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Ionizing radiation and plasma discharge mediating covalent linking of bioactive compounds onto polymeric substrate to obtain stratified composites for food packaging

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Biomedical Applications of Plasma Gas Discharge/gamma irradiation Processes

A. ***Plasma/Gamma irradiation Treatment*** (**Etching**)

1. Cleaning
2. Sterilization
3. Crosslink of surface layers

B. ***Plasma/Gamma Irradiation Polymerization*** (**Deposition**)

1. Form Barrier Film

a. Protective coating
b. Insulating coating
c. Reduce absorption from environment
d. Reduce release rate of leachables
e. Control drug delivery rate

2. ***Modify Protein and Cell Interactions***

a. Improve "biocompatibility"
b. Promote selective protein adsorption
c. Enhance cell adhesion
d. Improve cell culture surfaces
e. Provide non-fouling surfaces
f. Reduce surface friction

3. ***Provide Reactive Sites***

a. For grafting or polymerizing polymers
b. For immobilizing biomolecules

Purpose

- Self assembly and nano-organisation of different polysaccharides and polyphenols, which have the key influence on the effects of bio functionalization of the surfaces, and as such enabling the initial development of the novel concept of active packaging that will provide stability of food in food packaging.

- Encapsulation/immobilization of bioactive compounds (antimicrobial cationic or anionic polysaccharides and vegetable oils) by different techniques including plasma and gamma irradiation, emulsion and electrospinning techniques.

Materials

Substrates: Biodegradable polymers: Pol(lactic acid)

Synthetic non-degradable: Poly(ethylene)

Bioactive compounds: *Chitosan*

Four types of essential oils: Clove Oil, Thyme Oil, Rosemary Oil and Ti Tree Oil

and

Four types of cold pressed oils: Rosehip Seeds Oil, Grape Seeds Oil, Argan Oil and Apricot Oil

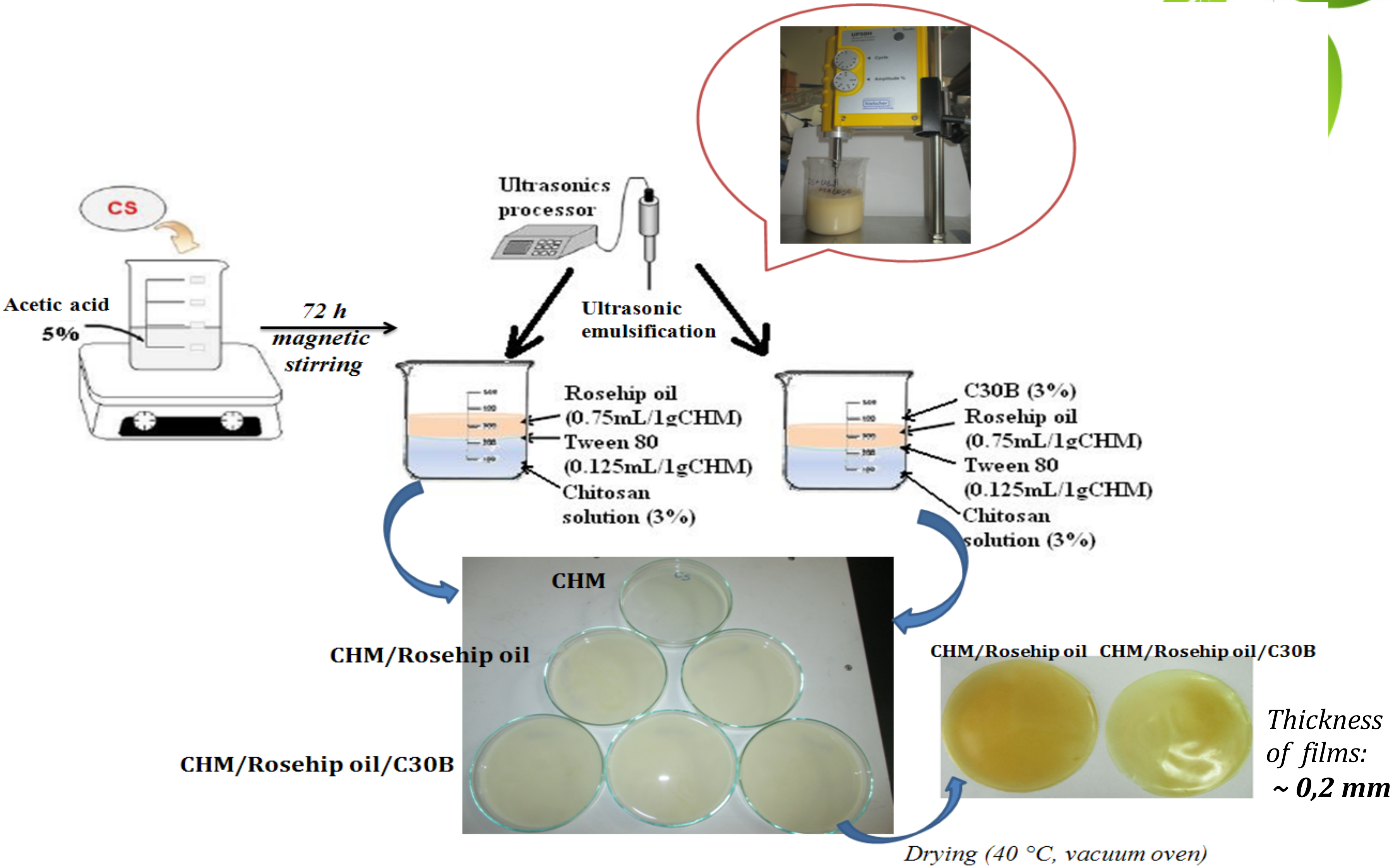
Health *benefits:*

- *Clove oil* - is strongest antioxidant on earth, antimicrobial, antifungal, antiseptic, antiviral, aphrodisiac and stimulating properties, it is *used* for diarrhea, hernia, and bad breath
- *Rosemary essential oil* can thicken hair, balance hormones, heal skin and improve memory.
- *Rosehip seed oil* is one of the top anti-aging oils, remove scars, heal sunburn, etc

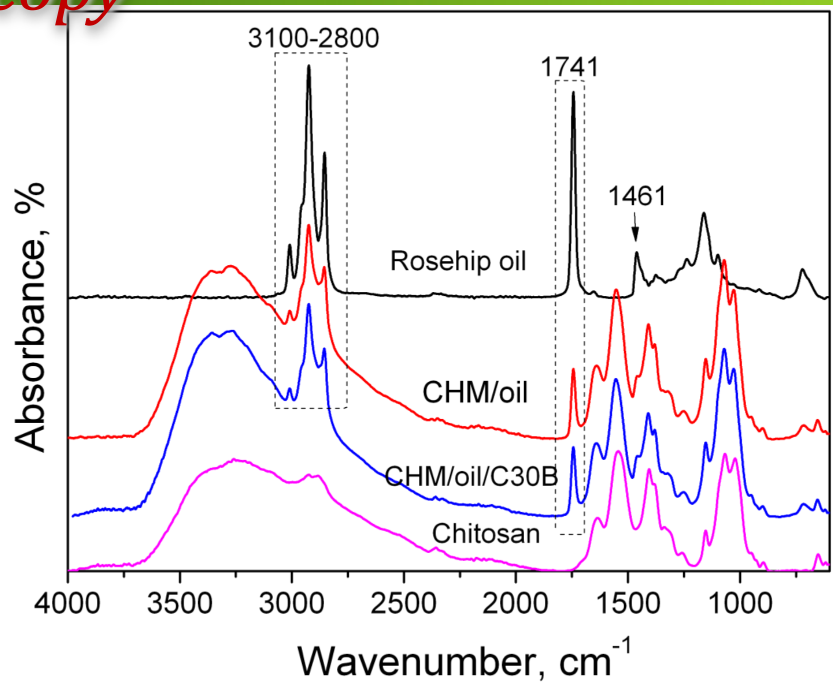
Emulsion/solvent casting technique.

- **Chitosan (CHM)** medium molecular from Sigma Aldrich, with 200-800 cP viscosity in 1% acetic acid, 75-85 % deacetylation degree and MW = 190,000-300,000 g/mol; - *biodegradable, nontoxic, antigenic, antimicrobial* - suitable for applications in food industry
- **Commercial Rosehip seeds oil** from Herbavit S.A., România; *antioxidant and antimicrobial* – flavoring and preservative agent
- **Montmorillonite (C30B)**, organically modified montmorillonite C30B was obtained from Southern Clay Products. C30B has a moisture content of < 2 % and a density of 1.98 g/cm^3; *improvement in mechanical, thermal properties with small amounts (1–5 wt %) –*
- **Tween 80 (T80), Polysorbate 80** – is a non-ionic surfactant, presented as an viscous yellow liquid, from Sigma Aldrich. T80 has Mw = 1310 g/mol and ≥ 58 % oleic acid - *stabilize the oil-based emulsions*

Oils encapsulation in chitosan by emulsion method



FT-IR Spectroscopy



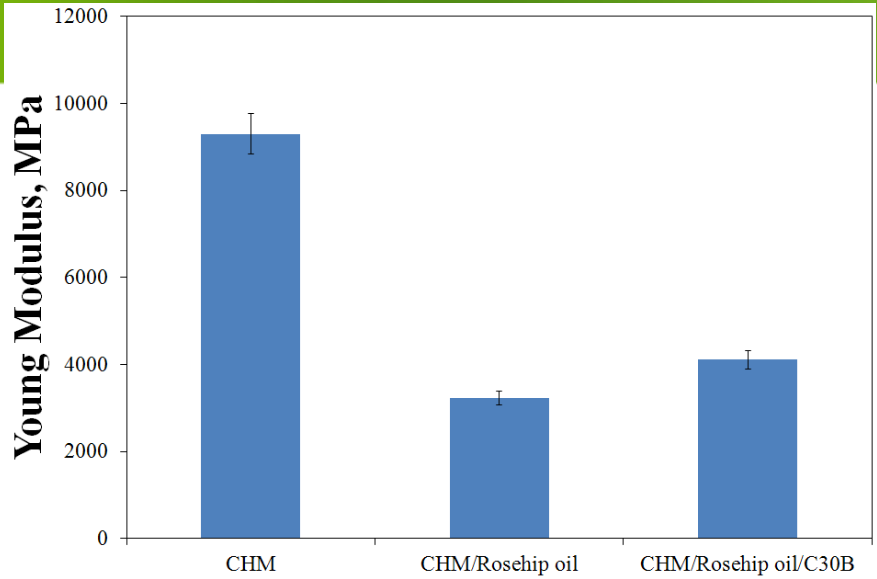
- The ATR-FT-IR spectra revealed that some characteristic bands for rosehip oil were evidenced also in the FT-IR spectra of CHM/Rosehip oil and CHM/Rosehip oil/C30B samples:

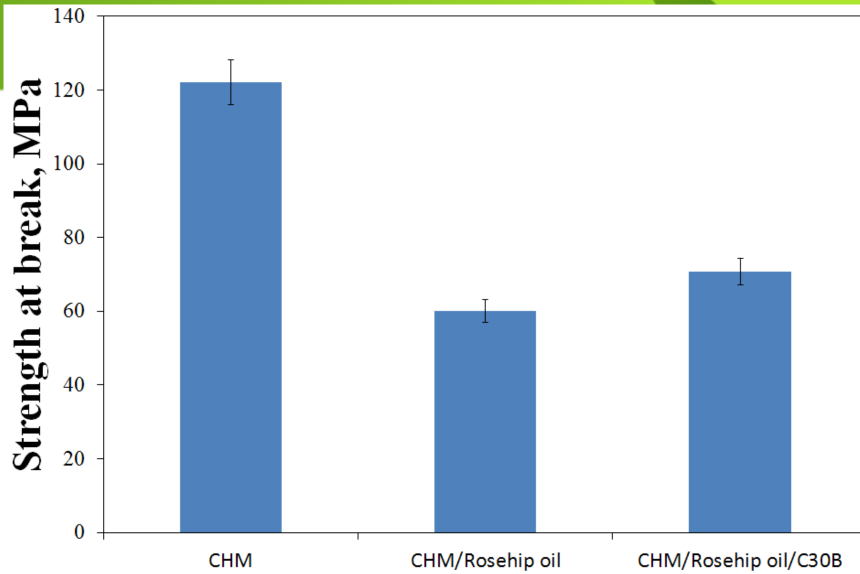
➢in the interval of 3100-2800 cm^{-1} - absorptions bands characteristic to the symmetrical and asymmetrical vibrations ν(C-H) of the CH_2 and CH_3 aliphatic groups from the alkyl rest of the triglycerides.

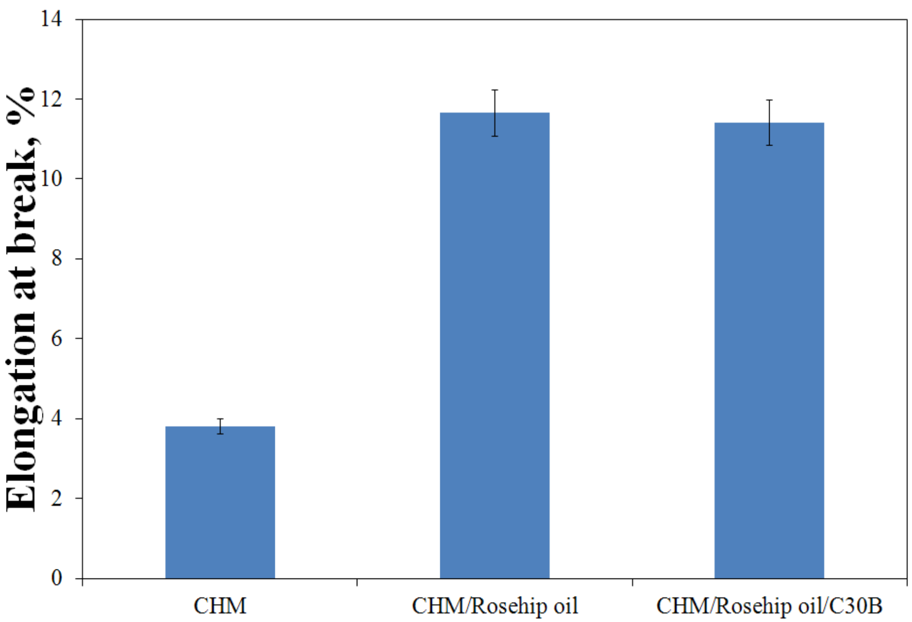
➢ 1741 cm^{-1} – characteristic band for oils with a high content in saturated fatty acids and short carbohydrate chain.

➢ 1461 cm^{-1} – attributed to C–H deformation vibration.

Tensile properties







The strength at break:
60 MPa CHM/rosehip oil
71 MPa CHM/rosehip oil/C30B
Elongation at break :
4 % pure CHM
12 % CHM/oil/C30B

Compared with pure chitosan films, vegetable chitosan-oil films are more flexible and could be recommended for obtaining packaging materials

Antimicrobial testing

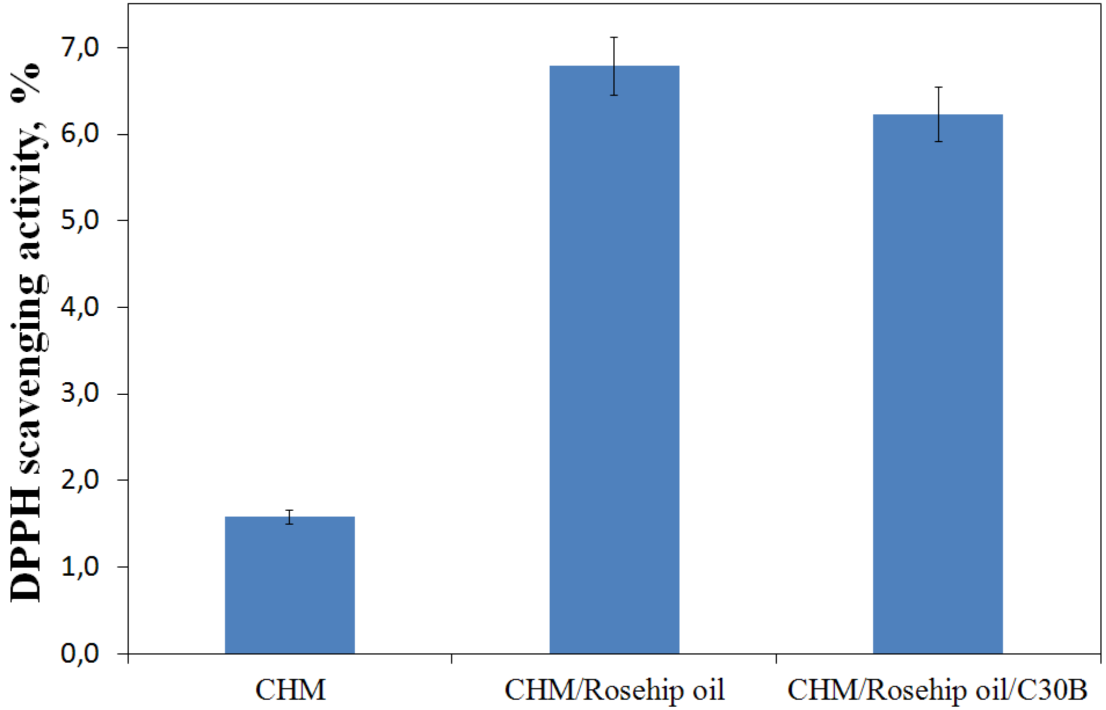
Inhibition of *Bacillus cereus Escherichia coli, and Salmonella typhymurium grown* **over CHM, CHM/Rosehip oil and CHM/Rosehip oil/C30B**

Sample	% Inhibition ATCC Bacillus cereus 14579		% Inhibition Escherichia Coli ATCC 25922		% Inhibition Salmonella typhymurium 14028	
	24h	48h	24h	48h	24h	48h
Commercial foil PE	0	27	0	29	0	39
Chitosan MM	82	100	73	96	65	100
Chitosan MM+T80	82	100	86	100	74	100
Chitosan MM/Rosehip oil/Rosehip oil/T80	59	100	86	100	61	94
Chitosan MM/Rosehip oil/Rosehip oil/T80/Cloisite C30B	82	100	90	100	68	100

- *Incorporation of rosehip oil and Cloisite 30B into chitosan improved the antimicrobial activity of chitosan film against E. coli.*

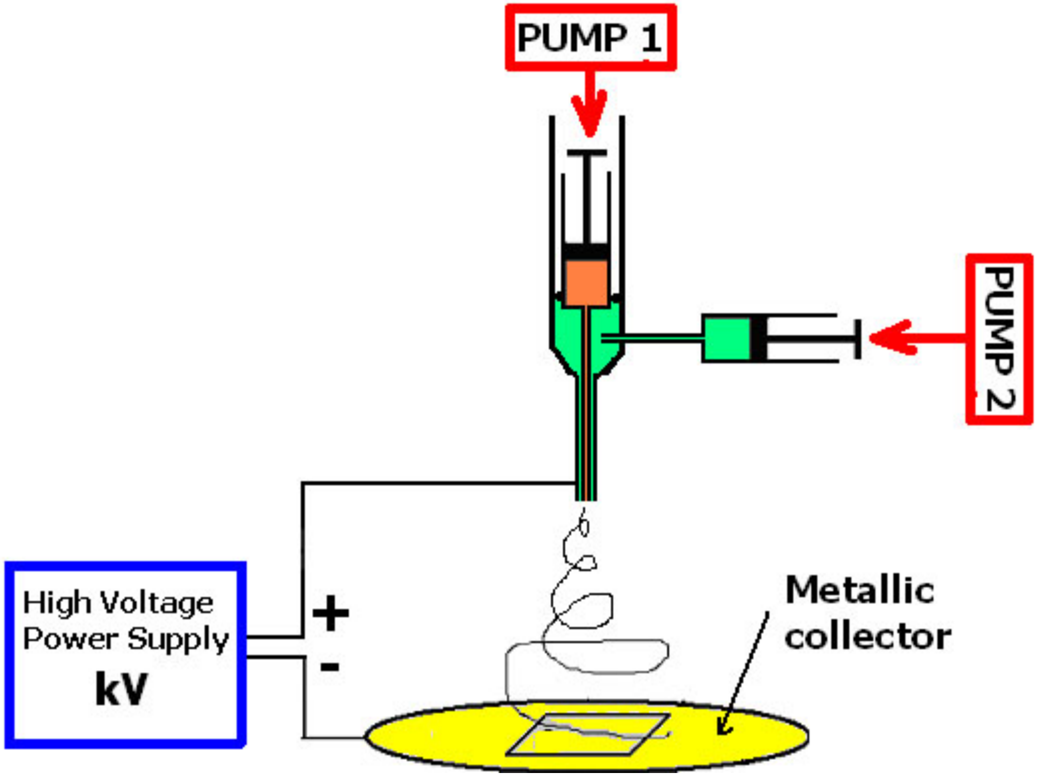
Antioxidant activity evaluation

❖ The radical scavenging activity of CHM/Rosehip oil and CHM/Rosehip oil/C30B (with solvent chloroform) was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical method.



Films with rosehip oil and C30B exhibited a higher level of radical scavenging activity with values of ~ 7% compared with 1% registered for CHM film.

Encapsulation of oils (CLOVE OIL / ARGAN oil) by Co-axial electrospinning



Electrospinning parameters

Voltage/needle collector distance:

9 kV / 9 cm

Flow rates 1.25 microliters/min

(for both core and shell)

(*approx 0.5 mg chitosan /30 min*)

Core: Oil solution 1.5 wt%

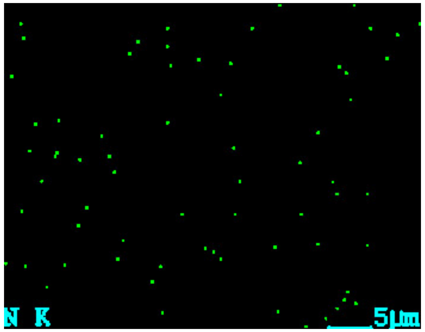
Shell : Chitosan solution 1.5 wt%

Oil solutions:
Argan Oil in Choloform
Clove Oil in Acetic Acid
both 1.5 wt%

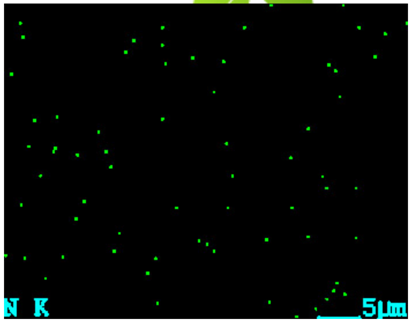
SEM/EDAX and TEM results

Sample	**C (wt%)**	**N (wt%)**	**O (wt%)**
Chit.High/Argan	68	**04**	27
Chit.High/Clove	64	**07**	28
Chit.Med./Argan	64	**03**	32
Chit.Med./Clove	75	**03**	25
Chit.Low./Argan	66	**05**	28
Chit.Low./Clove	61	**03**	34

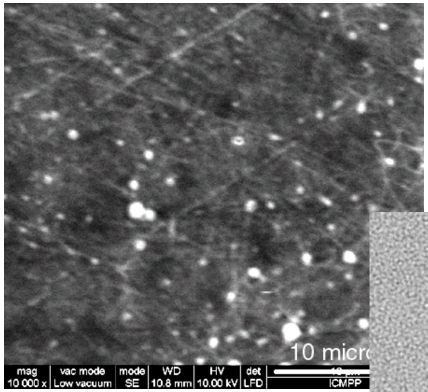
Due to the presence of N, the EDAX analysis confirms the presence of Chitosan onto PLA substrate.



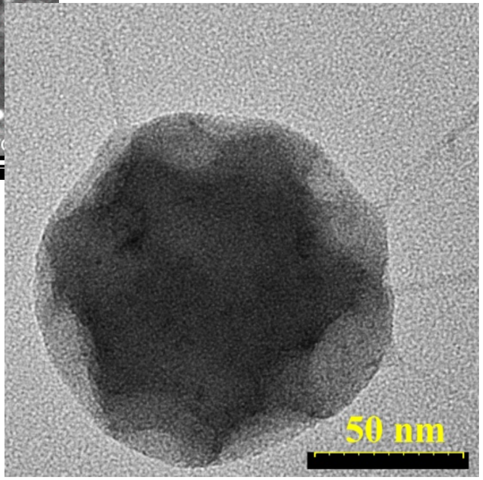
CHH/Clove oil

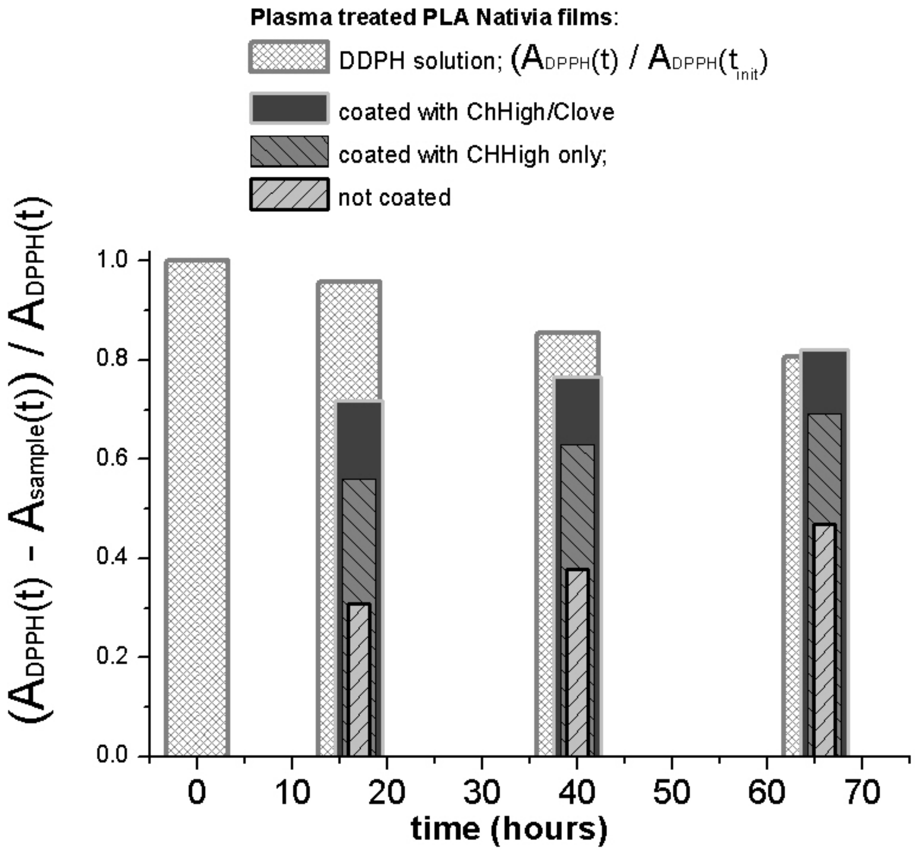


CHH/Argan oil



Only CHH is spinnable





-Antioxidant activity higher for the coated films

-Antioxidant activity higher for the CHH/Clove than for CHH coated films

- -for the DPPH solution
- $A_{DPPH}(t) / A_{DPPH}(t_{init})$
- For samples
- PLA,
- $PLA/CHH/N_2$
- $PLA/CHH/Clove/N_2$:
- $A_{DPPH}(t) - A_{SAMPLE}(t)] / A_{DPPH}(t)$

Coating of PLA : PlA/CH/vegetable oils stratified composites

Procedure

Cold high-frequency plasma: created inside a glass reactor, using: 0.5 mbar (50 Pa) vacuum, a source of high-frequency (1.3 MHz) and a 30 W discharge power;15 minutes; two different discharge gases were used: air and nitrogen;

Radiochemical processing: γ-irradiator M-38 GAMMATOR (USA), with ^{137}Cs source. in air at room temperature ; dose rate is 0.4 kGy/h. γ-doses were 5, 10, 15 and 20 kGy.

Coating by dipping method: chloroform solution (10 wt%) of rosehip seed oil (RO) and methanol solution (10 wt%) of clove oil (CO) for 60 minutes, on mechanic stirring.

Surface modification and surface coating.

Establishing of the optima conditions by comparing techniques of coating:
- ✓**immersion,**
- ✓**spreading and**
- ✓**electrospraying**

- Multi layer biodegradable polymer materials in a film structure with immobilized phenolic compounds (flavonoides) possessing oxygen-barrier, antimicrobial and antioxidative properties, at the beginning specially aimed for meat products.
- Development of non-toxic, biocompatible, biodegradable and novel antimicrobial and antioxidant formulations for different packaging materials.

- To the best of our knowledge self-assembled antimicrobial/antioxidant nano films constructed onto biodegardable matrices for food packaging ***were not developed*** and ***have not been patented yet***, thus this approach is of **high degree innovative**. It combines several benefits whilst producing of biodegradable and recyclable packaging material, it is part of green and healthy living that is the most important.

Immobilization strategies: *Covalent binding*

EDC and NHS

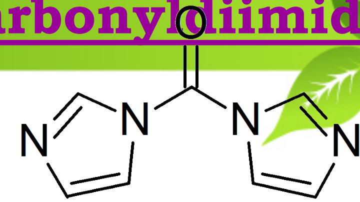
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)and *N-hydroxysuccinimide*(NHS)

- **EDC is zero-length crosslinking** agent used to couple carboxyl groups to primary amines;

-**EDC** reacts with a carboxyl to form an amine-reactive O-acylisourea intermediate;

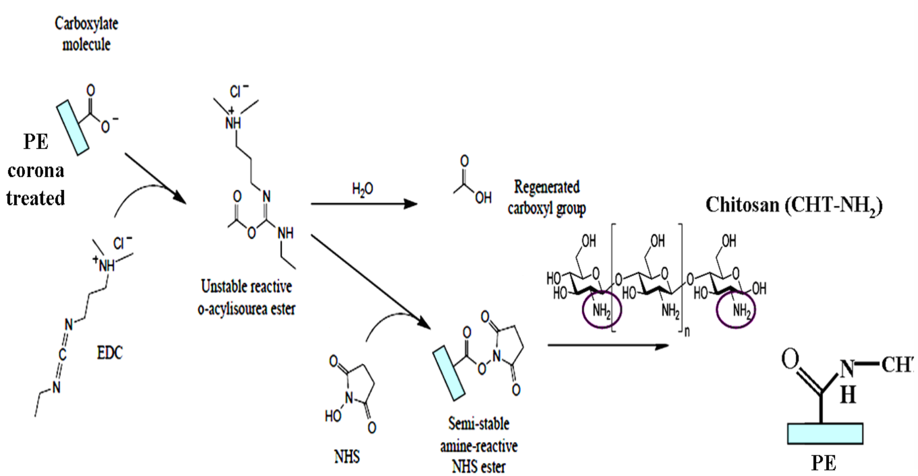
-**NHS** is included in EDC coupling protocols to improve efficiency or to create a more stable, amine-reactive intermediate.

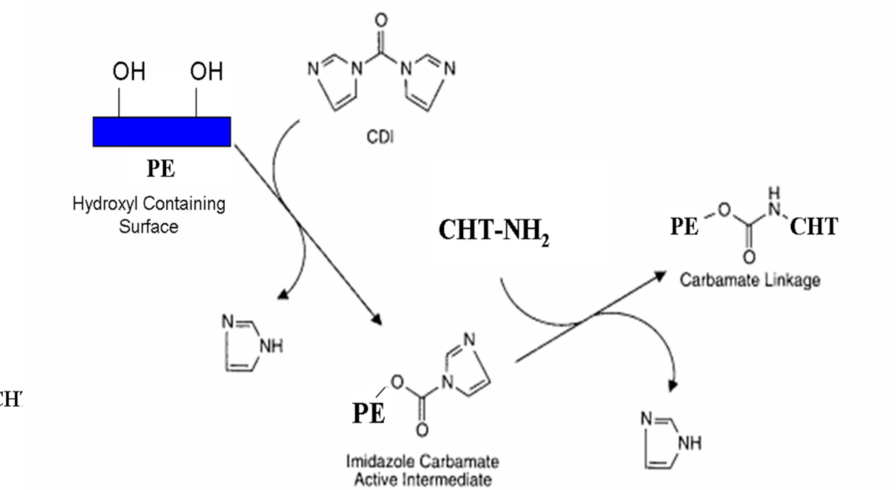
N, N’-Carbonyldiimidazole

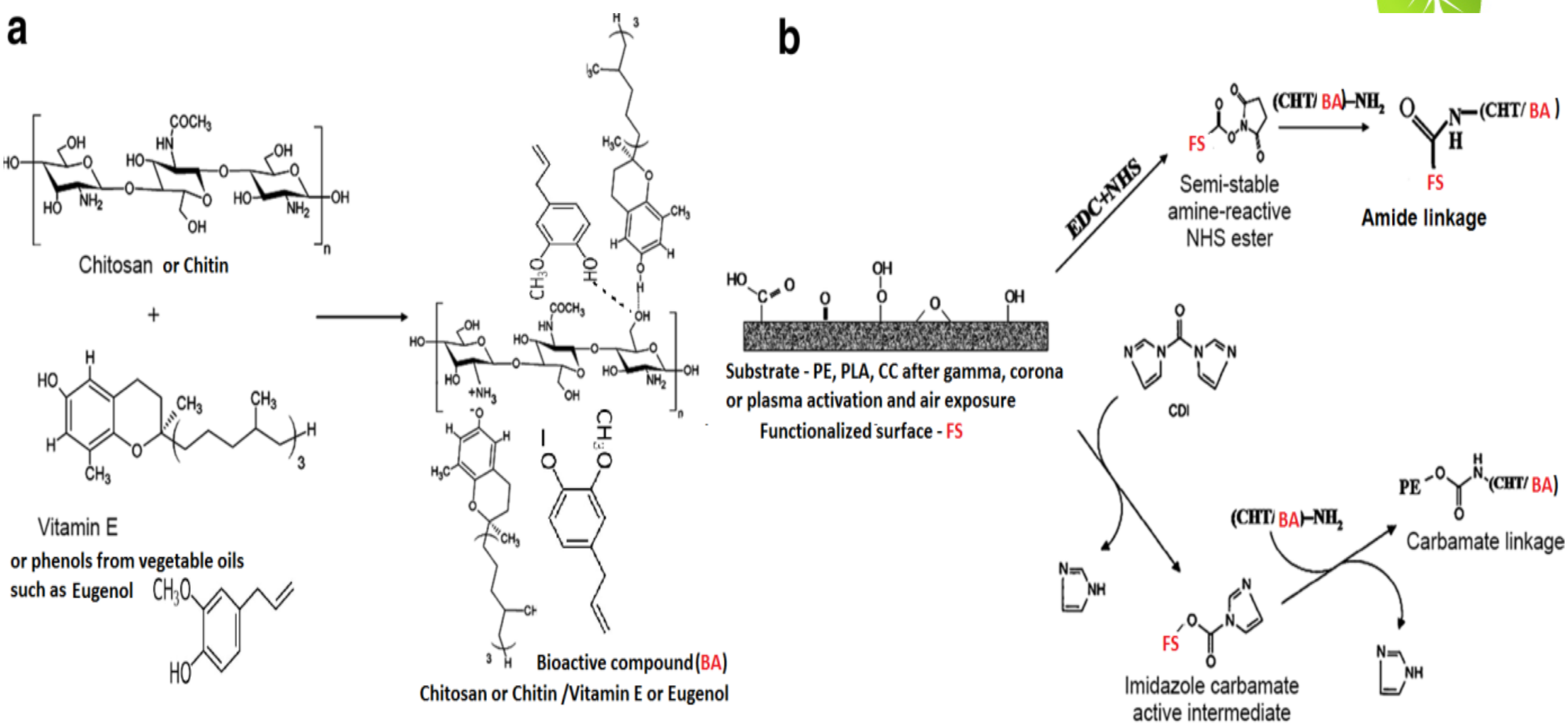


-**highly active carbonylating agent** that contains two acylimidazole leaving groups;

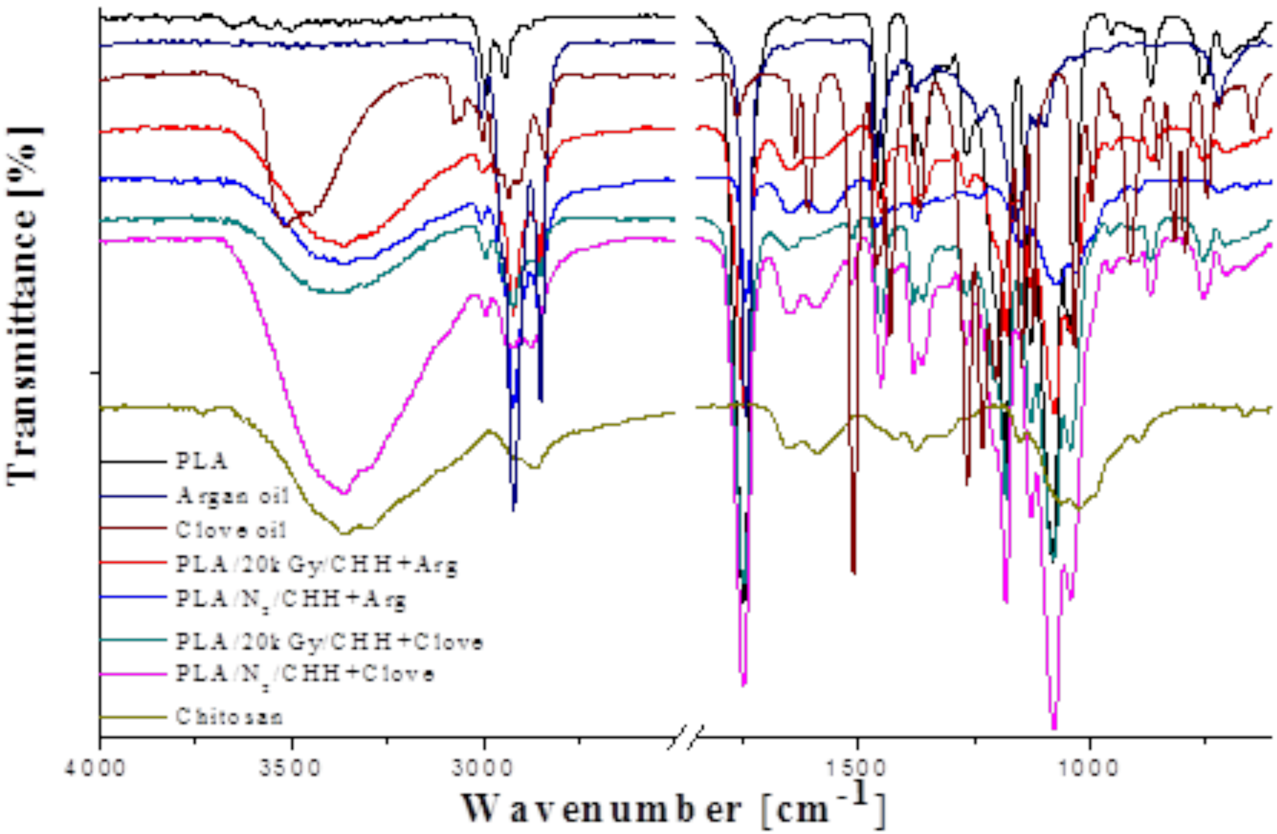
-**can activate carboxylic acids** or **hydroxyl groups** for conjugation with other nucleophiles, creating either zero-length amide bonds or one-carbon-length N-alkyl carbamate linkages between crosslinked molecules;



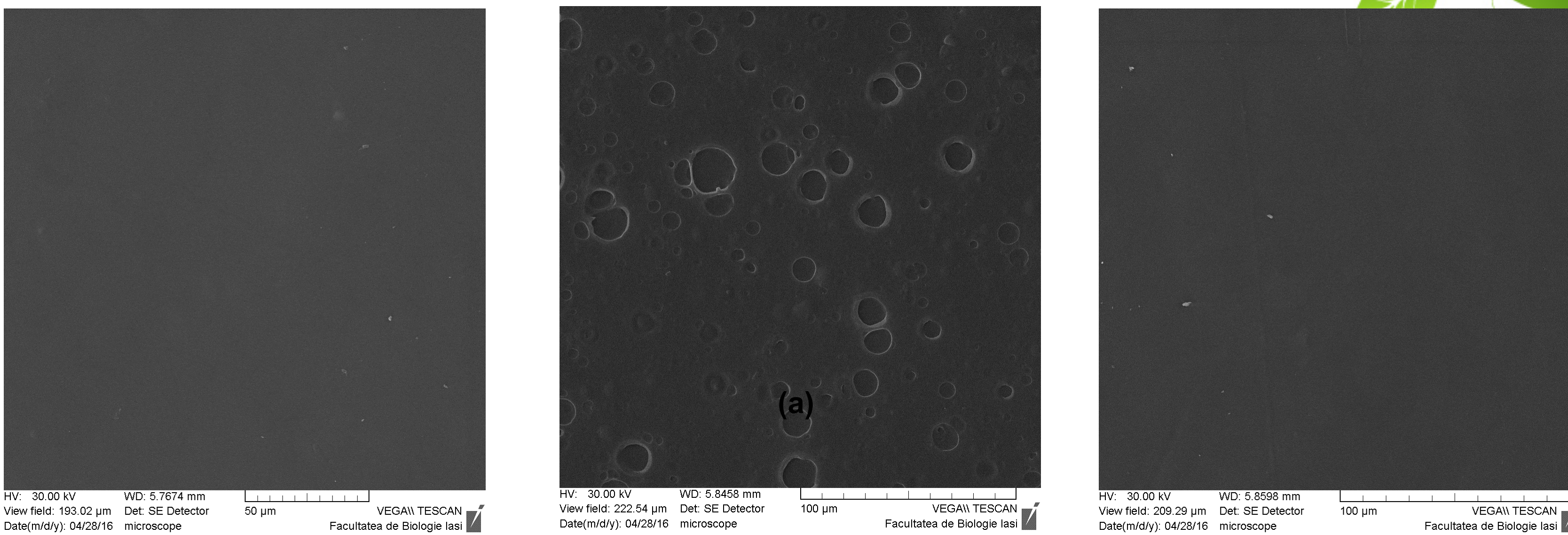




a
Chitosan or Chitin
+
Vitamin E
or phenols from vegetable oils
such as Eugenol
Bioactive compound(BA)
Chitosan or Chitin /Vitamin E or Eugenol
b
Substrate - PE, PLA, CC after gamma, corona
or plasma activation and air exposure
Functionalized surface - FS
EDC+NHS
Semi-stable
amine-reactive
NHS ester
Amide linkage
CDI
Imidazole carbamate
active intermediate
Carbamate linkage



The FTIR spectroscopy have demonstrated both the clove and argan oil incorporation into chitosan matrix and the surface immobilization of the chitosan-oil mixture onto PE and PLA substrates prior plasma treated and gamma irradiated.



(a) *(b)* *(c)*

SEM images of (a) PLA/N_2/CHH, (b) PLA/N_2/CHH+ARG and (c) PLA/N_2/CHH+Clove samples

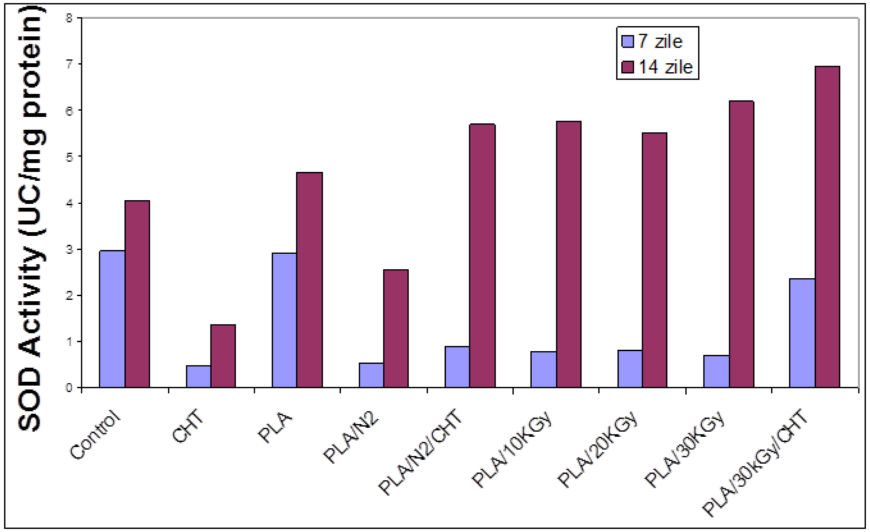
Efficient encapsulation of argan and clove oil by incorporation into chitosan matrix assure an homogeneous coating onto PLA surface

Biodegradation procedure

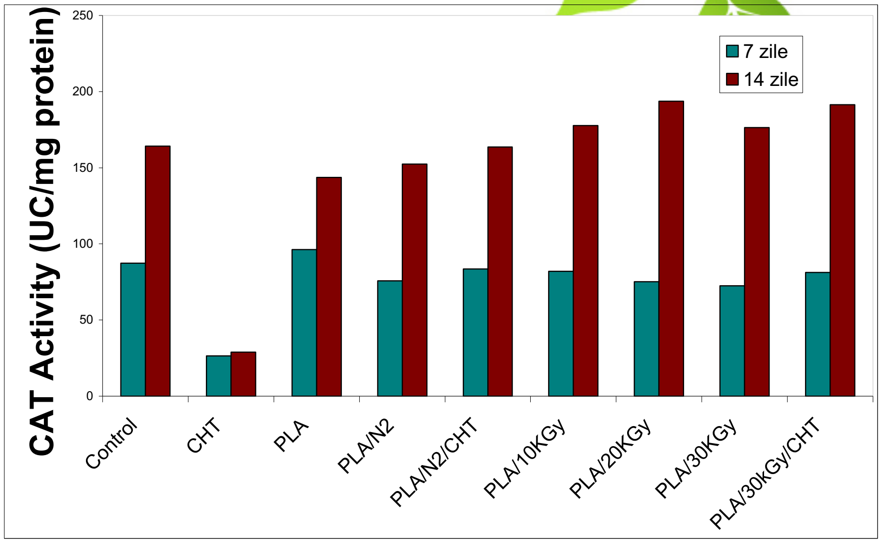
- The *Phanerochaete chrysosporium* was cultured in adequate Petri dishes on Sabouraud medium (peptone 10 g/L, glucose 35 g/L, agar 20 g/L). The fungus culture was placed for 7 days in thermostat room at 28.0 ±0.1°C. The liquid Sabouraud medium was inoculated with 0.8 cm diameter disks from 7 day culture of fungi. Chitosan-PLA based composites were supplied to fungus culture medium.
- **Biochemical investigation**: The assay of superoxide dismutase (SOD) activity in fungi mycelium samples was carried out by colorimetric Winterbourn's adapted method while the content of malondialdehyde (MDA) was assayed using thiobarituric acid (TBA) All resulted numerical values were expressed relatively to the amount of protein fungus mycelium assayed with Bradford method.
- **Gel Permeation Chromatography** – Variation of the average molecular weight
- **Atomic Force Microscopy (AFM)**

Polymeric substrate influence on *Phanerochaete chrysosporium* characteristics

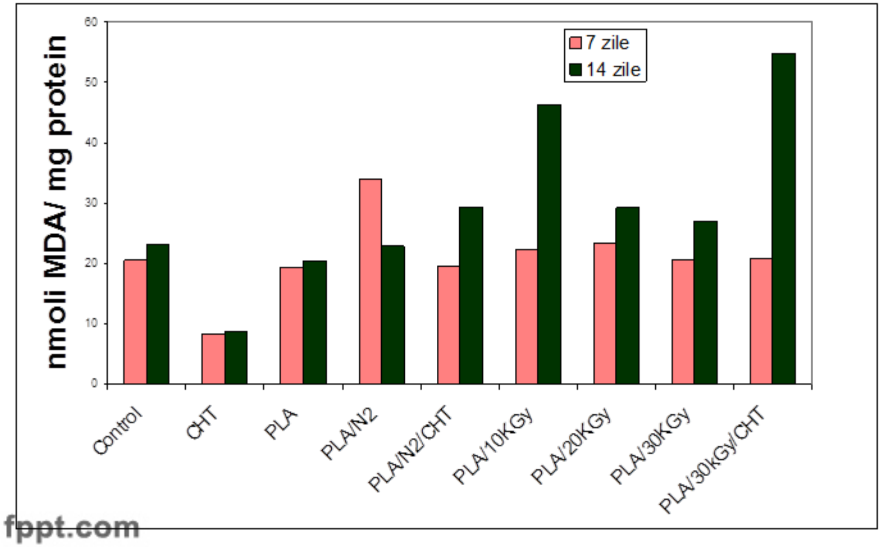
Superoxide dismutase



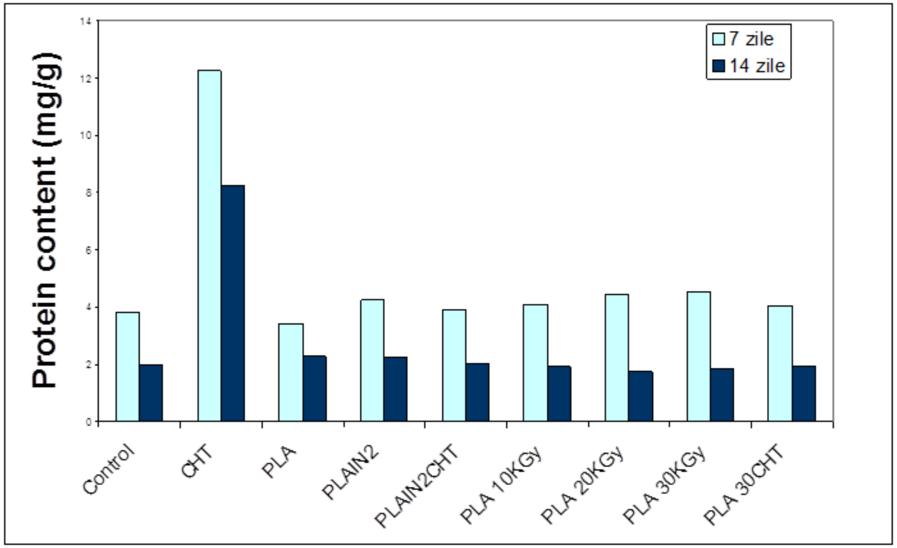
Catalase enzyme (CAT)



Malondialdehyde

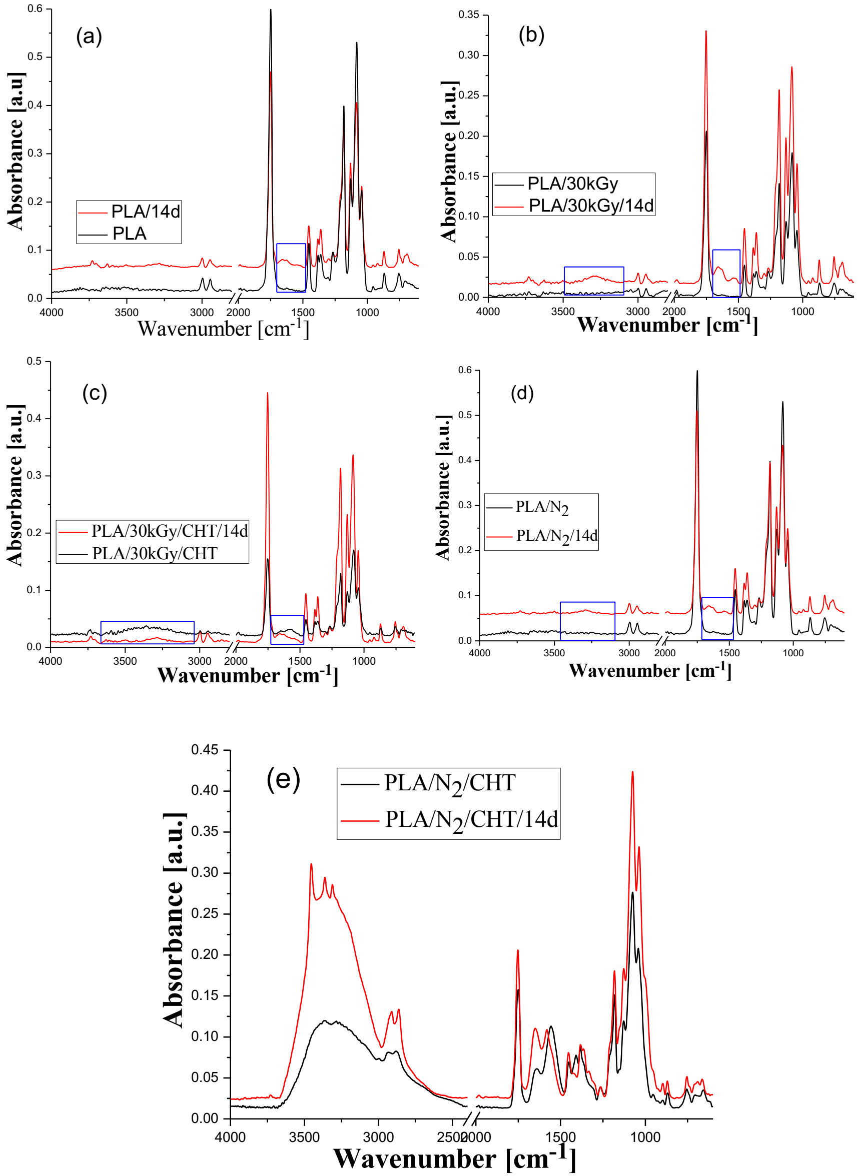


Extracellular protein of *P. chrysosporium*



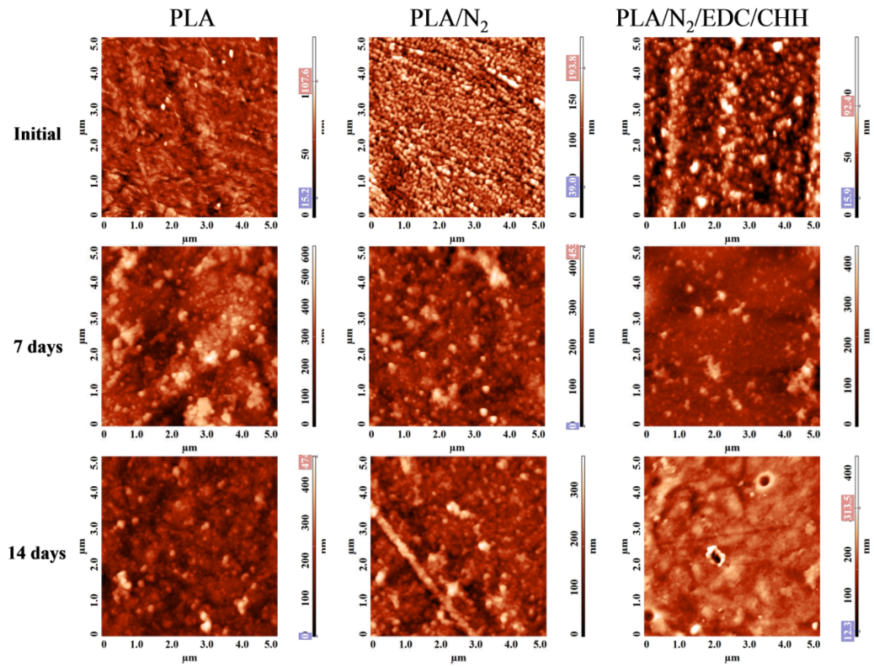
Average molecular weight change of enzymatically degraded PLA-based samples

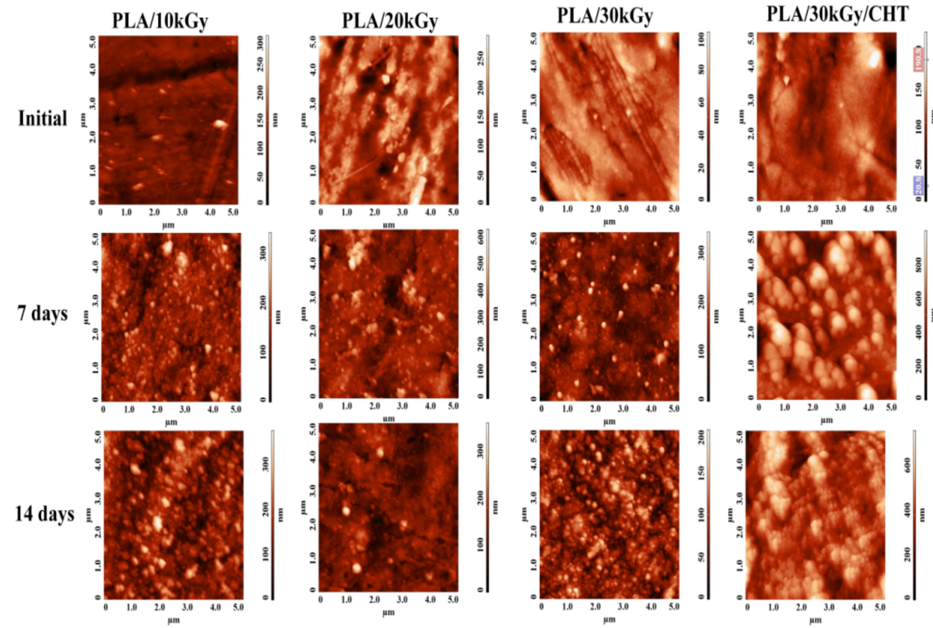
Sample	M_n ($x10^3$) g/mol	M_w ($x10^3$) g/mol	M_z ($x10^3$) g/mol	PDI M_w/M_n	[η] mL/g
PLA	299.7	451.4	678.7	1.507	199.2
PLA/7d	39.21	76.08	136.9	1.940	99.12
PLA/14d	48.56	85.02	144.6	1.751	101.5
PLA/30kGy	49.73	88.34	153.5	1.776	99.86
PLA/30kGy/7d	26.68	48.33	81.63	1.811	60.26
PLA/30kGy/14d	27.84	46.70	75.17	1.678	58.45
PLA/30kGy/CHH/7d	29.80	50.87	84.01	1.707	62.47
PLA/30kGy/CHH/14d	27.59	47.23	77.48	1.712	58.80
PLA/N2/CHH/14d	46.25	79.80	134.6	1.725	96.72

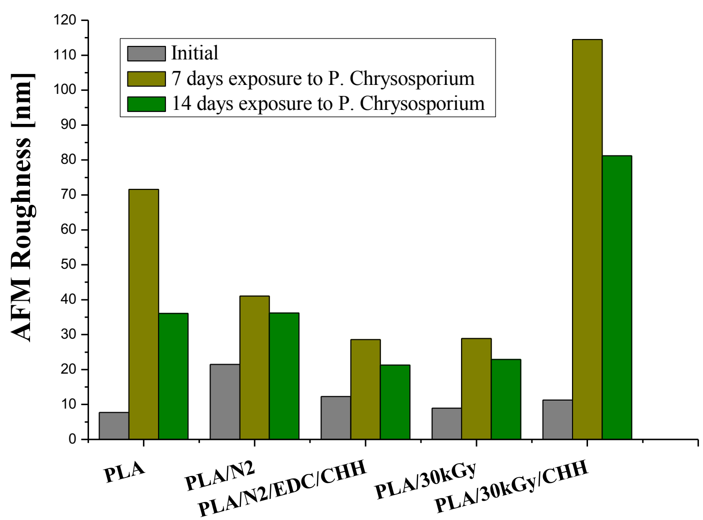


All the bands characteristic for chitosan are present in the spectra even after 14 days of biodegradation.

New bands appears in the spectra such as at 3455 cm^{-1} attributed to the free hydroxyl groups stretching.







Modification of surface is probably the formation of oligomers and other low-molecular biodegradation products as a result of random chain scission. Some of them can agglomerate at the surface creating the observed grains. The most significant topographical change in terms of morphology and roughness is observed for the PLA sample gamma irradiated and surface modified with chitosan

The plasma and gamma radiation exposed PLA and PLA/CHT stratified composites supported fungal growth resulting in their degradation, which is reflected from extracellular protein excretion and percent change in degradation and with change in polymeric substrate structure. The presence of bioaccessible material, i.e., PLA and chitosan, facilitated degradation. The plasma and gamma irradiated PLA samples show an increment in degradation due to secretion of protein.

Coating of LDPE

Consumption of meat in EU

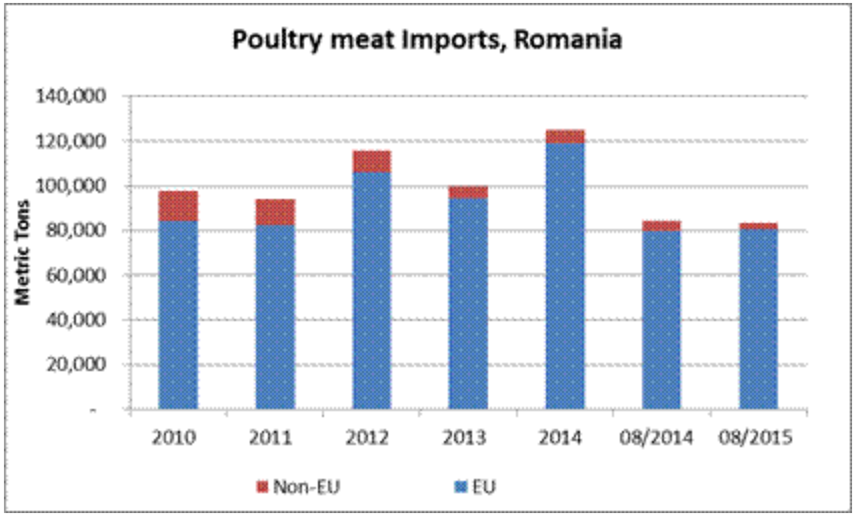
Table 1: Consumption of all kind of meat in EU-27 (kg/hab).

Meat origin	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022
Beef & Veal meat	16.37	16.32	15.90	15.33	15.29	15.58	15.82	15.96	15.92	15.86	15.83	15.78	15.74	15.71
Pork meat	40.56	41.55	41.48	41.15	40.26	40.33	40.66	40.76	40.79	40.66	40.63	40.74	40.74	40.79
Poultry meat	**23.07**	**23.47**	**23.63**	**23.92**	**24.19**	**23.98**	**23.86**	**23.71**	**23.89**	**23.96**	**24.00**	**24.02**	**24.05**	**24.11**
Sheep & Goat meat	2.50	2.37	2.33	2.20	2.17	2.14	2.12	2.08	2.06	2.02	1.99	1.97	1.96	1.95
Total EU-27	**82.51**	**83.70**	**83.34**	**82.61**	**81.92**	**82.03**	**82.45**	**82.52**	**82.65**	**82.50**	**82.45**	**82.52**	**82.49**	**82.56**

Source: European Commission, Prospects for agricultural markets and income 2012-2022.

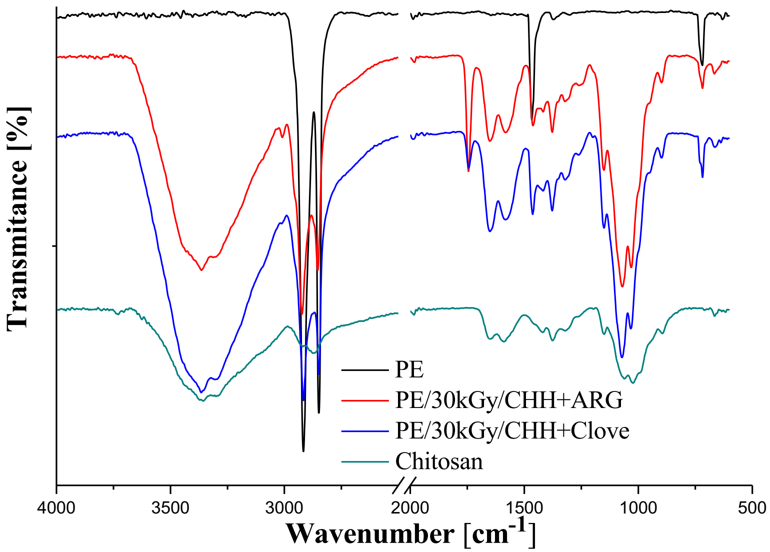
EU poultry meat consumption is expected to increase by 4,3% on average and reach 24.1 kg/capita by 2022, mainly driven by the increasing volumes consumed in the EU-N12.

Poultry meat consumption in Romania

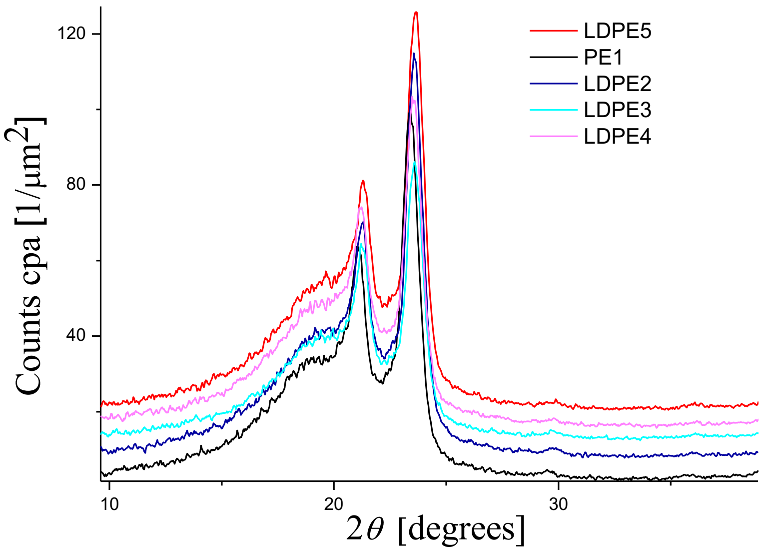


Per capita meat consumption, Romania (kg of meat/capita)

A large part of the Romanian population continues to have poor purchasing power, which impacts the dynamics of packaged food. The most important action taken by the government in 2015 was the reduction of VAT from 24% to 9% for all food products starting 1 June. This stimulated volume sales which in turn led to the value growth of packaged food overall. The secondary effect of the reduction of VAT was the orientation of consumers towards better quality brands, which they previously avoided as they regarded them as not being affordable.



By argan oil incorporation into chitosan-based emulsion a new band appears in the FTIR spectra of PE/30kGy/CHH+ARG at 1744.5 cm^{-1} that is assigned to ν(C=O) from RC=OOR structure.



The modified PE-based samples present a slight orientation. The patterns are very similar; only LDPE5 sample presents a shift of the peaks

XPS Analysis

Elemental composition and atomic ratios for PE and chitosan-coated/grafted samples.

Samples	Elemental composition (atomic %)				
	C	O	N	O/C	N/C
PE	99.2	0.8	-	-	-
PEcorona	88.54	9.22	0.72	0.104	0.008
PE,CHT+VitE	73.16	20.87	4.4	0.285	0.06
PEcor,CHT+VitE	69.94	23.54	4.5	0.336	0.064
PEcor,EDC+NHS, CHT+VitE	69.13	23.7	5.15	0.342	0.074
PEcor,CDI,CHT+ VitE	68.06	25.01	4.9	0.367	0.072

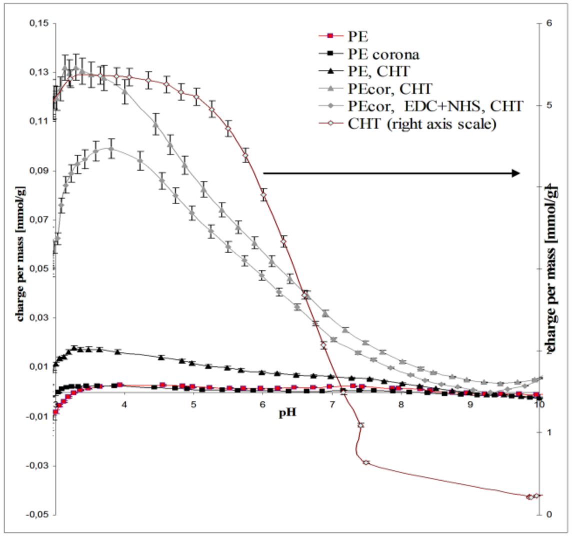
High-resolution XPS of C1s peak deconvolution and possible groups.

•**N-C=O / C-NH2** *atomic ratio increases after using both coupling routes but shows a larger increase for EDC+NHS case. Hence, it emphasizes that EDC+NHS coupling system is more effective than CDI.*

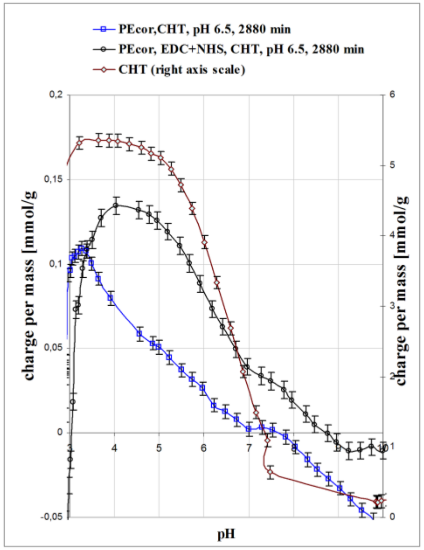
C1: C-C, C-H; **C2:** (C-NH_2 and/or C-O-C; **C3:** (C-O); **C4:** (N-C=O); **C5:** (O=C-OH)

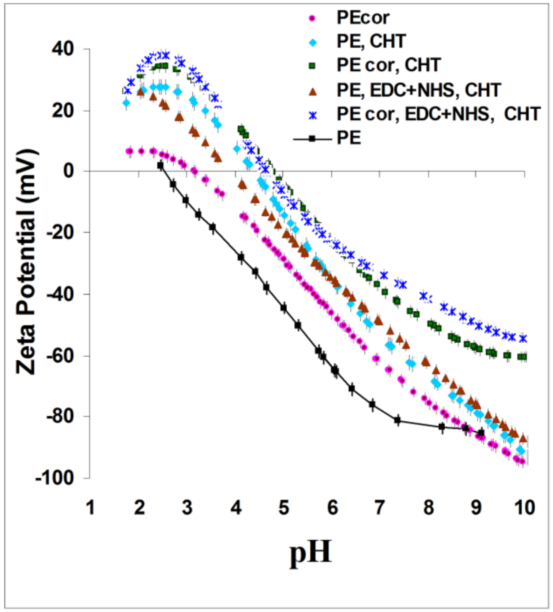
Potentiometric titration, zeta potential

Before

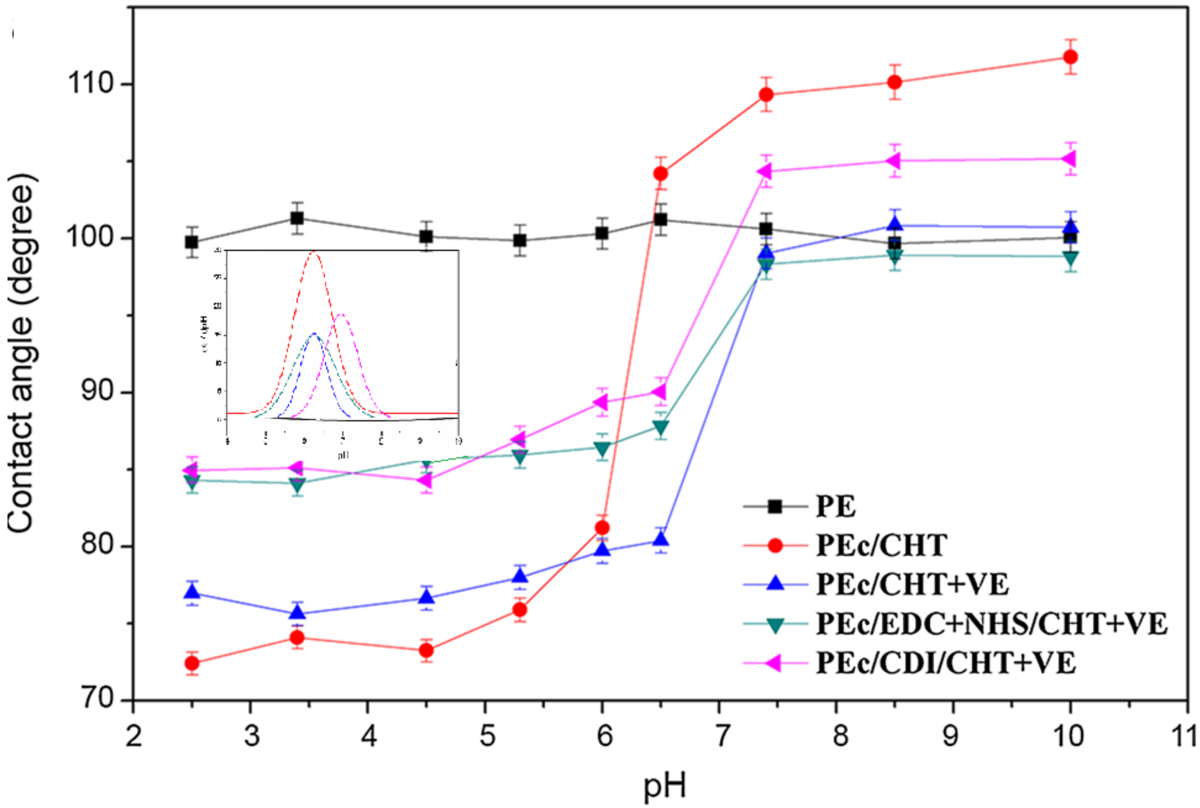


After desorption

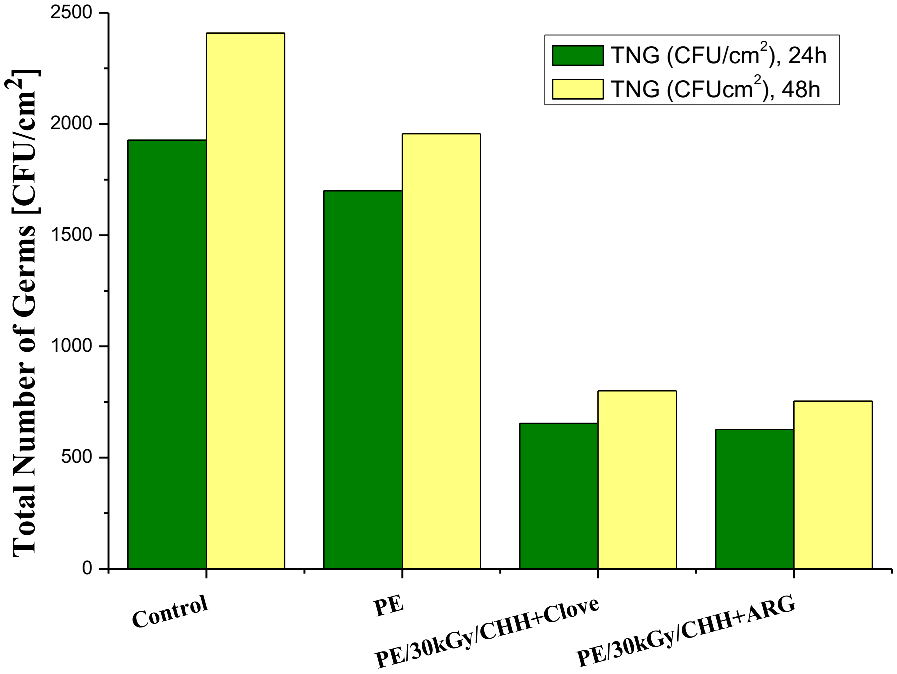




Using coupling agents (EDC+NHS and CDI) leads to obtaining stable chitosan layer and chitosan+vitamin E chemical bonded onto corona treated PE surface.

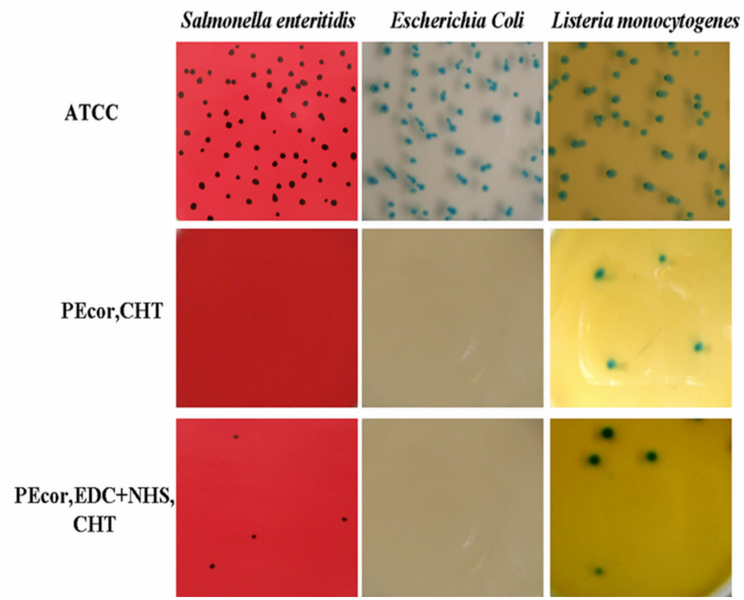


Contact angle (degree)
110
100
90
80
70
2
3
4
5
6
7
8
9
10
11
pH
PE
PEc/CHT
PEc/CHT+VE
PEc/EDC+NHS/CHT+VE
PEc/CDI/CHT+VE



Variation in time of Total Number of Germs for beef meat packed in polyethylene modified with chitosan and vegetable oils

Antibacterial and permeability tests

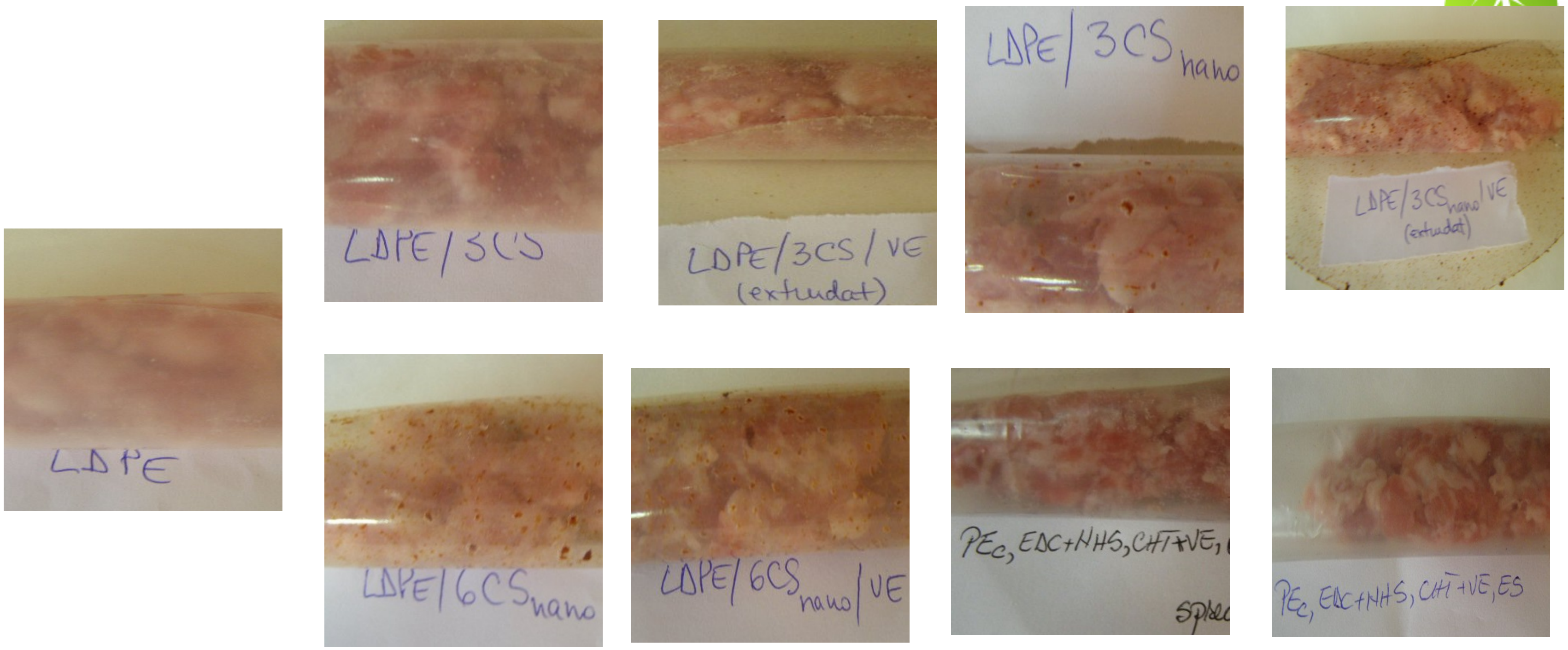


Oxygen permeability for chitosan-coated PE films.

Samples	O_2 Transmission Rate (mL/m^2*day)
PE	3833.36
Immersion	
PE,I,1CHT	3762.2
PEcor, I,1CHT	2150.04
PE,I,3CHT	3626.14
PEcor,I, 3CHT	1135.12
PE,I,5CHT	3612.32
PEcor,I, 5CHT	778.54
Spreading	
PE,S,1CHT	3800.25
PEcor,S, 1CHT	2142.52
PE,S,3CHT	3714.23
PEcor, S,3CHT	1310.00
PE,S,5CHT	3723.56
PEcor,S, 5CHT	1065.66
Electrospraying	
PEcor,ES,5CHT	2952.43

Parameters	Reference	CHARACTERISTICS AT 48h FROM EXPIRATION OF THE SELF LIFE **Legend:** ■ fresh meat; ■ relative fresh meat; ■ alterated meat														
		MARTOR	LDPE/3 CS	LDPE/3 CS nano	LDPE/3 CS/VE	LDPE/3 CS/IRG	LDPE/3 CS/VE	LDPE/3 CS NANO/VE	LDPE/6 CS	LDPE/6 CS nano	LDPE/6 CS/VE	LDPE/6 CS/IRG.	LDPE/6 CSnano/VE	PEc, EDC+NHS, CHT+VE, spread	PEc, EDC+NHS, CHT+VE, electrospraying	LD
Aspect	*Surface aspect homogeous, light pink, brigth*															
	Elastic consistence															
	In depth humid aspect, bright characteristic		.													
Smell	*Pleasant, characteristic of species*															
Bulion after boiling and sedimentation	*Transparent, clear, aromatic, at surface separates a compact layer and fat islands taste and pleasant smell.*															
pH	*5,7*	6,9	6,7	6,3	6,0	6,6	6,2	6,0	6,8	6,3	6,2	6,6	5,9	6,3	5,8	6
Reaction with H_2S	*negative*															
Total number of germs, ufc/g	*$1,9 \times 10^3$*	*$8,3 \times 10^9$*	*$2,9 \times 10^7$*	*$2,6 \times 10^5$*	*$5,1 \times 10^4$*	*$1,5 \times 10^8$*	*$8,4 \times 10^4$*	*$1,9 \times 10^7$*	*$8,7 \times 10^6$*	*$9,6 \times 10^4$*	*$1,7 \times 10^5$*	*$4,8 \times 10^8$*	*$2,4 \times 10^5$*	*$8,2 \times 10^7$*	*$9,4 \times 10^4$*	*5 ×*

Testing of new packaging materials directly as poultry meat packaging. Good results can be remarked for LDPE coated with CS and Vitamin E



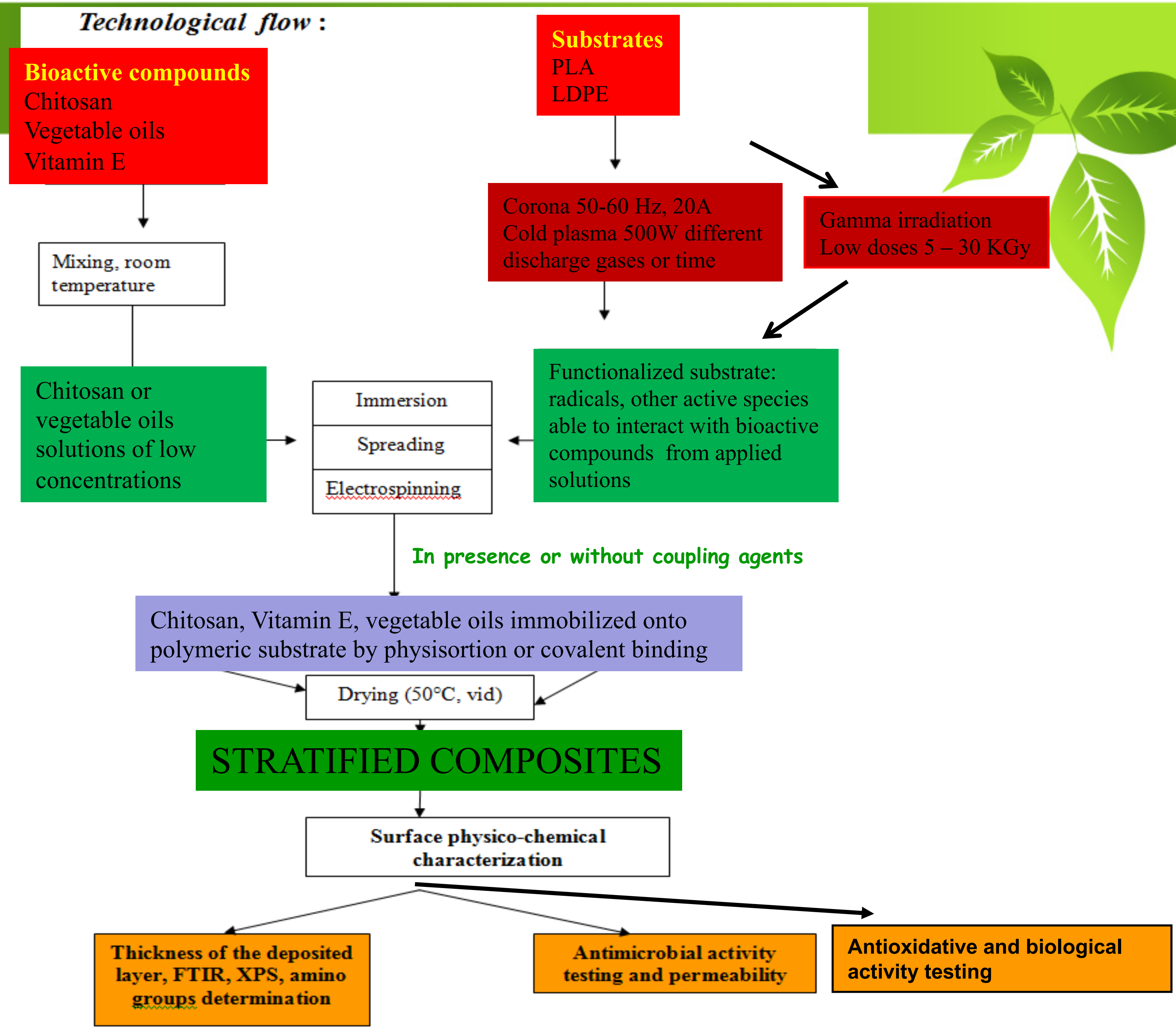
Microscopical aspects of the poultry meat packed in foils of LDPE/chitosan composites

Conclusions

- ✓ The incorporated additives derived from natural products do not negatively affect consumers health, some of them bringing benefits for health.

- ✓ Nanocomposites can not only passively protect the food against environmental factors, but they may enhance stability of foods.

Melt processing technique requires large amounts of bioactive agents, some of them could deteriorate during melt processing, therefore an other method should be found and this one will be applied where no technological changes are possible


Technological flow :
Bioactive compounds
Chitosan
Vegetable oils
Vitamin E
Substrates
PLA
LDPE
Mixing, room temperature
Corona 50-60 Hz, 20A
Cold plasma 500W different discharge gases or time
Gamma irradiation
Low doses 5 – 30 KGy
Chitosan or vegetable oils solutions of low concentrations
Immersion
Spreading
Electrospinning
Functionalized substrate: radicals, other active species able to interact with bioactive compounds from applied solutions
In presence or without coupling agents
Chitosan, Vitamin E, vegetable oils immobilized onto polymeric substrate by physisortion or covalent binding
Drying (50°C, vid)
STRATIFIED COMPOSITES
Surface physico-chemical characterization
Thickness of the deposited layer, FTIR, XPS, amino groups determination
Antimicrobial activity testing and permeability
Antioxidative and biological activity testing

Conclusions

•The coating/encapsulation can be performed by dipping, emulsion/solvent casting or electrospraying techniques in the last case very thin surface layers assure at minimum quantity for obtain both antimicrobial and antioxidant characteristics of packaging.

•ATR-FTIR, XPS proved and stability of layer and no migration in food product was assessed by potentiometric titration and spectroscopic methods.

•The EDC+NHS coupling system was proved to be more effective in grafting reaction, but not all NH_2 groups of chitosan participate to this reaction remaining active to impart antimicrobial characteristics to the coating

•In terms of efficiency and lower substances consumption electrospraying method is by far the most appropriate coating procedure.

- Bilateral project Romania (PPIMC) - Greece Aristotle University of Thessaloniki:"Smart, Safe, Health-promoting, Green Food Packaging" 2012-2014. (2-SHG FP) 2012-2014.

- Bilateral collaboration Romania (PPIMC) - Maribor University Slovenia: „Functionalization of synthetic polymers for development of new antimicrobial packaging". 2012-2014

- RCM Coordinated Research Project : Application of Radiation technology in the Development of Advanced Packaging Materials for Food Products 2014 -2017

IAEA
International Atomic Energy Agency

- **ACTIBIOSAFE Project** : Romanian - EEA Research Programme operated by MEN under the EEA Financial Mechanism 2009-2014/Contract No 1SEE/30.06.2014. 2014 - 2017

- **ERASMUS+:** Joint innovative training and teaching/learning program in enhancing development and transfer knowledge of application of ionizing radiation in materials processing" 2014-1-PL01-KA203-003611, 2014 -2017