Redox-Active Polymer Microcapsules for the Delivery of a Survivin-Specific siRNA in Prostate Cancer Cells

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ABSTRACT In this report, we describe the delivery of small interfering RNA (siRNA) using LbL-assembled microcapsules. The microcapsules are based on negatively charged poly(methacrylic acid) nanometer thin films containing cross-linking disulfide bonds. One system is polycation-free and another contains polylysine for siRNA complexation in the microcapsule void. When microcapsules containing a siRNA targeting survivin were delivered to PC-3 prostate cancer cells, a significant inhibition of the expression of the antiapoptotic protein was observed. However, down-regulation of survivin was also observed in PC-3 cells exposed to microcapsules embedded with a scrambled siRNA as well as in cells treated with empty microcapsules. These findings indicate a capsule-dependent off-target effect, which is supported by a reduction in the expression of other survivin-unrelated proteins. The microcapsules and their polymeric constituents do not affect cell proliferation, as determined by a metabolic assay, even after 4 days of exposure. In addition, in PC-3 cells exposed to microcapsules, we observed a marked accumulation of LC3b, a marker related to autophagy (i.e., self-digestion), a degradation pathway involved in the maintenance of cell homeostasis in response to different stresses. This evidence suggests that empty microcapsules can induce a perturbation of the intracellular environment, which causes the activation of a cell safeguard mechanism that may limit the therapeutic effect of the microcapsules in tumor cells.

KEYWORDS: autophagy · biocompatibility · layer-by-layer · microcapsules · siRNA · survivin

Small interfering RNAs (siRNA) are double-stranded RNA molecules approximately 21−25 base pairs (bp) long that act to inhibit gene expression through degradation of a sequence-matched mRNA.[1] siRNAs are promising therapeutics for the treatment of many disease states[2] by preventing the production of specific proteins but suffer from delivery problems. They are quickly degraded in the bloodstream and cannot efficiently cross the cell membrane unaided. A carrier is required to deliver the siRNA to its action site and improve its clinical potential. A number of different siRNA carrier systems have been proposed, including viruses,[3] cationic lipids,[4] and polymeric particles.[5,6] Most of the currently used siRNA delivery systems such as lipoplexes or polyethylenenimine (PEI) (polyplexes) are toxic and this severely limits their clinical applications. In addition, lipoplexes tend to be structurally more heterogeneous and unstable, aggregating over time in solution, and as a result, lipoplexes are typically prepared immediately before use. These represent major disadvantages from the standpoint of reproducibility, manufacturing, and drug administration. Given their inherent instability, especially when exposed to plasma proteins, the use of lipoplexes as an *in vivo* delivery vehicle for siRNA may be best suited to local direct administration, such as intranasal or orotracheal.[7] A number of approaches based on the use of polymers have been reported to effectively deliver siRNA in different experimental models. For example, a membrane-active polymer to which siRNA was covalently coupled *via* a disulfide bond and where both poly(ethylene glycol) (PEG, for stealth properties) and *N*-acetylgalactosamine (for hepatocyte targeting) groups are linked *via* pH-sensitive bonds has been used for the delivery of two different siRNA sequences (targeting apolipoprotein B and peroxisome proliferator-activated receptor alpha) into mouse liver *via* low-pressure intraveneous injection.[8] In addition, an electrostatic complex consisting of pH-responsive cationic micelles—formed from diblock copolymers of dimethylaminoethyl methacrylate and butyl methacrylate and siRNA—was found to sensitize multidrug resistant ovarian cancer cells to doxorubicin *via* knockdown of polo-like kinase 1.[9] Transferrin-targeted, cyclodextrin-containing polycation nanoparticles were also used for the encapsulation and delivery of siRNA in both animals and humans.[6,10] These nanoparticles demonstrated specific silencing of the *EWS-FLI1*

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gene in a murine model of metastatic Ewing's sarcoma, following long-term, low-pressure, low-volume tail-vein administrations.[10] These particles are currently in clinical trials, using siRNA targeting the M2 subunit of ribonucleotide reductase, an established anticancer target.[6]

Layer-by-layer (LbL) assembly has shown to be an emerging technique for the creation of drug delivery vehicles with nanometer thin polymer walls.[11] Microcapsules have a higher payload capacity than smaller nanoparticles, and can accommodate multiple types of drug molecules. The layered nature of the assembly makes surface modification relatively simple for inclusion of targeting or stealth properties. It also makes it possible to include different degradation mechanisms within a single film. When all of these properties are combined, LbL assembled microcapsules are a multifunctional platform for the release of different therapeutic agents and may be used for the dual delivery of anticancer drugs and siRNA.

LbL assembly has been investigated extensively for the encapsulation and delivery of DNA,[12] however there are few examples of siRNA delivery. One report details the LbL assembly of PEI and siRNA on gold nanoparticles.[13] The siRNA-coated nanoparticles were delivered to CHO-K1 cells stably expressing enhanced green fluorescent protein (EGFP) and reduced the production of EGFP to approximately 30%. In another study, siRNA-loaded calcium phosphate nanoparticles were incorporated into a LbL assembled film on a planar substrate.[14] Cells were seeded on the substrate and shown to reduce the expression of the protein osteocalcin.

In the present study, we deliver an siRNA targeting the cancer-related and antiapoptotic factor survivin, using two different types of microcapsules based on the LbL assembly of a reducibly degradable cross-linked poly(methacrylic acid) (PMA) film. The films are designed to maintain capsule integrity in the oxidizing bloodstream and in the extracellular environment (thus protecting the siRNA from denaturation) and then release the cargo in the reducing intracellular environment. The first type of capsules (polymer hydrogel capsules, PMA HCs) are composed of a multilayered film made with thiol-modified PMA (PMA_{SH}) that is alternately assembled with poly(vinylpyrrolidone) (PVPON) on 1 μm-diameter solid silica particles under hydrogen-bonding conditions at pH 4.[15] The thin hydrogel-like film is around 30 nm thick when complete.[16] Disulfide bridges are formed by oxidation and after core removal the film is washed into neutral pH solution, where the PVPON is released.[17] At neutral pH, the PMA HCs swell to approximately 1.5 μm. The siRNA cargo is preloaded onto the template particle prior to multilayer assembly. These PMA HCs have found several applications,[18] such as DNA encapsulation,[19,20] as well as the successful delivery of oligopeptides to white blood cells *in vitro* and *in vivo*,[21−23] and doxorubicin to cancer cells.[24] In addition to these microcapsules, a modified version has been included in the study, and are referred to as PMA_{SH}-polylysine (PLL) microcapsules. Instead of a solid particle, we used a nanoporous particle template into which PLL is infiltrated prior to PMA_{SH} multilayer assembly. The siRNA cargo is postloaded into these preformed microcapsules by diffusion through the multilayer film and complexation by the PLL.

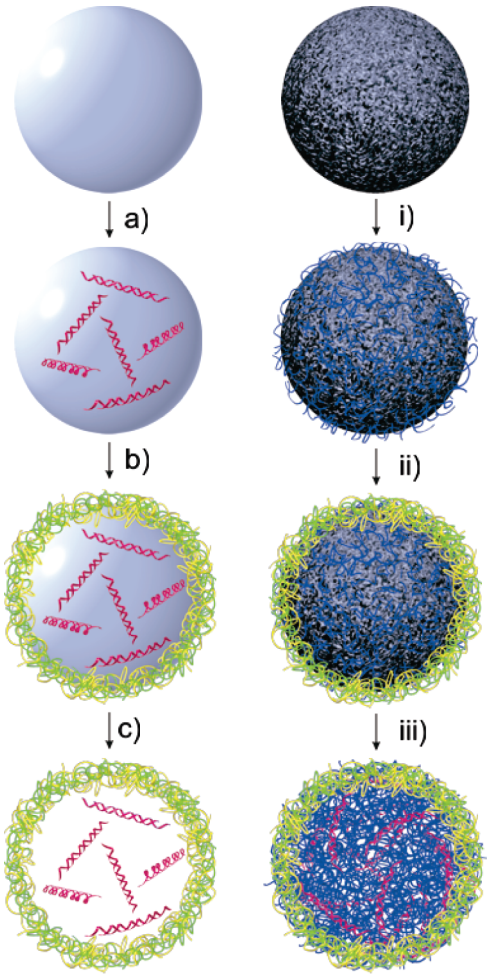

Figure 1. Schematic representation of siRNA loading methods. Preloaded (PMA_{SH}) microcapsules: (a) siRNA is adsorbed onto amine-functionalized silica particles; (b) LbL assembly of the PMA_{SH} film is performed around the siRNA-coated particles; (c) the film is cross-linked and the core removed. Postloaded (PMA_{SH}-PLL) microcapsules: (i) mesoporous silica particles are infiltrated with the polycation PLL; (ii) LbL assembly of the PMA_{SH} film is performed around the polymer filled particles; (iii) following core removal, the siRNA is infiltrated into the particles and sequestered by the polycation core.

RESULTS AND DISCUSSION

Loading siRNA into Microcapsules. Preloading of microcapsules with siRNA cargo (PMA HCs) has been achieved according to our method described for oligonucleotides[19] and dsDNA[20] and is summarized in Figure 1. The siRNA was incubated with amine-functionalized silica spheres

at 50% surface coverage, to ensure the subsequently formed polymer film maintains its integrity.[20] The siRNA used was a 22-mer duplex of RNA with a molecular weight of 13 000 g mol^{-1}. LbL assembly was performed at pH 4 *via* the alternate deposition of PMA_{SH} and PVPON until the nanometer sized film could retain the siRNA. When the silica core was dissolved away, the nucleic acids were displaced into the central void of the capsule. This technique results in microcapsules filled with nucleic acids that are uncomplexed. This is a distinguishing feature of this encapsulation method, as nucleic acids are typically complexed with polycations for gene or siRNA delivery.[25] The free state of the nucleic acids has allowed the application of these microcapsules for the encapsulated enzymatic production of RNA using a DNA template,[26] which is not possible when DNA is complexed with a polycation. The ability of the microcapsules to retain cargo is crucial for their use as drug delivery vehicles. If the microcapsules leak the contents of the cargo before reaching a desired destination the dose will be reduced, and the advantage of encapsulated delivery is negated. siRNA is one of the smallest types of nucleic acid cargo and is difficult to retain. An efficient and facile way to control the permeability of LbL thin films is to alter the thickness by changing the number of layers deposited. After encapsulation of a fluorescently labeled siRNA within PMA HCs of varying wall thickness, the PMA HCs were incubated in either pH 4 or pH 7 solutions for 24 h and the fluorescence of the siRNA remaining in the PMA HCs was measured using flow cytometry. At least 7-bilayers of PMA_{SH} (~14 mol % thiol groups) were required to retain 85% of the initially adsorbed siRNA in PBS (Figure 2A and B). Smaller biomolecular cargo (oligopeptides with a molecular weight of 1000 g mol^{-1}) freely diffuse through the capsule wall and have required covalent attachment to a polymer anchor to retain them inside the PMA HCs.[23] However, the permeability of the microcapsules is also dependent on the shape and charge of the cargo. For example, uncharged PVPON of 55 000 g mol^{-1} diffuses through the polymer film forming the capsules and is removed when 5-bilayer PMA_{SH} films are incubated in pH 7 solution.[17] It is likely that the negatively charged, stiff nature of the siRNA molecule reduces its permeability to the PMA_{SH} film, which provides both an electrostatic and steric barrier to leakage. The siRNA was encapsulated more effectively at pH 4, when both PVPON and PMA were present in the film (Figure 2A). However, even with both polymers present, the PMA HCs were significantly more permeable than traditional electrostatic LbL films, such as poly(styrene sulfonate)/poly(allylamine hydrochloride) microcapsules, which required only 3.5-bilayers to exclude a 30-mer ssDNA (a smaller and more flexible molecule).[27] This highlights that the properties of this film are significantly different to other films synthesized using

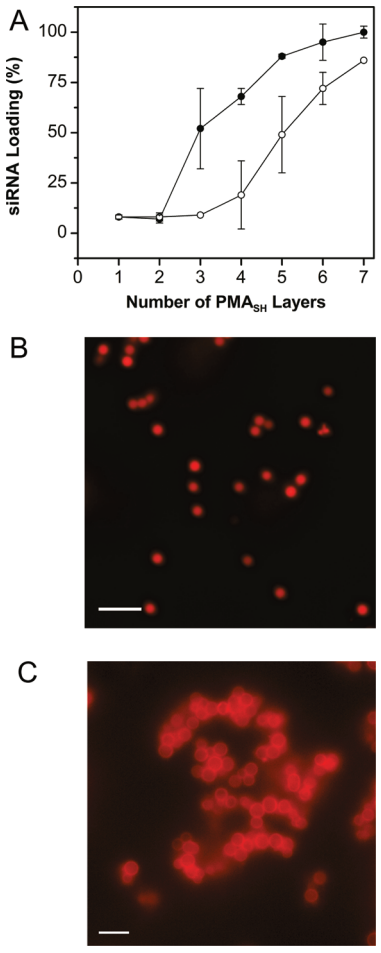

Figure 2. (A) Percentage of fluorescently labeled siRNA remaining inside PMA HCs as a function of the number of polymer layers (PVPON and PMA_{SH}), as assessed by flow cytometry at pH 4 (●) and pH 7 (○). (B) Representative image of fluorescently labeled siRNA (red) encapsulated within PMA HCs of ~1 μm diameter at pH 7. Scale bar: 5 μm. (C) Representative image showing fluorescently labeled siRNA (red) within PMA_{SH}-PLL microcapsules of ~1 μm diameter at pH 7. The observed clustering effect is due to sample drying. Scale bar: 3 μm.

the LbL method. The degree of thiol modification may also be used to alter the permeability of the microcapsules — this is the subject of ongoing research.

The postloading technique used to make PMA_{SH}-PLL microcapsules relies on sequestration of the siRNA inside the microcapsules after they are formed. The polycation PLL is infiltrated into the pores of nanoporous silica particles[28] prior to LbL assembly of the PMA_{SH} film, followed by subsequent core dissolution. The PMA_{SH} film is designed to be permeable to siRNA by using only 5-bilayers in the film, which is below the threshold of 7-bilayers for siRNA impermeability, as determined in Figure 2C. Therefore, the siRNA can diffuse through the film and is sequestered by the high concentration of encapsulated polycation in the central void (the loading of PLL is 10 mg per g of silica particles). The microcapsules are loaded with siRNA at pH 4 to minimize the electrostatic repulsion with the

PMA_{SH} multilayer film as in this state the polyelectrolyte is below its pK_a and therefore largely noncharged. At physiological pH the amino groups of PLL are positively charged and strongly interact with the negatively charged siRNA. As PLL is toxic at low concentrations,[29] we hypothesize that having the PLL/siRNA nanoassemblies within the microcapsule core is advantageous compared to uncoated PLL/siRNA because the multilayered film can shield the toxicity of the polycation.[30] PLL was selected as the polycationic component because it can be enzymatically degraded within cells into the amino acid lysine.

PMA_{SH}-PLL microcapsules containing fluorescently labeled siRNA at pH 4 and subsequently washed into pH 7 are shown in Figure 2C. PLL/siRNA nanoassemblies associate with the slightly negatively charged capsule wall at pH 4. Even after increasing the pH to 7, where the cross-linked multilayered film slightly swells (from 1.0 $\pm$ 0.1 to 1.2 $\pm$ 0.1 μm diameter), the loaded PLL/siRNA polyplex is retained inside the cavity (1.2×10^5 molecules of siRNA/capsule).

Each of the encapsulation techniques presented has its strengths and limitations. PMA_{SH}-PLL microcapsules have the capacity to load more siRNA as a function of PLL content than PMA HCs, which are restricted by the surface area of the template particles. However, both types of microcapsules have a high payload compared to other siRNA delivery systems. For example, a 70-nm nanoparticle made of cyclodextran-containing polycations can contain ~2000 siRNA molecules[31] whereas antibody conjugates have <10 siRNA molecules.[32] Conversely, a single PMA_{SH}-PLL capsule or PMA HC (size $\approx$ 1.5 μm) can carry 1.2×10^5 and 4×10^4 molecules of siRNA, respectively.

Capsule-Cell Interactions. The interaction of microcapsules with cells must be understood in terms of internalization, toxicity, and activity. Drug delivery vehicles must be nontoxic and nonimmunogenic. The interaction of PMA HCs with several cell lines has already been investigated, including whole blood,[21] colon cancer cells,[24,33] and a range of popular mammalian cell lines such as CHO and HEK.[34] These studies indicated that these microcapsules are nontoxic and that they can be internalized by certain cell types. The difference in the level of interaction of microcapsules with different cell types[21,34] highlights the need to investigate their interaction with each new cell line used. In addition, in the reducing intracellular environment these microcapsules are expected to deconstruct; therefore, we evaluated the effect of empty microcapsules and of PMA_{SH} polymer on the viability of an androgen-independent prostate cancer cell line using the tetrazolium salt (MTS). MTS is converted to formazan by mitochondrial dehydrogenases present in metabolically active cells, and the reaction is directly proportional to the number of living cells.

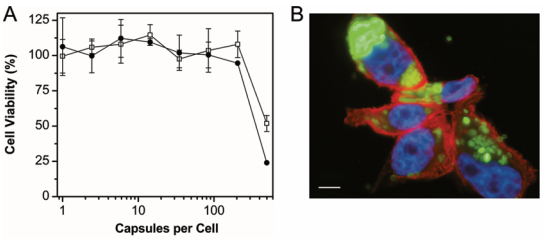

Figure 3. (A) Cell growth inhibition curves for PC-3 cells after incubation for 96 h. Data are reported as a percentage of viable cells exposed to PMA_{SH} (●) and PMA_{SH}-PLL (□) empty microcapsules compared with untreated cells. Data represent mean values $\pm$ s.d. (B) Fluorescence microscopy representative image showing the internalization of PMA HCs within PC-3 cells. PMA_{SH} was labeled with Alexa Fluor 488 dye (green); the immunostaining of the plasma membrane was performed using an appropriate antibody raised against the CD44 cell surface marker (red); nuclei were counterstained with 4′,6-diamidino-2-phenylindole (blue). Magnification 100×; scale bar: 10 μm.

Results from the MTS assay indicated that cell viability was essentially unaffected in PC-3 cells incubated with up to 200 microcapsules/cell for 96 h, whereas cell survival decreased abruptly above this threshold (Figure 3A). On the basis of this evidence, the microcapsules could be considered to have negligible toxicity to the PC-3 cells. These results agree with cell viability data obtained with oil-loaded PMA HCs, showing that the survival of colon cancer cells incubated with 100 microcapsules/cell for 24 h was not impaired,[24] and data for PMA HCs in various mammalian cell lines.[34] The surface chemistry is a defining property in cell viability studies. In this context, it is noteworthy that PMA_{SH} and PMA_{SH}-PLL microcapsules behaved similarly regarding cell viability (Figure 3A). PLL is known to be toxic at low concentrations and short incubation times;[29] however, for a 96 h incubation we observed no marked difference with the PMA HCs. As PLL is likely to remain complexed with PMA_{SH} in the extracellular environment, we hypothesize that this limits PLL from disrupting the cellular membrane. Although the microcapsules are degradable within the cellular environment, the polymer PMA_{SH} is not biodegradable. The capsule fragments are above the generally accepted size for renal excretion and are expected to be removed *via* biliary excretion in *in vivo* applications. To understand the effect of the capsule degradation products on cell viability, an MTS assay was performed in cells exposed to the PMA_{SH}. Results showed a marked decrease in PC-3 cell viability only at the highest (1 g L^{-1}) PMA_{SH} concentration tested (Figure S1, Supporting Information). Based on these findings, the degradation product might be considered devoid of any cytotoxic effect, given that concentrations above 0.2 g L^{-1} are far from the range of PMA_{SH} amounts expected to be released within a cell after degradation of a single 10-bilayer capsule. PMA_{SH} is significantly less cytotoxic than polycations commonly used for nucleic acid delivery, including PLL.

When the polycations PLL, PEI, protamine, and histone were incubated with cells at 1 g L^{-1} for 24 h, cell viability was reduced to less than 50% in brain microvessel endothelial cells, macrophages, hepatocytes and L929 mouse fibroblasts.[29,35] In our cell model, an equivalent decrease in cell viability was observed only after 96 h of incubation with 1 g L^{-1} PMA_{SH} (Figure S1, Supporting Information).

To determine whether microcapsules were efficiently internalized by tumor cells, their intracellular distribution was investigated by fluorescence and confocal laser scanning microscopy. Our analysis showed that 78 ± 5% of PC-3 cells had efficiently internalized one or more capsules after 24 h exposure to 100 fluorescently labeled microcapsules/cell (Figure 3B and Figure S2A and B, Supporting Information). This level of internalization is in agreement with a recent study using several cell lines showing ~80% of cells contained the PMA HCs.[34] It is clear from the images that degradation of the microcapsules has not continued to completion. The degradation process inside cells is slower than in experiments that mimic the cytoplasmic glutathione concentration, where degradation of PMA HCs occurred within 8 h.[16,17] These findings suggest that the large number of PMA HCs inside the cell may exhaust the available glutathione and thus do not completely degrade. Some capsules may remain partially degraded in cellular compartments. However, the thin film requires only holes and not complete destruction to allow the small siRNA cargo to leak from the capsule into the cell. This may make it difficult to achieve prolonged release (and transfection) from the PMA HCs; however, some temporal control over the cargo release may be achieved by increasing the density of the disulfide cross-links. We previously found that changing the density of the cross-links can alter the degradation rate,[16] which could potentially alter the time when cargo is released and the cell transfected. Compared to PMA HCs in solution (Figure 2B), the internalized PMA HCs are deformed, as indicated by the loss of their characteristic ring fluorescence, either due to osmotic pressure or mechanical force. Similar results were obtained with PMA_{SH}-PLL microcapsules, even though they were less efficiently taken up by tumor cells, as observed by fewer capsules inside the cell (Figure S2C, Supporting Information).

siRNA Delivery: On-Target versus Off-Target Effect. We next evaluated the ability of LbL assembled PMA HCs to deliver an siRNA (siSurv) able to decrease the expression of *survivin,* a cancer−related target gene.[36] Survivin is the smallest member of the Inhibitors of Apoptosis Proteins (IAP). As for other IAP family members, it acts as an endogenous antiapoptotic protein in response to different stimuli, such as death receptor activation, growth factor withdrawal, ionizing radiation, viral infection, and genotoxic damage.[37] Survivin is a multifunctional protein involved not only in apoptosis control but it orchestrates integrated cellular networks that are essential for tumor cell proliferation and viability.[38] In addition, some evidence suggests that survivin plays an important role in the drug and radiation resistant phenotype of human tumor cells, including prostate cancer.[37] Due to its activity within tumors and because survivin is abundantly found in virtually every human tumor, it has attracted much attention as a cancer relevant target. It has been proposed that the inhibition of survivin expression/functions in cancer may be a very useful and direct therapeutic strategy.[37]

For the intracellular delivery of siSurv we compared LbL microcapsules and a standard cationic lipid-mediated transfer system, namely Lipofectamine2000. As expected,[36] Western blot analysis showed that compared to a scrambled siRNA, 50 nM siSurv induced an almost complete abrogation of survivin expression levels (Figure 4A) when delivered to PC-3 cells using Lipofectamine2000. However, when PMA HCs were used to deliver siSurv, the extent of survivin inhibition was not as high (Figure 4A and Figure S3, Supporting Information). The survivin down-regulation from PMA HCs reached its maximum (~47%) at 96 h after 4 h transfection and was still appreciable 24 h later (Figure S3, Supporting Information). A down-regulation of survivin typically results in the activation of apoptosis.[36] Therefore, we examined the catalytic activity of caspase-3, the terminal effector caspase of the apoptotic machinery, which is expected to increase its activity when apoptosis is activated. As shown in Figure 4B, a 2.3-fold increase of caspase-3 catalytic activity was readily detectable in PC-3 cells at 72 h after the exposure to siSurv-filled microcapsules. However, the caspase-3 activation rapidly decreased at later time points and cells depleted for survivin did not show any morphological features of apoptosis (data not shown). We also did not observe any variation in the number of viable cells (data not shown), thus suggesting that the extent of survivin inhibition reached with capsule-delivered siRNA was not sufficient *per se* to overcome the threshold for the induction of spontaneous apoptosis.

One reason for the relatively low efficiency in the inhibition of survivin expression with encapsulated siRNA, compared to the transfection approach by lipoplexes, could be explained in terms of the amount of siRNA that was effectively delivered to PC-3 cells by microcapsules. In fact, the total amount of siRNA used in the transfection with microcapsules was ~150-fold less than in the standard transfection experiment with Lipofectamine2000 (0.33 nmol L^{-1} vs 50 nmol L^{-1}, respectively). To investigate this further, the same amount of siRNA (0.33 nmol L^{-1}) was delivered to PC-3 cells by both Lipofectamine2000 and LbL-assembled microcapsules. Under these experimental

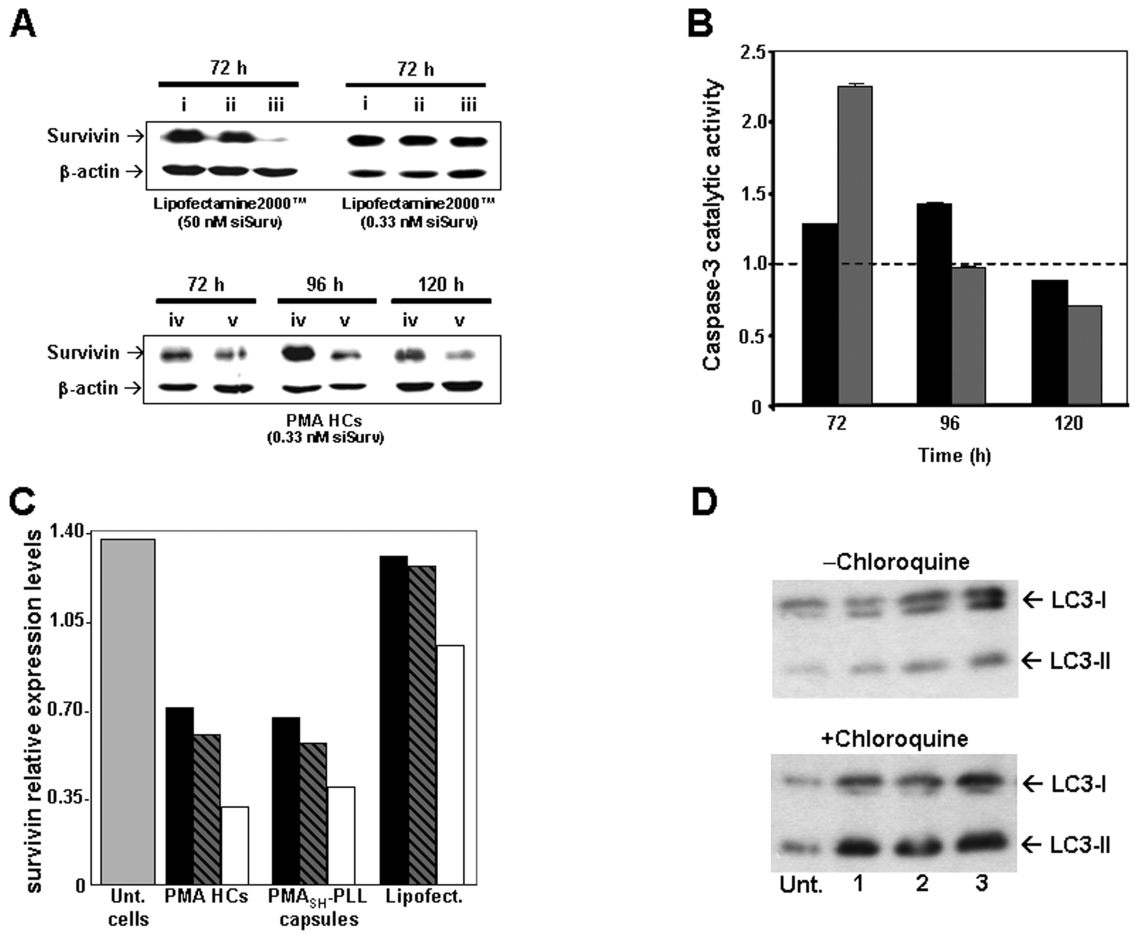

Figure 4. (A) Representative Western blot data showing survivin expression in PC-3 cells exposed to siSurv using either Lipofectamine2000 (according to the protocol reported in ref 36) or siSurv-filled PMA HCs. (i) Untreated cells; (ii) empty control (i.e., only Lipofectamine2000); (iii) 50 nM or 0.33 nM siSurv; (iv) empty microcapsules; (v) siSurv (0.33 nM) within PMA HCs. (B) Caspase-3 catalytic activity was assessed in PC-3 cells after the exposure to PMA_{SH} capsules in the presence (gray bars) or absence (black bars) of siSurv by hydrolysis of fluorogenic substrate *N*-acetyl-Asp-Glu-Val-Asp-pNA (DEVD-pNA). Data are reported as relative caspase-3 activity in treated samples compared to untreated cells (dashed line). (C) Quantification of survivin expression levels in PC-3 cells transfected with an equimolar amount (0.33 nM) of siSurv using PMA HCs, PMA_{SH}-PLL microcapsules or Lipofectamine2000. Data are reported as relative survivin expression levels in empty transfectants (black bars), in scrambled- and in siSurv-transfected cells (striped gray and white bars, respectively). Basal survivin expression levels in control (Unt.) cells are also displayed. (D) Representative Western blot data showing the expression levels of the autophagy markers LC3-I/LC3-II in untreated PC-3 cells (Unt.) and in cells exposed to empty microcapsules (1) as well as PMA HCs filled with scramble siRNA (2) or siSurv (3) ± Chloroquine.

conditions, a low down-modulation of survivin expression was observed when siSurv was delivered by Lipofectamine2000 compared to PMA HCs or PMA_{SH}-PLL microcapsules (Figure 4C and Table 1). However, a pronounced down-regulation of survivin also occurred in PC-3 cells exposed to either empty or scrambled-filled PMA HCs and PMA_{SH}-PLL microcapsules compared to cells exposed to Lipofectamine2000 (Figure 4C and Table 1). This suggests that exposure of PC-3 cells to microcapsules may also result in off-target effects. This result was supported by the evidence that other proteins not targeted by the siRNA, such as XIAP and Bcl-x_L were concomitantly down-regulated (data not shown). These findings may indicate that microcapsules or non biodegradable polymers can interact with intracellular components, forming supramolecular complexes (*e.g.*, protein aggregates) that could be selectively degraded in the cell *via* the autophagic pathway

To further understand the down-regulation of the off-target proteins in response to empty capsules, the cellular macroautophagy response was investigated. Macroautophagy has been recently described as a degradation pathway that can be activated to maintain cell homeostasis in response to different insults.[39] It is initiated by sequestering cytoplasmic cargoes (*e.g.*, long-lived, aggregated proteins and defective organelles) in double-membraned vesicles, called autophagosomes. The autophagosome is progressively acidified by fusion with lysosomes, to form an autolysosome where sequestered molecules are degraded and eventually recycled.[40]

The activation of macroautophagy was investigated by probing for an increase in the production of the

TABLE 1. Analysis[a] of Survivin Expression Data Obtained According to the Different Transfection Methodologies Using Equimolar Amounts of siSurv (0.33 nmol L^{-1})

	survivin/actin expression ratio[b]	% of survivin inhibition compared to untreated cells[c]	% of survivin inhibition compared to empty carriers[d]
PMA HCs			
Empty	0.70	49	—
Scrambled siRNA	0.59	57	16
siSurv	0.31	77	56 (48)
PMA_{SH}-PLL microcapsules			
Empty	0.66	52	—
Scrambled siRNA	0.56	59	15
siSurv	0.39	71	40 (30)
Lipofectamine2000			
Empty	1.30	5	—
Scrambled siRNA	1.26	8	3
siSurv	0.95	31	27 (25)

[a] The analysis has been performed according to data reported in Figure S3, Supporting Information. [b] The expression levels of survivn in each cell culture and is reported as the ratio between the densitometric values of survivin expression levels and those of β-actin, as detected by densimotetric analysis of Western immunoblotting data using ImageQuant software. [c] Data are reported as a percentage of inhibition of survivin expression levels in cell cultures as a function of the different treatment modalities compared to untreated (control) cells. Survivin/actin expression ratio in control cells = 1.37 (ImageQuant densitometric analysis). [d] Data are reported as a percentage of inhibition of survivin expression levels in cells exposed to siRNA according to the different delivery approaches compared to cells exposed to empty carriers. Numbers in brackets represent the inhibition of survivin expression levels reported as a percentage with respect to cells exposed to scrambled siRNA.

lipidated form of LC3b (LC3-II), a marker associated with the membrane of autophagosomes. This marker was increased in PC-3 cells exposed to microcapsules, and was independent of the presence of siRNA (Figure 4D).[41] To allow better characterization of LC3-II, chloroquine was applied to the cells to prevent degradation of the LC3-II in the autophagosomes by inhibiting autophagy *via* prevention of autophagosome acidification.[42] This showed a more marked accumulation of LC3-II in PC-3 cells exposed to microcapsules. This evidence indicates that an autophagic flux was activated in PC-3 cells, likely as a consequence of the internalization of microcapsules. Our recent study showed that PMA HCs internalized by colon cancer cells were indeed deformed in membrane-enclosed compartments, which further mature to late endosomes or lysosomes.[33] Given the close relationship between autophagosomes and lysosomes, it could be hypothesized that depending on the cellular context, the accumulation of microcapsules into intracellular vesicles could represent either the consequence of the activation of a cell defense pathway to external insults (*e.g.,* autophagy) or a potential mechanism to be exploited for the release of cargo. However, our results (Table 1) suggest that a small amount of siRNA is released from LbL assembled microcapsules into the cytoplasm. For example, the PMA_{SH} backbone might become more hydrophobic at low pH when the carboxylic acids are uncharged and may disrupt the endosomal lipid membrane, as previously demonstrated with poly(propylacrylic acid), which enhanced the intracellular delivery of DNA through this mechanism.[43] In addition, both siRNA and PLL-siRNA complexes should be released when the disulfide bonds in the membrane are broken by glutathione reduction, forming pores through which the cargo can escape. On the basis of our findings, a scenario could be set in which the intracellular fate of microcapsules and their cargos would depend on a fine balance between the activation of a defensive autophagic pathway and the ability to escape the lysosome compartment. This interesting phenomena is worthy of further investigation and is currently being expanded on in our laboratories.

CONCLUSION

LbL-assembled microcapsules with a reducibly degradable nanometer thin film wall have been successfully loaded with a siRNA by two different loading methods: preloading the template core, and postloading a preformed capsule filled with a polycationic sequestering agent. The siRNA cargo was able to decrease the expression of the antiapoptotic protein survivin, indicating that a certain degree of active siRNA release occurred. Future studies will attempt to quantify potential inactivation of siRNA during capsule synthesis, and the long-term stability of the encapsulated siRNA. There are several ways to improve the effectiveness of the siRNA delivery, for example, by increasing the amount of siRNA delivered by the microcapsules. This might be achieved by using a larger amount of polycation to sequester higher concentrations of nucleic acids. Additionally, surface functionalization of the microcapsules may promote cellular internalization *via* a pathway that improves cytoplasmic release. However, with our model, a pronounced down-regulation of survivin was also observed using microcapsules filled with a scrambled siRNA as well as with empty microcapsules. These findings suggest a capsule-dependent off-target effect, as also confirmed by the observed decrease in the expression of other proteins with survivin-unrelated mRNA sequences, such as XIAP and Bcl-x_L. On the basis of this evidence, it is possible that such perturbations in the cellular protein concentrations could also affect the enzymes belonging to the RNA interference apparatus, thus explaining the low efficiency by which siSurv acted when delivered by microcapsules compared to the lipid-mediated transfer. Such a capsule-dependent disturbance of the intracellular environment should be

expected to result in the impairment of cell survival. This was not the case when we assessed the biocompatibility of empty microcapsules and PMA_{SH} by the tetrazolium salt assay. In contrast, we were able to observe that tumor cells exposed to microcapsules, independent of the presence of siRNA, promptly initiated autophagy as a stress response pathway, thus remaining metabolically active. This suggests that the possible activation of cell defense pathways should be taken into account when biomaterials are used as carriers for therapeutic nucleic acids.

Overall, our findings indicate that further investigation into microcapsule-induced cellular changes at the molecular level is warranted. The efforts made in this research field will allow better understanding of the fate of microcapsules within cells and to develop strategies to circumvent the cell defensive responses, which may hinder the therapeutic effects of antisense oligomers.

MATERIALS AND METHODS

Reagents and Cell Lines. Roswell Park Memorial Institute medium (RPMI1640), fetal bovine serum (FBS), phosphate buffered saline (PBS), sodium dodecyl sulfate (SDS) and trypsin were obtained from BioWhittaker (Verviers, Belgium). The reagents for the cell viability assay, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) and phenazine methosulfate (PMS), were obtained from Promega (Milano, Italy). The primary antibodies, polyclonal to survivin (rabbit) and monoclonal to β-actin (mouse), were obtained from Abcam (Cambridge, U.K.). The anti-Mouse and anti-Rabbit horseradish peroxidase-linked secondary antibodies, and the ECL Western blotting detection reagents were obtained from GE Healthcare (Milano, Italy). Opti-MEM, FBS and Lipofectamine2000 were purchased from Invitrogen (San Giuliano Milanese, Italy). Poly(methacrylic acid, sodium salt) (PMA), (*M*w 15 kDa), was purchased from Polysciences (Warrington, PA). Cysteamine hydrochloride, *N*-hydroxysuccinimide (NHS), *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), polylysine (*M*w 90 kDa) and Chloroquine (30 mg L^{-1}) were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. Scrambled siRNA and siSurv, targeting specific consensus sequences [5′-AA(N19)UU-3′ within the survivin mRNA (Genbank accession no. NM_001168.1), were obtained as previously described.[36] Both siRNAs were purchased as preformed and purified duplexes and resuspended in water (50 μmol L^{-1}).

The human prostate carcinoma cell line PC-3 was obtained from American Type Culture Collection. Cells were resuscitated soon after arrival, and grown as a monolayer in RPMI 1640 containing 10% FBS. All cultures were maintained at 37 °C in a humidified 5% CO_2 incubator.

Encapsulation of siRNA. For PMA HCs, 1 μm-diameter SiO_2–NH_2^+ particles (0.5 g L^{-1}) in sodium acetate buffer (10 mM, pH 4) were incubated with siRNA corresponding to 50% of the surface saturation value.[20]

For PMA_{SH}-PLL microcapsules, infiltration of a TRITC labeled polylysine (1.2 g L^{-1}) in mesoporous silica particles (MS)[28] (5% w/w) with a series of pore sizes (4−40 nm) was first carried out. Ten mg of the MS particles were incubated with 2 mg of PLL solution in PBS buffer solution overnight. The amount of infiltrated PLL (10 mg g^{-1} of MS) was determined by monitoring the difference in the PLL-TRITC absorbance in solution before and after adsorption.

Loading of PLL in the nanopores was confirmed by fluorescence optical microscopy. The siRNA-coated particles and PLL-infiltrated particles were incubated alternately with PMA_{SH} (~18 mol % thiol) and PVPON (1 g L^{-1}) for 15 min. Three centrifugation/wash cycles (900 *g* for 30 s) with sodium acetate buffer (50 mM, pH 4) were conducted after deposition of each polymer layer. Polymers were added sequentially until 20 layers had been deposited. The core−shell particles were treated with chloramine T (2 mM) in MES buffer (10 mM, pH 6) for 2 min. The particles were suspended in sodium acetate buffer (10 mM, pH 4) to which HF (2 M) in NH_4F (8 M, pH 5) was added. *Caution! Hydrofluoric acid and ammonium fluoride are highly toxic. Extreme care should be taken when handling HF solution and only small quantities should be prepared.* The resulting microcapsules were washed *via* centrifugation (4500 *g* for 5 min) four times.

An aliquot of 100 μL of PLL-infiltrated microcapsules (1.2×10^6 microcapsules μL^{-1}) was incubated in sodium acetate buffer (50 mM, pH 4) with 100 μL of siRNA (250 nM) for 30 min, centrifuged and washed several times in PBS. The siRNA uptake into the microcapsule core was measured by using a Nanodrop spectrophotometer and measuring the absorbance at 260 nm of the solutions before and after adsorption.

Retention of siRNA Inside the Microcapsules. A sample was retained after each bilayer was deposited, and the thiols were oxidized using chloramine T (2 mM) in MES buffer (10 mM, pH 6) for 2 min. The particles were suspended in 50 μL of sodium acetate buffer (10 mM, pH 4) to which 100 μL of HF (2 M) in NH_4F (8 M, pH 5) was added. The resulting microcapsules were washed *via* four centrifugation/wash cycles (4500 *g* for 5 min). The microcapsules were analyzed in pH 4 and in pH 7 solutions using flow cytometry (laser power 100 mW, FL2 PMT 500 V).

Immunofluorescence Analyses. For immunofluorescence analyses, PC-3 cells grown on glass coverslips were exposed to fluorescently labeled microcapsules for the indicated time points. Cells were then fixed with 4% formaldehyde and successively probed with an anti-CD44 antibody (BD Bioscience, Buccinasco, Italy). AlexaFluor488 and AlexaFluor594 (Invitrogen) were used as secondary antibodies. Nuclei were counterstained with 0.1 μg mL^{-1} 4′,6-diamidino-2-phenylindole dye. Images were acquired with a Nikon Eclipse E600 microscope using ACT-1 software (Nikon) or, for confocal microscopy, with a Microradiance 2000 microscope (Bio-Rad Laboratories) equipped with Ar (488 nm) and HeNe (543 nm) lasers and processed with Adobe Photoshop Image Reader 7.0.

MTS Cell Proliferation Assay. Cell proliferation was assessed by the MTS proliferation assay (Promega), according to the manufacturer's protocol. Briefly, cells were seeded at the appropriate density into 96-well plates. The following day, the cells were treated with microcapsules or polymer for the indicated time points in a final volume of 100 μL of RPMI1640. Twenty microliters of the MTS solution was then added to each well. The absorbance was recorded using the Fluostar OPTIMA plate reader (BMG Labtech GmbH, Offenburg, Germany) at 492 nm after 4 h incubation at 37 °C in 5% CO_2.

Transfection Protocols. The day before transfection, PC-3 cells were seeded at a density of 5×10^4 cells per well in a 6-well plate. Transfection with Lipofectamine2000 was performed as described previously,[36] using a 50 nM final concentration of siRNA. Freshly prepared microcapsules filled with siRNA were delivered to PC-3 cells (125 capsules/cell) using a method similar to that used for standard transfection. Briefly, microcapsule/siRNA complexes were incubated with PC-3 cells for 4 h. Serum-free media was used to standardize the capsule transfection procedure with the control using Lipofectamine2000. After that time, standard growth media containing 10% FBS was added to the cells, and they were grown in an incubator for 72, 96, or 120 h. Cells were then harvested for successive analyses.

Western Immunoblotting and Caspase-3 Catalytic Activity Assay. For protein analysis, total cellular lysates were prepared from cell culture incubated for 30 min on ice in cell lysis buffer. The supernatant was collected after centrifugation (10 000× *g* for

10 min) and the total protein concentration was quantified using the Bio-Rad protein assay solution (Bio-Rad Laboratories, Segrate, Italy). Immunoblotting analysis was also performed to determine the amount of LC3-II, the phosphatidylethanolamine-conjugated form of LC3 (a marker of autophagy). Changes in LC3 electrophoretic mobility occur as a consequence of the conversion of the cytosolic form (LC3-I) to the autophagosome membrane-associated variant (LC3-II). As a consequence, on a SDS-polyacrilamide gel LC3-II migrates faster than LC3-I and on immunoblots probed with a LC3 antibody two bands are easily detectable: LC3-I (~18 kDa) and LC3-II (~16 kDa).[41]

40 μg of protein was resolved by a 15% SDS-polyacrylamide gel and transferred to nitrocellulose. The filters were blocked in PBS with 5% skim milk and incubated overnight with the primary antibodies specific for survivin, Bcl-X_L, X-linked inhibitor of apoptosis protein (Abcam) and LC3b (Sigma-Aldrich). The filters were then incubated with the secondary peroxidase-linked whole antibodies (GE Healthcare). Bound antibodies were detected using the enhanced chemiluminescence Western blotting detection system (GE Healthcare). An anti-β-actin monoclonal antibody (Abcam) was used on each blot to ensure equal loading of protein on the gel. The results were quantified by densitometric analysis using the ImageQuant software.

Cells were also analyzed for caspase-3 catalytic activity by means of an APOPCYTO/caspase-3 assay kit (Medical & Biological Laboratories, Naka-ku Nagoya, Japan) according to the manufacturer's instructions. Briefly, total protein extracts and the specific fluorogenic substrate *N*-acetyl-Asp-Glu-Val-Asp-pNA (DEVD-pNA) were mixed and incubated for 1 h at 37 °C. Hydrolysis of the specific substrates for caspase-3 was monitored by spectrofluorometry at 460 nm.

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Supporting Information Available: MTS assay for PMA_{SH} polymer. Confocal laser scanning microscopy images of PMA HCs and PMA_{SH}-PLL internalized microcapsules. Quantification of survivin expression levels in transfected cells. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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