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Supporting Information

Lumbrokinase and Earthworm Protein as Inhibitors on Crystallization of Calcium Oxalate Monohydrate

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Contents

S1. Details of peptide synthesis 2
S1-1. Peptide synthesis experiment 2
S1-2. Peptide purification experiment 3
S1-3. Peptide purity test results 4
S1-4. Effect of peptides derived from LRK 5
S2. Other information 8
Figure S1. Scanning electron micrographs of COM and COD crystals in the presence of 120 μg/ml LRK........ 8
Figure S2. Standard FTIR spectra of COM and COD crystals and FTIR spectra formed in the presence of LRK and EP. 8
Figure S3. Standard Raman spectra of COM and COD crystals and Raman spectra formed in the presence of LRK and EP. COM and COD represent calcium oxalate monohydrate and calcium oxalate dihydrate, respectively. 9
Figure S4. Structure and amino acid sequence of LRK. 9
Figure S5. The correlation between (v_0-v) and the respective concentration of EP, ... 9
Figure S6. Schematic of common modification mechanisms. (a) Schematic of modifier binding to the crystal surface via the step-pinning mechanism and (b) kink-blocking mechanism. 10

S1. Details of peptide synthesis

S1-1. Peptide synthesis experiment

1. Ten grams of CTC resin (degree of substitution 0.5 mmol/g) was weighed in a 50 ml reactor, and the resin was soaked in 10 ml of dichloromethane (DCM) for 1 hour.

2. The resin was washed 3 times the resin volume of nitrogen-dimethylformamide (DMF), drained, washed four times, and drained for later use.

3. Ten milliliters of 20% piperidine (piperidine/DMF) was added to the reactor and shaken on a decolorizing shaker for 20 minutes to remove the Fmoc group on the resin. After deprotection, wash with 3 times the resin volume of DMF twice, 3 times the resin volume of methanol 2 times, 3 times the resin volume of DMF 2 times, and drain the resin.

4. Take a small amount of resin and use the ninhydrin (Ninewell Ninhydrin hydrate) method to detect (two drops each for inspection A and inspection B, and react at 100°C for 1 min). The resin is colored, indicating successful deprotection.

5. A certain amount of the first amino acid at the C-terminus and 3 times the molar amount of 1-hydroxy-benzotriazole (HOBT) were weighed into a 50 ml centrifuge tube, 10 ml of DMF was added to dissolve it, and then 0.5 ml of N, N-diisopropylcarbodiimide (DIC) was added, shaken and shaken for 1 min to activate the amino acid. After the solution was clarified, it was added to the reactor, and then the reactor was placed in a shaker at 30°C for shaking.

6. After 2 hours, drain the reaction solution, wash it twice with DMF, cap it with a certain amount of methanol (methanol: DIEA: DCM=1:1:2) for half an hour, and then wash it with 3 times the resin volume of DMF four times, Drain and set aside.

7. A certain amount of 20% piperidine (piperidine/DMF=1:4) was added to the reactor and shaken on a decolorizing shaker for 20 minutes to remove the Fmoc group on the amino acid. After deprotection, wash with 3 times the resin volume of DMF twice, 3 times the resin volume of methanol 2 times, 3 times the resin volume of DMF 2 times, and drain the resin.

8. Take a small amount of resin and use the ninhydrin (Ninewell Ninhydrin hydrate) method to detect (two drops each for inspection A and inspection B, and react at 100°C for 1 min). The resin is colored, indicating successful deprotection.

9. Next, the amino acid and HOBT were weighed in a 50 ml centrifuge tube, 10 ml of DMF was

added to dissolve it, 0.5 ml of N, N-diisopropylcarbodiimide (DIC) was added, and the tube was shaken for 1 min to activate the amino acid. After the solution was clarified, it was added to the reactor, the reactor was placed in a shaker at 30°C and shaken for 1 hour. Take a small amount of resin for testing and use the ninhydrin method (two drops each for testing A and testing B, 100 Reaction at °C for 1 min). If the resin is colorless, the reaction is complete; if the resin is colored, the condensation is incomplete, and the amino acid needs to be redosed to react again.

10. After the reaction was complete, the resin was washed with DMF four times, drained, 20% piperidine (piperidine/DMF=1:4) was added to the reactor, and the mixture was shaken on a decolorizing shaker for 20 minutes. This removes the Fmoc group on the resin. After deprotection, wash 2 times with 3 times the resin volume of DMF, 3 times the resin volume of methanol 2 times, 3 times the resin volume of DMF 2 times, drain the resin, take a small amount of resin and use the ninhydrin method to test whether it is degraded To Fmoc group.

11. Follow steps 9-10 to connect the following amino acids in sequence.

S1-2. Peptide purification experiment

1 Purification test equipment: high-performance liquid chromatograph, freeze dryer

2 Purification reagents:

2.1 Analysis water: 0.1% TFA+100% pure water Analysis acetonitrile: 0.1% TFA+100% acetonitrile

2.2 Preparation water: 0.1% TFA+100% pure water Analysis acetonitrile: 100% acetonitrile

3. Crude product processing

Take a small sample to confirm the sample in MS. After the calculated molecular weight is correct, perform crude HPLC (analysis method 10-100%)

Confirm the peak time T of the target compound according to the crude HPLC chart

4 Crude product dissolution and filtration

Crude product dissolution: Acetonitrile + pure water, ultrasonic vibration to completely dissolve and then filter

5 Preparation of crude product

5.1 Preparation gradient setting: target peak time T*3.6-22 to obtain the sample preparation gradient

5.2 Crude product sampling and peak collection

5.3 Confirm the target peak by MS according to the preparative HPLC profile

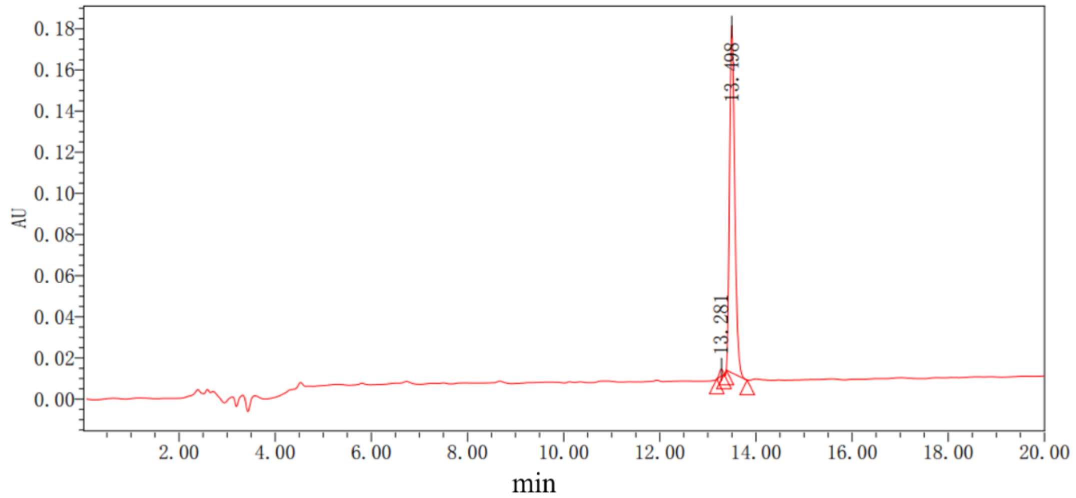
6 Perform analytical HPLC detection on the preparation target peak

7 Freeze-dry the samples that pass the test

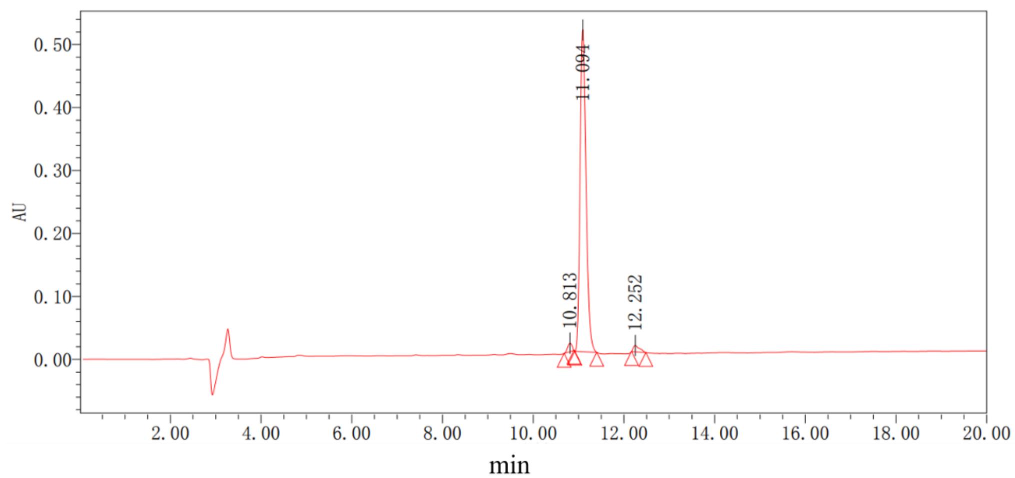
8. Lyophilize the sample again for HPLC detection and MS confirmation

S1-3. Peptide purity test results

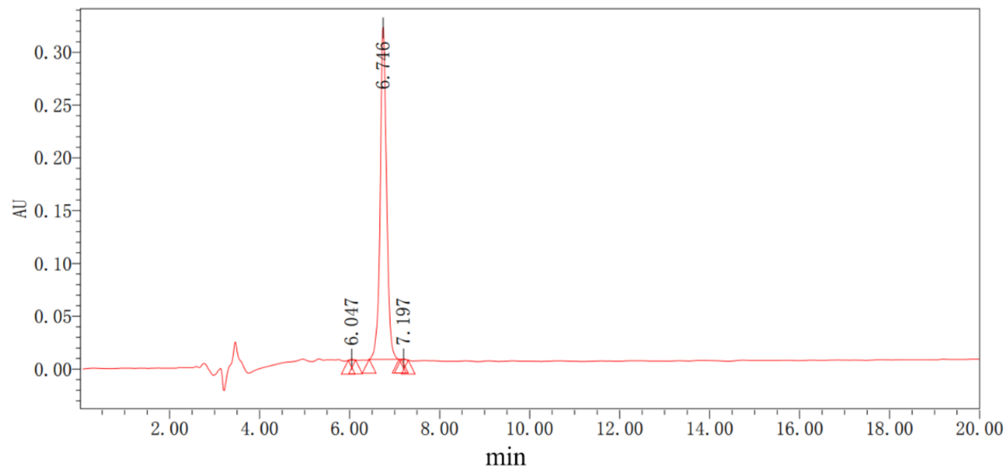
1. Peptide L1: DVDSIFVHED(>95%)



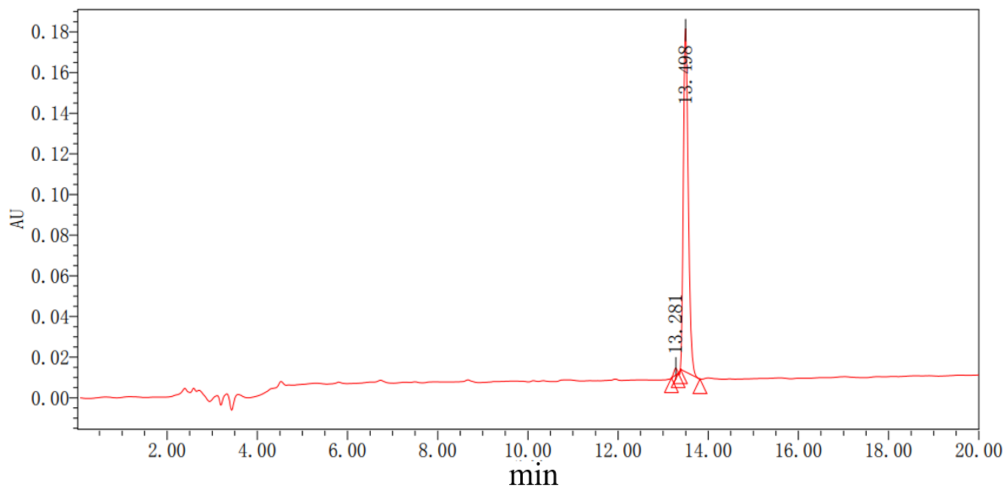
2. Peptide L2: DVYPLNSIFD(>95%)



3. Peptide L3: DSGGPLSVKD(>95%)



4. Peptide L4: YSDAGDMELP(>95%)



S1-4. Effect of peptides derived from LRK

According to previous studies, we can conclude that acidic amino acids can inhibit the formation of COM crystals, basic amino acids can promote the progress, and the presence of neutral acids has almost no effect on COM crystal growth [11]. Binding to calcium salts such as apatite or calcite (Table S-1) is mainly controlled by electrostatic interactions between calcium cations and anionic residues of peptides or proteins. To further explore the relationship between protein and peptide modification effects, as well as the influence of peptide types, including the number of acidic and basic amino acids and steric hindrance, on COM crystal modification effects, we synthesized four primary amino acid sequences from LRK that have never been studied before to study their effect on COM crystal growth. Cho et al. previously deduced the amino acid sequence of LRK (Figure S4) [34-35]. The chemical definitions and amino acid sequences of each of the six

isoenzymes of LRK with their respective fibrinolytic activities have previously been identified and characterized. LRK contains a total of 282 amino acids, including 28 acidic amino acids (D, E) and 21 basic amino acids (K, R, H). Herein, we selected four polypeptide chains with different acidic and basic amino acids (marked in green or red, respectively) from LRK, and each chains contained 10 amino acids in different spatial orders for exploration. Their sequence and related information are shown in Table S-1.

Table S-1. Peptides derived from contiguous segments of LRK

Name	Peptide sequences[a]	Number of acid amino acids	Number of basic amino acids	pI[b]
L1	DVDSIFVHED	4	0	3.84
L2	DVYPLNSIFD	2	0	3.56
L3	DSGGPLSVKD	2	1	4.21
L4	YSDAGDMELP	3	0	3.49

[a]Anionic amino with basic (green) and acidic (red) side groups. [b] Theoretical pI (ExPASy Bioinformatics Resource Portal).

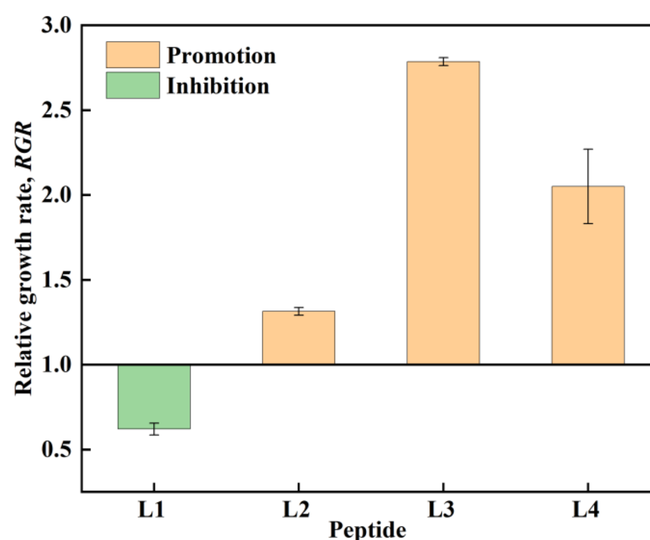


Figure S-1. Relative growth rate (RGR) in the presence of lysozyme peptide at a concentration of 50 μg/mL. Green and yellow bars refer to COM growth inhibition (RGR<1) and promotion (RGR>1), respectively. Data are the average of three or more separate measurements, and error bars are equal to 2 times the standard deviation.

Figure S-1 presents the RGR in the presence of lysozyme peptide at a concentration of 50

μg/mL. Interestingly, we observed that the L1 fragment was an inhibitor that could inhibit the growth rate of COM by 33%, while the L2-L4 fragment was a promoter, and the promotion effect ranked L2 < L4 < L3. L2 and L4 increase the growth rate of COM by approximately 31.4% and 105%, respectively. The most effective growth promoter, L3, can increase by 179%. What makes a great difference in the modification effect of these four peptides will be discussed next.

First, L1, as an inhibitor, has the largest ratio of acidic amino acids, 40%, and L2-L4 peptides are 20%, 20% and 30%, respectively, indicating that the total negative charge is one part of the contribution to the peptide efficacies. Although L4 contains more acidic amino acids than L2, L4 shows a 105% promotion effect on the growth rate of COM, which is much higher than that of L2. The generation of this phenomenon may be that all acidic amino acids in L4 are arranged in the middle of the peptide sequence, while both acidic amino acids in L2 are located at the end of the peptide, suggesting that steric hindrance may affect the binding of anionic residues to the COM crystal surface and thus determine the modification effect of the peptide. Compared with L2 and L4, L3, with only a basic amino acid next to the acidic amino acid at one end, presented the highest promotion effect on the growth rate of COM, which means that the presence of basic amino acids has a great influence on the modification effect of peptides. This further emphasizes the extremely important role of amino acid species in the modification of COM crystals by peptides.

Recently, some studies have suggested that there is a clear correlation between the RGR of the COM crystal and the peptide pI [11, 25]. Peptides rich in anionic side chains (i.e., low pI) are the most effective inhibitors of COM growth, and peptides rich in cationic side chains (i.e., high pI) are the most effective promoters of COM growth, especially almost all peptides with a pI value less than 6 that are COM growth inhibitors [11]. Interestingly, the pI values of the four peptides were also approximately 3.5-4.5 in our experiment (Table S-1), which is in the range of inhibitors. However, not all of them are inhibitors. This indicates that there is no obvious relationship between the RGR of the COM crystal and the pI of the peptide in our experiments. The properties of amino acids and steric hindrance are the main factors affecting the modification effect of peptides and even proteins.

In general, the effect of modifiers cannot be predetermined based on only one descriptor. Total negative charge, steric hindrance, charge distribution based on the peptide structure, and conformational changes caused by interface adsorption may also affect the modification effect of

peptides. Since these influencing factors are relatively independent and interdependent, they must be identified and seriously used to clarify the mechanism. In a broader context, the pathway of crystal growth promotion may allude to a more widespread mechanism of biogenic mineral formation (e.g., bone or exoskeletal structures).

S2. Other information

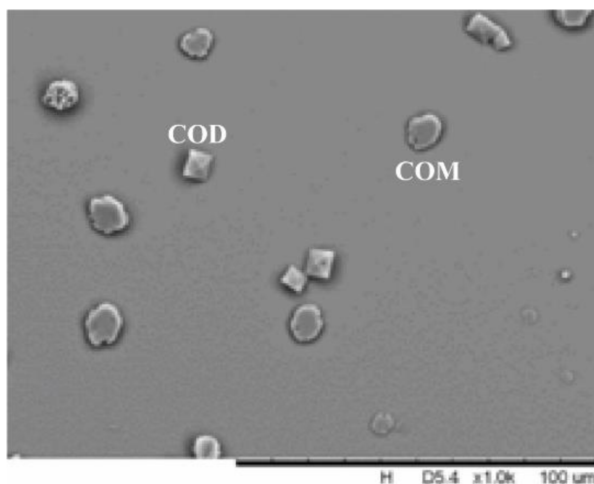


Figure S1. Scanning electron micrographs of COM and COD crystals in the presence of 120 µg/ml LRK. COM and COD represent calcium oxalate monohydrate and calcium oxalate dihydrate, respectively.

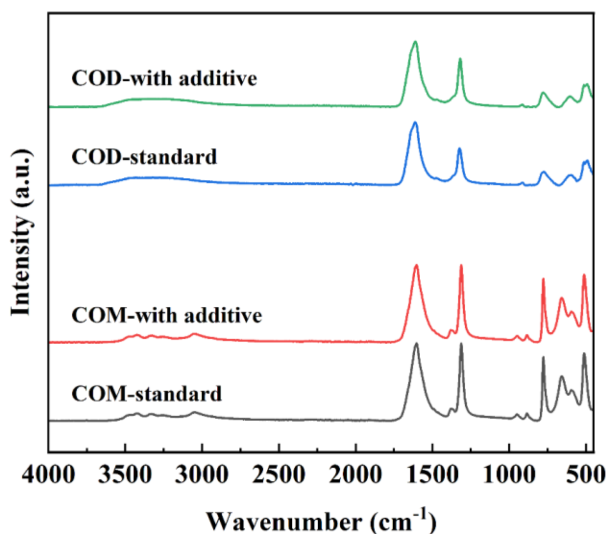


Figure S2. Standard FTIR spectra of COM and COD crystals and FTIR spectra formed in the presence of LRK and EP. COM and COD represent calcium oxalate monohydrate and calcium oxalate dihydrate, respectively.

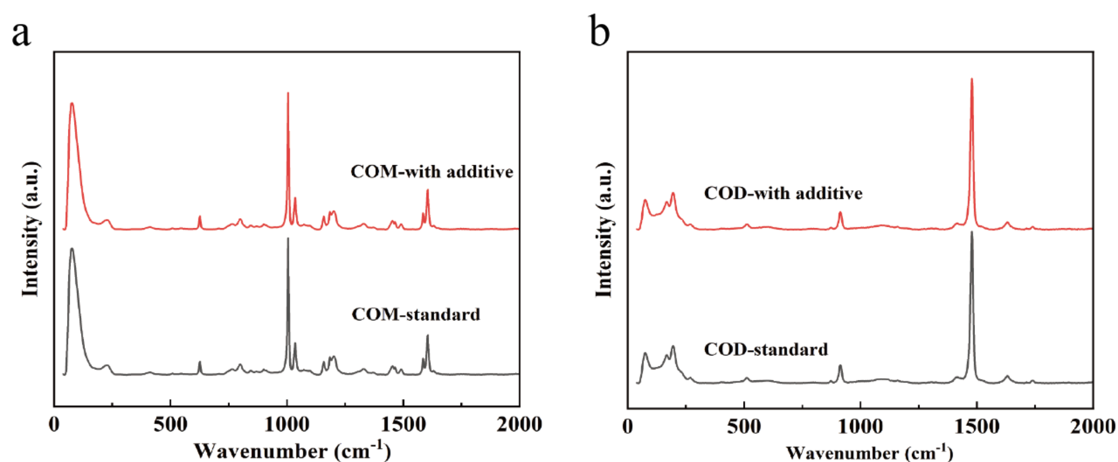


Figure S3. Standard Raman spectra of COM and COD crystals and Raman spectra formed in the presence of LRK and EP. COM and COD represent calcium oxalate monohydrate and calcium oxalate dihydrate, respectively.

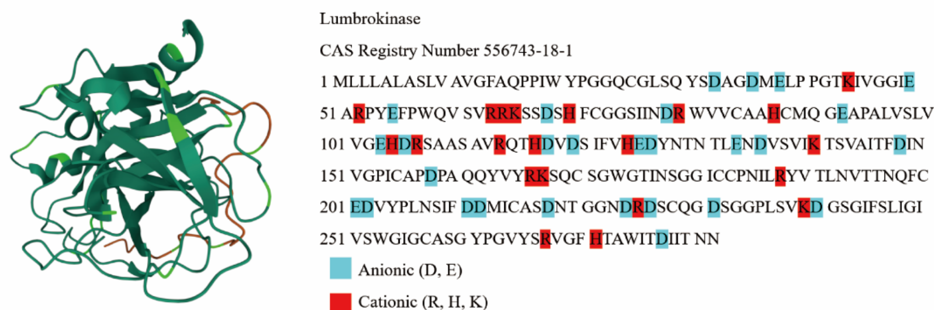


Figure S4. Structure and amino acid sequence of LRK. Amino acids with basic and acidic side chains are highlighted in green and blue, respectively.

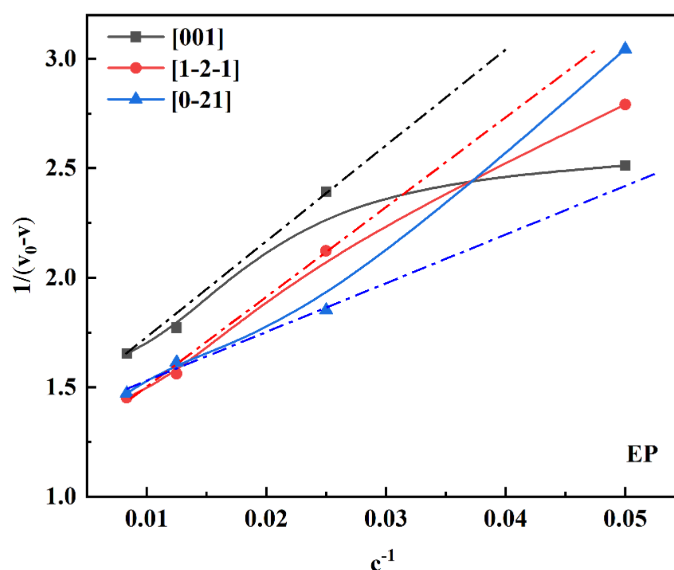


Figure S5. The correlation between (v_0-v) and the respective concentration of EP, *c*, in modified reciprocal coordinates. The deviating linear relationship between $1/(v_0-v)$ and $1/c$ shows that the

mode of EP molecules that inhibit the step growth in the [001], [021], and $[12\bar{1}]$ directions of the (100) surface does not follow the kink block mechanism.

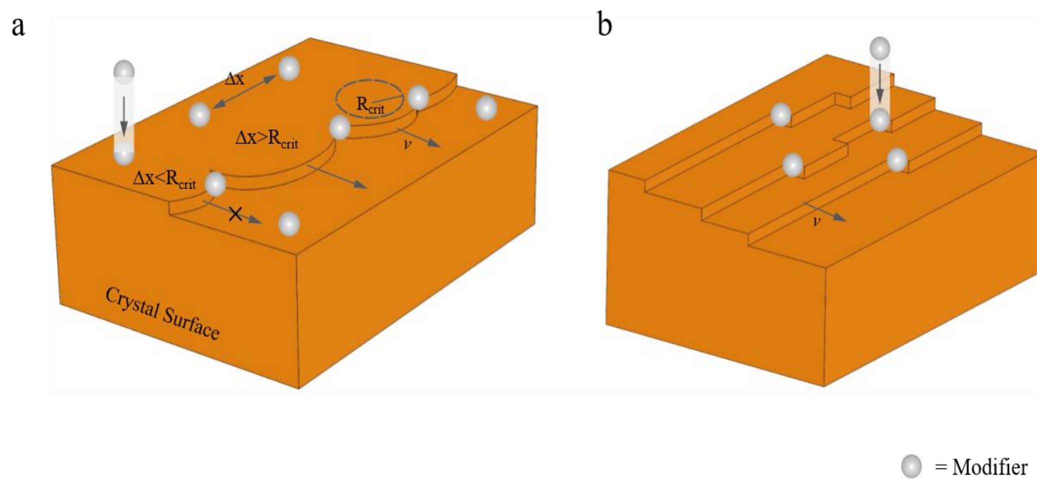


Figure S6. Schematic of common modification mechanisms. (a) Schematic of modifier binding to the crystal surface via the step-pinning mechanism and (b) kink-blocking mechanism.