

## Optimization of synthesizing leucine-binding nano-sized magnetite by a two-step transformation

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**Abstract**—The optimum conditions for synthesizing leucine (Leu)-binding nano-sized magnetite (NSM) particles by a two-step transformation (TST) process were studied. The formation and magnetization of as-synthesized NSM particles were investigated through variation of the acidity, the type of surface modifier, and the nature of the acid for pH adjustment. With increased acidity, the saturation magnetization of the NSM particles decreased, but the amount of Leu coated on the surface of NSM particles increased. After the influence of hydroxyl ( $\text{OH}^-$ ) groups on the surface of NSM particles was removed by using the dicarboxyl anion ( $\text{C}_2\text{O}_4^{2-}$ ) as a ligand in the first step, Leu was successfully bound with NSM particles in the second step. However, when polyethylene glycol (PEG) was used as a surface modifier, it was difficult to form the Leu-to-NSM particle complex. In terms of the acid used to modify pH,  $\text{H}_2\text{SO}_4$  was slightly less effective than HCl in achieving saturation magnetization because the coordination for  $\text{SO}_4^{2-}$  anions is stronger than that of  $\text{Cl}^-$  anions. The preparation of other amino acid-binding NSM particles can be optimized in an analogous manner.

Key words: Magnetite, Two-step transformation, Leucine, Amino acid, Nano-sized particle

### INTRODUCTION

Recently, surface functionalized nano-sized magnetite (NSM) materials have received great attention since they can provide interesting physical and chemical properties [1-3]. These particles can be applied to biomedical fields, such as cell, protein, DNA sorting or separation [4-7], medical diagnosis [8,9], and controlled drug delivery [10,11]. Furthermore, NSM particles encapsulated by certain amino acids (AAs) have good prospects for application in cancer therapy. AA imbalance therapy has been widely adopted for inhibition of cancer or tumor cells, and hyperthermia using magnetic fluid has been applied as a direct method for killing cancer and tumor cells [12].

Amino acids are attractive chemicals as ligands anchored with NSM particles. Therapy with amino acid imbalance (TAAI) or with amino acids as a dose of nutrients has been applied to treat cancer sufferers because some amino acids can reduce tumor cells [13,14]. It was also reported that leucine (Leu) would lead to the tumor cell shrinking or even dying out because some tumor cells are composed of Leu-zipper [15]. Moreover, leucine can be used in a diet, which repairs microtears that result from exercise. Hence, Leu-coated NSMs have a great potential in their applicability in cancer therapy and diagnosis. For that reason, we selected leucine as a representative of amino acids in this study.

In many studies, most functionalized NSMs were synthesized by encapsulating NSMs with polymers or oleates. In this case, however, the magnetic response can be reduced due to the low degree of NSM particulate contents. Therefore, to synthesize polymer-encapsulated NSM particles with high NSM content, improvement

in the synthesizing methods is needed [16-20]. Directly anchoring a hydrophilic or hydrophobic ligand to NSM particles can be an effective way to solve the limitations of the encapsulation methods [9].

Although direct chemical bonding of a ligand with  $\text{Fe}^{II,III}$  on the surface of NSM particles may be the optimum choice to obtain homogeneous functional NSM particles, directly anchoring a ligand to  $\text{Fe}^{II,III}$  is generally hindered by the surrounding  $\text{H}_2\text{O}$  molecules or  $\text{OH}^-$  groups [21,22]. Many researchers have studied some ways to replace the  $\text{H}_2\text{O}$  molecule and  $\text{OH}^-$  groups with a ligand such as oleate and some organic modifiers with carboxyl group [23,24]. The ligands were partly chemisorbed but mainly physisorbed on the NSM particles. However, the stability of functionalized NSM is very important because the NSM must carry its functional groups to the target. Therefore, the amino acid-coated NSM should be fabricated into a chemisorbed form rather than a physisorbed form for effective applications.

In our previous study [1], a two-step transformation (TST) process was reported to obtain amino acid-bound NSM (AA-NSM) particles. Using the TST process, a series of amino acid-coated NSM particles, including leucine (Leu), arginine (Arg), cysteine (Cys), cystine, aspartate (Asp), and tyrosine, (Tyr) were successfully synthesized. In addition, the role of each step was elucidated at a fixed condition and the amount of amino acid bound to NSM at each condition was compared to each other. However, because the synthesis of AA-NSM particles will be affected by various variables such as reagent, pH condition and surface modifier, the effect of the variables on synthesizing AA-NSM needs to be studied.

The purpose of this study is to investigate the optimum conditions for synthesizing Leu-NSM as a representative AA-NSM particle. The effects of acidity, surface modifier, and the nature of the acid used to adjust the pH on the formation and magnetization of

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as-synthesized NSM samples were studied in detail. In addition, the role of the dicarboxyl anion ( $C_2O_4^{2-}$ ), which served as the ligand in the first step of the TST process, was also investigated. All of the synthesized samples were analyzed by using Fourier-Transform Infrared Spectroscopy (FT-IR) and saturation magnetization.

## EXPERIMENTAL

### 1. Preparation of Samples

A series of NSM samples were prepared by the two-step transformation (TST) process, as reported in our previous work [1]. The whole synthesizing steps are illustrated in Fig. 1. The first step of the TST process is a simple chemical co-precipitation of  $Fe^{2+}$  and  $Fe^{3+}$  for 2 h in a concentrated ammonium hydroxide solution (28 wt%) at pH 8-9. A combination of  $FeSO_4 \cdot 7H_2O$  ( $Fe^{II}$  sulfate) and dehydrated  $Fe_2(SO_4)_3$  ( $Fe^{III}$  sulfate) was prepared as reagents with a stoichiometric molar ratio,  $Fe^{2+} : Fe^{3+} : L' = 1 : 2 : 0.1$  ( $L'$ =ligand 1). In this study, to investigate the effect of a surface modifier, the dicarboxyl anion ( $C_2O_4^{2-}$ ) from  $(NH_4)_2C_2O_4$  or polyethylene glycol (PEG, FW=5,000) was selected as the surface modifier ( $L'$ ).

The second step of the TST process is a transformation of  $L'$ -NSM to  $L''$ -NSM ( $L''$ =ligand 2) by mixing an aqueous suspension of  $L'$ -NSM with  $L$  ( $Fe_3O_4 : L = 10 : 1$ , in molar ratio) in an acidic

solution at pH 4-7, 75 °C. To investigate the effect of the nature of the acid, different kinds of acids such as HCl (37 wt%) and  $H_2SO_4$  (97 wt%) were used to adjust the pH value. At that point, Leu or  $C_2O_4^{2-}$  was selected as  $L''$ . The entire procedure was performed under a nitrogen atmosphere to control the size and oxidation of NSM particles [25].

To remove extraneous ligands from the suspended  $L''$ -NSM particles, the  $L''$ -NSM particles were washed five times with hot deoxygenated water accompanied by magnetic decantation. After filtration, the resulting dark black cakes were dried in a vacuum desiccator at room temperature for more than 2 weeks. The as-synthesized samples are characterized in Table 1.

### 2. Characterization of Samples

To measure the saturation magnetization of the samples, a vibration sample magnetometer (VSM; Lake Shore Model 7300) was used under magnetic fields up to 10 kOe. FT-IR spectra of the as-synthesized samples were recorded in transmission mode with a Perkin-Elmer infrared spectrometer (Model 1730). Powdered samples were ground with KBr and compressed into a pellet with a weight ratio of the powder sample : KBr = 1 : 300 mg. The FT-IR spectrum of a pellet composed of the same amount of pure dehydrated KBr was recorded as a blank. Additional analysis of NSM particles including TEM/HRTEM observations (Philips TEM-12, 120 kV/JEM-3010, 300 kV), XRD patterns (Rigaku X-ray diffractometer, model D/max-3A), and TGA measurements (TA instrument, model Q500) were previously reported in detail [1]. During the TST process, the particle size decreased and its distribution became narrow compared with that after the 1st step. The average particle size was about 9 nm, and they had good spherical or ellipsoidal shapes. Even though the saturation magnetization value of leucine-coated NSM decreased after the 2nd step of the TST process (from 64.59 to 58.21 emu/g), it was still higher than that modified with oleate acid or encapsulated by polymer layers.

## RESULTS AND DISCUSSION

### 1. Effect of Acidity

As reported in Table 1, the samples L25, L26, L27, and L32 were pre-modified by using the dicarboxyl anion ( $C_2O_4^{2-}$ ) in the first step of the TST process; however, to investigate the effect of acidity,  $C_2O_4^{2-}$  was substituted with Leu at various pH conditions (pH 4-7)

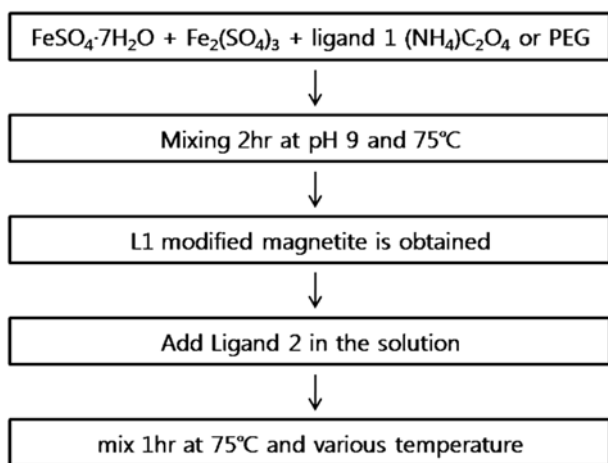


Fig. 1. Schematic diagram of synthesizing amino acid-coated NSM.

Table 1. Characterization of the as-synthesized NSM particles and resultant saturation magnetization

Sample No.	1 <sup>st</sup> step of TST process*		2 <sup>nd</sup> step of TST process		Saturation magnetization (emu/g)
	Reagent	$L'$	$L$	pH (acid)	
L24	$Fe^{II,III}$ sulfates	$C_2O_4^{2-}$	Leu	pH=6 ( $H_2SO_4$ )	63.97
L25	$Fe^{II,III}$ sulfates	$C_2O_4^{2-}$	Leu	pH=6 (HCl)	66.73
L26	$Fe^{II,III}$ sulfates	$C_2O_4^{2-}$	Leu	pH=5 (HCl)	58.21
L27	$Fe^{II,III}$ sulfates	$C_2O_4^{2-}$	Leu	pH=4 (HCl)	54.99
L29	$Fe^{II,III}$ sulfates	PEG	Leu	pH=5 (HCl)	70.80
L32	$Fe^{II,III}$ sulfates	$C_2O_4^{2-}$	Leu	pH=7 (HCl)	72.43
L37	$Fe^{II,III}$ sulfates	$C_2O_4^{2-}$	-	pH=5 (HCl)	65.51
L38	$Fe^{II,III}$ sulfates	$C_2O_4^{2-}$	$C_2O_4^{2-}$	pH=5 (HCl)	58.84
L40	$Fe^{II,III}$ sulfates	-	$C_2O_4^{2-}$	pH=5 (HCl)	65.81

\*The pH value of the solution at the 1<sup>st</sup> step (pH=8-9) was adjusted with a diluted ammonia solution.

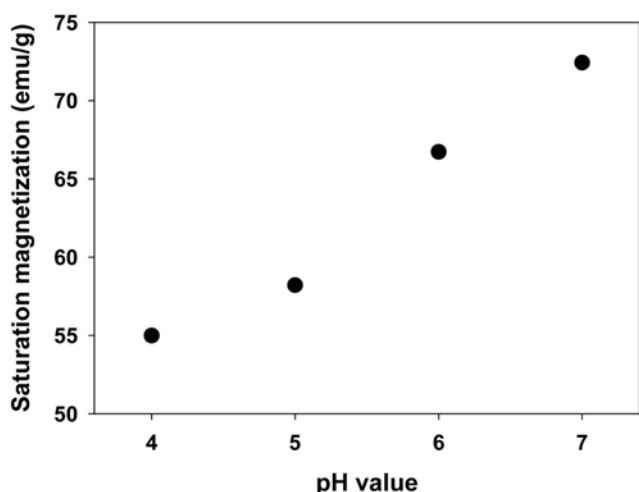


Fig. 2. Effect of acidity in the second step on the saturation magnetization of Leu-binding NSM particles; HCl was used to adjust pH.

in the second step.

Fig. 2 indicates the effect of acidity on the saturation magnetization of Leu-binding NSM particles. In the case of sample L32 (Table 1), when the L'-NSM particles were mixed with Leu at pH 7, Leu could not be bound to the NSM because the  $C_2O_4^{2-}$  was replaced by  $OH^-$ . Therefore, the saturation magnetization of sample L32 showed a maximum value, which is similar to the original NSM [1]. The substitution of Leu on the NSM particles was first observed at pH 6 (sample L25), which is close to its isoelectric point,  $pI=5.98$  [26]. In addition, it was reported that the maximum content of Leu on the NSM particles was observed at pH 5 (sample L26) [1]. At this condition, the saturation magnetization fairly decreased. It was reported that the saturation magnetization of NSM decreases when the particle surface is covered by ligands [27]. We had the same result in this experiment; the saturation magnetization of Leu-NSM particles decreased as the acidity increased. However, the saturation magnetization was still higher than that of particles modified with oleate acid or otherwise encapsulated by polymers [9,20]. On the other hand, at a high acidic condition such as pH 4 (sample L27), increased protonation of the carboxyl anion in Leu led to a weakening of the coordination of Leu with the NSM surface [1]. As a

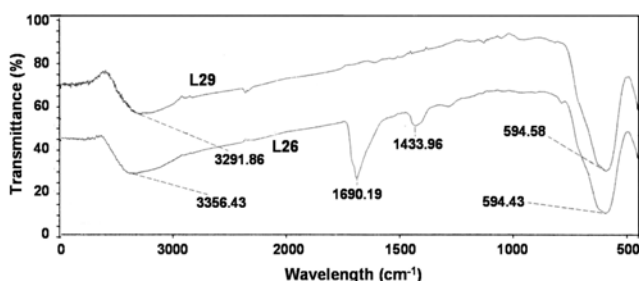


Fig. 3. Effect of surface modifiers on the FT-IR spectra of Leu-binding NSM particles; L29 ( $Fe^{II,III}$  sulfates as reagents) modified by PEG, L26 [1] ( $Fe^{II,III}$  sulfates as reagents) modified by  $C_2O_4^{2-}$  (in the second step, each sample was treated with Leu in an acidic solution at pH 5).

result, even though the Leu content was smaller than that of L26, the saturation magnetization decreased. Leu binding on the NSM will be discussed in the next FT-IR spectra section.

## 2. Effect of Surface Modifier

In Fig. 3, FT-IR spectra of samples L26 and L29 are compared to investigate the effect of the modifier (L') on the surface of the NSM particles. Each L' in the sample was substituted by Leu at the same pH condition (pH 5) in the second step of the TST process (Table 1).

When  $C_2O_4^{2-}$  was used as a surface modifier (sample L26), asymmetric and symmetric stretchings for the coordinated carboxyl anion were observed at 1,690 and 1,434  $cm^{-1}$ . The peak difference of  $\Delta\nu_{as-s}=256\text{ cm}^{-1}$  is specific for the unidentate coordination between the carboxyl anion and some metal cations. This indicates that the carboxyl anion in Leu was successfully combined with  $Fe^{II,III}$  on the surface of the NSM particles. Interpretation of the spectra implies that a Leu-to-NSM complex was formed during the TST process using  $C_2O_4^{2-}$  as a surface modifier (sample L26). In other words, the  $C_2O_4^{2-}$  group could remove the influence of the  $OH^-$  groups existing on the surface of the NSM particles during the co-precipitation of  $Fe^{II,III}$  from an aqueous solution.

However, in the FT-IR spectra of sample L29, which was pre-modified by PEG, no characteristic vibrations verifying the formation of a Leu-to-NSM complex were found. This indicates that NSM particles prepared by using PEG as a surface modifier did not provide any active position for Leu coordination during the TST process. This was similar to the result that the ligands were partly chemisorbed but mainly physisorbed on the NSM surface by direct mixing with the ligands [20,21].

In addition, the saturation magnetization of sample L26 was about 20% lower than that for sample L29 (Table 1). Complexes on the surface of sample L26, such as Leu, might lead to a decrease in the saturation magnetization.

## 3. Effect of the Nature of the Acid Used to Adjust the pH

Samples L24 and L25 were pre-modified by the dicarboxyl an-

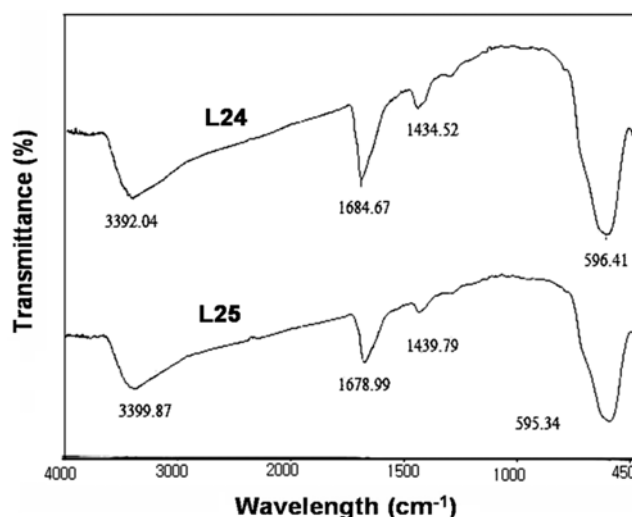


Fig. 4. Effect of acid type on the FT-IR spectra of Leu-binding NSM particles at pH 6 in the second step of the TST process, using  $H_2SO_4$  and HCl solutions for acidity adjustment of samples L24 and L25, respectively.

ion ( $\text{C}_2\text{O}_4^{2-}$ ) in the first step of the TST process, and the  $\text{C}_2\text{O}_4^{2-}$  of these samples was substituted with Leu at pH 6 in the second step (Table 1). Here, we used two kinds of acids,  $\text{H}_2\text{SO}_4$  (L24) and  $\text{HCl}$  (L25), to investigate the effect of the type of acid used to adjust the pH during the second step of the TST process.

In Fig. 4, the two peaks (near  $1,680$  and  $1,435\text{ cm}^{-1}$ ) of L24, which were caused by the asymmetric and symmetric stretchings resulting from the coordinated carboxyl anion, were more intense than those of L25. This may indicate that a greater amount of Leu is coated on the surface of the NSM particle in sample L24 or some of the  $\text{SO}_4^{2-}$  groups by forming complexation with  $\text{Fe(II,III)}$  on the surface of the NSM particles. The latter phenomenon is related to the coordination with  $\text{SO}_4^{2-}$  groups, which is stronger than that of  $\text{Cl}^-$  anions. In addition, as reported in Table 1, the saturation magnetization of sample L24 ( $63.97\text{ emu/g}$ ) was slightly lower than that of L25 ( $66.73\text{ emu/g}$ ). Although the nature of the acid used to adjust the pH appeared to have a slight effect on the saturation magnetization, this effect was relatively small compared to the other variables.

#### 4. Role of $\text{C}_2\text{O}_4^{2-}$

To investigate the role of  $\text{C}_2\text{O}_4^{2-}$  groups during the synthesis of Leu-binding NSM particles, sample L26 can be compared to samples L37, L38, and L40 (Table 1). Sample L37 was fabricated without the addition of Leu during the second step of the TST process, sample 38 was synthesized by adding  $(\text{NH}_4)_2\text{C}_2\text{O}_4$  instead of Leu during the second step, and sample L40 was co-precipitated without adding any surface modifier in the first step but adding  $(\text{NH}_4)_2\text{C}_2\text{O}_4$  in the second step. Otherwise, the procedures used to prepare samples L26, L37, L38, and L40 were identical.

In the case of L26, Leu was successfully formed to complexation with  $\text{Fe(II,III)}$  on the NSM surfaces. On the other hand, in the case of L37, L38, and L40, no characteristic peaks of dicarboxyl anion

were observed (Fig. 5). After the first step of the TST process,  $\text{C}_2\text{O}_4^{2-}$  groups were successfully coated on NSM surfaces when  $\text{C}_2\text{O}_4^{2-}$  was used as a surface modifier [1]. However, the FT-IR spectrum of sample L37 indicates that most of the  $\text{C}_2\text{O}_4^{2-}$  groups coated on the NSM surface in the first step were desorbed in the second step without Leu. In addition, the FT-IR spectra of samples L38 and L40 show that  $\text{C}_2\text{O}_4^{2-}$  groups were not well-coated on NSM surfaces even when  $(\text{NH}_4)_2\text{C}_2\text{O}_4$  was added in the second step. The saturation magnetizations of samples L37 and L40 were almost identical (near  $65\text{ emu/g}$ ) and were stronger than that observed for sample L26 (Leu-binding NSM), as reported in Table 1. The data for saturation magnetization support the conclusion that  $\text{C}_2\text{O}_4^{2-}$  groups on the surfaces of NSM particles were unable to form complexes irrespective of the presence of  $\text{C}_2\text{O}_4^{2-}$  in the second step of the TST process—as concluded from the FT-IR spectra. In the case of L38, the weak vibration located at  $1,634\text{ cm}^{-1}$  may have been caused by the interference of crystalline water. Consequently, the saturation magnetization observed for sample L38 was similar to that of sample L26.

The results indicate that  $\text{C}_2\text{O}_4^{2-}$  groups almost completely lose coordination on NSM surfaces at pH 5. Therefore, it is concluded that the carboxyl groups of the amino acid are able to occupy the spaces left by the desorption of the  $\text{C}_2\text{O}_4^{2-}$  groups on the surfaces of NSM particles at the condition of pH 5 during the second step of the TST process.

## CONCLUSIONS

The optimum conditions for synthesizing Leu-NSM particles, as representative AA-NSM particles, by using the TST process, were investigated through variation of the acidity, the surface modifier, and the nature of the acid used to adjust the pH. The saturation magnetization of the NSM particles prepared in this research decreased as the acidity at the second step increased because more Leu was coated on the surface of NSM particles.

Due to the high surface energy and hydrophilic properties of NSM particles, it is almost impossible to bind Leu directly to NSM particles through a one-step reaction. When  $\text{C}_2\text{O}_4^{2-}$  was used as a surface modifier, Leu could be successfully coated on the surface of NSM particles because the  $\text{C}_2\text{O}_4^{2-}$  group successfully competed with the large numbers of  $\text{OH}^-$  groups on the surface of NSM particles. However, when PEG was used as the surface modifier, the Leu-to-NSM complex could not be formed.

The carboxyl moiety in Leu cannot compete with both  $\text{OH}^-$  and  $\text{C}_2\text{O}_4^{2-}$  groups in coordination with  $\text{Fe(II, III)}$  in a basic solution, while coordination of the  $\text{OH}^-$  and  $\text{C}_2\text{O}_4^{2-}$  groups, especially  $\text{C}_2\text{O}_4^{2-}$  groups (protonation), decreased sharply. In addition, due to the specific acid-base character of Leu, its coordination complex with  $\text{Fe(II, III)}$  remained intact when the acidity increased. Therefore,  $\text{C}_2\text{O}_4^{2-}$  groups lose the ability to coordinate with NSM surfaces at pH 5 during the second step of the TST process. As a result, when the acidity increased, the  $\text{C}_2\text{O}_4^{2-}$  groups on the NSM particles were replaced with Leu. Therefore, until the acidity increased from pH 7 to pH 5, the quantity of Leu complexed with NSM particles was increased while the saturation magnetization was decreased. Conversely, when the pH value was less than 5, the coordination complex weakened, and a corresponding decrease in saturation magnetization was observed resulting from the protonation of the carboxyl anion in Leu.

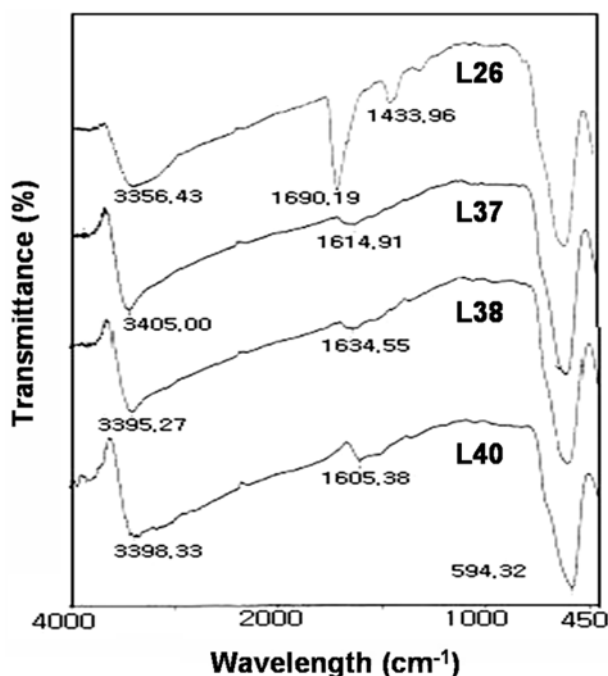


Fig. 5. Effect of  $\text{C}_2\text{O}_4^{2-}$  on the FT-IR spectra of Leu-binding NSM particles, L26 [1], L37, L38, and L40.

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