppm and 71.5 ppm appear carbonyl of acylamino, carbonyl and carbon of citric acid linked to amide groups respectively. The -CH₂-NH- (50.41 ppm), -CH₂- of citric acid (34.41 ppm) and -CH₂- of aspartic acid (18.27 ppm) carbons were observed from ¹³C NMR spectrum of PAC, which confirmed that the copolymer used in this study was PAC.

¹H NMR spectra of PAC is shown in **Figure 4**. The proton resonance peaks of -NH (-CO-NH), the methene (-CH₂) of citric acid and the methene (-CH₂) of aspartic acid were seen at the chemical shifts from 1.45-1.58 ppm, 2.06-2.13 ppm and 4.46-4.78 ppm, respectively. Shielding area appeared up and down direction of double bond. The chemical shift of C=O was at 2.98-3.03 ppm in high magnetic field. 2.06-2.13 ppm and 4.46-4.78 ppm in the formant indicated that PAC existed isomer, which further indicated the structure of copolymer.

Biodegradability of poly (aspartic acid-citric acid) copolymers (PAC)

As shown in **Figure 5**, it is seen that the degradation percentages of PAC (Mw: 16242) in 10 days and 28 days were 28.05% and 79.73%, respectively. The degradation percentages of glucose were 38.91% and 98.99% respectively and the degradation percentages of aniline were 29.12% and 60.15%, respectively. According to the evaluation standards on biodegradability of

water soluble matters of OECD301B, PAC belongs to the easy biodegradable water treatment agent. The reason for different biodegradation percentages is caused by the chemical structure difference. In aerobic conditions, glucose can be directly degraded and completely oxidized to CO_2 and H_2O with molecular oxygen through chemical reactions. However, Aniline, containing benzene ring, was oxidized catechol firstly, at the same time release of NH_4^+ . The catechol degradation could be open loop by two ways, namely catabolic pathways of meta position and ortho position. Catalysis is through catechol 1, 2 dioxygenase and catechol 2, 3 dioxygenase, respectively. By the ortho position catabolic pathways, catechol was cut in between its two hydroxyl, and then succinic acid and acetyl coenzyme (the middle metabolites of tricarboxylic acid cycle) were produced through multi-step reactions. By the meta position catabolic pathway, catechol was cut in one side of the hydroxyl, which generated pyruvic acid and acetaldehyde [15-17]. The PAC does not have benzene ring but its structure is more complex than that of glucose. PAC has a structure similar to the amide bond of proteins and its degradation is a multistep process. Firstly, it requires microbial extracellular enzymes to decompose PAC into short peptides, which will be then decomposed into small molecules of amino acids. Finally it will produce CO_2 and N_2 through deamination and decarboxylation. Therefore, biodegradability of PAC is better than that of aniline but is slightly worse than that of glucose.

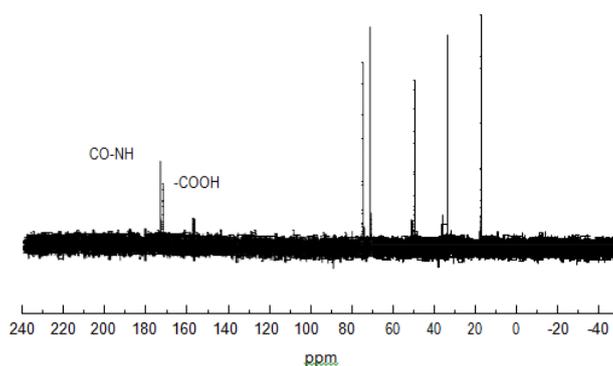


Figure 3. ^{13}C NMR spectra of PAC.

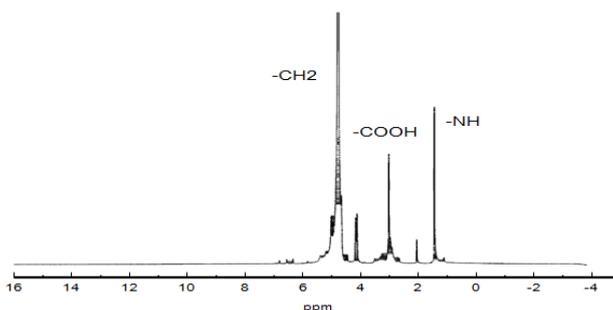


Figure 4. ^1H NMR spectra of PAC.

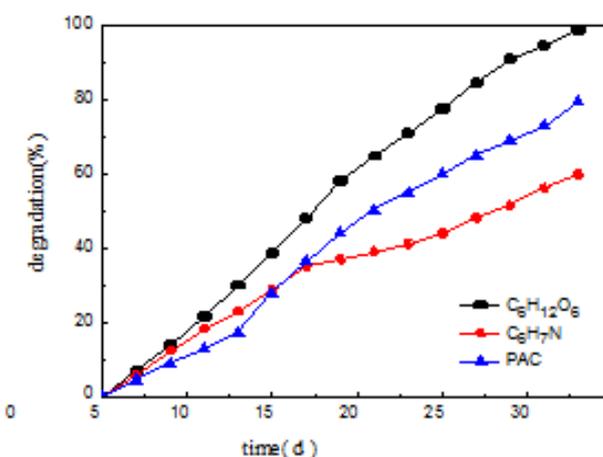


Figure 5. Biodegradability of PAC.

Influence of the content of citric acid on the biodegradability of PAC

Figure 6 shows the influence of the content of citric acid on the biodegradability of PAC. The biodegradability of PAC was significantly enhanced with the increase in the ratio of citric acid in the copolymer, because properties, position and number of the functional group in the molecular structure of PAC could influence the microbial biodegradability [18-19]. Citric acid belongs to polycarboxylic acid and it is a safe, non-toxic and biodegradable common raw material. Its molecule contains a hydroxy group (-OH) and three carboxyl groups (-COOH), a hydrophilic group, which can interact with synthetic polymer, and chelate with a variety

of metal ions, and carboxyl group can form hydrogen bonds with water [20]. Therefore, the increase in the ratio of citric acid in the copolymer could enhance the hydrophilicity of the polymer and provided a comfortable living environment for microorganisms and more active sites for degradation enzymes. With the increase of citric acid content in the copolymer, the number of carboxyl group increased. The copolymer became more susceptible to microbial decomposition and offered the nutrition for microorganisms. Thus it will be more conducive to the growth of microorganisms and the microbial utilization of exogenous substances. The degradation percentage was enhanced.

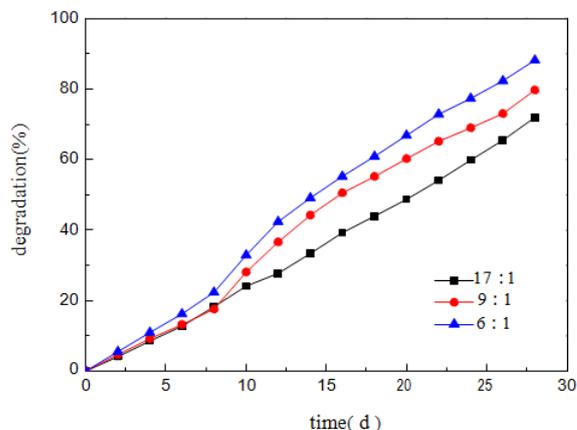


Figure 6. Influence of the content of citric acid on the biodegradability of PAC.

Influence of the copolymer molecular weight on the biodegradability of PAC

As shown in Figure 7, the degradation percentage of the copolymer, varied with molecular weight of PAC (Mw: 16242, Mw: 16958, and Mw: 26764), were 28.05%, 17.7%, 15% on 10 days and 79.73%, 51.75%, 36.15% on 28 days, respectively. It can be observed that with molecular weight of copolymers increasing, the biodegradability of the copolymer was weakened and the incubation period was prolonged. The relatively poor biodegradation performance of high molecular weight polymers may be attributed to the diffusion of microorganisms and enzyme molecular into the internal of copolymer which hinders the attack of enzyme or bacteria to the most sensitive reaction. In addition, according to the specified evaluation standard of water soluble biodegradability in OECD301B, the theoretical degradation rates of easily biodegradable water treatment agent for 10 days and 28 days were 10% and 60%. According to this standard, copolymers with molecular weight of 16958 and 26764 cannot meet the degradation rate standard of easy-biodegrading water treatment agent. It may be attributed to the fact that there is an optimum molecular weight range for the copolymer to exhibit its inhibition performance effectively. Once the molecular weight exceeds a certain range, the inhibition capacity of PAC would be weakened significantly. Hence, on the condition of similar scale inhibition performance, lower molecular-weight products should be used as water- treatment agent by considering the environmental compatibility.

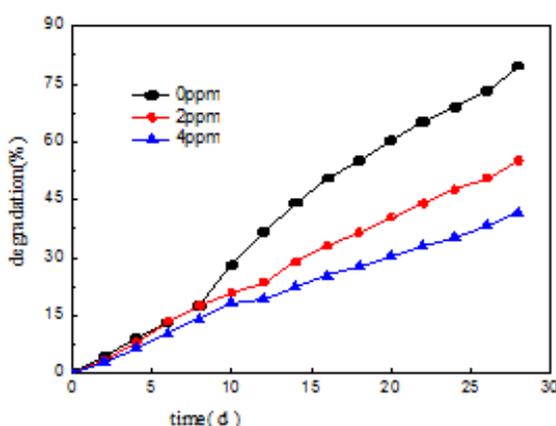


Figure 7. Influence of the copolymer molecular weight on the biodegradability of PAC.

Influence of Cu²⁺ on the biodegradability of PAC

Figure 8 shows that with the gradual increase of Cu²⁺ concentration, the degradation rate of PAC was declining. The reason may be that biodegradation is an enzyme catalytic reaction and Cu²⁺ binding with -SH base of enzyme could make enzyme lose its activity, thus affecting the bond between the substrate and enzyme active center, and the chemical reaction capacity [21]. In addition, Cu²⁺ positively charged ion, which is inclined to bind to the negatively charged bacteria protein, enhances its sterilization performance [22]. Hence, increasing the concentration of Cu²⁺ can kill partial bacteria and thus the degradation rate will decrease.

The comparison of biodegradability among PAC, ATMP and PASP

From Figure 9, it is observed that the degradation percentages of PAC, PASP and ATMP in 10 days and 28 days were

28.05%, 38.31%, 38.09% and 79.73%, 93.56%, 79.32%, respectively. According to the evaluation standards on biodegradability of water soluble matters of OECD301B, PAC, PASP and ATMP belong to easily biodegraded water treatment agents; however, the biodegradability of the three agents varied significantly. The reason may be assigned to the chemical structure difference between PAC, PASP and ATMP. Just structure of copolymer weakens the cohesive strength between bacteria and active sites for biodegradability of exogenous substances, thus lowering the activity of microbial degradation enzyme and subsequently affecting their biodegradable properties. ATMP molecular contains three phosphonic acid groups with negative charges, and thus repels the negatively charged bacteria protein molecule, which results in an average degradation performance. PASP is a kind of polyamino acid with two different isomers of alpha and beta, which are shown in **Figure 10**. In addition, there were both -COOH and C-N bond in the structure of PASP and PAC, but it may be ascribed to the fact that the -COOH group of PAC structure was connected with a long carbon chain substituents, which had negative effect on biodegradability. Finally, the biodegradability of PASP was a little higher than PAC.

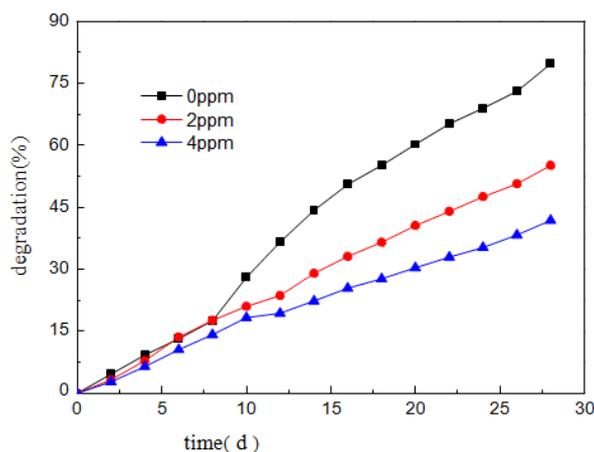


Figure 8. Influence of Cu²⁺ on the biodegradability of PAC.

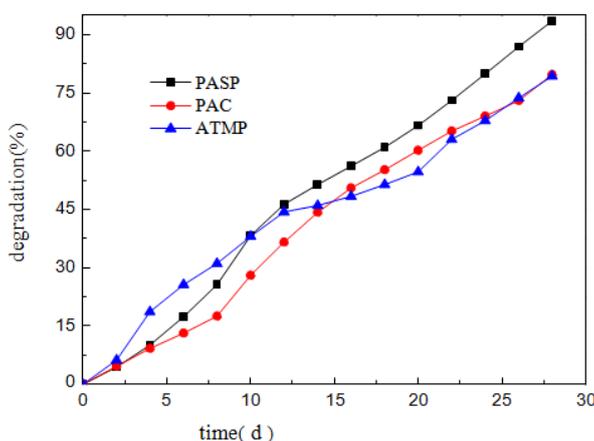


Figure 9. Biodegradability of PAC, ATMP, and PASP.



Figure 10. Two kinds of configuration on PASP.

CONCLUSIONS

Poly (aspartic acid-citric acid) copolymer (PAC) is a novel water-soluble agent and can serve as the biodegradable water treatment agent. PAC has a structure similar to the amide bond of proteins and its degradation is a multistep process. Microbial extracellular enzymes can decompose PAC into short peptides, which produce CO₂ and N₂ through deamination and decarboxylation. With the increase in the ratio of citric acid in copolymerization process and the decrease in the molecular weight of copolymer, the biodegradability of PAC was significantly enhanced. The increase in the ratio of citric acid in the copolymer

can enhance the hydrophilicity of the polymer and provides a comfortable living environment for microorganisms and more active sites for degradation enzymes. The relatively poor biodegradation performance of high molecular weight polymers may be attributed to the diffusion of microorganisms and enzyme molecular into the internal of copolymer and thus hindering the attack of enzyme or bacteria to the most sensitive reaction. Cu²⁺ could decrease the degradation rate through inhibiting enzymatic activities. In addition, compared with other commercial scale inhibitors, biodegradable property of PAC was excellent due to the presence of more carboxyl groups (-COOH) and peptide bonds (-CO-NH-).

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