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The obtaining of nanocomposite slurries with practical aplication in real time biological fixing

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The present paper presents the fixing mechanisms of microorganisms on support carrying agents based on Fe_3O_4 biochemically activated nanoparticles. The processing of biological fixing carrying agents with undifferentiated action is utterly important because it enlarges the type and the number of the fixed biological structures with a view to subsequent separation and identification.The biological fixing carrying agents based on nanoparticles having a mixed and controllable structure of the Fe_3O_4-{n[$SiO_{1.5}$-$(CH_2)_3$-(n-m)NH_2]-mNH-$C_5H_8O_2$} type (m= biochemical activation factor), offer a series of advantages in the field of analytical microbiology, among which : external modulated control unselective biochemical fixing, separation opportunities through controlled discrimination, etc.

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1. Introduction

Biochemical fixing carrying agents [1] represent those class of materials designed for the fixing of microorganisms on the surface or in the depth of their own structures. Due to the complexity and structural diversity of microorganisms these composite materials have a decisive role in the biological research and detection, as they favour the undestructive retention of the biological material to be examined.

The classical biological fixing carrying agents facilitate the fixing of microorganisms in the whole fixing volume through the insertion in their own structure of macromolecular structures having a role in nutrition. These types of fixing carrying agents have two great disadvantages: a) they retain a small number of microorganisms; b) the subsequent biochemical and biological applications are limited due to the difficulties of the biological separation and purifying process, etc.

The disadvantages generated by the classical fixing carrying agents and the present requirements in the field of biochemical research, detection and identification directed the efforts in the field towards the obtaining of 'intelligent' composite materials, which should fulfill at the same time the functions of biochemical fixing, separation and identification. In accordance with the necessities, requirements and the opinion of the field specialists, the new types of composite materials, recognized in the field as 'support carrying agents of biochemical fixing', must enlarge the classical applicability limit through the embedment of the following functions: i) undifferentiated fixing for a large number of microorganisms, which differ from the viewpoint of the membership class and biochemical structure; ii) the undestructive fixing of microorganisms; iii) to allow the handling and the external control of the fixed microorganisms for those applications that require the separation and discrimination according to the physical, chemical and biological characteristics.

''The intelligent support carrying agents'' of biological fixing allow the fulfillment of the present requirements in the field, but they encounter a series of difficulties in the issue of the biological analysis and detection. A real solution in the case of biochemical detection and identification [2,3] consists of the modeling of the internal physical and chemical structure of the support carrying agent, even since the synthesis stage, so that this could allow the control of the fixed biological structure according to an external modulation factor. The most common and important difficulties are: a) the incompatibility between 'the intelligent support carrying agent', the structure responsible for the biochemical fixing and the characteristic structure of the fixed microorganism b) the incompatibility between the external physical modeling and control factor, the biochemical structure of the microorganism and the observable which is useful to the identification c) the lack of a unitary analytical model which should describe in a coherent and unitary manner the biological separation, detection and identification.

This paper presents the experimental data of free fixing of microorganisms in the environment, with a view to the checking of the properties, characteristics and chemical and biochemical fixing mechanisms of the synthesized 'biological fixing carrying agent'. The synthesized fixing carrying agent consists of a Fe_3O_4

nanoparticle slurry [4] coated in polymeric layers of the $[nSiO_{1.5}\text{-}(CH_2)_3NH_2]$ **(L)** type and biochemically activated with glutaraldehyde. Fe_3O_4 core shells are synthesized in such a manner as to accurately integrate the magnetic moment factor which should respond bijectively to the external modulating and control stimulus, $\vec{H}$. At the same time, the undifferentiated fixing and controlled biological separation carrying agent based on Fe_3O_4-$\{n[SiO_{1.5}\text{-}(CH_2)_3\text{-}(n\text{-}m)NH_2]\text{-}mNH\text{-}C_5H_8O_2\}$ nanoparticle slurry, must fulfill the previously mentioned terms, which define a good 'intelligent support carrying agent'.

The glutaraldehyde, as a biological cross-linked molecule [5], assures through bidirectional action on microorganisms and on **(L)** layer [6], the existence of unspecific chemical interactions of the anorganic-organic type, similar to the biochemical ones, of the epitope-epitope type, with a high degree of specificity.

2. Experimental

2.1 The synthesis of the fixing carrying agent

The biological fixing carrying agent was synthesized in several stages, as follows:

2.1.1 The synthesis of Fe_3O_4 nanoparticles

The magnetite nanoparticles were obtained through the coprecipitation of $FeCl_3$ and $FeCl_2$ (8,58/23,275g - Acros Organics) [7], using as base for coprecipitation the ammonia (60ml, 25% - Reactivul Bucureşti). Fe_3O_4 was obtained according to chemical equation 1:

$$FeCl_2 + 2FeCl_3 + 8NH_3 + 4H_2O \rightarrow Fe_3O_4 + 8NH_4Cl \quad (1)$$

The reaction time was 30 minutes [8,9] (after the beginning of the coprecipitation reaction), the stirring rate was of 55 KHz (with ultrasounds), and the chemical stabilization of the nanoparticles was performed through nitric acid treatment (100 ml, 2,3 M) for 15 minutes [10]. Additionally, the magnetite slurry was treated for 15 minutes with sodium citrate tribasic dehydrate (100ml, 50%, Merck) [11,12].

2.1.2 The coating of Fe_3O_4 nanoparticles

The Fe_3O_4 nanoparticles were coated in $nSiO_{1.5}\text{-}(CH_2)_3NH_2$ through (3-aminopropyl)-triethoxysilane cohydrolyse ($C_9H_{23}NO_3Si$, 14ml, Merck) [13, 14] , according to chemical reaction 2:

$$Fe_3O_4 + nNH_2(CH_2)_3Si(OEt)_3 \xrightarrow{H_2O} Fe_3O_4 - n[SiO_{1.5}\text{-}(CH_2)_3NH_2] \quad (2)$$

2.1.3 The biological activation of Fe_3O_4 nanoparticles

The nanoparticles obtained in the previous stage were activated with glutaraldehyde solution (mixture formed from:7 ml glutaraldehyde, 4ml NaOH, ~920 ml distilled water), according to chemical equation 3:

$$Fe_3O_4 - n[SiO_{1.5}\text{-}(CH_2)_3NH_2] + mC_5H_8O_2 \xrightarrow{H_2O} Fe_3O_4 - \{n[SiO_{1.5}\text{-}(CH_2)_3(n\text{-}m)NH_2] - mNH\text{-}C_5H_8O_2\} \rightarrow (N_F) \quad (3)$$

The reaction lasted 60 minutes at a temperature of 59^0C and at a stirring rate of 55 KHz. The glutaraldehyde activated slurry was treated with NaCl (0.2M), washed and kept in distilled water.

2.2 The free fixing of saprophyte bacteria from the environment

The nanoparticles obtained according to the previous procedures were kept in a Berzelius glass, in distilled water, in direct contact with the air, for 30 days, without any stirring or external influence [fig.4 a), b), c), d)]. The glass in which the free fixing took place was placed above two magnets presenting a high value of intensity of the magnetic field (H).

3. Results and discussions

The aim of this research is to check the following hypotheses: a) the undifferentiated fixing of N_F nanoparticles on the biological structure surface b) the marking out of unspecific links of the chemical-biochemical type, which theoretically, should be established between the biochemically active surface of N_F and the biological structure surface (including macromolecules with biochemical functions, e.g. proteins, enzymes, etc.) with which the nanoparticles get in touch, regardless of the biochemical and morphological surface specificity [15] of the target biostructures c) the study of the intimate interaction mechanisms between N_F and the biochemical structure on the surface or in the depth of which it was fixed.

The study of the fixed microorganisms morphology (fig.3) and of the intimate biochemical interaction mechanisms was done using the electronic transmission microscope (TEM) Philips S208, having the following working parameters: acceleration tension 80kV, spot 3, beam current 19μA, Olympus camera.

The images attached in fig.3 can be interpreted in accordance with the general model of interaction between the electrons from the scanning camera of the microscope and the nano/microstructures on whose surface N_F nanoparticles were fixed (fig.1a, fig.1b and fig.2a, fig.2b).

The TEM images analysis clearly indicates that the fixed biostructures are part of the bacterial class [16], as it was expected, taking into account the conditions in which the experiment was performed.

The morphology analysis of the bacteria presented in the images in table no.1 indicates that N_F were fixed on at least three different types of bacteria.

The bacteria surface contains a large number of N_F nanoparticles, coated in silicone polymer and activated with glutaraldehyde.

Additionally, on bacteria surface a certain N_F arrangement pattern in compact groups distinguishes itself.

Most likely, the N_F distribution on the bacterial surface is due to the existence in the fixing areas of epitopes specific to the fixed bacterium, presenting high

reactivity in relation to the biochemical cross-linking centres inserted on N_F surface. The bacteria in fig.3 **b)**, **c)**, **e)**, **g)** and **i)** show well delimitated patterns, characteristic to the N_F biochemical fixing centres. Thus, one can determine the surface shape and topology, the areas which are likely to present different biochemical structures and functions (antigens, nutrition, cilia, etc.), the thickness of the cellular membrane, the bacterium size, etc.

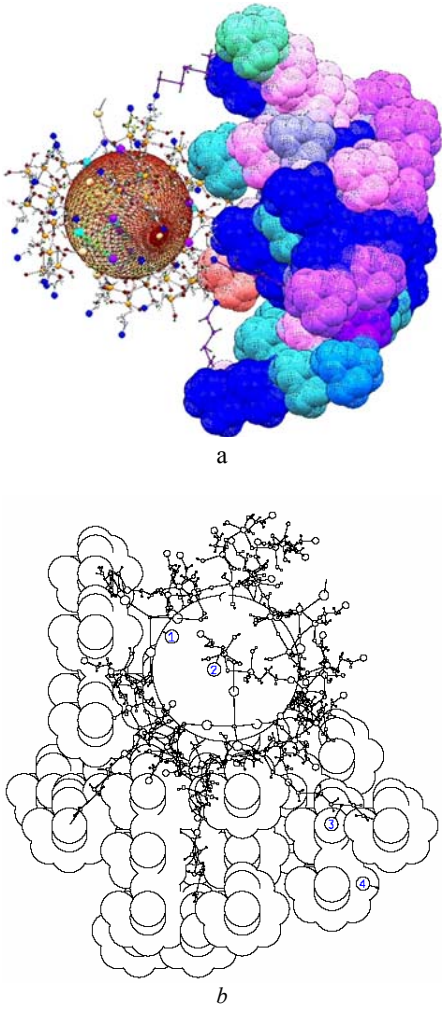


Fig.1. a 3D model of crosslinking biostructure with molecular functional groups (associate projection attached in fig.1b); b 3D rear view projection of model of crosslinking biostructure with molecular functional groups. 1 Nonometric magnetite core, 2 Silicon layer doped with amino-groups, 3 Glutaraldehide crosslinking molecules with carbonilic functional groups, 4 Fixed biostructure.

From a physical and phenomenological point of view, the electron interaction with the biological material is reduced to the following adsorption processes of the incident electrons on the target structure: a) collisions with the N_F biochemical fixing centres having a complex structure of the [semiconductor]–[metallic oxide] type; b) electric conduction due to the high water content, followed by the attenuation of the electron flux which passes through the sample through their retention in the sample mass (or the deviation toward the support grid) and its electrostatic charging; c) electron collision with biostructure molecules, followed by processes of molecular excitation, adsorption and quantum emission.

The result of these interactions is concretized in the electron number which reach the detector surface, forming an image of the crossed medium, image which is bijective in relation to the biochemical structure of the circulated – through medium. The average penetration length [17] in the sample layer is of several hundred nanometers, this parameter influencing significantly the establishment of those biostructure areas which present a high degree of affinity of N_F fixing and their manner of distribution on bacteria surface and in their depth.

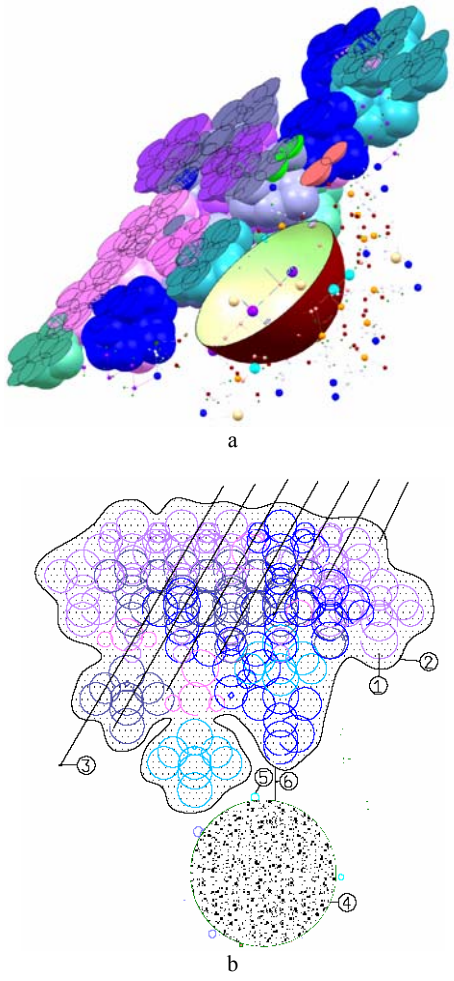


Fig.2a 3D general model of a cutting plane crossing biostructure at a certain point. b General model of a projecting plane at a Z level point axi, 1. molecular macrostructure of biostructure, 2. frontier of biostructure (cellular membrane), 3. beam electron, 4. Fe_3O_4 nanoparticle, 5. Fe_3O_4-{n[$SiO_{1.5}$-$(CH_2)_3$-(n-m)NH_2]-mNH structure contact space betwin frontiers of biostructure and Fe_3O_4 (induced by crossed - link molecules).

Image f) in fig.3 and the images in fig.4 suggest the existence of cellular division processes [16], directly in the fixing slurry. The bacterial colonies developed, most likely through the following successive evolutional processes: *N_F bacteria capture on the separation surface between the fixing slurry and the air→the transport through diffusion* or *magnetic attraction among the N_F centres→selective sedimentation according to the structure and biochemical characteristics*. Bacterial colonies can be clearly differentiated through visual inspection, but they cannot be identified [fig.3 a), b), c), d)].

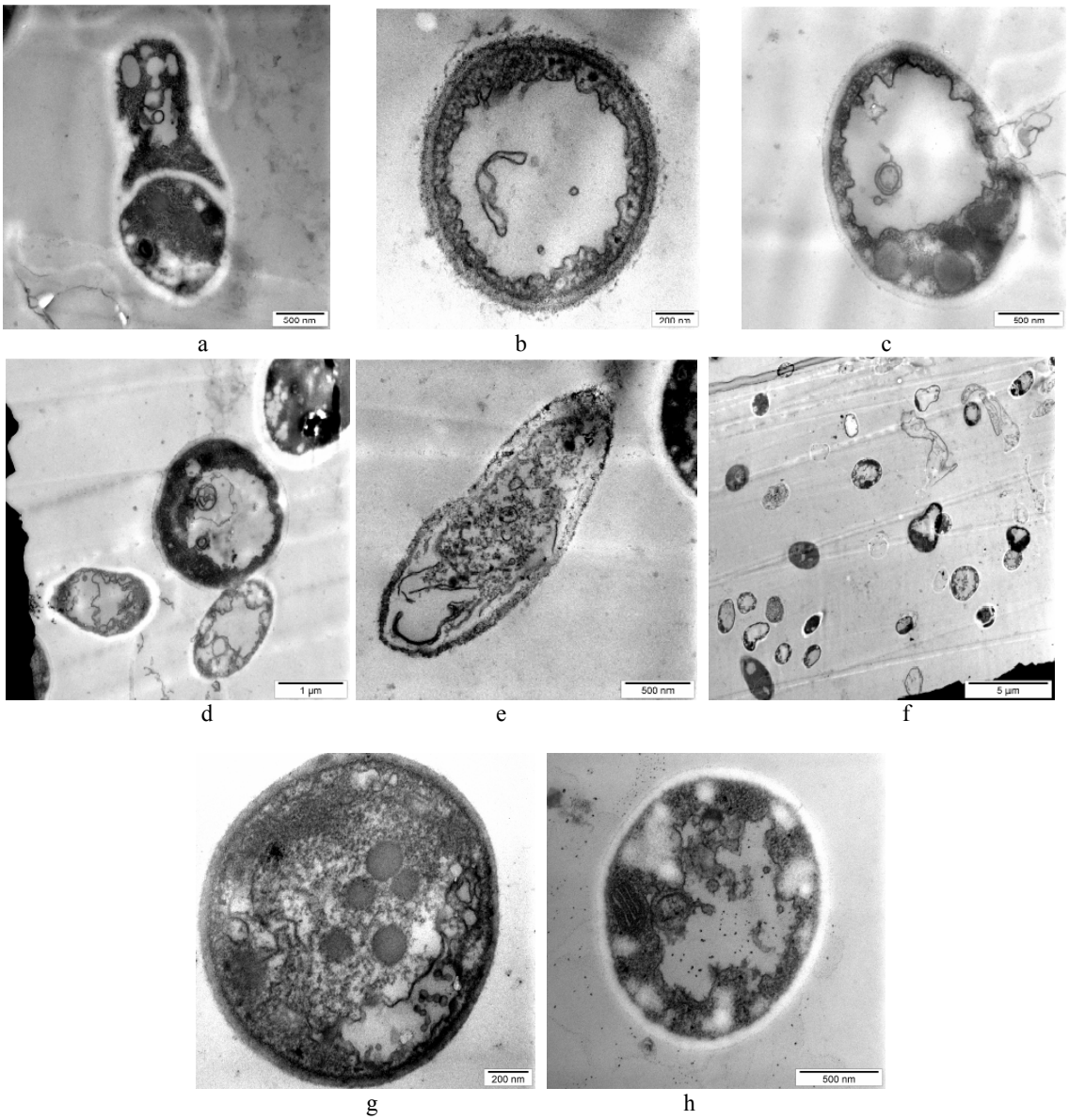
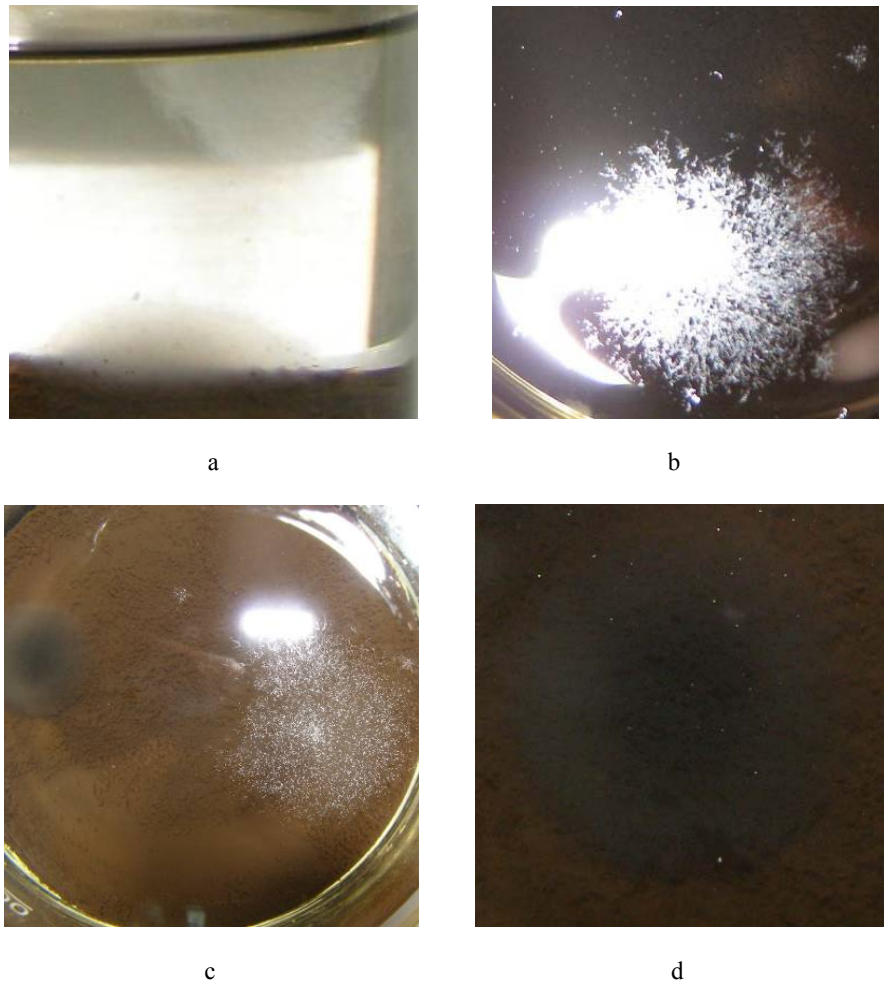


Fig. 3 Images of bacterial flora from the environment, on whose surface N_F nanoparticles were fixed. The images were taken with the electronic transmission microscope, Philips S208

Bacterial colonies development is allowed only if we suppose that the biochemical fixing structure is not toxic for the biochemical structure which it reaches. Because the functional groups that are responsible with the biochemical cross fixing (O=CH-) are generally toxic, the following intimate mechanisms of N_F fixing on the surface, in the immediate vicinity and in the depth of the target bacterium, are valid: the N_F biochemically active surface is chemically inactivated by biomolecules with small molecular mass which are subsequently absorbed specifically on the microorganism surface (cellular membrane) b) N_F fixes on the surface and in the depth of the cellular membrane, through unspecific biochemical links of the O=CH- type [proximity epitope], being

blocked at this level c) N_F has the appropriate dimensions to penetrate through the cellular membrane, fixing itself subsequently, through mechanisms of the a) and b) type, at the level of the areas with high specificity in relation to the previously mentioned mechanisms. The N_F nanoparticles patterns distribution on the contour and in the depth of the image of the analysed bacteria indicate the presence of all the three types of biochemical fixing mechanisms previously presented in details.

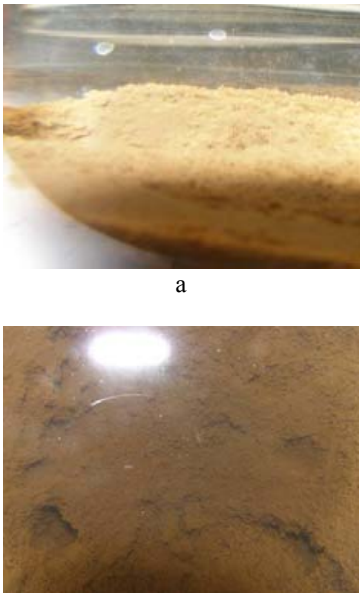
The shape and the size of the bacteria colonies are shown in the images in fig.3 and fig.4. The colonies were developed both on the surface and in the depth of the deposited N_F underlayer (fig.5). From this point of view difficulties of phenomenological interpretation occur, as deep traces were left in the underlayer where the samples were extracted from. These traces indicate that the bacterial colonies might feed directly on the N_F.



a b c d

Fig. 4. Images of bacterial flora colonies on whose surface N_F nanoparticles were fixed, before taking analysis samples. The images were taken with an Olympus SP-500UZ digital camera.

One should take this aspect into account in the process phenomenology; however, it does not explain other aspects of the biochemical fixing process which is being examined, as for instance: a) cellular division (involves metabolic processes which require a large spectrum of organic nutrients) b) the development of bacterial colonies in the liquid in which the slurry was present, separately from the N_F sediment c) the ingestion manner of complex mineral structures of the N_F type etc.



a

b

Fig. 5. Images of the N_F nanoparticle underlayer on which the bacterial flora was fixed after having taken the sample for analysis. The images were taken with an Olympus SP-500UZ digital camera.

3.1 The most probable chemical mechanism which influence the biological fixing process

After having morphologically and structurally analysed the patterns formed on the microorganism surface, one can draw the following conclusions: i) the microorganism surface presents areas which have high chemical affinity towards the **(L)** chemically activated surface ii) the **(L)** fixed on the microorganism surface have a discrete structure [fig.3, a)÷i)].

The explanation of these experimental data compels one to admit that the biochemically active surface of **(L)** structures is saturated with chemical groups of the same type (most of the aldehyde type), so that between the **(L)** particles electostatic repulsion should appear or stable chemical bindings should not be formed.

The acceptance of the saturation hypothesis of the active surface of the coating layer (L) with biochemically active chemical groups and with chemical groups meant to mask the chemical groups of the i) hydrolysable –OH type ii) ≡Si-O-Si≡, ≡Si-(ONH_3)-Si≡, b≡Si-NH_2, ≡Si-(ONH_3)-Si≡ [which would induct the creation of undesired crossed linked chemical bonds between the neighbouring (L) layers] demands that the covering layer (L) should be modelled by a complex physico-chemical structure of the type proposed in fig.6. Under these circumstances the structure of the polymeric network of the (L) layer is modelled by a relation of the $n[SiO_{1.5\gamma}\text{-}(CH_2)_3(NH_2)](NH_2)_{n\delta}(C_5H_8O_2)_\varepsilon$ (fig.6, $C_5H_8O_2$=R^1) type.

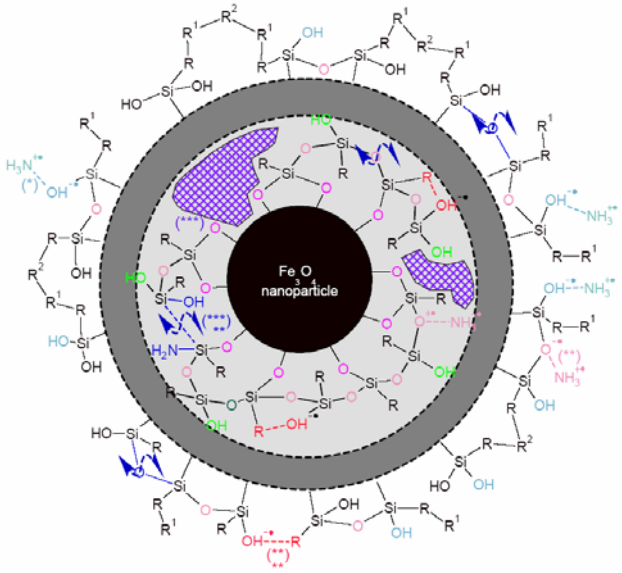


Fig.6 The type of chemical links that are likely to be established during the synthesis and growth of the C1 layer processes, and wich have an essential role in the distribution of the physical and chemical characteristics on the surface and in the deph of the C1 layer (*) adsorbtion points of the NH_3 through electrostatic links and the formation of new ones, of the NH_3-OH tupe (**) electrostatic adsorbtion points of the NH_3 around the ≡Si-O-Si≡ links and the formation of others of the ≡Si-(ONH_3)-Si≡ type (***) freely formed space in the polymerization network (****) formation points of hydrogen links between $–NH_2$ and -OH (*****) ≡Si-(ONH_3)-Si≡ link breaking points and the formation of new ones of the ≡Si-OH and ≡Si-NH_2 type under local electrostatic tension pressure and polymer network inner energy R = $[-(CH_2)_3\text{-}NH_2]$; R^1 = $[CHO\text{-}(CH_2)_3\text{-}CHO]$; R^2 = $[=CH\text{-}(CH_2)_3\text{-}CH=]$

The γ and δ parametres have been subsequently introduced to explain deviations from the theoretical structure model noticed as a result of the analyses.The γ parameter has been introduced to correct the deviations from the theoretical condensation process of (3-aminopropyl)-triethoxysilane. The insertion of this parameter has been imposed by the analysis of the characteristic X ray spectra which show a significant difference between the oxygen and the silicone quantities identified in the nanostructure.

The δ parameter models the sum of the amino groups, of the -NH_2, NH_3 şi NH_4OH type coming from the ≡Si-$(CH_2)_3NH_2$ links which cannot be hydrolised and the NH_3 fixed in the structure flaws of the polymeric layer through hydrogen links, elctronic polarization, difussion etc (fig.6).

The ε (ε≤δ) parameter models the superficial density of biological cross-linked molecules on the (L) surface.

This parameter expresses directly the $–NH_2$ group quantity wich are available for the fixing of a biologically crossed linked molecule . In this case, the number of the available $–NH_2$ groups is given by the group sum i) $-NH_2$ existing on the surface of the (L) layer ii) $–NH_2$ unmasked or blocked through process of the *, **, ***, ⁂ and ⁂* type.

These inconsistencies can be explained if one admits the fact that in the tridimensional network of (L) electrostatic tensions are generated due to the bonds of the ≡Si-OH---NH_3, ≡Si-(ONH_3)-Si≡, -NH_2---(HO)- (fig.6 / *, **, ⁂) type. An important role in the molecular chemosorbtion with low molecular mass is played by the free space bags (fig.6 / ***), unoccupied by the atoms or the molecules which make up (L). The holes in the molecular network of the (L) layer occur due to the surface flaws (e.g. the specific surface orientation), the group number – OH which can be hydrolised and which determine the policondensation degree of $C_9H_{23}NO_3Si$, the orientation in space of the links of the ≡Si–$(CH_2)_3NH_2$ which cannot be hydrolised type.

The ≡Si–C≡ bonds are responsible for the fluctuation of the condensation degree and for the Si/O variation ratio, according to the physical and chemical synthesis parameters, the length and the ramification degree of the carbon chain which bonds directly to the silicone. The internal tensions generated by the bonds which cannot be hydrolised model the γ [$\gamma \in (0, 1.5)$] ratio concerning the ≡Si–O–Si≡ bond formation through the reorientation of the adjacent silicone atoms. Some of the ≡Si–O–Si≡ bonds can break under the action of the electrostatic and mechanical tensions, forming new bonds of the ≡Si–OH and ≡Si–NH_2 ⁂* type. The bonds of the *, ** and ⁂ type are hydrogen bonds established in the growth stage of the (L) layer and subsequently integrated along with its growth. This type of bonds can also be established through NH_4OH diffusion in the depth of the already deposited polymer layer, or through internal structural reshaping occurring along with the variation of the local physical factors.

The variation of the physical and chemical absorbtion degree of amino groups can be explained through tha fact that along with the volume growth of (L) the number and size of vacant spaces where these groups can fix through infiltration also grow.

The introduction of auxiliary physical and chemical processes, according to the theoretical diagram in fig.6, is necessary in order to explain the experimental results which are being presented in fig.3. In other words, the acceptance of this type of processes (identified in the diagram in fig.3 under the form of [*, **, ***, ⁂, ⁂*]) explains and models a series of physical and chemical processes, such as: i) excellent structural time stability of structures of the (L) type ii) excellent chemical stability of the (L) structure in relation to the secondary hydrolysis processes iii) chemical time stability of the biologically activated surface, relative to the „self biological linking" of (L) neighbourhood.

4. Conclusions

The experimental data presented in this paper represents the theoretical and experimental support of future methods and techniques regarding the biological marking, analysis, detection and identification based on superparamagnetic nanometric composite slurries of the Fe_3O_4-{n[$SiO_{1.5}$-$(CH_2)_3$-(n-m)NH_2]-mNH-$C_5H_8O_2$} type.

The unconventional methods and techniques concerning the biochemical marking and control present a series of advantages over the classical ones, among which: a) a large spectrum of fixed biological structures b) the control possibility of the fixing, separation and identification processes in magnetic field [18] c) the study of the external topology and of the internal biochemical structure of the fixed biostructures [19] d) reduced costs, etc.

The determination of the main biochemical fixing mechanisms which come between the target microorganisms and the composite structures of the N_F type, will represent a real support in the elaboration of theoretical models of dynamics and kinematics determination [20, 21] of the biological structures marked with N_F in external control magnetic fields.

As a result of the biochemical fixing experiment presented above, the following issues were established: a) N_F fixes a large number of microorganisms (unlimited theoretically), without presenting affinity for a certain class or group of microorganisms b) the N_F fixing manner on microorganisms presents specific special morphism regarding the biochemical structure of the microorganism on which it was fixed d) the N_F dimensions allow these nanostructures to arrange themselves discretely in the distribution areas in the microorganism, allowing the continuous investigation of chemical, biochemical and physical processes existent at this level in the organism.

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