

COLLOQUIA: Nanoforum 2012

Smart magnetic nanovesicles for theranostic application: Preparation and characterization

C. MARIANECCI⁽¹⁾(*), F. RINALDI⁽¹⁾, C. INGALLINA⁽²⁾, D. PASSERI⁽³⁾, A. SORBO⁽⁴⁾,
M. ROSSI⁽³⁾ and M. CARAFA⁽¹⁾

⁽¹⁾ *Department of Drug Chemistry and Technologies, University of Rome Sapienza
Piazzale A. Moro 5, 00185, Rome, Italy*

⁽²⁾ *Center for Life Nano Science@Sapienza, Istituto Italiano di Tecnologia
Viale Regina Elena, 291, 00161, Roma, Italy*

⁽³⁾ *Department of Basic and Applied Sciences for Engineering, University of Rome Sapienza
Via A. Scarpa 16, 00161, Rome, Italy*

⁽⁴⁾ *European Union Reference Laboratory for Chemical Elements in Food of Animal Origin,
Istituto Superiore di Sanità - Viale Regina Elena 299, 00161, Rome, Italy*

ricevuto il 24 Febbraio 2013

Summary. — Nanomedicines are submicrometer-sized carrier materials designed to improve the biodistribution of systemically administered (chemo)therapeutic agents. By delivering pharmacologically active agents more effectively and more selectively to the pathological site nanomedicines aim to improve the balance between the efficacy and the toxicity of systemic (chemo)therapeutic administrations. Nanomedicine formulations have also been used for imaging applications and, in recent years, for theranostic approaches, that is, for systems and strategies in which disease diagnosis and therapy are combined. On the one hand, “classical” drug delivery systems are being co-loaded with both drugs and contrast agents. Actually, nanomaterials with an intrinsic ability to be used for imaging purposes, such as iron-oxide-based magnetic nanoparticles (MNPs), are increasingly being loaded with drugs or alone for combining disease diagnosis and therapy. In this study, non-ionic surfactant vesicles loaded with lipophilic and hydrophilic MNPs have been prepared. Vesicles have been characterized in terms of dimensions, ζ -potential, time stability, bilayer characteristics and overall iron content. The encouraging obtained results confirm that Tween 20 and Span 20 vesicles could be promising carriers for the delivery of hydrophilic and lipophilic MNPs, respectively, thereby prompting various opportunities for the development of suitable theranostic strategies. The analyzed formulations confirm the importance of surfactant chemical-physical characteristics in entrapping the MNPs of different polarity, highlighting the high versatility of niosomal bilayer and structure; property that make them so appealing among drug delivery nanocarriers.

PACS 81.07.-b – Nanoscale materials and structures: fabrication and characterization.

PACS 82.70.Uv – Surfactants, micellar solutions, vesicles, lamellae, amphiphilic systems, (hydrophilic and hydrophobic interactions).

PACS 61.46.Df – Structure of nanocrystals and nanoparticles.

(*) E-mail: carlotta.marianecci@uniroma1.it

1. – Introduction

The use of nanoparticulate pharmaceutical carriers to enhance the *in vivo* efficiency of many drugs well established itself over the past decade both in pharmaceutical research and clinical setting. Surface modification of pharmaceutical nanocarriers, such as solid lipid particles, polymeric nanoparticles, nanocapsules, liposomes, niosomes, micelles and others [1, 2] is normally used to control and modulate biological properties in a desirable fashion and make them to simultaneously perform various therapeutically or diagnostically important functions. The most important results of such modification allow an increased stability and half-life of nanocarriers in the circulation, a better biodistribution, passive or active targeting into the required pathological zone. Furthermore, surface modification can enable to obtain responsiveness to local physiological stimuli such as pathology-associated changes in local pH and/or temperature, and ability to serve as imaging/contrast agents for various imaging modalities (gamma-scintigraphy, magnetic resonance imaging, computed tomography, ultrasonography). Nanoparticle formulations have demonstrated clinical relevance by increasing drug efficacy and decreasing toxicity and numerous targeted formulations are under clinical evaluation. There are unique opportunities to use multifunctional formulations for both diagnostic and targeting purposes [3]. Vesicles formed by surfactants are known as Niosomes[®] or non-ionic surfactant vesicles (NSVs). The self-assembly of non-ionic surfactants into vesicles was firstly reported in the seventies by researchers in the field of cosmetics [4]. Niosomes are analogous to liposomes; in fact the NSVs can be prepared following the same procedures, under a variety of conditions, leading to the formation of unilamellar or multilamellar vesicular structures [5]. When compared to phospholipid-based vesicles, the surfactant vesicles have several advantages such as greater stability, thus lesser care in handling and storage and lower cost, attractive for industrial applications both in the field of pharmaceuticals and cosmetics [5-9]. Moreover niosomes, like liposomes, are capable of encapsulating both hydrophilic and lipophilic drugs [10]. The superior magnetic properties of iron oxide magnetic nanoparticles (MNPs) along with their non-toxicity, biodegradability and inexpensiveness, have made them a material of choice in many bioapplications, such as contrast probes in magnetic resonance imaging (MRI). In order to confer colloidal suspendability to the particles, additives, typically hydrophilic polymers are added during the particle formation process, which passivate the nanocrystal surface and protect against particle aggregation [11]. In this study, the preparation and characterization of MNPs loaded niosomes that offer the opportunity for targeted, non-invasive diagnosis by MRI will be described. For this purpose, iron oxide MNPs have been encapsulated into surfactant vesicles to develop niosomal contrast agents, *i.e.*, magnetic niosomes (MNs). Here, we have focused our attention on iron oxide NPs with a hydrodynamic diameter of 50 nm or less. Such MNPs consist of a magnetic core coated by various materials (hydrophilic coating, *e.g.*, dextran, or lipophilic coating, *e.g.*, lipid or polymaleic acid co-olefin) to improve the solubility and/or stability of the MNPs in colloidal suspension. This particular feature makes MNs suitable as MRI contrast agents for several biomedical applications and at the same time for active targeting strategies and for hyperthermia based treatments. However, for *in vivo* applications, the circulation time of MNs in the bloodstream should be long enough to ameliorate target-ligand interaction. This goal can be achieved by steric stabilization of the niosomes (*e.g.*, by adding a poly-ethylene glycol [PEG]-based surfactant to the formulation). Tween 20 is a natural pegylated hydrophilic surfactant (HLB=16.7), it possesses 20 PEG units in its molecule and is able to prevent the rapid recognition and consequent uptake of MNs via the mononuclear phago-

TABLE I. – *Samples composition. The MNPs in samples A₁ and B₁ were supplied by Colorobbia, while those in sample A₂ by Chemicell.*

Sample	Tw20 (mM)	Sp20 (mM)	Chol (mM)	hMNPs (mg/mL)	lMNPs (mg/mL)
A ₀	15	–	15	–	–
A ₁	15	–	15	3.58	–
A ₂	15	–	15	3.58	–
B ₀	–	15	15	–	–
B ₁	–	15	15	–	3.58

cyte system. Tween 20 could be an useful surfactant to prepare vesicles, as previously reported [12-15] able to entrap hydrophilic MNPs. To entrap lipophilic MNPs, Span 20, a lipophilic surfactant (HLB=8.6) without PEG units in its molecule have been used to prepare MNPs loaded vesicles. The prepared vesicular formulations have been analyzed in terms of chemical and physical characteristics, including iron oxide loading capacity, encapsulation efficiency, particle size, ζ -potential, time stability, and the influence of the magnetic cores on surfactant membrane polarity and microviscosity.

2. – Materials and methods

2.1. Materials. – Tween 20 (Tw20), Span 20 (Sp20), cholesterol (Chol), Sephadex G75, Hepes salt {N-(2-idroxyethyl) piperazine-N-(2-ethanesulfonicacid)} and pyrene were Sigma-Aldrich products (Sigma-Aldrich SRL, Milan, Italy). Hydrophilic magnetic nanoparticles (hMNPs) (fluidMAG-CMX) were Chemicell products (Chemicell, Berlin, Germany). Other kind of hMNPs and lipophilic magnetic nanoparticles (lMNPs) were a kind gift of Ce.Ri.Col. (Colorobbia, Vinci, Italy). All other chemicals used throughout this investigation were of analytical grade and no additional purification was carried out. Double-distilled water was used throughout the study.

2.2. Vesicle preparation and purification. – Unilamellar vesicles were obtained from a non-ionic surfactant/MNPs aqueous dispersion (Hepes pH 7.4) by means of the “film” method as previously reported [5,16], according to the compositions in table I. Tw20 and Sp20 concentration in the samples was always remarkably above their CMC (in water at 25 °C: Tween 20 = 0.006 g/dL; Span 20 = not detectable). For this purpose, Tw20 or Sp20, Chol, lMNPs and pyrene (4 mM in niosomes dispersion), when necessary, were dissolved in a CHCl₃/CH₃OH (3:1) mixture in a round bottomed flask. After evaporation of the solvents, the dried film containing lMNPs and/or pyrene was hydrated by addition of 5 mL (0.01 M) Hepes pH 7.4 solution while in the case of dried film without lMNPs and/or pyrene by addition of 5 mL of hMNPs in Hepes buffer (0.01 M) at pH 7.4. The dispersion was vortexed for about 5 min and then sonicated for 5 min at 60 °C using a tapered microtip operating at 20 kHz at an amplitude of 16% (Vibracell-VCX 400-Sonics, USA). In order to separate MNPs-loaded vesicles from untrapped substances, for the evaluation of entrapment efficiency (e.e.), the vesicle dispersion was purified by gel-filtration on Sephadex G75 (glass column 50 × 1.2 cm), using Hepes buffer as eluent in the case of MNPs prepared with hMNPs from Chemicell, while for MNPs with lMNPs and hMNPs obtained by Ce.Ri.Col., the untrapped material was eliminated by filtration with 1.2 μ m filter and then dialysis method.

2'3. Light scattering measurements. – Dynamic light scattering (DLS) was used to determine mean size and size distribution of non-ionic surfactant vesicles and to control IMNPs and hMNPs dimensions. The vesicle dispersions were diluted 100 times with the same buffer used for their preparation to avoid multiscattering phenomena. Buffer solutions used for dynamic light scattering experiments were filtered through $0.45\ \mu\text{m}$ cellulose filters to eliminate dust particles. Vesicle mean size and size distribution (polydispersity index, PDI) were measured at $25\ ^\circ\text{C}$ using a Malvern Nano ZS90 light scattering apparatus (Malvern Instruments Ltd., Worcestershire, UK) at a scattering angle of 90.0° . The same apparatus was used for the evaluation of ζ -potential of vesicles and of MNPs, which were appropriately diluted (1:10) in distilled water at $25\ ^\circ\text{C}$. The laser Doppler anemometry was used and hence the electrophoretic mobility of non-ionic surfactant vesicles was measured. The ζ -potential value was calculated from the electrophoretic mobility in the Smoluchowsky approximation. Reported data represent mean of the ζ -potential and of the hydrodynamic diameter (d_h) for the surfactant vesicles. Results of DLS experiments are given as the average values obtained using samples from three different batches \pm standard deviation (SD).

2'4. Vesicle physical stability. – Physical stability studies of MNPs loaded vesicles were carried out to investigate if significant changes of d_h and ζ -potential of surfactant vesicle dispersion occur during storage. For colloidal stability at different temperatures, the vesicle formulations were stored at $4\ ^\circ\text{C}$ and $25\ ^\circ\text{C}$ for a period of 90 days. Samples from each batch were withdrawn at definite time intervals (1, 30, 60 and 90 days) and the ζ -potential and d_h of vesicles were determined.

2'5. Determination of Iron entrapment efficiency (e.e.). – Fe e.e. within non-ionic surfactant vesicles was determined using inductively coupled plasma mass spectrometry (ICP-MS) on purified MNs. Samples were transferred into TFM vessels and digested by means of a microwave oven (Ethos, Milestone, FKV, Sorisole, Bergamo, Italy) equipped with a probe for the temperature control. The digestion mixture was constituted of 2 mL of H_2O ultrapure (up), 4 mL of HNO_3 65% (v/v) (Merck, Darmstadt, Germany), 0.5 mL of HCl and 1 mL of H_2O_2 40% (v/v) (Merck, Darmstadt, Germany). The digestion programme was preceded by a soft pre-digestion treatment of the samples in order to avoid any strong reaction during the final dissolution process. After cooling, the digested solutions were quantitatively transferred in 50 mL Falcon tubes by adding high purity deionized water up to 20 mL. These solutions were further diluted (1 + 9) before the instrumental analysis. Measurements were performed by means of a dynamic reaction cell (DRC) ICP-MS (Elan DCR II, Perkin Elmer SCIEX, Norwalk, CT, USA) operating in DRC mode with ammonia as reaction gas (purity of 99.999%) so as to overcome the likely interferences on the Fe determination. The cell parameters were the following: gas flow = 0.6 mL/min and $\text{RPq} = 0.6$. ^{56}Fe was the isotope used for quantification purpose with external calibration approach. Fe e.e. was calculated as the ratio between the mass of the incorporated Fe and that of the Fe used for vesicle preparation. Magnetite content in hMNPS Chemicell is 87% (w/w) of the total mass, while in hMNPs and IMNPS Cericol the Fe content is 0.0496 M and 0.0190 M of the starting suspension. Results are the average of three different batches \pm SD.

2'6. Measurements of vesicular micropolarity. – The fluorescence experiments on vesicles incorporating pyrene were carried out (Perkin-Elmer LS55 spectrofluorometer with excitation wavelength 319 nm) to evaluate the micropolarity of the hydrocarbon bilayer in vesicles. Pyrene allows the investigation of the lateral distribution and the dynamics

TABLE II. – *Samples characterization. The hydrodynamic diameter (d_h), polydispersity index (PDI), ζ -potential and Fe entrapment efficiency (e.e.) are reported.*

Sample	d_h (nm)	PDI	ζ -potential (mV)	e.e. (%)
A ₀	197.0 ± 5.1	0.304	-13.7 ± 0.8	0
A ₁	185.1 ± 9.4	0.244	-11.7 ± 0.4	53 ± 2
A ₂	211.2 ± 5.5	0.371	-26.2 ± 0.4	55.8 ± 2.3
B ₀	170.2 ± 2.0	0.192	-37.8 ± 0.5	0
B ₁	187.6 ± 2.8	0.131	-31.1 ± 1.0	15 ± 3

of membrane compounds. Pyrene in the bilayer is present as excimers at high concentrations; when the excimer fluorescence decrease, there is an increase in monomer fluorescence. The monomer and the excimer have different fluorescent signals and the ratio of the fluorescence intensities is directly related to the probe distribution in the lipid network. The pyrene monomer fluorescent spectrum consists of five peaks. It is well established that the ratio I_1/I_3 between the intensities of the first (I_1) and third (I_3) vibration bands of the pyrene fluorescence spectrum (corresponding to 372 nm and 382 nm, respectively) is related to the polarity of the pyrene environment [17]. Low values of the I_1/I_3 ratio correspond to a non-polar environment. This ratio increases as the polarity of the medium rises [17]. Since pyrene is solubilized inside the hydrocarbon chain of vesicles, the information obtained from fluorescence of pyrene in our systems refers to the bilayer of the vesicle pigeon-hole [18]. Pyrene may form intramolecular excimers. The process depends on the rate of conformational change of the molecule which is sensitive to the viscosity of the probe microenvironment [19]. Hence the I_E/I_M ratio, where I_M and I_E stand for the intensity of the monomer and the excimer fluorescence, respectively, is used to estimate the microviscosity. Because of its high hydrophobicity, the solubilization zone of pyrene is in the vesicle bilayer, as was established in the case of polymeric micellar solutions [20]. The pyrene probe may also evidence (only qualitatively) the micropolarity variation in the solubilization region, by the change in the ratio of monomer vibronic bands intensities measured at 377 nm and 397 nm [21].

3. – Results and discussion

The measurements carried out by DLS indicate that selected surfactants in presence of Chol form vesicular structures with no significant different d_h , while differences in ζ -potential (table II) are evident between Sp20 and Tw20 empty vesicles. The higher (in absolute value) ζ -potential of Sp20 vesicles is probably due to the absence of PEG external coating. ζ -potential values allow to foresee the stability of the prepared formulations. A high negative value of ζ -potential is important in preventing aggregation. It has been reported that a physically stable nanosuspension solely stabilized by electrostatic repulsion will have a minimum ζ -potential of -30 mV [22]. hMNPs Chemicell and Colorobbia have dimensions of 50 nm and 20 nm, respectively, while lMNPs have dimensions of 30 nm. There is not a significant increase/decrease of vesicular dimensions after entrapment of MNPs. In particular Tw20 vesicles, hydrated with solutions of hMNPs that are probably placed inside vesicular aqueous core, show unchanged dimensions com-

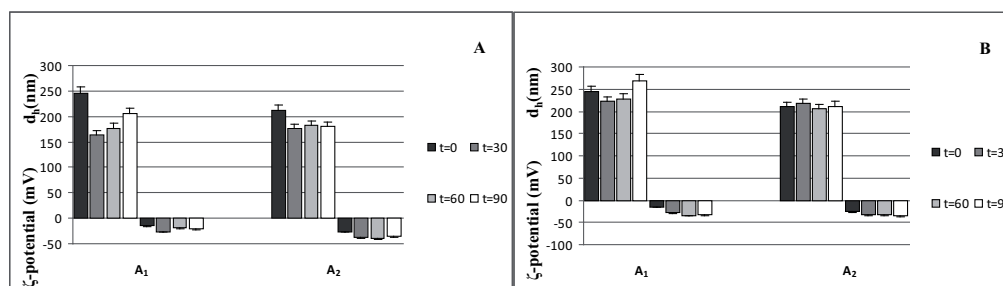


Fig. 1. – Physical stability of hMNPs entrapped in surfactant vesicle (samples A₁ and A₂). Vesicle hydrodynamic diameter (d_h) and ζ -potential values were evaluated by DLS at definite time intervals (1, 30, 60, 90 days) at 25 °C (panel A) and 4 °C (panel B). Reported data are the means of three experiments \pm SD.

pared to empty ones, while in Sp20 vesicles the insertion of IMNPs in surfactant bilayer is responsible of a slight increase in vesicle dimension. Only in the case of Chemicell MNPs loaded Tw20 vesicles there is a decrease in ζ -potential values; the MNPs could be placed also on the vesicle surface, inside the PEG coating, eliciting a variation in ζ -potential values respect empty vesicles. Apart from providing d_h and ζ -potential values, DLS provides also valuable information on the homogeneity of the suspension. A single sharp peak in the DLS profile implies the existence of a single population of scattering particles. DLS and PDI measurements indicated that the empty vesicles showed a significant yield of homogeneous vesicular structures. The physical stability studies of empty NSVs are widely reported in literature [23,24]. From the analysis of obtained results it is evident that Tw20 vesicles hydrated by hMNPs both of Colorobbia and of Chemicell in 10 mM Hepes buffer pH 7.4 are stable for at least 3 months when stored at 4 °C and at 25 °C (fig. 1 A and B). The same results have been obtained in the case of Sp20 vesicles prepared in presence of IMNPs. Even ζ -potential values remain constant indicating that the analyzed formulations possess a good physical stability (fig. 2).

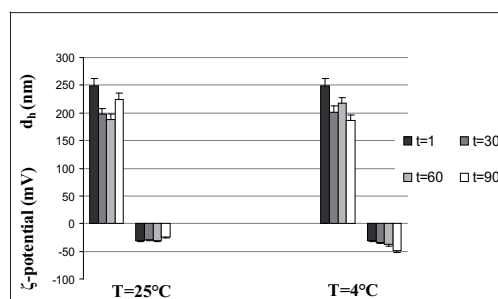


Fig. 2. – Physical stability of sample B₁. Vesicle hydrodynamic diameter (d_h) and ζ -potential values were evaluated by DLS at definite time intervals (1, 30, 60, 90 days) at 25 °C and 4 °C. Reported data are the means of three experiments \pm SD.

TABLE III. – Vesicle bilayer polarity (I_1/I_3) and microviscosity (I_E/I_M), evaluated by fluorescence studies.

Sample	I_E/I_M microviscosity	I_1/I_3 polarity
A ₀	0.420	1.120
A ₁	0.524	1.052
A ₂	0.438	1.180
B ₀	1.160	0.304
B ₁	1.490	0.420

The e.e. data (table II) show a good capability of the two type of vesicle of entrap both hydrophilic and lipophilic MNPs. It is evident that the hMNPs are better entrapped than the lMNPs ones probably due to the higher volume occupied by the aqueous core respect to the the lipophilic bilayer. To better evaluate the influence of MNPs entrapment on vesicle bilayer, fluorescence analyses were performed in order to evaluate bilayer polarity and microviscosity (table III). The MNPs encapsulation led to different behaviour in vesicle bilayer respect to empty vesicles; the I_E/I_M values are rather constant in the case of Tw20 vesicles-hMNPs Chemicell and Tw20 empty vesicles. This could be related to a non-insertion of hydrophilic MNPs in vesicular bilayer, but in the case of Colorobbia MNPs a slight, not significant variation is present. The unchanged values of the microviscosity parameter confirm the location of hydrophilic MNPs in vesicular aqueous core. lMNPs loaded Sp20 vesicles show an increase in polarity and microviscosity values with respect the empty vesicles, showing a more hydrophilic and viscous pyrene microenvironment. This fact could be explained by the presence of the lMNPs inside the bilayer which permits a partial water penetration, leading to an increase in polarity and in microviscosity. The insertion of lMNPs in the bilayer could be responsible of the slight increase of vesicle dimensions (table II). The low e.e., also with increasing lMNPs loading amount, could be related to an excess of MNPs inside the bilayer, in some cases leading to the inhibition of vesicular structure formation.

The reported results are very encouraging and confirm that Tw20 and Sp20 vesicles could be promising carriers for the delivery of hydrophilic and lipophilic MNPs, respectively, thereby prompting various opportunities for the development of suitable theranostic therapeutic strategies. The analyzed formulations confirm the importance of surfactant chemical-physical characteristics in entrapping the MNPs of different polarity, highlighting the high versatility of niosomal bilayer and structure; property that make them so appealing among drug delivery nanocarriers. In the future it could be very interesting to deepen chemical-physical characterization of these structures together with their magnetic properties analyses and try to entrap a model drug in these structures to evaluate their theranostic potentiality.

* * *

Financial support from Sapienza University of Rome, Ateneo Funding (2010), is acknowledged. The authors are grateful to Colorobbia Industry and in particular to DR. BALDI for MNPs supply.

REFERENCES

- [1] MÜLLER R., *Carriers for Controlled Drug Delivery and Targeting* (CRC Press, Boca Raton, FL) 1991.
- [2] ALONSO M. J., *Biomed. Pharmacother.*, **58** (2004) 168.
- [3] TORCHILIN V. P., *Adv. Drug Deliver. Rev.*, **64** (2012) 302.
- [4] HANDJANI-VILA R. M., RIBIER A. and VANLERBERGHE G., *Nonionic surfactant vesicles*, in *Liposome Technology*, edited by GREGORIADIS G. (CRC Press, Boca Raton, FL) 1993, pp. 201–213.
- [5] UCHEGBU I. F. and FLORENCE A. T., *Adv. Colloids Interface Sci.*, **58** (1995) 1.
- [6] VAN HAL D. A., BOUWSTRA J. A., VAN RENSEN A., JEREMIASSE E., DE VRINGER T. and JUNGINGER H. E., *J. Colloids Interface Sci.*, **178** (1996) 263.
- [7] PAOLINO D., MUZZALUPO R., RICCIARDI A., CELIA C., PICCI N. and FRESTA M., *Biomed. Microdevices*, **9** (2007) 421.
- [8] TERZANO C., ALLEGRA L., ALHAIQUE F., MARIANECCI C. and CARAFA M., *Eur. J. Pharm. Biopharm.*, **59** (2005) 57.
- [9] MARIANECCI C., RINALDI F., MASTRIOTA M., PIERETTI S., TRAPASSO E., PAOLINO D. and CARAFA M., *J. Control. Release*, **164** (2012) 17.
- [10] UCHEGBU I. F. and VYAS S. P., *Int. J. Pharm.*, **172** (1998) 33.
- [11] FRASCIONE D., DIWOKY C., ALMER G., OPRIESSNIG P., VONACH C., GRADAUER K., LEITINGER G., MANGGE H., STOLLBERGER R. and PRASSL R., *Int. J. Nanomed.*, **7** (2012) 2349.
- [12] MARIANECCI C., CARAFA M., DI MARZIO L., RINALDI F., DI MEO C., ALHAIQUE F., MATRICARDI P. and COVIELLO T., *J. Pharm. Pharmaceut. Sci.*, **14** (2011) 336.
- [13] DI MARZIO L., ESPOSITO S., RINALDI F., MARIANECCI C. and CARAFA M., *Colloids Surface B*, **104** (2013) 200.
- [14] DI MARZIO L., MARIANECCI C., RINALDI F., ESPOSITO S. and CARAFA M., *Lett. Drug Des. Discov.*, **9** (2012) 1.
- [15] MARIANECCI C., RINALDI F., DI MARZIO L., POZZI D., CARACCILO G., MANNO D., DINI L., PAOLINO D., CELIA C. and CARAFA M., *Biomed. Microdevices* (2013).
- [16] CARAFA M., MARIANECCI C., LUCANIA G., MARCHEI E. and SANTUCCI E., *J. Control. Release*, **95** (2004) 67.
- [17] KALYANASUNDARAM K. and THOMAS J. K., *J. Am. Chem. Soc.*, **99** (1977) 2039.
- [18] VANDERKOOI J. M. and CALLIS J. B., *Biochemistry*, **13** (1974) 4000.
- [19] ZACHARIASSE K. A., *Chem. Phys. Lett.*, **57** (1978) 429.
- [20] VASILESCU M., BANDULA R. and LEMMETYINEN H., *Colloids Polym. Sci.*, **288** (2010) 1173.
- [21] VASILESCU M., ANGELESCU D. G., BANDULA R. and STAIKOS G., *J. Fluoresc.*, **21** (2011) 2085.
- [22] MULLER R. H., JACOBS C. and KAYSER O., *Adv. Drug Deliv. Rev.*, **47** (2001) 3.
- [23] MARIANECCI C., PAOLINO D., CELIA C., FRESTA M., CARAFA M. and ALHAIQUE F., *J. Control. Release*, **147** (2010) 127.
- [24] CARAFA M., DI MARZIO L., MARIANECCI C., CINQUE B., LUCANIA G., KAJIWARA K., CIFONE M. G. and SANTUCCI E., *Eur. J. Pharm. Sci.*, **28** (2006) 385.