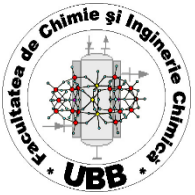

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FACULTY OF CHEMISTRY AND CHEMICAL ENGINEERING
DEPARTMENT OF CHEMICAL ENGINEERING
SCIENTIFIC RESEARCH CENTER IN PHYSICAL CHEMISTRY

PhD thesis summary

THE PHYSICO-CHEMICAL STUDY OF SOME NATURAL COMPOUNDS WITH BIOLOGICAL ACTIVITY

PhD candidate:
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SCIENTIFIC ADVISOR:
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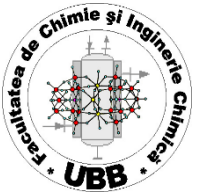
CLUJ-NAPOCA
2022



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Defense date: 28 September 2022

TABLE OF CONTENTS:

KEYWORDS

curcumin, whey protein concentrates, PEG4000, PEG6000, silymarin, resveratrol, antioxidant activity, stability, solubility, molecular docking

INTRODUCTION

Curcumin (CCM) is the predominant component (70%) of turmeric extract, it is a natural polyphenol that has antioxidant, anti-inflammatory and anti-carcinogenic action. It is a compound that shows sensitivity to light, air and heat, and also has low solubility in water. Its pharmacological action is diminished due to sensitivity to light, hydrophobic structure and chemical instability, respectively. The main purpose of this doctoral thesis is to determine some preparation techniques in order to improve the solubility and stability of curcumin in various aqueous environments of pH 1.5 (simulated g astric environment), pH 7.4 (simulated physiological environment) and pH 8.0 (simulated intestinal environment) and at different temperatures: 296 K, 301 K, 309 K and 313 K.

In this context, in **Chapter 1** several strategies were developed, namely: encapsulation of CCM in protein, for example in whey protein concentrate, by complexation in various environments with controlled pH and depending on temperature, by co-precipitation and drying by spraying, SD, or by lyophilization, FD.

The main purpose of this doctoral thesis is to determine some preparation and characterization techniques in order to improve the solubility and stability of curcumin, described in **Chapters 2 and 3**. In order to determine the relationship between the preparation method and the properties/applications of the obtained product, depending on the characteristics of the material investigated, a large number of physico-chemical characterization methods were used.

Chapter 4 opens an interesting topic, namely the use of nanostructured nanomaterials for the transport of physiologically essential ions or biologically active molecules in vitro or in vivo. The aim of this study was to analyze the response of cultured osteoblasts on strontium-substituted hydroxyapatites (HAP-Sr) of well-defined high crystallinity deposited as thin films on glass slides. To date, this aspect has not been carefully investigated in the context of bio-ceramics. In this study, we present osteoblast activity on HAP-Sr synthesized for different amounts of strontium substituting calcium within the hydroxyapatite network ($Ca_{10}(PO_4)_6(OH)_2$, HAP), namely HAP-5%Sr, HAP-10 %Sr, HAP-15%Sr and HAP-59.2%Sr (Sr-HAP, with the formula $Sr_{10}(PO_4)_6(OH)_2$), in comparison with pure stoichiometric HAP, chosen as a control. Each bioceramic was deposited as a thin self-assembled multilayer substrate (scaffold) and chemically bonded to the surface of the glass plates. The use of these nanomaterials as a curcumin transport vehicle is in progress in the Physical Chemistry Research Center for use in orthopedic implants against infections.

In **Chapter 5**, the solid dispersed system with a high curcumin content was prepared with whey protein concentrate in a molar ratio of 5:1 by spray drying. X-ray powder diffraction and DSC analysis show the formation of the solid dispersed system in the amorphous state, and FTIR analysis identifies the presence of weak hydrogen

bonding interactions between the system components. SEM images show a homogeneous morphology of spherical microparticles.

Chapter 6 describes the molecular interaction between curcumin, CCM, and whey protein concentrate, WPC, which was studied using fluorescence measurements. The binding number (n), binding constant (KS) and thermodynamic parameters (ΔG, ΔH and ΔS) were determined at different temperatures and pH values of the aqueous solutions. The thermodynamic and structural approach of the binding mechanism between CCM and WPC constitutes a first in the specialty literature. The highest stability of this CCM-WPC complex was revealed in acidic pH environment (e.g. gastric fluid with pH 1.5), decreasing at alkaline pH value (e.g. small intestinal fluid with pH 7.5 to 8) and thus indicating a good stability of this complex in the simulated gastric environment. The CCM-WPC complex as a spray-dried powder was further investigated by XRD, FTIR and AFM imaging. The interaction and binding mechanism was also deepened by molecular docking.

Chapter 7 discusses the stability and solubility of curcumin, CCM, which can be improved by complexing with whey protein concentrate (WPC). CCM-WPC of 1:1 and 1:0.5 molar ratio complexes were encapsulated by two methods, namely spray drying (SD) and freeze/freeze drying (FD). The major objective of this work is to determine the stability and solubility of encapsulated CCM-WPC complexes by SD and FD method. X-ray diffraction and thermogravimetric analysis were performed on crude CCM and WPC as well as their complexes. The stability of these CCM-WPC complexes is studied by UV-Vis spectra and the half-life, $t_{1/2}$, determined by 2nd order kinetics. The IC_{50} antioxidant activity of curcumin was also studied both in the pure state and in the CCM-WPC complexes of various molar ratios.

Chapter 8, uses atomic force microscopy (AFM) to reveal the surface topography of adsorption films for pure curcumin, pure whey protein and their complex (CCM-WPC). The obtained results show that the individual nanoparticles were stabilized in aqueous dispersion and successfully adsorbed on the glass slides in the form of well-organized and smooth thin films with a surface roughness of about 5 nm.

Another strategy is developed in **Chapter 9** and consists in the use of the synthetic polymer, polyethylene glycol, PEG, biocompatible with varied molecular mass: PEG400, PEG4000 or PEG6000, in the melt. By incorporating curcumin into the PEG melt, a stable bicomponent system results. We found that the introduction of whey protein in the bicomponent PEG-CCM composite led to another tri-component PEG-CCM –WPC composite with the improvement of curcumin stability in aqueous media; by adding silymarin, SiL, the resulting composite was: PEG-CCM –WPC-SIL; PEG-CCM –WPC-RESV was also prepared by the same method. Therefore, 8 new materials were prepared, called composites or complexes, multi-component systems containing curcumin. Complex formation was investigated by Fourier Transform Infrared Spectroscopy (FTIR). The thermal stability of all synthesized complexes was investigated compared to the precursors by thermogravimetric (TG) and differential scanning calorimetry (DSC) analysis. Stability studies of CCM were carried out in various aqueous environments and it was found that the composite materials led to a substantial increase in the half-life - of curcumin - contributing at the same time to the

realization of very good quality polycomponent systems that can be used in the food industry or in the pharmaceutical one.

Chapter 10 describes the General Conclusions of the research from the Doctoral Thesis.

Chapter 11 presents the achievements of Drd. Eng. Chem in the scientific research work included in this Doctoral Thesis.

Papers published by the PhD candidate

1. S. Rapuntean, P. T. Frangopol, I. Hodisan, Gh. Tomoaia, D. Oltean-Dan, A. Mocanu, C. Prejmerean, O. Soritau, **L. Z. Racz**, M. Tomoaia-Cotisel, In vitro response of human osteoblasts cultured on strontium substituted hydroxyapatites, Rev. Chim. (Bucharest), 69(12), 3537-3544, (2018). **I.F. 1.605**

2. **L. Z. Rácz**, M. Tomoaia-Cotișel, Cs.-P. Rácz, P. Bulieris, I. Grosu, S. Porav, A. Ciorîță, X. Filip, F. Martin, G. Serban, I. Kacso, Curcumin-Whey Protein Solid Dispersion System With Improved Solubility And Cancer Cell Inhibitory Effect, Stud. Univ. Babes-Bolyai, Chem., 66(3), 209-224, (2021). **I.F. 0.447**

3. **L. Z. Rácz**, G.-A. Paltinean, I. Petean, Gh. Tomoaia, L. C. Pop, G. Arghir, E. Levei, A. Mocanu, Cs.-P. Rácz, M. Tomoaia-Cotisel, Curcumin and Whey Protein Binding And Structural Characteristics of Their Complex Evidenced by Atomic Force Microscopy, Stud. Univ. Babes-Bolyai, Chem., 2022, 67(3), in press. **I.F. 0.447**

4. **L. Z. Rácz**, Cs. P. Rácz, L. C. Pop, Gh. Tomoaia, A. Mocanu, I. Barbu, M. Sárközi, I. Roman, A. Avram, M. Tomoaia-Cotisel, V.-A. Toma, Strategies for improving bioavailability, bioactivity, and physical-chemical behavior of the curcumin, Molecules, 2022, Manuscript ID-1919555. Under review. **I.F. 4.927**

5. **L. Z. Rác**z, Cs.-P. Rácz, O. Horovitz, Gh. Tomoaia, A. Mocanu, I. Kacso, M. Sárközi, M. Dan, S. Porav, G. Borodi, M. Tomoaia-Cotisel, Complexation of Curcumin using Whey Proteins to Enhance Aqueous Solubility, Stability and Antioxidant Property, Stud. Univ. Babes-Bolyai, Chem., 2022, 67(3), in press. **I.F. 0.447**

CHAPTER 1. STRATEGIES FOR IMPROVING THE BIOAVAILABILITY, BIOACTIVITY AND PHYSICO-CHEMICAL BEHAVIOR OF CURCUMIN

Curcumin is one of the most frequently explored plant compounds with various biological actions such as antibacterial, antiviral, antifungal, antineoplastic and anti-oxidant/anti-inflammatory properties. Laboratory data and clinical studies have demonstrated that the bioavailability and bioactivity of curcumin are influenced by the characteristics of the types of curcumin molecular complex. Curcumin has a high capacity to form molecular complexes with proteins (such as whey proteins, bovine serum albumin, β-lactoglobulin), carbohydrates, lipids, and natural compounds (eg, resveratrol, piperine, quercetin). These complexes increase the bioactivity and bioavailability of curcumin. The current review ascertained these derivatization strategies for curcumin in terms of biological and physicochemical aspects, with a strong focus on whey proteins, characterization methods, and thermodynamic characteristics of protein–curcumin complexes. The literature review provides a comprehensive approach to curcumin-biomolecule interactions with regard to strategies to improve curcumin bioactivity as well as bioavailability.

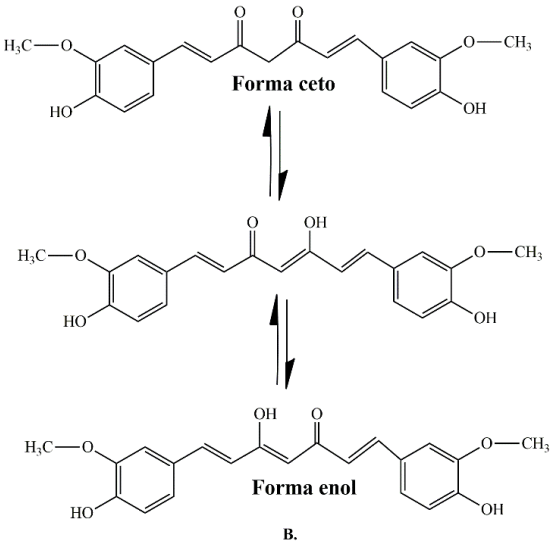

Figure 1. B: CCM enol and ceto forms

Most chemotherapeutic drugs have high toxicity, through their systematic administration they are distributed in the non-targeted body, randomly reaching different organs/tissues, causing multiple side effects. Therefore, the use of compounds with a good safety profile, non-toxic and with high bioavailability would be imperative. Curcumin is a hydrophobic polyphenolic compound [21], with a wide range of pharmacological properties (Figure 2): antiproliferative, anticancer [22-33],

antidiabetic, antioxidant [34], anti-inflammatory [35-37], antifungal and antimicrobial [38], which is why the attention of researchers turned to its study in the biomedical field. In addition, CCM as an antiviral [38-40] and anti-inflammatory agent [41] may be useful for the prevention and treatment of COVID-19. This aspect is very well documented in literature: CCM prevents inflammatory diseases, inhibits carcinogenesis, controls neurological, respiratory, cardiovascular, metabolic, autoimmune diseases and some cancers [38-40].

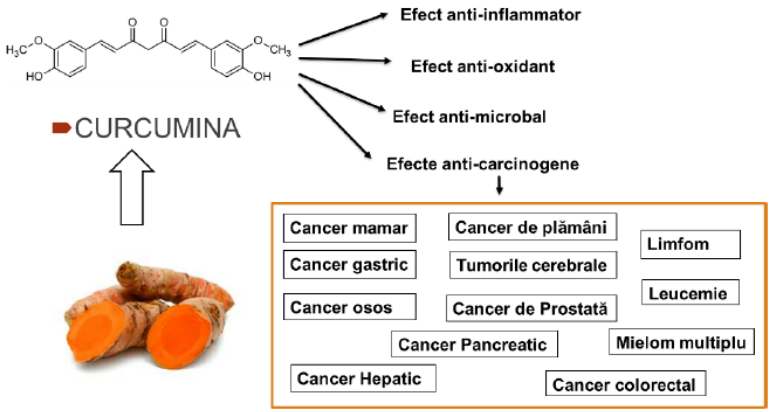

Figure 2: The therapeutic effects of CCM were reflected by a wide spectrum of action, as observed by the literature. The antitumor action of CCM has been extensively explored under clinical and experimental conditions.

The biggest disadvantage of CCM is its low solubility in water, hence its low bioavailability; there are many publications on this topic. The methods to eliminate these disadvantages of CCM were exposed by: obtaining solid dispersions of the second and third generation, complexing CCM with proteins, adding different flavonoids or adjuvants, like piperine, to CCM. PEG-ylation can improve the deficiencies of CCM, but combination by other techniques or other polymers has further improved its properties. Another alternative is the complexation of CCM with proteins, which greatly improves the water dispersibility, stability and bioavailability of CCM. The best results for improving CCM properties can be obtained by combining all the listed techniques. Identifying the correct method of formulation and use of appropriate excipients in relation to CCM can result in effective pharmacological treatment by administering a lower dose, which can have a significant cost implication.

CHAPTER 2. THE ENGINEERING OF DRYING FOOD AND PHARMACEUTICAL PRODUCTS

The development of pharmaceutical products has proven to be a challenge, due to the complexity of their production and purification, but also their physical and chemical stabilization. In order to have an acceptable shelf life as a pharmaceutical product, for a product in solid form it is necessary to combat instability under the action of environmental factors. The most important factor in the deterioration of product quality is the water included in medicinal products and food, so a drying method must be used to remove it. Well-known technologies are lyophilization, spray drying and low pressure drying [1].

Lyophilization is the most common method to produce solid-state proteins, but this procedure can bring many drawbacks during freezing and drying, such as changes in pH, ice crystal formation, change in dissolution concentration, and others. These problems can cause significant denaturation in proteins, so the need arises for the use of stabilizers during cooling and drying procedures [1, 4, 5].

Spray drying is a known method for producing small-sized particles by transforming a fluid material into dry particles in a hot gaseous drying environment [11, 12]. Since its discovery, the spray drying technique has been improved in terms of its design and operational applications. The transformation of the fluid into dry particles has a very weak effect on its quality, the method being common and widespread as an encapsulation technology in the food industry [12, 14]. The process is suitable for heat-sensitive substances such as phenolic compounds because the spray drying temperature is relatively low (generally the inlet temperature is less than 200 °C) and the droplet/particle residence time is very short (within seconds). This method consists of complex interactions of equipment components, processing parameters and physical properties of materials, which all together influence the characteristics of the final product [14]. It should be noted that the difference between the parameters of the drying process and the characteristics of the materials that make up the complex, regulates the microencapsulation process, which ultimately influences the properties of the obtained powder, such as fluidity, stability, solubility, shape, size, moisture content, efficiency process and bulk density [14, 15].

CHAPTER 3. COMPLEX CHARACTERIZATION METHODS

In order to determine the relationship between the preparation method and the properties/applications of the obtained product, depending on the characteristics of the investigated material, a large number of physico-chemical characterization methods were used. In the following, the methods and devices used are presented - for the most complex characterization of the obtained products:

• Morphological analysis performed by transmission electron microscopy (TEM), scanning electron microscopy (SEM) and atomic force microscopy (AFM)
• Structural analysis: powder X-ray diffraction (PXRD) and Fourier transform infrared spectroscopy (FTIR)
• Thermal analysis: thermogravimetry (TGA), derivative thermogravimetric curve (DTG) and differential scanning calorimetry (DSC)
• Analysis of the interaction between WPC and CCM – from fluorescence spectroscopy studies
• Stability and solubility testing of curcumin – from research using UV-Vis spectrophotometry
• Thermodynamic analysis – ΔG, ΔH and ΔS
• Cytotoxicity research on A375 and A549 cell cultures
• MTT assay for cell viability
• Biocompatibility of nanomaterials using osteoblast culture

CHAPTER 4. IN VITRO RESPONSE OF HUMAN OSTEOBLASTS CULTURED ON STRONTIUM SUBSTITUTED HYDROXYAPATITES

The aim of this study was to analyze the response of cultured osteoblasts on strontium-substituted hydroxyapatites (HAP-Sr) of well-defined high crystallinity deposited as thin films on glass slides. To date, this aspect has not been carefully investigated in the context of bioceramics. In this study, we present osteoblast activity on HAP-Sr synthesized for different amounts of strontium replacing calcium within the hydroxyapatite network ($Ca_{10}(PO_4)_6(OH)_2$, HAP), namely HAP-5%Sr, HAP-10 %Sr, HAP-15%Sr and HAP-59.2%Sr (Sr-HAP, with the formula $Sr_{10}(PO_4)_6(OH)_2$), in comparison with pure stoichiometric HAP, chosen as a control. Each bioceramic was deposited as a thin self-assembled multilayer substrate (scaffold) and chemically bonded to the surface of the glass plates. These coatings revealed by AFM and SEM imaging a granular texture consisting of bioceramic nanoparticles. They possess a high degree of crystallinity, i.e. 68% to 86%, depending on the amount of Sr in the HAP network, as seen by XRD analysis. Osteoblasts were cultured for up to 21 days and showed enhanced adhesion and proliferation, particularly evident at relatively high strontium contents (especially 5 and 10 wt%, as determined by SEM-EDX), where alkaline phosphatase activity and collagen type I were strongly highlighted.

These bioceramics showed a high biocompatibility in vitro stimulating the activity of osteoblasts in the bone formation process. These nanobiomaterials may have applications in orthopedic and dental surgery, improving osseointegration as bone implant coatings, as well as for bone repair and regeneration. High-purity stoichiometric nanohydroxyapatite, HAP: $Ca_{10}(PO_4)_6(OH)_2$ and strontium-substituted nanohydroxyapatites, HAP-Sr, for different amounts of Sr: HAP-5% Sr; HAP-10% Sr; HAP-15% Sr; HAP-59.2%Sr (i.e., Sr-HAP: $Sr_{10}(PO_4)_6(OH)$), were synthesized by template compd.-assisted wet chemical methodology. After lyophilization, the organic templates were removed by calcination in two steps, at 550 ^oC for 6 h and then at 850 ^oC for 4 h.

Hydroxyapatite is used as a vehicle for the transport of physiologically essential ions.

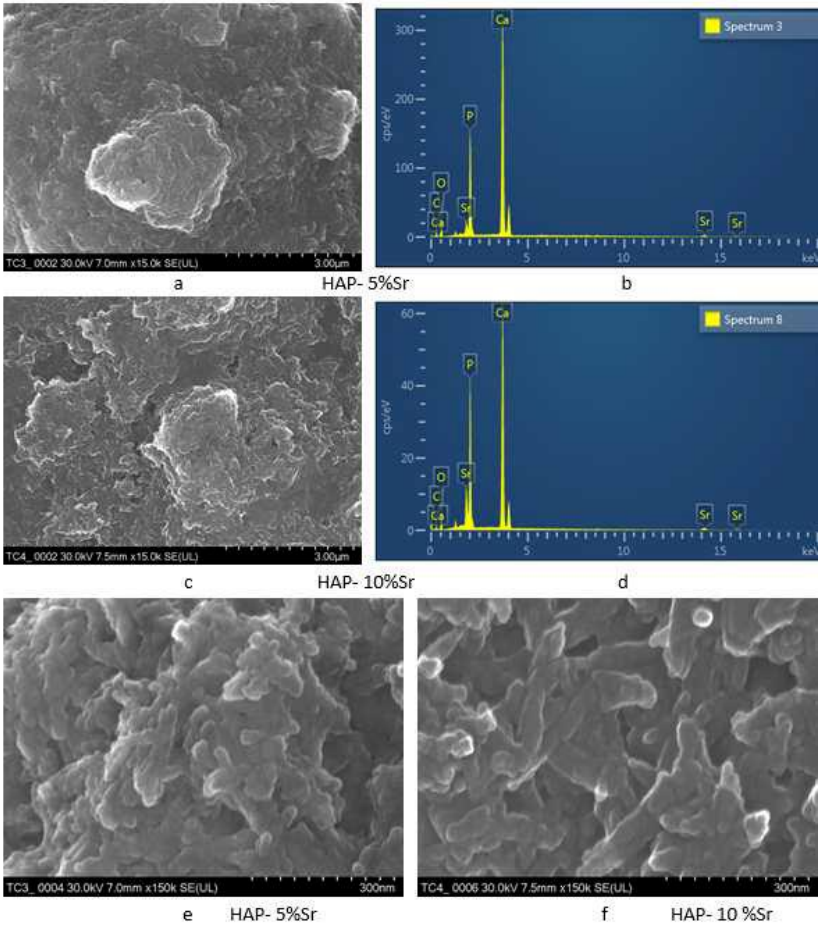

Figure 1. SEM images and EDX spectra for freeze-dried, calcined HAP-5%Sr (a, b, e) and HAP-10%Sr (c, d, f) substrates

CHAPTER 5. CURCUMIN-WHEY PROTEIN SOLID DISPERSIONS WITH ENHANCED SOLUBILITY AND CELL-INHIBITING EFFECT

The high curcumin solid dispersed system was prepared with whey protein concentrate in a molar ratio of 5:1 by spray drying method. X-ray powder diffraction and DSC analysis show the formation of the solid dispersed system in the amorphous state, and FTIR analysis identifies the presence of weak hydrogen bonding interactions between the system components. SEM images for the system show a homogeneous morphology of spherical donut-like microparticles. The solubility of curcumin in the dispersed system was improved compared to pure curcumin, which is practically insoluble in water, reaching a value of 70 μg/mL in buffer aqueous solution at pH 8, similar to the intestinal environment. The synthesized material shows a more pronounced cytotoxic effect on skin melanoma cells compared to lung adenocarcinoma cells, but in both cases the effect is promising, and through more complex analyzes the antitumor potential of CCM-WPC_SD could be further exploited.

To demonstrate the formation of the CCM-WPC_SD solid dispersed system obtained by incorporating CCM into WPC, in an optimized 5:1 molar ratio, it was first analyzed by XRPD.

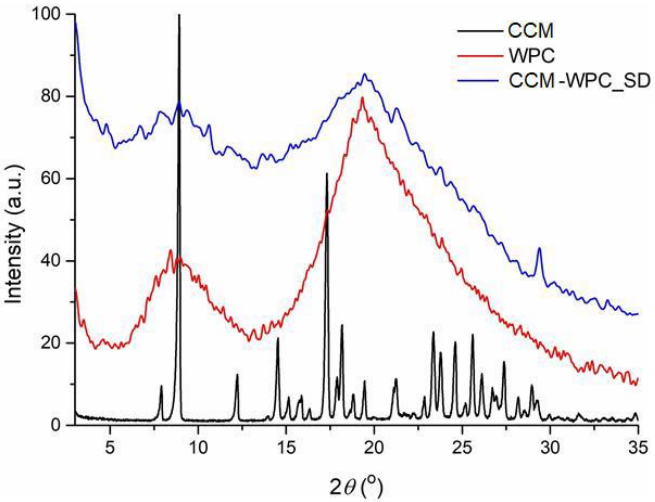

Figure 2. XRPD spectra for CCM, WPC and CUC-WPC_SD

Analyzing the DSC curve of CCM-WPC_SD a broad endothermic signal of low intensity can be observed between 40 and 90°C attributed to the removal of residual unbound water, the melting signal of CCM is missing, indicating the loading of curcumin into WPC at a stoichiometric ratio of 5:1.

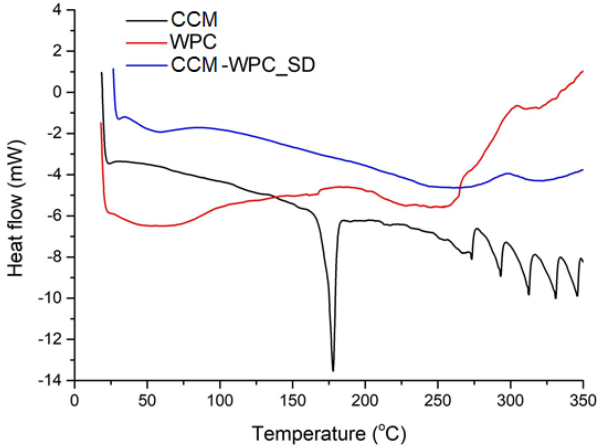

Figure 3. DSC curves for CCM, WPC and CCM-WPC_SD
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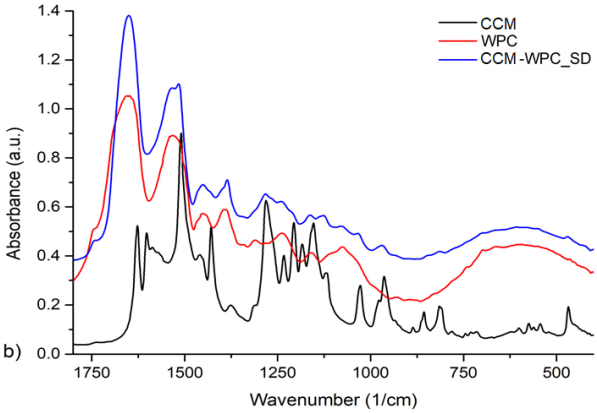

Figure 4. FTIR spectra for CCM, WPC and CCM-WPC_SD, spectral domain a) 3750-2750 cm^{-1} and b) 1800-400 cm^{-1}
Studia UBB Chemia, 66(3), 209-224 (2021)

The changes undergone by some vibrational bands, especially those characteristic of the OH and COOH groups, suggest the existence of weak hydrogen bonds and electrostatic interactions between the two components of the CCM-WPC_SD dispersed system.

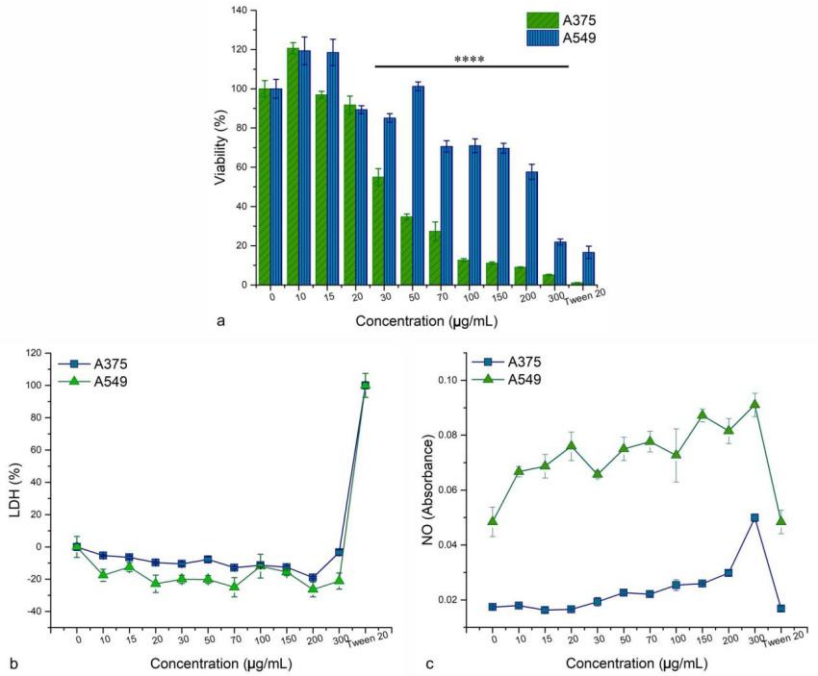

Figure 8. Cytotoxicity assays performed on A375 and A549 cells treated with CCM-WPC_SD. a) The MTT assay shows the degree of deterioration of cell viability in 24 hours. b) The LDH test shows the amount of LDH released into the culture medium of cells treated with the drug for 24 hours. c) The nitric oxide (NO) test shows the amount of nitrogen oxide released into the culture medium of the treated cells.**Studia UBB Chemia, 66(3), 209-224 (2021)**

The calculated LDH values show no significant difference between the two cell lines (Figure 8b). Compared to cells treated with Tween 20, no necrosis was observed, however, the negative values recorded could indicate a membrane blockage [6]. A direct dose-dependent response was observed in A375 cells by recording high values of nitric oxide (NO) at concentrations above 30 µg/mL. The results are consistent with the effects observed in the MTT assay. A dose-dependent response was also observed for A549 cells, however the trend was more chaotic (Figure 8c), compared to skin melanoma cells. However, the highest value recorded for nitric oxide concentration was associated with the lowest value recorded for the MTT assay, in both cell types.

CHAPTER 6. THE RELATIONSHIP BETWEEN CURCUMIN AND WHEY PROTEIN CONCENTRATE. THERMODYNAMIC AND STRUCTURAL APPROACH

The molecular interaction between curcumin, CCM, and whey protein concentrate, WPC, was studied using fluorescence measurements. The binding number (n), binding constant (K_S) and thermodynamic parameters (ΔG, ΔH and ΔS) were determined at different temperatures and pH values of the aqueous solutions. Thermodynamic approach revealed the highest stability of this CCM-WPC complex in acidic pH medium (e.g. gastric fluid with pH 1.5), decreasing at alkaline pH value (e.g. small intestinal fluid with pH 7.5 to 8) and thus indicating a good stability of this complex in the simulated gastric environment. Then, in the alkaline pH that is characteristic of the small intestine environment, this complex can release CCM to enter the bloodstream and be directed to its site of action. The spray-dried CCM-WPC complex was further investigated by XRD, FTIR and AFM imaging. X-ray powder diffraction shows that due to the chemical interaction between CCM and WPC, a new amorphous compound is formed. The FTIR spectra provide strong evidence for the interaction of CCM with WPC and the formation of the CCM-WPC complex. AFM images revealed the nanostructure of CCM, WPC and CCM-WPC complex with an average particle size of 30 nm to 60 nm. Docking studies showed the hydrophobic interactions between CCM and WPC, in agreement with the formation, driven by the entropic component, of CCM-WPC complexes, leading to a better understanding of the binding mechanism between these components. Therefore, this chapter confirmed that WPC is a promising carrier for CCM, which can lead to a functional dietary supplement through the incorporation, distribution and high stability of these bioactive compounds, together enhancing their biological effects.

The binding parameters, the binding number (n, the number of binding sites) and the association constant (K_S, the binding constant) [57, 58, 61, 62], characterizethe equilibrium:

$$n\ CCM + WPC \rightleftharpoons (CCM)_nWPC$$

$$K_s = \frac{[(CCM)_nWPC]}{[CCM]^n[WPC]}$$

where the square brackets indicate the equilibrium concentration of the respective species. They can be calculated according to the following equation (Eq.1) [57, 64], for static quenching:

$$\log\frac{F_0-F}{F} = \log K_S + n\log[CCM] \quad (1)$$

where: F – fluorescence intensity, F_0 - maximum intensity (intrinsic fluorescence of pure WPC) and [CCM]—molar concentration of CCM.

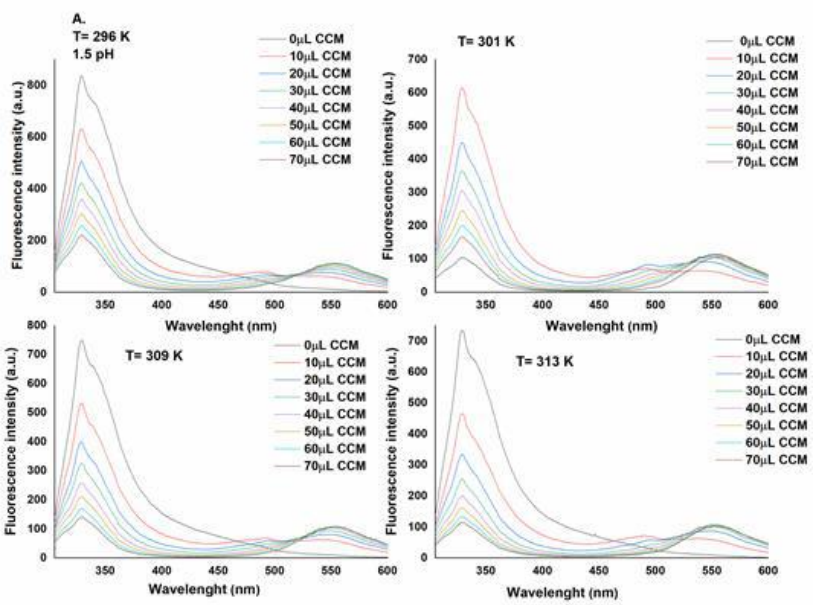

Figure 1. Fluorescence emission spectra of WPC solutions (2 mL solution, concentration $2x10^{-6}$M) obtained by gradual titration with 10µL of CCM solution ($2x10^{-3}$ mol/L), resulting in CCM concentrations between 10^{-5} – $6.8x10^{-5}$ M (see Table 1), pH 1.5 at four constant temperatures (296, 301, 309 and 313K), indicated on the graphs. The maximum of the fluorescence spectra is at the wavelength of 328 nm; spectra were recorded in the range of 300-600 nm with an excitation wavelength of 295 nm.

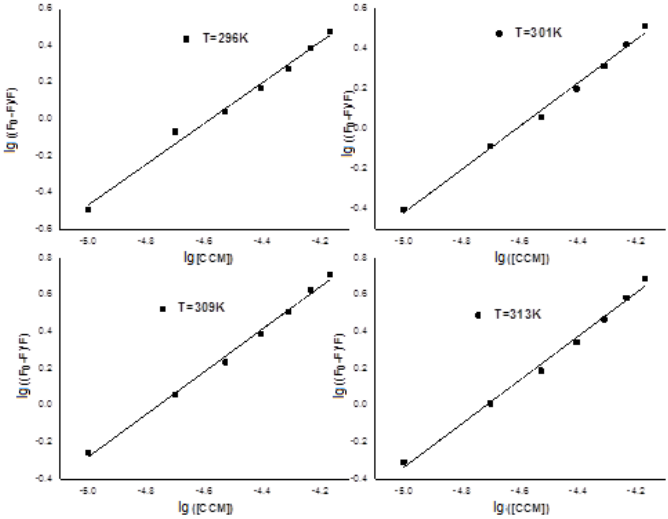

Figure 2. Regression lines y = a + bx of lg((F$_0$-F)/F) vs lg([CCM]) at pH value 1.5 and at the studied temperatures: 296, 301, 309 and 313K. Decimal logarithm is denoted by lg.

The change in enthalpy (ΔH) and entropy (ΔS) were calculated from the Van`t Hoff equation (Eq. (2), by plotting log K$_S$ versus 1/T (T-absolute temperature) [57, 58, 62, 63].

$$\ln K_S = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (2)$$

Where, K$_S$ - the equilibrium association constant (L/mol) at the corresponding temperature (T in K) and R - the universal gas constant [J/(mol·K)]. The temperatures used were between 296 and 313 K. ΔH and ΔS were determined from the slope and y-intercept, respectively, of the Van`t Hoff regression line. The free energy change (ΔG) was calculated using Eq. (3) [62] and Eq. (4) [63, 64]:

$$\Delta G = \Delta H - T\Delta S \quad (3)$$

$$\Delta G = -RT\ln K_S \quad (4)$$

The negative value of ΔG reveals that the interaction process is spontaneous (Table 4) [61]. The positive ΔH and ΔS values of the interaction between CCM and whey protein concentrate (Table 4) emphasize that hydrophobic forces play a major role in the binding process [65, 66], binding is mainly determined by entropy, and enthalpy is unfavorable [67].

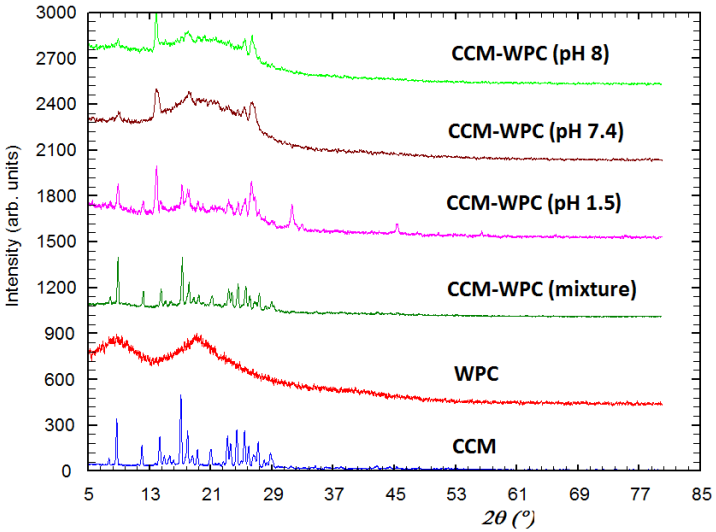

Figure 5C. X-ray diffraction for CCM, WPC, their CCM-WPC physical mixture and CCM-WPC complexes (molar ratio 35:1) at different pH values: 1.5, 7.4 and 8.0.

Figure 5C shows the X-ray powder diffractograms of CCM, WPC, CCM-WPC (mechanical blend), and three other samples obtained by complexation at pH 1.5, 7.4, and 8.0. The diffractograms on the powders highlight the fact that by complexation different compounds were obtained compared to the initial ones. The 3 most intense diffraction peaks for curcumin are at 2θ diffraction angles: 8.850°, 17.300°, and 25.160°, while the most intense diffraction peaks for the complexes are found at 2θ: 9.130°, 18.140°, and 26.260°. Each of the three complexed samples has an intense diffraction halo, demonstrating that the complexes also have an amount of amorphous phase. The halo in the sample that was prepared at pH 1.5 is less intense, compared to the halos corresponding to the samples that were prepared at pH 7.4 and pH 8.0, which are very prominent.

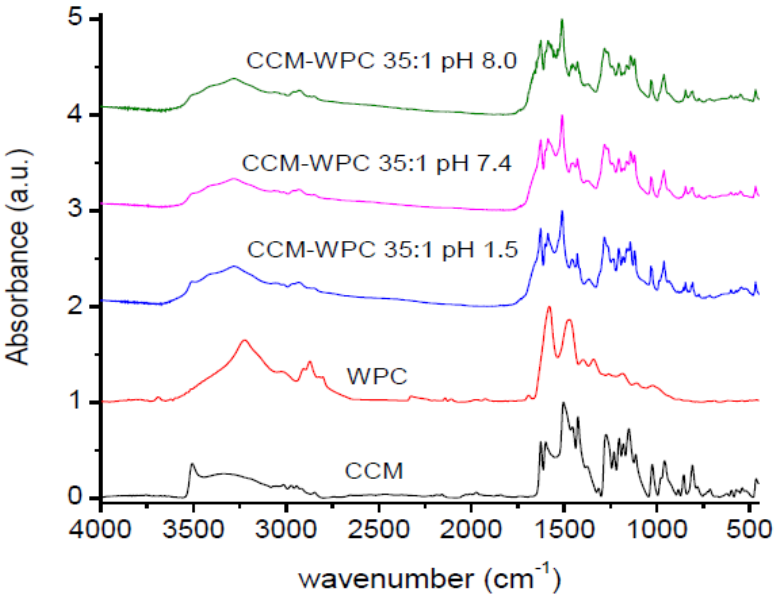

Figure 6B. FTIR spectra for CCM, WPC and CCM-WPC complexes with a molar ratio of 35:1 at pH 1.5, 7.4 and 8.0. The spectra is normalized to 1.

In conclusion, there is strong evidence for the interaction between CCM and WPC and the formation of new supramolecular complexes. In particular, the change of the absorption band in the spectral range 3600-3000 cm^{-1} may be due to the formation of hydrogen bonds between CCM and WPC.

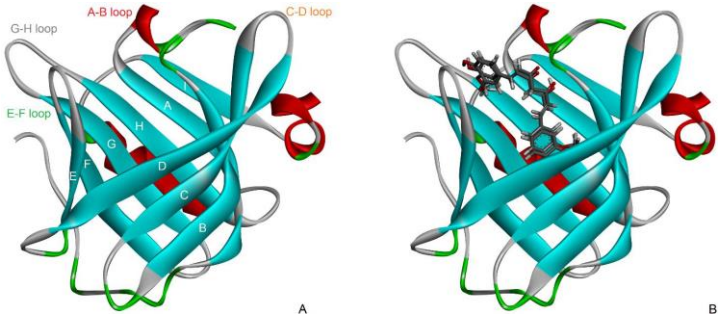

Figure 8. (A) Bovine β-lactoglobulin. The central cup is constituted by the eight antiparallel β chains (A-H) and is guarded by flexible loops: A-B, C-D, E-F, G-H; (B) Superimposed docking conformations of keto-enol and diketo curcumin tautomers having the highest binding energy.

In our docking analysis we considered as host molecule the monomeric β-lactoglobulin 3NPO, deposited in the RCSB database. Figure 8A shows this structure in which the eight antiparallel β-strands (A-H) and the flexible loops: A-B, C-D, E-F, G-H surrounding the central calyx have also been labeled. Remembering the previously mentioned Tanford transition, it can be noted in this crystallographic conformation that the EF loop is in an open state. In Figure 8B, the conformations with the maximum binding energies calculated for the keto-enol and diketo tautomers of curcumin are also plotted. It can thus be observed that the two conformations differ only slightly. Their binding energies are: -9.11 kcal/mol for the keto-enol and -8.82 kcal/mol for the diketo form. In the following, these results will be presented in detail.

CHAPTER 7. COMPLEXATION OF CURCUMIN WITH WHEY PROTEIN TO IMPROVE THE SOLUBILITY IN AQUEOUS SOLUTIONS, STABILITY AND ANTIOXIDANT EFFECT OF CURCUMIN

The stability and solubility of curcumin, CCM, can be improved by complexing with whey protein concentrate (WPC). CCM-WPC complexes in molar ratio 1:1 and 1:0.5 were prepared by two methods, namely spray drying (SD) and freeze drying (FD). The major objective of this work is to determine the stability and solubility of encapsulated CCM-WPC complexes by SD and FD method. X-ray diffraction and thermogravimetric analysis were performed on crude CCM and WPC as well as their complexes. Changes in thermal behavior and crystallinity suggest the formation of these CCM-WPC complexes. Scanning electron microscopy images showed that the methods used influenced the morphology and properties of the complexes formed. The stability and solubility of curcumin is improved by complexation in both encapsulation methods. It was observed by comparing the molar ratio used and the drying methods used, that the results obtained are different. The solubility of the samples obtained by the FD method was higher than that obtained by the SD method, which may explain the difference in their morphology (FD samples are more amorphous than SD samples). The antioxidant property of curcumin and protein complexes compared to vitamin C showed that CCM has a lower IC50 value than vitamin C, that is, the antioxidant effect of CCM is higher (Table 5). The addition of WPC enhanced the antioxidant activity of CCM, probably due to its protein encapsulation.

Table 5: Half of maximum inhibitory concentration (IC50) determined for ascorbic acid, CCM and its complexes with WPC

Materials	**The line equation from the graphs in Excell**	**Molecular weight (g/mol)**	**IC50** (μg/mL)	**IC50 (M)**
Ascorbic acid	y=8.429x – 7.0714	176.24	6.771	$3.842* 10^{-5}$
Curcumina	y = 4.3234x + 2.1078	368.38	11.077	$3.007* 10^{-5}$
1CCM:1WPC_SD	y = 2.9895x + 2.2429	18768.38	15.975	$8.511* 10^{-7}$
1CCM:1WPC_FD	y = 2.7424x – 2.3095		19.074	$1.016* 10^{-6}$
1CCM:0.5WPC_SD	y = 2.3338x + 2.2733	9568.38	20.298	$2.121* 10^{-6}$
1CCM:0.5WPC_FD	y = 2.6255x – 2.7293		18.004	$1.881* 10^{-6}$

CCM has a crystalline structure, which by complexing with the whey protein concentrate becomes amorphous. This amorphous nature of the complexes contributes to the significant increase in solubility. The morphology of the complexes is different depending on the drying techniques used. The antioxidant properties, solubility and stability of CCM were significantly increased by complexation.

CHAPTER 8. BINDING OF WHEY PROTEIN TO CURCUMIN AND THE STRUCTURAL CHARACTERISTICS OF THEIR COMPLEX HIGHLIGHTED BY ATOMIC FORCE MICROSCOPY

Curcumin (CCM) has beneficial effects on human health due to its pharmacological activity, having a protective role against many diseases. Whey protein concentrate (WPC) is a product from the dairy industry that is often used to enhance and stabilize various foods. Whey protein favors curcumin to improve its water solubility, poor bioavailability, stability and efficacy. Atomic Force Microscopy (AFM) was used to reveal the surface topography of the adsorption films for pure curcumin, pure whey protein and their complex (CCM-WPC). The obtained results show that individual nanoparticles were mobilized in aqueous dispersion and successfully adsorbed on glass slides in the form of thin films. Their shape is rounded and the diameter differs from one sample to another: 30 nm for CCM, 55 nm for WPC and 40 nm for the CCM-WPC complex. It is demonstrated that both CCM and WPC form a complex that incorporates them into a compact structure. The surface roughness was also monitored, and pure curcumin produces a smooth and uniform film, while the presence of WPC causes more pores to appear on the film surface, which increases the roughness value. The obtained results provide useful evidence for the application of WPC as an effective carrier of CCM, a bioactive polyphenolic compound. In addition, this work supports the use of the CCM-WPC complex as a dietary supplement with a role in health maintenance.

The nanostructures of the CCM–WPC system were successfully investigated by atomic force microscopy, which is a fundamental method for nanocharacterization of materials. The initial CCM and WPC powders were able to form nanoparticles in the aqueous medium. These nanoparticles were adsorbed on glass substrate forming thin films. Their shape is round and the average diameter differs from one sample to another: 30 nm for CCM, 55 nm for WPC.

Complexation between CCM and WPC leads to an intermediate diameter of about 40 nm. CCM nanoparticles are uniformly adsorbed on the glass surface generating a smooth thin film with little roughness. WPC tends to generate pores in the adsorbed film, which leads to relatively higher roughness values. The CCM-WPC complex forms a smooth thin film in which the influence of the whey protein concentrate produces some pores in the composition that affect the roughness values.

These results could support the development of functional foods, including curcumin and whey proteins, with new uses in yogurt and nutritional supplements.

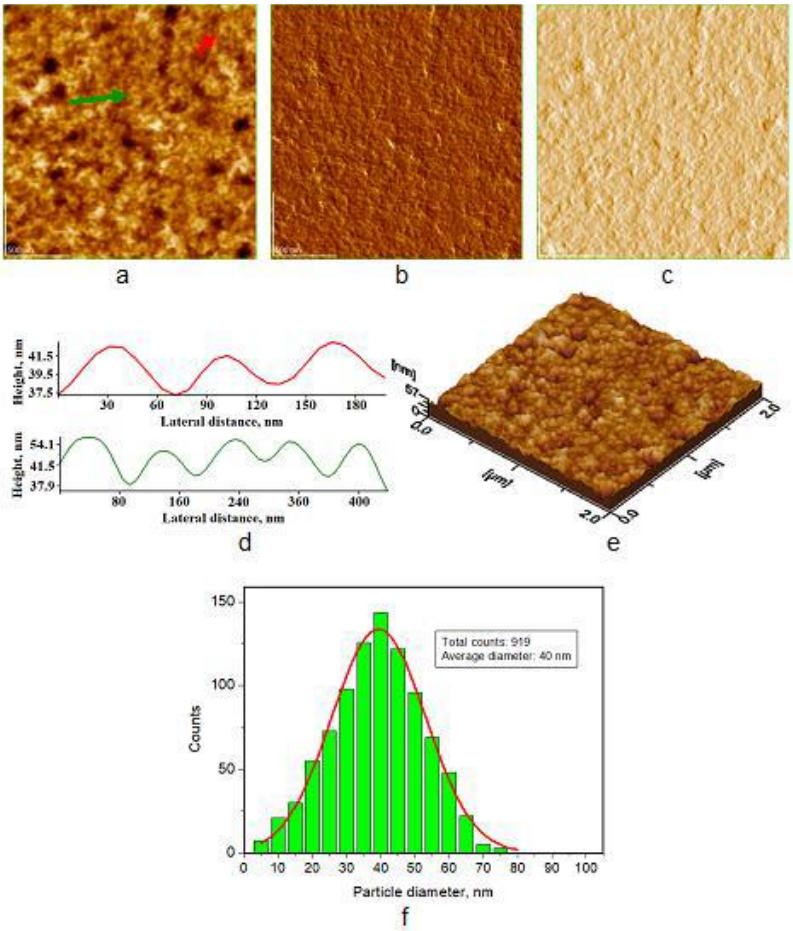

Figure 3. AFM images for CCM-WPC complex, pH 7.4 adsorption on glass and natural drying: a) topographic image, b) phase image, c) amplitude image, d) profiles along arrows in panel (a), e) three-dimensional image, and f) distribution histogram of particles. Scanning area 2 µm x 2 µm.

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CHAPTER 9. SYNTHESIS AND CHARACTERIZATION OF PEG, CURCUMIN AND WHEY PROTEIN COMPOSITES – IMPROVED THERMAL STABILITY

Curcumin (CCM) is a natural polyphenol with promising pharmaceutical potential. However, this potential is restricted due to its limited bioavailability. To solve this aspect new composites (new complexes) of curcumin-PEG (PEG4000 or PEG6000) were synthesized, with or without an addition of whey protein (WPC), resveratrol (RES) or silymarin (SIL). The thermal stability of all synthesized composites was investigated compared to the precursors by thermogravimetric (TG) and differential scanning calorimetry (DSC) analysis, revealing a thermal stability up to 200 °C. The formation of the composites and the interaction between the components was investigated by Fourier transform infrared (FTIR) spectroscopy. The kinetic study demonstrates an increased stability of curcumin in the studied composites, especially for the composites: PEG4000-CCM-WPC-SIL and PEG6000-CCM-WPC-SIL which present the highest stability of curcumin in the aqueous phase. This strategy for designing composites based on PEG-CCM and WPC is useful because it leads to new materials with increased stability of curcumin and the design of various food supplements.

The present study describes a series of complexes based on two types of polyethylene glycol (PEG4000 and PEG6000) and curcumin, with or without an addition of protein in the form of whey protein concentrate (WPC) or a secondary polyphenol, trans-resveratrol (RES) or silymarin (SIL). RES and SIL were chosen due to the synergistic effect reported in the presence of curcumin – trans-resveratrol [Du et al. 2013, Chen et al. 2017]; silymarin [Montgomery et al. 2016].

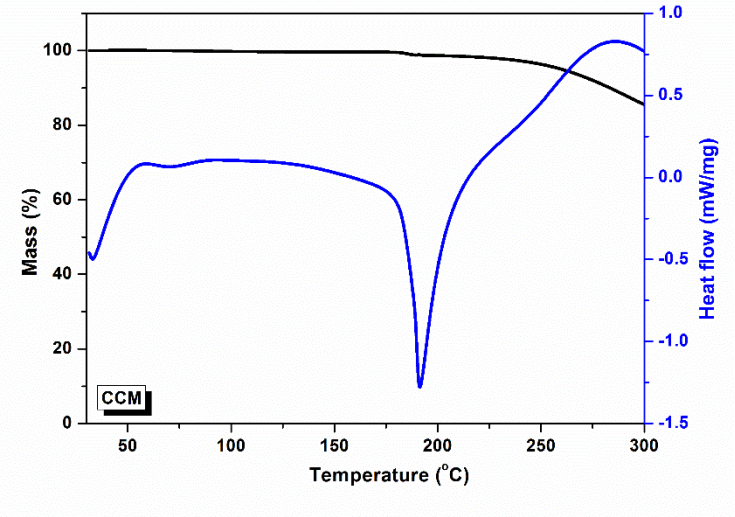

Figure 1. Curcumin TG and DSC curves

The thermal stability of all synthesized composites was investigated compared to the precursors by thermogravimetric (TG) and differential scanning calorimetry (DSC) analysis, revealing a thermal stability up to 200 °C.

Tabel 4. The half-life ($t_{1/2}$) of curcumin and composites (of the complex) with PEG4000 or PEG6000 using the second-order kinetic model.

Material	**$t_{1/2}$ (min)**		
	pH 1.5	**pH 7.4**	**pH 8.0**
CCM	78	74	4
PEG4000-CCM	292	146	16
PEG4000-CCM-WPC	3266	4072	1113
PEG4000-CCM-WPC-RES	3395	4872	2524
PEG4000-CCM-WPC-SIL	4248	5176	2569
PEG6000-CCM	327	170	13
PEG6000-CCM-WPC	1983	2017	1228
PEG6000-CCM-WPC-RES	3867	5378	2532
PEG6000-CCM-WPC-SIL	5050	5534	2099

The stability of curcumin in aqueous solutions for the composites developed in this study was comparatively investigated, by determining the half-life according to a 2nd-order kinetics. An increased stability of curcumin was found in all the new composites obtained, especially for the composites: PEG4000-CCM-WPC -SIL and PEG6000-CCM-WPC-SIL showing the highest stability of curcumin in aqueous media (Table 4).

CHAPTER 10. GENERAL CONCLUSIONS

The biggest disadvantage of CCM is its low solubility in water, hence its low bioavailability.

To combat this shortcoming, several strategies have been developed, namely: encapsulation of CCM in protein, for example in whey protein concentrate, by complexation in various media with controlled pH and depending on temperature, by co-precipitation and spray drying , SD, or by lyophilization, FD. Identifying the correct method of formulation and use of appropriate excipients in relation to CCM can result in effective pharmacological treatment by administering a lower dose, which can have a significant cost implication.

For example, the high curcumin solid dispersed system was prepared with whey protein concentrate in a molar ratio of 5:1 by the spray drying method. X-ray powder diffraction and DSC analysis show the formation of the solid dispersed system in the amorphous state, and FTIR analysis identifies the presence of weak hydrogen bonding interactions between the system components. SEM images show a homogeneous morphology of spherical microparticles.

Molecular interaction between curcumin, CCM and whey protein concentrate, WPC, which was studied using fluorescence measurements. The binding number (n), binding constant (KS) and thermodynamic parameters (ΔG, ΔH and ΔS) were determined at different temperatures and pH values of the aqueous solutions. The thermodynamic and structural approach of the binding mechanism between CCM and WPC constitutes a first in the specialty literature. The highest stability of this CCM-WPC complex in acidic pH medium (eg, gastric fluid with pH 1.5) was revealed.

Several PEG-based complexes (either PEG4000 or PEG6000) were also synthesized with or without the incorporation of whey protein or a secondary polyphenol, either trans-resveratrol or silymarin. Addition of the protein leads to a more stable complex through hydrogen bonding. This correlates with the dramatic increase in water solubility in complexes with PEG, curcumin, and whey protein. The addition of a secondary polyphenol decreases water solubility. However, the cumulative antioxidant and anti-inflammatory potential might be more rewarding in a supplement. In terms of thermal stability, all complexes are quite stable up to about 200 oC. This is more than adequate with respect to any further processing regarding a potential pharmaceutical application. In contrast, in terms of pH stability, the results clearly highlight the beneficial effect of WPC and flavonoids on increasing the half-life of CCM. While PEG-CCM complexes tend to degrade rapidly at all pH values, the most complex ones, including at least whey protein, greatly stabilize curcumin.

In vitro tests of human osteoblasts cultured on strontium-substituted hydroxyapatites were performed demonstrating a high osteogenic potential of these ceramics and their strong involvement in bone formation and mineralization. These ceramic substrates stimulated bone cells to grow on their surface and actively participate in the formation of new bones and favored bone growth on their surface, leading osteoblasts to enter terminal stages of differentiation triggering the process of mineralization of the newly formed bone.

All tested ceramic substrates were shown to be biocompatible with significant differences in cell adhesion, proliferation and differentiation. The best biocompatibility and excellent osteoconductivity was shown by HAP-Sr, especially those with Sr amount of 5%, 10% and 15%.

These promising results recommend the mentioned complexes for potential pharmaceutical applications. Future studies are underway to examine their prospective effects both in vitro and in vivo.

CHAPTER 11

The scientific research activity is embodied by 5 ISI-quoted scientific works: 2 published works and 2 works accepted for publication and 1 work in progress of publication, in Molecules journal. We mention that we still have two scientific papers in manuscript form that are being finalized for publication.

LIST OF ISI PAPERS

1. S. Rapuntean, P. T. Frangopol, I. Hodisan, Gh. Tomoaia, D. Oltean-Dan, A. Mocanu, C. Prejmerean, O. Soritau, ***L. Z. Racz***, M. Tomoaia-Cotisel, In vitro response of human osteoblasts cultured on strontium substituted hydroxyapatites, Rev. Chim. (Bucharest), 69(12), 3537-3544, (2018). **I.F. 1.605**

2. ***L. Z. Rácz***, M. Tomoaia-Cotișel, Cs.-P. Rácz, P. Bulieris, I. Grosu, S. Porav, A. Ciorîță, X. Filip, F. Martin, G. Serban, I. Kacso, Curcumin-Whey Protein Solid Dispersion System With Improved Solubility And Cancer Cell Inhibitory Effect, Stud. Univ. Babes-Bolyai, Chem., 66(3), 209-224, (2021). **I.F. 0.447**

3. ***L. Z. Rácz***, G.-A. Paltinean, I. Petean, Gh. Tomoaia, L. C. Pop, G. Arghir, E. Levei, A. Mocanu, Cs.-P. Rácz, M. Tomoaia-Cotisel, Curcumin and Whey Protein Binding And Structural Characteristics of Their Complex Evidenced by Atomic Force Microscopy, Stud. Univ. Babes-Bolyai, Chem., 2022, 67(3), in press. **I.F. 0.447**

4. ***L. Z. Rácz***, Cs. P. Rácz, L. C. Pop, Gh. Tomoaia, A. Mocanu, I. Barbu, M. Sárközi, I. Roman, A. Avram, M. Tomoaia-Cotisel, V.-A. Toma, Strategies for improving bioavailability, bioactivity, and physical-chemical behavior of the curcumin, Molecules, 2022, Manuscript ID-1919555. Under review. **I.F. 4.927**

5. ***L. Z. Rácz***, Cs.-P. Rácz, O. Horovitz, Gh. Tomoaia, A. Mocanu, I. Kacso, M. Sárközi, M. Dan, S. Porav, G. Borodi, M. Tomoaia-Cotisel, Complexation of Curcumin using Whey Proteins to Enhance Aqueous Solubility, Stability and Antioxidant Property, Stud. Univ. Babes-Bolyai, Chem., 2022, 67(3), in press. **I.F. 0.447**

List of conferences and symposiums

The dissemination of the scientific results from the Doctoral Thesis was carried out in 4 oral presentations at the national conferences of the Romanian Academy of Scientists [1-4], one of which was in English [4] at NanoBioMat 2022 with international participation.

1. **L. Racz**, A. Mocanu, Cs.-P. Racz, I. Kacso, O. Horovitz, M. Tomoaia-Cotisel, RO: „Sistem de dispersie solidă de curcumină-proteine din zăr cu solubilitate îmbunătățită și efect inhibitor al celulelor canceroase”/ ENG : „Curcumin-whey protein solid dispersion system with improved solubility and cancer cell inhibitory effect”, Prezentare orală, Academia Oamenilor de Știință din România, Conferința Științifică Națională de Toamnă „Tradiții și Progrese în Știința Românească”, Secțiunea Științe Biologice, 18 – 20 noiembrie, on line platforma Zoom, Volum rezumate pag. 21 (2021).

2. **L. Racz**, Cs.-P. Racz, A. Mocanu, I. Kacso, M. Tomoaia-Cotisel, RO : „Potențialul terapeutic al curcuminei și tehnici de a-i îmbunătăți solubilitatea și bioactivitatea in vivo”/ ENG : „Therapeutic potential of curcumin and techniques for improving its solubility and bioavailability in vivo”, Prezentare orală, Academia Oamenilor de Știință din România, Conferința Științifică Națională de Toamnă „Tradiții și Progrese în Știința Românească”, Secțiunea Științe Biologice, 18 – 20 noiembrie, on line platforma Zoom, Volum rezumate pag. 30-31 (2021).

3. **L. Racz**, Cs. P. Racz, O. Horovitz, A. Mocanu, M. Tomoaia-Cotisel, RO: “Perspective asupra interacțiunilor curcuminei cu proteinele din zăr: abordare termodinamică a legării curcuminei de proteine și potențiale efecte biologice” / ENG: “Insights into curcumin and whey protein interactions: Thermodynamic approach of curcumin - protein binding and potential biological effects”, Prezentare orală, Academia Oamenilor de Stiinta din Romania, Conferința Științifică Națională de Primăvară „Era digitală – Provocări și Oportunități pentru Societatea Contemporană”, 6 – 7 Mai 2022, sectiunea Biologie, on-line platforma Zoom, Volum de rezumate, pag. 103-104 (2022).

4. **L. Racz**, Cs.-P. Racz, O. Horovitz, A. Mocanu, M. Tomoaia-Cotisel, ENG: „Thermodynamic approach of curcumin and whey protein binding”, Virtual International Scientific Conference on “Applications of Chemistry in Nanosciences and Biomaterials Engineering” NanoBioMat 2022 – Summer Edition, 22-24 June 2022, Oral presentation on Session I, Advanced Techniques for Material Design and Processing, on line platforma TEAMS (2022).

LIST OF SCIENTIFIC RESEARCH GRANTS

Contract **PN2 Partnership 241/2014-2016** Development of innovative nanomaterials based on advanced nanotechnology with applications in dental and periodontal disease prophylaxis, InovaMat, Coordinator: Babeș-Bolyai University, Director: Prof. Dr. Aurora Mocanu

Obtaining a supplement-type food product, on a natural substrate of Apium Graveolens L. nutritionally optimized by enrichment with selenium and vitamins, in order to improve the quality of life.
SMIS CODE 119675

Nutritional optimization of food products based on grapes and berries, by enrichment with resveratrol, in order to increase the intake of antioxidants in the diet.
SMIS CODE 119601

Member of the Center for Scientific Research in Physical Chemistry

Ph.D. Eng. Chem. **Rácz Levente Zsolt** is a member of the Center for Scientific Research in Physical Chemistry since 2015. CECHIF: Founder (2006) and Director (2006-present): Prof. Univ. Dr. M. Tomoaia-Cotișel, Faculty of Chemistry and Chemical Engineering at Babeș-Bolyai University in Cluj-Napoca.