ADSORPTION OF CARTILAGE OLIGOMERIC MATRIX PROTEIN BY EDTA-COATED MAGNETITE

A. Furqon1,3,*, T. Gusdinar1, S. Ibrahim2 and E. Julianti1
1School of Pharmacy, Bandung Institute of Technology, Bandung, Jawa Barat, Indonesia
2Faculty of Pharmacy, Universitas Jenderal Achmad Yani, Cimahi, Jawa Barat, Indonesia
3Faculty of Health Science, Universitas Jenderal Achmad Yani Cimahi, Indonesia
*Corresponding Author: ayifurqon@gmail.com

ABSTRACT
In the cartilage and tendon of vertebrates, the oligomeric cartilage matrix protein (COMP) was an acidic pentameric extracellular protein. Available COMP extraction from biological fluids required a significant amount of EDTA buffer and solvent. When calcium is depleted, the conformation of the molecule changes. Other acidic protein solid phase extraction by magnetic solid phase extraction exhibited corona formation with low solvent consumption and followed the Langmuir model of isotherm in a short time process. In a buffered system, COMP as a model of an acidic protein interacted with EDTA as a chelating agent. There is no available isotherm model study for COMP to magnetically functionalized EDTA. The objective of this study was to examine the adsorption model of magnetically coated EDTA to recombinant COMP. In a chamber containing a ferric chloride electrolyte and EDTA salt, magnetically coated particles were synthesized by electrodeposition. Characterization of the material included F-TIR spectroscopy, X-ray diffraction, magnetization analysis, and scanning electron microscopy. In the adsorption model, the Langmuir, Freundlich, and Temkin equations were utilized. The FT-IR analysis detected amine and carboxyl groups, as well as iron oxide groups at wavenumber 424.3 cm⁻¹. The analysis of X-ray diffraction revealed eight peaks with a distinct magnetite profile. However, it was discovered that a reversible S-shaped magnetization was anti-paramagnetic, suggesting a weak electrostatic interaction with the recombinant protein COMP. At room temperature, magnetite-coated EDTA can efficiently adsorb COMP.

Keywords: Recombinant COMP, Thrombospondin-5, Magnetite-EDTA, Iron Oxide Adsorbent, Solid Phase Extraction, COMP Adsorption.

INTRODUCTION
Acidic biomacromolecule Cartilage oligomeric matrix protein (COMP) is expressed in vertebrate cartilage and long bones, also known as thrombospondin 5 (TSP-5).
In pathological conditions associated with the diagnosis of osteoarthritis (OA) disease, rheumatoid arthritis (RA), skeletal anomalies (pseudo dysplasia). In prognosis, it acts as a biomarker for potential breast cancer metastasis to articular cartilage as well as for non-invasive hepatic fibrosis-induced viral infection. Major problems in the preanalytical process are low levels of COMP in body fluids that required high volume samples, need high volume EDTA buffer and solvents when extracted from cartilage tissues, and laboratory intensive. The EDTA molecule is a chelating agent, one of the ligands of COMP protein, and had a good affinity for the extraction and purification of human cartilage-derived COMP. It reported that depleted calcium will change the conformation of COMP. Solid-phase extraction (SPE) has become preferable to liquid-liquid extraction (LLE) for the extraction of inorganic and organic analytes in environmental and biological fluids due to restated privileges, for instance, the ease of operation, high enrichment factors, suitable selectivity, facile automatization, enough flexibility, low needs of solvents and hence being less expensive and toxic to the environment in addition to being fast. In the other study, albumin was used as a model of acidic protein with an isoelectric point around four (pI=4), and monolayer adsorption followed the Langmuir model of isotherm when solid phase extraction was done using magnetic solid phase extraction. In a two-hour extraction process, it formed a corona while using a little solvent in bare magnetic particles. Meanwhile, the co-precipitation method and electrodeposition of the synthesis of iron oxide-coated EDTA (mag@EDTA) are widespread, while the cathodic electrodeposition of mag@EDTA synthesized faster and reported a better crystallinity property compared to the co-precipitation method, whereas the cathodic
electrodeposition method synthesized mag@EDTA faster and reported a better crystallinity property than the co-precipitation method. Before actual sample testing is performed in samples rich in mineral and trace element matrices such as human cartilage, blood, and urine in the future, it is important to synthesize EDTA-coated magnets and predict an adsorption model for COMP. In this report, we optimized the electrodeposition plate size and carbon electrode type for large-scale electrodeposition to further investigate the adsorption of mag@EDTA to recombinant human COMP.

EXPERIMENTAL

Material and Methods
Material: Ferric and ferrous chloride were analytical grade from Merck, disodium EDTA from Loba Chemie AR 99% (No. Cat. 0585800500). Pure human COMP recombinants (catalog no. PKSH031804) and ELISA reagent for quantifying COMP (catalog no. E-EL-H0654) were from elabscience™. The 316L 2B stainless steel cathode and graphite-carbon anode were from a local source.

Synthesis of Magnetic Adsorbent
Electrodeposition Magnetite
Electrochemical methods made synthesized magnetic particles. The chamber assembly position consists of a cathode stainless steel plate between anode graphite rods, or carbon graphite plates spaced 5-7 cm apart. A mixture of 0.005 M ferric chloride and 2:1 M ferrous chloride in demineralized water gives a suitable electrolyte without additives at room temperature. The DC electric power supply ran at ten mA/cm² for 15 to 30 minutes. The cathode plate is cleaned with demineralized water, and the black crust is taken out and dispersed in methanol, then centrifuged at 3000 rpm for 20 minutes and cleaned with water. The precipitate was attracted using a neodymium magnet and evaporated at 90 °C for 4-12 hours to remove water. The black powder obtained is referred to as the uncoated particle.

Electrodeposition Magnetite Coated EDTA
An electrolyte mixture of 1 gram of EDTA per liter of demineralized water with a stirring magnet for 10 minutes. Next steps, FeCl₃ and FeCl₂, are added to the EDTA solution and stirred with a magnet for 10 to 15 minutes. Electrodeposition experiments were performed on an electrolytic device with a current of 8 to 10 mA/cm² for 30 minutes. The plate containing debris was washed gently in demineralized water until free of hematite and excess EDTA. Rinse with methanol and centrifuged at a speed of 3000 rpm for 20 minutes, the dark deposits are ultimately distinguished from suspension using a neodymium magnet bar and dried in an oven for 2 to 4 hours at 90°C.

Characterization Magnetite Coated EDTA
Particle form was visualized using a scanning electron microscope (Hitachi SU-3500, voltage 20 kV). X-ray diffraction determines the Co Ka radiation of the crystal powder phase at 1.789 (Bruker D8 Advanced). Analysis of graph pattern peaks are standardized to the JCPDS map for magnetite, then predict crystal size based on the Scherrer formula. The diameter size of particle distribution and ζ zeta potential were studied using dynamic light scattering (DLS, Horiba SZ 100), and measurements were taken to prepare functional and non-coated particle dispersions with a concentration of 0.1 mg iron per liter of distilled water. F-TIR verifies the detection of functional groups of EDTA attached to the surface of particles. The F-TIR spectrum is examined with the Shimadzu 8400S spectroscope tool. Every F-TIR spectrum was acquired from 4000 to 250 cm per cm wavenumber after ten scans at broad spectra of 4 wavenumbers. Determination of magnetic properties of both coated and non-coated particles are studied in the range of below 10,000 oersteds (Vibrato meter, model: OXFORD VSM1.2H). Magnetic hysteresis and magnetization saturation measurements were performed on both EDTA-coated and non-EDTA-coated particles.

Detection Method
COMP was quantified by calculating the optical density of the sample using a linear regression curve fitted to a serial standard COMP solution. Recombinant concentrations of COMP were prepared by stepwise dilution from 40 ng/ml to 0 ng/ml. Each solution concentration was added to five adjacent wells (100 µL per well) while samples were added to other wells (100 µL per well). The plate was covered with the sealant provided in the kit and incubated at 37°C for 90 minutes. Biotinylated Detection Ab/Ag was performed by
removing the liquid from each well without washing, then adding 100 µL of biotinylated Detection Ab/Ag working solution to each well and covering with the plate sealer. After mixing, it was incubated at 37°C for 1 hour. Washing is performed by adding 350 µL of wash buffer to each well three times. 100 µL of HRP conjugate working solution was added to each well, covered and incubated at 37°C for 30 minutes. Wash five times, then add 50 µL of substrate to each well, cover and incubate at 37 °C for approximately 15 minutes. The reaction should not exceed 30 minutes before adding 50 µL of Stop Solution to each well. The optical density (OD value) determination of each well at once with a microplate reader was set at 450 nm.

RESULTS AND DISCUSSION

Functional Groups Analysis
The scanning of the wavelength spectrum starts at wave number 4000 to 250 1/cm. Functional group analysis is observed in hydroxyl, amine, carboxyl groups, and iron oxide (Fe-O), as shown in Fig.-1.

Fig.-1: The FTIR Spectra Analysis of EDTA and Iron Oxide Groups

The hydroxyl groups' spectrum stretches vibrations from 3450 to 3225 cm\(^{-1}\) wavenumber. At the carboxyl groups, vibrational suppression occurs in the wavelength range of 1605 to 1560 per cm and 1440 to 1360 cm\(^{-1}\) wavenumber. The N-H stretch group occurs at a wavenumber range of 3400 to 3270 cm\(^{-1}\) wavenumber. The C-N stretch group ranges from 1145 to 1040 cm\(^{-1}\) wavenumber. However, the deformation N-H group was strong in 805 to 705 cm\(^{-1}\) wavenumber. The iron oxide groups were seen at a stretch observed at about 424.3 cm\(^{-1}\) wavenumber for the spinel structure. This spectra consistent with magnetite coated EDTA in aqueous solution.\(^{15,14,18}\)

XRD Analysis
Braggs angle \(\Theta\) using the equation:

\[
\text{Tan}2\Theta = \frac{R}{D}
\]

Where D is the specimen to film distance and 2R is the diameter of specimen

XRD results showed that mag@EDTA had 18 peaks typical of JCPDS magnetite material standard #01-088-0315. Crystallinity has been calculated to range from 10,000 to 89,993 theta degrees. The mag@EDTA crystallinity percentage is approximately 69.3%, while magnetite is approximately 67.4%, with amorphous percentages of 30.7% and 32.6%, respectively. The Scherrer equation data showed that the particle size was about 52 to 56 nanometers.

Fig.-2: Mag@EDTA Peak Analysis from the Electrochemistry Process
This finding showed that hematite and goethite phases are still major problems in aqueous based short periods synthesis. The time and pH dependence of the transformation phase is a non-stoichiometric mechanism. At a transition pH of 7.00, ferrihydrite converted to goethite. Conversely, at a pH of at least 7.50, ferrihydrite converted to magnetite within only 1.5 hours, with a structural Fe$^{3+}$: Fe$^{2+}$ molar ratio of 1:0.4.

**Magnetization Measurement Results**

Figure-3 showed the shapes of uncoated and EDTA-coated particles at 20-25°C temperature. The magnetic possession of the particles synthesized by electrodeposition utilized a vibrating sample magnetometer (VSM). Both hysteresis patterns have entirely adjustable S shapes supporting the extremely paramagnetic variety of one and the other. The particle remnant and coercivity standard can be figured at the magnetic field conditions (as visualized in Fig.-3). The mag@EDTA particles show magnetic saturation of 51.86 emu per gram, the magnetic remnant of 8.46 emu per gram, and remanent coercivity of 2.3 oersted’s for the uncoated magnetic saturation of 65.76 emu per gram and remanent magnetization of 9.58 emu per gram with remanent coercivity 1.1 oersted. The presence of an antiferromagnetism plane can indicate the decrease in the Ms. of the EDTA-capped particle. It matched several EDTA coated with magnetic nanoparticles.

**Particle Morphology**

The scanning electron microscope showed that the magnetite crystal structure presented an amorphous form and varied in size at submicron. The mag@EDTA crystals in Fig.-4B appear to be more evenly distributed and larger than uncoated magnetite, with fewer clusters of similar particles. The increased concentration of iron oxide EDTA in the solution leads to an accumulation of this molecule on goethite (α-FeOOH). In the pH 4-7 range, this dissolution and reabsorption process increases the total EDTA concentration on the surface complexes stable during this process.

**Charge Measurement and Particle Distribution**

The optimization study evaluated the magnetite electrodeposition electrode and mag@EDTA design setup using the indicator of particle size in the water medium (z-average) from deposition scrap material from the
cathode of 316L 2B stainless steel plate. For pure magnetite electroplating, optimal cathode size is seen from plate length and width of 4 x 10 cm, while the optimal graphite-carbon anode is cylindrical, with the measured average particle size (z-average) 4417.1 nanometer. The mag@EDTA electrodeposition, which has a functional molecule-tagged EDTA-optimized run-on anode plate with a width of 4 cm and a length of 10 cm, has a particle deposition size of 4085.7 nanometers. Particle distribution size in water ranging between 1 and 1000 microns, which could be used as a protein adsorbent in protein purification referred to common high scale production magnetic beads carrier.

Table-1: Electrode Optimization of Magnetite@EDTA Synthesis at Room Temperature

<table>
<thead>
<tr>
<th>Name of plat</th>
<th>Stainless steel size (cm)</th>
<th>Graphite size (cm)</th>
<th>Current (mA)</th>
<th>Zeta (mV)</th>
<th>pI</th>
<th>Z-average (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe₃O₄ Plat (1)</td>
<td>4 x 10</td>
<td>4 x 10</td>
<td>400</td>
<td>-37.7</td>
<td>2.222</td>
<td>6699.4</td>
</tr>
<tr>
<td>Fe₃O₄ Rod (2)</td>
<td>4 x 10</td>
<td>1 x 10</td>
<td>400</td>
<td>-53.9</td>
<td>1.775</td>
<td>4417.1</td>
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<tr>
<td>Fe₃O₄ Rod (3)</td>
<td>20 x 20</td>
<td>1 x 20</td>
<td>2000</td>
<td>-47.2</td>
<td>1.977</td>
<td>5888.4</td>
</tr>
<tr>
<td>Fe₃O₄ EDTA Plat (1)</td>
<td>4 x 10</td>
<td>4 x 10</td>
<td>400</td>
<td>-58.8</td>
<td>1.463</td>
<td>4085.7</td>
</tr>
<tr>
<td>Fe₃O₄ EDTA Rod (2)</td>
<td>4 x 10</td>
<td>1 x 10</td>
<td>400</td>
<td>-46.7</td>
<td>1.945</td>
<td>5901.2</td>
</tr>
<tr>
<td>Fe₃O₄ EDTA Rod (3)</td>
<td>20 x 20</td>
<td>1 x 20</td>
<td>2000</td>
<td>-48.8</td>
<td>2.245</td>
<td>7846.8</td>
</tr>
</tbody>
</table>

Adsorption Studies Mag@EDTA COMP

Freundlich equation (1906):

$$\log q_e = \log K_F + \frac{1}{n} \log C_e$$

(1)

Where $C_e$ is the equilibrium liquid phase concentration (mg/L), $C_0$ is the initial liquid phase concentration (mg/L), $k_1$ is the first-order adsorption rate constant (1/min), $k_2$ is the second-order adsorption rate constant (g/mg 1/min), $K_F$ is the Freundlich isotherm constant related to the adsorption capacity [(mg/g) (1/mg)1/n]. $K_L$ is the adsorption energy constant of the Langmuir adsorption isotherms (1/mg) while 1/n is the Freundlich isotherm constant related to the adsorption intensity. $q_e$ is the equilibrium concentration of solid-phase adsorbate (mg/g), $Q_m$ is the maximum surface coverage (monolayer formation) of the sorbent (mg/g) $q_t$ is the amount of adsorption at time t (mg/g), $R^2$ is the correlation coefficient, $R_L$ is the dimensionless separation factor $V$ The volume of the solution (mL), while W is the mass of the adsorbent (g).

Langmuir's equation (1917)

$$\frac{C_e}{q_e} = \frac{C_e}{Q_m} + \frac{1}{Q_m K_m}$$

(2)

Temkin's equation:

$$q_e = \frac{RT}{b} \ln K_T + \frac{RT}{b} \ln C_e$$

(3)

Where $K_T$ is the equilibrium binding constant (L/mol) corresponding to the maximum binding energy, $b$ corresponds to the adsorption heat, R is the universal gas constant (8.314 J/K 1/mol) and T is the temperature (K). Stitching $q_e$ with respect to ln($C_e$) (Equation III.3) yields a straight line of slope $RT/b$ and intercept ($RT \ln K_T/b$).

From Table-3. The regression coefficient value of 0.9370 showed that the Freundlich model equation was the best-fitting model for adsorption and desorption compared to Langmuir and Temkin. The Freundlich constant value of 1.09313 x 10⁻³¹ explains that interaction between adsorbent and protein was weak and multilayer or heterogeneous. In this case, the recombinant COMP was bound to an amorphous magnetic EDTA particle. This low binding affinity could indicate the differential biological activity of these particles towards COMP. In protein immobilization, considering the pH and ionic strength of the solution, only weak interactions such as hydrophobic and van der Waals interactions and hydrogen bonds keep the protein molecule on the surface of the magnetic adsorbent.

Table-2: The Results of the COMP and mag@EDTA Adsorption Study

<table>
<thead>
<tr>
<th>Concentration Mag.EDTA</th>
<th>C_i (mg/L)</th>
<th>C.e (mg/L)</th>
<th>1/C_e</th>
<th>Log C_e</th>
<th>Ln C_e</th>
<th>q_e (mg/g)</th>
<th>1/q_e</th>
<th>Log q_e</th>
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<tr>
<td>0.01</td>
<td>0.00120</td>
<td>0.00023</td>
<td>4443.8</td>
<td>-3.6478</td>
<td>-8.3993</td>
<td>0.000195</td>
<td>5136.4</td>
<td>-3.7107</td>
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<td>0.02</td>
<td>0.00184</td>
<td>0.00021</td>
<td>4708.3</td>
<td>-3.6729</td>
<td>-8.4571</td>
<td>0.000652</td>
<td>1532.9</td>
<td>-3.1855</td>
</tr>
</tbody>
</table>

ADSORPTION OF CARTILAGE OLIGOMERIC MATRIX

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Heterogeneous and multilayer COMP adsorption at the surface of the mag@EDTA model was relevant to the other study of acidic and hydrophobic protein (bovine serum albumin), which reported that the distribution of nanoparticle sizes in aqueous medium ranging from 30 to 1000 nm affected conformational changes, formation of a double layer at the surface, and multiple functional layers in the structure of magnetic particles in which the magnetite phase is easily oxidized to maghemite. However, these properties have been advantages of faster and easier desorption of the human low abundant protein from matrix sample such as urine, synovial fluids, and bloods by customizing the ionic strength elution, pH of the buffered system at room temperature as sample extraction and enrichment platform to further support the purification-based chromatography analysis, and proteomic analysis.

**CONCLUSION**

FT-IR spectrum analysis demonstrated baseline agreement that magnetite particles had successfully bound EDTA. The magnetite EDTA phase has been standardized with the JCPDS chart and has an average crystal size of 60.6 nanometers. Magnetite-EDTA particles are dispersed in aqueous solvents with submicron particle sizes range of 4085.7 nanometers to 7846.8 nanometers and a potential zeta of -58.8 mV to -48.8 mV. It allows electrostatic and hydrogen bonding with the acidic recombinant protein COMP. Magnetite-EDTA can rapidly adsorb COMP and potential platform in protein extraction at room temperature.

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**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

**AUTHOR CONTRIBUTIONS**

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

- A. Furqon [https://orcid.org/0000-0002-5072-6972]
- T. Gusdinar [https://orcid.org/0000-0002-2478-2022]
- S. Ibrahim [https://orcid.org/0000-0002-2726-0692]
- E. Julianti [https://orcid.org/0000-0002-5504-6140]

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