Nanostructures with liposomes and carbon nanotubes

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Our work was focused on obtaining biocomposites from liposomes and carbon nanotubes in order to find the best way for preparing functionalized nanostructures to be used as vectors for therapeutic molecules. Unilamellar liposomes were prepared by thin film hydration method. Chlorophyll *a* embedded into some of the lipid bilayers was used as a spectral marker to monitor the changes occurred in the liposomal membrane. Single walled carbon nanotubes have been added to liposomes and the biocomposites were studied by spectral methods (UV-VIS absorption and emission, DLS). The nano-biocomposites stability was checked by ξ-potential measurements and by luminol chemiluminescence experiments.

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1. Introduction

In recent years the interest in nanomedicine is rapidly growing in order to find more efficiently drug delivery systems (DDS) as promising tools for cancer treatment [1-4].

Liposomes are nanosized vesicles consisting in an aqueous core (hydrophilic compartment) surrounded by one or many phospholipidic bilayers (lipophilic compartment), structure very similar with that of natural membranes.

The importance of liposomes is related to their use as models for biomembranes and as DDS, with many biomedical applications [5-7].

Liposomes are considered excellent vehicles for the delivery of hydrophobic and hydrophilic anticancer drugs to tumor tissues [8].

Carbon nanotubes (CNTs) have attracted significant attention in the last decade due to their interesting properties and biomedical applications [9 - 11].

CNTs are the most studied nanostructures and their functionalization is aiming at making them good candidates for nanomedicine therapy.

"Materials of the future" – carbon nanotubes have a wide variety of applications in many fields: electronics (transistors, wires, switches, memory storage devices), optoelectronics (LED - light-emitting diodes), lasers, sensors, fibers and especially in biology and medicine (diagnosis and therapy, drug delivery systems, delivery of genes, vaccines, fluorescent markers for cancer treatment) [12-14], in biotechnology (biosensors), neuroscience, tissue engineering [15, 16].

Functionalization of the outer surface of CNT with biomolecules (nucleic acids, proteins, peptides, lipids) [17-19] is reported in the literature as leading to their internalization into the cell [20].

The functionalization of carbon nanotubes with phospholipidic model membranes was recently reported [21, 22].

The spectral features of chlorophyll *a* (Chl*a*) - a "valuable" molecule with antioxidant properties, allow its use as a sensor to monitor the changes occurred in the biomimetic membranes [23-26].

The aim of this study was to design new nanobiomaterials based on carbon nanotubes (CNTs) functionalized with artificial lipid bilayers, as promising carriers for an extensive range of biomedical applications.

2. Experimental part

2.1. Reagents

The lipids used for liposome preparation were dipalmitoyl phosphatidylcholine (DPPC) and soybean lecithin, purchased from Sigma Aldrich (Germany).

KH_2PO_4, Na_2HPO_4, luminol (5-amino-2,3-dihydro-phthalazine-1,4-dione), Tris, HCl, H_2O_2 were supplied from Merck (Germany).

Solvents of analytical grade used for Chl*a* extraction (ethanol, methanol, n-propanol, petroleum ether, acetone, ethyl ether) and for lipid film preparation (chloroform) were purchased from Merck (Germany).

Single walled carbon nanotubes (SWCNTs) were purchased from Aldrich (Germany).

2.2. Liposome and bionanocomposite preparation procedures

Liposome preparation procedure

Unilamellar liposomes (ULVs) were prepared by thin film hydration method [27] with little modifications [28], followed by an ultrasound treatment using a titanium probe sonicator (15 min, Hielser, UP 100 H) above the critical temperature (T_c) of phase transition of lipids

resulting in liquid crystalline lipid bilayers. Different types of liposomes were obtained by using various lipids: soybean lecithin and dipalmitoyl phosphatidylcholine (DPPC).

In some samples of liposomes, Chl*a* was inserted into artificial membranes during the lipid film preparation (Chl*a*/lipid molar ratio=1/100), as a spectral marker.

Chl*a* was prepared from fresh spinach leaves by a chromatographic method according to Strain & Svec method [29] and checked for purity.

In the case of the samples with Chl*a*, the experiments were carried out in dark due to the photosensitivity of this photopigment.

Preparation of biocomposites liposomes/carbon nanotubes

Single walled carbon nanotubes (SWCNTs) have been added to liposome suspensions in different SWCNT/liposomes ratios. Massic ratios are specified for each sample.

Two variants were used as follows:

Variant V1: specific aliquots from a stock suspension SWCNTs were added to suspension of MLV (*Multilamellar Lipid Vesicles*) or ULV (SUV – *Small Unilamellar Vesicles* or IUV - *Intermediate Unilamellar Vesicles*). The mixed suspensions were further sonicated by using a sonicator with Ti probe.

Variant V2: specific aliquots from a stock suspension of SWCNTs were added in the hydration phase of liposome preparation. The mixed suspension was further sonicated by using a sonicator with Ti probe.

2.3. Characterization methods

UV-VIS spectroscopy analysis

The absorption spectra of liposomes and nano-biocomposites were recorded on a double beam UV-VIS spectrophotometer Lambda 2S Perkin Elmer (PECSS software), in the wavelength range of 200-800 nm.

Fluorescence analysis

The fluorescence emission spectra of Chl*a* in liposomes and nano-biocomposites were performed on a PERKIN-ELMER LS55 spectrophotofluorimeter.

The samples were illuminated with a 430 nm excitation light.

DLS technique

The evolution of size distribution of the prepared liposomes with different lipids and the hydrodynamic diameters of liposomes suspended in phosphate buffer (pH 7.4) were determined by dynamic light scattering (DLS) technique using a Zetasizer Nano ZS (Malvern Instruments Ltd., U.K.), at a scattering angle of 90^o and 25^{o}C temperature. The particle size analysis data were evaluated using intensity distribution. The measuring range of dynamic light-scattering particle-size analyzer is 0.6 nm – 6 μm. The average diameters (based on Stokes-Einstein equation) and polydispersity index (which indicates the width of the size distribution and has a value between 0 and 1) were calculated from the three individual measurements.

ξ-potential determination

The measurement of electrokinetic potential is used to assess the charge stability of a disperse system. The measurement of ξ-potential was realized by using the appropriate dispositive of Zetasizer Nano ZS (Malvern Instruments Ltd., U.K.). Zeta potential is measured by applying an electric field across the analyzed aqueous dispersion. All measurements were performed in triplicate.

Chemiluminescence method

The *in vitro* antioxidant activity of liposomes and nano-biocomposites has been determined by chemiluminescence method using a Chemiluminometer Turner Design TD 20/20, USA.

A cyclic hydrazide (luminol) has been used as light amplifying substance which emits light when oxidized and is converted into an excited aminophtalate ion in the presence of oxidizing species such as superoxide (O^{2-}), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH) and singlet oxygen ($^1O_2$).

The luminol increases the detection sensitivity of activated oxygen species in a sample. H_2O_2 in TRIS-HCl solution buffer (pH = 8.6) has been used as a generator system for free radicals.

3. Results

Chl*a* (extracted from fresh spinach leaves) embedded into some of the artificial lipid bilayers was used as a spectral marker to monitor the changes occurred in the liposomal membrane, at molecular level.

Single walled carbon nanotubes (SWCNTs) have been added to liposome suspensions and the resulted composite nanostructures were studied by spectral methods (UV-VIS absorption and emission, DLS).

The physical stability of nano-biocomposites was checked by performing ξ-potential measurements and the chemical stability was checked by luminol chemiluminescence assays.

Characterization of Chla - liposomes and nano-biocomposites by UV-VIS absorption spectroscopy

Fig. 1 shows the absorption spectra of the biocomposites Chl*a*-liposomes/SWCNTs. The absorption spectra in VIS of biocomposites are due to Chl*a* absorption.

It is observed a wavelength dependence of light scattering. It is known that particles in suspension exhibit a Rayleigh type scattering, more pronounced when the particle size is smaller than the wavelength ($R<\lambda/20$). All spectra were corrected (see Fig. 2) as regarding the Rayleigh scattering and then normalized against the absorption at the maximum in red (OD at 670 nm).

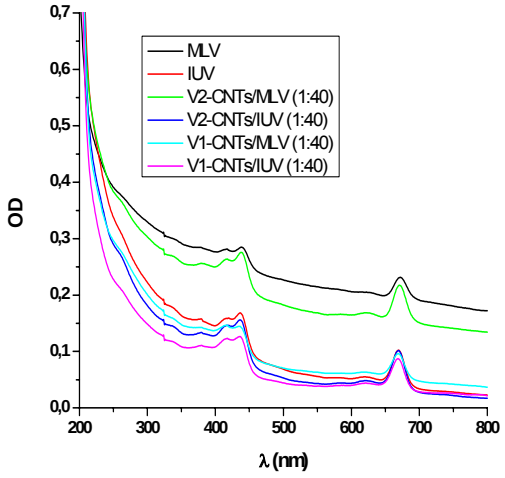


Fig. 1. Absorption spectra (uncorrected) of the biocomposites Chla- DPPC (0.5 mM) liposomes/SWCNTs.

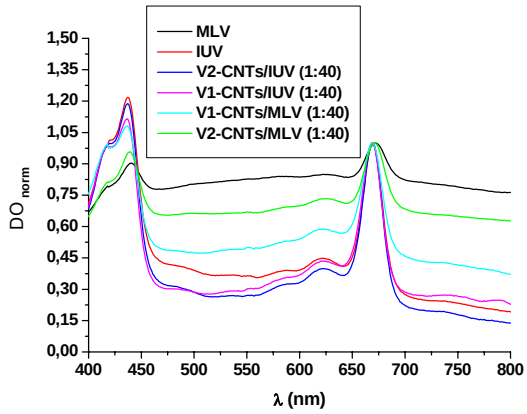


Fig. 2. The VIS absorption spectra (corrected) of Chla in DPPC (0.5 mM) - liposomes and in biocomposites DPPC (0.5 mM) - liposomes/SWCNTs.

Greater absorbance values were observed in the case of milky MLV suspensions (due to their higher dimensions) as compared to those of clear IUV suspensions (due to their smaller dimensions).

Fluorescence behavior of liposomes and nano-biocomposites

Fig. 3 reveals the emission spectra of chlorophyll *a* in Chl*a*-liposomes/CNTs biocomposites obtained using excitation wavelength of 430 nm.

Chl*a* placed at the lipid bilayer/aqueous medium interface, in the vicinity of polar heads of lipids, was used as a fluorescent marker.

The variations in emission peak position are within the resolution working limits. The main fluorimetric peak was about 678 nm.

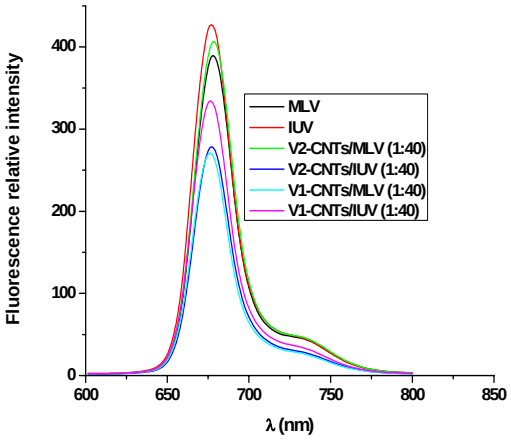


Fig. 3. Fluorescence emission spectra of Chla in DPPC (0.5 mM) -liposomes and in biocomposites Chla-DPPC (0.5 mM) liposomes/SWCNTs (excitation wavelength: 430 nm).

There is a net difference between the fluorescence intensities of IUVs-based biocomposites and those based on MLVs.

Characterization of liposomes and bionanocomposites by DLS technique

Liposomes are specially used as drug carriers, so the main factor that influences their *in vivo* behavior is the vesicle size.

The Table 1 illustrates the hydrodynamic diameters, $z_{average}$ (the particle diameter plus the double layer thickness) and polydispersity index, PdI (the measure of the distribution of nanoparticle population) of each Chl*a*-DPPC liposomes/CNTs biocomposites prepared. The DLS results indicated that the prepared biocomposites exhibited different behavior, with monomodal and bimodal particle size distributions and polydispersity indices ranging from 0.437 ÷ 0.673. The high values for PdI indicate a large size distribution with multiple liposome population. However, after SWCNTs addition to liposomal suspensions, followed by ultrasound irradiation steps the values of mean particle size and of the polydispersity index (PdI) are decreasing.

Table 1. DLS data of the Chla-DPPC (0.5 mM) liposomes/CNTs biocomposites prepared.

Sample	Z average (nm)	PDI	Peak1 (nm)	Peak2 (nm)	Peak3 (nm)
V1-Chl*a*-DPPC-MLV	810.1	0.480	847.5 (100%)	-	-
V1-Chl*a*-DPPC-SUV	331.3	0.487	592.5 (53.9%)	115.8 (46.1%)	-
V1-Chl*a*-DPPC-MLV/CNTs	481.4	0.673	561.8 (57%)	118.9 (43%)	-
V1-Chl*a*-DPPC-SUV/CNTs	197.8	0.406	149 (72.3%)	835.4 (24.1%)	5138 (3.6%)
V2-Chl*a*-DPPC-MLV/CNTs	248.2	0.560	450.1 (58%)	112.0 (40.5%)	5560 (1.5%)
V2-Chl*a*-DPPC-SUV/CNTs	330.7	0.437	127.5 (66.1%)	576.1 (33.9%)	-

A size distribution profile is exemplified in Fig. 4 for soybean lecithin vesicles. The MLV liposomes exhibited a monomodal particle size distribution, with a good polidispersity index of 0.279.

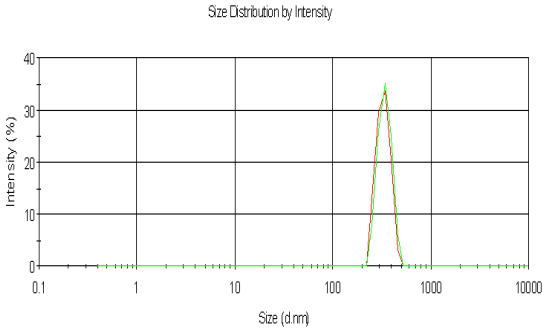


Fig. 4. The particle size distribution of the suspensions of soybean lecithin (0.5 mM) MLV liposomes, suspended in phosphate buffer (pH 7.4)

Evaluation of physical stability of liposomes/CNTs biocomposites

The physical stability of the lipid nanostructures was evaluated in terms of ξ potential. The electrokinetic potential reflects the electric charge on the particle surface, being a key factor to evaluate the physical stability of colloidal dispersions. When the absolute value of ξ is higher than ± 25 mV for colloidal formulation, the particles are electrochemically stable under the investigated conditions.

The physical stability of liposomes/CNTs biocomposites was dependent on the variant used for preparation and on the massic ratio liposomes/CNTs.

The surface charge of all the lipid nanostructures was negative, ranging from -4.71 to -30.3 mV (Table 2).

In two cases the zeta potential values were less than -25 mV (Table 2). These values demonstrated that the V1-CNTs/SUV obtained at a molar ratio of 1:25 and 1:120, respectively, are physically stable systems.

A moderate physical stability (with ξ = -23.3 mV) manifests the sample prepared with the second variant of preparation and at a massic ratio of 1:160, whilst the multilamellar and SUV liposomes do not show a physical stability.

Table 2. ξ potential values of the soybean lecithin (0.5 mM) liposomes/CNTs biocomposites (for each sample the massic ratios are specified in brackets).

Sample	**Zeta potential [mV]**
V1-CNTs /SUV (1:25)	-27.5 ± 5.20
V1-CNTs /SUV (1:120)	-30.3 ± 1.67
V1-CNTs /MLV (1:25)	-12.9 ± 1.45
V2-CNTs /SUV (1:160)	-23.3 ± 2.40
SUV	-4.71 ± 0.43

The oxidative stress behavior of nanobiocomposites

It is well known that *oxidative stress* is a disturbance in the prooxidant-antioxidant balance in favor of the

prooxidant species, leading to potential damage [30].

In this work, the oxidative stress was simulated *in vitro*, on different types of nanostructures with liposomes and carbon nanotubes, using the luminol chemiluminescence (CL) system.

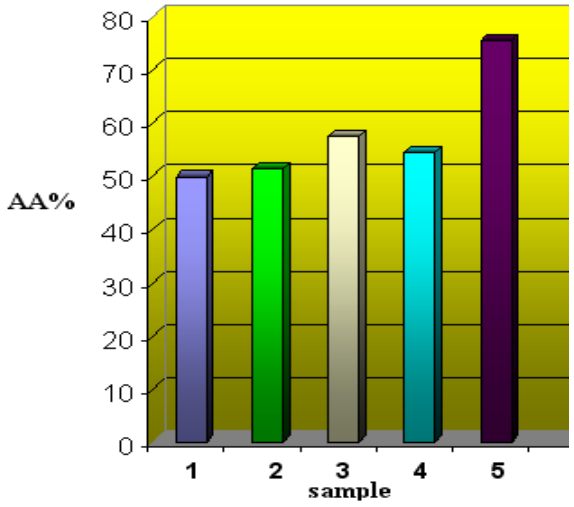
A large range of free radicals of oxygen were produced by the oxidative degradation of luminol in presence of H_2O_2 in alkaline buffer.

The antioxidant activity (percentage of free radical scavenging) was calculated for each sample using the relation:

$$AA = \frac{I_0 - I}{I_0} \cdot 100\%$$

where I_0 is the maximum CL intensity for standard at t = 5 s and I is the maximum CL intensity for sample at t = 5 s. The *standard* represents the reaction mixture without the sample.

Fig. 5 shows that the biocomposites obtained from SUVs by V2 preparation variant are more stable against oxidative stress than the other samples.



Legend
1 = V1-CNTs/SUV (massic ratio 1:120)
2 = V1- CNTs/SUV (massic ratio 1:25)
3 = V1- CNTs/MLV (massic ratio 1:25)
4 = V1- CNTs/MLV (massic ratio 1:120)
5 = V2- CNTs/SUV (massic ratio 1:160)

Fig. 5. Antioxidant activity of soybean lecithin (0.5 mM) liposomes/CNTs biocomposites prepared.

4. Discussion

The presence of the carbon nanotubes affected the dimensions of the biocomposites liposomes/ SWCNTs obtained by the both variants of preparation. Position of the Chl*a* absorption maximum does not change, so the Chl*a* location in lipid bilayers is maintained in the same place and is not affected by the interaction lipid/nanotube.

The fluorescence quenching in the case of multilamellar liposomes (MLVs) towards the unilamellar liposomes (SUVs or IUVs) can be explained by efficient energy transfer between chlorophyll molecules of MLV lamella. A decrease in fluorescence relative intensities it is observed in the case of liposomes/CNTs biocomposites compared to simple liposomes (MLV and ULV, respectively). One explanation could be a more efficient energy transfer between chlorophyll molecules of lipid bilayers of the liposomes ordered along the carbon nanotubes. The ULV size does not exceed 100-200 nm and the MLV size does not exceed 1000 nm, so the lipid vesicles can be ordered over micrometer length carbon nanotubes, due to interactions lipid / nanotube.

The zeta potential analysis revealed that the presence of carbon nanotubes has a benefic effect on the stability of the liposome suspensions. Variant V1 of nanoconstruct preparation resulted in obtaining of more stable biocomposites. However, the nanocomposites obtained by variant V2 provide a short-term stability.

The chemiluminescent assay revealed a better stability against oxidative stress for biocomposites obtained from SUVs by V2 preparation variant than the other samples.

5. Conclusions

In this work two types of preparation have been developed for biocomposites based on liposomes and carbon nanotubes.

The results obtained by monitoring the biocomposite spectra using chlorophyll as a molecular sensor revealed the existence of interactions between carbon nanotubes and artificial lipid bilayers. Interaction with carbon nanotubes has significant effects on the structure of the liposomes.

Important factors in determining the stability to physical and chemical stress proved to be: the size and lamellarity of liposomes, the relative concentration of carbon nanotubes/liposomes in the process of biocomposite preparation.

Zeta potential analysis and chemiluminescence measurements showed that there are optimal massic ratios liposomes/carbon nanotubes to increase the physical and chemical stability of the nano-biocomposites.

The noncovalent functionalization of carbon nanotubes by building nano-biocomposites with liposomes, presented in this study, is a promising technique for biomedical (nanovectors) and biotechnological applications (biosensors).

A future task is also to improve the liposome stability by incorporating different antioxidants with synergistic interactions and by modifying the bilayer composition.

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