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Abbreviated running title: β-casein-hydrophobic bioactive compound binding

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Abstract

β-casein, a phosphoprotein representing 37 % of the bovine milk caseins, has specific features promoting its application as a nanocarrier for hydrophobic bioactives. In this study, the interactions of β-casein with curcumin and vitamin D3 under the same physico-chemical conditions were investigated. The interaction kinetics have been studied by surface plasmon resonance (SPR) and fluorescence spectroscopy. The K_D value for curcumin-β-casein interaction has been successfully evaluated ($4.1 \pm 0.7 \times 10^{-4}$ M) using SPR by fitting data to a 1:1 Langmuir interaction model. Conversely, the SPR responses obtained for vitamin D3 show that the interactions between this hydrophobic compound and the β-casein immobilized on the sensor chip were below the sensitivity of the SPR apparatus. Moreover, the fluorescence quenching data show that curcumin has higher affinity to β-casein ($K_A = 23.5 \pm 1.9 \times 10^4$ M^{-1}) than vitamin D3 ($K_A = 5.8 \pm 1.1 \times 10^4$ M^{-1}).

Highlights:

- The interactions between monomeric β-casein and two hydrophobic bioactive molecules, curcumin and vitamin D3, were evaluated.
- These interactions were investigated by surface plasmon resonance and fluorescence quenching.
- Curcumin has a higher binding affinity to β-casein compared to vitamin D3.

Keywords: β-casein, curcumin, vitamin D3, surface plasmon resonance, fluorescence quenching, binding parameters.

Chemical compounds studied in this article

Curcumin (PubChem CID: 969516); Vitamin D3 (PubChem CID: 5280795)

1. Introduction

Over the past years, there has been a high demand for incorporation of biologically active natural ingredients into functional food to contribute to consumer's well-being (Vieira da Silva, Barreira, & Oliveira, 2016). However, most of these bioactives display low solubility, poor stability during processing, and low permeability in the gastrointestinal tract. Therefore, different encapsulation strategies have been developed in order to avoid these limitations and protect these molecules during processing and storage, maintaining their active molecular form until consumption and delivery to the physiological target within the organism (Okuro, Furtado, Sato, & Cunha, 2015). Milk proteins, especially caseins, have unique and interesting functional properties making them effective nanocarriers for bioactives (Ranadheera, Liyanaarachchi, Chandrapala, Dissanayake, & Vasiljevic, 2016). As casein micelles is a natural self-assembly, some studies investigated the binding of hydrophobic compounds such as curcumin to native casein micelles (Sahu, Kasoju, & Bora, 2008) or to modified casein micelles (Benzaria, Maresca, Taieb, & Dumay, 2013). Among the four different caseins, α_{s1}-, α_{s2}-, β- and κ-caseins, a particular interest is paid to β-casein. This phosphoprotein, composed of 209 amino acids and with a molecular weight ~ 24 kDa, constitutes about 37% of the caseins in bovine milk and is the most hydrophobic casein thanks to its hydrophobic C-terminal tail rich in proline residues. Consequently, several studies have shown that β-casein can be used as a natural nanocarrier for hydrophobic bioactive molecules in order to increase their bioavailability (Semo, Kesselman, Danino, & Livney, 2007; Shapira, Davidson, Avni, Assaraf, & Livney, 2012). Particularly, the separation between its hydrophobic C-terminal domain and its hydrophilic highly charged N-terminal domain

containing an anionic phosphoserine cluster makes it highly amphiphilic allowing the β-casein self-association in small oblate micelles (Holt & Sawyer, 1993; Swaisgood, 2003). The intermolecular interactions to form β-casein micelles occur at concentrations above the critical micellization concentration (CMC) of ~ 0.5 - 2 mg/mL at milk pH (6.8) and room temperature (O'Connell, Grinberg, & De Kruif, 2003). Several studies have investigated the encapsulation of hydrophobic molecules inside the hydrophobic core of β-casein micelles and have shown an improvement of the efficiency of poorly water-soluble compounds using this nanocarrier system (Esmaili et al., 2011; Mehranfar, Bordbar, Fani, & Keyhanfar, 2013). At the same time, some studies have focused on the investigation of the binding between β-casein and hydrophobic bioactive molecules such as lipophilic vitamins (Forrest, Yada, & Rousseau, 2005), flavan-3-ol derivatives (Bohin, Vincken, Van Der Hijden, & Gruppen, 2012) or naringenin (Moeiniafshari, Zarrabi, & Bordbar, 2015). The fluorescence spectroscopy is a commonly method used in these studies to evaluate the interaction between the hydrophobic bioactive compound and the protein. Indeed, fluorescence quenching analysis, recognized as a highly sensitive methodology with a fast and easy implementation, evaluates the changes in the local environment of the fluorophore and tryptophan residues, as a consequence of molecular interaction (Lakowicz, 2006).

Molecular interaction affinities can also be investigated using surface plasmon resonance (SPR). SPR is recognized as a technique giving the ability to detect highly dynamic complexes being difficult to investigate using other techniques (Douzi, 2017). This well-suited label-free optical technique for affinity characterization provides real-time monitoring of binding kinetics. The method is based on the immobilization of one of the binding partners on a functionalized sensor surface. The other partner, free in solution, is then injected over the surface containing the immobilized molecule. The

101 binding is monitored by subsequent changes in the refractive index at the biosensor
102 surface (Homola, Yee, & Gauglitz, 1999).
103 SPR has been used more and more in the last decade to characterize the interactions
104 between proteins and low molecular weight molecules in biochemical, biophysical and
105 biomedical fields (Vachali, Li, Nelson, & Bernstein, 2012; Watrelot et al., 2016; Xiao,
106 Jiang, Zhou, Li, & Liu, 2011). Some studies investigated SPR interactions between
107 caseins (Marchesseau, Mani, Martineau, Roquet, Cuq, & Pugnière, 2002) or between
108 caseins and polysaccharides (Thompson, Singh, & Dalgleish, 2010). However, up to
109 now, SPR has not been used to study and quantify the interaction between caseins and
110 hydrophobic low molecular weight biomolecules.
111 Curcumin is a low-molecular weight, natural polyphenolic phytoconstituent isolated
112 from the dried rhizome of the plant *Curcuma Longa*. It has been used for centuries as a
113 naturally occurring medicine for disease treatment and displays potent anti-
114 inflammatory, antitumor and antioxidant activities (Hewlings & Kalmen, 2017).
115 However, curcumin bioavailability is limited due to its low solubility in aqueous
116 solution (2.99×10^{-8} M) and it is characterized by a poor stability towards oxidation and
117 light. Vitamin D3, one of the major forms of vitamin D, is hydrophobic in nature and
118 highly susceptible to environmental conditions such as temperature and light. It is
119 implicated in active intestinal calcium absorption but it was also shown recently that its
120 role also includes immune function, cardiovascular health and cancer prevention
121 (Gupta, Behera, Pawdal, Rawat, Baldi & Gupta, 2019). Nowadays, vitamin D 3
122 deficiency is a public health problem in many countries. Therefore, the supplementation
123 of food products with curcumin or vitamin D3 could offer additional source of these
124 essential hydrophobic bioactive compounds.
125

The objective of the present work was to study the interactions of β-casein with curcumin and vitamin D3 using surface plasmon resonance and fluorescence quenching spectroscopy. The binding kinetics were investigated using both methodologies by gradually adding higher concentrations of hydrophobic bioactives to β-casein. A comparison of binding affinities was then performed for the two different hydrophobic molecules.

2. Materials and methods

2.1 Materials and chemicals

β-casein from bovine milk (> 98%), curcumin (PubChem CID: 969516, ≥ 65%), chloramphenicol (PubChem CID: 5959), absolute ethanol (PubChem CID: 702), dimethyl sulfoxide (DMSO, PubChem CID: 679) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, PubChem CID: 23831) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Vitamin D3 (PubChem CID: 5280795, 99%) was obtained from Thermofisher Acros Organics (Geel, Belgium). Sodium acetate was purchased from Merck (Darmstadt, Germany). CM5 sensor chips, HBS-EP buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.005% v/v surfactant P20), HBS-EP+ buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.5% v/v surfactant P20), HBS-N buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl), N-ethyl-N'-(dimethylaminopropyl)carbodiimide hydrochloride (PubChem CID: 2723939, EDC), N-hydroxysuccinimide (PubChem CID: 80170, NHS) and 1M ethanolamine-HCl pH 8.5 were all sourced from GE Healthcare Life Sciences (Velizy-Villacoublay, France). All solutions were prepared using Milli-Q water (Millipore®).

2.2 Surface plasmon resonance (SPR) studies

SPR analyses to evaluate the interaction of curcumin or vitamin D3 (analyte) with β-casein (ligand) immobilized onto a carboxymethylated dextran sensor surface (CM5 sensor chip) were performed using a Biacore T200 apparatus at 25°C (GE Healthcare Life Sciences, Uppsala, Sweden). During the analyte-ligand interaction, the change of the refractive index was measured in real time that allowed to plot the results of interaction as response units (RU) versus time (one RU represents the binding of 1 pg of protein per square mm).

2.2.1 Covalent immobilization of β-casein on CM5 sensor chip

β-casein was covalently immobilized on the CM5 sensor chip by a standard amine-coupling chemistry according to the manufacturer's protocol (GE Healthcare). Briefly, the surface was functionalized with 0.4 M EDC and 0.1 M NHS mixture. 40 µM β-casein stock solution was prepared in HBS-EP buffer (pH 7.4). The ß-casein monomeric state in the experimental conditions was checked by dynamic light scattering analysis (data not shown). The solution was vortexed and placed in an ultrasonic bath for 10 min. Prior to covalent immobilization, a preconcentration test at different pH values was performed to obtain the optimum pH for the immobilization procedure. Thus, β-casein stock solution was diluted at 20-30 µg/mL in 10 mM acetate buffer at pH 3.6 and was injected at 10 µl/min on two flow cells to reach two different immobilization levels of 2700 RU and 6900 RU, respectively. These two different β-casein immobilization levels were explored in order to monitor the effect of mass transport hindrance caused by the surface binding sites located at different points (Schuck & Zhao, 2010). The unreacted carboxyl groups of the dextran were blocked with 1 M ethanolamine-HCl, pH 8.5 and the surface was washed with HBS-N buffer. One other flow cell, treated with the same procedure but without protein was used as a control surface. Coated surfaces were stable for at least 2 days.

2.2.2 Kinetic analysis of β-casein-hydrophobic bioactive compound interaction

Bioactive compound stock solutions were prepared by dissolving curcumin (34 mM) in DMSO and vitamin D3 (53 mM) in ethanol. For each bioactive compound, a specific solvent was selected to ensure a high solubility of the bioactive compound; curcumin solubility in DMSO is 68 mM and vitamin D3 solubility in ethanol is 78 mM. Stock solutions were filtered through a 0.2 µm cellulose-acetate membrane (Sartorius, Goettingen, Germany) and protected from light during experiments. The bioactive compound stock solution concentrations were spectrophotometrically determined at 420 nm for curcumin using a molar absorption coefficient (ε) of 49,000 AU/mol/cm in DMSO (Khumsupan, Ramirez, Khumsupan, & Narayanaswami, 2011) and at 265 nm for vitamin D3 using a molar absorption coefficient (ε) of 18,300 AU/mol/L in ethanol (Vieth, Kimball, Hu, & Walfish, 2004). Prior to SPR analysis, bioactive compound stock solutions were diluted using HBS-EP+ buffer to obtain bioactive compound concentrations between 3 and 1000 µM. The final concentration of DMSO and ethanol did not exceed 3% (v/v) in diluted bioactive compound solutions injected in flow cells. HBS-EP+ containing 3% (v/v) DMSO or 3% ethanol was used as running buffer. The diluted bioactive compound solutions at various increasing concentrations were successively injected. The analysis cycle for each bioactive compound concentration consisted, for the control cell and both β-casein coated flow cells, in the injection of the running buffer at a flow rate of 30 µL/min, then the diluted bioactive compound solution for 60 s (association phase) and finally the running buffer for 100 s (dissociation phase). The signal of the control surface flow cell was subtracted from the signal of the ß-casein flow cells. Chloramphenicol, a negative control, was also injected at 500 and 1000 µM in HBS-EP+ containing 3% (v/v) DMSO. Each experiment series included blanks (running buffer), injection of bioactive compound, negative control, and

200 solvent corrections. Experiments were repeated three times. Sensorgrams were analyzed
201 using BIA evaluation 3.0 software by steady-state fitting with the one-to-one Langmuir
202 binding model to obtain K_D value. When a bioactive compound, injected in the flow
203 system as the analyte, reacts with ß-casein, immobilized as the ligand, to form a
204 complex, the rate of the complex formation depends on the free concentrations of the
205 bioactive compound ([Bioactive]) and ß-casein ([ß-casein]) as well as the stability of the
206 formed complex ([Bioactive-ß-casein]) and can be described by the Langmuir binding
207 model equation (Eq. 1) (Myszka, Jonsen, & Graves, 1998):

208 $$d[\text{Bioactive-ß-casein}]/dt = K_a\,[\text{Bioactive}]\,[\text{ß-casein}] - K_d\,[\text{Bioactive-ß-casein}] \quad (1)$$

209 where K_a is the association rate constant and K_d is the dissociation rate constant.
210 In SPR experiment, the Langmuir binding model equation (Eq. 1) can be written as Eq.
211 2:

212 $$dR/dt = K_a\,C\,(Rmax-R) - K_d\,R \quad (2)$$

213 where the bound bioactive concentration ([Bioactive-ß-casein]) is directly proportional
214 to the SPR response (R), the concentration of the analyte ([Bioactive]) is the injected
215 concentration (C) and the free ß-casein concentration ([ß-casein]) is obtained as the
216 difference between Rmax, maximum response due to analyte binding directly
217 proportional to the total ß-casein concentration, and R.
218 At steady state, dR/dt = 0 and Eq. 2 can be written as Eq. 3:

219 $$C\,(Rmax-R) = K_D\,R \quad (3)$$

220 where $K_D = k_d/k_a$.
221 The effectiveness of the fitting is evaluated by the χ^2 value.

222 **2.3 Evaluation of β-casein-hydrophobic bioactive compound interaction by**
223 **fluorescence spectroscopy**

Fluorescence measurements were performed on a Cary Eclipse spectrofluorimeter at 25°C using a 1 cm quartz cuvette (101-QS, Hellma Analytics). A 20 µM β-casein stock solution was freshly prepared in a 10 mM HEPES buffer (pH 7.1). The solution was then vortexed and placed in an ultrasonic bath for 1 h before being filtered through a 0.2 µm cellulose-acetate membrane (Sartorius, Goettingen, Germany). The β-casein concentration was checked by absorbance measurement at 280 nm using an extinction coefficient E (1%/1 cm) of 4.65 (De Kruif & Grinberg, 2002). Concurrently, curcumin and vitamin D3 stock solutions (3 mM) were prepared in absolute ethanol then filtered through a 0.2 µm cellulose-acetate membrane (Sartorius, Goettingen, Germany). The bioactive compound concentrations in ethanol were evaluated by measuring the absorbance at 427 nm for curcumin solution and at 265 nm for vitamin D3 solution with a molar extinction coefficient of 61,864 AU/mol/L/cm (Majhi, Rahman, Panchal, & Das, 2010) and 18,300 AU/mol/L/cm (Vieth et al., 2004) for curcumin and vitamin D3, respectively. For both bioactive compounds, β-casein intrinsic fluorescence was measured at a concentration of 8 µM in the presence of increasing concentrations of curcumin and vitamin D3by adding 1 to 100 µL of bioactive compound solution to β-casein dispersion. The mixture was immediately vortexed for 15 s and allowed to equilibrate for 5 min before fluorescence measurements. The emission spectra were recorded from 290 to 500 nm with an excitation wavelength of 280 nm. Both the excitation and emission slit widths were set at 5 nm. A control experiment was performed on β-casein without an addition of bioactive compound and treated exactly as the sample containing bioactive compound. The measurements have been repeated three times and the mean values were reported.

2.4 Statistical analysis

All measurements were made in triplicates at least. The differences were considered significant at $p < 0.05$ level.

3. Results and discussion

3.1 Surface plasmon binding experiments

3.1.1 β-Casein immobilization on CM5 sensor chip

To minimize the effects of mass transport, steric hindrance, crowding and aggregation of high capacity surface, it is recommendable to have low protein density surface and high running flow rate for kinetics evaluation by SPR (Xiao et al., 2011). Nevertheless, for the binding analysis with low molecular weight molecules, such as curcumin and vitamin D3, the mass transport effect is minimized and a high density of protein immobilization is necessary to enhance the binding between β-casein and hydrophobic compounds in order to obtain a maximum of response in RU.

Two experimental conditions require special attention to optimize the immobilization of β-caseins on CM5 sensor chip. Firstly, the β-casein dispersion has to be prepared at a low concentration (20-30 µg/mL) in order to prevent its self-association occurring at concentrations above 0.5 to 2 mg/mL at milk pH (6.8) and 25°C prior to immobilization (O'Connell et al., 2003). Besides, the highest level of immobilization of a protein on a carboxymethyl dextran chip (CM5) is obtained when the running buffer has a pH approximately one unit below the protein isoelectric point (pI). At this pH, the protein has a net positive charge and, consequently, electrostatic interactions between the positively charged protein and the negatively charged carboxymethyl dextran matrix increase the pre-concentration of the protein at the chip surface for the amine coupling establishment (Johnsson, Löfås, & Lindquist, 1991). Consequently, it is impossible to immobilize β-casein at milk pH (~6.8). Thus, the pH was lowered to 3.6 since the β

casein pI is 4.8 (Walstra, Wouters, & Geurts, 2006). These optimized conditions have enabled high β-casein immobilization densities (i.e.6900 RU and 2700 RU, the two different levels tested) on CM5 sensor chip. The N-hydroxy succinimide esters activated by the functionalization of the CM5 carboxyl groups by EDC/NHS have enabled protein binding to the surface by amine coupling. As primary amines are highly reactive, there is a strong probability that β casein was covalently linked to the CM5 chip thanks to the N terminus hydrophilic region. Besides, according to the amino acid sequence of β-casein, the other primary amines from lysine are mostly located on the hydrophilic region close to the N-terminus region (Fischer, 2010; Holt & Sawyer, 1993). Consequently, the hydrophobic fragment of β casein should be more available for hydrophobic interactions with hydrophobic molecules.

3.1.2 Surface plasmon resonance kinetic analysis

The sensorgrams and the concentration-dependence of the specific binding of curcumin to immobilized β-casein after DMSO and double blank correction are presented in Fig. 1A-B and Fig. 1C-D, respectively. These results are shown for two β-casein immobilization levels, 6900 RU for Fig. 1A and C and 2700 RU for Fig. 1B and D. No association was observed for either the control flowcell or the negative control. As expected for small molecular weight molecules, the box-shape of the sensorgram revealed fast association and dissociation rates, which makes difficult to quantify them.

As curcumin is poorly soluble in aqueous solutions (2.99×10^{-8} M) (Letchford, Liggins, & Burt, 2008), different organic solvents like dimethyl sulfoxide (DMSO) and ethanol were considered. As the solubility information for curcumin according to the supplier was of 10 mg/mL and 25 mg/mL in ethanol and DMSO, respectively, DMSO was chosen as solvent in order to reach higher concentrations of curcumin in the diluted solution injected during the association phase. Unlike curcumin, vitamin D3 shows

298 higher solubility in ethanol (30 mg/mL) than in DMSO. Therefore, ethanol was used as
299 a solvent for vitamin D3 prior to SPR study. However, vitamin D3 was unsuccessfully
300 analyzed using the same procedure as for curcumin tested in HBS EP+ containing 3%
301 ethanol (Fig. 2) and in 3% DMSO (v/v) (data not shown). The responses of vitamin D3
302 under these both conditions were under the limit of T200 Biacore apparatus sensitivity.
303 It clearly means that no interaction was observed between β-casein immobilized on
304 CM5 chip and vitamin D3 under these SPR conditions. According to the manufacturer
305 (GeHealthcare), the sensitivity of Biacore T200 apparatus enables precise affinity
306 analysis of low molecular weight molecules with a maximal dissociation constant (KD)
307 value in the millimolar range.
308 Concerning curcumin binding to β-casein and in order to obtain accurate estimations of
309 the binding constants, SPR data have been analyzed by fitting the SPR sensorgrams
310 using non-linear fitting of the SPR signal at the steady state with a Langmuir binding
311 isotherm model (De Mol & Fischer, 2008). The response in RU is then considered as
312 directly proportional to the injected analyte concentration (C) (Fig. 1 A-B). The curves
313 shown in Fig. 1 C-D represent steady-state affinity analysis of curcumin binding to β-
314 casein fitted to 1:1 Langmuir interaction model. The K_D value obtained from the SPR
315 analyses is 4.1 ± 0.7 × 10^{-4} M for curcumin in DMSO. The calculated χ^2 value (χ^2 =
316 4.54) indicates a good accuracy of the fitting. Figure 2 shows a comparison of dose-
317 effect of curcumin and vitamin D3 in different solvents. The kinetic study obtained for
318 curcumin dissolved in ethanol reveals similar k_D value 6.9 ± 0.9 × 10^{-4} M as that
319 obtained in DMSO. However, vitamin D3 gave too low responses to be analyzed in 3%
320 ethanol. Although the study of interactions between low molecular weight molecules
321 and bovine proteins starts to be carried out by SPR technique (Park, Kim, Lim, & Lee,
322 2015; Watrelot et al., 2016), this analytical technique has not been used previously to

study interactions between β-casein and hydrophobic low molecular weight molecules such as curcumin. To further explore the binding of β-casein to curcumin and vitamin D3, fluorescence spectroscopy was also performed to complement the SPR analyses.

3.2. Fluorescence spectroscopy analysis of β-casein interactions with curcumin and vitamin D3

Fluorescence spectroscopy dealing with transitions from the excited state to the ground state has a widespread application in the field of biophysics, pharmaceuticals but also food science (Williams & Daviter, 2013). In order to get an additional insight on the interaction between small hydrophobic molecules and β-casein, the intrinsic fluorescence emission of β-casein solution excited at 280 nm wavelength was investigated after a progressive addition of each bioactive compound (vitamin D3 and curcumin). As for SPR study, the β-casein concentration is below its CMC in order to prevent its self-association. Fluorescence emission spectra of β-casein display a peak at 345 nm as shown in Fig. 3. The protein excitation at 280 nm caused the emission of mainly tryptophan residues and, in aqueous solution, the emission maximum wavelength of free tryptophan is close to 352 nm. A lower λ_{max} of 345 nm was observed for the single tryptophan of individual β-casein located in its hydrophobic fragment. This blue shift could be attributed to the apolar environment location of this residue (Benzaria et al., 2013). The emission spectra of the β casein solution containing increasing content of curcumin (Fig. 3A) or vitamin D3 (Fig. 3B) show that the increase of bioactive compound concentration induced a gradual decrease of β-casein fluorescence intensity. This fluorescence quenching is attributed to curcumin-β-casein or vitamin D3-β-casein complex formation reducing the fluorescence intensity of the tryptophan residues residue. The maximum of the fluorescence emission spectra (λ_{max}) was plotted against curcumin and vitamin D3 concentration in Fig 3C and 3D,

348 respectively. Curcumin-β-casein binding generates a blue shift of fluorescence emission
349 maximum wavelength by ~12 nm in the presence of curcumin (Fig. 3C). This λ_{max} shift
350 points out the change in the polarity of the β-casein tryptophan residue
351 microenvironment, as consequence of curcumin binding which strongly suggests the
352 hydrophobic character of curcumin and β-casein interactions. This hydrophobic contact
353 of curcumin with hydrophobic domain of β-casein was also reported by Bourassa,
354 Bariyanga, & Tajmir-Riahi (2013). Conversely, no shift was observed for vitamin D3-β-
355 casein complexation (Fig. 3B) suggesting that the transfer of vitamin D3 from the polar
356 aqueous environment to the apolar hydrophobic region of β-casein does not modify the
357 hydrophobicity region close to the tryptophan residue as concluded for curcumin. This
358 was in accordance with a previous study showing that the interactions of β-casein with
359 vitamin D3 are mainly hydrophobic inducing a disturbance in the phenylalanine
360 residues located in the hydrophobic domain of β-casein (Forrest et al., 2005). Besides,
361 as the phenolic hydrogens of curcumin have pKa values of 8.38, 9.88, and 10.51 in
362 aqueous solutions, at experimental pH (~7.1), curcumin is in a neutral form and its
363 binding due to charge interactions can be neglected (Sahu et al., 2008).
364 The binding constants were evaluated from the fluorescence quenching of β-casein in
365 presence of bioactive compound. In order to check the presence of static or dynamic
366 quenching in bioactive compound-β-casein complexes, Eq. 4 was used to evaluate the
367 Stern-Volmer constant (Lakowicz, 2006):
368 $$S_0/S = 1 + K_{sv}[Q] \quad (4)$$
369 where S_0 and S are the fluorescence areas in the absence and presence of bioactive
370 compound, respectively, [Q] is the bioactive compound concentration and K_{sv} is the
371 Stern-Volmer quenching constant. The Stern-Volmer plots for β-casein quenching by
372 curcumin and vitamin D3 are presented in Fig. 4A and 4B, respectively. A linear

dependence at bioactive compound concentrations lower than 10 µM clearly appeared for the two hydrophobic bioactive compounds indicating that the quenching is mainly static in nature with some degree of dynamic quenching at high bioactive compound concentrations. The Stern-Volmer quenching constants (K_{SV}) for curcumin and vitamin D3 were evaluated to $13.5 \pm 2.0 \times 10^4$ M^{-1} and $3.4 \pm 0.3 \times 10^4$ M^{-1}, respectively.

When small molecules are bound independently to a set of equivalent sites on a macromolecule, the binding constant and the number of binding sites can be comparatively calculated assuming the static quenching by Eq. 5:

$$\mathrm{Ln}[(S_0\text{-}S)/S] = \ln K_A + n \ln[Q] \qquad (5)$$

Here, S, S_0, and [Q] are the same as the parameters in the Stern-Volmer equation (Eq. 4), K_A is the association constant and n is the number of binding sites. The plots of ln[(S_0-S)/S] versus ln[curcumin] and ln[vitamin D3] are shown in Fig. 4C and Fig. 4D, respectively. The n, K_A, and K_D estimated values for the β-casein-bioactive compound complexes are summarized in Table 1. The obtained K_A and the number of binding sites value (n) for curcumin were $23.5 \pm 1.9 \times 10^4$ M^{-1} and 0.74 ± 0.05, respectively. This association constant is in good agreement with that reported for bisdemethoxycurcumin of about $2.01 \pm 0.03 \times 10^5$ M^{-1} (Mehranfar, Bordbar, Keyhanfar & Behbahani, 2013). However, a four times lower K_A value of $4.4 \pm 0.03 \times 10^4$ M^{-1} was obtained for diacetylcurcumin-ß-casein interactions evaluated by fluorescence quenching in another previous study (Mehranfar, Bordbar, Fani, & Keyhanfar, 2013). These results suggest that the phenolic OH groups of curcumin and bisdemethoxycurcumin play an important role in the interaction with ß-casein (Mohammadi, Bordbar, Divsalar, Mohammadi, & Saboury, 2009). Lower K_A values in the order of 1.10^3 M^{-1} were obtained for other polyphenols (Hasni, Bourassa, Hamdani, Samson, Carpentier, & Tajmir-Riahi, 2011). The fluorescence quenching results obtained in a previous study investigating the

interaction between curcumin and whole casein showed a higher binding constant (13.43×10^5 M^{-1}) than that obtained in the present study (Nadi et al., 2014). This difference could be attributed to the presence of α and κ-caseins in the whole casein dispersion tested. The thermodynamic parameters evaluated in this study confirmed the hydrophobic nature of casein-curcumin interactions. Besides, the fluorescence results suggested that curcumin was located at the vicinity of the caseins tryptophanyl side chains due to a fluorescence energy transfer from the fluorophores (tryptophans) to the quencher (curcumin).

Concerning vitamin D3, a previous study on vitamin D3 and bovine β-casein interactions (Forrest et al., 2005) reported significantly higher K_A values ($3.8\text{-}7.7 \times 10^6$ M^{-1}) compared to the K_A presented in Table 1 ($5.8 \pm 1.1 \times 10^4$ M^{-1}). This difference could be attributed to the ionic strength effect on the protein charges by the ions enhancing hydrophobic interactions due the decrease of the protein solubility.

Curcumin has clearly a significantly higher affinity to β-casein than vitamin D3. Firstly, the hydrophobicity of vitamin D3 (logP = 7.9) is significantly higher than that observed for curcumin (logP = 3.6), as evaluated by Costache, Sheihet, Zaveri, & Knight (2010), potentially limiting its flexibility in the aqueous medium during fluorescence measurements. Besides, the higher interaction obtained for curcumin could be attributed to its phenolic hydroxyl groups playing an important role in bioactive compound-protein interactions. In fact, the two phenyl rings of curcumin are involved most likely in π-π stacking interaction with the aromatic side chains of β-casein (Hobani, Jerah, & Bidwai, 2017; Mcgaughey, Gagne, & Rappe, 1998).

Comparing the binding kinetic results obtained using SPR and fluorescence quenching, it clearly appears that the equilibrium association constant (K_A) obtained from fluorescence quenching is significantly higher than that evaluated by SPR (Table 1).

Same observations were previously reported by studies comparing SPR and fluorescence quenching for bimolecular interaction determination highlighting also lower association constants for SPR (Plesa et al., 2011; Xiao et al., 2011). This difference may be attributed to the different experimental conditions of these two techniques. The fluorescence quenching method is based on quenching of tryptophan residues when the excitation wavelength is set at 280 nm. That is to say, this method focuses on the interaction between tryptophan and hydrophobic molecules. When the fluorescence intensity decreases with the bioactive molecule addition, it means that the microenvironment of the tryptophan changed and the binding occurred between β-casein and the hydrophobic bioactive. Thus, the fluorescence quenching method does not consider the interaction between bioactive compounds and other sites on β-casein. Concerning SPR method, it is based on refractive index change on the sensor chip surface. The entire signal is caused by any interaction recorded without consideration of the binding sites. However, in the case of our experiments, the β-casein monomers were immobilized on the sensor chip surface by amine coupling involving the binding of free amine groups mainly present on its hydrophilic tale which could favor the hydrophobic fragment available to interact with other molecules. The SPR allows the description of binding events of a number of different molecules to the immobilized ß-casein, which can be monitored in real-time without labeling requirements. The major advantage of SPR is the evaluation of specific interactions since the unspecific binding is evaluated on a control channel and thus removed from the measured signal. Therefore, both methods can be used to evaluate the interactions between hydrophobic bioactive compounds and β-casein while bearing in mind that the fluorescence method is focused on binding sites around the fluorophore residues while SPR is more accurate for all kinds of binding sites investigation.

448

449 **4. Conclusion**

450 ß-casein, the most hydrophobic casein in bovine milk, displays an open conformation
451 which is easily accessible to gastric proteases. Hence, it may be used as an oral delivery
452 vehicle to release hydrophobic bioactive molecules such as curcumin and vitamin D3.
453 The investigation of the affinities and kinetics of the interaction between ß-casein and
454 two hydrophobic compounds was performed to understand how these bioactive
455 compounds could be associated to ß-casein prior to their delivery in the organism. We
456 have evaluated the interactions between curcumin and vitamin D3, as model
457 hydrophobic nutraceutical compounds, and ß-casein by a multimethodological approach
458 using surface plasmon resonance (SPR) biosensor in comparison with fluorescence
459 spectroscopy as a traditional and classical method to evaluate the association constants.
460 The fluorescence quenching data show that curcumin has higher affinity to β-casein (K_A
461 $= 23.5 \pm 1.9 \times 10^4\ M^{-1}$) than vitamin D3 ($K_A = 5.8 \pm 1.1 \times 10^4\ M^{-1}$). The K_A value for
462 curcumin-β-casein interaction has been successfully evaluated ($2.45 \pm 1.4 \times 10^3\ M^{-1}$)
463 using SPR by fitting data to a 1:1 Langmuir interaction model. Conversely, the SPR
464 responses obtained for vitamin D3 show that the interactions between this hydrophobic
465 compound and the β-casein immobilized on the sensor chip were below the sensitivity
466 of the SPR apparatus. It clearly appears that the equilibrium association constant (K_A)
467 obtained from fluorescence quenching is significantly higher than that evaluated by
468 SPR. This difference may be attributed to the different principles of these two
469 techniques. The fluorescence quenching method is based on quenching of tryptophan
470 residues when they interact with hydrophobic molecules without consideration of the
471 interactions with other sites on β-casein. Unlike fluorescence quenching, SPR, a
472 powerful non-destructive and label-free technique, offers a direct and real-time

473 monitoring of the interaction kinetics regardless of the binding sites. The fluorescence
474 quenching is more accurate to study the binding sites around the tryptophan residues
475 while the SPR technique offers the opportunity to study all kinds of binding sites. In
476 summary, both SPR and fluorescence results provided evidence about the higher
477 affinity of curcumin with β-casein compared to vitamin D3. The SPR developed method
478 is versatile for measuring the binding of β-casein to other bioactive molecules,
479 promising applications in many other protein-small molecules interactions.
480
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484
485 **References**
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633
634

635 **Figure captions**

636 **Figure 1:** Binding sensorgrams for curcumin interaction with immobilized β-casein on
637 CM5 biosensor at 25°C (A and B) and steady-state affinity analysis of curcumin
638 binding to β-casein fitted to a 1:1 interaction model (C and D). Two β-casein
639 immobilization levels of 6900 RU (A and C) and 2700 RU (B and D) were tested.
640 Curcumin was prepared at 34 mM in DMSO then injected in the flow system at
641 different concentrations (a) 0, (b) 3.9, (c) 7.8, (d) 15.6, (e) 31.2, (f) 62.5, (g) 125, (h)
642 250, (i) 500 and (j) 1000 µM. The experiment was repeated three times.

643

644 **Figure 2:** Steady-state affinity analysis of β-casein binding to curcumin and vitamin D3
645 respectively, prepared in different solvents for stock solutions then diluted in HBS-EP+.
646 β-casein immobilization level was 2 700 RU.

647

648 **Figure 3:** β-casein intrinsic fluorescence emission spectra (T=25°C, λ_{ex}=280 nm) and
649 shift of the maximum peak in the presence of increasing concentration of curcumin and
650 vitamin D3 added to 8 µM β-casein. Curcumin was prepared at 3 mM in absolute
651 ethanol then added to β-casein solution at different concentrations (a) 0, (b) 0.7, (c) 1.4,
652 (d) 2, (e) 2.8, (f) 3.5, (g) 5.5, (h) 6.9, (i) 10.4, (j) 13.9, (k) 20.8, (l) 27.7, (m) 34.7 and
653 (n) 69 µM. Vitamin D3 was prepared at 3 mM in absolute ethanol then added to β-
654 casein solution at different concentrations (a) 0, (b) 0.8, (c) 1.7, (d) 2.5, (e) 3.4, (f) 4.2,
655 (g) 5.1, (h) 6.8, (i) 8.5, (j) 12.7, (k) 17, (l) 25.5, (m) 34 and (n) 85 µM. The experiment
656 was repeated three times.

657

Figure 4: Sterne-Volmer plots of the fluorescence quenching of the β-casein by curcumin (A) and vitamin D3 (B) and the plot of ln[(S_0-S)/S] versus ln[curcumin] (A) and ln[Vitamin D3] (B) for the binding parameter evaluation with β-casein at 25°C.

Figure 1

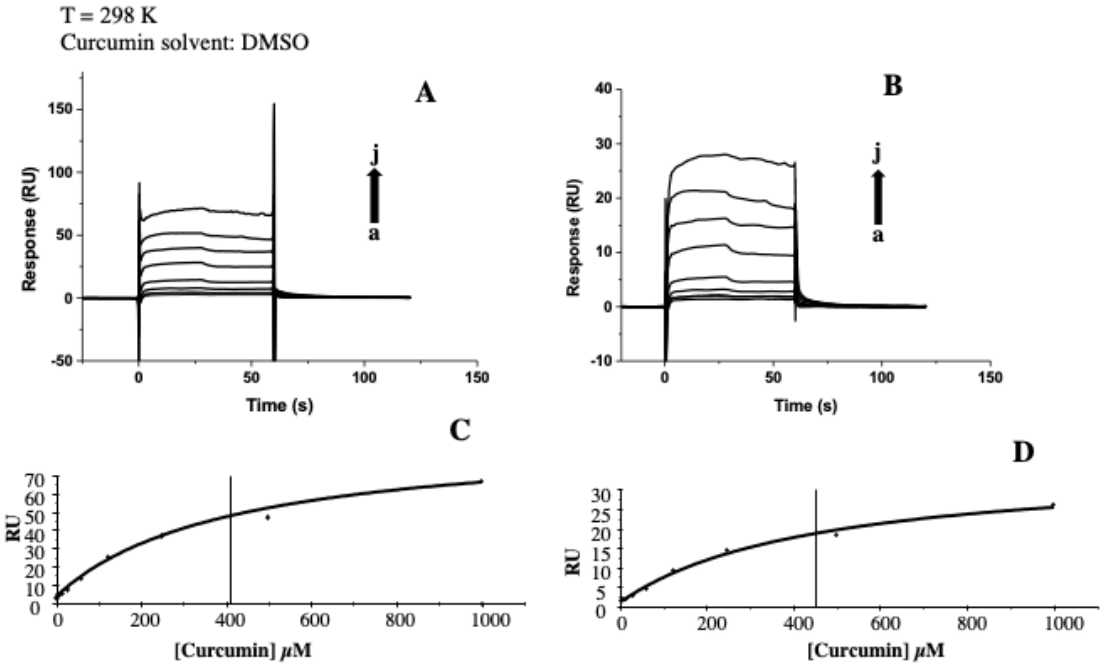
T = 298 K
Curcumin solvent: DMSO
A
B
C
D
Response (RU)
Time (s)
j
a
RU
[Curcumin] μM

Figure 2

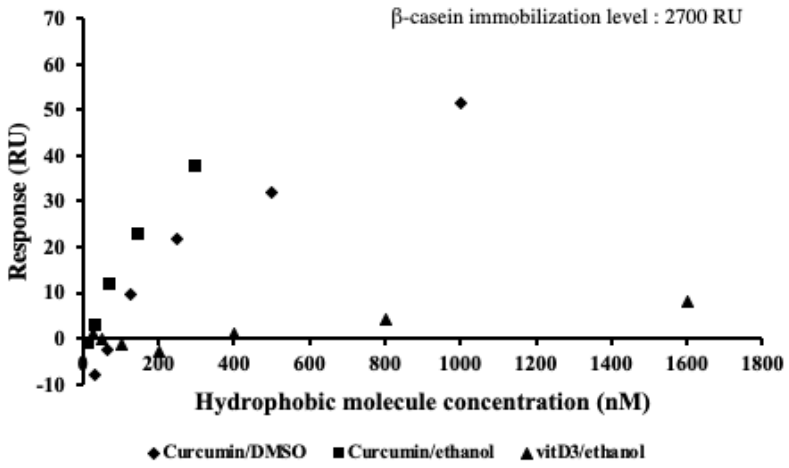
β-casein immobilization level : 2700 RU
Response (RU)
70
60
50
40
30
20
10
0
-10
200
400
600
800
1000
1200
1400
1600
1800
Hydrophobic molecule concentration (nM)
Curcumin/DMSO
Curcumin/ethanol
vitD3/ethanol

Figure 3

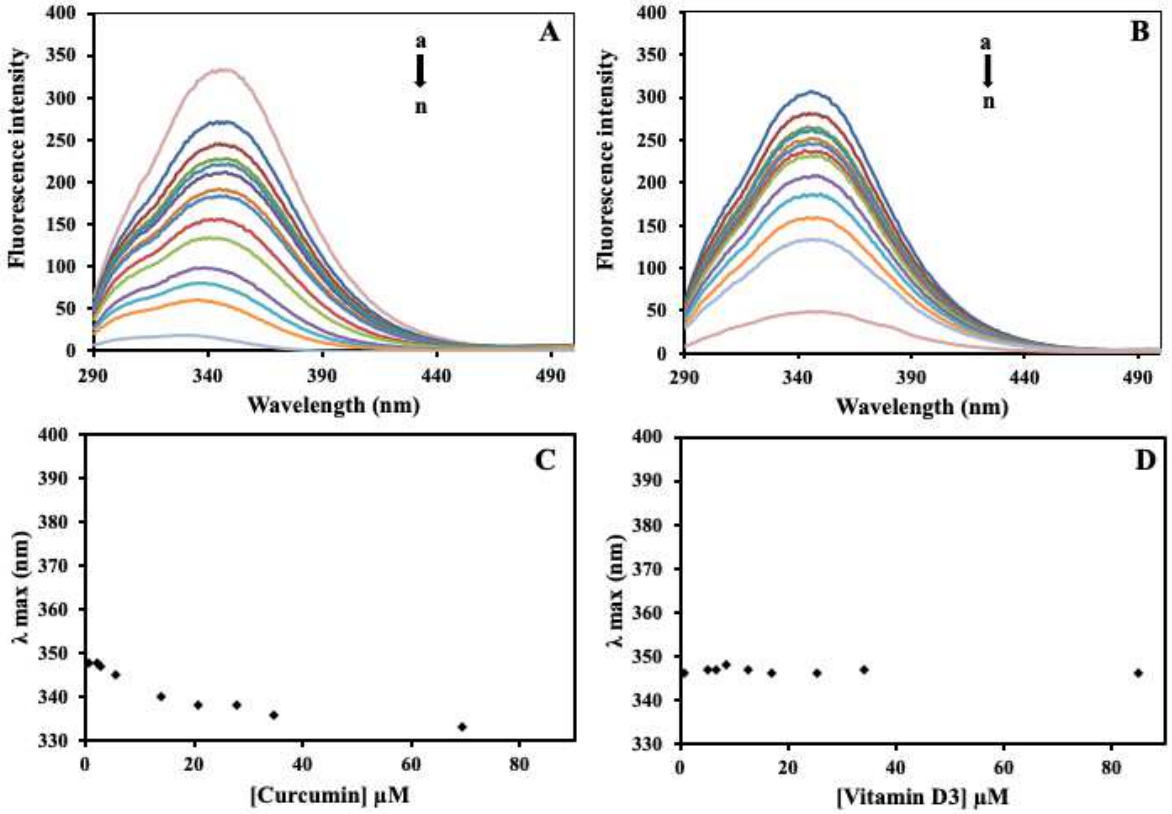
A
B
C
D
a
n
Fluorescence intensity
Wavelength (nm)
λ max (nm)
[Curcumin] µM
[Vitamin D3] µM

Figure 4

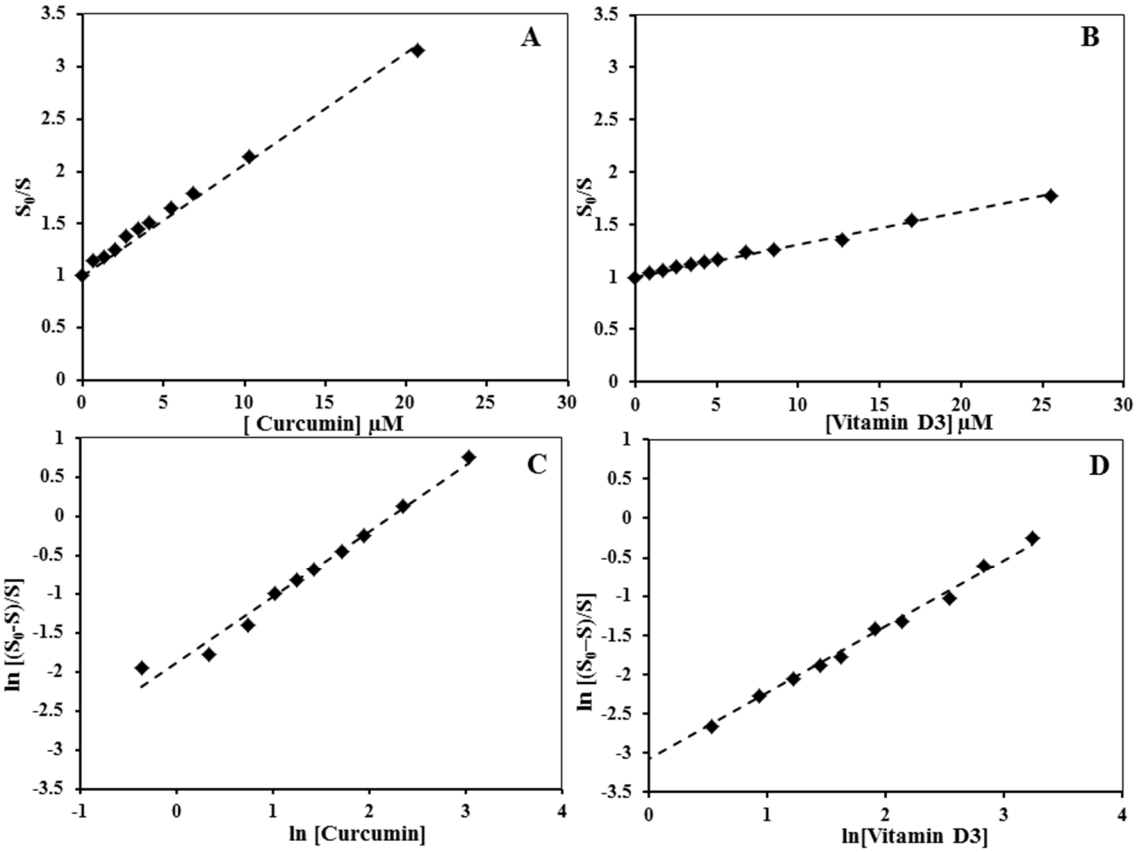
A
B
C
D
S_0/S
[Curcumin] µM
[Vitamin D3] µM
ln [(S0-S)/S]
ln [Curcumin]
ln[Vitamin D3]

Table 1: Binding parameters of the interactions between curcumin or vitamin D3 and β-casein at 25°C obtained by SPR and fluorescence quenching.

Method	SPR	Fluorescence quenching		
	K_A	K_A	n	K_{sv}
	($\times 10^3 M^{-1}$)	($\times 10^4 M^{-1}$)		($\times 10^4 M^{-1}$)
Curcumin	2.45 ± 1.42	23.5 ± 1.9[a]	0.74 ± 0.05[a]	13.5 ± 2.0
Vitamin D3	-	5.8 ± 1.1[b]	0.82 ± 0.04[a]	3.8 ± 0.3

Results are expressed as the means ± standard deviations of three replicates.

Different superscript letters presented in the same column indicate significant differences ($p < 0.05$)