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Cellular communication via nanoparticle-transporting biovesicles

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Abstract

Aims—Endothelial cells are dynamic cells tasked with selective transport of cargo from blood vessels to tissues. Here we demonstrate the potential for nanoparticle transport across endothelial cells in membrane-bound vesicles.

Materials & methods—Cell-free endothelial-derived biovesicles were characterized for cellular and nanoparticle content by electron microscopy. Confocal microscopy was used to evaluate biovesicles for organelle-specific proteins, and to monitor biovesicle engulfment by naive cells.

Results—Nanoparticle-laden biovesicles containing low-density polyethyleneimine nanoparticles appear to be predominately of endosomal origin, combining features of multivesicular bodies, lysosomes and autophagosomes. Conversely, high-density polyethyleneimine nanoparticles stimulate the formation of biovesicles associated with cellular apoptotic breakdown. Secreted LAMP-1-positive biovesicles are internalized by recipient cells, either of the same origin or of novel phenotype.

Conclusion—Cellular biovesicles, rich in cellular signals, present an important mode of cell-to-cell communication either locally or through broadcasting of biological messages.

Keywords

biovesicle; endothelia; exocytosis; iron oxide; microvesicle; nanoparticle

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To be effective, intravascularly injected drugs or cargo-loaded drug-delivery vehicles must exit the vasculature at sites appropriate for transport to their intended target. The tumor - associated vasculature presents unique characteristics that can be exploited for drug delivery, such as enhanced permeability and altered expression of surface molecules [1,2]. These traits enhance the entry of nanoparticles that are able to pass through tumor endothelial fenestrations [3,4] and provide a mechanism for increased retention of nano- and microparticles based on engagement of endothelial surface proteins. Understanding the mechanisms of transport, interaction of nanoparticles with the endothelium, cellular internalization, subcellular location, and long-term fate and compatibility of nanoparticles is critical in order to evaluate the efficacy and safety of nanoparticle-based therapeutics [5–7].

While many studies have investigated the mechanisms of nanoparticle internalization and intracellular trafficking [5,6,8,9], their long-term fate is less well established. Several studies have observed the cellular secretion of particles, including superparamagnetic iron oxide nanoparticles (SPIONs) [8,10,11], single-wall carbon nanotubes, gold [12], poly(lactic-co-glycolic acid) [13], silica [14] and silicon particles [15], in a variety of cell lines, including macrophages, endothelia, fibroblasts and cancer cells. The secretion of particles is influenced by cell type, making transfer between cells potentially asymmetric [14]; for example, while both human umbilical vein endothelial cells (HUVECs) and HeLa cancer cells can internalize newly introduced or cell-secreted silica nanoparticles, only HUVECs secrete the nanoparticles [14]. Therefore, the cell-to-cell transfer of nanoparticles in cocultures of HeLa cancer cells and HUVECs is unidirectional.

Endothelial cells have phagocytic potential and are able to internalize both synthetic and cell-derived microparticles [7,16,17]. The authors recently reported that human microvascular endothelial cells (HMVECs) internalize and secrete silicon microparticles and are able to transfer these microparticles between cells through direct cell-to-cell transfer, including through cellular connections known as tunneling nanotubes [15]. The authors have also reported the existence of cell-free biovesicles carrying SPIONs in nanoparticle-treated J774 murine macrophages [6] and HMVEC cultures [16]. In this study, the architecture and subcellular origin of HMVEC cell-derived biovesicles are explored. Furthermore, the authors investigate the internalization of isolated biovesicles by recipient cells of the same or unique phenotype.

Materials & methods

Isolation of cell-free SPION-loaded biovesicles from HMVEC cultures

HMVECs, a gift from Rong Shao (Baystate Medical Center/University of Massachusetts, MA, USA), were grown in EBM® medium containing supplements and growth factors (Lonza, MD, USA). HMVECs were incubated with 2 µg/ml 15 nm polyethyleneimine (PEI)-SPIONs (Ocean Nanotech, AR, USA) for 12–18 h following vortex mixing and brief sonication of the nanoparticles. The surface potential of each particle type in 0.1 M phosphate buffer was determined using a Malvern Zetasizer (Worcestershire, UK). Two varieties of PEI-SPIONs with different PEI surface densities were utilized (zeta-potentials: 4.5 and 7.7 mV). Following particle incubation, the treated cells were washed and supplemented with new media. Conditioned medium from the cells was collected after 24 h

and cell-free biovesicles were collected by high-speed centrifugation ($21,000 \times g$, 10 min each). The pellet obtained was suspended in phosphate-buffered saline (PBS; pH 7.4) and further purified for biovesicles that contained SPIONs using magnetic separation by overnight incubation in a magnetic cuvette holder (Ocean Nanotech).

Transmission electron microscopy imaging of cells & cell-free biovesicles

HMVECs, seeded into six-well plates, were incubated with either amine-SPIONs or PEI-SPIONs (2 $\mu\text{g}/\text{ml}$) for 6 h. Cells and/or cell-free biovesicles were fixed with a solution of 2% paraformaldehyde and 3% glutaraldehyde (Electron Microscopy Science, PA, USA) in PBS. Samples were then washed and incubated with 0.1% cacodylate-buffered tannic acid, followed by incubation with 1% buffered osmium tetroxide for 30 min. The samples were subsequently dehydrated in increasing concentrations of ethanol and embedded in EPONTM resin (Miller-Stephenson, CA, USA). Ultrathin sections were cut with a Leica Ultracut microtome (Leica, IL, USA), stained with uranyl acetate and lead citrate in a Leica EM Stainer, and examined in a JEM 1010 transmission electron microscope (JEOL USA, Inc., MA, USA) with a voltage of 80 kV. Digital images were obtained using the AMT Imaging System (Advanced Microscopy Techniques Corp., MA, USA).

Confocal imaging of free SPIONs & biovesicle uptake by endothelial & cancer cells

For imaging primary uptake of SPIONs, HMVECs were seeded in eight-well glass-bottom confocal chamber slides (BD Falcon, CA, USA), incubated with Alexa Fluor[®] 594-wheat germ agglutinin (Invitrogen, CA, USA) for 10 min at 37°C, and then incubated with DyLight 488[®] (Pierce, IL, USA)-conjugated PEI-SPIONs (5 $\mu\text{g}/\text{ml}$) overnight. The samples were then washed and fixed with 4% paraformaldehyde, mounted in ProLong[®] Gold (Life Technologies, NY, USA), and imaged with a Nikon A1 confocal microscope (Nikon Instruments, Inc., NY, USA).

For imaging secondary SPION uptake (i.e., biovesicles), harvested biovesicles were washed with 1% bovine serum albumin in PBS, and then incubated with fluorescein isothiocyanate (FITC)-conjugated LAMP-1 antibody (clone H5G11, raised against the whole protein); 1:200 dilution (Santa Cruz Biotechnology, CA, USA). The biovesicles were then washed with PBS and incubated overnight with MCF7, 4T1 or HMVEC cells seeded in glass-bottom plates (Becton Dickinson, NJ, USA). Cells were fixed with 4% paraformaldehyde, mounted with Vecta-Shield[®] (Vector Laboratories, Inc., CA, USA) with 4',6-diamidino-2-phenylindole, and examined with a Nikon A1 confocal microscope. Reflected light (640 nm) was used to visualize the SPIONs.

Purified vesicles were also stained with annexin V (Life Technologies). Biovesicles were resuspended in 100 μl annexin-binding buffer with 5 μl Alexa Fluor 488-annexin V for 15 min and then washed. Stained and unstained vesicles were imaged using a confocal microscope as previously described.

Lysosome colocalization studies were performed using LysoTracker Red[®] (Life Technologies). HMVECs were treated with LAMP-1-stained vesicles (as aforementioned) or free PEI-SPIONs (0.5 $\mu\text{g}/\text{ml}$; 7.7 mV) overnight. Samples were rinsed, stained with 75

nM LysoTracker for 2 h and then fixed with 4% paraformaldehyde in PBS. Slides were mounted with VectaShield with 4',6-diamidino-2-phenylindole and imaged as aforementioned.

Cellular proliferation in the presence of PEI-SPIONs

HMVECs were seeded into 96-well plates (5000 cells/well in 200 μ l medium) and left to adhere overnight. Cells were then incubated with 15 nm PEI-coated SPIONs (4.5 mV) at a concentration of 0 (control), 2, 5 or 7 μ g/ml for 24 h. The medium was then replaced with a fresh medium and cell proliferation was measured at 24, 48 and 72 h postparticle addition. Proliferation was measured by adding 50 μ l/well of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, MO, USA) diluted in 100 μ l/well fresh media. After 3 h the solution was replaced with 250 μ l dimethyl sulfoxide and absorbance was measured at 570 nm using a Synergy H4™ plate reader (BioTek Winooski, VT, USA).

Results

Endocytosis of PEI-SPIONs by HMVECs

Evidence for the uptake of SPIONs in membrane-bound vesicles was supported by confocal micrographs (Figure 1A). Dylight 488-labeled PEI-SPIONs (4.5 mV) were incubated with Alexa Fluor 594- wheat germ agglutinin-stained cells. Wheat germ agglutinin selectively binds to *N*-acetylglucosamine and *N*-acetylneuraminic acid and thereby labels the cell membrane that subsequently contributes to the endosomal membrane. Yellow coloration of the endosomes in nanoparticle-treated cells supports evidence of the mixing of green and red channel dyes from the nanoparticles and from newly formed endosomes, respectively, indicating 'apparent colocalization', with an average overlap coefficient of 0.98 (derived using the Nikon NIS-Elements software; Nikon Instruments, Inc.).

Transmission electron microscopy (TEM) micrographs of HMVECs incubated with PEI- (Figure 1B; 4.5 mV) and amine-modified (Figure 1G) SPIONs for 6 h confirmed nanoparticle internalization by endothelial cells and their localization within membrane-enclosed vesicles. PEI-SPION-carrying vesicles were seen both within and surrounding HMVECs (Figures 1B–1D). As seen in Figures 1C, 1E & 1F, not all biovesicles secreted from PEI-SPION-treated cells contained visible SPIONs. In addition, not all extracellular PEI-SPIONs were membrane enclosed. Biovesicle entities were also seen surrounding HMVECs treated with amine-modified SPIONs (Figures 1G–1I). The surface potentials of PEI and amine modified nanoparticles were 4.5 and –4.0 mV for the PEI- and amine-SPIONs, respectively.

Maturation and fusion between endosomal organelles can be associated with the movement of the vesicles toward the perinuclear region of the cell [18]. In both confocal and TEM micrographs, the presence of SPIONs near the perinuclear region of the cells supports normal maturation and trafficking of nanoparticle-loaded endosomes. Internalization of the PEI-SPIONs (4.5 mV) did not impact cell viability, based on cellular proliferation across 3 days at 2, 5 and 7 μ g/ml (Figure 2).

Structure of cell-free biovesicles

To characterize cell-free biovesicles, conditioned medium from HMVECs following 3 days of incubation with PEI-SPIONs (4.5 mV) was collected. Following high-speed centrifugation and isolation using a high magnetic field separator, we recovered a dark pellet, which supported the presence of SPIONs. Ultrastructural characterization of the isolated material by TEM revealed the presence of membrane-bound vesicles containing SPIONs (Figure 3). The vesicles had an average size of approximately 1000 ± 100 nm and exhibited components characteristic of multivesicular bodies, autophagosomes and primary lysosomes. These biovesicles contained recycled membrane whirls, vesicular bodies and partially degraded rough endoplasmic reticulum and ribosomes, the latter seen as electron-dense amorphous masses.

Intercellular transfer of nanoparticles through biovesicles

To examine whether endothelial cell-derived biovesicles may be involved in intercellular communication between cells, the recovered endothelial biovesicles were incubated with naive HMVECs, 4T1 or MCF-7 breast cancer cells for 24 h, followed by TEM analysis of the cells. The resulting micrographs revealed the presence of SPIONs confined within membrane-bound compartments inside HMVECs (Figure 4A), as well as within the two breast cancer cell lines (Figures 4B & 4C).

In order to support the role for lysosomes as the cellular origin of the HMVEC-derived biovesicles, isolated vesicles were labeled with anti-LAMP-1 FITC-conjugated antibody. HMVECs were treated with low PEI density SPIONs (4.5 mV) for 24 h prior to the isolation of biovesicles from the culture media and the resulting biovesicles were imaged by confocal microscopy. Using reflectance following excitation at 640 nm to visualize SPIONs (Figure 5A,III), the presence of the nanoparticles within LAMP-1-positive biovesicles was confirmed.

Since cationic PEI nanoparticles with a high positive charge density are cytotoxic [19], the potential for cellular apoptosis and membrane blebbing exists in treated cells. HMVECs were incubated with high PEI density SPIONs (7.7 mV) for 24 h and the resulting biovesicles were isolated from the culture media. A population of discrete and clustered vesicles were positive for Alexa Fluor 488 annexin V labeling (Figure 5B), supporting the presence of phosphatidylserine (PS) within the biovesicle membrane and supporting origins within the plasma membrane. Figures 5B,I & 5B,III show SPIONs using light reflectance, while Figures 5B,II & 5B,IV are overlays of the reflectance and the FITC channel.

While cell-free PEI-SPION-laden biovesicles were positive for expression of LAMP-1, supporting a late endosome/lysosome origin, intracellular labeling of vesicles with LysoTracker Red after treatment of HMVECs with high-density PEI-SPIONs was negative (Figure 6C). PEI reportedly disrupts acidification of the endosome based on buffering of incoming protons. However, HMVECs treated with membrane-encapsulated SPIONs resulted in shielding of the PEI nanoparticles, enabling endosomal acidification and permitting LysoTracker labeling of the vesicles (Figure 6B). Untreated control cells labeled with LysoTracker are shown in Figure 6A.

Human endothelial-derived LAMP-1 FITC-labeled biovesicles were introduced to naive HMVECs or to human MCF-7 or mouse 4T1 breast cancer cells for 24 h (Figures 6D–6F). Using reflectance to visualize SPIONs and bright-field microscopy for cell imaging, colocalization of the newly internalized biovesicles and SPIONs supported cellular uptake of SPION-carrying biovesicles by both endothelial and cancer cells, regardless of the species or origin.

Discussion

Rather than serving as static barrier cells, endothelial cells are highly dynamic and interactive with their surrounding milieu. One of the primary functions of the endothelium is selective transport of material across the barrier. Established mechanisms of passage from the luminal side to the interstitial space include trans cellular, transcytosis and interendothelial transport. During the process of transcytosis, plasma membrane receptors mediate the internalization of cargo; for example, activation of the GP60 receptor stimulates signaling pathways leading to the transcytosis of albumin across the endothelial cell monolayer [20].

The potential for transendothelial transport of SPIONs to the tumor microenvironment was examined. First, it demonstrated that PEI-SPIONs are internalized into endocytic vesicles that traffic to the perinuclear region of the cell. The authors and others have previously demonstrated localization of amine- and PEI-SPIONs within lysosomes [21,22]. In this study, treatment of HMVECs with PEI-SPIONs with a low cationic charge density (4.5 mV) did not alter cellular proliferation at three concentrations (2, 5 and 7 µg/ml) over 3 days. Cellular morphology and migration of nanoparticle-carrying endosomes to the perinuclear region of the cell provided additional support for normal cellular functioning. However, it is well established that high-density PEI-SPIONs are cytotoxic. Treatment of HMVECs with a low concentration (0.5 µg/ml) of high-density PEI-SPIONs (7.7 mV) resulted in cellular uptake of the nanoparticles, but inhibited acidification of the endosome needed for LysoTracker staining. This is most probably due to buffering of protons by PEI and potential disruption of the lysosomal membrane.

The presence of biovesicles in HMVEC conditioned media was observed after incubation of cells with amine- and PEI-SPIONs. Ultrastructural analysis of the secreted biovesicles revealed the presence of membrane-enclosed clusters of nanoparticles. Based on the morphology of the amine- and low-density PEI-SPION-loaded vesicles, they appeared to be endosome (e.g., multivesicular bodies) or autophagosome (intracellular organelles and other cytoplasmic components) derived, or hybrid structures created by fusion with either lysosomes or autophagosomes, such as amphisomes and autolysosomes [23,24]. In Figure 7, a possible pathway for the release of membrane-enclosed nanoparticles from endothelial cells is outlined. The first part summarizes the well-established cellular uptake of nanoparticles through endocytosis, while the second part describes the routing and cellular secretion of mature nanoparticle-containing compartments.

Biovesicles isolated from low-density PEI-SPION-treated HMVECs were introduced to cancer cells and naive endothelial cells, and the ability of the acceptor cells to internalize the

biovesicles was confirmed by electron and confocal microscopy. Human MCF-7 and murine 4T1 cancer cells, as well as naive HMVECs, were able to internalize the endothelial-derived biovesicles. These data support the concept that transport of nanoparticle-loaded biovesicles is a potential mechanism for crossing the endothelial barrier and a means to disseminate therapeutic cargo and/or broadcast signals to distant locations.

Cellular biovesicles are heterogeneous and are generated by diverse biological mechanisms, including microenvironmental stimulation, stress and death [25]. The composition of both the contents and the membrane depend on the stimulus triggering their release. Three major cellular events lead to the formation of biovesicles: release of exosomes from multivesicular bodies; cellular apoptotic breakdown; and membrane blebbing. During blebbing, microparticles are formed by bleb detachment from the cell body [26]. PS, normally found on the cytosolic face of the plasma membrane, translocates to the extracellular face. The translocation of PS is reported to be a key event that triggers microparticle formation and release [27].

The authors recently reported that the cellular exchange of microparticles between endothelial cells is enhanced by cellular stress [15]. Stress-induced secretion of microvesicles is triggered by exogenous stimuli, including pathogens and events leading to cell death [28]. These events involve vesicular/membrane intermediates and may include membrane blebbing. Golgi reassembly and stacking proteins have been indicated in unconventional secretion events, mediating the tethering or fusion of vesicles to the plasma membrane in lower eukaryotic cells, with similar roles proposed in vertebrate cells [29]. For example, amphisomes can fuse with the plasma membrane, resulting in the release of their contents into the extracellular space [30]. These studies support secretion events in the presence of foreign or pathogenic entities, providing a potential mechanism for secretion and/or transfer of nanoparticle-loaded vesicles.

Since highly positive PEI-nanoparticles induce apoptosis in cells, the potential for apoptotic blebs to incorporate SPIONs into microvesicles was explored. A population of high-density PEI-SPION-loaded vesicles isolated from treated HMVECs were positive for annexin V labeling. While large clusters of microvesicles or membrane aggregates were seen, individual single microvesicles existed, both carrying SPIONs. The potential for cellular uptake of PS-positive microvesicles is supported by reports that endothelial cells internalize these vesicles using developmental endothelial locus-1 as a bridge to the endothelial cell. Developmental endothelial locus-1 contains the canonical Arg-Gly-Asp motif, which binds to integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ on the cell surface [15]. Therefore, both lysosomal and plasma membrane-derived biovesicles have the potential to transport SPIONs and potentially other types of nanoparticles between cells. For the delivery of nanoparticle-based therapeutic agents, the potential for microparticle-based transport poses questions regarding its impact on drug dissemination and secondary targeting through biovesicle acquisition of novel surface components.

Conclusion

Recognition of the importance of cellular microvesicles as mediators of intercellular communication is expanding. Cellular exchange of microvesicles has the ability to influence cellular functions and the capacity to alter the phenotype of other cell types through the transfer of material, such as growth factors, DNA and miRNAs [25,31]. Nanoparticle-loaded biovesicles have the added complexity of delivering intrinsic and extrinsic signals.

Future perspective

The existence of synthetic nanoparticles (i.e., drug-delivery vehicles) within cell-free biovesicles creates a need to study its impact on signal broadcasting and dilution of therapeutic cargo. Future studies will provide further insight on the influence of particle properties, nature of the cargo, and biological stressors on the rates and impact of biovesicle secretion, trafficking and cellular uptake.

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Executive summary

Endocytosis of polyethyleneimine-superparamagnetic iron oxide nanoparticles by human microvascular endothelial cells

- Polyethyleneimine- (PEI) and amine-superparamagnetic iron oxide nanoparticles (SPIONs) are internalized by endothelial cells into membrane-bound vesicles.
- SPION-laden endosomes are transported to the perinuclear region of the cell.
- Human microvascular endothelial cell proliferation was unaltered following uptake of PEI-SPIONs with a surface potential of 4.5 mV.
- SPION-laden biovesicles exist outside the cell.

Structure of cell-free biovesicles

- Cell-free biovesicles have characteristics of endosomes and autophagosomes.
- High charge density PEI-SPIONs cause endothelial cells to secrete phosphatidyl serine-positive microvesicles, some functioning as shuttles for nanoparticles.

Intercellular transfer of nanoparticles through biovesicles

- Extracellular endothelial-derived biovesicles are candidates for uptake by naive endothelial cells and cancer cells, providing a mechanism for transport of therapeutic cargo across the endothelium.

Figure 1. Cellular uptake of superparamagnetic iron oxide nanoparticles and secretion of microvesicles by human microvascular endothelial cells

(A) Confocal images of Alexa Fluor® 594-wheat germ (Invitrogen, CA, USA) agglutinin-labeled human microvascular endothelial cells incubated overnight with DyLight 488® (Pierce, IL, USA)-conjugated PEI-SPIONs are displayed as (i) separate channels and (ii) merged images. (iii) The bright field image is included to show that the green dye overlaps with the location of the nanoparticles. (B–I) Transmission electron micrographs of human microvascular endothelial cells incubated with PEI-(B–F) or amine- (G–I) SPIONs for 6 h. Cells and surrounding extracellular entities were imaged at (B, E & G) 10,000× and (C, D, F, H & I) 75,000× magnification.

PEI: Polyethyleneimine; SPION: Superparamagnetic iron oxide nanoparticle.

Figure 2. The influence of polyethyleneimine-superparamagnetic iron oxide nanoparticles on human microvascular endothelial cell proliferation

Human microvascular endothelial cells were incubated with 2, 5 and 7 µg/ml polyethyleneimine-superparamagnetic iron oxide nanoparticles and cell density was determined at 24, 48 and 72 h using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation assay. Absorbance of formazan at 570 nm is plotted against time.

Figure 3. Transmission electron micrographs of superparamagnetic iron oxide nanoparticle-loaded cell-free biovesicles

An array of images showing cell-free human microvascular endothelial cell-derived biovesicles, emphasizing the diverse nature of the biovesicles and the presence of polyethyleneimine-superparamagnetic iron oxide nanoparticles, membrane bundles, multiple vesicular bodies and electron dense regions (100,000 $\times$ magnification).

Figure 4. Transmission electron micrographs of recipient cells following overnight incubation with isolated biovesicles

Transmission electron micrographs show (A) recipient human microvascular endothelial cells, (B) MCF-7 and (C) 4T1 cells after overnight incubation with endothelial cell-derived biovesicles. The boxed regions show increasing magnifications (20,000, 40,000 and 100,000 $\times$). At the highest magnification (right column), polyethyleneimine-superparamagnetic iron oxide nanoparticles are visible inside membrane-bound compartments.

Figure 5. Confocal images of human microvascular endothelial cell-free biovesicles labeled with anti-LAMP-1 fluorescein isothiocyanate-conjugated antibody or fluorescent annexin V (A,i & A,ii) Confocal images of a cell-free biovesicle, shown as a bright field image and a fluorescent image labeled with anti-LAMP-1 fluorescein isothiocyanate-conjugated antibody. (A,iii & A,iv) Polyethyleneimine-superparamagnetic iron oxide nanoparticles (4.5 mV) are visualized by light reflectance following excitation at 640 nm (reflectance plus merged reflectance and fluorescein isothiocyanate channels). (B) Cell-free biovesicles were isolated from the media of human microvascular endothelial cells treated with polyethyleneimine-superparamagnetic iron oxide nanoparticles (7.7 mV). (B,i & B,ii) Control biovesicles (no staining), and (B,iii & B,iv) annexin V-labeled biovesicles. (B,i & B,iii) an overlay of bright-field microscopy and light reflectance, and (B,ii & B,iv) An overlay of the fluorescein isothiocyanate channel and light reflectance.

Figure 6. Confocal images of polyethyleneimine-superparamagnetic iron oxide nanoparticle and biovesicle uptake within cells

(A–C) HMVECs were either ([A], control) untreated, (B) incubated with biovesicles labeled with LAMP-1 fluorescein isothiocyanate (FITC)-conjugated antibody or (C) incubated with free PEI-SPIONs (0.5 µg/ml, 7.7 mV) for 24 h. (A,i, B,i & C,i) An overlay of bright-field microscopy, 4',6-diamidino-2-phenylindole and FITC channels. (A,ii, B,ii & C,ii) Reflected light used to visual SPIONs. (A,iii, B,iii & C,iii) LysoTracker® Red staining (Life Technologies, NY, USA). (D–F) Naive HMVECs, MCF-7 and 4T1 cancer cells were incubated with anti-LAMP-1 FITC antibody-labeled HMVEC biovesicles for 24 h. Cells were imaged by bright-field microscopy and 4',6-diamidino-2-phenylindole fluorescence of labeled nuclei and are shown as overlays with the FITC channel to visualize internalized biovesicles. Reflected light was used to visualize PEI-SPIONs within the biovesicles (left, 4.5 mV). (D,ii, E,ii & F,ii) Merged reflectance and FITC channels only. HMVEC: Human microvascular endothelial cell; PEI: Polyethyleneimine; SPION: Superparamagnetic iron oxide nanoparticle.

Figure 7. The proposed mechanism for the generation and release of the biovesicles containing superparamagnetic iron oxide nanoparticles

(1) Superparamagnetic iron oxide nanoparticle internalization through endocytosis and localization of the nanoparticles in early endosomes. (2) Maturation of the early endosome to MVBs. (3) Fusion of the MVBs containing superparamagnetic iron oxide nanoparticles with lysosomes. (4) Fusion of MVBs with autophagosomes to form amphisomes. (5) Fusion of amphisomes with lysosomes to form autolysosomes. (6) Possible secretion of lysosomes and autolysosomes containing superparamagnetic iron oxide nanoparticles from the cell. MVB: Multivesicular body.