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Article

Fast computational chemistry methods applied to new anti-Ebola virus entry drugs - application for new therapeutic targets

***Ana-Maria Udrea*[1], *Alin Puia*[1♣], *Maria Mernea*[1] ,**
***Iulia Alexandrescu*[1], *Speranta Avram*[1]**

University of Bucharest, Faculty of Biology, Anatomy Dept., Animal Physiology and Biophysics, 91-95 Spl. Independentei, Bucharest-076201, Romania

ABSTRACT

Ebola virus is responsible for severe symptoms and has fatality rates up to 90%. Some approved drugs among antipsychotics and Selective Serotonin Reuptake Inhibitors (SSRI) antidepressants appear to be efficient inhibitors, with fewer secondary effects. There is an immense pressure to use fast research methods to discover new antivirus-drugs or, detect new antivirus applications of clinically used drugs. We have generated quantitative structure–activity relationship (QSAR) models on drugs used to treat genetic disorders, with various inhibitor concentrations (IC50) on Ebola virus. We evaluated the predicted affinity at Ebola virus glycoproteins of other efficient SSRI antidepressants, antipsychotics and anticancer drugs.

Keywords: QSAR, Ebola virus, Antipsychotic, Computational Chemistry.

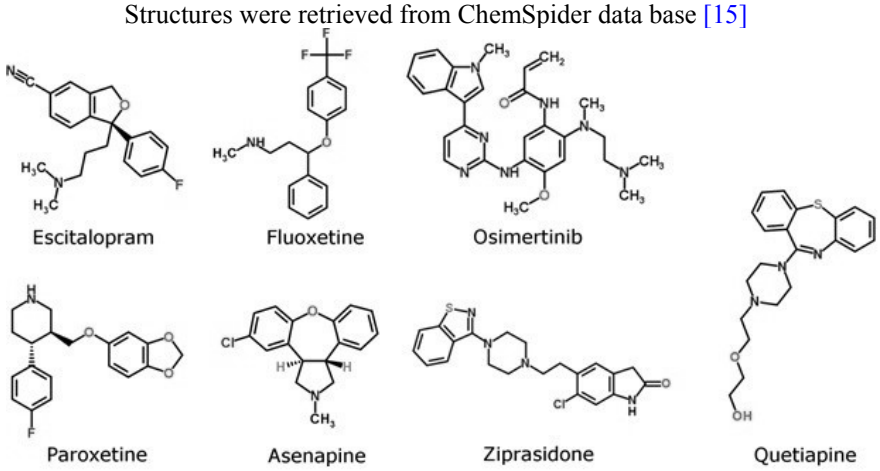
♣ Correspondent author: e-mail: alin2391@gmail.com Telephone: +40720542786

1. INTRODUCTION

Ebola virus (Ebov), belonging to Mononegavirales Order, Filoviridae family, causes a critical hemorrhagic fever disease with fatality rates up to 90% [1]. Typical symptoms occurring in the 21st day after Ebov infection include fever, fatigue, diarrhea, headache, abdominal pain, cramping, nausea and vomiting [2]. The very first Ebov outbreak was in 1976, in Democratic Republic of Congo [3]. The virus flourished in Africa due to the general instability of the region and poor levels of healthcare.

In spite of the high mortality due to Ebov infections, there is no viable treatment available [4].Previous attempts to fight the infections included: (i) whole blood from surviving Ebov patients [5]; (ii) IgGs isolated from horses hypervaccinated with Ebov [6]; (iii) humanized-mouse antibodies [7]; (iv) inhibitors of protein production based on RNA -polymerase inhibitors and interfering RNA nanoparticles; (v) gene-silencing using small interfering RNAs [8]. A promising approach in identifying an Ebov treatment is based on the repurposing of approved drugs. In this direction, Kouznetsova et al [9] identified 53 compounds with the capacity to block Ebola virus-like particle entry which included commonly used antidepressants and antipsychotics [9]. Ebola virus is composed from: a glycoprotein (subdivided in sGP, GP1, GP2 and ssGP), a protein matrix (VP24, VP40, VP35, and VP30), and a nucleoprotein [10]. In their study Kouznetsova et al. and Johansen [9,11] assessed the effect of 2816 compounds from a NCATS-approved drug collection assembled for drug repurposing tests .[9] on inhibiting the entry of Ebola virus - like particles (VLP), composed of a glycoprotein and the matrix VP40 protein fused to a beta-lactamase reporter enzyme into cultured HeLa cells. In the end, 53 compounds were identified to have the potential to block Ebola VLP entry into cells. These compounds belong to different drug classes, including microtubule inhibitors, estrogen receptor modulators, antihistaminics, antipsychotics, pump/channel antagonist and anticancer/antibiotics.

Figure 1.Chemical structures of the drugs taken into account as possible Ebola medication. Structures were retrieved from ChemSpider data base [15]



The aim of our study was to expand Kouznetsova's study by predicting the inhibitory reaction of other widely used antipsychotics, antidepressants and new anticancer drugs, with minimal side effects. Hereby, based on our expertise in computational biology[12,13,14] we conducted a Quantitative Structure-Activity Relationship (QSAR) study on the viability as Ebola medication of three potent antidepressants belong to SSRI's (Selective serotonin reuptake inhibitors), namely Escitalopram, Fluoxetine and Paroxetine; three atypical antipsychotics namely Asenapine, Quetiapine and Ziprasidone; and a new anticancer compound represented by Osimertinib (Figure 1).

2. METHODS

2.1. Dataset for Analysis

In the present study we used a set of 21 drugs identified from the literature that were clinically proven to inhibit Ebola VLP entry into HeLa cells [9]. These drugs covered large classes of pharmaceutical compounds used in depression, psychosis, viral infections and cancer. These chemical compounds are represented by 5 antidepressants: Bifemelane, Clomipramine, Imipramine, Maprotiline and Sertraline; 4 antipsychotics: Piperacetazine, Thioproperazine, Thiothixene, Trifluoperazine, 9 anticancer drugs: Bosutinib, Daunomycin, Raloxifene, Sunitinib, Topotecan, Toremifene, Vinblastine, Vincristine, Vinorelbine, one antiviral compound Tilorone, one anticholinergic compound namely Benztropine and one antiallergic compound namely Clemastine. Their inhibitory activities expressed as the half maximal inhibitory concentration (IC50) vary between 0.048 microM (Vinblastine) and 13.7 microM (Imipramine). Biological activities were evaluated as pIC50 using the following formula: -logIC50 (uM)* 10-6 Eq.(1) [16].

2.2. Molecular modeling and the minimum potential energy calculation of compounds

Molecular modeling of the compounds was performed using their 3D structures retrieved from the ChEMBL database [17] (the database code of each compound is listed in Table 3). Minimum energy evaluation of compounds was performed by AM1 semi-empirical method, Conjugate-Gradient algorithm, convergence 0.01. After energy minimization, Gasteiger partial charges were used [18].

2.3. QSAR methodology

Initially, we calculated 23 descriptors belonging to two categories: (i) 2D descriptors including physical properties: steric (subdivided van der Waals surface and volume, solvent accessible surface and volume), atom and bond counts (hydrophobic/polar, donor /acceptor

atoms, rigid and rotatable bonds) and electronic descriptors (molecular polarizability, molar refractivity, dipole moment); and (ii) 3D molecular descriptors including potential energy descriptors and globularity. Descriptors calculation was performed with MOE software.
Descriptors evaluation was followed by overlapping descriptors removal using Pearson correlations. Afterwards, the most useful 3 descriptors for the QSAR model were selected by performing a SLR (Simple Linear Regression) [19]. The selected descriptors are: (i) ASA – describing the water accessible surface induced by negative atoms, determined using a probe radius of 1.4 Å that rolls over all atoms with negative partial charges (less than 0);(ii) LogP - represents the logarithm of the octanol/water partition coefficient [20]; (iii) SMR - molecular refractivity calculated using an atomic contribution model that assumes the correct protonation state (washed) structures [21].

Table 1 Pearson correlation matrix

Pearson correlation matrix			
	LogP	ASA-	SMR
LOGP	1		
ASA	0.051	1	
SMR	0.061	0.058	1

Furthermore, the selected molecular descriptors were used for developing several QSAR models. The Pearson correlation matrix of these descriptors is presented in Table 1.

2.4. Chemometric analysis

The QSAR models developed in Volsurf software were statistically analyzed in order determine their reliability. The statistical parameters that we calculated are: correlation coefficient of the regression between the predicted and observed activities of compounds (R^2), cross-validated r^2 (Q^2), the root mean square error (RMSE) and cross-validated RMSE. In practice, a QSAR model can be validated if R^2 and Q^2 exceed 0.8 and 0.5 [17]. The following QSAR model: pIC50 = 2.85442 -0.00157 * (ASA-) +0.18857 * logP(o/w)+0.21406 * SMR Eq.(2) presented the best statistical parameters, therefore it was used to predict the biological activities of the 7 drugs that we considered as candidates for repurposing.

2.5. Training and testing sets

The composition of the training and test sets is very important, as it has the ability to influence the consistency of resulting QSAR models. Here, the molecules were randomly distributed in the training (16 molecules) and test set (5 molecules). The predictive power of

our QSAR model was used for predicting the Ebov inhibitory activity of the seven drugs we considered for repurposing.

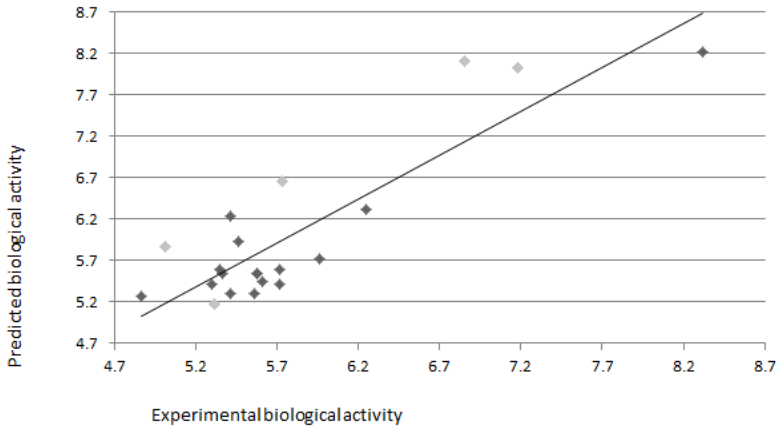
3. RESULTS AND DISCUSSIONS

In the present study, we generated a QSAR model that associates the molecular descriptors of several drugs proved to inhibit Ebov VLP entry into cultured cells with their inhibitory activities. The dataset we considered includes antidepressant, antipsychotic, anticancer, antiallergic, anticholinergic and antiviral drugs. The power of prediction of the developed model was used to predict the Ebov antagonistic activity of 7 compounds already used in clinics for different purposes: 3 antidepressants, 3 antipsychotics and 1 anticancer. The advantages of identifying Ebov inhibitors among these drugs are that they are already used in clinics and have minimal side effects.

Table 2. Summary of QSAR statistical parameters for the best model

ROOT MEAN SQUARE ERROR (RMSE)	0.22
CORRELATION COEFFICIENT (R^2)	0.90
CROSS-VALIDATED RMSE	0.42
CROSS-VALIDATED R^2 (Q^2)	0.73

Figure 2. Correlation between predicted and experimental values of the pIC50 obtained by QSAR model (q^2=0.73 r^2 =0.90). Dark grey – Values from the training set. Light grey – Values from test set.



Initially, several QSAR models were developed based on the three molecular descriptors we identified as being significant (non-overlapping and non-redundant) for the inhibition of Ebov VLP particles entry into the cells. From these, we selected the model that presented the best statistical parameters (Table 2) for predicting the anti-Ebov activity of the repurposing candidate drugs.

The QSAR model has a good predictive power, as showed by the predicted pIC50 values (pIC50 pred) of the 21 drugs taken in testing and training sets and by the residuals calculated as experimental pIC50 values (pIC50exp) minus the predicted pIC50 values (Table 3).The good correlation between predicted biological activities and experimental biological activities is presented in Figure 2.

Table 3. Comparison between observed and predicted activities (pIC50) and the residual values differences between experimental and predicted biological activities for training and test (Bolded) sets.

Approved indication	Drugs	ChEMBL code	pIC50 exp	pIC50 pred	Residual
Antidepressants	**Bifemelane**	**CHEMBL1192517**	**5.31**	**5.17**	**-0.13**
	Clomipramine	CHEMBL415	5.30	5.42	0.11
	Imipramine	CHEMBL11	4.86	5.27	0.41
	Maprotiline	CHEMBL21731	5.61	5.44	-0.16
	Sertraline	CHEMBL809	5.56	5.30	-0.25
Antipsychotics	**Piperacetazine**	**CHEMBL1584**	**5.01**	**5.87**	**0.85**
	Thioproperazine	CHEMBL609109	5.36	5.54	0.18
	Thiothixene	CHEMBL1201	5.71	5.59	-0.11
	Trifluoperazine	CHEMBL422	5.34	5.59	0.24
Anticancer	Bosutinib	CHEMBL288441	5.41	6.24	0.82
	Daunomycin	CHEMBL178	5.58	5.53	-0.04
	Raloxifene	**CHEMBL81**	**5.73**	**6.64**	**0.91**
	Sunitinib	CHEMBL535	5.71	5.41	-0.30
	Topotecan	CHEMBL84	5.41	5.30	-0.11
	Toremifene	CHEMBL1655	6.24	6.31	0.07
	Vinblastine	**CHEBI:27375**	**8.31**	**8.21**	**-0.10**
	Vincristine	**CHEBI:28445**	**6.85**	**8.10**	**1.25**
	Vinorelbine	**DB00361(*)**	7.18	8.03	0.84
Antiallergic	Clemastine	CHEMBL1626	5.95	5.72	-0.23
Anticholinergic	Benztropine	**CHEBI:3048**	5.57	5.54	-0.03
Antiviral	Tilorone	CHEMBL47298	5.46	5.93	0.47

*Vinorebline mol file was acquired from drugbank.ca database [22]

After validating the QSAR model, we used it for predicting the Ebov infection inhibitory activity of the seven drugs we considered for repurposing. Their predicted biological activities are presented in Table 4. As can be seen, the highest pIC50 value that should correlate to a higher biological activity was obtained in the case of Osimertinib, an anticancer compound. This result is in agreement with the observation that, in the dataset we considered, experimental pIC50 values of some anticancer compounds are higher and even significantly higher than that of antipsychotic and antidepressant drugs (Table 3). According to its predicted pIC50 value (6.41), Osimertinib should have an Ebov inhibitory activity higher than that of Toremifene (6.24) and lower than that of Vincristine (6.85). The following activities are those of antipsychotic drugs Ziprasidone (5.72) and Quetiapine (5.37). Zisprasidone has a similar predicted pIC50 value to that of the antipsychotic drug Thiothixene (5.71) and the anticancer compounds Raloxifene (5.73) and Sunitinib (5.71). Quetiapine should have similar inhibitory activities with Thioproperazine (5.36) and Trifluoperazine (5.34), two antipsychotic drugs.

Table 4: Predicted biological activities of the drugs with potentially inhibitory effect on Ebola VLP virus

Approved indication	Drugs	ChEMBL code	Predicted pIC50
Antidepressant	Escitalopram	CHEMBL1508	5.27
	Fluoxetine	CHEMBL41	5.04
	Paroxetine	CHEMBL490	5.11
Anticancer	Osimertinib	31042598 (**)	6.4
Antipsychotic	Asenapine	CHEMBL1201756	4.91
	Quetiapine	CHEMBL716	5.37
	Ziprasidone	CHEMBL708	5.72

**Osimertinib file was acquired from Chemspider.com database

The three antidepressants that we considered for repurposing, namely Escitalopram, Paroxetine, Fluoxetine, have predicted pIC50 values of 5.27, 5.11, 5.04. By comparing these values with the experimental pIC50 values of the compounds in training and testing sets, we identified that: (i) Escitalopram should have an Ebov inhibitory activity similar with the antidepressants Bifemelane (5.31) and Clomipramine (5.30); (ii) Paroxetine and Fluoxetine might inhibit Ebov entry into cells in a similar manner with the antipsychotic Piperacetazine (5.01), Paroxetine being slightly more active. The lowest predicted pIC50 value was calculated for the

antipshychotic Asenapine (4.91). This value is similar to the experimentally determined pIC 50 value for Imipramine (4.86)

Results presented above show that the compounds we considered for repurposing should exert a moderate effect of inhibiting Ebov VLP entry into host cells. The limitations of our study are those raised by the measurements performed by Kouznetsova et al, 2014.: experiments were performed using Ebov VLP, therefore their results should be further confirmed in Ebov infection assays and in animal models. Nevertheless, our study brings new information on the potential of antipsychotics and antidepressants to be repurposed even for fighting serious infections, such Ebov infections.

4. CONCLUSION

Here we investigated the possibility of repurposing three antidepressants (Escitalopram, Fluoxetine and Paroxetine), three antipsychotics (Asenapine, Quetiapine and Ziprasidone) and one anticancer compound (Osimertinib) as anti Ebov medication. Our idea is supported by the fact that in screening for anti-Ebov compounds, Kouznetsova et al. identified antipsychotic and antidepressant drugs that could inhibit Ebov entry into host cells. The effectiveness of our molecules of interest was predicted using QSAR. A reliable QSAR model (Q^2=0.73,R^2=0.90) was obtained by considering three non-overlapping and non-redundant molecular descriptors: ASA, LogP (including implicit hydrogen atoms) and SMR (including implicit hydrogen atoms). By applying the derived QSAR equation in the case of our target molecules, we identified that considered antipsychotic and antidepressant drugs should present a medium anti-Ebov effect, comparable with Thiothixene, Thioproperazine, Trifluoperazine, Bifemelane, Clomipramine, Piperacetazine or Imipramine. In the case of the anticancer compound considered here, we observed that it should have a higher anti-Ebov activity than the considered antipsychotic and antidepressant drugs, higher than that of the anticancer drug Toremifene and lower than that of Vincristine.

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Article

OIL WASTEWATERS COAGULATION USING AIDS

Smaranda Masu [1♣] ***Mariana Albulescu*** [2]***, Maria Popa*** [3]

[1]National R & D Institute for Industrial Ecology ECOIND,Branch of Timisoara, 300004, 1 Regina Maria Square, Timisoara, Romania

[2] West University of Timisoara, Advanced Environmental Research Laboratory, 4 Oituz Street, Timisoara, Romania

[3] "1 Decembrie 1918" University of Alba Iulia, Faculty of Sciences, Nicolae Iorga, 11-13, Alba Iulia, 510009, Romania

ABSTRACT

Oil is composed of organic compounds with varied molecular structure; in water suppress the respiration of plants and animals, and have toxicity effects on flora and fauna. An important pretreatment step for the water containing oil is coagulation with optimal dose of coagulant such as polyaluminum chloride (PAC). The addition of coagulating aids had reduced the PAC optimal dose for by 25-50%. The UV analysis of the treated/untreated water showed significant preferential adsorption for total petroleum hydrocarbons at 254nm wavelength (A254) and the correlation of this parameter with conventional control parameters - total organic carbon (TOC) and chemical oxygen demand (COD).

Keywords: Total petroleum hydrocarbons, wastewater, polyaluminum chloride, coagulant aids.

1. INTRODUCTION

Water polluted with petroleum hydrocarbons cannot be discharged into the environment, they must be treated by a pretreatment plant which generally provides a stage of decantation containing three successive settling basins where hydrocarbons are separated and recovered. Volatile petroleum hydrocarbons that reach the water evaporate quickly. Their toxicity cannot therefore manifest acutely, but these compounds present a particular disadvantage because they alter the taste, or, if in larger amounts, they pose the risk of explosion and fire. Heavy petroleum hydrocarbons are very slightly soluble, present as films and/or emulsions thus

♣ Correspondent author: tel.: +4035600822:, Fax:+40256220369, E-mails:smarandamasu@yahoo.com

having significant, dangerous toxicity. Their toxicity is manifested up to the living cell level. Internal rules on water protection impose a frequent control of the operation of clarifiers if having frequent rainfall. If the decantation step is not sufficient to remove total petroleum hydrocarbons (TPH) from the wastewater so that when released into the emissary be respected internal rules of NTPA.002, then, in the pretreatment process there must be introduced additional treatment steps such as coagulation. Protection of surface and ground water is severely regulated as the penetration in the layers of soil to groundwater causes changes in water taste. For the groundwater protection must be carried out works to optimize the working conditions regarding the security against loss of petroleum hydrocarbons in the environment [1-3]. Waters polluted with petroleum hydrocarbons contain organic/inorganic suspensions and stable droplets of oil-in-water emulsions. Suspension systems and emulsions can be destabilized by acidification or by adding aluminum or iron salts or suitable poly-electrolytes. There have been numerous experiments that have confirmed the effectiveness of aluminum and iron salts. The addition of Al^{3+} salts (pre-hydrolyzed, pre-polymerized) has major effects; they neutralize the negative charges of the colloids and destabilize droplets of non polar products. At the introduction of the coagulation agent based on Al in the aqueous phase, heavy, sedimentable flocks are formed and a whole variety of complex ions. A net result of this action causes destabilization of the emulsion, their coalescence and destabilization of colloids. In the case of a low pH, the precipitate of aluminum hydroxide in aqueous solution forms a complex that raises turbidity. At alkaline pH greater than 8, the species present at equilibrium is gibbsite which is an anionic monomer $Al(OH)^{4-}$. At pH<6, the dominant species at equilibrium with the gibbsite is the monomeric cation Al^{3+}. At pH<6.5 the solubility of the compounds of Al is less than 10^{-6} mol $\cdot L^{-1}$. If the pH were adjusted to 6.5 then the solubility of the aluminum hydroxide and turbidity would determine an optimum in the chemical pre-treatment [3-8]. The addition of natural materials with adsorbent characteristics as coagulation aids result in the increase of the removal efficiencies of petroleum compounds from wastewater [9-10]. Analytical control of influents and effluents plays an important role in the management and optimization of processes involved in treatment plants of industrial wastewater containing petroleum hydrocarbons. It is obvious that in the design of these technologies, the real possibilities for reducing pollutants in each stage have to be known. Control parameters are pH, TPH, organic load parameter expressed by chemical oxygen demand (COD), biochemical oxygen demand (BOD), suspensions and others [8, 11]. UV analytical results for petroleum hydrocarbons have led to the identification of a spectro-photometric parameter, A254, which can evaluate globally, qualitatively and quantitatively, the petroleum compounds. This parameter refers to the absorption of a large number of petroleum hydrocarbons at 254nm wavelength. A254 analyzes allowed the characterization of global pollution from wastewater, six times faster than COD analyzes. A254 values for influent and effluent water must be measured on unfiltered and filtered (filter 0.45) water. Correlations between A254 and turbidity showed an average growth of unfiltered A254 of 0.004 cm^{-1} per1 ˚NTU [12]. This study monitored the efficiencies of TPH removal from wastewater, by coagulation with:

1. The optimal dose (OD) of Al^{3+} using polyaluminum chloride (PAC),
2. Reduced doses of Al^{3+} using polyaluminum chloride (PAC), *vs.* OD and the addition of aids with adsorbent properties: indigenous volcanic tuff, Kieselgur charcoal, biological sludge,

3. The correlation of the conventional control parameters of treated / untreated waters with the unconventional spectrophotometers parameter - absorbance at 254 nm wavelength, A254.

2. METHODS

Materials:

1. Coagulation agents: prepolymerized aluminum salt *i.e.* polyaluminum chloride coagulant (PAC), molar ratio OH / Al = 2.4;

2. Coagulation aids: indigenous volcanic tuff from Marsid/Zalau quarry with size < 0.2 mm, Kieselgur (Dicalite 4200 Agromar 99 SRL, Focsani), charcoal (Letea Energo Prest SRL. Pitesti), biological sludge (Sewage sludge with 8.3 $mg \cdot L^{-1}$ DM from municipal wastewater treatment plant);

3. Waters A1- A3 wastewaters taken from decantation basin of a PETROM deposit.

Methods: Coagulation has been performed with a stirrer equipped with variable speeds (Phipps & Bird Company, USA). The optimal dose of coagulation agents, in the absence / presence of adjuvant for maximum pollutant removal, has been assessed by Jar Test method. The coagulation pH was the pH of the studied wastewater samples. (Coagulation required no corrections with acids or bases). The wastewater used for treatment was in volumes of 250 ml in coagulation vessel; time for rapid stirring = 3 min; slowly agitation= 15 minutes; gravitational sedimentation = 30 minutes. In the supernatant separated from the treated samples, conventional parameters were analyzed according to the standardized norms: pH determined by pH-meter model 290A ORION RESEARCH USA, turbidity with Micro 100 Laboratory Turbid meter, Scientific Inc. USA; COD by hot K dichromate oxidation in strongly acidic medium; TOC by TOC Analyzer with Multi N/C 2100 Analytic Jena, Germany. The absorbance – the unconventional parameter, at 254 nm wave length, A245, was analyzed by UV VIS spectrophotometer, Specord 205, Analytic Jena, Germany. Samples filtered through paper filter Sartorius filter papers FT 2-206. Total petroleum hydrocarbons (TPH) have been determined according to the Romanian standardized norms by extraction with tetrachlorethylene, Merck (SR 7877-2/95), *i.e.* TPH is extracted from a volume of wastewater corrected to pH<5 with hydrochloric acid, (1:3 vol:vol) (V), by mixing with solvent. Extracts number is four. Solvent extracts dried by passing through a silicagel filter the determination was performed at a wavelength of 2930 cm^{-1} with Infrared Spectrophotometer Model 500, Buch Science. The optimal dose was determined according to standard procedure, depending on the minimum turbidity obtained in the sample treated with coagulating agent. Studied waters must have the characteristics required by national norm HG 352/2005 - NTPA 002 to be discharged into the sewerage networks of localities and directly in wastewater treatment plants [13]. pH = 6.5-8.5, TPH $\leq$5 $mg \cdot L^{-1}$, C$\leq$ 500 $gO_2 \cdot L^{-1}$.

3. RESULTS AND DISCUSSIONS

3.1. Results

Table 1 shows the characteristics of the investigated A1, A2 and A3 wastewaters. Wastewater containing petroleum hydrocarbons, (TPH), are brownish-yellow apparent color and slight yellow real color. Waters have a strong smell of petroleum products. They present petroleum hydrocarbons spots and/or emulsified stable droplets. Waters had turbidity ranging from 27.2 to 47.5˚NTU, pH in the range of 7.15 -7.68 and TPH in the range of 6.03-8.33mg·L^{-1}. TPH exceed the permissible value of the current standards NTPA 002/2005 of 5 mg/l for discharging them in the local sewerage networks and wastewater treatment plants directly. The organic load expressed by COD parameter exceeds the values allowed by the norms for waters A2 and A3, with 18-26%.

Table 1. Characteristics of the investigated wastewaters

No.	**Parameters**	Oil wastewater		
		A1	**A2**	**A3**
1	Apparent color	Brownish-yellow	Yellow	Brownish
2	Real color	Slightly yellow	Slightly yellow	Slightly yellow
3	Smell	Petroleum products	Petroleum products	Petroleum products
4	Aspect	Film and emulsions	Film and emulsions	Film and emulsions
Wastewater characteristics after gravitational separation				
5	pH	7,68	7,15	7,56
6	Turbidity[^{o}NTU]	27,2	34,9	47,5
7	COD [$mgO_2 \cdot L^{-1}$]	221,5	676,4	608,8
8	TPH [$mg \cdot L^{-1}$]	6,03	8,33	7,42
9	TOC [$mgC \cdot L^{-1}$]	21,2	89,7	72,0
10	* Absorbance A254 [cm^{-1}]	0,80	1,65	2,59

* Samples filtered

The organic load was expressed by TOC parameter, ranging from 21.2 to 89.7 mg C/l. The spectra drawn in the UV-VIS were performed on filtered water. Analyzing the spectra, these waters show a range of absorption in the ultraviolet range at 240-260 nm and in the visible range at 430-450 nm. Absorbance at a wavelength of 254 nm, referred to as A254 has been selected for the study as a control parameter. This wavelength has been selected as a number of organic compounds with conjugated double bonds or aromatic nature absorb water in the bands B and K. The values of the parameter for the studied waters had A254 in the range of 0.80 to 2.78 A.U. (Absorbance units). A254 values were taken as such without correction due to a computational color because the signals in the visible domain 430-450, represent 2-5% of the 254 [12].

Table 2-4 show the characteristics of the water treated samples A1-A3 by:

1. The optimal dose (OD) of Al^{3+} using polyaluminum chloride (PAC),
2. Reduced doses of Al^{3+} using polyaluminum chloride (PAC) and coagulant aids with adsorbent properties: indigenous volcanic tuff, Kieselgur charcoal, biological sludge

Table 2. Treated samples A1 characteristics with the optimal dose and low dose of PAC+ aids

No.	Parameters	PAC Optimal dose OD=3,6 [mgAl ·L^{-1}]	Low dose of PAC + aids			
			0,7 OD PAC + volcanic tuf 0,5 [mg·L^{-1}]	0,75 OD PAC + kieselgur 0,5 [mg·L^{-1}]	0,7 OD PAC+ caorchal 0,4 [mg·L^{-1}]	0,7 OD PAC+ biological sludge 20 [ml·L^{-1}]
1	Turbidity [^{o}NTU]	9,5	10,5	9,5	6,5	9.5
2	COD [mgO_2·L^{-1}]	152	128,0	117,5	126,5	146
3	TPH [mg·L^{-1}]	3,97	4,19	4.07-	3.87	3.5
4	TOC [mgC·L^{-1}]	15,8	13,2	15,2	12,6	15.3

Table 3. Treated samples A2 characteristics with the optimal dose and low dose of PAC+ aids

No.	Parameters	PAC Optimal dose OD=16,4 [mg Al ·L^{-1}]	Low dose of PAC + aids			
			0,5 DO PAC+ Volcanic tuf 0,5 [mg·L^{-1}]	0,75 DO PAC+ kieselgur 0,5 [mg·L^{-1}]	0,5 DO PAC+ caorchal 0,4 [mg·L^{-1}]	0,5 DO PAC + biologic sludge ,20 [ml·L^{-1}]
1	Turbidity [^{o}NTU]	9,2	6,5	7.2	3.3	9,0
2	COD [mgO_2·L^{-1}]	465	426,5	425	312	386,2
3	TPH [mg·L^{-1}]	5,48	5,05	4.88	3.6	4,16
4	TOC [mgC·L^{-1}]	73,2	71,3	63.3	54.7	65,5

Table 4. Treated samples A3 characteristics with the optimal dose and low dose of PAC+ aids

No.	Parameters	PAC Optimal dose OD=8,4 [mg Al·L^{-1}]	Low dose of PAC + aids			
			0,5 DO PAC + Volcanic tuf 0,5 [mg·L^{-1}]	0,75 DO PAC + kieselgur 0,5 [mg·L^{-1}]	0,5 DO PAC+ caorchal 0,4 [mg·L^{-1}]	0,5 DO PAC + biologic sludge 20 [ml·L^{-1}]
1	Turbidity [^{o}NTU]	7,5	9,7	9,5	5,5	4,0
2	COD [mgO_2·L^{-1}]	436	426,5	478	316,5	416,2
3	TPH [mg·L^{-1}]	3,8	3,8	4,0	3,15	2,56
4	TOC [mgC·L^{-1}]	53,8	56,3	57,8	53,9	65,3

3.2. Discussion

Tables 2-4 show that the optimal dose of PAC was determined by the composition and concentration of pollutants in the investigated wastewater. Optimal doses of coagulant for the 3 waters were between 3.6-16.8 mg Al·L^{-1}. Tables 2-4 show that the reduction of the dose of PAC was determined by the nature of coagulant aids. For the

wastewater A1, PAC quantity reduction was of 25-30% compared to the optimal dose in the absence of the aids and for the wastewaters A2 and A3, PAC reduction was of 25-50% compared to the optimal dose. The residual values from samples treated with PAC in the presence/absence of the aids are similar and rank, generally, in the standardized norms.

The exception was water A3 when treating it with the optimal dose of PAC, the TPH supernatant containing an amount of TPH which exceeded by 8.75% the amount allowed by standards. The use of aids determined that substances by their adsorbing properties to reduce TPH from the investigated water under the limits imposed by standards. When using charcoal as aid, the supernatant of the treated samples contains suspended coal, for which a further filtering is required if this option is chosen.

From the aids used, the biologic sludge stands out, determining highest efficiencies of removal of TPH in the investigated waters. Figure 1a and 1b show the compared efficiencies to reduce TPH and COD load of water treated with CAP in the presence/ absence of aids, sewage sludge.

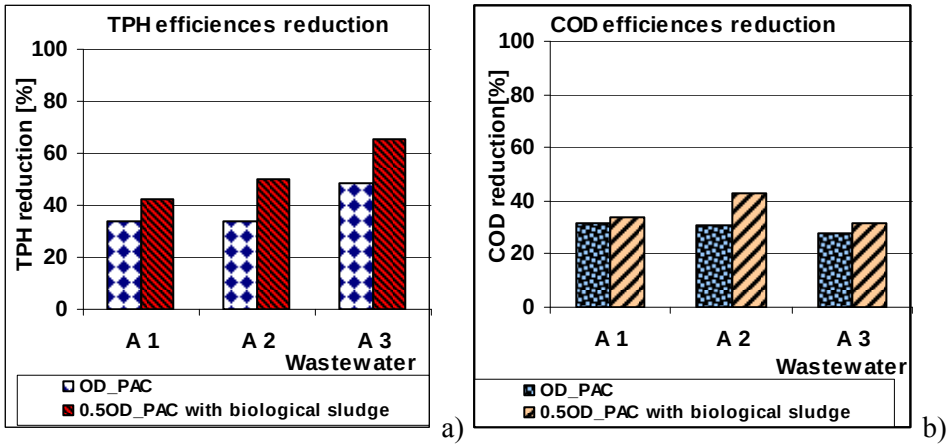
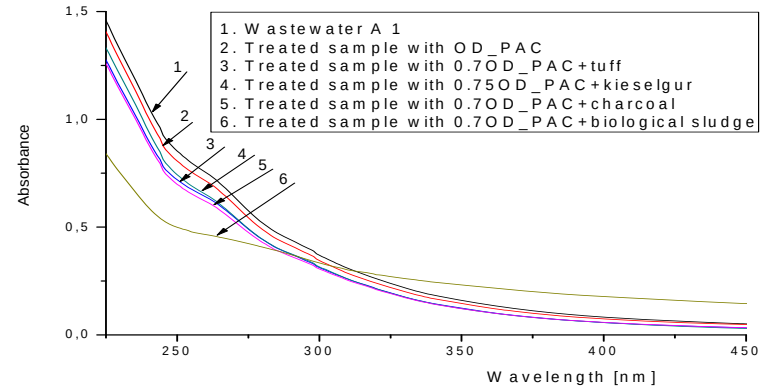
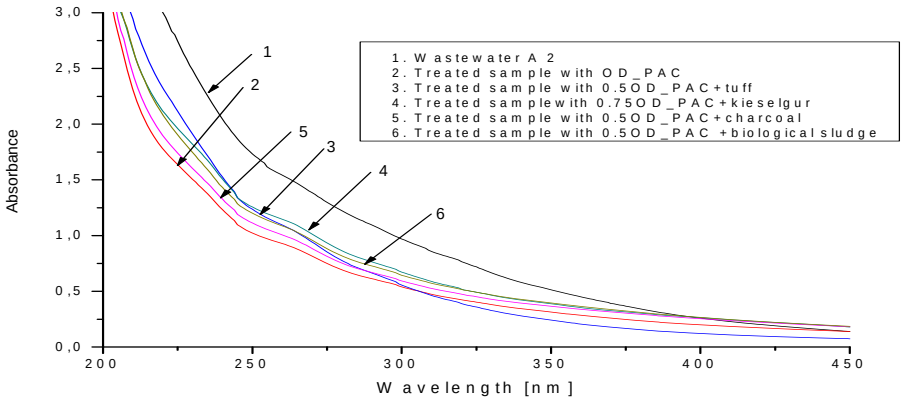


Figure 1. Reduction efficiency of the organic load of wastewater when using the optimal dose of PAC, of the reduced dose of PAC and of sewage sludge as aids
a) TPH, b) COD

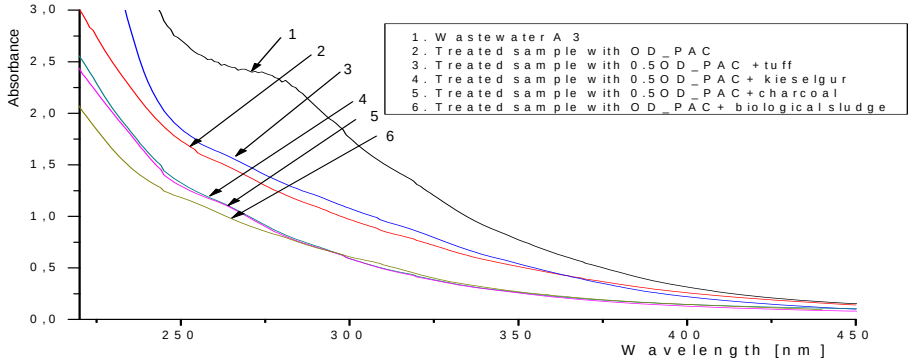
TPH reduction efficiencies in the treated samples were between 34-48.8% when using the optimal dose of PAC and 42.0-65.5% for low-dose of PAC and sewage sludge aid. COD decreased by 28.4-31.4% when using the optimal dose of CAP and between 31.5-43.0% when using the reduced dose of PAC and the sewage sludge as aids. Figure 2 shows the variation of absorbance determined for the untreated/treated samples. The use of coagulation agents caused the decrease in the absorbance of the analyzed UV domain. Studying these nonspecific global parameters one can assess how the coagulating agents interact with dissolved organic matter in wastewaters. The absorbance at a wavelength of 254 nm, as shown by the curves in Figure 2a, 2b and 2c, indicates the formation of a concentration plateau A 254 nm parameter is an indicator that can be used in monitoring the efficiency to remove TPH.



a)



b)



c)

Figure 2. Selective UV VIS spectrum untreated/treated waters with PAC in aids absence/presence: a) wastewater A 1, b) wastewater A 2, c) wastewater A3.

Studies have been conducted in this regard on wastewaters from a motorway service station in California. Wastewaters contain a mixture of petroleum hydrocarbons, natural organic material, soil, sand, road salt, rainfall water and microorganisms. In this context, the control parameter of the treated water, analyzed as an indicator of absorption of total petroleum hydrocarbons (TPH) was the absorbance in UV at a wavelength of 254 nm (A254) [12]. Figures 2a-2c show that from the aids used, the organic materials, i.e. sewage sludge and charcoal, led to better efficiencies to reduce the oil content in the wastewater. It demonstrates that these adsorbent materials exercise in the case of studied wastewaters their capabilities to adsorb TPH.

CONCLUSIONS

The use of a coagulation stage in the pre-treatment process of the studied wastewaters with variable content of oil, was accomplished with prepolymerized salts of Al, polyaluminum chloride with a ratio OH/Al = 2.4. The used coagulation agent, in optimal doses, resulted in a reduction of the organic load, expressed as chemical oxygen demand, total organic carbon and total petroleum hydrocarbons. Reduction efficiencies were between 34.0-48.8% for TPH, 28.4-31.4% for COD and 34.1-49.7. % for TOC. The residual TPH concentrations in the treated wastewaters have not been in all cases below the maximum allowed by current standards of 5 $mg \cdot L^{-1}$. The use of coagulation aids, with adsorbent properties of organic nature *i.e.* sewage sludge, has reduced the optimal dose of polyaluminum chloride by 30-50%. TPH reduction efficiencies were higher, between 42.0 and 65.5% and between 31.6 and 42.9 for COD. TPH and COD amounts in the treated waters were in all cases below the maximum allowed by current regulations. By the study of some global nonspecific parameters, such as UV absorbance at 254nm wavelength, A254, it has been possible to assess how the coagulation agents interact with the dissolved organic matter in wastewaters, and their reduction in the treated waters. Parameter A254 may be an indicator used more quickly and effectively in monitoring the efficiency to remove petroleum compounds.

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Article

STRUCTURE AND REACTIVITY OF SOME AROMATIC COMPOUNDS WITH ACIDIC AND BASIC CHARACTER

Daniela Dascălu [(1,2)♣], ***Simona G. Muntean*** [3], ***Laura Pitulice*** [1,2], ***Delia Isac*** [1]
[1]*Department of Biology-Chemistry, West University of Timisoara, 16 Pestalozzi, 300115 Timisoara, ROMANIA*
[2]*Advanced Environmental Research Laboratories, West University of Timisoara, 4 Oituz, 300086, Timisoara, ROMANIA*
[3]*Institute of Chemistry Timisoara of Romanian Academy, 24 Mihai Viteazul, 300223, Timisoara, ROMANIA*

ABSTRACT

Energetic and structural indices established with quantum-mechanics calculations by Hückel molecular orbital method (HMO) have been used to correlate the structure and reactivity of some diazotable and coupling compounds. The investigated molecules are used to obtain azo dyes.

Keywords: Amines, Dyes, Quantum chemical calculations, Structure, Reactivity of Aromatic Compounds

1. INTRODUCTION

The present investigation resides in our interests for aromatic compounds involved in substitution reactions [1]. The molecules selected for the study illustrate their role in diazotation and coupling processes as substrates and electrophilic reactants, respectively. Therefore, the aromaticity of some amines and their derivatives, also hydroxy arenes, can be correlated with both energetic and π electron density repartition indices, calculated by HMO method [2]. Some of the presented systems fit the general Ar-Y model (Y: ^{-}OH, $^{-}NH_2$, $^{-}NH_3^{+}$, ^{-}NHR, $^{-}NR_2$), highly correlated with the electronic effects (σ and π) and substituent constants, too [3].

♣ Correspondent author: Tel: +40256592625, Fax:+40256592620, E-mail: daniela.dascalu@e-uvt.ro

In order to contribute to the knowledge of the structure-reactivity relationship in diazotization reaction, electronic spectra (200-800 nm) were recorded for sulfanilic acid, sulfanilamide and the corresponding hydrochlorides, for diazonium salts and hydrolysis products. Molecular orbital calculations using the HMO method were performed for the above mentioned molecules [4].

The diazonium salts were obtained through diazotization in acidic solution with sodium nitrite, the nitrous acid excess was destroyed with ammonium sulfamate. The concentrations and the preparation technique were those described in the specific literature [5].

Solid sulfanilic acid can also be treated with gaseous NOCl in order to obtain solid diazonium chloride with a 100% yield. The water produced within the reaction is removed *in situ* with the NOCl excess [6]. Diazonium salts as diazotization products, on the one hand, and electrophilic reactants in azo coupling processes, on the other hand, are also involved in thermal decomposition reactions [7]. The kinetics of thermal decompositions of diazonium salts of sulfanilic acid and sulfanilamide were monitored in solution by spectrophotometry. The following activation energies (E) and pre exponent (A) values were obtained for thermal decompositions of benzene diazonium salts: E = 127.19 kJ mol^{-1}, $A = 6.0 \cdot 10^{15}$ s^{-1} for the sulfanilic acid derivative, and E = 124.20 kJ mol^{-1}, $A = 4.0 \cdot 10^{15}$ s^{-1} for the sulfanilamide derivative [8]. For these quick reactions in which C-N bonds break, a value of $A > 10^{14}$ s^{-1} indicates that, within the activated complex, the interaction of π electrons between the nitrogen atoms and atoms of the molecule residue is weaken or even cancelled [9].

The correlation diagram of molecular orbitals and the correlation diagram of electronic states for the decomposition reaction of the benzene diazonium cation were established using Hückel molecular orbital calculations [10]. The activation energy of the thermal decomposition must be of purely thermodynamic nature, without a certain barrier, as the diazonium cation is more stable than the decomposition products [7]. The following activation enthalpies ($\Delta H^{\neq}$ / kJ mol^{-1}) and activation entropies ($\Delta S^{\neq}$ / J K^{-1} mol^{-1}) were obtained for thermal decompositions of benzenediazonium salts: for the sulfanilic acid derivative $\Delta H^{\neq}$ = 124.683, $\Delta S^{\neq}$ = 48.116 for the sulfanilamide derivative $\Delta H^{\neq}$ = 121.336, $\Delta S^{\neq}$ = 44.350 [8]. The activation enthalpies were quite high instead the corresponding activation entropies have high positive values.

The experimental data illustrate that stability of substituted diazonium ions, precipitated as diazonium fluoroborates, can be increased by complexation with crown ethers [11]. Diazonium cations are not strong electrophilic reactants due to delocalization of their positive change. Instead, a higher reactivity is encountered at those diazonium cations possessing electron attractive substituents in para positions [12]. The azo coupling process of these compounds may occur only with strong nucleophiles such as: phenolates, naphtholates, free aromatic amines, in which case it has an elevated selectivity [13]. Therefore, the azo coupling reactions occur almost unitary at positions with the highest electron density (ρ_q) of the coupling compounds [14]. The reactivity of coupling compounds, variously substituted at the same position, falls within the series $-O^- > -NH_2 > -OH$, and the substitution orientation at multifunctional components is governed by pH through protolithic equilibriums in which hydroxyl and amino groups are engaged [15].

2. RESULTS AND DISCUSSIONS

Over time, performed calculations have shown that the HMO method correctly illustrates the variation of electronic density repartition and energy levels within a series of molecules, not too different from each other [2]. The method success is related to the use of parameters based on experimental results, fact that conducts to a significant compensation of errors arisen from applied simplifications [14]. Selecting the Streitwieser parameters (Table 1), the energetic and structural indices of conjugated systems possessing a certain number of electrons (n_π) have been calculated using computational techniques.

Table 1: The HMO parameters used in calculations (after Streitwieser) [16]

Group	h_x	*Bond*	$K_{Y\text{-}X}$
$-NH_2$	1.50	Ar-NH_2	0.80
$-\overset{+}{N}\equiv, \equiv N$	1.25	Ar-N=	1.00
-OH	2.00	$-\overset{+}{N}\equiv N$	1.00
$-N(CH_3)_2$	1.10	Ar-OH	0.80
$-NH_3^+$	0.50	Ar-COOH	0.90
=O	1.00	$-\underset{\vert}{C}=O$	1.00

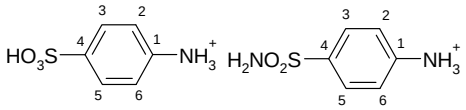
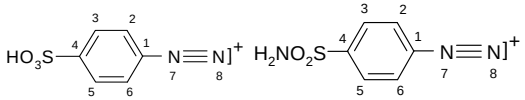
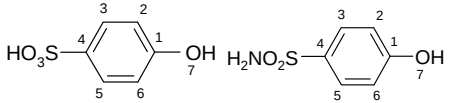
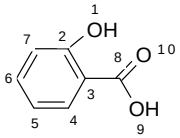
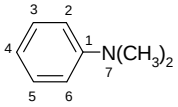
The presence of solubilizing ($-SO_3H$) and ($-SO_2NH_2$) groups does not interfere with the electronic state of the carbon atom to which they link. Moreover, these groups do not participate in conjugation with the aromatic ring [17,18].

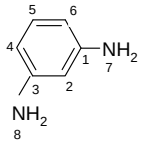
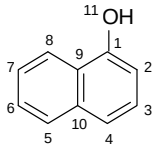
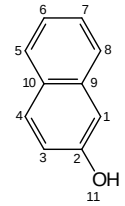
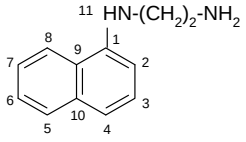
For a certain compound, the values of delocalization energy (DE), the highest occupied molecular orbital energy (ε_{HOMO}), the lowest unoccupied molecular orbital energy (ε_{LUMO}) facilitate the estimation of compound stability, and its reducing or oxidizing character. With regard to the absorption bands appearing in UV-VIS domain, the first band (λ_{max}) corresponds either to transition between ε_{LUMO} and ε_{HOMO} of the highest conjugated system or to transition from a non-participating atomic orbital to ε_{LUMO}. The $n\text{-}\pi^*$ transition needs a lower energy than the $\pi\text{-}\pi^*$ transition and thus it occurs at higher wavelengths [14,19].

Although, the HMO method does not adequately consider the electron repulsion, which opposes their agglomeration at a given atom, there is a certain consistency between parameters characterizing electron distribution and chemical reactivity of the corresponding atoms. Thus, the electronic densities (ρ_q) are correlated with atoms basicity, with their ability to participate in the electrophilic substitutions [2,14].

The values obtained by HMO method sustain the chemical reactivity interpretation given for the 12 investigated organic molecules (Table 2).

Table 2 Energetic indices calculated by HMO for the organic compounds used in diazotization and coupling reactions

Compound	n_π	*DE(β)*	ε_{LUMO}	ε_{HOMO}	λ *(nm)*
 (I) (II)	6	2.0490	α–0.8409·β	α+β	282
 (III) (IV)	8	2.6073	α+0.1786·β	α + β	633
 (V) (VI)	8	4.1968	α-0.999·β	α+0.8274·β	284
 (VII)	12	5.1408	α-0.5351·β	α+0.8386·β	378
 (VIII)	8	3.7320	α - β	α+0.7437·β	298

 (IX)	10	3.4650	α-1.0404·β	α+0.6824·β	302
 (X)	12	5.8872	α-0.6606·β	α+0.5382·β	434
 (XI)	12	5.8812	α-0.6332·β	α+0.5807·β	428
 (XII)	12	5.4264	α-0.6698·β	α+0.5022·β	444

Tabel 3. Electronic densities (ρ_q) calculated by HMO for the atoms of some coupling components of sulfanilic acid and sulfanilamide diazonium salts

Compound	ρ_1	ρ_2	ρ_3	ρ_4	ρ_5	ρ_6	ρ_7	ρ_8	ρ_9	ρ_{10}	ρ_{11}
V, VI	0.9547	1.0400	0.9980	**1.0292**	0.9980	1.0400	1.9400	-	-	-	-
VII	1.9306	0.9035	1.0572	0.9523	**1.0288**	0.9574	1.0417	0.6394	1.8953	1.5939	-
VIII	0.9538	1.0484	0.9977	**1.0367**	0.9977	1.0484	1.9172	-	-	-	-
IX	0.9521	1.0989	0.9521	**1.0835**	0.9953	**1.0835**	1.9173	1.9173	-	-	-
X	0.9442	1.0561	0.9958	**1.0407**	1.0071	0.9983	1.0095	0.9934	1.0210	0.9993	1.9346
XI	**1.0566**	0.9530	1.0259	0.9957	0.9983	1.0097	1.0000	1.0096	0.9984	1.0138	1.9399
XII	0.9418	1.0685	0.9952	**1.0514**	1.0092	0.9980	1.0127	0.9923	1.0248	0.9993	1.9076

• The hydrochlorides of sulfanilic acid and sulfanilamide (I), (II), and also their diazonium salts (III), (IV) present low values of delocalization energy (DE), as these compounds are double substituted in para positions with electron attractive groups. The hydrolysis products of sulfanilic acid and sulfanilamide diazonium salts (V), (VI), and also the salicylic acid (VII) are more aromatic than N,N-dimethyl aniline (VIII) and m-phenylene diamine (IX), in accordance with TOPAZ aromaticity [20]. Moreover, using the Hückel theory (HT) it can be seen that 1-naphthol (X) and 2-naphthol (XI) are more stable than 1-naphthyl ethylenediamine (XII), and the (V), (VI) compounds are more aromatic than (VIII) molecule.

• The ε_{LUMO} and ε_{HOMO} values indicate that (I), (II), (III), (IV) compounds are the most resistant to oxidation, and the (VIII), (IX) molecules are the most resistant to reduction. The (III), (IV) compounds should be stronger oxidizing agents, and the (XII) polynuclear amine should be stronger reducing agent.

• For the (I), (III), (V) compounds that are present in the acidic solution when sulfanilic acid diazotization occurs, and also the (II), (IV), (VI) compounds present in the medium when sulfanilamide is diazotized, the correlations between $\lambda_{calculated}$ and spectral measurements ($\lambda_{experimental}$) are satisfactory [4].

• Regarding the electronic densities calculated with HMO method for the atoms of (V) to (XII) molecules (Table 3), it should be mentioned that their relative values are more important than their absolute ones within this series of similar compounds, or for different atoms of the same substance.

• The atoms electronic densities follow the $\rho_O > \rho_N > \rho_C$ relationship being in good agreement with the known electronegativity tendency [21,22]. The usage of phenolates and naphtolates, strong nucleophiles, in azo coupling reactions is based on the fact that the oxygen atom in the $-O^-$ anion is less electronegative than in the -OH group due to its negative charge [14].

• The calculated values in Table 3 place the coupling components upon reactivity as follow:

(IX) > (VIII) > (V), (VI) > (VII); (XII) > (X); (XI) > (X), according to their experimental behavior.

• The diazonium cations (III), (IV) perform electrophilic attacks to the following atoms: C(4) of (V), (VI), (VIII), (IX), (X), (XII), C(5) of (VII) and C(1) of (XI) coupling components. It should be mentioned that the ($-SO_3H$) group of (V) molecule and the ($-SO_2NH_2$) group of (VI) molecule are removed through these reactions [12].

• Even though the C(2) atoms of (V), (VI), (VIII), (IX), (X), (XII) compounds and C(7) atom of (VII) molecule are positions with the highest electronic density, the electrophilic attacks do not occur at these atoms due to steric impediments.

The motivation of choosing these molecules is mainly linked to the azo dyes that can be synthesized from these (Table 4).

Table 4: Aromatic amines, hydroxy arenes and corresponding azo dyes [13]

Diazotable compounds	*Coupling compounds*	*Azoic dyes*
I	VIII	Methyl orange
	XI	β-naphthol orange
	X	α- naphthol orange
	VII	Chromatable acid yellow
II	IX	Red prontosil

4. CONCLUSIONS

The calculation of energetic indices by HMO method for the investigated conjugated systems enables their comparison regarding aromaticity and oxidizing/reducing character. The performed analysis underlines that as the bonds conjugated system extends the maximum absorption band moves towards visible domain. In this context, naphtolates are preferred as coupling components. We found a good agreement between structural indices and also between them and reactivity of atoms of series of similar compounds.

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Short Communication

STUDY OF THE TOPOLOGICAL INDICES OF THE LINE GRAPHS OF H-PANTACENIC NANOTUBES

***Mohammad Reza Farahani*(♣)[1], *Muhammad Faisal Nadeem*[2], *Sohail Zafar*[3], *Zohaib Zahid*[3], *Mohamad Nazri Husin*[4],**
[1]Department of Applied Mathematics of Iran University of Science and Technology (IUST), Narmak, Tehran 16844, Iran.
[2]Department of Mathematics, Comsat Institute of Information Technology, Lahore, Pakistan.
[3]School of Sciences, University of Management and Technology (UMT), Lahore Pakistan.
[4]School of Informatics and Applied Mathematics of University Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia.

ABSTRACT

In this paper, we computed the edge version of atom-bond connectivity and geometric arithmetic indices of H-Pantacenic nanotube. We also computed ABC4 and GA5 indices of the line graph of H-Pantacenic nanotube.

Keywords: Atom-bond connectivity index, geometric-arithmetic index, line graph, phenylenes, H-Pantacenic nanotube.

1. INTRODUCTION

Let *G* be a simple graph, with vertex set V(G) and edge set E(G). The line graph L(G) of graph *G* is the graph whose vertices are the edges of *G* and two vertices e and f are incident if and only if they have a common end vertex in G. The degree d_u of a vertex u is the number of vertices adjacent to u. Also, the degree d_e of an edge e of E(G) is the number of its joining vertices in V(L(G)). For a natural number l, we define $V_l(G)=\{u\in V(G)|d_u=l\}$.

♣ Correspondent author: Tel: +98-919-247-8265, E-mails: Mr_Farahani@Mathdep.iust.ac.ir & MrFarahani88@gmail.com faisal.nadeem@ciitlahore.edu.pk, sohailahmad04@gmail.com, zohaib_zahid@hotmail.com,naz_reyhusin@yahoo.com

A molecular graph is a collection of points symbolize the atoms in the molecule and set of lines denotes the covalent bonds. These points and lines are known as vertices and edges in graph theory. A topological index is a map from the collection of chemical compounds described by molecular graphs to the set of real numbers. The clear contenders for topological indices are number of vertices and the number of edges. Topological indices are invariant under the graph isomorphisms. The benefit of topological indices are that they may be used directly as a straightforward arithmetical descriptors in a comparison with physical, chemical or biological parameters of molecules in Quantitative Structure Property Relationships (QSPR) and in Quantitative Structure Activity Relationships (QSAR).

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The edge adjacency matrix of a molecular graph is identical as vertex adjacency matrix of the line graph of this molecular graph. That's why the edge connectivity index of molecular graph is identical as Randić vertex connectivity index of the corresponding line graph**.** This remark opens new trends in the subject of topological indices: (i) an approach based on creating topological indices depends on the line graph of molecular graph; (ii) computation of the topological indices of the line graph of molecular graph.

The use of the line graphs in chemistry was initiated from the very beginning of structural chemistry. The first topological index based on line graph is introduced by Bertz in 1981, [1] in the advancement of the theory of molecular branching and complexity. There are various topological indices based on the line graph of molecular graph (see [2-4]). For more details about the applications of line graphs in chemistry, we refer the articles (see [5-7]).

Estrada et al. proposed a topological index based on the degrees of vertices of graphs in [8], which is named as the atom-bond connectivity index (*ABC*). The *ABC* index of a graph *G* is defined as

$$ABC(G)=\sum_{uv\in E(G)}\sqrt{\frac{d_u+d_v-2}{d_u\times d_v}}$$

In beginning, the close connection among the heats of formation of alkanes and the *ABC* index is experienced. After that the *ABC* index turned out to be a resourceful tool to model the thermodynamic properties of organic chemical compounds (see [9]).

The fourth member of the class of *ABC* index was launched by M. Ghorbani et al. in [10]:

$$ABC_4(G)=\sum_{uv\in E(G)}\sqrt{\frac{S_u+S_v-2}{S_u\times S_v}}$$

where $S_u=\sum_{v\in N_u}d_v$ and $N_u=\{v\in V(G)|uv\in E(G)\}$.

In [11], the edge version of *ABC* index was introduced:

$$ABC_e(G)=\sum_{ef\in E(L(G))}\sqrt{\frac{d_e+d_f-2}{d_e\times d_f}}.$$

Note that $ABC_e(G)=ABC(L(G))$.

In [12], D. Vukicevic and B. Furtula introduced the geometric arithmetic (GA) index. The *GA* index for graph *G* is defined by

$$GA(G)=\sum_{uv\in E(G)}\frac{2\sqrt{d_u\times d_v}}{d_u+d_v}$$

The 5th *GA* index was introduced by Graovac et al. in [13]:

$$GA_5(G)=\sum_{uv\in E(G)}\frac{2\sqrt{S_u\times S_v}}{S_u+S_v}.$$

The edge version of *GA* index was introduced in [14]:

$$GA_e(G)=\sum_{ef\in E(L(G))}\frac{2\sqrt{d_e\times d_f}}{d_e+d_f}.$$

It is easily seen that $GAe(G)=GA(L(G))$. For more information on degree-based topological indices, we pass on to the articles [15-45].

The following lemma is helpful for computing the degree of a vertex of line graph.

Lemma 1. Let *G* be a graph with $u,v\in V(G)$ and $e=uv\in E(G)$. Then:

$$d_e=d_u+d_v-2.$$

In order to calculate the number of edges of an arbitrary graph, the following lemma is significant for us.

Lemma 2. Let *G* be a graph. Then

$$\sum_{u\in V(G)}d_u=2|E(G)|.$$

This is also known as handshaking Lemma.

In this paper, we computed two versions of degree-based indices *ABC* and *GA*, for the case of line graph of H-Pantacenic nanotube.

2. RESULTS AND DISCUSSION

The H-Pantacenic nanotube *K[p,q]* and its line graph are shown in Figures 1 and 2, respectively.

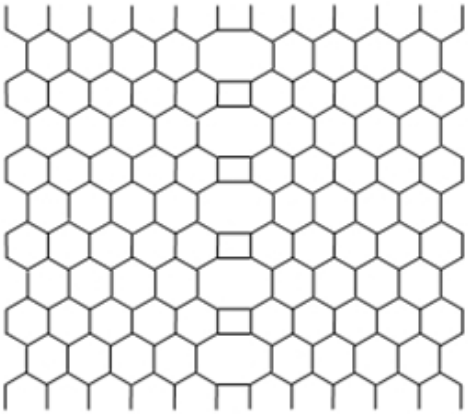
Theorem 1. Let *G=K[p,q]* be a graph of H-Pantacenic nanotube with 22pq vertices and *33pq-2q* edges. Then

$$(1)\, ABC_e(G) = \frac{33}{2}\sqrt{6}pq + q\left(2\sqrt{2} + \frac{4}{3}\sqrt{15} - 5\sqrt{6}\right).$$

$$(2)\, GA_e(G) = 66pq + q\left(\frac{8}{5}\sqrt{6} + \frac{32}{7}\sqrt{3} - 20\right).$$

Proof. In L(G),there are 33pq-2qvertices. It is easily seen from Fig. 2 and Lemma 1 that $|V_2(L(G))| = 2q$, $|V_3(L(G))| = 4q$ and $|V_4(L(G))| = 33pq-8q$. By using Lemma 2, we get $|E(L(G))| = 66pq-8q$.

Fig. 1. The H-Pantacenic nanotube *K[p,q]*.



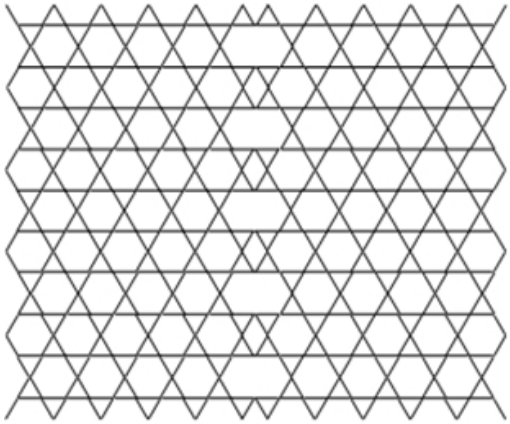
The edge set E(L(G)) divides into three edge partitions based on degrees of the end vertices, i.e. $E(L(G))=E1\,(L(G)) \cup E2\,(L(G)) \cup E3\,(L(G))$. The edge partition E1(L(G)) contains 4q edges uv, where du=2 and dv=3, the edge partition E2 (L(G)) contains 8q edges uv, where d_u=3 and d_v=4 and the edge partition E_3 (L(G)) contains 66pq-20q edges uv, where $d_u=d_v=4$.

$$\mathrm{ABC_e(G)} = \sum_{ef \in E(L(G))} \sqrt{\frac{d_e + d_f - 2}{d_e \times d_f}},$$

then

$$\mathrm{ABC_e(G)} = 4q\sqrt{\frac{2+3-2}{2\times 3}} + 8q\sqrt{\frac{3+4-2}{3\times 4}} + (66pq-20q)\sqrt{\frac{4+4-2}{4\times 4}}.$$

Fig. 2. The line graph of H-Pantacenic nanotube *K[p,q]*.



After simplification we get,

$$\mathrm{ABC_e(G)} = \frac{33}{2}\sqrt{6}pq + q\left(2\sqrt{2} + \frac{4}{3}\sqrt{15} - 5\sqrt{6}\right).$$

Similarly one can find the expression of $GA_e(G)$.

$$GA_e(G) = 66pq + q\left(\frac{8}{5}\sqrt{6} + \frac{32}{7}\sqrt{3} - 20\right). \blacksquare$$

Theorem 2. Let G_L=*L(K[p,q])* be a line graph of H-Pantacenic nanotube. Then:

$$(1)\ \mathrm{ABC_4}(G_L) = \frac{33}{8}\sqrt{30}pq + q\left(\frac{6}{35}\sqrt{70} + \frac{2}{15}\sqrt{210} + \frac{2}{35}\sqrt{770} + \frac{2}{15}\sqrt{138} + \frac{2}{15}\sqrt{435} - 2\sqrt{30}\right)$$

$$(2)\ \mathrm{GA_5}(G_L) = 66pq + q\left(\frac{95}{31}\sqrt{15} + \frac{2}{3}\sqrt{35} + \frac{8}{5}\sqrt{6} + \frac{8}{29}\sqrt{210} - 32\right).$$

Proof. The edge partition of G_L based on the degree sum of vertices lying at the unit distance from end vertices of each edge is given in Table 1.

Since

$$ABC_4(G_L) = \sum_{uv\in E(G_L)} \sqrt{\frac{S_u + S_v - 2}{S_u \times S_v}},$$

then after doing some calculations by using Table1, we get

$$ABC_4(G_L)=\frac{33}{8}\sqrt{30}pq+q\left(\frac{6}{35}\sqrt{70}+\frac{2}{15}\sqrt{210}+\frac{2}{35}\sqrt{770}+\frac{2}{15}\sqrt{138}+\frac{2}{15}\sqrt{435}-2\sqrt{30}\right)$$

Table 1.

$(S_u,S_v);uv\in E(G_L)$	*Number of Edges*
(6,10)	*4q*
(10,14)	*4q*
(10,15)	*4q*
(14,15)	*4q*
(15,16)	*8q*
(16,16)	*66pq-32q*

Similarly we can find the expression of $GA_5(G_L)$ by using Table 1.

$$GA_5(G_L)=66pq+q\left(\frac{95}{31}\sqrt{15}+\frac{2}{3}\sqrt{35}+\frac{8}{5}\sqrt{6}+\frac{8}{29}\sqrt{210}-32\right).\blacksquare$$

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Review

ANTIBACTERIAL PROPERTIES OF CHITIN AND CHITOSANS

Adina MATICA, Gheorghița MENGHIU, Vasile OSTAFE ♣
West University of Timisoara, Department of Biology – Chemistry (Pestalozzi 16, Timisoara, 300315) & Advanced Environmental Research Laboratories (Oituz 4, Timisoara 300086, Romania)

ABSTRACT

Chitosan and chitosan based-materials are products with proved antimicrobial activity that have found many applications in medicine, pharmacy, food and textile industries. The antibacterial activity of chitosan can be explained by (i) ionic interaction of positive charges of the chitosan based-materials with negative molecules located on the surface of bacterial cells; (ii) penetration of chitosan chains into the cells and interaction with negatively charged molecules like mRNA, inhibiting protein synthesis; and (iii) realization of an external coating that chelate essential metals involved in microbial growth. Depending on the bacterial strain, all these events can take places, but with different strengths. Although there are high differences between the chemical structure of surfaces of gram-positive and gram-negative bacteria, the effectiveness of chitosans in reducing microorganism growth and multiplication seems to be similar. The antibacterial propertied of chitosan based-materials depend on molecular weight and degree of acetylation (abundance of positive charges). In general, at lower molecular weight and lower degree of acetylation the chitosans present a higher antibacterial activity. Derivatization of amino and hydroxyl groups of chitosan chains, usually provide a higher efficiency against all types of bacteria.

Keywords: chitin, chitosan, chitosan derivatives, antibacterial activity

♣ Correspondent author: +40-755240153, vasile.ostafe@e-uvt.ro

INTRODUCTION

Chitin, a biopolymer synthetized by crustacean [1,2], fungi [3,4], mushrooms [5,6] and insects [7], is, after cellulose, the second most abundant substance on biosphere[8]. Although chitin is synthetized by many categories of organisms, the main sources for chitin extraction are crab and shrimp shells. The difference between cellulose, that is a biopolymer made from D-glucose units linked via β (1→4) bonds, and chitin is that the units in chitin is N-acetyl-glucosamine (more exactly 2 – acetamide – 2 deoxy – D – glucopyronose) linked by β (1→4) bonds [9] (Figure 1). In fact, not all the units in natural chitins are N-acetyl-glucosamine, some of these units are deacetylated. Chitosan is obtained by deacetylation of chitin, either in alkaline conditions (chemical deacetylation in concentrated NaOH) using chitin deacetylase (enzymatic deacetylation) [10]. When the ratio of acetylated units ((1 → 4)-2-acetamide-2-deoxy-β-D-glucan versus (1 → 4)-2-amine-2-deoxy-β-D-glucan)) is higher than 40% the product is considered to be chitin, but when the acetylated units decrease under 40%, the polymer is named chitosan [11]. Chitin can be extracted from producing organisms by chemical methods (demineralization with strong acids and deproteinization with strong bases)[12] and biological (enzymatic) methods [10].

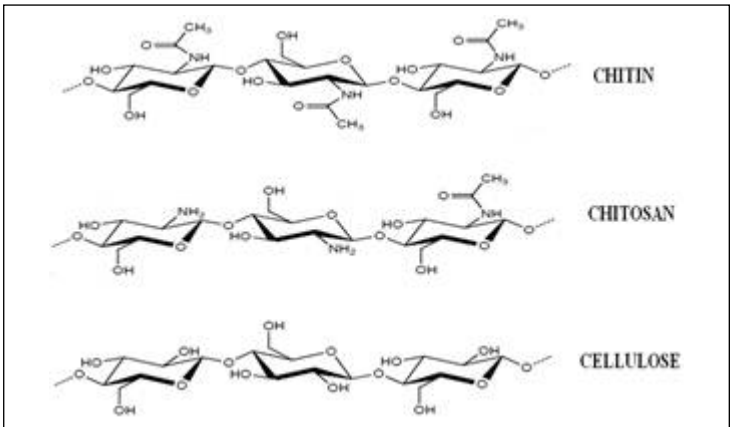

Figure 1. Chemical structures of chitin, chitosan and cellulose

Extracted chitin has a highly ordered crystalline structure, poor solubility and a relatively low reactivity. Chitosan, at least in acidic media (solution below its pKa ≈ 6.3), is more soluble than chitin and, for this reason, preferred for use as starting material in various types of applications. The solubility of chitosan varies on biological origin, molecular weight and degree of acetylation [13]. As in other cases of natural biopolymers, the characterization of chitin and chitosan is not standardized. In majority of the cases when chitosan based materials are used, the product is characterized by its molecular weight (MS)

and degree of acetylation (DA). Although there are not standards to categorize the chitosan based materials, it is accepted to be considered as low molecular weight (LMW) when the polymer has a molecular weight smaller than 50 kDa, medium (MMW), between 50 and 150 kDa and high molecular weight (HMW) when the molecular weight is higher than 150 kDa [11].

It is thought that microbes, like bacteria, fungi and parasites, being the major cause of infectious diseases, kill more peoples than other malady [14]. The compounds that kill or inhibit the growth of microbes are called antimicrobial agents. Among them, the study of antimicrobial polymers have conduct to many industrial applications, like stimuli-responsive polymeric materials for human health applications [15], antimicrobial polymers for anti-biofilm medical devices [16], antimicrobial peptides and enzymes [17,18], antimicrobial polymers with metal nanoparticles [19]. Compared with antimicrobial agents that are small molecules, the antimicrobial polymers seems to have superior efficacy, reduced toxicity, lower impact on environment and are less prone to decrease their efficacy due to development of microbial resistance [20].

In order to adhere to a substrate, the microbes excrete extracellular polymeric substances that form a matrix where the cells can better develop themselves. This matrix, also called biofilm is a polymeric conglomeration composed of polysaccharides, proteins and nucleic acids [21]. Destruction or malfunction of these biofilms is one of the major target of antibacterial strategies [16]. For instance, antimicrobial peptides can disrupt the bacterial cell membrane [18]. Inhibition of biofilm formation is a hopeful strategic alternative to killing microbes, as inside the matrix, bacteria are better protected than in solution free state [22].

Chitosans are largely used as antibacterial agents [23]. The degree of acetylation and the molecular weight have a major role in the antibacterial activity of chitosan based products. In order to increase its low solubility the raw chitosan is chemically modified either at its primary amino or at the primary alcohol groups [24]. Due to incomplete characterization of chitosan-based materials, it is rather complicate to compare them and to control the influence of various types of factors that affect the antibacterial activity and mode of action of chitosans. Even the activity of chitosan was investigated as antimicrobial agent against a large range of organisms, like bacteria, yeasts, fungi or algae, in experiments involving in vitro or in vivo interactions, it is not yet clear if chitosan has a bacteriocidal (kills the live bacteria) or bacteriostatic (obstructs the growth of bacteria) activity [11]. Only recently there were made some attempts to introduce some rules and limits in classification of chitosan samples according to the molecular weight and the degree of deacetylation [23].

Apart the influence of MW and DA of chitosan samples, which has to be characterized with precision in order to can compare the antibacterial effect (like minimal inhibitory concentration MIC), other factors like pH, temperature, salinity can play a significant role in the antibacterial activity. Due to chitosan solubility at lower pH values, chitosan based products have a higher antibacterial activity in acidic environment [25]. The experiments have proved that, in most of the cases, the antibacterial activity of chitosans is increased at higher temperature values (until 40°C) and lower pH values (between 4 and 6) [23,26].

Probably due to solubility issues, it seems that the molecular weight (MW) of chitosans has a greater influence on antibacterial activity than the degree of acetylation (DA). Studies on *Bacillus cereus, E. coli, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella*

enterica, B. subtilis, Listeria monocytogenes and *Klebsiella pneumoniae* have shown that the chitosans with smaller MW have a higher antibacterial activity, as small polymers have higher mobility and stronger interactions with the bacterial walls, than the chitosans with high molecular weights [11]. Studies on some Gram-positive and Gram-negative bacteria have revealed that antibacterial activity is higher at lower DA [10].

PASSIVE OR ACTIVE ACTION

Passive Action

Antibacterial polymers, like chitosan, can act as antibacterial agent passively, i.e. can reduce protein adsorption on its surface that conducts to impairing the adhesion of bacteria. That means that these polymers do not kill bacteria but repel them. Repelling process can be realized by (1) hydrophilic / hydrophobic repulsion; (2) electrostatic repulsion, or (3) to have a low surface free energy [27]. For example, poly(ethylene glycol) was used as neutral polymer brush system to prevent protein and cell adhesion against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* [28] and charged polyampholytes, like phosphobetaine and phospholipid polymers were used against *Staphylococcus aureus*[29]. Use of albumin-glycerol and whey-glycerol no cell growth was observed in the case of *Bacillus subtilis* and *Escherichia coli* [30]. Polyphenols were effective against periodontal bacteria (*Streptococcus mitis, Fusobacterium nucleatum, Porphyromonas gingivalis*) reducing the film formation [31].

Active Action

In the category of active polymers are the compounds that actively kill bacteria that stick to the polymer surface, as in the case of cationic biocides, antimicrobial peptides, or antibiotics. In this case, between bacteria and polymer can be electrostatic and / or biocidal interactions [27]. For example, the polymers functionalized with positively charged quaternary ammonium groups can interact with the cell wall and cell membrane, conduction to leakage of intracellular content and cell destruction. Acrylamide polymers with quaternary ammonium have proved active action against *Staphylococcus albus, Escherichia coli, Rhizoctonia solani, Fusarium oxysporum* [32], and polyurethane containing quaternary ammonium groups were active against *Staphylococcus aureus, Escherichia coli* [33]. Combination of two active compounds, an antimicrobial cationic monomer (bearing tertiary amine) and an antioxidant and antimicrobial hydrophobic monomer has provided a synergistic action against biofilms (of *Staphylococcus epidermidis* and *Staphylococcus aureus*) and suppress reactive species oxygen [34].

Polymeric Biocides

Antimicrobial polymers can be included in the following categories: polymeric biocide, biocidal polymers and biocide-releasing polymers [35]. In the case of polymeric biocides, various types of monomers with antimicrobial activity, i.e. monomers bearing amino, carboxyl, or hydroxyl groups are linked to a polymeric matrix and used to form the final product [35]. There are situations when the final polymer is less active than the monomers, due to the fact that the polymer is less soluble than monomers and / or because the biocidal groups do not reach their target [36]. Polymerization of antibiotics (Penicillin V and Cephradine) with PEG-Lysine via a hydrolytically stable bond conducted to an inactive polymer [37]. If the active monomers were linked to the matrix via a labile bond, the conjugates exhibited full antimicrobial activity. Polymeric materials with quaternary ammonium and phosphonium salts were used with success against *Staphylococcus aureus* and *Escherichia coli* [38]. Other polymeric biocides were constructed with benzimidazole (active against *Micrococcus luteus, Staphylococcus aureus, Bacillus subtilis*), halogenated monomers (*Staphylococcus aureus* and *Escherichia coli*) or various types of quaternary ammonium groups [27].

Biocidal Polymers

In the case of biocidal polymers, the active chemical functions are part of the polymer itself. The polymers contain quaternary ammonium groups, or phosphonium, tertiary sulfonium or quanidinium groups that interact with the negative charged groups from the outer membrane of the bacteria. Due to their membrane proteins, teichoic acids of Gram-positive bacteria, and negatively charged phospholipids at the outer membrane of Gram-negative bacteria, the cationic polymers can lead to destabilization of cell surface and induce the bacterial death. The effectiveness of these cationic polymers is in direct relation with the charge density of the cationic function on the polymeric backbone [27,39].

There are many type of biocidal polymers: quaternary ammonium polyethyleneimine, quaternary phosphonium modified epoxidized natural rubber, arginine – tryptophan rich peptide, guanylated polymethacrylate, ammonium ethyl methacrylate, metallo-terpyridine carboxymethyl cellulose, poly(*n*-vinylimidazole) modified silicone rubber, heparin, poly-ε-lysine, and gramicidin A, chitosan and others [27]. Among them chitosan, due to its nontoxicity, biodegradability and biocompatibility, is the most common natural biocidal polymer exhibiting inherent antimicrobial activity. Other group of very common biocidal polymers comprise antimicrobial peptides. More than 1000 peptides have been screened for antimicrobial activity. These polymers, beside disrupting the bacterial membranes and inhibiting the cellular processes, act as immunomodulatory agents stimulating the noninflammatory host immune response [40]. The unwanted side effects of antimicrobial peptides include antimicrobial resistance, low stability and high production costs [27].

Biocide-Releasing Polymers

In the category of biocide-releasing polymers, the products are realized by polymerization of biocide molecules together with the polymeric backbone or by creation of composites between polymer and biocide molecules. In fact, in biocide-releasing polymers the polymer itself is a carrier for biocides molecules, and the product in its polymeric form exhibit antibacterial activity due to incorporation of antibiotic or antiseptic compounds. The

controlled release of biocidal molecules from the polymer has the advantage of releasing of active molecules that have in vivo short half-lives for a certain time, maintaining in this way a high local biocide concentration in the vicinity of bacteria [27]. Example of such biocide-releasing polymers are dextran containing gentamicin [41], poly-L-lysine, polyethylene glycol containing staphylolytic LysK enzyme [42], poly(octanediol-co-citrate) having choline chloride, tetraethylammonium bromide, hexadecyltrimethylammonium bromide, methyltriphenylphosphonium bromide, cyclodextrin with triclosan, poly(methyl methacrylate) with silver [43], or polycaprolactone with silver [44].

The antimicrobial polymers can be classified also in surface-bound or solution-base polymers categories. While surface-bound polymers have direct antimicrobial activity on the polymer surface, the solution-based polymers should be dissolved or dispersed in solution in order to manifest antimicrobial properties. Most of the biocidal polymers enter in the surface-bound polymers category, while biocide-releasing polymers should be solubilized in order to release the biocidal molecules. Polymeric biocides may be categorized in both categories, surface-bound or solution-base polymers, depending of the chemical structure of bioactive repeating units [27].

THE ANTIMICROBIAL MODELS OF CHITOSAN

The fact that there are some evidences that the leakage of intracellular components produced by chitosan in gram-negative bacteria is superior to that produce in gram-positive bacteria can be explained by the difference in composition of membranes and walls of these groups of bacteria, i.e. the outer membranes of gram-negative bacteria contain mainly lipopolysaccharides with phosphate and pyrophosphate groups that make a higher density of negative charges on bacterial surface comparing with gram-positive bacterial cells, where the membranes are composed by peptidoglycans associated to polysaccharides and teichoic acids. On the other hand, there are publications where the antibacterial effects of chitosans are stronger on gram-positive bacteria (*Listeria monocytogenes, Bacillus megaterium, B. cereus, Staphylococcus aureus, Lactobacillus plantarum, L. brevis, L. bulgaris*, etc.) than on gram-negative bacteria (*E. coli, Pseudomonas fluorescens, Salmonella typhymurium, Vibrio parahaemolyticus*, etc.) [11]. This would suggest that the antibacterial mode of action of chitosan is dependent upon the host microorganism [45].

Another mechanism of chitosan antimicrobial activity is the property of chitosan to bind metals, i.e. the amine groups has the capacity to uptake the metal cations by chelation [46]. Contrary to the situation when chitosan acts at low pH with the negative charges from the bacterial cell surface, the chelation process is more efficient at high pH, when the positive metal ions can bound to chitosan, to unprotonated amino groups and the electron pair on the amine nitrogen is available for donation to metal ions. At pH lower than 6, the metal can interact with only one amino group and three hydroxyls or water molecules. At pH between 6 and 7 the metal ion can interacts with two amino groups from two different chains. When the pH is higher than 7, the predominant complexation is ruled by two amino and two hydroxyl deprotonated groups. In the process of chelation of metal ions by chitosan, it is possible to be involved some essential nutrients for bacterial cells, that being extracted from their normal sites, contribute to cell death. Although possible, the metal chelation

mechanism seems to have a reduced influence to the overall antibacterial activity of chitosans [11].

Another possible mechanism of action of chitosan against bacteria is its binding with microbial DNA and / or mRNA, interfering with transcription and translation processes [47], although not all the authors agree with this possibility [48]. The dominant argument is that chitosan acts principally as an external membrane disruptor rather than as a penetration material [11].

SENSITIVITY OF MICROORGANISM STRAINS TO CHITOSAN

There are numerous reports about the minimum inhibitory concentration (MIC) for chitin, chitosan, their derivatives or combination, with diverse results for different microorganisms. MIC is defined as the smallest concentration of an antimicrobial that will inhibit the observable development of a microorganism after overnight cultivation. Unfortunately, the non-standardized protocols make difficult to compare MIC results from different authors. For example, MIC of chitosan for *Escherichia coli* vary from 20 [49] to 1000 ppm [50], for *Vibrio parahaemolyticus* from 150 [51] to 1000 ppm [52] and for *Staphylococcus aureus* from 20 [49] to 1250 ppm [53].

Gram-positive bacteria

The explanation of the antibacterial effect of chitosan on gram-positive bacteria is the non-covalent binding of chitosan to teichoic acid incorporated in the peptidoglycan layer [54]. The surface localized teichoic acid molecules are important for cell division and interaction with chitosan can impair this process and possible other processes equally important for the bacterial growth. The roles of teichoic acids are to protect the cells against environmental stress, to control the enzyme activity and to assure a cationic concentration of the cell surface to facilitate the binding of the cell to receptors. The significance of teichoic acid towards chitosan action was shown on *S. aureus* mutants in genes involved in teichoic acids biosynthesis. The mutant species of *S. aureus* were more resilient compared to the wild type. This proves that polyanionic teichoic acids are the target site of chitosan antibacterial activity towards gram-positive bacteria. At least in the case of small molecules of chitosan (smaller than 5 kDa) it was advocated that the polymer can enter in the bacterial cell and block the synthesis of DNA [55], emphasizing the fact that the molecular weight of chitosan is an important factor that can affect the mode of action of this polymer [23].

There are articles describing the antibacterial activity of chitosan in the form of nanoparticles. At least for S. aureus, the nanoparticles of chitosan proved to have a lower bactericidal concentration (4 μg/mL) compared to soluble chitosan (32 μg/mL). The antimicrobial activity is improved when chitosan nanoparticles are loaded with cupper (2 μg/mL) [56].

There are reported cases when chitosan films have not antimicrobial activity, at least against *Staphylococcus aureus* and *Staphylococcus epidermidis*, although the chitosan

solutions were very effective as an antimicrobial agent [57]. Against other types of bacteria (like *Lactobacillus plantarum* and *Listeria monocytogenes*) chitosan films proved to be active [23]. In the form of films from quaternary chitosan, the antibacterial properties are manifested even toward *Staphylococcus sp*. cells [58].

Gram-negative bacteria

One mechanism that is believed to be involved in the interaction of chitosan with gram-negative bacteria is correlated to the chelation effect of chitosan with cations when the pH is above pKa [59]. Another mechanism of action of chitosan is the electrostatic interaction of chitosan with anionic parts of lipopolysaccharides from the outer membrane of gram-negative bacteria [11]. It is also possible that chitosan (at least polymers with low molecular weight) pass through membrane and interferes with DNA/RNA synthesis [55]. Which of these mechanisms is prevailing remains unclear. Taking into account the difference in MW of the chitosan-based products, it appears that oligo-chitosan have a lower antibacterial activity than low, medium and high MW chitosans [23]. In fact, the differences in antibacterial activities of different chitosans, with different MW is rather small and seems to be largely dependent on the bacteria.

Considering the fact that it was observed a higher antimicrobial activity with increasing the degree of deacetylation, electrostatic interactions could be the major factor determining the antibacterial activity of chitosans. Chitosan (pKa6.3–6.5) has the highest antibacterial activity at low pH due to the protonated amino groups. This explains why quaternized chitosan derivatives are more effective than chitosan and why chitosan is more effective than chitin. Quaternized chitosan derivatives have a better solubility than chitin and raw chitosan and an improved antibacterial activity, due to permanent positive charges [60].

Other non-covalent interactions between chitosan and molecules from the bacterial surface can be considered to explain the mechanism of chitosan antibacterial activity. For example, chitosan can interact with cholesterol molecules and destabilize the bacterial membrane [61].

N-SUBSTITUTED CHITOSAN DERIVATIVES

Although it was confirmed that chitin and raw chitosan have antibacterial activities, the use of these polymers is limited due their low solubility in aqueous solutions. Water soluble chitosan based materials can be realized by introduction of stable positive charges in the polymer chains. The resulted cationic polyelectrolyte derivative presents antibacterial properties that are independent of the pH of the environment. Quaternization of the nitrogen atoms of the amino groups of raw chitosan is one possibility to obtain soluble derivative of chitosan. This can be realized by extensive methylation (with dimethylsulfate in strong alkaline environment) conducting to N,N,N-trimethylchitosan derivative [62].

Reports with quaternary salts of chitosan shown that the antibacterial activity is higher than that of raw chitosan [63]. For example, the activity of N-propyl-N,N-dimethyl chitosan

against *E. coli* is 20 times higher then that of raw chitosan proving the importance of cationic permanent charges for the antibacterial activity [64]. An important characteristic of the chitosan derivatives is the data that the alkyl moiety have an significant role in the antimicrobial activity, promoting hydrophobic interactions with hydrophobic residues from the bacterial membrane. That means between chitosan derivatives and molecules from the bacterial surface can take place hydrophobic and hydrophilic interactions, favoring the structural affinity between the bacteria cell wall and the polymer derivative [11]. This was confirmed by the works of Rabea [65] that confirmed that antimicrobial activity increases with the chain length of the alkyl substituent. Hydroxypropyl and carboxymethyl chitosans derivatives have also antibacterial activities. Hydroxypropyl chitosans, grafted with maleic acid are soluble derivatives of chitosan and at neutral pH present an antibacterial activity higher than that of raw chitosan [66]. Although carboxymethyl chitosan derivatives can have both negative and positive substituent groups, it seems that the influence of carboxymethyl part is less important than the presence of positive charges on the polymer chain, or its molecular weight [11]. Other types of chitosan derivatives have also shown improved antibacterial activities. For example, acyl thiourea chitosan derivatives have higher antimicrobial activity against *S. aureus* and *Sarcina sp*. [67]. Similarly, thymine-chitosan [68], sulfonated chitosan [69] and alkyl sulfonated chitosan [70] showed a superior antimicrobial activity against *S. aureus*.

N,O-SUBSTITUTED CHITOSAN DERIVATIVES

Diverse thiosemicarbazone chitosans [67] and hydroxylbenzene-sulfonanilide chitosan derivatives have shown antimicrobial activities against *S. aureus* and *Sarcina sp*., while quaternary carboxymethyl chitosan derivatives have presented antibacterial activity against *B. subtilis* and *S. pneumonia* [71]. Quaternary chitosan derivatives showed antibacterial activity against *E. coli* with MIC values ranging between 0.006 till 0.3 mg/mL [72]. 2-Hydroxypropyl dimethyl-benzyl-ammonium N,O-(2-carboxyethyl) chitosan chloride with a varying degree of quaternization showed an increased antibacterial activity against *S. aureus* [73].

APPLICATIONS OF POLYMERS WITH ANTIBACTERIAL PROPERTIES

Medical Industry

Medical industry is one of the major beneficiary of any kind of materials that present antibacterial activity. The surface of any kind of medical instruments is susceptible to microbial infection. Although there are notable progresses in materials and procedures, most hospital-acquired infections derive from medical devices. To diminish biofilm development and to increase the long-term use of medical devices a coating copolymer of 4-vinyl-n-hexylpyridinium bromide (VP) and dimethyl(2-methacryloyloxyethyl) phosphonate

(DMMEP) active against several pathogenic bacteria (*Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus sanguinis, Escherichia coli*) was proposed [74].

Antimicrobial peptides, that are also involved in modulation of the immune response [75], antimicrobial wound-dressing containing antimicrobial peptides grafted to chitosan (polycationic polymer) or to alginate (polyanionic polymer) presented a high antimicrobial effect (in the range of 4–6 log reduction) for *Staphylococcus aureus* and *Klebsiella pneumonia* [76] with no toxic effect against human dermal fibroblasts [27].

Composite gels based on chitosan and ZnO, containing gentamicin that was slowly released under planktonic and surface-attached conditions have shown highly effective antimicrobial activities against Pseudomonas aeruginosa and Staphylococcus aureus [77]. The system has the advantages to be transferred to any other soluble antibiotic or any other type of drugs as the active molecules remain trapped in the chitosan-ZnO composite gel, and, most important, when used in a wound dressing device, it maintained a moist environment to the wound.

Impregnated catheters with triclosan, rifampicin and sparfloxacin have shown to be active against *Proteus mirabilis, Escherichia coli* and *Staphylococcus aureus*, providing a solution to reduce catheter-associated urinary tract infection in both short-term and long-term urinary catheter use. The active chemicals were released from the polymeric coating of catheters during several weeks, preventing colonization of wound and catheters with bacteria [78].

Food Industry

Another industry that can benefit from the use of antimicrobial polymers is food industry. Here, the major application of polymers with antibacterial properties is realization of packages that prevent the development of microbial cells. Because of its favorable properties of negligible human toxicity and antibacterial effectiveness, nisin was approved to be used as a food preservative. Nisin was impregnated in films created from chitosan – poly-lactic acid, from where was slowly released during the period of validity of food products. These films have shown to have a high antimicrobial activity against *Staphylococcus aureus* [79].

Chitosan based products were used to enhance fish preservation during storage [80], to improve the quality of fresh cut broccoli [81] or to control bacterial contamination during brewing [82]. Another advantage of using chitosan based-product in food packages is the fact that it was noticed an improvement in the sensory quality during storage. This was observed for packages of chicken meat [83], of cherry tomato fruits [84], jujube fruits [85] or red table grapes [86].

Textile Industry

Another industry that benefits from development of antibacterial polymers is textile industry. Under suitable conditions of temperature and humidity, cloths are good substrates

for microbial growth. Nanocomposite coatings with high thermal stability and high antimicrobial activity, based on Ag:ZnO/chitosan were developed using a modified sol-gel method with 3-glycidyloxypropyltrimethoxysilane and tetraethoxysilane as functionalization agents and were applied to make antimicrobial fabrics from textile blend of cotton/polyester (50%/50%) [87]. Silk coated with chitosan showed an antibacterial activity [88]. When chitosan was mixed with dyes, beside an improved antimicrobial activity it was observed and improved dye-ability of silk [89].

4. CONCLUSIONS

Chitosan is a chitin derived biopolymer with many interesting applications. Many of its applications in medicine, pharmacy, textile or food industries derive from antibacterial activities of chitosan based-materials. The effectiveness of antimicrobial properties can be modulated by selecting the range of molecular weight of the polymeric chains and the degree of acetylation of amino groups. Further improvement of antibacterial efficiency of chitosan based-materials can be realized by derivatization of amino and / or hydroxyl groups of monomeric units of the polymeric chain. Depending on the application, chitosan based-materials can be presented in the soluble form, films or nanoparticles dispersed in a suitable environment. Having so many parameters that can be modified, one can produce chitosan based-materials with antibacterial activities against gram-positive and gram-negative bacteria.

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Review

ANTIFUNGAL PROPERTIES OF CHITOSANS

Adina MATICA, Gheorghița MENGHIU, Vasile OSTAFE ♣
West University of Timisoara, Department of Biology – Chemistry (Pestalozzi 16, Timisoara, 300315) & Advanced Environmental Research Laboratories (Oituz 4, Timisoara 300086, Romania)

ABSTRACT

Chitosan, a natural polymer derived from chitin by deacetylation, has proved antifungal activity against numerous type of fungi. The most accepted mechanisms of action of chitosan and chitosan based materials against fungal cell are briefly presented. The influence on antifungal activity of molecular weight of the polymer, the degree of deacetylation, pH, the influence of various type of derivatization and forms of presentation were also considered. Due to the lack of consistency of the published paper in this field, but especially because the antifungal activity of a product depend greatly on the tested specie, a prediction of the efficacy of chitosan based material cannot be made.

Keywords: chitosan, antifungal activity, chitosan derivatives, nanochitosan

♣ Correspondent author: +40-755240153, vasile.ostafe@e-uvt.ro

INTRODUCTION

Literature is abundant in examples of antifungal effects of chitosan and chitosan derivatives ([1]) The mode of action of these chitosan based-materials depend of various types of factors, among them the most important being the type of microorganisms. In this respect, there are two type of fungi - chitosan-sensitive fungi and chitosan-resistant fungi[2].

As in the case of antibacterial activity of chitosan [3] antifungal activity of chitosan can be explained by several mechanisms. The positive charges from chitosan chains affects the cell membranes due to electrostatic interactions with negatively charge phospholipids [2]. Depending on the molecular weight (MW) of polymer, chitosan can enter in the cell after disturbance of the membrane [4]. Once in the cell, chitosan can interact with DNA and / or RNA synthesis [5] and with protein synthesis [6]. In the case of fungi that are resistant to chitosan, it seems that the polymer is incapable to disturb enough the cell membrane and cannot penetrate the cell [2]. In the case of this type of fungi, it was not observed the leakage of the intracellular material due to destruction of the membrane, as it was proved in the case of sensitive fungi [7]. Due to the variances in fluidity of the cell membranes of sensitive and resistant fungi, the action of chitosan on these two types of fungi is different. It was revealed that the higher the amount of unsaturated fatty acids in the fungi cell membrane, the more sensitive are these types of cells. Using mutants with a reduced amount of unsaturated fatty acids, it was observed a decreased antifungal activity of chitosan, compared with the wild types of the same species. These experiments leaded to the conclusions that the action of chitosan on fungi depend on membrane composition and fluidity [8].

The fungi manifesting different sensibility to chitosan belong to different fungal family, taxonomical classification being realized based on the fatty acid composition [9]. It was hypothesized that one can predict or explain the antifungal activity of chitosan using the taxonomy based on fatty acid composition. Similarly, it was assumed that the uptake of chitosan by the cell is ATP dependent, at least for those fungal species that are sensitive to chitosan. By monitoring the chitosan uptake in the presence of azide or at low temperature, when the production of ATP is inhibited, it was shown that chitosan was unable to pass the plasma membranes. Comparing these results with the standard situation, i.e. at room temperature and in the absence of the azide inhibitor, it was possible to conclude that chitosan uptake by fungal sensitive cells is diffusion and ATP dependent [2].

ANTIFUNGAL MECHANISM OF CHITOSAN

Likewise to antibacterial activity of chitosan, the activity of this polymer and of chitosan based-derivatives against fungi is supposed to be rather fungistatic than fungicidal [10]. It seems that chitosan can communicate regulatory changes in both fungus and host [11,12]. In the presence of chitosan, there were started some biological processes, like induction of synthesis of chitinases together with activation of action on vesicular arbuscular mycorrhizal fungi and on entomopathogenic fungi [13].

Interaction of chitosan with various types of fungi depend on the cell wall composition,

and when the interaction can take place, the polymer interfere with fungal growth. There are microscopic evidences that show that chitosan oligomers may diffuse inside hyphae, where may interfere with the enzymes involved in fungus growth. The extent of degradation of fungal cells depend on several factors, among them most important being the concentration of the chitosan, its molecular weight (MW), its degree of deacetylation (DD) and the local pH [14].

Regarding the influence of chitosan concentration, besides the obvious observation that the antifungal activity increase with polymer concentration, it was observed the concentration of chitosan modify the length of the lab phase. As the pH of the medium increase the effectiveness of chitosan decrease[14].

Although the antifungal activity of chitosan was studied from more than 20 years now and many articles on this subject were published, it is rather hard to compere these results due to unclearly characterization of chitosans and to differences in experimental factors. Even so, some conclusions can be drown. The minimum inhibitory concentration of chitosans, against various type of fungi, vary in the range of 0.01 till 8 mg/mL. The antifungal activity of chitosan depend on MW, DD and pH. Unfortunately, the data is cumbersome and no clear relation cu MW and DD can be considered in all cases. It is surer to consider that the antifungal activity of chitosan is dependent on the type of fungi [1].

Theoretically, the source for chitosan production should not be a factor that influence the antifungal activity. Perhaps the manufacture procedure can have a small influence, due to the presence of some by-products or residual compounds, but none of the published articles has treated this subject. Nevertheless, there are published results that, at least indirectly, can conduct to the conclusion that the source of chitosan may affect the antifungal activity. For example, chitosan from shrimps inhibits fungus *Aspergillums niger* [15], whole chitosan produce from *Rhizomucor miehei* and *Mucor racemosus* present antifungal properties against *Candida albicans* and *Candida glabrata* [16]. Species like *Rhizoctonia solani, Thanatephorus cucumeris, Sclerotinia sclerotiorum* and *Sclerotium rolfsii*, among other species, are inhibited by chitosan extracted from larvae of the housefly [17]. However, the source of chitosan production cannot modify the mechanism of action of the polymer, as long as, the chitosans with the same purities, same MW and DD and in similar conditions (pH, temperature, etc.) are used.

When chitosans with different MW were tested against several species, there were noticed differences in susceptibility and influence of MW. For example, *Puccinia asparagi* and *Fusarium oxysporum* are sensitive to chitosan with low molecular weight (LMW), while against *Stemphylium solani* chitosan with high molecular weight (HMW) was more effective [18]. In another study is was reported that LMW chitosan was more active against mycelial growth Rhizopus stolonifera, but inhibition of spore development of the same mold was better realized by HMW chitosan [19].

It seems that LMW chitosan is more successful against mycelial growth, as LMW chitosan is more efficient in interacting and further disturbing the function of cellular membranes [1]. LMW chitosan is also more effective in inhibition major metabolic processes once penetrating the fungal cell. Probably both these mechanisms of action take place simultaneously, but the extent of each depend on the fungal strain. It appear also to be a synergy in the case when both these mechanisms take place, which might explain the maximum antifungal activity of LMW chitosan, compared to oligo-chitosans and HMW chitosan. In same articles there is not a clear difference between oligo-chitosans and LMW

chitosans, and the efficiency of oligo-chitosans is quite common mentioned [20].

Degree of deacetylation (DD) is also an essential factor for the antifungal activity. Generally, it is accepted that as the DD is increased the antifungal activity is also increased [21]. Combining the two factors, DD and MW, one may say that the antifungal activity is higher at higher DD and lower MW. This conclusion was proved on various species: *Candida albicans* [21], *Fusarium oxysporum, Aspergillus parasiticus* [22], *Aspergillus fumigatus,* [23] and *Aspergillus flavus* [24]. These results sustain the mechanism of action of chitosan based on electrostatic interaction of positive charges of the polymer with negatively charged phospholipids from cell membranes. Increasing DD means to increase the number of free amino groups that can interact with phospholipids heads.

N-substituted chitosan derivatives

Even though raw chitosan have an antifungal activity by itself, there are researches to improve this activity by chemical modification of chitosan. The addition to the polymer chain of substituents like heterocycles of aromatic moieties can lead to an increase of the antifungal activity. An increased activity against *Pythium debaryanum* and *Fusarium oxysporum* was obtained using different N-benzyl chitosan derivatives prepared by reductive amination [25]. Against Botrytis cinerea and Pyricularia grisea an improved antifungal activity was obtained for aliphatic N-substituted and N-benzyl chitosan derivatives [25]. Improved antifungal activity against *Aspergillus niger* was obtained with thymine based chitosan derivatives [26]. Imino-chitosan derivatives presented improved antifungal activity against *Aspergillus fumigatus* [27]. Other chitosan derivatives with improved antifungal activities are thiosemicarbazone-chitosan, 2-(α-arylamino phosphonate)-chitosan, hydroxylbenzenesulfonanilide chitosan [1].

Beside the new functional groups, the antifungal performances of these chitosan derivatives, still depend on MW and DD. Unfortunately, rarely the authors of new chitosan based products take into consideration the degree of substitution (DS), i.e. the number of the new functions introduced on the polymeric chain, although most of them determine the DS. But seldom they consider this as an important factor that influence the antimicrobial properties of the new product. The antifungal activity do not depend linearly on DS. For example, N-octyl-chitosan with DS 12% was the best antifungal product, when compared with similar products, but with DS of 8%, 21% and 22% [25]. From this study, it was concluded that it is necessary to find a good balance between the hydrophilicity and hydrophobicity of the chitosan derivatives. The new functions introduced on the polymeric chain may increase the hydrophobicity which may change the type of interaction with the fungal cell wall. That means that for N-substituted chitosan derivatives, it will be necessarily to find an optimal ratio between free amino groups and substituted amino groups, as these functions have an important role in the mechanism of action of chitosan derivatives in interaction with fungal cells. It has to be taken into consideration the fact that by quaternization the chitosan become more soluble in aqueous solutions, and for this reason a better interaction with all kind of molecules are expected to be more facilitated [28]. Amphiphilic derivatives, containing dodecyl and propyltrimethylammonium substituents, were soluble at neutral pH and presented a 4 times increased antifungal activity against *A. flavus* and *A. parasiticus* [29].

O-substituted chitosan derivatives

The derivatization of chitosan can be realized either at amino groups, by quaternization, when the positive charges remain charged at neutral pH, or by derivation the hydroxyl groups presented on the polymeric chain. Most of the articles presenting new O-substituted chitosan derivatives do not make a clear comparison of the properties of the new product and samples of raw chitosan, with the same MW and DD. In these cases is hard to decide if the new product is really a better antifungal product and if the derivatization was worth. For example, some different type of O-acyl chitosan derivatives were analyzed regarding the antifungal properties against *Botrytis cinerea* and *Pyricularia grisea* [30] and the antimicrobial activity was better compared with raw chitosan. If one take into consideration the fact that the reaction to synthesize O-acyl chitosan was realized in an acidic environment that can lead to a depolymerization, resulted that the new product have, in fact, a lower MW that the initial polymer. At least in this example, it cannot be considered that the improved antifungal activity is only due to derivatization. Another example when the depolymerization should be taken into consideration is the case when O-substituted chitosan derivatives were synthesized via protection of the amino group with methanesulfonic acid [31]. For all derivatives synthetized in this work, an increased activity was noticed, in top being O-benzyl chitosan derivative.

N,O-substituted chitosan derivatives

Derivative of chitosan containing N-benzyl carboxymethyl groups prepared by reductive amination of O-carboxymethyl chitosan manifested increased antifungal activity against *Alternaria solani* and *Fusarium oxysporum* [32]. Hydroxylbenzenesulfonanilide chitosan derivatives that were tested against *Fusarium oxysporum* and *Colletotrichum gloeosporioides* have also presented improved antimicrobial activity compared with the starting chitosan material [33]. Similarly, a quaternary carboxymethyl chitosan presented a higher activity against *Geotrichum candidum* and *Candida albicans* than the original chitosan [34].

Nanoparticles, films and application of chitosan coatings

In the last decade chitosan nanoparticles, nano-rods and films have attracted the interest of researchers and industries as there were developed many applications of these type of materials. One of the major interest is the used of chitosan nanoparticles as antifungal agent due to the fact that chitosan based materials in nanoforms and films presented an increased antifungal activity. For example, chitosan nanoparticles at concentration of 1 mg/mL inhibited mycelial growth with 82.2% for *Alternaria alternata*, with 87,6% for *Macrophomina phaseolina* and with 34.4% for *Rhizoctonia solani* [35]. The raw chitosan inhibited the mycelial growth with only 20% for all three fungi. The inhibition of spore germination for A. alternate was 87.1% compared with only 21% when raw chitosan was tested.

Films of chitosan and of chitosan derivatives also possess an improved antifungal activity when compared with soluble form of chitosans [36]. For example, films of chitosan and soluble chitosans with different molecular weight (LMW and HMW) presented and average radial inibhition of 88% against *Aspergillus niger*, 57% for *Alternaria alternate* and 0% for *Rhizopus oryzae*. In these cases it was not observed a influence of the molecular weight on the antifungal activity against the tested fungi. Beside the molecular weight, there are also other factors that can be considered when the nano-forms of chitosan based materials are tested, like viscosity, surfactants, procedures of assay and others. Most important factor seems to be the type of fungus. At least from the results published until now, it is impossible to predict if a certain type of film from chitosan or a chitosan derivative will have antifungal properties and the extent of these [1].

An application that is considered more often in the last years is to coat with chitosan films directly the vegetables and fruits. At least in the case of tomatoes, grapes and citrus fruits, this application proved to be beneficial for the extend of preservation of the merchandise. When table grapes were coated with films of chitosan the growth of *Botrytis cinerea* [37] and of Penicillium expansum [38] was reduced. Chitosan films were used on postharvest blueberries, when increased freshness, increased firmness and reduced weight loss were obtained compared with control samples [39]. At least in the case when the films of chitosans were used on citrus, a distinction was made between oligo-chitosan and HMW chitosan. While oligo-chitosan films retarded the ripening process more than HMW films, the high molecular weight form of chitosan was more effective against tested fungi (*B. cinerea, P. digitatum, P. italicum and B. lecanidion*) [40].

To control gray mold caused by *Botrytis cinerea*, the tomatoes were coated with different molecular weight films of chitosan (5 kDa; 37 kDa; 57 kDa; 290 kDa). The most efficient was chitosan coatings of 57 kDa chitosan, that means that, at least for tomatoes, when the molecular weight of chitosan used to coat the vegetable increased or decreased, the antifungal activity decreased as well. In experiments realized in vitro, the most effective against *B. cinerea* was chitosan with the lowest molecular weight [31]. Nevertheless, it is proved that chitosan coating and films have a certain capacity in retarding the ripening process and the potential to reduce the infection of fruits or vegetables during the period between harvest and consumption [1].

CONCLUSIONS

Chitosan, a natural polymer, with many applications in various fields of research and industries, have proved to be a good agent against various types of fungi. This antifungal activity was tested for various types of chitosans, with different molecular weight, different degree of deacetylation, with different functional groups added to the polymeric backbone, in different forms (soluble, nanoforms, films) and against different types of fungi. Most of published results tend to conclude that the activity against fungi can be increased by increasing the degree of deacetylation. This conclusion is in accordance with the mechanism of action of chitosan, which is believed to be the electrostatic interaction between the positive charges from the chitosan chains and the negative charges from the components of the cell wall and membrane.

Regarding the molecular weight, the most effective chitosan against fungi was the

polymer with LMW, although the differences in antifungal efficacy was rather low, when chitosans with LMW, MMW and HMW were tested. In the case of LMW chitosan, a dual mode of actions can be assumed – after electrostatic interaction with cell membrane and disruption of it, the LMW chitosan can enter in the cell and can interact with other molecules negatively charged like RNA, DNA, proteins. The published results seem to agree that the antifungal activity is highly dependent on the type of fungi.

Chitosan derivatives and nano-forms of chitosan based materials have better antifungal properties than neat chitosan, and this activity can be influence by the molecular weight, degree of deacetylation and nature of functional groups added to the polymeric chain. Most of the chitosan derivative and nano-chitosans are active at neutral pH.

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Review

TOXICITY OF CHITOSAN BASED PRODUCTS

Adina MATICA, Gheorghița MENGHIU, Vasile OSTAFE ♣
West University of Timisoara, Department of Biology – Chemistry (Pestalozzi 16, Timisoara, 300315) & Advanced Environmental Research Laboratories (Oituz 4, Timisoara 300086, Romania)

ABSTRACT

Chitosan is a natural polysaccharide with great biocompatibility and low toxicity. Most important factors that define its properties are the acetylation degree and molecular mass. This polysaccharide can be used in preventing or treating wound and burn infections due to its intrinsic antimicrobial properties, and also because of its ability to deliver extrinsic antimicrobial agents to wounds and burns. It can be used also as a slow-release drug-delivery vehicle for growth factors that help improving wound healing. Chitosan it is a natural polymer used in various application, from agriculture, cosmetic and food industry to medical and pharmaceutical fields. Over the past years, chitosan became an important tool in drug delivery therefore it is important to evaluate the safety profile of this biopolymer.

Keywords: chitosan toxicity, drug delivery, biocompatibility, cytotoxicity, natural polysaccharides

1. INTRODUCTION

Chitosan is one of the most used biopolymer in research fields and it is obtained from alkaline deacetylation of chitin [1, 2].

For many years now, chitosan represents a major scientific interest. This natural polysaccharide shows promise for safe use in biomedical applications. Being chemically versatile and also possessing many beneficial properties (biodegradability, biocompatibility, muco-adhesive and antimicrobial properties, antioxidant and hemostatic effect) it is considered a biologically compatible and possibly non-toxic material [3-5].

♣ Correspondent author: +40-755240153, vasile.ostafe@e-uvt.ro

Regarding hemostatic effect of chitosan, it is believed that it has the ability of activating macrophages and also causing cytokine stimulation, which has led to a great interest in wound healing applications [6-8].

Due to its high killing rate against microorganisms, particularly Gram-positive and Gram-negative bacteria and also because of the advantage of showing low toxicity towards mammalian cells, chitosan has attracted great attention from researchers [9].

2. SOURCE OF CHITOSAN

Marine organisms like shrimp, crabs, squid, and lobsters are the most important source of chitin. Present also in insects, mollusks and fungi, chitin is the most abundant biopolymer, after cellulose and lignin. Millions of tons of crustacean waste are generated every year from the seafood processing industry thus crustacean waste represents a renewable source of chitin [10].

Chitin is the precursor of chitosan and is a polysaccharide synthesized by numerous microorganisms and also higher plants. Chitinases from microorganisms, generally hydrolyze randomly N-acetyl-β-1,4-glucosaminide linkages, that means they are endochitinases [11, 12].

One of the biggest disadvantage of chitosan is the poor solubility at neutral pH, so in order to overcome this, scientist have developed various chitosan derivatives, by chemically modifying hydrophilic groups or by grafting on water soluble functional groups [13].

By improving water solubility of chitosan derivatives it was also enhanced the cationic nature of chitosan, which allowed a better interaction with antigens or cell membranes [14].

3. CHITOSAN TOXICITY

When it comes to chitosan toxicity, published studies reveal ambiguous information and various explanations that are not totally confirmed by the results. Some analysis of the toxicity of chitosan and chitosan derivatives show that regardless of molecular weight, toxicity of chitosan enhances with an increase in the acetylation degree. Another observations show that cytotoxicity is directly proportional to molecular mass [15].

Because of the already known beneficial properties, chitosan has become increasingly used as scaffold in drug delivery and tissue engineering applications [16].

Although there are many studies published, chitosan drug delivery products are not approved by the FDA, thereby very few biotech companies are using chitosan as raw material [17].

Interest in using chitosan as a pharmaceutical excipient is not new, however, this polysaccharide still does not appear to be present in any drug existing on the market. In drug delivery, excipients play a vital role, but developing new ones it is mostly a slow process which is related to cost and regulatory problems and also demonstrating that these kind of products are safe for human use [6, 17].

Still, there are reports that this polymer is under investigation for use in many pharmaceutical formulations including drug delivery [18]. Published studies have reported the use of chitosan as granulations, gels, coating agents [6].

For all that, chitosan reached human body not as a pharmaceutical excipient, but through its use as a dietary supplement. In addition, chitosan exists on the market as medical device on various forms and formats: from hemostatic dressing for the treatment of bleedings, to bandages and as a coating agent for contact lens [19]. In the food industry however, chitosan has been listed as a GRAS product (Generally Recognized As Safe) in the U.S. and furthermore, in Japan, Italy and Finland chitosan is recognized as food additive [20].

Most studies show that chitosan toxicity is related to degree of deacetylation and molecular weight [21].

Guangyuan et al showed that 50% acetylated chitosan was efficiently degraded by lysozyme. After 4 hours of incubation the polymeric solution has lost 66% of his viscosity. The degradation rate was dependent on the degree of acetylation, so the more acetylated form of chitosan was degraded faster [22].

3.1. *In vitro* toxicity

Chitosan nanoparticles may be an alternative that can facilitate drug delivery. Because they could pass from the gastrointestinal tract, alveolar sac or nasal cavity into the systemic circulation, they could cause some toxicity to the human body. There are some studies that reported the cytotoxic effect of chitosan nanoparticles [23].

It was observed that chitosan nanoparticles of 40 nm in size showed inhibitory effects on the proliferation of a tumor cell line and chitosan nanoparticles were more toxic then chitosan itself [24].

Other researchers have used live human cells in order to examine the cytotoxicity of chitosan nanoparticles. The results showed that they are able to penetrate the cell and thus reducing the cell viability, the proliferation and compromising the cell membrane [25, 26].

Beside lysozyme, which can degrade easily chitosan, there are three forms of human chitinase that were thoroughly studied and that showed hydrolytic activity against chitosan and its derivative: acidic mammalian chitinase (AMCase), di-N-acetylchitobiase, and chitotriosidase. They have been identified in plasma, liver and gastrointestinal tract [27].

Studies have revealed that degradation rate depend on MW and DD [28] thus a higher reaction rate was observed for degradation of high MW chitosan while low DD chitosan was degraded faster [12, 29].

Cytotoxicity of chitosan is dependent on the MW, DD, concentration of the polymer and some other unspecific factors. The half maximal inhibitory (IC50) is approximately 0.2 – 2.0 mg/mL in most cell models [30].

It was reported that three commercialized types of chitosan with various DD and MW possessed different levels of adjuvant activity and these are caused not only by a single factor but rather a combination of multiple factors such as MW, DD, solubility and particle size [28].

Chitosan toxicity analysis have shown that toxicity enhances with the increase of the acetylation degree, regardless of molecular weight, but also it has been reported that chitosan toxicity depends directly on molecular mass [15, 31].

The results of experiments testing the cytotoxicity and genotoxicity of chitooligosaccharides upon lymphocytes revealed that even though these chitooligosaccharide

did not possessed genetic toxicity, they have demonstrated a great cytotoxic effect which was dependent on molecular mass [32].

The information on cytotoxicity of chitosan derivatives differ dramatically and this may be explained by the fact that polysaccharide derivatives are very diverse and so are the cell lines on which the studies were performed. One hypothesis, presented by Ahmed [33] shows that low molecular weight chitosan derivatives with increased acetylation degree are more efficiently degraded.

3.2. *In vivo* toxicity

The most important aspect in the use of polymers, mainly chitosan, as drug delivery system, is the biodegradation or metabolic fate in the human body. It is important for chitosan to have a suitable molecular weight for renal clearance, or, if the polymer has a larger size, then it should undergo degradation, and so, it would become suitable for renal clearance [34].

When entering the vertebrate bodies, chitosan seems to accumulate mainly in the liver, but also kidney and stomach [35]. It is presumed that the liver is the most significant site of accumulation because this organ is the primary site of metabolism [17].

Some scientists believe that the rate of biodegradation of chitosan in living organisms is dependent on the degree of deacetylation (DD), the degradation rate decreases with the increasing of DD. It is possible that, if the studies are conducted given appropriate conditions and also adequate time, chitosan would be degraded enough to be excreted [5, 36, 37].

Chitosan may be hydrolyzed chemically by acids present in the stomach and when referring to enzymatic hydrolysis, chitosan can be degraded by breaking linkages like glucosamine–glucosamine, glucosamine–N-acetyl glucosamine and N-acetyl glucosamine–N-acetyl glucosamine [17].

There are enzymes existing in vertebrates that can degrade chitosan, most reported being lysozyme[38, 39] and also bacterial enzymes found in the colon. Moreover, scientists have identified eight human chitinases belonging to the glycoside hydrolase 18 family from which three of them have shown enzymatic activity on chitosan [40].

Strange is the fact that there were reported to be some proteases that can degrade chitosan films, but maybe more relevant to know is that scientists showed that chitosan can be digested by rat colonic and cecal bacterial enzymes and also porcine pancreatic enzymes [7].

It should be mentioned that the majority of enzymes used in assays that investigate the enzymatic activity are not from vertebrates. Therefore, taking into account that the specific activity of enzymes differ from species to species, it must be considered that there is a major variability between organisms and the way they interact with different substances [41].

Another study on determining chitosan nanoparticles toxicity was performed using the zebrafish embryo model. Researchers have exposed zebrafish embryos to chitosan nanoparticles for 96 hours and induced a dose-dependent inhibition of embryo by chitosan nanoparticles of different size. When comparing with the control group, they observed a significant decrease in hatching rate at 20 mg/L and 40 mg/L chitosan nanoparticles of 200 nm. In addition, they detected an increased mortality rate among zebrafish embryos, therefore at high concentrations (30 mg/L for 200 nm nanoparticles and 40 mg/L for 340 nm nanoparticles), chitosan nanoparticles had toxic effect, leading to the death of embryos within 96 hours of exposure. The surviving zebrafish embryos from the 5 mg/L group (200 nm

nanoparticles) showed signs of malformations. The rate of malformations seems to increase when increasing chitosan nanoparticles concentration. Interesting was that the embryos treated with chitosan nanoparticles of 340 nm size showed decreased malformations rate when compared with the 200 nm particles treated group. Therefore, smaller chitosan particles showed higher toxic effect on zebrafish embryos [42].

Lagarto performed acute toxicity studies in order to assess the safety of chitosan and chitosan acid salts from *Panurilus argus* lobster and observed no signs of toxicity during the experimental period and concluded that the approximate lethal doses of chitosan are higher than 2000 mg/kg in female rats. In addition, no major changes were observed regarding the body weight of control versus chitosan treatment groups. Macroscopic examinations of organs showed no abnormalities in treated rats [43].

Signs of toxicity or even mortality were not observed neither in the case of chitosan repeated oral dose toxicity. Instead, erythrocyte count was increased at a dose of chitosan of 300 mg/kg/day for males and at 1000 mg/kg/day for females. Apparently no other biochemical parameters were affected by chitosan doses [43].

Some studies reported similar cytotoxic effects of different chitosan derivatives [44, 45]. Others studies have shown good tolerance and safety performance of chitosan products administered orally [46]. Baldrick performed *in vivo* tests on rats and concluded that very few side effects appeared both in mouse and rat models after oral intake of chitosan (1 – 15 g/kg/day for 3 months) [6].

In addition, there were no relevant clinical signs found in human volunteers after taking oral doses of chitosan up to 6.75 g/day [44].

Villacis reported that people with shrimp allergies developed no allergic reaction after oral administration of shellfish derived glucosamine [47].

All these many studies and results suggest that chitosan may be a possible biocompatible and biodegradable polysaccharide that exhibits no, or minor toxicity, and can become a potential safe pharmaceutical material [6, 44].

3.3. Toxicity against microorganisms

Although *in vivo* tests have shown low toxicity of chitosan, this polymer and its derivatives seem to be toxic to bacteria [48-52], fungi [53-56] and parasites [57, 58].

Researchers have concluded that antimicrobial effects of chitosans depend mainly on molecular weight, small molecular weight chitosan being more toxic than larger chains against microorganisms [9, 59, 60]. The antimicrobial properties of chitosan represent a great advantage in treating infectious diseases [9, 61, 62].

Chitosan can be used in preventing or treating wound and burn infections because of its intrinsic antimicrobial properties, and due to its ability to deliver extrinsic antimicrobial agents to wounds and burns in order to protect the wounds from microorganisms infections [63].

Another useful advantage of chitosan is the ability of being a slow-release drug-delivery vehicle for growth factors that can help by accelerating wound healing processes. The large number of published papers in this area suggests that chitosan will continue to play an important role in the management of wounds and burns [9].

Interactions between different pathogenic microorganisms and chitosan were investigated by Andres [62]. Several bacterial strains as *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Staphylococcus saprophyticus* were used for mortality constant rate determination. A hypothesis regarding antibacterial mechanism was the influence of free amino groups present in chitosan on disruption of cell wall.

Comparative studies were realized on antimicrobial activity of chitosans and chitosan oligomers against Gram-negative and Gram-positive bacteria [64]. The oligomers of chitosan showed low antimicrobial activity while chitosans inhibited growth of most bacteria tested. Likewise 0.1% chitosan showed antimicrobial activity on Gram positive bacteria. 1% acetic acid used as a solvent for preparation of chitosan solution inhibits almost all tested bacteria growth, except lactic acid bacteria [65].

Muzzarelli [66] highlighted the antibacterial efficiency of N-carboxybutyl chitosan obtained from crustacean chitosan with 73% DD, against different strains of Gram positive and Gram negative pathogenic bacteria. In this study was showed that N-carboxybutyl chitosan was active against Gram positive bacteria and *Candida spp*. Electron microscopy experiments proved that N-carboxybutyl chitosan decomposed external part of the cell wall in Staphylococci and duplication has also been affected. An abnormally expanded periplasmic space was detected close to the N-carboxybutyl chitosan pads in Gram –negative microorganisms. N-carboxybutyl chitosan pads affected also cell wall of *Candida albicans* strains. Intracellular structures changed their distributions or characteristics [67].

In another study, Raafat [68] detected antibacterial activity of chitosan dose-dependent. However, for *Staphylococcus simulans,* chitosan treatment showed that the cells membrane and cell wall remained intact. For *Staphylococcus aureus* SG511 the treatment with chitosan leads to changes in expression profile of genes in charged with autolysis, regulation of stress and metabolism.

4. CONCLUSIONS

Considering those many factors that influence *in vivo* and *in vitro* toxicity (MW and DD) systematic studies, regarding various chitosans and chitosan based products, should be performed. Describing chitosan structure and correlating this with safety profile would provide data that will help regulatory scientists to understand the toxicity or non-toxicity of chitosan and chitosan derivatives.

Amongst MW and DD, there are many other factors to consider that influence the toxicity: salt form, source, purity, polydispersity.

Until now, studies have shown low toxicity of chitosan, (μg/mL), but when it comes to chitosan derivatives, the modification will produce new chemical properties, so the studies should be performed individually, taking into account each modification [17].

To ensure chitosan safety, scientist must eliminate protein, metal or other contaminants that could cause potential toxic effects. There are enzymes that show activity against chitosan, at least *in vitro*, but when it comes to derivatives it may be more difficult to generalize or to extrapolate the conclusions, because they could give indigestible molecules. It is necessary for these kind of compounds to be small enough to be clinically viable and to be renally excreted [17].

Amongst purity, MW and DD, researchers should be aware of the physiochemical and biological properties of chitosan, the properties of raw material, the biological source and manufacturing procedure.

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Review

BIODEGRADABILITY OF CHITOSAN BASED PRODUCTS

Adina MATICA, Gheorghița MENGHIU, Vasile OSTAFE ♣
West University of Timisoara, Department of Biology – Chemistry (Pestalozzi 16, Timisoara, 300315) & Advanced Environmental Research Laboratories (Oituz 4, Timisoara 300086, Romania)

ABSTRACT

The biodegradation of chitosan is catalyzed by enzymes or chemicals *in vitro* or *in vivo* and it refers to the breakdown of the polymer substance into smaller fractions such as monomers (D-glucosamine, N-acetyl-glucosamine). When using chitosan in drug-delivery systems and tissue engineering, the biodegradation rate is particularly crucial. The degree of deacetylation (DD) and molecular weight (MW) are the key factors for controlling the biodegradation rates of chitosan. In addition, chemical modifications of chitosan will significantly influence the biodegradation rate. Lysozyme is one of the most common used enzymes for in vitro degradation studies. The study of chitosan degradation *in vivo* can be conducted either by injecting chitosan intravenously or by implanting chitosan subcutaneously in laboratory animals. Oral administration can give valuable results and this technique can be used to study the degradation of chitosan in the gastrointestinal tract. Despite the fact that are numerous studies made so far, the mechanism of chitosan degradation is not yet fully understood.

Keywords: chitosan biodegradation, chitosan derivatives, *in vitro* biocompatibility

♣ Correspondent author: +40-755240153, vasile.ostafe@e-uvt.ro

INTRODUCTION

Chitosan is a natural polyaminosaccharide composed of randomly distributed β-(1→4)-linked D-glucosamine and N-acetyl-D-glucosamine, having a very similar structure to that of cellulose. This polymer is increasingly used in biomedical and industrial fields. During the last decades, chitosan has become an important tool in gene therapy due to its biocompatibility and low cytotoxicity. However there are some disadvantages such as low solubility in aqueous media, and regarding drug delivery, it shows low transfection efficiency, and low specialty on targeted disease [1, 2].

As such form, chitosan is not present in nature, but it can be easily extracted from chitin through alkali deacetylation of this natural polymer. To „become" chitosan, chitin should be at least 60% deacetylated. The deacetylation of chitin can be performed in two ways: (1) by chemical hydrolysis, which happens under severe alkaline conditions, or (2) by enzymatic hydrolysis through particular enzymes such as chitin deacetylase [3, 4]. Chitosan has a rigid crystalline structure due to hydrogen bond which are formed inter- and intra-molecular because of the amine and hydroxyl groups [5].

Being the second most abundant polymer found in nature (after cellulose), chitin is found as a component part of many organisms, from fungi to crustaceans, to algae and insect cuticles [6, 7]. At industrial scale, however, the two main sources are represented by fungal mycelia and crustacean shells [8]. From these two, the mushroom source is considered more advantageous if considering the controlled production environment, this leading to a better reproducibility of the final product [9]. Another important aspect from which the fungal source is more appreciated is the allergenic point of view. The resulted chitosan is considered to be more suitable for biomedical applications [10].

As a natural polymer, the characterization of chitosan is very important because chitosan may be contaminated with organic and inorganic compounds. One significant drawback of chitosan is the poor solubility in aqueous solutions, except in acidic medium, and this makes the analyses slightly difficult to perform. The most used methods in characterizing chitosan are UV-spectroscopy, pH-potentiometric titration, IR-spectroscopy, NMR spectroscopy colloidal titration and also enzymatic degradation and viscosimetry and size exclusion chromatography [11, 12].

Chitosan solubility limits greatly its applications. One of the most important water soluble derivatives of chitosan are made by alkylation of amino group, or derivatization with charged functions of hydroxyl groups from the chitosan backbone. Compared to chitosan, these by-products have enhanced physicochemical and biological properties with great potential in applications such as tissue engineering and drug delivery. [13].

In the last years, there were many attempts to replace petrochemical products with renewable, biological components. Natural occurring polymers such as cellulose, chitin, starch, collagen, gelatin, and alginate [14] are abundant candidates that represent a sustainable source as they could reduce the consumption of fossil fuels, and thus having a positive impact over the environment.

Maybe the most challenging work is equipping these biomaterials with the same properties of the synthetic products, from a functional perspective. Chitosan is regarded like

a unique biopolymer, and because of its intrinsic properties it is most valuable than any petrochemical equivalent.

Chitosan is the only naturally occurring positively charged biopolymer [15] and also exhibits many important intrinsic features such as antibacterial along with antifungal activity [16], mucoadhesive, hemostatic and analgesic properties [17].

Because in biomedical fields are used so many by-products of chitosan, it is very important to study their biodegradability. Researchers showed that chitosan can be biodegraded into non-toxic residues [18] and it is believed that the rate of its degradation is highly dependent on the molecular mass and also its deacetylation degree. Furthermore, there are studies that showed that, at some extent, biocompatibility of chitosan based-materials can take place in physiological medium [19, 20]. These important features make chitosan an appropriate and outstanding candidate for biomedical applications.

CHITOSAN BASED PRODUCTS

Chitosan derivatives for gene therapy

Gene therapy is a biomedical field that attracts many scientists attention. This application uses genetic materials (DNA, RNA), as a pharmaceutical agent in order to treat numerous diseases. The main mechanisms underlying gene therapy are: delivering missing genes, replacing the defective ones and disabling undesired gene expression [21]. Thereby, gene therapy has the ability of treating various diseases and the interest regarding this application is constantly increasing. However, there are some disadvantages when using genetic material. First of all, nucleic acids can be rapidly degraded by nucleases, also they have larger size, high anionic charge, poor cellular uptake, and non-specificity [22].
Trying to overcome these shortcomings, researchers use vectors in gene therapy for safely delivering genetic materials.

Cationic polymers, like chitosan, are considered suitable carriers for delivery of non-viral genetic materials [23]. These polymers can condense with genetic materials due to electrostatic interactions and then form polyplexes in order to facilitate the cellular uptake [24]. Moreover, scientist observed that polyplexes escape the lysosome degradation by triggering an osmotic swelling effect, this being possible due to amino group of polyplexes which are quick in enabling the cell to absorb protons [25].

Chitosan has various bioactivities due to the primary amino groups present in this polymer main chain and for this reason chitosan is extensively used in many biomedical fields, such as drug delivery and gene therapy and also the industrial fields, such as water treatment, heavy metal flocculants and foods industry benefit from it [26].

Chitosan is a polysaccharide soluble in acidic solution, the optimum pH is about 6.5 [26]. Its solubility it is highly dependent on the degree of deacetylation (DD). If DD is around 40%, chitosan can be soluble to a pH value equal to 9, whereas if the DD of chitosan is approximately 80%, then this polymer it is soluble only at a maximum pH value of 6.5. There are some other factors that influence chitosan solubility, such as molecular weight (MW) and ionic strength of the solution [1].

Due to chemical properties described above, chitosan was extensively used as a gene

carrier for gene therapy. In pharmaceutical and medical applications, chitosan is used as a non-viral cationic polymer because of its biodegradability to normal body constituents, biocompatibility, bacteriostatic, fungistatic, hemostatic, non-toxic, anticancerogen, anticholesteremic properties. Being a cationic polymer, chitosan protects negatively charged genetic material against nuclease degradation.

Various studies presented that chitosan/DNA polyplexes have been able to transfect into various cell types: human embryonic kidney cells (HEK293), cervical cancer cells (HeLa cell) [25], primary chondrocytes [27], Chinese hamster ovary cells (CHO-K1) [28], fibroblast cells (NIH 3T3) [29], epithelioma papulosum cyprinid cells (EPC) [30].

Chitosan 3D-scaffolds for tissue engineering

When fabricating implantable scaffolds scientist should pay attention to body compatibility, scaffold mechanical properties, porosity, morphology, as well as healing and tissue replacement ability [31].

Very important data presented in published research concluded that tissue engineering scaffolds should not induce acute or chronic effects, should be biodegradable, because the new tissue should be able to replace the biopolymer used. Another important feature that scaffolds should possess is surface properties that promote cell attachment, differentiation and proliferation [32, 33]. Research in this direction are so valuable and chitosan could be a relevant candidate for 3D-scaffolds knowing that the preparation of such biomaterials could lead to substitution for damaged tissue and organs [34-36].

Chitosan hydrogels

Hydrogels are very interesting biomaterials because their high content of water it makes them compatible with the majority of living tissues. These gels are formed of a liquid phase, usually water (sometimes adjuvants) and a solid phase (less than 10% of the total volume of the gel) that ensures the consistency of the gel [10]. Moreover, the hydrogels must be soft and flexible simulating soft body tissues, so it can minimize the damage that is made during implantation [37]. Besides biomedical scaffolds for tissue replacements, hydrogels are also used as drug and growth factor delivery [38-41].

There were three main types of hydrogels developed, one of them being *physically associated chitosan hydrogels*. These physically associated hydrogels are formed by reversible interactions (hydrogen bonds, hydrophobic interactions or electrostatic interactions) between polymer chains. The structure of the gel is given by these interactions, fewer interactions give a softer gel and a higher number of interactions will result in a tighter and stiffer gel.

One remarkable property of chitosan is that it can form gels all by itself, not needing any additive.

This process is possible due to the neutralization of chitosan amino groups, which leads to the inhibition of the repulsion between chitosan chains. As such, the chitosan hydrogel formation occurs due to hydrogen bonds, hydrophobic interactions and chitosan crystallites

[40, 42-44].

Chitosan hydrogels can also be formed by mixing chitosan with other water-soluble non-ionic polymers such as PVA (polyvinyl alcohol) [45-48]. Thermo-sensitive chitosan hydrogels can be formed by mixing raw chitosan with polyol salts such as glycerol phosphate disodium salt [49-52]. More stable hydrogels can be formed with polyethylene glycol) [53, 54].

When forming gels, positively charged chitosan interacts with negatively charged molecules (sulfates, phosphates and citrates ions) [55, 56]. The swelling capacity of the obtained hydrogel is given by the number of D-glucosamine units vs. N-acetyl-D-glucosamine units, as well as by the concentration and size of the anionic species presented in the hydrogel.

Studies have described chitosan hydrogels in combination with larger negatively charged molecules such as proteins (albumin, gelatin, keratin, collagen and fibroin), anionic polysaccharides (alginate, pectin, hyaluronic acid, heparin, xanthan, dextran sulfate, chondroitin sulfate, fucoidan), glycosaminoglycans and carboxymethyl cellulose [51, 57-66].

Chitosan sponges

Chitosan sponges, obtained by freeze drying, are very useful as wound healing materials, because of their ability of soaking wound exudates, and in the meantime inducing tissue regeneration. In bone tissue engineering, chitosan sponges are used as a filling material [67]. Such examples of chitosan sponges are chitosan/tricalcium phosphate (TCP) [68] and chitosan/collagen sponges [67], chitosan–ZnO composites which have a good swelling ration and show hemostatic activity [69].

Chitosan films

Chitosan films are easily prepared by wet casting chitosan salt solutions followed by drying, either using oven or infrared (IR) drying [70, 71]. One example is Hem-Con bandage [72, 73] which is an engineered chitosan acetate derivative designed as a haemostatic dressing.

Chitosan porous nanofibers

Chitosan fibers can be produce in several ways, one of the first examples was reported in the early 1930, fibers were produce from acetic acid using dry and wet spinning [74-76]. In order to decrease the costs and improving fiber properties, chitosan was blended with other polymers such as sodium alginate [77], polyacrylic acid [78], sodium chondroitin sulfate, sodium heparin, sodium hyaluronat, cellulose [79].

In the recent years, the most used technique for preparations of nanofiber membranes is electrospinning (ESP). This is a versatile technique which produces polymer fibers from a few nanometers to microns in diameter [4].

BIODEGRADABILITY MECHANISM

When explaining chitosan biodegradability, it is important to know that chitosan, besides being a polymer bearing amino groups, it is also a polysaccharide, thus containing breakable glycosidic bonds. Chitosan seems to be degraded in vivo by unspecific enzymes, but mainly lysozyme was reported to have a such property [37, 80].

Biodegradation of chitosan leads to non-toxic oligosaccharides formation. It is possible that these oligosaccharides, with variable lengths, can be either incorporated in metabolic pathways or be excreted.

Zhang et al. observed that there is a link between degradation rate and molecular mass, deacetylation degree (DD) and the distribution of N-acetyl D-glucosamine residues [81]. The relation between chitosan biodegradability and DD is also dependent on crystallinity, chitosan being a semi-crystalline polymer. Crystallinity has its maximum at a DD equal to 0 (chitin form) or 100% (fully deacetylated chitosan), and it decreases at an intermediate DD. The biodegradation rate is increasing when crystallinity decreases, and this happens closely to 60% DD of chitosan.

Acetyl residues distributed along chitosan chain also affect the crystallinity of chitosan, and consequently, the biodegradation rate. It is appropriate to conclude that the smaller chitosan chains are more efficiently biodegraded that higher molecular mass chitosans [12, 82].

Although many tests have shown that chitosan is biocompatible and presents low toxicity [83, 84], FDA (Food and Drug Administration) only approved the use of chitosan as wound dressing [85].

Preparation method is crucial in determining biocompatibility of chitosan with physiological medium. One of the major concern related with biocompatibility it to avoid allergic reactions, that can be caused by residual proteins. The biocompatibility seems to be influenced by the density of amino groups on the polymeric chain, i.e. the biocompatibility increases with DD. Compared to chitin, chitosan proved to be more biocompatible *in vitro*. Apparently, with the increase in the number of positive charges, the interaction between chitosan and cells increases also, thus improving biocompatibility [86].

Being a natural biodegradable biopolymer, chitosan undergoes enzymatic degradation to non-toxic components.

In vivo, chitosan may be degraded by several enzymes, first of all by lysozyme which is a non-specific enzyme present in all mammalian tissues. Degradation products are non-toxic oligosaccharides which can be afterwards either excreted or incorporated to glycosaminoglycans and glycoproteins [87].

In vitro degradations of chitosan happens via oxidation, chemical, or enzymatic hydrolysis. These methods are commonly used for the preparation of low molecular chitosan under controlled conditions [88]. A crucial role in degradation rate is represented by molecular weight, deacetylation degree, polydispersity, purity level and moisture content.

The possible mechanism of degradation of chitosan usually begins with random splitting of β-1,4-glycosidic bonds (depolymerization) followed by hydrolysis of N-acetyl linkage (deacetylation). Consequently, molecular weight decreases and an increase in deacetylation degree is observed. Simultaneously, the cleavage of chitosan functional groups (amino, carbonyl, amido, and hydroxyl) may occurs, depending of the chemical and / or enzymatic

conditions. In addition, chitosan depolymerization may induce formation of free radicals which can lead to oxidation processes [89].

The polymer structure is altered by strong intermolecular interactions between formed fragments of chitosan, thus leading to the irreversible loss of its physicochemical properties.

Although numerous data have been published regarding chitosan application in biomedical fields, very few studies and review articles have investigated the long term stability of chitosan based products [90].

4. CONCLUSIONS

When using chitosan in applications for controlled drug delivery and tissue engineering it is required that, this polymer must be not only biocompatible but also biodegradable. Understanding of the biodegradation of chitosan is not only crucial but also necessary, because it involves performance and safety issues. Degradation rate of chitosan-based products depends on their applications. For example, when using chitosan-based product as a template in tissue-engineering applications, the degradation rate of the chitosan derivative should be relatively slow, because it should maintain its mechanical strength until tissue regeneration is almost complete. On the other side, if chitosan is used as a drug delivery carrier, then it should degrade relatively quickly but in a controlled manner in order to release continuously the drug to the target. The chemical structure also influences the biodegradation rate of chitosan, and also the MW, and the surrounding media to which it is applied. The degradation kinetics could affect the cell growth, tissue regeneration, and host response.

Biodegradation is even more important when chitosan derivatives are to be used in humans as drug delivery carriers or as tissue-engineering scaffolds. Biodegradation can be investigated using *in vitro* biodegradation methods, followed by *in vivo* models. The hydrolysis of chitosan and its derivatives, and thus biodegradation of the biomaterials in humans, is mainly catalyzed by lysozymes, chitosanases, chitinases, and chitin deacetylase enzymes. The MW and DD of chitosan are the dominant factors affecting the rate of biodegradation. High MW and DD it is believed to contribute to slow degradation, whereas low MW and DD enhances the biodegradation rate.

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Research Article

ON THE GENERALIZED ZAGREB INDEX OF DENDRIMER NANOSTARS

***Mohammad Reza Farahani* (♣)[1], *M.R. Rajesh Kanna*[2], *and R. Pradeep Kumar*[3]**
[1]Department of Applied Mathematics of Iran University of Science and Technology (IUST), Narmak, Tehran 16844, Iran.
[2]Post Graduate Department of Mathematics, Maharani's Science College for Women, Mysore- 570005. India.
[3]Department of Mathematics, The National Institute of Engineering, Mysuru-570008, India.

ABSTRACT

In this paper, we focus on the structure of molecular graph "Dendrimer Nanostars $D_3[n]$" $(\forall n \in \mathbb{N} \cup \{0\})$ and present some new results about the Generalized Zagreb index of Dendrimer Nanostars.

Keywords: Molecular graph; Dendrimer Nanostars; Zagreb indices; Generalized Zagreb Index.

1. INTRODUCTION

Let $G=(V,E)$ be a simple connected graph of finite order n and the sets of vertices and edges of G are denoted by $V=V(G)$ and $E=E(G)$, respectively. In such a simple molecular graph, vertices represent atoms and edges represent bonds. We denote degree and distance by d_v and $d(u,v)$, that the degree of a vertex v of G which is defined as the number of edges incident to v and the distance $d(u,v)$ between the vertices u and v of the graph G is equal to the length of (number of edges in) the shortest path that connects u and v A general reference for the notation in graph theory is [1-6].

♣ Correspondent author: Tel: +98-919-247-8265, E-mails: Mr_Farahani@Mathdep.iust.ac.ir & MrFarahani88@gmail.com, mr.rajeshkanna@gmail.com, pradeep.mysore@gmail.com

In chemistry, graph invariants are known as topological indices. In graph theory, we have many different topological indices of arbitrary graph *G*. A topological index of a graph is a number related to a graph which is invariant under graph automorphisms. Obviously, every topological index defines a counting polynomial and vice versa.

The Wiener index *W(G)* is the oldest based structure descriptor introduced by *Harold Wiener* in 1947[7], is the first topological index in chemistry. The Wiener index of *G* is defined as the sum of distances between all pairs of vertices of *G* and is equal as follow:

$$W(G)=\frac{1}{2}\sum_{u\in V(G)}\sum_{v\in V(G)} d(u,v)$$

where *d(u,v)* is distance between the vertices *u* and *v* of the graph *G* [7-11].

More than forty years ago by *I. Gutman* and *N. Trinajstić* introduced the *First Zagreb index* $M_1(G)$ [4,5]. It is defined as the sum of squares of the vertex degrees d_u and d_v of vertices *u* and *v* in *G*. Recently, we know the *Second Zagreb index* $M_2(G)$. The first and second Zagreb indices of *G* are denoted by $M_1(G)$ *and* $M_2(G)$, respectively and defined as follows:

$$M_1(G)=\sum_{v\in V(G)} (d_v^2) \text{ or } \sum_{e=uv\in E(G)} (d_v+d_v)$$

$$M_2(G)=\sum_{e=uv\in E(G)} (d_v\times d_v)$$

where d_u and d_v are the degrees of *u* and *v*, respectively.

Also, we know that their polynomials (the *First Zagreb polynomial* $M_1(G,x)$ and the *Second Zagreb polynomial* $M_2(G,x)$) as follow:

$$M_1(G,x)=\sum_{e=uv\in E(G)} x^{(d_v+d_v)}$$

$$M_2(G,x)=\sum_{e=uv\in E(G)} x^{(d_v\times d_v)}$$

On the other hands, one can see that the First Zagreb index $M_1(G)$ and the Second Zagreb index $M_2(G)$ are equal to first derivative of its polynomial (at $x=1$) $\forall i=1,2$, respectively as:

$$M_i(G)=\frac{\partial M_i(G,x)}{\partial x}|_{x=1}$$

The readers interested in more information on the Zagreb indices and topological indices can be referred to [11-29] and to the references therein.

In 2011, *A. Iranmanesh et.al* [30] introduced the generalized Zagreb index of a connected graph *G*, based on degree of vertices of *G*. The *Generalized Zagreb index* is defined as:

Definition 1: [30] Let *G* be a graph with the set of vertices *V*(*G*) and the set of edges *E*(*G*). The Generalized Zagreb index of *G* is defined for arbitrary non-negative integer *r* and *s*

as follows $(\forall r,s\in \mathbb{N})$:

$$M_{\{r,s\}}(G)=\sum_{e=uv\in E(G)} (d_u^r d_v^s+d_u^s d_v^r)$$

Corollary 1. [30,31] Let G be a graph with the vertex and edge sets $V(G)$ and $E(G)$. Some of the properties of the generalized Zagreb index of G are as

$$M_{\{0,0\}}(G)=2\sum_{v\in V(G)} =2|E(G)|$$

$$M_{\{1,0\}}(G)=M_1(G)$$

$$M_{\{r\text{-}1,0\}}(G)=\sum_{v\in V(G)} d_v^{\,r}$$

$$M_{\{1,1\}}(G)=2M_2(G)$$

$$M_{\{r,r\}}(G)=2\sum_{uv\in E(G)} (d_u\times d_v)^r$$

The goal of this paper is computing the *Generalized Zagreb index* of an infinite class of

Dendrimer Nanostars $D_3[n]$ $(\forall n\in \mathbb{N}\cup\{0\})$.

2. RESULTS AND DISCUSSION

A Dendrimer Nanostars an artificially manufactured or synthesized molecule built up from branched units called monomers and is one of the main objects of Nano biotechnology that is prepared in a step-wise fashion from simple branched monomer units, the nature and functionality of which can be easily controlled and varied. The first terms of this family of Nanostars are shown Fig.1 and Fig.2. For more study about Dendrimer Nanostars, we encourage the reader to consult papers and books [32-39].

Now, we present some new results about this general version of Zagreb indices an

infinite class of Dendrimer Nanostars $D_3[n]$ $(\forall n\in \mathbb{N}\cup\{0\})$.

By these terminologies, we can present the main results of this paper in following theorem.

Theorem 1. Let $D_3[n]$ be the n^{th} growth of Dendrimer Nanostar $(\forall n\in \mathbb{N}\cup\{0\})$. Then, the

generalized Zagreb index of $D_3[n]$ is equal to $(\forall r,s\in \mathbb{N})$:

$$M_{\{r,s\}}(D_3[n])=3(2^n)(3^r+3^s-2(3^{r+s}))+4\zeta_n(3^{r+s}+3^r2^s+3^s2^r+2^{r+s})$$

Proof of Theorem 1. Consider the Dendrimer Nanostar $D_3[n]$ $(\forall n\in \mathbb{N}\cup\{0\})$. Now, for

achieve our favorite topological indices of this class of Dendrimer Nanostars, we present the following notations.

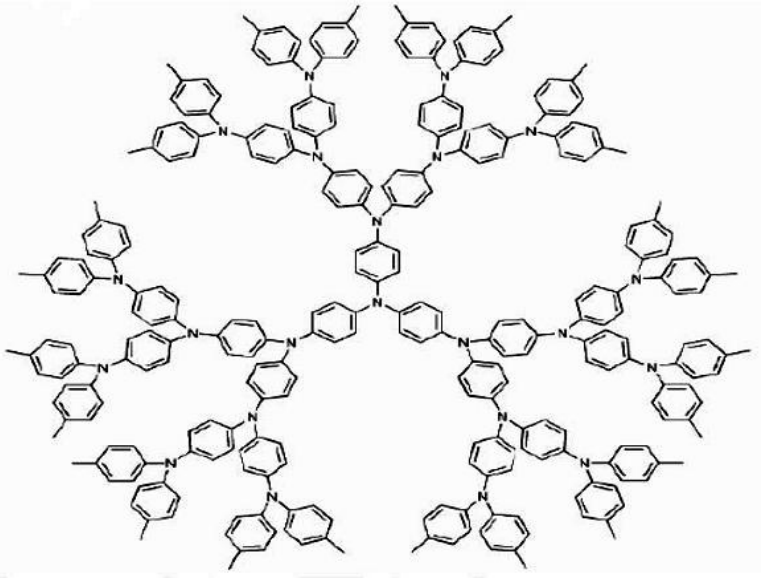
For a graph $G=(V;E)$, we have several partitions of the vertex set $V(G)$ and the edge set $E(G)$ of G, as follow [20]:

$$\forall k:\ \delta\le k\le \Delta,\ V_k(G)=\{v\in V(G)|\ d_v=k\}$$
$$\forall i,j:\ \delta\le i,j\le \Delta,\ E_{\{i,j\}}(G)=\{uv\in E(G)|d_u=I\ \&\ d_v=j\}$$

such that $V(G)=\bigcup_{i=\delta}^{\Delta} V_i(G)$, $E(G)=\bigcup_i E_{\{i,j\}}(G)$, where $\delta=Min\{d_v|v\in V(G)\}$ and $\Delta=Max\{d_v|v\in V(G)\}$ be the minimum degree and the maximum degree of vertices of G, respectively.

From the 2-Dimensional structure of Dendrimer Nanostar $D_3[n]$ (depicted in Figure 1) and a "Core $D_3[0]$" and "*Leaf*" (depicted in Figure 2), we see that $D_3[n]$ create by add $3(2^n)$ leafs to $D_3[n-1]$ in the n^{th} growth of Dendrimer Nanostar. Thus, there are $\zeta_n=3\sum_{i=0}^{n}(2^i)=3\left(\frac{2^{n+1}-1}{2-1}\right)=3(2^{n+1}-1)$ leafs (C_6) in Dendrimer $D_3[n]$.

Figure 1. [37-39] The 2-Dimensional of $D_3[3]$ denotes the 3^{th} growth of Nanostar Dendrimer.

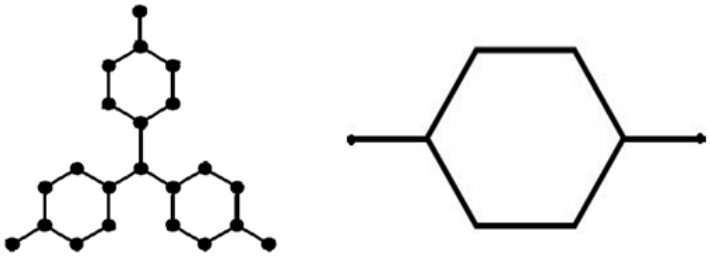


Therefore, by using above notations and reference [37-39], we have

$$V_1=\{v\in V(D_3[n])|\ d_v=1\}\rightarrow |V_1(D_3[n])|=2\times|V_1(D_3[n-1])|=3(2^n)$$
$$V_2=\{v\in V(D_3[n])|\ d_v=2\}\rightarrow |V_2(D_3[n])||=V_2(D_3[n-1])|+4\times 3(2^n)=12(2^{n+1}-1)$$
$$V_3=\{v\in V(D_3[n])|\ d_v=3\}\rightarrow |V_3(D_3[n])|=15(2^n)$$

Thus $V(D_3[n])=V_1 \cup V_2 \cup V_3 \rightarrow |V(D_3[n])|=4(3(2^{n+1})-5)$.

Figure 2. [37-39] A "Core $D_3[0]$" is the primal structure (A) and a "Leaf" is the added graph in each branch of Dendrimer Nanostar $D_3[n]$.



Also, from the structure of Dendrimer Nanostar $D_3[n]$ in Figure 1) [37-39], one can see that

$$E_{\{1+3\}}=\{ uv\in E(D_3[n])|\ d_u=1 \& d_v=3\} \rightarrow |E_{\{1+3\}}|=3(2^n)$$
$$E_{\{2+2\}}=\{ uv\in E(D_3[n])|\ d_u=d_v=2\} \rightarrow |E_{\{2+2\}}|=2\zeta_n=6(2^{n+1}-1)$$
$$E_{\{2+3\}}=\{e=uv\in E(D_3[n])|\ d_u=3\ \&d_v=2\} \rightarrow |E_{\{2+3\}}|=4\zeta_n=12(2^{n+1}-1)$$
$$E_{\{3+3\}}=\{e=uv\in E(D_3[n])|\ d_u=d_v=3\} \rightarrow |\ E_{\{3+3\}}|=2\zeta_n-3(2^n)=9(2^n)-6$$

And these imply that

$$E(D_3[n])=E_{\{1+3\}} \cup E_{\{2+2\}} \cup E_{\{2+3\}} \cup E_{\{3+3\}} \rightarrow |E(D_3[n])|=8\zeta_n=24(2^{n+1}-1)$$

Now, we have following computations for the generalized Zagreb index of the n^{th} growth of Dendrimer Nanostar $D_3[n]$ $(\forall n\in \mathbb{N}\cup\{0\})$ as:

$$\begin{aligned}
M_{\{r,s\}}\left(D_3[n]\right) &= \sum_{e=uv\in E\left(D_3[n]\right)} \left(d_u{}^r d_v{}^s + d_u{}^s d_v{}^r\right) \\
&= \sum_{uv\in E_{\{1,3\}}} \left(3^r 1^s + 3^s 1^r\right) + \sum_{e=uv\in E_{\{2,2\}}} \left(2^r 2^s + 2^s 2^r\right) + \sum_{e=uv\in E_{\{2,3\}}} \left(3^r 2^s + 3^s 2^r\right) + \sum_{e=uv\in E_{\{3,3\}}} \left(3^r 3^s + 3^s 3^r\right) \\
&= \sum_{uv\in E_{\{1,3\}}} \left(3^r + 3^s\right) + \sum_{uv\in E_{\{2,2\}}} 2\left(2^{r+s}\right) + \sum_{uv\in E_{\{2,3\}}} \left(3^r 2^s + 3^s 2^r\right) + \sum_{uv\in E_{\{3,3\}}} 2\left(3^{r+s}\right) \\
&= \left(3^r + 3^s\right) \times (3(2^n)) + 2\left(2^{r+s}\right)\times(6(2^{n+1}-1)) + \left(3^r 2^s + 3^s 2^r\right)\times\left(12(2^{n+1}-1)\right) + 2\left(3^{r+s}\right)\times\left(9(2^n)-6\right) \\
&= \left(3^r + 3^s\right)\times(3(2^n)) + 2\left(2^{r+s}\right)\times(2\zeta_n) + \left(3^r 2^s + 3^s 2^r\right)\times(4\zeta_n) + 2\left(3^{r+s}\right)\times\left(2\zeta_n - 3(2^n)\right) \\
&= 3(2^n)\left(3^r + 3^s - 2\left(3^{r+s}\right)\right) + 4\zeta_n\left(3^{r+s} + 3^r 2^s + 3^s 2^r + 2^{r+s}\right).
\end{aligned}$$

Finally, the generalized Zagreb index of Dendrimer Nanostar $D_3[n]$ is equal to

$$M_{\{r,s\}}(D_3[n])=3(2^n)(x+y-2xy)+4\zeta_n(xy+xt+yz+tz)$$

In which $x=3^r$, $y=3^s$, $z=2^r$, $t=2^s$ and $\zeta_n=3(2^{n+1}-1)$ and this completed the proof of the Theorem1.■

Corollary 2. Consider the Dendrimer Nanostars $D_3[n]$ for all non-negative integer number *n,* thus the first and second Zagreb indices of $D_3[n]$ are equal to

$$M_1(D_3[n])=M_{\{1,0\}}(D_3[n])=6(39(2^n)-20)$$

and

$$M_2(D_3[n])=\tfrac{1}{2}M_{\{1,1\}}(D_3[n])=6(47(2^n)-25)$$

also $$|E(D_3[n])|=\tfrac{1}{2}\, M_{\{0,0\}}(D_3[n])=8\zeta_n=24(2^{n+1}-1).$$

Proof of Corollary 2. $\forall n\in\mathbb{N}\cup\{0\}$, Consider the Dendrimer Nanostars $D_3[n]$, by using Theorem 1 and Corollary 1 the proof is obvious.

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