Release of coumarin encapsulated in chitosan-gelatin irradiated films

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Abstract

Chitosan and fish gelatin, by-products from marine industry, were used to prepare active biobased films containing an antioxidant (coumarin). After drying, the films were irradiated at 40 and 60kGy by electron beam accelerator. The effect of irradiation on the film properties as well as the antioxidant release mechanism were investigated and compared with the control. -Electron Spin Resonance (ESR) unravelled free radical formation during irradiation in films containing coumarin. After antioxidant addition and/or irradiation treatment, only a shift of amide A, and amide B peak was observed for all the films, and a shift of amide II band for the control film after 60kGy irradiation dose. Irradiation allowed to improve the thermal stability of the control films. Both addition of coumarin and irradiation increased the surface wettability (increase of the polar component of the surface tension). From the water barrier analysis, neither irradiation nor coumarin addition influenced the permeability at the lower RH gradient used (0-30% RH). Using the higher RH gradient (30-84%) induced a rise of the WVP of all films (containing or not coumarin) after irradiation treatment. At 60kGy, the tensile strength of only the control films increased significantly. Finally, even if functional and structural properties are only weakly affected, it is enough to modify the release kinetics of the antioxidant into aqueous medium. The apparent diffusion coefficient of coumarin is two times reduced after irradiation. Irradiation also allowed to better protecting the encapsulated antioxidant. Indeed, the amount of coumarin in the non-irradiated film was significantly lowered Compared to the initial quantity, which is probably due to degradation. Coumarin in irradiated films is more protected considering this aspect.

Key Words: Electron beam irradiation; coumarin; chitosan-fish gelatin edible film; functional and structural properties, release properties

1. Introduction

Maintaining food quality, improving safety, and reducing storage losses waste are key objectives of a sustainable food system. Nowadays, the modern food industry is facing new challenges one of which being related to the food packaging to extend shelf life. Currently, a great number of research works are focused on the use of bio-based films with good water and oxygen barrier properties to protect food (Fabra et al., 2011). Since the consumer demand has shifted to safe materials, especially from renewable agriculture by-products and food processing industry wastes (Tharanathan, 2003), natural polymers (proteins, polysaccharides) are the main components of edible films (Gontard & Guilbert, 1994). They are considered as active packaging when they incorporate active compounds, such as antimicrobials, preservatives or antioxidants, which allow to improve food quality and safety (Han, 2002). Chitosan is a natural polymer from fish industry waste obtained by the deacetylation of chitin. It is a nontoxic material, biocompatible, and biodegradable that manifests antibacterial properties. In acidic environment the amino groups are protonated and their positive charges can interact with polyanions such as alginate, carrageenan, gelatin, etc. forming polyelectrolyte complexes (PEC) increasingly used in the encapsulation of various biocomponents. Due to these characteristics, chitosan has been widely used for the production of edible films as well as bio-compatible polymeric materials (Aider, 2010; Rivero et al., 2010). Gelatin is another widely used bio-based material obtained by the controlled hydrolysis of the insoluble fibrous collagen present in the bones and skin generated as waste during animal slaughtering and fish processing. Its excellent film forming ability is well-known (Hoque et al, 2011). Gelatin-based films are used for coating or packaging in order to maintain the quality of foods during storage, due to its good barrier to oxygen, light and prevention of dehydration and lipid oxidation (Jongjareonrak et al, 2006). Most research on gelatin film has focused on gelatin derived from mammalian sources such as bovine and porcine. Recently, there has been more interest in using fish as alternative sources of gelatin, due to

religious considerations or fear of bovine spongiform encephalopathy (Pérez-Mateos et al., 2009).

Chitosan and gelatin have been shown to be compatible due to the ability to associate through electrostatic and hydrogen bonding. Specifically, when chitosan is positively charged and gelatin is negatively charged under appropriate conditions of pH. This is particularly important to improve the final network properties as compared to those obtained from the pure polymers (Benbettaieb et al., 2015a). Thus, many investigations focused on their possible use as a matrix to obtain bio-packaging materials. In this sense and to make films even more useful, functional edible films that contain active compounds have been developed, to enhance food quality and product shelf-life (Suppakul et al, 2003). The incorporation of antioxidants in these biodegradable and edible polymers is an interesting alternative to food preservation, since oxidation is one of the major problems affecting food quality as well as film biopolymer stability during ageing (Martins et al, 2012). The use of natural, non-toxic antioxidants such as ferulic acid or α-tocopherol to preserve the consumer health has been investigated (Fabra et al, 2011). Several researchers have previously reported on the potential benefits of using naturals antimicrobials and antioxidants compounds in edible and bio-based films for extending food shelf life (Oussalah et al., 2004; Ouattara et al., 2000). However, little information exists about the influence of these compounds on films structural and physicochemical properties.

Recently, Tammineni et al.(2014) reported that mechanical and barrier properties of bovine gelatin films were improved after tannic acid incorporation. Furthermore, crosslinking bovine gelatin films with tannic acid results in reduction of film solubility by about 80% (Zhang et al., 2010). Kavoosi et al. (2014) studied antioxidant and antibacterial activity of gelatin films incorporated with *Zataria multiflora* essential oil (2 to 8% w/w of gelatin). They reported that beside their excellent antibacterial properties against both Gram-positive and Gram-negative bacteria, bioactive films have new functional properties. Peng and Li. (2014) demonstrated that

94 water vapour permeability of chitosan films decrease while the tensile strength inversely
95 increases when essentials oils are incorporated. Therefore, we were interested in encapsulating
96 these natural compounds into chitosan-gelatin blend edible films. Moreover, physical methods
97 including dehydrothermal treatment, ultraviolet, heat and gamma irradiation (Bigi et al., 1998)
98 help to modify the polymeric network through the cross-linking of the polymer chains and also
99 help to improve the functionality of polysaccharide (Sabato et al., 2000) or protein (Vachon et
100 al., 2000) based films. Indeed, irradiation treatments have been described to enhance water
101 barrier and mechanical properties of protein-polysaccharides complexes (Lacroix et al., 2002;
102 Lee et al., 2004; Jo et al., 2005; Inamura et al., 2013). The structural modifications induced by
103 irradiation could increase the capacity of cross-linked edible films to control the release of
104 embedded active compounds. In the current literature there is a lack of detailed-studies dealing
105 with the effects of polymers structure, in particular chitosan-gelatin films, on the retention and
106 release properties of the added antioxidant compounds (Papadokostaki et al, 1997). Very few
107 studies have been published on the impact of irradiation on the release of active compounds from
108 natural biopolymers. Indeed, Tin Wui et al. (2002) showed that the release-retarding property of
109 alginate and alginate–chitosan beads is significantly enhanced after the beads irradiated by
110 microwave. In the same way, Lacroix et al. (2002) displayed that gamma-irradiation induces
111 cross-links in calcium caseinate edible films and thus allows a better control of enzyme and
112 active compounds release. Previous works displayed that ferulic acid, quercetin or tyrosol
113 addition affected differently the functional properties of gelatin-chitosan films according the
114 irradiation dose (Benbettaieb et al, 2015b).Irradiation accentuated the wettability and the
115 hydrophilicity of the film containing antioxidants whereas oxygen barrier and thermal stability
116 were enhanced.
117 The aim of this study is to further investigate the effect of coumarin addition and electron beam
118 irradiation on the mechanical, thermal, barrier and structural properties of chitosan-fish gelatin

edible films. The effect of irradiation on the coumarin release in liquid medium was also investigated.

2. Materials and methods

2.1. Materials and reagents

Commercial grade chitosan (CS) (France Chitine, , MW=165 kDa, low viscosity, 85%, deacetylation degree, France) and commercial grade fish gelatin (G) (Rousselot 200 FG 8 with a 180 Bloom degree, a viscosity of 4 mPa.s at 45°C and with the concentration of 6.67% ,in water, and a pH=5.4) were used as film-forming matrix. Anhydrous glycerol (GLY) (Fluka Chemical, 98% purity, Germany) was used as a plasticizer in order to improve the mechanical properties of the films. Glacial acetic acid (Sigma, 99.85% purity) was used to prepare the solvent for chitosan and helped to improve their solubility. Silica gel and potassium chloride saturated salt solution (KCl, Sigma-Aldrich, France) were used to fix the relative humidity at <2% and 84% for water vapour permeability measurements. Coumarin (minimum purity 99%, Sigma Aldrich, molecular weight = 146 $g.mol^{-1}$, molar volume = 117 $cm^3.mol^{-1}$, melting point = 70°C, LogP = 1.39, solubility in water = 1.9 $g.L^{-1}$ (at 25°C), boiling point =298°C (à 101325 Pa), surface tension= 46.4 $mN.m^{-1}$ (at 25°C), saturated vapour pressure = 1.33 Pa (at 25°C), data from Chemspider.com,) was used as a model ~~of~~ natural antioxidant molecule.

2.2. Film formation

20 grams of the chitosan powder was dispersed in 1 L of a 1% (v/v) aqueous acetic acid to obtain a 2% (w/v) film forming solution. The solution was homogenized at 1200 rpm with high shear homogeniser (Ultra Turrax (RW16 basic- IKA-WERKE) at 25°C. As a clear film-forming solution was obtained, no more treatment has been applied to the chitosan solution for improving the solubilisation. Then, 2.2 grams of glycerol (10% w/w dry matter) was added to this solution,

under stirring. The pH of the chitosan solution was about 4.9±0.2. A 60 grams of fish gelatin powder was separately solubilized in 1 L of distilled water under continuous stirring and heating at 70°C for 30 min to obtain a 6% w/v solution (pH≈6.5). 6.6 grams of Glycerol (10% w/ dry matter) was added to this film forming solution after complete solubilisation of gelatin.

Subsequently, equal weight of the respective solution was mixed at 1:1 ratio and stirred for 30 min and pH was adjusted to 5.6 with acetic. This condition was specifically designed to obtain a polyelectrolyte complex between chitosan and gelatin since the iso-electric point of gelatin (Ip= 4.5-5.2) while the pKa of the amino group (pH=6.2-6.5) of chitosan. At this condition, therefore, gelatin is negatively charged while chitosan is positively charged thus avoiding any phase separation upon mixing. Coumarin was added to the final film forming solution at a concentration of about 50 mg /g polymer (corresponding to a 47 mg/g total dry weight of film). The aqueous dispersions were homogenized at 1200 rpm using the Ultra Turrax until complete dissolution.

30 mL of the film forming solution (FFS) in the presence and absence of coumarin was then poured into plastic Petri dishes (13.5 cm diameter). A minimum of 30 films (ie 30 Petri dishes) have been prepared for each formulation. Aqueous solvent was removed by drying in a ventilated climatic chamber (KBF 240 Binder, ODIL, France) at 25°C and 45% RH for 18 to 24h. After drying, films were peeled off from the surface and stored up to equilibrium in a ventilated climatic chamber (KBF 240 Binder, ODIL, France) at 50% RH and 25°C before each measurements.

2.3. Radiation treatment

Irradiation was carried out in the AERIAL pilot plant (Innovation Park, Illkirch, Strasbourg, France), using a linear electron accelerator at ambient temperature (20±0.5ºC). Thin dried films (70 to 85µm thickness) were irradiated for 40 and 60kGy using a 2.2 MeV energy electron beam

at a dose rate of 0.3 kGy/sec. One batch of film formation was preserved as non-irradiated reference. We selected a maximum of 60kGy doses that can yield a high density of crosslinking in proteins and in the range of authorized doses in food and packaging. Moreover, according to the Codex General Standard for Irradiated Foods (CAC, 2003), ionizing radiations foreseen for food processing are recommended to be limited to 60kGy. Dosimetry was performed using alanine pellet dosimeters calibrated according to ASTM/ ISO 51607 (2004).

2.4. Film characterizations

2.4.1. Thickness measurement

Film thickness was measured with an electronic gauge (PosiTector 6000, DeFelsko Corporation, USA). Five measurements were taken for each film sample, one from the center and four close to the perimeter. Mean value was used in further calculations. The film thickness according the sample and formulation ranges between 70 and 85

2.4.2. Mechanical properties

A universal traction testing machine (TA.HD plus model, Stable MicroSystems, Haslemere, England) with a 300 N load cell was used to determine tensile strength (TS, MPa), Young's modulus (YM, MPa) and percentage of elongation at breakpoint (E, %), according to ASTM method D882 (1992). Rectangular film samples (2.5×8 cm^2) were cut using a special precision sample cutter (Thwing-Albert JDC Precision Sample Cutter) in order to get tensile test piece with an accurate width and parallel sides throughout the entire length. Before testing, all samples were equilibrated for two weeks at 25°C and 50% relative humidity (RH). Equilibrated film samples were then placed in the extension grips of the testing machine and stretched uniaxially at a rate of 50 mm/min until breaking. TS, YM and E were determined from stress–strain curves. Measurements were carried out at room temperature (25±2°C) and five samples for each formulation were tested.

2.4.3. Water vapour permeability

The water vapour permeability (WVP) was determined according to the gravimetric method described in the ASTM E96-80 (1980) and adapted to edible and bio-based films by Debeaufort, Martin-Polo and Voilley (1993). Two relative humidity gradients were used: 0-30% and 30-84%. Prior to WVP measurements, all film samples were equilibrated at 25 ± 0.5°C and 30% relative humidity for 72 h. The average value of five thickness measurements per type of film was used in the WVP calculations (statistical error on the film thickness was taken into account in WVP uncertainty). The film samples (6.44 cm^2 discs) were placed between two teflon rings on the top of the glass cell containing silica gel (0% RH) for the first RH gradient (0-30%) or a salt solution of KCl (84% RH) for the second RH gradient (30-84%). The second RH gradient was selected to obtain an average water content of film at the stationary state of permeation that corresponds to the RH of 50% used for mechanical property characterizations and FTIR analysis. Those permeation cells were then introduced into a climatic chamber (KBF 240 Binder, ODIL, France) maintained at 30% RH and 25±0.5°C and periodically weighed.

The WVP ($g.m^{-1}.s^{-1}.Pa^{-1}$) calculation was based on the change in the absolute value in weight loss of the permeation cell versus time once the steady state was reached (Benbettaieb et al, 2015a). Five replicates for each film formulation were performed.

2.4.4. Surface properties

2.4.4.1. Water contact angle

The sessile drop method was used for contact angle measurement, with a DGD-DX goniometer (GBX, Romans-sur-Isere, France), equipped with the DIGIDROP image analysis software (GBX, Romans-sur-Isere, France), according to Karbowiak et al. (2006). A droplet of liquid (~ 1.5 µL) was deposited on the film surface with a precision syringe. Then, the method is based on image processing and curve fitting for contact angle measurement from a theoretical meridian drop profile, measuring contact angle between the baseline of the drop and the tangent at the

drop boundary. The contact angles with water and diiodomethane at 0 and 20 s were measured from both sides of the drop and averaged. The measurement was carried on over 120 s. Five replicates per film were carried out.

2.4.4.2. Surface tension

The surface tension (γ_S) of the film and its polar (γ_S^p) and dispersive (γ_S^d) components were calculated using diiodomethane ($\gamma_L = 53$ mN/m; $\gamma_L^d = 50.8$ mN/m and $\gamma_L^p = 0$ mN/m) and water ($\gamma_L = 72$ mN/m ; $\gamma_L^d = 21.8$ mN/m and $\gamma_L^p = 51$ mN/m) knowing their surface tension (γ_L) and respective dispersive (γ_L^d) and polar components (γ_L^p) given by Strom et al.(1987) and according the following equations established by Owens and Wendt (1969) :

$$\gamma_S = \gamma_S^d + \gamma_S^p \quad \text{..... Eq.1}$$

$$\gamma_{Li}(1 + cos\theta i) = 2[\sqrt{\gamma_S^d \times \gamma_{Li}^d} + \sqrt{\gamma_S^p \times \gamma_{Li}^p}\,] \quad \text{.....Eq.2}$$

where the subscripts S and L refers to the solid (the film surface) and the liquid, respectively. As only two liquids have been used for the regression, the accuracy of values obtained from this analysis has been considered at the p-level of 0.01.

2.4.4.3. Swelling index and swelling rate with water

Swelling index was obtained from the water drop volume kinetics using the following equation:

$$\text{Swelling index (\%)} = \left(\frac{\Delta V}{V_0}\right).100 = \left(\frac{V_{max} - V_0}{V_0}\right).100 \quad \text{Eq.3}$$

where ΔV is the droplet volume variation (µL) measured on the film sample (over the first 20s). V_{max} is the maximal apparent volume (µL) of the droplet and V_0 is the initial volume (µL) of the droplet.

Swelling rate was obtained from the drop volume kinetics using the following equation:

$$\text{Swelling rate (µL/s)} = \left(\frac{\Delta V}{\Delta t}\right) = \frac{V_{max} - V_{ini\ Sw}}{t_{max} - t_{ini\ Sw}} \quad \text{Eq.4}$$

Where ΔV is the droplet volume variation (μL) during Δt time (s) measured on the film sample, assuming a linear regression.

V_{max}: maximum swelling volume, $V_{ini\ Sw}$: initial volume of swelling, t_{max}: time of maximum swelling, $t_{ini\ Sw}$: initial time corresponding to the beginning of swelling

2.4.5. GPC-MALLS system

The GPC-MALLS system consisted of a degasser ERC-3215- α (ERC, Japan), a constametric ® 3200 MS pump (Thermo Separation Products, FL), an injection valve with 100 μL loop (Reodyne 7725i) fitted inside a temperature regulated oven (Gilson, Model 831, UK) maintained at 40^{o}C ±1^{o}C and a DAWN-DSP multi-angle light scattering photometer (Wyatt Technology, Santa Barbara, CA, USA) equipped with He–Ne laser (λ= 633 nm). Simultaneous concentration detection was performed using a calibrated differential refractometer (RI 2000, Schambek, Germany). A refractive index increment dn/dc value of 0.180 mL/gm was used in the calculations

The mobile phase was made to contain 0.15M ammonium acetate, 0.2M acetic acid and 0.1M sodium chloride and was filtered through 0.2 μm pore size cellulose nitrate membrane. The samples injected were subjected to prior filtration through a nylon filter of 0.45 μm pore size. A set of two columns SB-803HQ and SB-806HQ (8 mm × 300 mm, Shodex OHpak, Japan, exclusion limits 1×10^5 and 2×10^7 g/mol) , housed inside the oven, was used for the separation. The flow rate for the eluent was 0.45 mL/min. The Berry fitting method with linear fit was used for data processing in ASTRA software (Version 4.90.08). For the measurement of molecular weight, about 27mg (30mg of films containing about 12% water content) of chitosan-gelatin film incorporated with coumarin 5% (w/w polymer) was dissolved in 10mL of mobile phase. The solution was then heated in a water bath for 20 min at 45°C and subsequently centrifuged for

5min at 25000 rpm at 25°C. The soluble fraction was removed from the respective solution and was heated at 45°C for 5 min prior to injection into the GPC-MALLS system.

The entire GPC-MALLS system was maintained at 40 ± 1°C. Temperature control was achieved using the in-house heating methodology provided thermostatic heating to all pipework between the detectors.

2.4.6. Thermogravimetric analysis

Thermogravimetric analysis (TGA) was used to evaluate the thermal stability of the samples. Measurements were performed using a TA instrument (TA instruments Discovery TGA New-Castle, USA), from 25 to 800°C, at a heating rate of 20°C/min, under nitrogen atmosphere. The weight of the film sample (initially around 8 mg) was constantly measured with an accuracy of 0.01 mg. Films were stored at 25°C and 50% RH for two weeks before TGA measurements.

2.4.7. Attenuated Total Reflectance - Fourier Transforms Infrared (FTIR-ATR) spectroscopy

The Fourier Transform Infrared spectra from each film were obtained using a spectrometer (Perkin-Elmer, Spectrum 65, France) using Attenuated Total Reflectance (ATR) with ZnSe crystal. For each measurement, 32 scans in the wave length range 400-4000 cm^{-1} with a 4 cm^{-1} resolution were co-added before the Fourier transform. The spectra were collected in duplicate. This analysis aimed at determining the modifications at the molecular scale induced between active molecules and polymers after electron beam irradiation.

2.4.8. Electron spin resonance (ESR)

The ESR technique allows measuring the presence of free radicals. ESR signals were recorded at room temperature on a Bruker EMX spectrometer (Bruker, Berlin, Germany) controlled with a Bruker ER 041 XG microwave bridge operating at X-band (~9 GHz). 50mg of each film was cut and placed in the sampling tube. ESR spectra were carried out using 100 kHz magnetic field with 6G modulation amplitude, 20.12 mW microwave power, 9.49 GHz frequency and 5 scans.

2.5. Release of coumarin in aqueous medium

Prior to release experiments, the real concentration of coumarin in the films was determined. A film sample of about 60 mg (about 5x5 cm²) was immersed up to fully dispersion in 100 mL of acetic acid solution at pH 4 under stirring at 50°C in order to fully solubilized the polymers and the coumarin. The amount of coumarin in the liquid medium was determined by UV-vis spectrophotometry (Biochrom Libra S22) at 278 nm (previously determined from the absorbance spectrum of the pure coumarin in water). A series of standard solutions for this antioxidant (1, 2, 4, 5, 10, 25 and 50 mg/L) was used for calibration, according to the Beer-Lambert's law. This concentration was then compared to the theoretical content introduced in the films when prepared. The measured initial concentration was used to calculate the percentage of retention in the film after the release kinetics.

The release of the coumarin was carried out in triplicate using the rotating paddle dissolution apparatus (AT7 Smart type II, Sotax, Basel). 600 mg of each film were incorporated at time 0 in 1 L of the dissolution medium (water adjusted at pH=7 using 0.1 M of NaOH). 3 mL samples were withdrawn and assayed for antioxidant release periodically up to equilibrium. The amount of coumarin in the release medium was determined by UV-vis spectrophotometry (Biochrom Libra S22) as previously described.

The effective diffusion coefficient of coumarin in the film (D) was also determined from the release kinetics assuming a Fick's law (Eq.5), and considering the transient state of the transfer.

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2} \qquad \text{Eq.5}$$

Where C is the concentration of this antioxidant in the film over the time t, x is the thickness of the film and D is the effective diffusion coefficient.

The experimental method chosen corresponds to the case of diffusion of the solute from a plane sheet (film) into a stirred solution of limited volume (Crank, 1975). As the solution is constantly stirred, we assumed there is no boundary layer effect on diffusion. Therefore, the concentration of coumarin in the solution, initially zero, is considered to be uniform in the release medium

according to Crank (1975). The concentration of this antioxidant in the film is also assumed to be uniformly distributed within the film at time zero. We also consider a unidirectional diffusion of the coumarin in the film, and a D which does not depend on the concentration or on the time. This mass transfer equation (Eq.5) can thus be solved using the following analytic solution to the second Fick's law applied to transient state (Crank, 1975):

$$\frac{C_t}{C_\infty} = 1 - \sum_{n=1}^{\infty} \frac{2\alpha(1+\alpha)}{1+\alpha+\alpha^2 q_n^2} \exp\left(-\frac{D q_n^2 t}{l^2}\right) \qquad \text{Eq. 6}$$

Where, Ct is the concentration of coumarin determined in the dissolution medium over time, as previously detailed; C∞ is the maximum concentration of this antioxidant determined in the dissolution medium when equilibrium is achieved; $\alpha = V_s/(K \times V_f)$ with V_s the volume of solution (m^3), V_f the volume of the film (m^3) and K the partition factor; q_n are the non-zero positive roots of $\tan(q_n) = -\alpha q_n$ using n values between 1 and 6.

D is the effective diffusion coefficient ($m^2.s^{-1}$), and l is the half thickness of the film (m).

This model was applied to the release experimental kinetics (up to equilibrium) in order to determine the effective diffusion coefficient of coumarin in the film, by minimising the sum of the square of the differences between measured and predicted values, using the Levenberg–Marquardt algorithm, and taking D as adjustable parameter. Modeling was performed using Matlab software (The Mathworks, Natick, MA).

2.6. Statistical analyses

The data were analyzed using an independent sample t-test with the statistical software SPSS 13.0 (SPSS Inc., Chicago, IL). A standard deviation (p-value < 0.05) at the 95% confidence level was used to compare all parameters analysed (water vapour permeability, mechanical properties, surface properties and parameters of the kinetics of release) between irradiated and non-irradiated films in the presence and absence of coumarin.

3. Results and discussion

3.1. How irradiation and coumarin addition influence chemical and structural organisation of polymer-blend network

3.1.1. Free radical generation

Electron spin resonance (ESR) is an appropriate tool to detect and identify the generation of free radicals in a polymer structure. The ESR spectra of chitosan-gelatin blend film incorporated with coumarin before and after irradiation (60kGy) are displayed in **Fig.1**. A very weak ESR signal is detected, at 3365 G, in the case of non-irradiated films. Contrarily, just irradiated film displays a prominent peak at the same position suggesting the presence of free radical species induced by the irradiation treatment. This peak remains visible even after 3 months of storage revealing that free radicals are still present in the films.

Recently, we reported that the peak intensity, determined at 3500 G, for irradiated chitosan-gelatin film increases with increasing the irradiation dose Benbettaieb et al. (2015a).

The analysis of the peak to peak amplitude between film containing or not containing coumarin shows that, after irradiation at 60kGy, the peak amplitude is 2 to times higher for irradiated films compared to non-irradiated ones, for film without coumarin and for film containing coumarin, respectively.

The detection of free radicals generated within the films may contribute to clarify the role of these reactive species to initiate the reaction sequence inducing change in the chemical and structural organisation of the biopolymer-blend network. A new arrangement in the structure is expected after irradiation, which could favour the linkage between the biopolymers chains or between the biopolymers chains and the active compound. Even if the water content of film was quite low (about 12%), possible hydroxyl-coumarin obtained after hydroxylation reaction by OH radical produced from water radiolysis could react with polysaccharide and protein network. As

364 coumarin is a phenolic substance made of fused benzene and alpha pyrone ring, their phenolic
365 groups can be easily converted to quinone in presence of peroxide radicals (produced by
366 irradiation), via quinone-mediated reactions. Indeed, ahe slight change in the film colour after
367 irradiation is observed. This could be more probably attributed to quinone generation from
368 antioxidants more than Maillard reaction because the temperature involved in the film making
369 and irradiation process remained lower than 50°C. In addition to providing a source of stable free
370 radicals, quinones are known to complex irreversibly with nucleophilic amino acids in protein.
371 The reaction mechanism involves an initial oxidization of phenolic structures to quinones, which
372 can readily react with nucleophiles from reactive amino acid groups in protein: sulfuhydryl
373 group in cystein, amino group of lysine and arginine, amide group from aspartic and glutamic
374 acids, indole ring of tryptophan and imidazole ring from histidine (Zhang et al., 2010).This tends
375 to be confirmed as our films gain a yellowish colour after irradiation. Sahu et al. (2009) showed
376 the efficiency of microwave irradiation for the oxidation of phenol to quinone after free radicals
377 generation in the presence of hydrogen peroxide. Casimiro et al. (2010) showed that, in acidic
378 medium, NH_3^+ groups (from deacetylated units) of chitosan are able to be involved in some
379 interactions during irradiation. As displayed by Madeleine-Perdrillat et al. (2015), even at low
380 water content, molecular mobility in chitosan films remains quite high and allows the supposed
381 free radical mobility in the film. Knowing free radicals are present after irradiation, they could
382 originate sequences of reactions within and between polymer chains and antioxidant which could
383 in turn affect the structural properties of the final network. These structural and functional
384 properties modifications have been assessed in the followings.
385
386 **3.1.2. Changes in chemical structure by molecular interaction analysis**
387 GPC-MALLS was used to determine the molecular weight parameters of chitosan-gelatin film
388 before after irradiation and in the presence of coumarin. The typical elution profile (15 – 19 mL)

of chitosan-gelatin film was monitored by the light scattering (detector 90° degree), refractive index and UV at 280nm detectors (data not shown). The results are tabulated in **Table 1**. The weight average molecular weight for chitosan gelatin film in the presence and absence of coumarin was similar and almost an identical value was obtained following irradiation at 60kGy. The results given in **Table 1** show that there is no significant difference of for the molecular weight, the polydispersity and the z-radius between irradiated (and non-irradiated films are observed for the control). So irradiation induce neither covalent reticulation nor biopolymer degradation under the conditions employed in this study. The incorporation of coumarin has no additional effect on the molecular size of the biopolymers even when coupled with irradiation process

Fourier transform infrared spectroscopy (FTIR) was used in order to assess the possibility of interactions and the nature of linkage between polysaccharide-protein network and coumarin following irradiation treatment. FTIR spectra of irradiated and non-irradiated chitosan-fish gelatin blend films in the presence and absence of coumarin are displayed in **Fig. 2**. The spectrum of non-irradiated control film showed characteristic peaks: 3300-3360 cm^{-1}, assigned to ν_{OH} stretching of free water and ν_{NH} stretching of amide A, 2915-2935 cm^{-1}, assigned to ν_{CH} asymmetric/symmetric stretching of amide B, 1550-1680 cm^{-1}, assigned to C=C and C=O stretching of primary and secondary amine NH band of amide I, 1550-1610 cm^{-1}, assigned to δNH of amide II and 1240-1340 cm^{-1}, assigned to aromatic primary amine, CN stretch of amide III (Coates, 2000; Benbettaieb et al., 2015a). The peak observed in 1034 cm^{-1} is related to possible interactions arising between plasticizer (OH group of glycerol) and polymer structure via hydrogen bonds (Cerqueira et al, 2012). The amide B ν_{CH} (2932 cm^{-1}) is slightly shifted to higher wavenumber (2941 cm^{-1}) after incorporation of coumarin. However, no change is observed on amide-I, amide-II and amide-III peak position. Similar results was founded by

414 Benbettaieb et al.(2015b) after incorporation of ferulic acid in chitosan-gelatin film. Contrarily,
415 other author found a shift on amide I band, attributed to interaction between chitosan (amide)
416 and starch or ferulic acid (hydroxyls) in the case of starch-chitosan blend film incorporating
417 ferulic acid (Mathew and Abrahman, 2008); or to caffeic acid oxidation inducing protein
418 crosslinking (Nuthong et al, 2009); or to crosslinking in gelatin gel after UV irradiation (Bahat
419 and Karim, 2009). Therefore, FTIR analysis only shows a weak modification in the structure of
420 the final network without new significant linkage. Only the amide A and amide B (and amide II
421 for control film) groups exhibit a shift after both antioxidant addition and irradiation treatment.
422
423 **3.1.3. Thermo-gravimetric analysis (TGA)**
424 Thermo-gravimetric analysis was performed in order to study the effect of coumarin addition
425 before and after irradiation on the thermal stability of blend chitosan-fish gelatin films. **Fig. 3**
426 shows two main stages of weight loss events for all films. The first stage occurs over a
427 temperature range of around 51-121°C and results in a weight loss (Δw1) of approximately 5.2-
428 7.4%. It is associated with the loss of acetic acid and free water sorbed in the film. These results
429 are in agreement with Inamura et al. (2013), who observed similar behavior for biocomposite
430 films prepared with gelatin and nut wastes as fiber source (46-140°C). Barreto et al. (2003) and
431 Pena et al. (2010) also showed similar results for gelatin film, from 25 to 200°C. These
432 temperature ranges differences can be attributed to the variation of the initial water content as
433 well as the plasticizer used. The second stage of weight loss (Δw2= 42.3-53.8%) occurs in the
434 temperature range from 215 up to 330°C. This is most likely due to to the degradation of the
435 polysaccharide and protein backbones as well as the evaporation and thermal degradation of
436 glycerol (from 177-211°C up to 450°C (Maturana and Pagliuso, 2011)) and also structurally
437 bound water evaporation. Pure coumarin (powder) exhibits a single stage in weight loss, with
438 decomposition starting at 160-180°C and finishing at about 230°C **(Fig. 3)**. Only weak

difference was observed regarding thermal decomposition temperatures and weight loss (Δw1 and Δw2) when control film is compared to the films with coumarin due to the low coumarin content (47mg/g). Opposite results were observed in the case of skin gelatin after addition of star anise extracts (Hoque et al., 2011) and green tea extract (Wu et al., 2013). They suggested that interactions occurring between phenolic compounds and gelatin yielded to a stronger film network and therefore a higher heat resistance of the films. The above studies are comparable to those we recently reported on the same gelatin- chitosan film which showed improved thermal stability following the addition of quercetin (Benbettaieb et al., 2015b). After irradiation, thermal degradation temperature of the control film is improved, associated with a decrease observed on weight loss (Δw1 and Δw2). This result suggests the apparition of new bonds, thermally more resistant to heat than initial bonds existing before irradiation which enhances the thermal properties. Similar result was found by Inamura et al. (2013) in the case of composite gelatin-nut shell fiber after 40 kGy irradiation dose. Inversely, we cannot observe any significant modification of thermal stability for film containing coumarin after irradiation. Benbettaieb et al. (2015b) showed a reverse tendency for chitosan-gelatin film containing ferulic acid after 60kGy irradiation dose. Finally, from the above structural and thermal analysis, we can conclude that the interaction between coumarin and polymer chains is very weak and no covalent or strong linkage occurred following irradiation. For this reason, a complementary analysis must be undertaken to better understand the effect of both irradiation and coumarin addition on functional properties of films.

3.2. Impact of both irradiation and coumarin addition on functional film properties

3.2.1. Surface properties and wettability

The contact angle (θ) value obtained after deposition of a water drop on the film surface indicates the surface hydrophobicity. To estimate the resistance of films to liquid water, the

swelling index and swelling rate were also determined from the droplet volume kinetics, along with the contact angle values at the initial time of deposit (0 s) and at a considered metastable equilibrium (20 s). Results for all films are summarized in **Fig. 4**. For untreated films, the contact angles (at 0 and 20s) significantly decrease ($p<0.05$) after incorporation of coumarin. Furthermore, no swelling is observed for the control film. Swelling index and swelling rate significantly ($p<0.05$) increase to 52±4% and to 68±24x10^{-3} µL/s, respectively after addition of coumarin. After irradiation, the contact angles (at 0 and 20s) significantly decrease for all films (decreases is not significant only for the contact angle at 20s for coumarin film). Whereas, swelling index and swelling rate tend to increase for all films, but they are only significant ($p<0.05$) for the control films. To better understand the effect of coumarin on film surface properties under electron beam irradiation, the surface tension was also determined. Surface tension does not show significant modification after incorporation of coumarin. However, a slight increase is observed in the polar component. The presence of this antioxidant seems to slightly contribute to the hydrophilicity of the film. Similar behaviour was recently reported by Benbettaieb et al.(2015b) in our study on the same film but in the presence of ferulic acid. Irradiation induces a decrease of the contact angle value with water, concomitant to an increase in the polar component of the surface tension for all films. It can be attributed to a reorientation of polar groups at the film surface, hence increasing the polar component of the surface tension. Thus irradiation increases wettability of the films.

3.2.2. Water vapour permeability

Table 2 displays the WVP of non-irradiated and irradiated films in the presence and absence of coumarin for the two RH gradients studied (0-30 % and 30-84 %). For the 0-30%RH gradient, the WVP of non-irradiated film containing coumarin (0.47±0.03 x10^{-11} g.m^{-1}.s^{-1}.Pa^{-1}) is in the same range to that of control film (0.52±0.1x10^{-11} g.m^{-1}.s^{-1}.Pa^{-1}). Inversely, Wu et al. (2013) noticed a

decrease of 16 % in the WVP for films composed of silver carp (*Hypophthalmichthys molitrix*) skin gelatin containing green tea extract (0.7%). Other authors did not observed any change in the water vapour permeability when ferulic acid was added to to gelatin films (Cao et al. 2007), to soy protein films (Ou et al. (2005), or to caseinate based films (Fabra et al., 2011). After irradiation we did not observed any modification in WVP (0-30% RH gradient) for control film and for film containing coumarin. Furthermore, the 0-30% RH gradient correspond to water activity average equal to 0.15, which is in the BET domain (water contained in the film is only involved in the structure organisation and not available for reaction), thus we propose that only water involved in structure with weak plasticization of the network by water. In this domain, neither irradiation nor coumarin addition affects the water barrier properties of the films. On the other hand, higher RH gradient of 30-84% induces a rise of the WVP of all films (with or without coumarin addition) after irradiation treatment. WVP increases from 2.41±0.44 to 23.06±0.85 $x10^{-11}$ $g.m^{-1}.s^{-1}.Pa^{-1}$ and from 2.23±0.65 to 24.8±2.11 $x10^{-11}$ $g.m^{-1}.s^{-1}.Pa^{-1}$, respectively for control film and film with coumarin after 60kGy irradiation dose. Due to the effect of irradiation, the barrier properties are mainly related to the increasing the water content that induces the plasticization of the film during permeation. The 30-84% RH gradient corresponds to a mean water activity average equal to 0.57, which is in the plasticization domain of the network by water. But the effect of coumarin addition in the network on the transfer phenomena is not significant.

3.2.3. Mechanical properties

Mechanical parameters (tensile strength (TS), Young's Modulus (YM) and elongation at break (E)) of all studied films are given in **Table 2**. TS, YM and E of control film (non-irradiated, without coumarin) were 25.9±3.9 MPa, 1523±266MPa and 2.2±0.4%, respectively. Jridi et al. (2014) found similar value of %E (2.7±0.5%) but higher value of TS (44.3±1.2 MPa) for

chitosan-skin fish gelatin blend film (50:50 w/w) that could be due to film thickness or molar mass of polymer. Compared to the control film, no significant modification is observed on the mechanical parameters (TS, YM and %E) after coumarin addition. An opposite tendency was observed by Benbettaieb et al. (2015b) who showed that TS increases significantly when ferulic acid was added in to the chitosan-gelatin film. Only the TS of control film increases significantly with the increasing dose at ~~a~~ 60kGy. When irradiation doses (40 and 60kGy) were applied on film containing coumarin, no significant ($p<0.05$) modification of mechanical parameters was observed. This increase of film stiffness and resistance is in accordance with the improvement of thermal stability of control film after 60kGy irradiation dose, previously observed from TGA analysis.

Finally, results from functional and structural properties are still less consistent to make any hypothesis related to the crosslinking reaction between polymers chains and coumarin under irradiation. Only few interactions occur (probably modified by hydrogen bonds) between the different reactive compounds and some orientation of polar groups to the surface, which enhance the film wettability and hydrophilicity. The interactions between polymer and coumarin after irradiation, even if they seem to be weak could nevertheless affect the release of the antioxidant into aqueous medium.

3.3. Influence of irradiation on the coumarin release in aqueous media

The release experiments were performed three months after film irradiation. All data are summarized in **Table 3.** The amount of coumarin in the non-irradiated film (10.1±1.5 mg/g of film) determined after complete solubilisation of films in acetic acid solution (experimental value) was significantly ($p<0.05$) lower than the theoretical value (47.1±4.7 mg/g of film) calculated from the film formulation. This means that, about 80% of coumarin disappeared, probably due to oxidation during storage (**Table 3)**. After irradiation, measured (real) amount of

539 coumarin in the films is far less reduced, as only 35% is lost. Thus, irradiation protects coumarin
540 against degradation during the time of storage (3 months). It could be that coumarin make some
541 interaction with polymers and/or with free radical and thus is less available to be oxidized during
542 storage period. This result suggests that irradiation may act as a safeguard method of antioxidant
543 when this later is encapsulated in hydrocolloid films. This can be considered as a good way to
544 protect active compound and to ensure its quality until final consumer. As the difference
545 observed between theoretical and experimental content of coumarin in the film, only the
546 experimental concentration is considered for the study of the release. The release kinetics of
547 coumarin from chitosan-gelatin based films (non-irradiated and irradiated at 40 and 60kGy) in
548 water medium (at pH=7) are displayed in **Fig.5.** Release kinetics of coumarin exhibited the
549 typical shape of non-time-dependent and non-concentration-dependent diffusion. The content of
550 coumarin remaining in the film after release significantly ($p < 0.05$) increased from 1.7±0.6 to
551 7.17±0.5 and to 12.6 ±1.7 mg/g of film, respectively after 40 and 60kGy irradiation doses. This
552 could be explained by the interaction between the polymer chains and this antioxidant, favoured
553 by the irradiation process and because the initial concentration is higher. This could therefore
554 modify the film structure organisation and the release mechanisms of antioxidant from the film.
555 Furthermore, the effective diffusion coefficients (D) of the coumarin in the films were calculated
556 from the release kinetics by fitting experimental data using Eq.6 and are given in **Table 3**. The
557 diffusivity is related to the molecular mobility within the polymeric network and could be related
558 to several factors such as molecular weight, structural characteristics of the matrix and solubility
559 of this antioxidant. We considered here that the partition coefficient did not affect the transfer as
560 the concentrations involved are always much lower that the solubility limit of the coumarin in
561 water media. As it can be observed, the effective diffusion coefficient of coumarin significantly
562 decreased ($p<0.05$) from 3.26±0.74 to 1.87±0.48 and to 2.04 ±0.05x$10^{-11}$$m^2$.$s^{-1}$ respectively after
563 40 and 60kGy irradiation doses. This is also in agreement with the increase of the coumarin

content remaining in the film after the release (by chemical or physical entrapment).This decrease of diffusion coefficient could be related to the irradiation treatment which limits the mobility of coumarin and therefore decreases the apparent diffusivity. Tin Wui et al. (2002), worked on the influence of microwave irradiation on the drug release properties of polysaccharide beads and showed that the release-retarding property of alginate and alginate–chitosan beads was significantly enhanced by subjecting the beads to microwave irradiation. They showed that microwave technology can be employed in the design of solid dosage forms for controlled-release application without the use of noxious chemical agents. In our case, irradiation could favor the interaction between coumarin and biopolymer via free radical mediated mechanism. Hence, coumarin is more linked and consequently, more protected and less mobile. The effect of irradiation also modified the surface properties by increasing its polarity and then swelling phenomenon occurs too quickly to affect the diffusion determination.

Despite the swelling, the film remains intact and no dissolution or network structure destruction was observed during the kinetic of release. In non-irradiated film, as the structure is less dense, water can easily enter into the network and favour the polymeric chain mobility and thus the coumarin diffusion through the hydrated films is greater. Irradiation allowed to delay by 50% the release time. So, film irradiation after optimization, would be an effective process for controlled release of active naturals antioxidants in aqueous foods or even for medical applications.

4. Conclusions

Chitosan and fish gelatin films encapsulating coumarin were prepared as an active biobased film. After film drying, irradiation using electron beam was applied at 40 and 60kGy. This work aimed to investigate the coupled effect of irradiation and of the presence of the active compound on the structure and functional properties of the films. Electron Spin Resonance (ESR) displayed the free radical formation during irradiation in films. Coumarin did not affect the thermal stability of films whereas irradiation slightly improved it. Both addition of coumarin and

irradiation decreased the contact angle with water and increase the polar component of the surface tension of films, as well as the swelling index and rate. This is attributed to a reorientation of polar groups at the film surface. From water barrier analysis, neither irradiation nor coumarin addition affected the water vapour permeability at low RH gradient. However, a higher RH gradient (30-84%) induced a rise of the WVP of all the films after irradiation treatment that is mostly related to the surface properties and film wettability. Incorporation of coumarin did not affect the mechanical properties of films on the contrary to irradiation, but very weakly. The interactions between biopolymers and coumarin after irradiation affected the release of the antioxidant into the aqueous medium. The content of coumarin remaining in the film at equilibrium after release significantly increased when film were irradiated, from 17% to 32% mg/g of film, inversely, the effective diffusion coefficient of coumarin decreased by 1.6 times. Irradiation, also displayed that it is an efficient process to prevent coumarin degradation during the storage of films, as more than 60% of the antioxidant was preserved compared to non-irradiated films.

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Figure captions

Figure 1: ESR spectra of chitosan-fish gelatin films containing coumarin and irradiated at 60kGy at the dose rate of 300Gy/sec. Measurements were performed at 25°C and 50% RH, 3 months after irradiation.

Figure 2: FTIR spectra of non-irradiated and irradiated (60kGy) chitosan-fish gelatin film containing or not coumarin. All films were previously equilibrated at 50% RH and 25°C.

Figure 3: TGA thermograms of irradiated and non-irradiated chitosan-fish gelatin film with and without coumarin. All films were previously equilibrated at 50% RH and 25°C.

Figure 4: Water contact angle at 0 and 20s, swelling index, swelling rate and surface tension (γ_S) with dispersive (γ_S^d) and polar components (γ_S^p) of irradiated and non-irradiated chitosan-fish gelatin film with and without coumarin. Measurements were done at room conditions (~20°C, ~50%RH).

Figure 5: Kinetic release of coumarin in water medium at pH=7 and 25°C, for control (0kGy) and irradiated (40 and 60kGy) chitosan-fish gelatin films. Ct: concentration of coumarin released in the aqueous dissolution medium at time t; C∞: the maximum concentration of coumarin released. Symbols are experimental values (mean+standard deviation) and solid line corresponds to a Fickian data modeling using Eq (6).

Tables captions

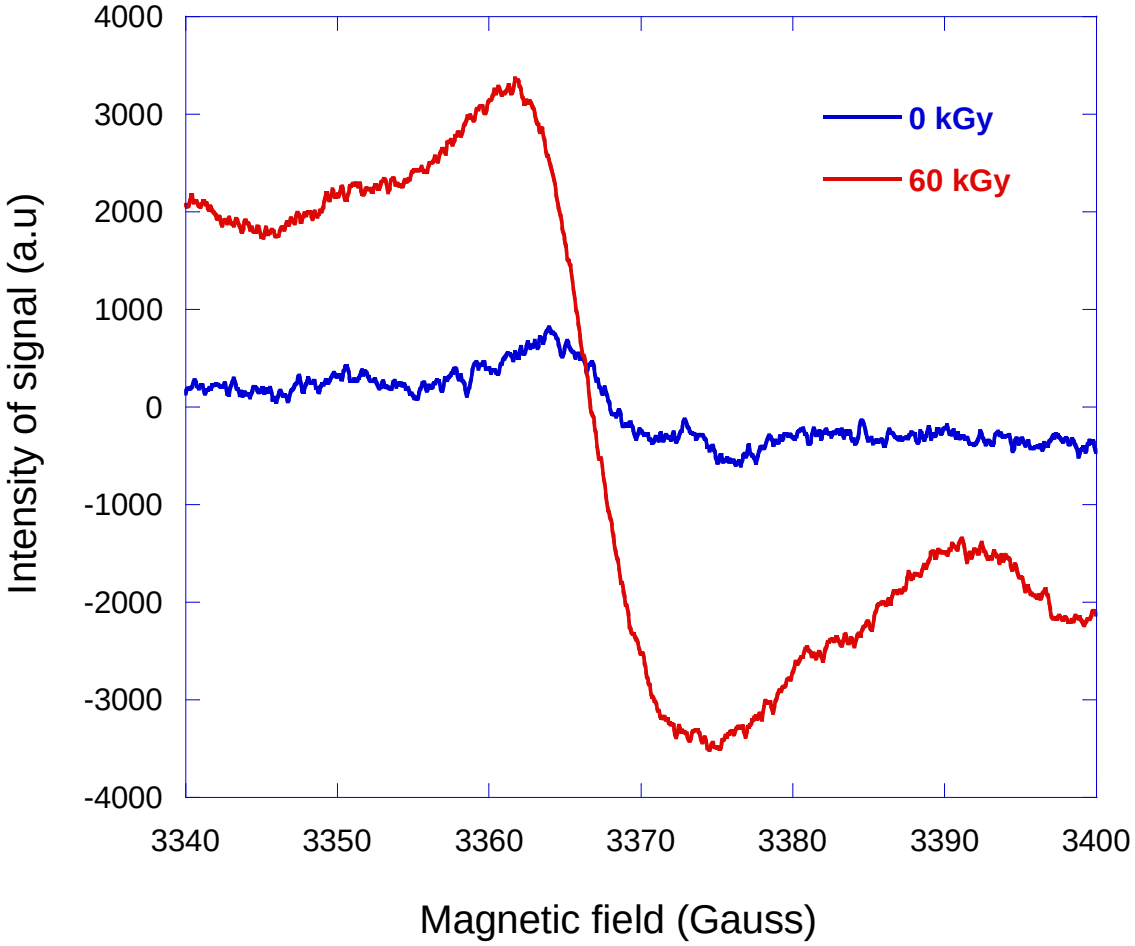
Table 1: Number and weight average molar mass (Mn, Mw), polydispersity (Mw/Mn) and z-average mean square radius (Rz) for irradiated and non-irradiated control films and for irradiated coumarin films, determined from SEC-MALLS analysis.

Table 2: Thickness, water vapour permeability (WVP) and mechanical properties (Tensile strength (TS), Young's Modulus (YM) and elongation at break (% E)) of irradiated and non-

irradiated chitosan-fish gelatin film with and without coumarin. Water vapour permeability was measured at 25ºC under (0-30) % and (30-84) % RH differentials. Mechanical properties were measured at 25°C and 50% RH.

Table 3: Kinetics release parameters of coumarin from irradiated and non-irradiated chitosan-fish gelatin film. All parameters were determined during release and from release kinetics profile (up to total release) of coumarin from irradiated and non-irradiated films. Dissolution medium is water at pH=7 and 25°C.

Figure 1


4000
3000
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1000
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-4000
Intensity of signal (a.u)
3340
3350
3360
3370
3380
3390
3400
Magnetic field (Gauss)
0 kGy
60 kGy

Figure 2

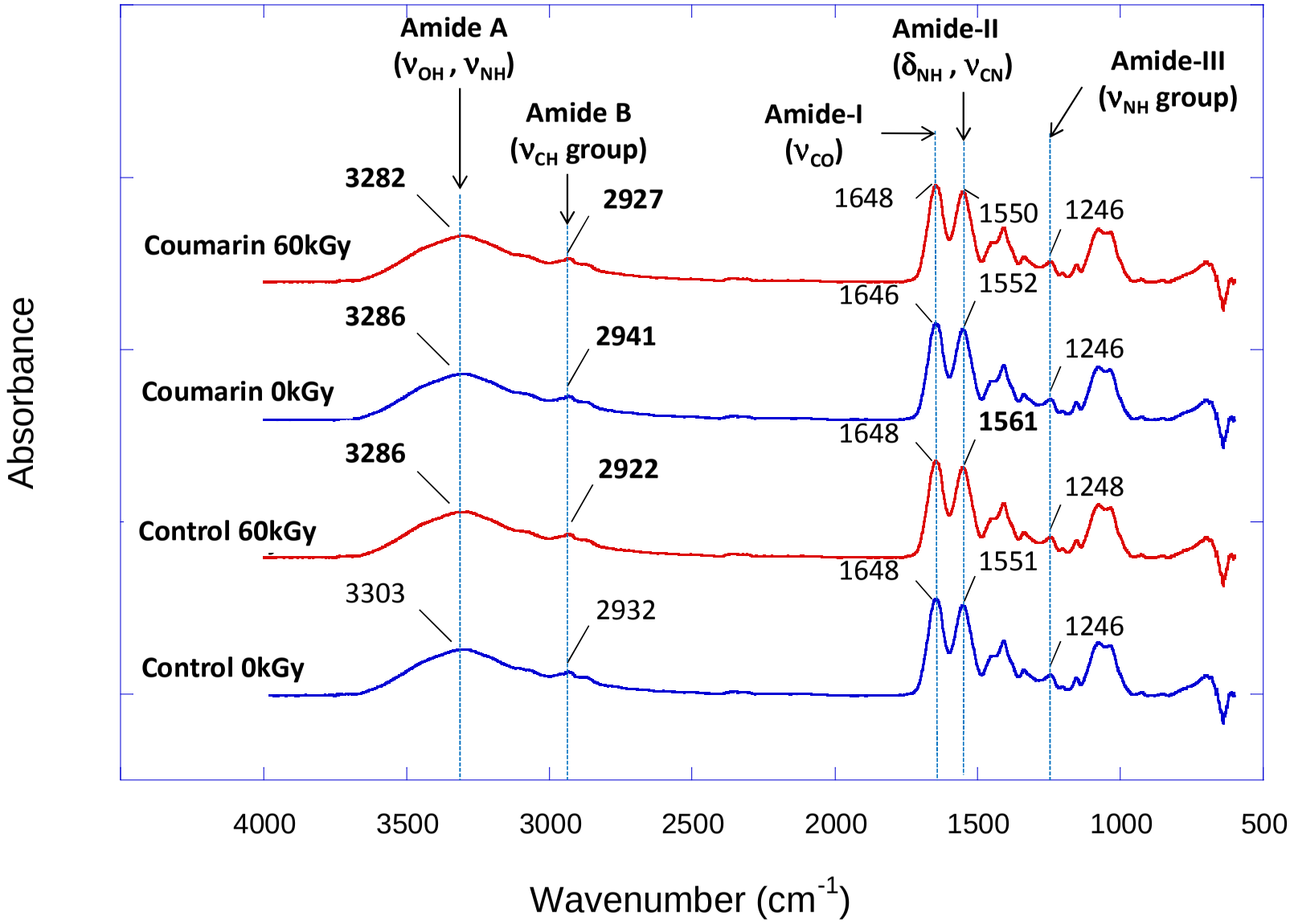
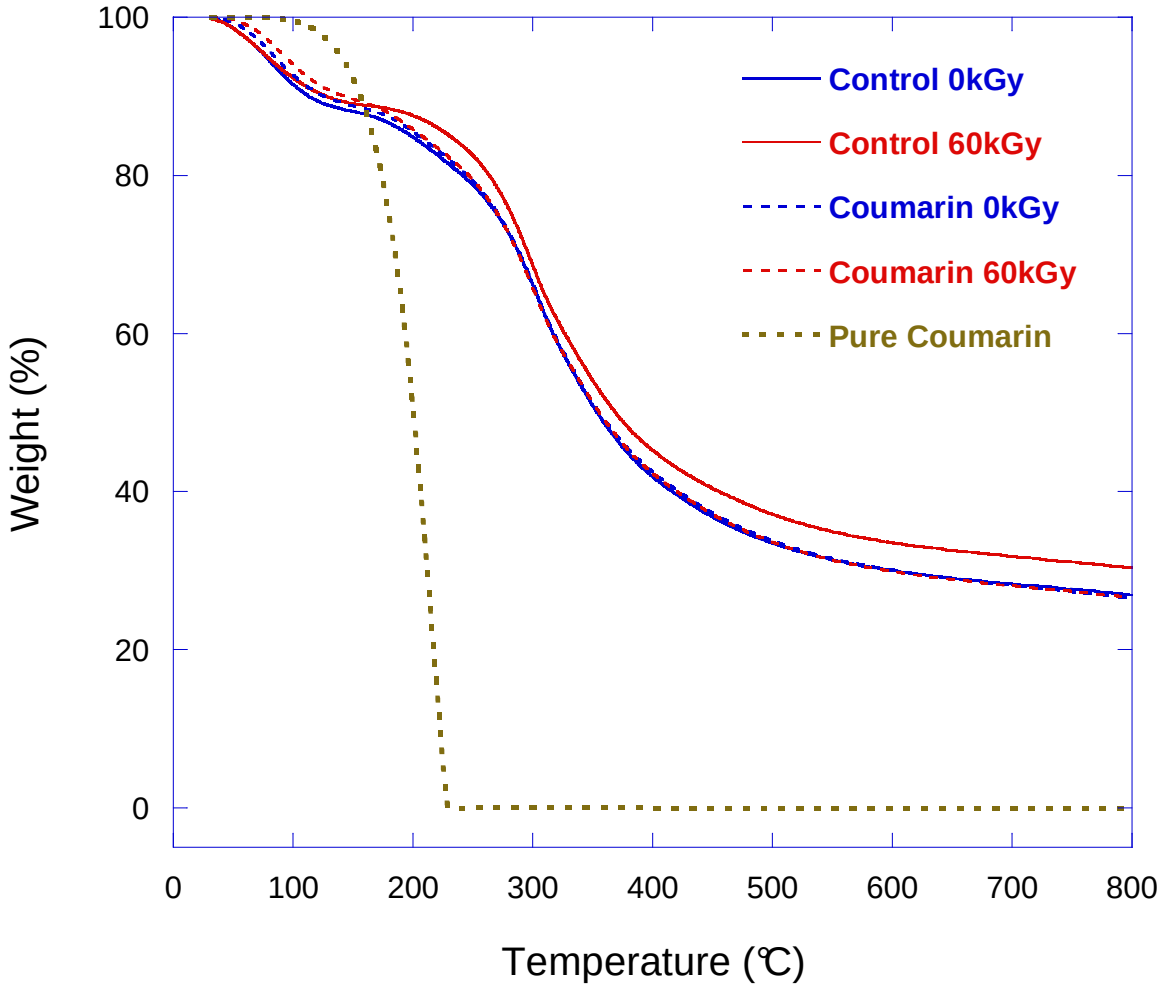
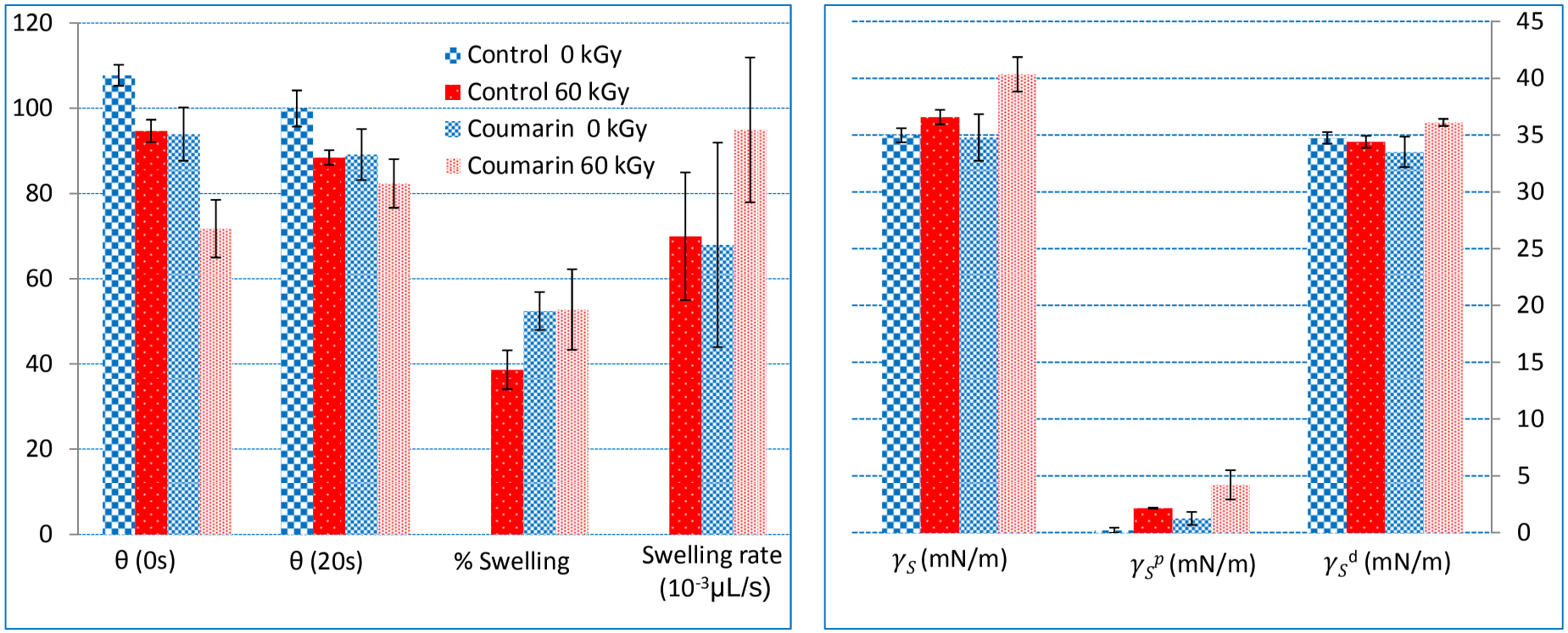

Amide A
(ν_{OH} , ν_{NH})
Amide B
(ν_{CH} group)
Amide-I
(ν_{CO})
Amide-II
(δ_{NH} , ν_{CN})
Amide-III
(ν_{NH} group)
Coumarin 60kGy
3282
2927
1648
1550
1246
Coumarin 0kGy
3286
2941
1646
1552
1246
Control 60kGy
3286
2922
1648
1561
1248
Control 0kGy
3303
2932
1648
1551
1246
Absorbance
4000
3500
3000
2500
2000
1500
1000
500
Wavenumber (cm^{-1})

Figure 3


Control 0kGy
Control 60kGy
Coumarin 0kGy
Coumarin 60kGy
Pure Coumarin
Weight (%)
Temperature (℃)
100
80
60
40
20
0
0
100
200
300
400
500
600
700
800

11 **Figure 4**



12

Figure 5

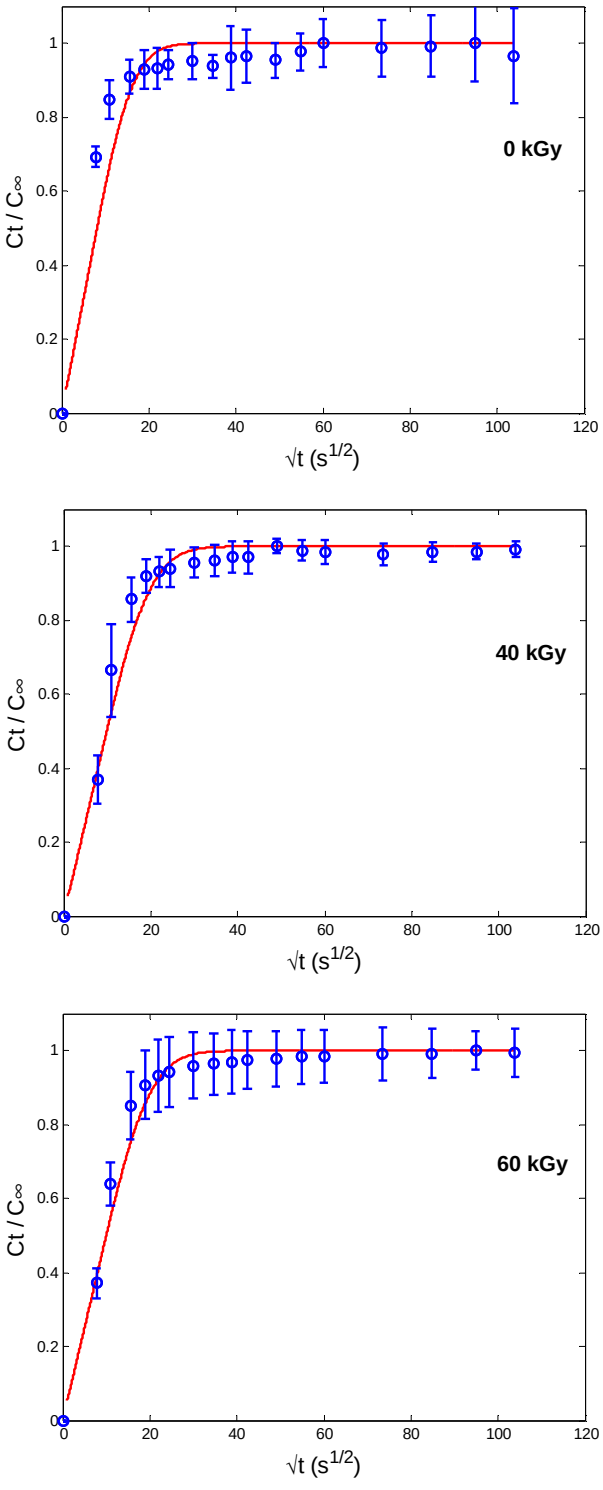


Table 1

Films	Mw (10^5 g/mol)	Mn (10^4 g/mol)	Polydispersity	Rz (nm)
Control 0kGy	1.36 (2%)	8.39 (2.4%)	1.62 (3.2%)	17.3 (24%)
Control 60kGy	1.20 (1.9%)	5.37 (5%)	2.23 (5%)	17.9 (21%)
Coumarin 60kGy	1.35 (2%)	5.09 (6%)	2.66 (7%)	18.1 (22%)

Mean (relative error%)

Table 2

Films	Irradiation dose (kGy)	Thickness (µm)	WVP (10^{-11} g.m^{-1}.s^{-1}.Pa^{-1})		TS (MPa)	YM (MPa)	%E
			ΔRH= (0-30)%	ΔRH= (30-84)%			
Control	**0**	65±8 [a]	0.52±0.10 [a,b]	2.41±0.44 [a]	25.89±3.92 [a]	1523±266 [a]	2.2±0.4 [a]
	40	65±8 [a]	0.59±0.04 [b,c]	22.46±0.7 [b]	28.54±3.76 [a]	1221±235 [a,b]	3.1±0.9 [a,b]
	60	65±8 [a]	0.56±0.03 [b,c]	23.06±0.85 [b,c]	39.20±8.32 [b]	1270±23 [a,b]	4.1±2.1 [a,b]
Coumarin	**0**	77±12 [a]	0.47±0.03 [a,d]	2.23±0.65 [a]	30.95±0.83 [a,b]	1328±185 [a,b]	4.7±0.5 [a,b]
	40	77±12 [a]	0.47±0.02 [a,d]	25.13±1.25 [d]	28.65±5.02 [a]	1006±115 [b]	3.2±0.3 [a,b]
	60	77±12 [a]	0.50±0.04 [a,c]	24.80±2.11 [d,c]	31.35±5.64 [a,b]	1187±201 [a,b]	3.0±0.4 [a,b]

Values are given as mean ± standard deviation. Means with the same Arabic letter in the same column are not significantly different at $p<0.05$.

Table 3

	0kGy	40kGy	60kGy
Theoretical content of coumarin in film (mg/g of film)	47.1±4.7	47.1±4.7	47.1±4.7
Initial Content of coumarin in film prior to release (mg/g of film)	10.1±1.5 [a]	31±0.4 [b]	39.2±4 [c]
Content of coumarin remaining in the film after release at equilibrium (mg/g of film)	1.7±0.6 [a]	7.2±0.5 [b]	12.6±1.7 [c]
Diffusion coefficient (10-11 m2/s)	3.26±0.74 [b]	1.87±0.48 [a]	2.04±0.05 [a]

Values are given as mean ± standard deviation. Means with the same Arabic letter in the same line are not significantly different at $p<0.05$.