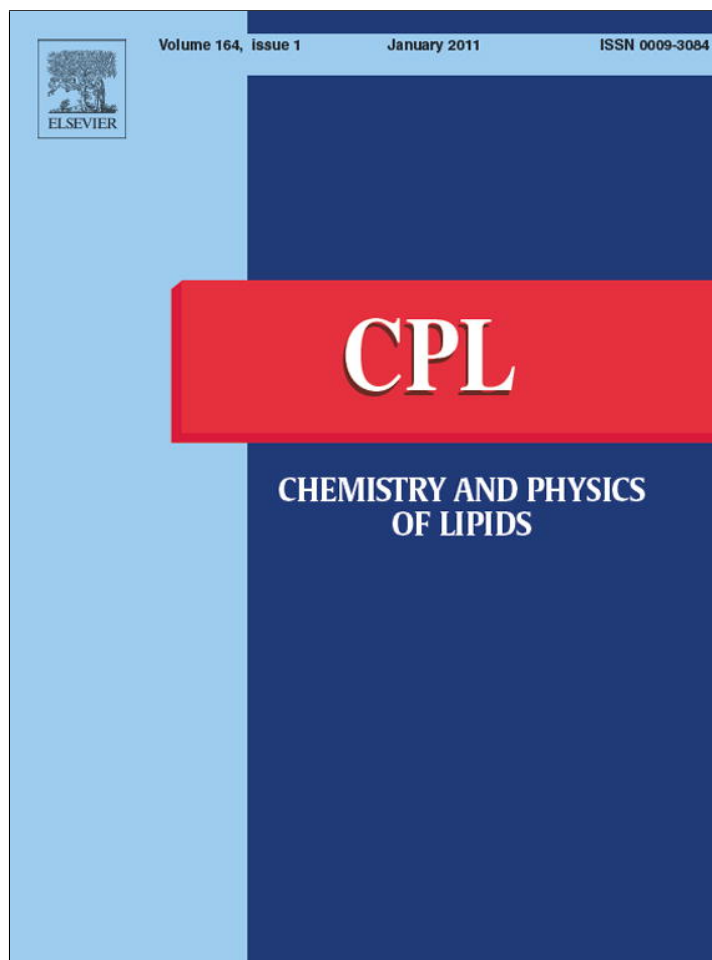


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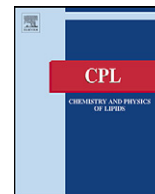
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Chemistry and Physics of Lipids

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Short communication

Electric-field controlled liposome formation with embedded superparamagnetic iron oxide nanoparticles

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ARTICLE INFO

Article history:

Received 29 September 2011

Received in revised form

10 November 2011

Accepted 11 November 2011

Available online 23 November 2011

Keywords:

Nanoparticles

Giant liposomes

Encapsulation

Electroformation

ABSTRACT

Liposomes are one of the most promising biomaterial carriers to deliver DNA,¹ proteins, drugs and medicine in human bodies. However, artificially formed liposomes have to satisfy some crucial functions such as: (i) to efficiently carry drugs to targeted systems, (ii) to be biologically stable until they are removed from human body, (iii) to be biodegradable, and (iv) to be sufficiently small in size for effective drug delivery. Here, we report an efficient and novel method to simultaneously manufacture and incorporate super-paramagnetic iron-oxide nanoparticles (efficient target finder in the presence of external magnetic field) into the liposome's interior and its bilayer. In this technique, we use electric field to control the formation of liposomes and the incorporation of iron oxide nanoparticles. Our preparation procedure does not require any chemical or ultrasound treatments. Apart from that, we also provide further experimental investigations on the role of electric fields on the formation of liposomes using XPS² and the magnetic-optical microscope.

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1. Introduction

Liposome is bilayered membrane structure composed of phospholipid molecules (Torchilin, 2005). The liposome interior consists of internal aqueous solution (sucrose in our case) and external aqueous solution (in our case a mixture of sucrose and glucose solution). The liposome's bilayer could also consist of a mixture of phospholipids into which the cholesterol could be incorporated. The phospholipids are amphiphilic molecules consisting of a polar part, i.e. head, and a non-polar part, i.e. tails.

Liposomes have already been used before, as pharmaceutical carriers as a result of their unique surface chemical property that can be modified with specific ligands (Torchilin, 2005; Ismailova et al., 2005). By combining them with magnetic nanoparticles (also called magnetoliposomes or magnetosomes for short), one could effectively manipulate the flow of magnetoliposomes in human body with external magnetic field. This is one of the important reasons why magnetosomes have been applied to deliver genes, drugs, proteins and various types of medicines to targeted tissues. Such applications are possible due to the existence of bilayers, containing a hydrophilic and a hydrophobic part that can capture either the polar (water) soluble compounds inside the liposome (core)

or nonpolar (oil) soluble compounds between the bilayers (Sharma and Sharma, 1997). The possible techniques to produce the magnetosomes are: (i) ultrasound treatment, which produces small sized liposomes (diameter of ≈ 200 nm) (Lapinski et al., 2007), (ii) extrusion method which also produces small liposomes (less than $5 \mu\text{m}$) (Zhang and Granick, 2006), (iii) electroformation method which uses an alternating electric field applied between two conductive electrodes (Angelova and Dimitrov, 1986; Dimitrov and Angelova, 1988; Giria et al., 2005; Sangregorio et al., 1999; De Cuyper and Valtonen, 2001; De Cuyper and Joniau, 1988; Fortin-Ripoche et al., 2006; Sabate et al., 2008). By changing the parameters of electric field (amplitude and frequency), we were able to systematically study the effect of electric field on the size of the liposomes.

We chose iron oxide nanoparticles since they are biocompatible, and due to their superparamagnetic property-high degree of magnetic susceptibility in the presence of external magnetic field below the Curie temperature (Wei et al., 2008). Therefore, one can easily manipulate the flow directions of the magnetosomes in blood vessels. The manipulation of magnetic nanoparticles via external magnetic field is one of the fastest developing technique in biomedicine. For example, they are widely used in cellular therapy, cell labelling, cancer cell targeting and as a "tool" in cell biology research to separate and purify cell populations or extract solutions (cell sorting), tissue repair, drug delivery, MRI,³ hyperthermia and magnetofection (Gupta and Gupta, 2005).

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E-mail address: janez.pavlic@fe.uni-lj.si (J.I. Pavlič).¹ DNA – deoxyribonucleic acid.² XPS – X-ray photoelectron spectroscopy.³ MRI – magnetic resonance imaging.

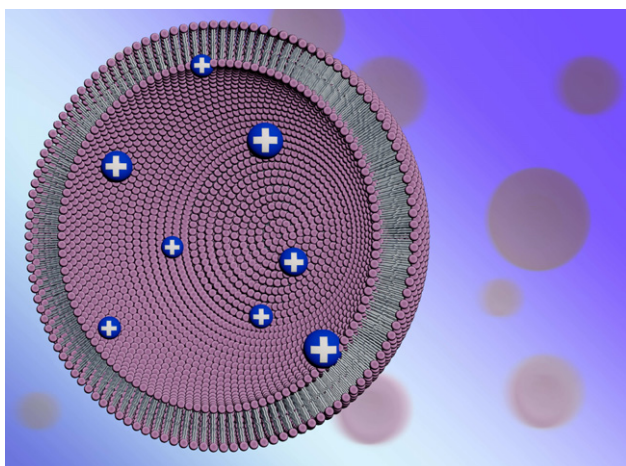


Fig. 1. Schematic figure of a 3D model of liposome with encapsulated positively charged superparamagnetic nanoparticles.

Specifically functionalised magnetic nanoparticles can be used as a sensitive detection procedure for bacteria via magnetic relaxations (Kaittani et al., 2007). The magnetosomes having encapsulated iron oxide nanoparticles require high magnetisation values, with uniform size, and most importantly, have stable and functionalisable surfaces. They should work specifically and have to be stable at different temperatures and not agglomerate in buffer solutions. The benefits of magnetosomes are: (i) nanoparticles can be functionalised without toxic chemicals, (ii) the size of liposomes can be controlled with electric field, (iii) the liposomes are well known to be biocompatible, biodegradable and have complete absence of toxicity (including the absence of antigen and allergic reactions in the human body) (Torchilin, 2005).

In this work, we will describe the influence of surface properties (electric charge) of magnetic nanoparticles during the encapsulation of iron oxide nanoparticles into the liposomes' core or also possibly into the bilayer, in the presence of electric field as shown in schematic Fig. 1. Subsequently, we shall discuss and explain why our procedure to prepare magnetosomes is reliable, straightforward and does not require a multi-step chemical processes. For example, electric fields when applied in short-time pulses have significant effects on living organism (Huelsheger et al., 1983; Favre and Schueler, 2008).

2. Materials and methods

2.1. Synthesis of magnetic nanoparticles

The synthesis of superparamagnetic maghemite nanoparticles ($\gamma\text{-Fe}_2\text{O}_3$) has been carried out via a controlled chemical coprecipitation approach (Slomkowski, 1998; Dauphas et al., 2009; Legeay et al., 2006; Velzenberger et al., 2008). Prior to synthesis an aqueous mixture of ferric and ferrous salts and sodium hydroxide as an alkali source were prepared separately as stock solutions. In this method the corresponding metal hydroxides were precipitated during the reaction between the alkaline precipitating reagent and the mixture of metal salts. The metal hydroxides were subsequently oxidised with air resulting in the formation of the $\gamma\text{-Fe}_2\text{O}_3$ spinel product.

Superparamagnetic $\gamma\text{-Fe}_2\text{O}_3$ nanoparticles obtained after the synthesis cannot be directly dispersed in a liquid medium to obtain stable colloid suspension (called ferrofluid). In this regard, it is necessary to chemically modify the particle surface in order to prevent particle aggregation and subsequent sedimentation. The surface of prepared $\gamma\text{-Fe}_2\text{O}_3$ nanoparticles were stabilised electrostatically,

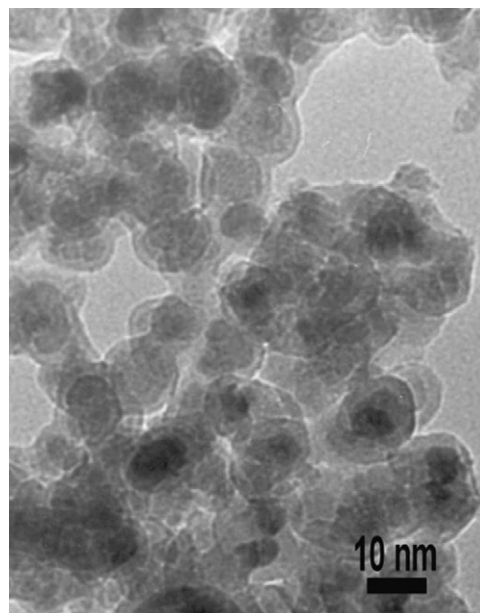


Fig. 2. TEM image of iron oxide nanoparticles. The size of nanoparticles is 10 ± 2 nm.

with positive and negative surface charge, and sterically, with amino ($-\text{NH}_2$) and hydroxyl ($-\text{OH}$) alkyl groups. Thus, four different aqueous based colloidal suspensions were prepared.

Prepared nanoparticles were characterised using XRD⁴ and TEM.⁵ Specific surface area of powders was determined by BET method (Brunauer, Emmett and Teller) (Brunauer et al., 1938). A specific magnetisation of the prepared nanoparticles was also measured using VSM.⁶ The size of $\gamma\text{-Fe}_2\text{O}_3$ nanoparticles was about 10 ± 2 nm as can be seen in Fig. 2.

2.2. Synthesis of magnetic liposomes – magnetoliposomes

A modified method of electroformation was used for formation of giant phospholipid vesicles, i.e. liposomes (Angelova and Dimitrov, 1986). The liposomes were prepared at room temperature (25°C). A synthetic lipid POPC⁷ and cholesterol (both purchased from Avanti Polar Lipids) were used. Adequate volumes of POPC and cholesterol, both diluted in chloroform or mixture of chloroform:methanol (volume ratio 2:1) were first mixed together in a volume ratio 4:1 and then thoroughly mixed by a vortex, as can be seen on the left side of Fig. 3.

About $20\ \mu\text{l}$ of the obtained lipid mixture was applied to each of the two platinum electrodes. The electrodes were then put to an exicator, from which air and volatile solvent were extracted by vacuum pump for 60 min. The electrodes coated with a lipid mixture were put into a plastic vial for electroformation, to which 1.6 ml of 0.2–0.3 M sucrose solution was added. To sucrose solution ferrofluid was simultaneously added. The ferrofluid in sucrose solution was diluted by 1600-times in volume. Nanoparticles were embedded into the interior of the lipid bilayer and liposome itself during electroformation of phospholipid vesicles.

Two platinum electrodes were connected to an AC function generator with an output voltage amplitude of 5 V, wherein the equivalent electric field intensity was 1000 V/m at the frequency of 10 Hz for 60 min. The voltage and frequency were then lowered to

⁴ XRD – X-ray diffractometry.

⁵ TEM – transmission electron microscopy.

⁶ VSM – vibrating sample magnetometer.

⁷ POPC – 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine.

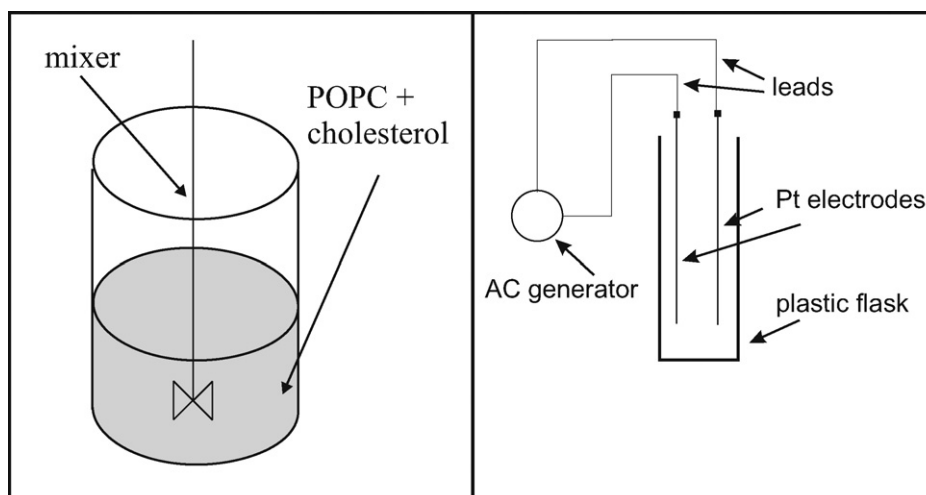


Fig. 3. Schematic figure of experimental setup: left figure shows preparation of lipid mixture; right figure shows set-up for formation of liposomes in an electric field.

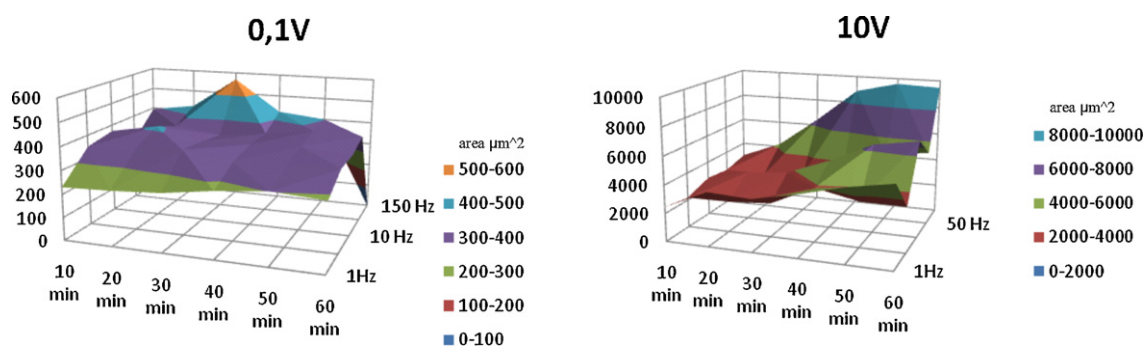


Fig. 4. Size dependence of time and frequency at amplitude voltage of 0.1 V in left figure and amplitude voltage of 10 V during electroformation of magnetoliposomes is presented.

1 V, wherein the equivalent electric field intensity was 200 V/m at frequency of 2 Hz for 30 min.

2.3. Characterisation of magnetosomes

Magnetic liposomes which were formed according to procedure mentioned above were analysed by phase contrast optical microscope to see their structure, size and response to an external magnetic field. Liposomes were observed by an inverted optical microscope Zeiss Axiovert 200 with phase contrast optics (optical magnification was 1000 \times) and recorded by the Sony XC-77CE video camera. The evidence of encapsulation of nanoparticles in liposomes was their movement in the presence of external magnetic field generated by external permanent magnets. Liposomes having no magnetic particles were not moving.

In our experiments we also investigated the effect of the applied sine voltage signal to the Pt electrodes at different frequencies and amplitudes using positively charged magnetic nanoparticles. The

effects of different applied voltage signals were observed via the measurements of size (radii) of several vesicles. From Fig. 4 effect of changes in the electric field parameters (voltage and frequency) on the vesicle size (radius) can be clearly seen. The sizes (radii) were measured using ImageJ software.

Voltage signal was applied at constant voltage and frequency until the end of one electroformation procedure. In the following experiments we changed the frequencies, but kept the voltage amplitude constant. Then we increased the voltage and changed the frequencies at the same voltage amplitude. This was conducted throughout all the voltages (from 1 V to 5 V by an increment of 1 V) and frequencies (from 1 Hz to 10 Hz by an increment of 2 Hz). Fig. 5 shows a micrograph of time sequence of magnetosomes growing during electroformation process at 5 V an 3 Hz.

Liposomes were further analysed by XPS to see their chemical composition. Samples were analysed with an XPS instrument TFA XPS Physical Electronics. Since XPS analyses are performed in vacuum, a drop of solution containing magnetic liposomes was put on

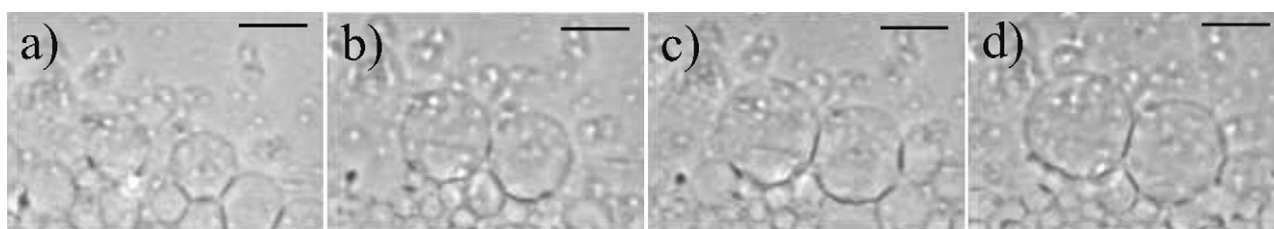


Fig. 5. Micrographs depict solution containing magnetoliposomes with positively charged magnetic nanoparticles prepared at 5 V and 3 Hz in time (a) after 10 min of electroformation, (b) 20 min, (c) 30 min and (d) 40 min. Bar is 10 μ m, optical magnification is 1000 times.

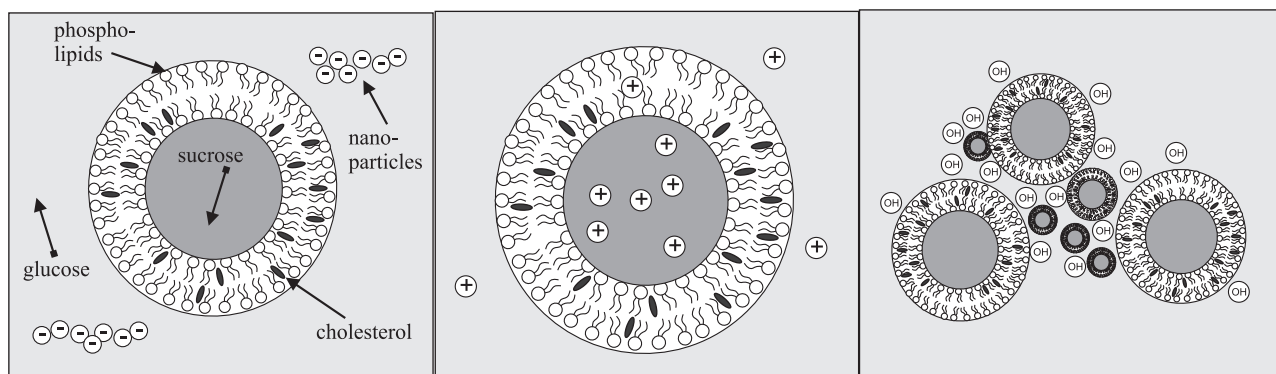


Fig. 6. Models of interaction of various nanoparticles with phospholipid vesicles are schematically presented. Left cartoon shows liposomes with negatively charged magnetic nanoparticles; middle cartoon shows liposomes with positively charged magnetic nanoparticles; right cartoon shows liposomes with magnetic nanoparticles functionalised with $-OH$ groups on the surface.

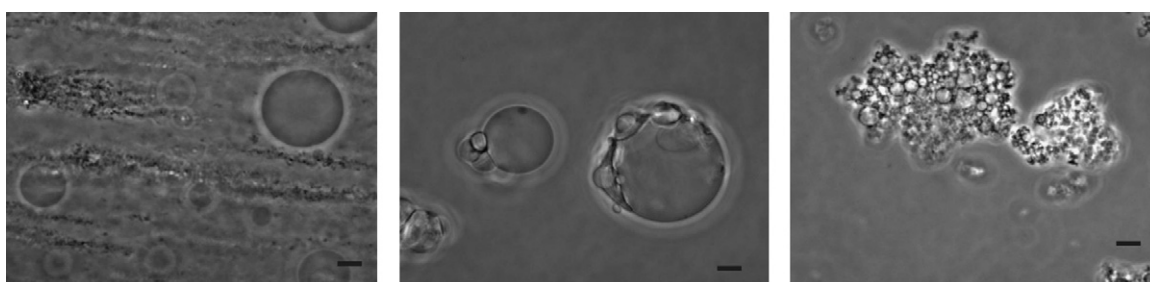


Fig. 7. Left micrograph presents solution containing liposomes with negatively charged magnetic nanoparticles. Middle micrograph presents solution containing liposomes with positively charged magnetic nanoparticles. Right micrograph presents solution containing liposomes with $-OH$ groups on the surface of nanoparticles. Bar is 10 μm , optical magnification is 1000 times.

Si slice and dried. Such sample was then placed to XPS chamber and excited with X-rays with a monochromatic $Al K_{\alpha 1,2}$ radiation at 1486.6 eV. Photoelectrons were detected with a hemispherical analyser positioned at an angle of 45° with respect to the normal to the sample surface. Survey-scan spectra were made at a pass energy of 187.85 eV and 0.4 eV energy step. The concentration of elements was determined by using MultiPak v7.3.1 software from Physical Electronics, which was supplied with the spectrometer (Moulder et al., 1992).

3. Results

In Fig. 6 models of interaction among liposomes with different functionalised magnetic nanoparticles, which were formed by the described method, is schematically shown.

In Fig. 7 micrographs of electroformed giant magnetosomes are presented. Left figure shows agglomerate of negatively charged magnetic nanoparticles arranged around electroformed liposome. Middle figure shows the liposomes, into which positively charged magnetic nanoparticles are encapsulated. The magnetosomes rotated under the influence of the external rotating magnetic field. Right figure shows agglomerates of very small liposomes (less than 5 μm) with magnetic nanoparticles that have $-OH$ groups on the surface. We believe, that vesicle agglomerates are formed, due to the binding of nanoparticles to their surfaces.

4. Discussion

The schematic Fig. 6 implicates various ways of incorporation of magnetic nanoparticles into the liposomes as a function of their surface functionalisation. Schematic Fig. 2 indicates that negatively charged magnetic nanoparticles are repelled from the phospholipid

bilayer, which is partially negatively charged as well. Schematic Fig. 3 implicates how positively charged magnetic nanoparticles get encapsulated into the liposome. Fig. 4 shows a system of magnetic nanoparticles with superficially bound $-OH$ groups and liposomes. Such magnetic nanoparticles might induce the binding to the polar part of amphiphilic lipid molecules, thus forming small micelles. In order to be stable in the solution of glucose and sucrose, the micelles start clumping together and form large agglomerates. The incorporation of magnetic nanoparticles into liposomes was confirmed by an external rotating magnetic field, since the liposomes started to rotate under the influence of the external magnetic field. This is not the case for liposomes formed with nanoparticles having bound $-NH_2$ groups and nanoparticles having negative surface charge. In the case of nanoparticles having negative surface charge, the liposomes were stationary in the presence of external magnetic field, meaning that they were not encapsulated by a liposome or incorporated into its bilayer. As mentioned above the negatively charged magnetic nanoparticles were repelled from the phospholipid bilayer, which is partially negatively charged as well. This has hindered their incorporation into the liposomes. The formation of magnetosomes was hindered in the case of nanoparticles having bound $-NH_2$ groups, as no liposomes were formed at all. It seems that this inhibits the liposome formation.

To further prove the presence of magnetic particles in the liposomes, the samples were analysed by XPS to obtain their chemical composition. In Fig. 8 we can see an example of the chemical composition of a liposome containing magnetic nanoparticles and the composition of an empty liposome having no nanoparticles. In the first case, we can see oxygen and carbon originating from phospholipids as well as high peaks due to Si and Fe which are constituents of magnetic nanoparticles, while in the second case, we can observe only oxygen and carbon originating from phospholipids.

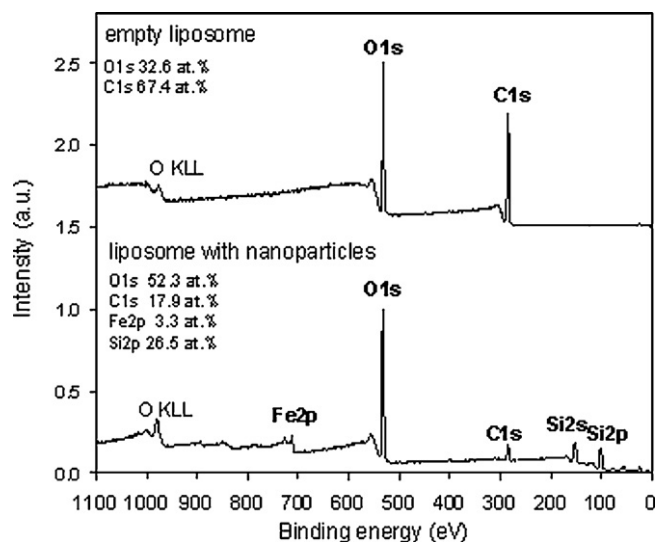


Fig. 8. XPS survey spectrum of synthesised magnetosomes showing their chemical composition (at.%).

5. Summary

We succeeded to encapsulate nanoparticles in giant magnetosomes formed by modified electroformation method. We showed the dependence of liposome size with respect to frequency of applied electric field.

Our new method of electroformation was successfully applied to form magnetic liposomes that consist of phospholipid vesicles and magnetic nanoparticles trapped inside and within a phospholipid bilayer. A stable suspension of different nanoparticles was added to solution used for electroformation of phospholipid vesicles. Nanoparticles with positive surface charge and nanoparticles with $-OH$ groups were successfully embedded into liposomes. While nanoparticles with negative surface charge and nanoparticles with $-NH_2$ groups failed to be encapsulated into liposomes. With positively charged nanoparticles we were able to control the size of magnetosomes during the synthesis by applying different electric field parameters (voltage amplitude and frequency). With this simple method of encapsulating magnetic nanoparticles into giant liposomes or their phospholipid bilayers is suitable for different ranges of applications.

Acknowledgement

The research was supported by Ministry of Higher Education, Science and Technology of the Republic of Slovenia (project

L2-2047, Synthesis and functionalization of composite nanobeads for early diagnosis of neurodegenerative diseases).

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