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ORIGINAL ARTICLE

Molecular properties of lysozyme-microbubbles: towards the protein and nucleic acid delivery

Sonia Melino · Meifang Zhou · Mariarosaria Tortora · Maurizio Paci · Francesca Cavalieri · Muthupandian Ashokkumar

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Abstract Microbubbles (MBs) have specific acoustic properties that make them useful as contrast agents in ultrasound imaging. The use of the MBs in clinical practice led to the development of more sensitive imaging techniques both in cardiology and radiology. Protein-MBs are typically obtained by dispersing a gas phase in the protein solution and the protein deposited/cross-linked on the gas–liquid interface stabilizes the gas core. Innovative applications of protein-MBs prompt the investigation on the properties of MBs obtained using different proteins that are able to confer them specific properties and functionality. Recently, we have synthesized stable air-filled lysozyme-MBs (LysMBs) using high-intensity ultrasound-induced emulsification of a partly reduced lysozyme in aqueous solutions. The stability of LysMBs suspension allows for post-synthetic modification of MBs surface. In the present work, the protein folded state and the biodegradability property of LysMBs were investigated by limited proteolysis. Moreover, LysMBs were coated and functionalized with a number of biomacromolecules (proteins, polysaccharides, nucleic acids). Remarkably, LysMBs show a high DNA-binding ability and protective effects of the nucleic acids from nucleases and, further, the ability to transform the bacteria cells. These results highlight on the possibility of using LysMBs for delivery of proteins and nucleic acids in prophylactic and therapeutic applications.

Keywords Microbubbles · Lysozyme · Lactoferrin · Nucleic acid delivery · Ultrasounds · Proteolysis

Abbreviations

BSA	Bovine serum albumin
DNase I	Deoxyribonuclease I
DTT	Dithiothreitol
EtBr	Ethidium bromide
FITC	Fluorescein isothiocyanate
IPTG	Isopropyl-β-D-thiogalactoside
Lf	Lactoferrin
MBs	Microbubbles
OD	Oxidation degree
pWW_f	pQE30-ww_f
RP-HPLC	Reversed-phase chromatography
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy

Introduction

Microbubbles (MBs) are small gas-filled colloidal particles, with a size range of 0.5–5 μm, that have specific acoustic properties that make them useful as contrast agents in ultrasound imaging. The MB's shell plays an important role in preventing the exchange of gases between the bubble core and the medium. The shell is primarily made up of protein, polymer or lipid-based coatings. The use of MBs in

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S. Melino (✉) · M. Tortora · M. Paci · F. Cavalieri (✉)
Department of Sciences and Chemical Technologies, University of Rome "Tor Vergata", via della Ricerca Scientifica, 00133 Rome, Italy
e-mail: melinos@uniroma2.it

F. Cavalieri
e-mail: francesca.cavalieri@uniroma2.it

M. Zhou · F. Cavalieri · M. Ashokkumar
School of Chemistry, The University of Melbourne, Parkville, Melbourne, VIC 3010, Australia

clinical practice led to the development of more sensitive imaging techniques both in cardiology and radiology, including subharmonic and multipulse imaging, pulse inversion and harmonic power Doppler (de Jong et al. 2000). Gas-filled MBs play an expanding role in therapeutic applications due to their unique cavitation (Ng and Liu 2002; Liu et al. 2006; Unger et al. 2001a, b) and sonoporation (Mehier-Humbert and Guy 2005; Wu et al. 2006) properties. Acoustic cavitation phenomenon has been put into practical use in achieving several therapeutic interventions. An intriguing issue and a very promising future application is the development of site-targeted microbubble contrast agents that can be used to image molecular events in vivo. Moreover, MBs have been proposed as a new vehicle for delivery of therapeutic agents such as plasmid DNA and drug directly to tissue by ultrasound-mediated destruction of payload-bearing MBs (Mayer and Grayburn 2001; Unger et al. 2001a, b, 2002; Porter et al. 1995a, b). MB-mediated therapy has been used to deliver genes or drugs to specific tissues (Ng and Liu 2002; Bekeredjian et al. 2003; Hauff et al. 2005; Shimamura et al. 2004; Taniyama et al. 2002; Li et al. 2009; Hernot and Klibanov 2008; Deelman et al. 2010; Yuan et al. 2011) utilizing acoustic cavitation and sonoporation effects, including neural tissues, skeletal muscles, myocardium, kidneys, vessels, and tumors. As MBs are constrained to the intravascular space and are too large to exit the vasculature only disease sites located either in the bloodstream or on the vascular endothelial surface can be targeted. For instance, endothelial cells and receptors associated with angiogenesis vascular inflammation are readily accessible to MBs (Kaufmann and Lindner 2007).

A variety of drugs and therapeutic molecules were incorporated into the MBs and effectively released in the thrombolytic, atherosclerotic therapy (Unger et al. 2004; Ferrara 2008) and for cardiac gene transfection (Bekeredjian et al. 2003; Tsunoda et al. 2005; Browning et al. 2011). Drugs can be conjugated to the MB shell with the use of non-covalent binding such as electrostatic or hydrophobic interactions, van-der-Waals forces or merely by physical encapsulation. Large negatively charged molecules, like nucleic acids, are suitable for attachment to positively charged shells. According to the drug properties, an appropriate shell composition must be developed to bind drug molecules. The incorporation of the drug in the shell is a process significantly influenced by the nature of the drug, in terms of lipophilicity and hydrophilicity.

Protein-based MBs are also obtained by creating (e.g., sonication) a microscopic dispersion of gas into the protein solution. The protein is subsequently deposited on the gas–liquid interface stabilizing the gas core with a thin shell (Grinstaff and Suslick 1991; Porter et al. 1995a, b). The chemical nature of the shell and its mechanical properties are crucial for therapeutic applications and MBs coated with protein shell can be stable enough to not require lyophilization or bedside in situ preparation/reconstitution.

Air-filled MBs with human albumin shells (Albunex®) are used as ultrasound contrast agents and have been suggested as magnetic resonance susceptibility contrast agents for tumor imaging (Moseley et al. 1991). The first in vivo investigation of susceptibility contrast induced by MBs was reported in liver using Optison®, MBs with human albumin shell with perfluorocarbon as core gas, at 7 T in rat liver (Wong et al. 2004). Moreover, a peculiarity of the albumin-coated MBs is the ability to adhere only to the vessel walls in the setting of endothelial dysfunction and not to normally functioning endothelium, thus suggesting also the use of these MBs as markers of endothelial integrity (Villanueva et al. 1997). This peculiarity could lead the delivery of drugs or genes bound to albumin-shelled MBs selectively concentrated at the site of vascular injury in the presence (Deng et al. 2004; Hashiya et al. 2004) or absence of ultrasound application (Porter et al. 2001). Transient pore formation on cell membranes was demonstrated by electron microscopy after destruction of MBs (Meijering et al. 2009); such cavitation effects might facilitate entry of genes into cells. The transfection efficiency of the genes is limited by the negative surface potential of the albumin-shelled MBs. Thus, coating with poly(allylamine hydrochloride) (Lentacker et al. 2006) or, more recently, with polyethylenimine (Dang et al. 2011), that make the surface charge of the MBs positive, was used to make them a useful non-virus gene delivery method in vitro.

Recently, growth factor-releasing scaffolds have also been fabricated by incorporating growth factor-releasing bovine serum albumin (BSA) MBs in the scaffold-manufacturing processes. BSA-MBs have been used as a new porogen to produce BSA-coated cell-friendly surfaces in preserving the bioactivity of loaded IGF-1 growth factor (Nair et al. 2010).

These innovative applications of protein-MBs initiate the investigation on the properties of MBs obtained using different proteins that are able to confer them specific properties and new potentiality. Although albumin-shelled MBs exhibit interesting molecular properties, the thin monolayer shell that is stabilized by protein dimerization, compromises the MBs stability and consequently the reliable use for drug loading and release which require a longer lifetime (Lentacker et al. 2006). In our previous work, we have indeed synthesized stable lysozyme MBs (LysMBs) using high-intensity ultrasound-induced emulsification of a partially reduced lysozyme in aqueous solution (Cavalieri et al. 2008). Both the hydrophobic nature of the enzyme, to provide foaming properties, and the formation of disulfide bonds are requirements for the formation of stable protein-MBs. Compared to air-filled

BSA MBs, lysozyme MBs exhibit a longer shelf life (months/years). The remarkable stability of the lysozyme MBs, is ascribed to the thicker (100–200 nm) and elastic cross-linked protein shell (Zhou et al. 2011a, b). The cross-linked protein coating provides a high degree of control over the MBs surface properties and ultrasound acoustic response (Zhou et al. 2011a, b).

At sufficiently high power levels (0.5 W/cm^2), MBs can be broken resulting in the release of the gas core that could act as bubble nuclei. The acoustic MBs destruction could promote the release of therapeutic agents loaded onto the MBs surface (Zhou et al. 2011a, b; Cavalieri et al. 2010). Interestingly, the size distribution of LysMBs can be finely tuned using the ultrasound technique (Zhou et al. 2011a, b).

In the present work we demonstrated LysMBs biodegradability property and the possibility to functionalize them with proteins and a polysaccharide. In addition, the cationic surface of these MBs makes them able to bind to DNA and to protect the DNA by nucleases without additional coating with cationic polymers. The data reported here show possible applications of these MBs as multifunctional carriers for therapeutic proteins and gene therapies.

Materials and methods

Microbubble preparation

Lysozyme (5% w/v) (Sigma-Aldrich) was denatured in 50 mM Tris–HCl, pH 8.3, buffer containing 100 mM DTT or 10 µl/ml of β-mercapto-ethanol for 60 s. 20 kHz ultrasound was applied for 30 s at the air–water interface using a Sonics and Materials ultrasound generator (Branson) with a 3-mm-diameter horn at an applied acoustic power of 160 Wcm^{-2}. MBs were separated from the remaining protein and broken microparticles by flotation and repeated washing. Scanning electron microscopy (SEM) images were recorded on air-dried MBs sputter-coated with a thin gold film using a FEI Quanta operated at an acceleration voltage of 10 kV. All experiments were carried out at least three times with different preparations of LysMBs.

Limited proteolysis of LysMBs

Three different forms of lysozyme were analysed for resistance to trypsin proteolysis, native, thermally denaturated by heating for 2 min at 100°C and LysMBs. The concentrations of the three forms were estimated by Bradford assay.

The lysozyme solutions (0.6 mg/ml) in 25 mM Tris HCl buffer, pH 7.84, were proteolyzed with 1% trypsin (Sigma) (E/S 1:100 w/w) at 23°C or 37°C and the proteolysis was monitored during the time by SDS-PAGE. Time course of the trypsin digestion of BSA-coated LysMBs was performed using 80 µM BSA and 40 µM LysMBs and 1:100 E/S w/w ratio at 37°C. All reactions were stopped by the addition of 10 µl SDS-PAGE sample buffer and freezing. The trypsin digestions were performed for the times indicated in figure legends. Gels were run by the method of Laemmli (1970) using 20% or 15% acrylamide in the separating gels and 4% in the stacking gels at 200 V constant. The gels were stained with Coomassie brilliant blue (Bio-Rad). Time course of the trypsin proteolysis of LysMBs (1 mg/ml lysozyme) (E/S 1:100 w/w) in 30 mM Tris HCl buffer, pH 8.3, at room temperature) was also evaluated using RP-HPLC system chromatography. The soluble fraction was picked up at different times and analysed by RP-HPLC using a C18 column. Before the analysis the proteolysis was stopped by added of 10% acetic acid at the solutions and after stored by freezing, except the reaction after over-night that was analysed immediately by RP-HPLC (mod. LC-10AVP Shimadzu equipment, Milan, Italy) with a solvent B gradient (0 min, 0%; 0–40 min, 60%; 40–45 min, 60%, and 50–55 min, 90%), using 0.1% TFA as solvent A and 80% CH_3CN, 0.1% TFA as solvent B and a Brouwnlee C-18 column (OD-300, 250 mm $\times$ 4.6 mm, 7 µm). Eluate was monitored at 220 nm by UV detector (Shimadzu).

Coating of LysMBs with proteins and dextran

LysMBs were loaded with proteins using aqueous solutions of BSA (Sigma-Aldrich) and lactoferrin (Lf, Sigma-Aldrich) at 1/0.5, 1/1, 1/2, 1/5 lysozyme/protein molar ratios. After 10 min of incubation at room temperature, LysMBs were separated from the excess protein by flotation and repeated washing.

The interaction between LysMBs and proteins were assessed using an inverted Olympus IX71 wide field fluorescence microscope with a 60$\times$ objective lens and green filter cube and ζ potential measurements (Malvern Zetasizer). FITC-BSA and FITC-Lf solutions, which were obtained using a FITC conjugation protocol (1 µl of 10 mg/ml FITC in DMSO solution was added to 100 µl of (3 mg/1 ml) BSA or Lf in 15 mM sodium bicarbonate, pH 8.0 buffer and the mixture incubated at room temperature for 2 h), were added to the LysMBs solution using a 1/1 lysozyme/protein molar ratio and the coated MBs were viewed under fluorescent microscope after washing. An aqueous solution of dextran (Mw 40,000 Da, Sigma-Aldrich) was oxidized with sodium periodate (Sigma-Aldrich) for 3 h at room temperature, to yield theoretical oxidations of 5% glucose units. The oxidation degree (OD) of dextran is defined as the number of oxidized residues per 100 glucose residues. The resulting solution was dialyzed against MilliQ water for 3 days and freeze dried.

LysMBs coating with oxidized dextran was performed by adding 0.5 ml of oxidized-FITC dextran (0.5 mg/ml) to

0.5 ml LysMBs suspension (0.5 mg/ml) at pH 5.0. After 8 h of incubation at room temperature LysMBs were separated from the unreacted dextran by flotation and repeated washing.

DNA-binding properties of LysMBs

The interaction between LysMBs and plasmid DNA, pDNA, was assessed using fluorescent microscope, the pDNA was stained with EtBr (ethidium bromide, Bio-Rad) and mixed with the LysMBs solution, incubated for 30 s and the solution was viewed under fluorescent microscope with a 100× objective. The interaction between LysMBs and pDNA was also assessed using the gel retardation shift assay after 15 min of incubation of the pQE30-ww$_f$ (pWW$_f$) plasmid with increasing amounts of LysMBs (from 0 to 40 LysMBs/pDNA w/w ratio) and gel electrophoresis was carried out on 1% agarose slab at 60 V for 80 min. The gel was stained with EtBr and examined by UV light using an ultraviolet transilluminator. The experiments were carried out at least three times.

LysMBs protection of DNA by nuclease digestion

The protective effects of the LysMBs on DNA by nucleases were assayed using pWW$_f$ plasmid and the deoxyribonuclease I (DNase I; GE Healthcare, Italy) and BamH1 (Fermentas-M-Medical, Italy) nucleases. The pWW$_f$-LysMBs complexes were obtained incubating the mixture for 10 min at room temperature. After incubation 0.2 µl of DNase I (0.2 U) or 0.2 µl of BamH1 (0.2 U) were added to the pWW$_f$-LysMBs and pWW$_f$ solutions in 1/20 or 1/40 w/w ratios and after incubation the solutions were assayed on agarose electrophoresis using 1% agarose gel and 60 V constant.

To evaluate the integrity of the pDNA in the pWW$_f$-LysMBs complexes after nuclease treatment, 10 µl of pWW$_f$ (about 78 µg/ml) was incubated with or without 10 µl LysMBs (0.6 mg/ml) for 10 min at room temperature and then 0.3 µl of DNase I (0.3 U) was added. After 10 min of incubation at room temperature, the solutions were used for a heat shock transformation of XL1-blue super-competent bacteria cells. In brief, 100 µl of XL1-blue *E. coli* cells were added to the solutions and incubated in ice after 30 min, after 90 s at 42°C the solutions were incubated in ice for 2 min. 900 µl of LB medium was added to each solution and after 45 min of incubation at 37°C the cultures were centrifuged and the pellet put on petri dishes with LB agar and ampicillin (100 µg/ml). The WW$_f$ protein expression *E. coli* strain transformed with pWW$_f$-LysMBs complexes was induced by addition of 1 mM IPTG to the culture in LB medium with 100 µg/ml ampicillin at 0.6 OD$_{600nm}$ and the over-expression was assayed by SDS-PAGE. The experiments were carried out at least three times.

Results and discussion

Preparation and characterization of LysMBs by limited proteolysis

Lysozyme-MBs prepared as described in the experimental section had a size distribution of 2.8 ± 0.8 µm. Figure 1 shows a SEM image of the LysMBs obtained using β-mercapto-ethanol as the reducing agent. The thick protein shell (150 nm) (Zhou et al. 2011a, b) ensures that LysMBs are stable and can be stored at room temperature for months. It is known that egg-white lysozyme was found to form amyloid fibrils when incubated in vitro at pH 2.0 for several days (Krebs et al. 2000). Thus, it is important to understand the nature of LysMBs for their use in medical applications. In previous years, the analysis of the molecular features of the protein aggregates has attracted considerable attention particularly to understand the process of the amyloid fibrils formation. Amyloid fibrils are self-assembled filaments of peptides or proteins and these aggregates are associated with severe debilitating diseases, such as Alzheimer's, type 2 diabetes, prion, Parkinson's, senile systemic amyloidosis and Huntington's (Sacchettini and Kelly 2002; Dobson 2003; Stefani and Dobson 2003; Selkoe 2003). The amyloid fibril formation from native proteins is usually due to a conformational change leading to the formation of partly folded intermediates and subsequent association of these protein species to form prefibrillar species given by soluble oligomers that subsequently associate into well-ordered mature fibrils (Dobson 2003). The nature of protein intermediates appears to be critical for the onset of fibril formation, since these species

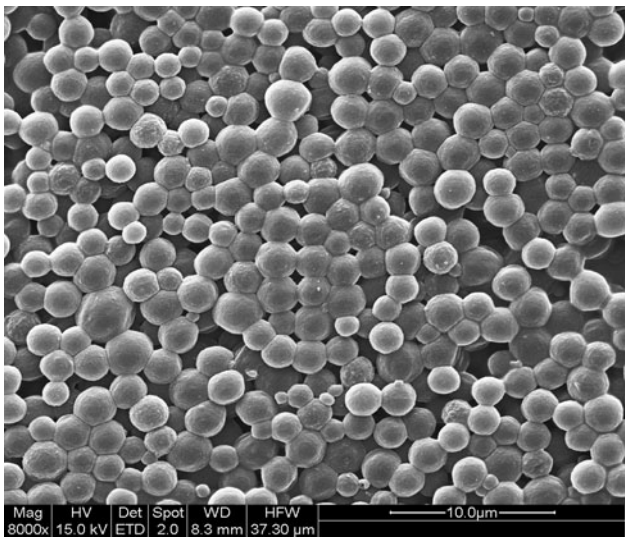


Fig. 1 SEM image of LysMBs obtained using a 5% lysozyme solution with 10 µl/ml of β-mercapto-ethanol in 50 mM Tris HCl, pH 8.3, buffer. The average MB size is 2.5 ± 0.8 µm

show strong intermolecular interactions due to the exposure of hydrophobic patches otherwise buried in the folded structure of the native protein (Fink 1998). Peculiar characteristics of amyloid fibrils include the presence of cross-β structural motif (Sunde and Blake 1997) and the unusual resistance to proteolytic degradation. In fact, the more defined amyloid fibrils that are formed over longer periods of time are completely resistant to proteolysis. In the absence of a good structural characterization of these protein micro-complexes, we have investigated in this work on the protein-folded state and on the biodegradability of LysMBs using the limited proteolysis. Limited proteolysis experiments have been used in the past for probing molecular features of proteins in their native or partially folded states (Fontana et al. 1986, 1997; Aceto et al. 1998; Melino et al. 2004). Local chain flexibility is required for a native protein to be attacked by a proteolytic enzyme, thus a specific region of the folded protein is sufficiently exposed to bind at the active site of the protease. Instead, enhanced flexibility appears to be the key feature of the site(s) of limited proteolysis.

The denaturation and ultrasound treatment of the lysozyme during the LysMBs preparation could lead to a partly unfolded state of the protein and the formation of amyloid fibrils. Generally, lysozyme is digested by pepsin but it is resistant to trypsin (During et al. 1999) and it had been

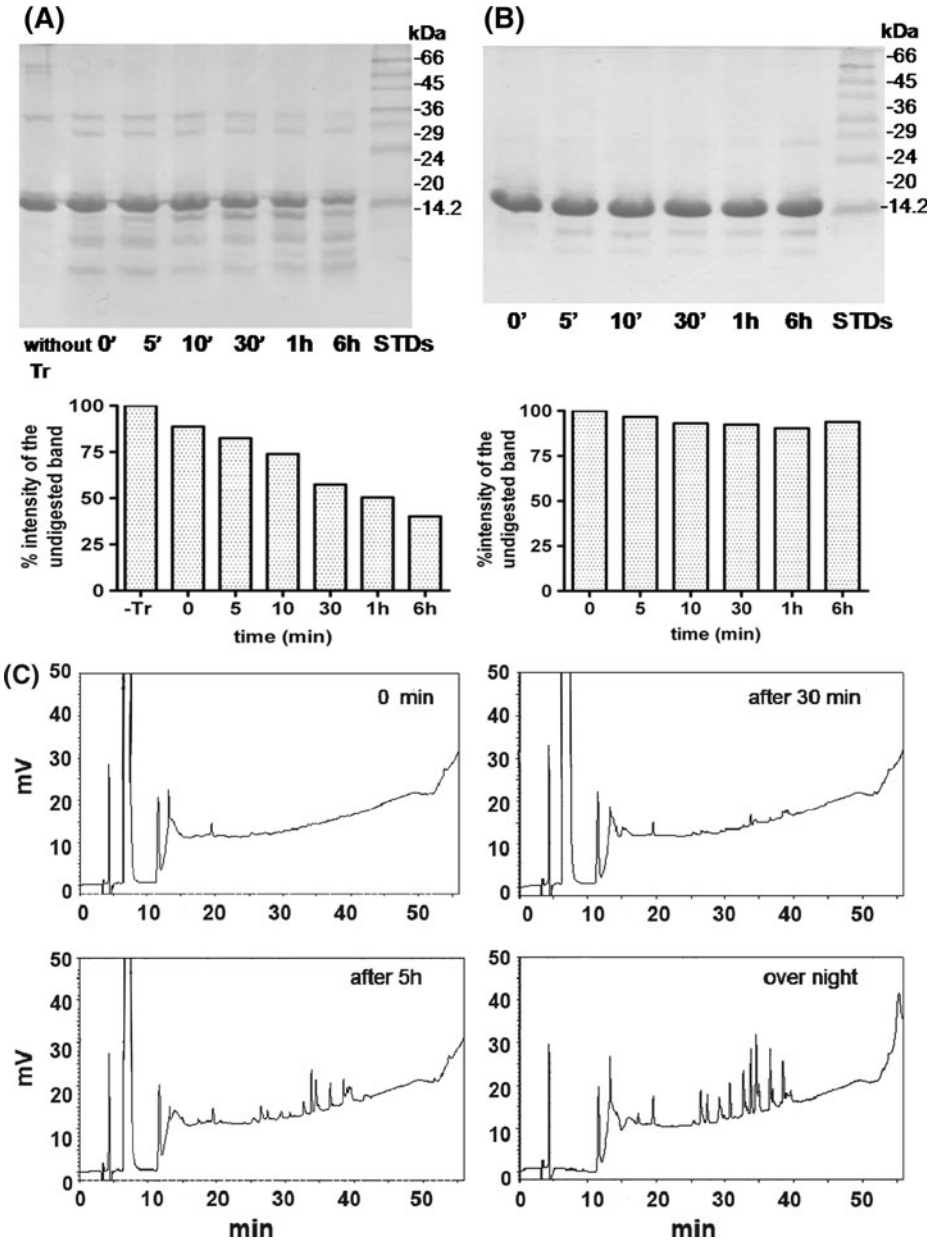


Fig. 2 Biodegradability of the LysMBs. Time course of the Trypsin proteolysis of **a** LysMBs (0.6 mg/ml lysozyme) or **b** lysozyme heated for 2 min at 100°C solution (E/S 1:100 w/w) in 25 mM Tris HCl buffer, pH 7.84, at room temperature. The reactions were arrested by the addition of 5 µl SDS–PAGE sample buffer and freezing prior to SDS-PAGE in 20% acrylamide gel was performed. The histograms of the intensities of the undigested band during the proteolysis are shown below: the data are normalized for the band intensity before the proteolysis (100%). **c** Time course of the Trypsin proteolysis of LysMBs evaluated by RP-HPLC using E/S 1:100 w/w ratio in 30 mM Tris HCl buffer, pH 8.3, at room temperature. The soluble fraction was picked up at 0′, 30′, 5 and 16 h and analysed by RP-HPLC

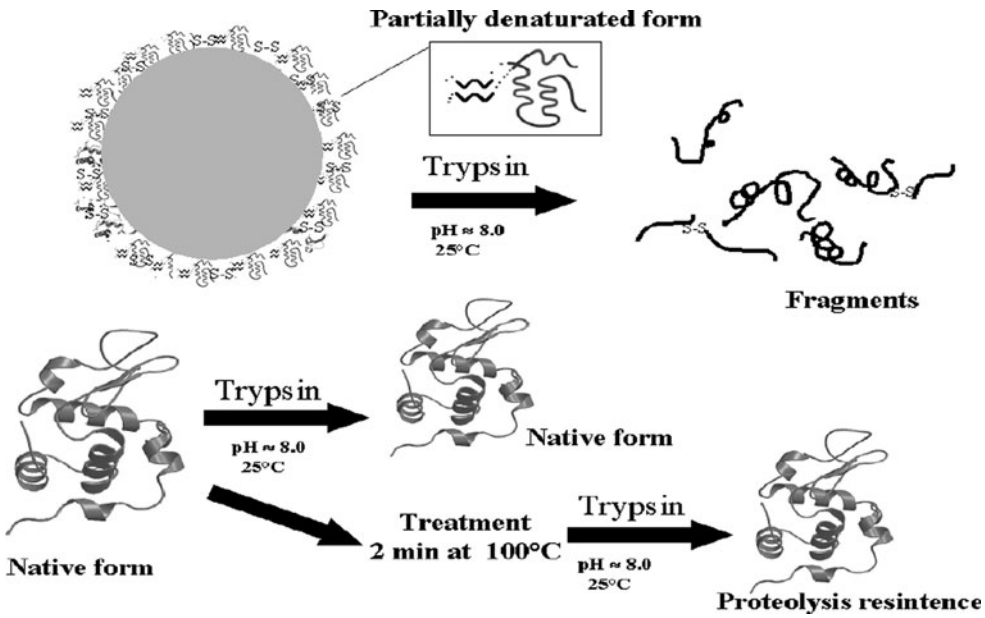
demonstrated that it is proteolyzed by trypsin only after denaturation by heat treatment at 95°C for 20 min (Mine et al. 2004). Thus, we have performed trypsin digestion of LysMBs to investigate the folded state of the protein and possibly the formation of protofibrils.

Figure 2 shows the time courses of the trypsin-limited proteolysis of the LysMBs. The SDS-PAGE of the products during the proteolysis of the LysMBs shows the disappearance of the principal band, which corresponds to lysozyme, and the appearance of new bands at lower molecular weights (Fig. 2a). Moreover, in agreement with these results the HPLC analysis of the soluble fraction during the proteolysis (Fig. 2c) shows the formation of the soluble fragments during the proteolysis. These results demonstrate the biodegradability of the LysMBs suggesting the absence of amyloid-like structure of the LysMBs and demonstrating also a partially unfolded state of the lysozyme in the MBs that is suitable to the digestion with trypsin (Scheme 1). These data also indicate that the previously observed antimicrobial activity of LysMBs (Cavalieri et al. 2008) could also be due to not only the non-catalytic bacterial inhibition of the lysozyme, but also a direct interaction of the regions of the molecule with conserved secondary structure with bacterial surface leading to the damage of the membrane integrity, as described by Mine et al. (2004).

Interaction of the LysMBs with other proteins

Another critical aspect for the in vivo use of the MBs is the interaction with other proteins and the possibility to obtain other-proteins coated LysMBs. It has been shown (Cavalieri et al. 2008) that the multilayer deposition of polyelectrolytes onto LysMBs is a straightforward approach to modify the surface properties of the MBs. Lysozyme air-filled MBs are positively charged colloidal particles (ζ-potential $+40 \pm 3$ mV) and provide a good template for assembly of negatively charged biomolecules such as BSA. In a biological fluid, proteins can readily associate with LysMBs surface leading to a protein corona that largely controls the biological in vivo response of MBs. Thus, we have investigated the adsorption of one layer of BSA, the most abundant plasma protein in mammals, and to lactoferrin (Lf) onto LysMBs surface. Lf is an iron-glycoprotein belonging to the innate immune system which plays a relevant role in the iron metabolism (Bellamy et al. 1993; Muller et al. 1999; Wakabayashi et al. 2002; Superti et al. 1997; Swart et al. 1998; Semba et al. 1998; Ikeda et al. 2000; Inoue et al. 2001), and has several anti-inflammatory properties (Elrod et al. 1997); in fact, the human Lf has also been shown to be protective in inflammation induced by allergic asthma (Mela et al. 2010).

This study provides information on the colloidal stability of LysMBs once MBs are injected in the blood stream. Our protein binding experiments show that at low molecular ratios BSA/LysMBs the albumin induces aggregation of the LysMBs, but at molar ratios higher than 1:1, it is possible to obtain BSA-coated LysMBs (see Figure 1S Supporting information). This may suggest that at the high concentration of albumin in plasma a typical bolus of injected LysMBs (10^6 MBs/mL) is possibly stabilized by a protective layer of BSA. However, the relative concentration of the LysMBs to the albumin at the site of mixing with blood



Scheme 1 Schematic representation of the Trypsin digestion of both lysozyme and LysMBs

could be an important factor to consider using the LysMBs. Thus, the coating of MBs with albumin or other proteins prior in vivo injection is a possible strategy to pursue. Loading of BSA and Lf onto MBs surface, carried out at pH 7.0 and using a 1/1 protein/LysMBs molar ratio, was studied by measuring the particles ζ-potential.

Upon BSA and lactoferrin adsorption the LysMBs surface potential of the coated particles was reversed to -24 ± 8 mV and -7 ± 1 mV, respectively. This confirms the presence of the proteins layer on the MBs surface. In addition, the fluorescence microscopy images of the LysMBs coated with fluorescent labeled FITC-BSA (Fig. 3a) and FITC-Lf (Fig. 3b) show the presence of a protein layer homogeneously distributed on the surface of the MBs.

Interestingly, contrary to BSA, Lf is one of the proteins, which carries a net positive charge at physiological pH (pI 8.0–8.5); therefore, in principle we cannot expect the electrostatic interaction to be an effective driving force for the protein layer build up. The efficient coating of LysMBs with Lf may result from the charge asymmetry of the Lf molecules which behave like a Janus particles (Bennett et al. 1981). The adsorption process of Lf on lysozyme surface could be driven by domains that are negatively charged, whereas positively charged domains face towards the solvent. However, such orientation of Lf Janus particles would prevent the effective MBs surface charge from reverting. The formation of nanometer-sized Lf aggregates, with an opposite charge to the constituent monomers, has been also observed at very low ionic strength. Lf self-association in biological fluids is widely reported and Lf tetramers were also found in human serum (Mantel et al. 1994). It was demonstrated that tetramer is the dominating form of lactoferrin under physiological conditions (Kanyshkova et al. 2001) and many Lf functional properties are ascribed to the oligomeric forms (Tanaka et al. 1998). Lf molecules within the aggregate orient themselves with the positively charged domain towards the interior of the aggregate and negatively charged domain being exposed towards the outside of an aggregate particle (Bennett et al. 1981). Hence, the experimental data suggest that Lf aggregates, bearing a negative charge, assemble on the LysMBs surface. Nevertheless, we cannot rule out a possible rearrangement of the biomacromolecule on the surface to accommodate the electrostatic interactions.

These data indicate that the LysMBs could be potentially used as a carrier of a number of therapeutic proteins whose release can be triggered by the ultrasound irradiation. Particularly, the considerable range of activities of Lf suggests that the Lf-coated LysMBs might be developed for a variety of prophylactic and therapeutic applications. With its excellent safety profile and encouraging animal efficacy results, Lf holds promise as an effective and safe drug for the treatment of diabetic ulcers, and for antimicrobial and anticancer therapies. In fact, the protection by

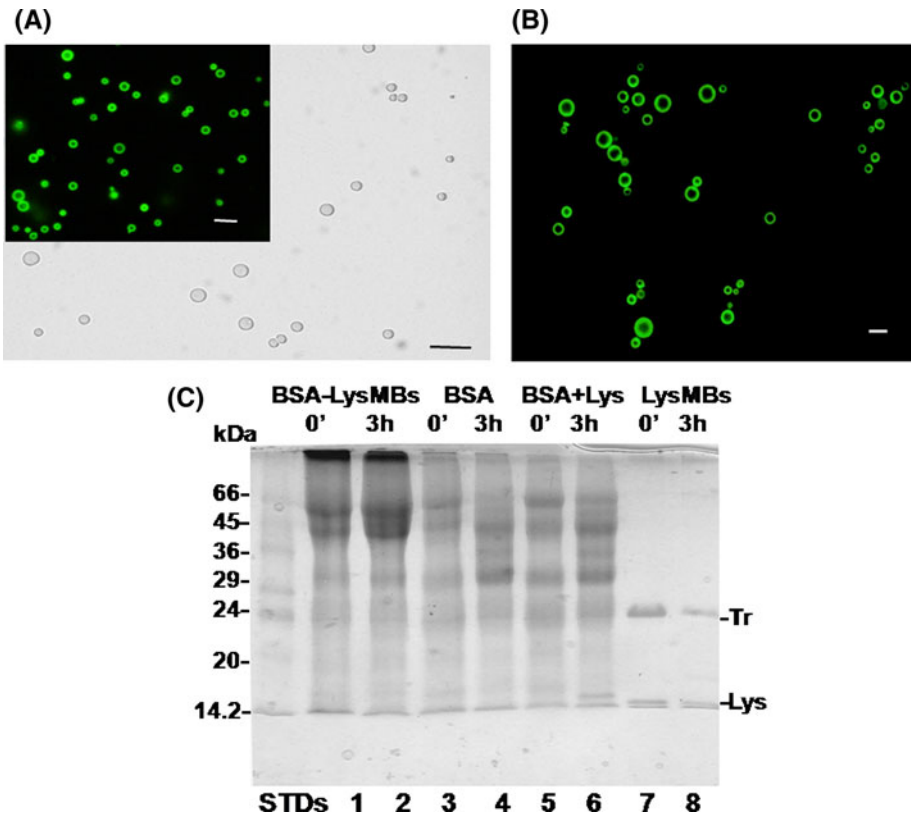


Fig. 3 Interaction of the LysMBs with BSA and Lf. **a** Optical microscopy image of BSA-coated LysMBs using a molar ratio BSA/LysMBs 1:1. *Inset*: fluorescence microscopy image of FITC-BSA-coated LysMBs, *scale bar*: 5 μm. **b** Fluorescence microscopy image of FITC-Lf-coated LysMBs using a molar ratio Lf/LysMBs 1:1, **c** trypsin proteolysis of 80 μM BSA and 40 μM LysMBs (*lines 1* and *2*), 80 μM BSA (*lines 3* and *4*), 80 μM BSA and 40 μM lysozyme (*lines 5* and *6*) and 40 μM LysMBs (*lines 7* and *8*) solutions, pH 7.3, using 1:100 E/S w/w ratio after 0′ and 3 h at 37°C

bovine Lf against mortality induced by several infections in mice has also been described (Bhimani et al. 1999) and prophylactic administrations of intravenous and oral Lf have been shown to protect against bacterial infections (Dial et al. 2000). Anticancer effects of Lf have been also demonstrated (Bezault et al. 1994) and discovered the link between the oral administration of Lf, induction of IL-18, immunomodulation, and protection against experimental cancer metastasis (Kuhara et al. 2000). Thus, the Lf-coated LysMBs could represent a new possible system for the delivery of the holo-lactoferrin for therapeutic applications. Generally, peptides and proteins are rapidly excreted from the body in a combination of events that include proteolysis, renal ultra-filtration, liver clearance, and potential immunogenicity (Caliceti and Veronese 2003). Herein, we have investigated the protective effect of the LysMBs against the protease digestion of the proteins loaded on the MBs shell. Trypsin digestions of BSA linked to LysMBs or only in presence of lysozyme were performed and the results are discussed below.

In Fig. 3c is shown the SDS-PAGE of the proteolytic products after 3 h at 37°C. The gel electrophoresis shows a different protein band pattern in the case of the proteolysis of the BSA-coated LysMBs with respect to the BSA alone or in the presence of lysozyme. The absence of characteristic "daughter" bands of the BSA after 3 h of trypsin digestion of the BSA-LysMBs indicates a major resistance of the BSA to the proteolytic cleavage when loaded on the LysMBs. A time course of the BSA-LysMBs proteolysis was performed (see Figure 2S Supporting information) and also after overnight, only low levels of BSA degradation was observable, demonstrating a high resistance to the proteolysis. A variety of peptides, proteins, and antibody fragments have recently been screened for pharmaceutical applications in virtue of their high biological activity and specificity, thus the increased resistance to protease digestion of the proteins loaded on LysMBs is a relevant benefit that can make the LysMBs an attractive delivery system for improving the in vivo stability of protein of interest for therapeutic applications.

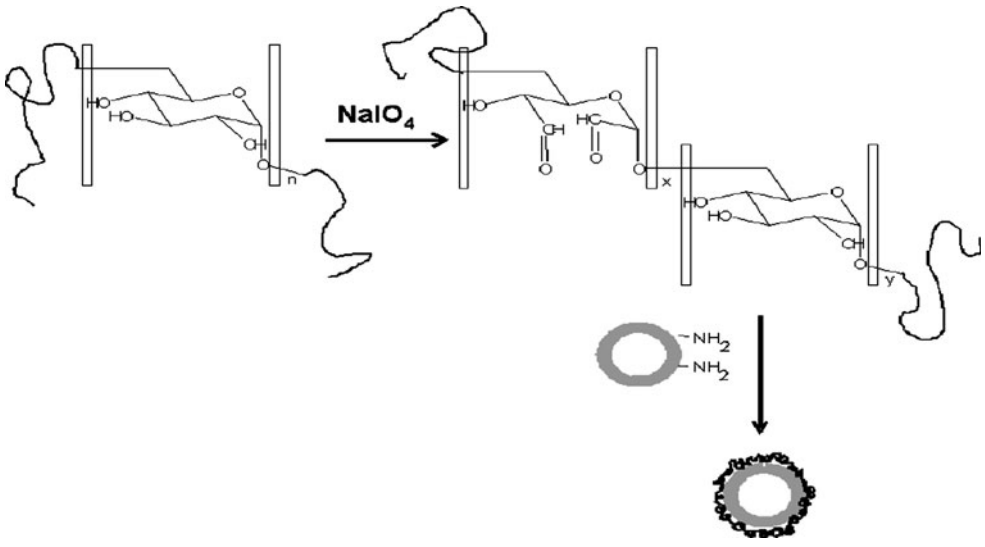
Surface modification of LysMBs with oxidized dextran

MBs in vivo circulation life is typically increased incorporating poly(ethylene glycol) chains into the shell of MBs in order to form a steric barrier against coalescence and adsorption of macromolecules to the microbubble surface. Surface functionalization with oxidized dextrans has been shown to be a viable alternative to pegylation for non-fouling applications leading to a significant reduction in protein adsorption (Martwiset et al. 2006). Due to their non-toxicity and high water solubility, dextran and its derivatives have been widely used as plasma volume expander and drug vector (Mitra et al. 2001).

Covalent attachment of oxidized dextrans is accomplished via reactions between the LysMBs surface amine groups and the aldehyde groups of dextrans (see Scheme 2). The surface decoration of FITC-labeled oxidized dextran is shown in Fig. 4. The fluorescence microscopy image of FITC dextran-coated LysMBs demonstrates that MBs can be also functionalized exploiting the chemical conjugation of other macromolecules.

Interaction of the LysMBs with DNA

The inability to deliver nucleic acids to target cells via systemic delivery is the biggest rate-limiting barrier in gene therapy applications. Targeted gene delivery by co-injection of plasmid DNA with MBs can be effective for producing detectable levels of gene expression, but it often



Scheme 2 Surface modification of LysMBs with oxidized FITC dextran

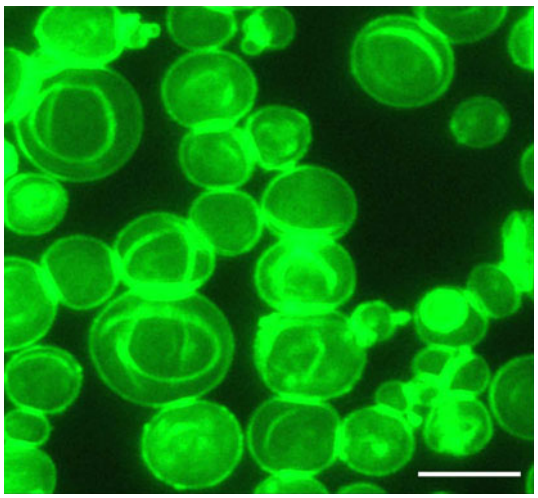


Fig. 4 Fluorescence microscopy image of FITC dextran-coated LysMBs. *Scale bar*: 3 μm

requires large amounts of DNA in order to produce quantifiable results (Bekeredjian et al. 2003). This is due to the fact that poly(nucleic acids) are prone to nuclease mediated degradation and rapid excretion by the reticuloendothelial system when introduced into the blood stream (Bielinska et al. 1997).

Therefore, carriers are required to facilitate delivery. Several methods have been developed to utilize MBs as carriers for nucleic acids in order to increase their circulation time in blood stream, protect them from degradation, and improve specificity of targeted delivery. The relative simplicity of the formulation procedure lends this class of MBs to be an attractive tool for both drug and gene delivery applications. The 110-nm-thick protein shell of MBs can accommodate the loading of nucleic acids or other macromolecules without compromising the acoustic properties of the microbubble. These macromolecules may be fully or partially incorporated within the shell by covalent cross-linking of proteins during the formulation stage. Alternatively, the charged protein surface is amenable to adsorption of nucleic acids without significantly altering the acoustic response. The mechanisms of gene attachment may be attributed to its adsorption to the microbubble shell due to electrostatic interaction. One of the aims of this work was to develop ultrasound responsive MBs that are able to bind and protect the DNA against nucleases, and stay stable during several hours. Incubation of pDNA and LysMBs results in fusion of the DNA to the protein shell. In Fig. 5a is shown the gel retardation shift assay at different pDNA/LysMBs w/w ratios, using pQE30-ww_f (pWWf) as plasmid demonstrating that at 1:20 pDNA/LysMBs w/w ratio there is a good interaction between the nucleic acid and the MBs and at 1:40 w/w ratio no free pDNA bands are detectable on the agarose gel electrophoresis. Moreover, fluorescence microscopy images of the complexes LysMBs/pDNA, which were obtained by using 40:1 w/w ratio and stained with EtBr, show the integrity of LysMBs and a uniform coating of the microbubble surface with pDNA (see Fig. 5b).

Nuclease protection of DNA by LysMBs

In Fig. 5c is shown the cleavage of pDNA by endonuclease Bam H1. This restriction enzyme has only one recognition site on the pWW_f and the cleavage leads to a linear form of the pWW_f. The agarose gel electrophoresis of the samples after nuclease treatment show the absence of a linear form of pWW_f in presence of LysMBs demonstrating the absence of detectable free pDNA and the possible protective effect of the LysMBs on pDNA.

The ability of the LysMBs to protect pDNA against nuclease cleavage was also tested using DNase I, an endonuclease that cleaves preferentially next to pyrimidine nucleotides and it is active on both single and double-stranded DNA. The LysMBs were able to prevent pWW_f degradation by DNase I as shown using two different experimental approaches described hereafter. In Fig. 5d is shown the agarose gel electrophoresis of the pWW_f and pWW_f-LysMBs complexes. After treatment with DNase I, 2.5 M NaCl was added to the samples before the electrophoresis for inducing dissociation of pDNA-LysMBs complexes. The smearing of the DNA band is noticed due to the high salt concentrations used and the complete degradation of the pDNA was observed in absence of MBs. No complete dissociation of the complexes occurred under these conditions indicating a strong interaction between the macromolecules. The resistance of pDNA-coated LysMBs to DNA degradation with nuclease digestion enzymes is a result, which is consistent with other literature reports utilizing cationic polymers to bind and protect DNA (Lentacker et al. 2006; Nishikawa and Huang 2001; Bielinska et al. 1997; Boussif et al. 1995; Midoux et al. 2008). Moreover, the ability of the LysMBs to inhibit the pDNA degradation by DNase I was also evaluated by transformation of bacterial cells with the pWW_f after treatment with DNase I in the presence and absence of the LysMBs. In Fig. 5e are shown the petri dishes obtained after transformation of *E. coli* cells with pWW_f and pWW_f-LysMBs solutions after incubation for 10 min with an excess of DNase I at room temperature. The presence of the *E. coli* colonies only in the case of the bacteria transformed with pWW_f-LysMBs demonstrates the resistance of pDNA loaded LysMBs to nuclease and the integrity of the pDNA further demonstrating that the interaction between pDNA and LysMBs does not induce changes on the pDNA that could interfere on the transformation property of nucleic acid molecules. Furthermore, a good regulation and

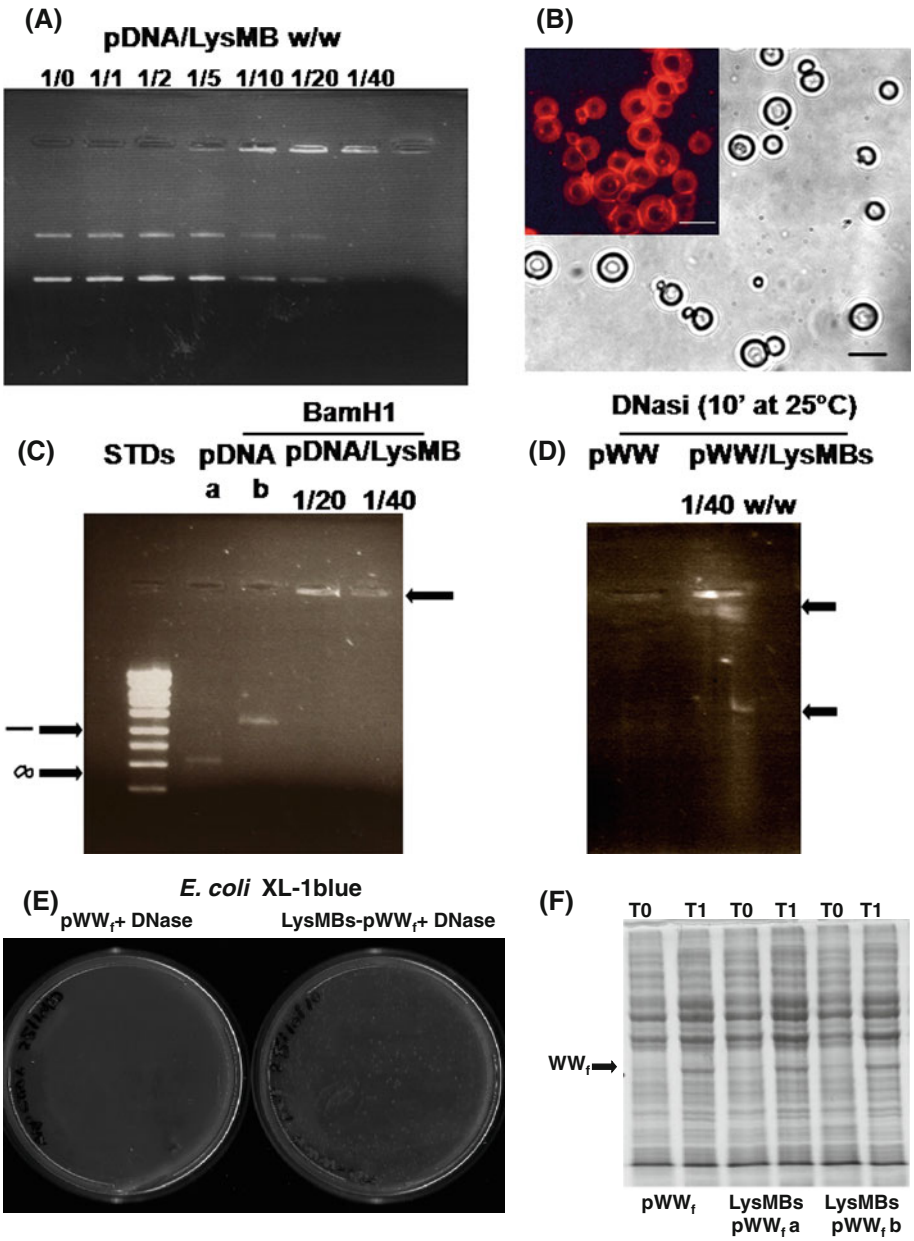


Fig. 5 Interaction of the LysMBs with pDNA. **a** Gel retardation shift assay at different pWW_f/LysMBs w/w ratios, using 0.5 μg pWW_f and 1% agarose gel, the a band is due to the retardation shift effect of the peptide on the pDNA, **b** Optical microscopy image of pWW_f-LysMBs. *Inset*: fluorescence microscopy of pWW_f-LysMBs stained with 2 μl of EtBr solution (0.004 mg/ml). Scale bar: 3 μm, **c** Agarose gel electrophoresis (1% agarose) of the 0.25 μg pWW_f after cleavage with BamH1 (0.2 U) for 1 h at 37°C, **d** Agarose gel was run after addition of 2.5 M NaCl for inducing dissociation of pDNA. Smiling appeared due to the high salt concentrations used, **e** the petri dishes of LB agar medium with 100 μg/ml ampicillin obtained after overnight incubation at 37°C with *E. coli* cells transformed with pWW_f (**a**) and pWW_f-LysMBs (**b**) solutions, which were previously treated with 0.3 U of DNase I for 10 min at 25°C, **f** gene expression of ww_f in *E. coli* transformed with pWW_f-LysMBs complexes. T0 and T1 are, respectively, before and after induction of the expression by addition of 1 mM IPTG

expression of WW_f protein in BL21 *E. coli* cells transformed with pWW_f loaded LysMBs was also observed (see Fig. 5f). These results indicate that the LysMBs show a good efficiency to interact and protect the pDNA without affecting the properties of nucleic acid to transfer the genetic information and show the prospective to use them as an efficient system to the delivery of the nucleic acids to the cells or in bacterial biofilms.

Innovative applications of protein-MBs initiate new studies on their chemical-physical properties and to use proteins that are able to confer them specific properties and new potentiality. In summary, in the present work the functional properties of LysMBs have been evaluated demonstrating their biodegradability and the absence of proteolytically resistant protein structures. Different coatings can modify the cellular interactions of MBs in terms of their adhesion, internalization and intracellular fate following internalization. Here we presented evidence that LysMBs can be functionalized by other proteins (e.g., albumin and lactoferrin), polymers (e.g., dextran) and nucleic acids. The resistance to the proteolysis of proteins loaded on the shell of LysMBs has also been demonstrated indicating the possibility of new potential applications of these MBs as carrier of proteins in prophylactic and therapeutic applications and in tissue engineering. In addition, the resistance of pDNA-loaded LysMBs to the DNA degradation by nucleases and their ability to transform the bacteria cells suggest their possible use in gene delivery.

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