Abstracts of the UK-Russia Researcher Links Workshop: Extracellular vesicles – mechanisms of biogenesis and roles in disease pathogenesis

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Cardioprotection by plasma exosomes
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Introduction: Myocardial infarction is the acute event that causes morbidity and mortality in patients with coronary artery disease. Currently early reperfusion (restoration of normal coronary flow) remains the mainstay of treatment for those suffering an acute myocardial infarction. However, reperfusion also causes injury to heart muscle in what is termed “lethal reperfusion injury.” Therefore, a major aim of our research is to find ways to reduce lethal reperfusion injury. Exosomes from stem cells have been shown to protect the heart. We hypothesized that endogenous plasma exosomes can communicate signals to the heart and provide protection against ischemia and reperfusion injury, and we examined the mechanism of this protection. Methods: We isolated exosomes from rat and human plasma and characterized them by electron micrography, nanoparticle tracking analysis and Western blot analysis. We tested cardioprotection using an in vivo rat model of myocardial infarction, as well as in an isolated, perfused rat heart model, and in isolated cardiomyocytes subject to hypoxia and reoxygenation. We investigated the cardioprotective signalling pathway using Western blot analysis and specific inhibitors. Results: Plasma exosomes were cardioprotective in all models tested. Cardioprotection was blocked by neutralizing antibody against HSP70 on the exosome surface and by inhibitors of TLR4 and ERK1/2 protein kinase. Cardioprotection correlated with phosphorylation of heat shock protein HSP27. Conclusions: Plasma exosomes are able to activate TLR4 in cardiomyocytes, leading to ERK1/2-mediated phosphorylation of the protective heat shock protein HSP27, which protects cells from ischemia and reperfusion injury.

Invadoplysin – an odyssey from chromosomes to lipid droplets to serum protease
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We have utilized Drosophila as a model system to identify novel and conserved genes essential for progression through the cell cycle and normal cellular physiology. Invadoplysin is a zinc-metalloprotease that we have shown to link cell division and cell migration in D. melanogaster (1). Invadoplysin localizes to lipid droplets in mammalian cell lines, and Drosophila invadoplysin mutants have a decreased triglyceride: protein ratio (2). Invadoplysin additionally interacts with mitochondrial ATP synthase subunits (3) and plays a role in angiogenesis (4). As many proteases function in catalytic pathways, it is intriguing that the first genetic interactor of invadoplysin is an ubiquitin protease – targeting histone H2B, and thereby linking to the chromosome defects we observe (5). Invadoplysin is the first metalloprotease localized to lipid droplets, and physiological functions remain at this point speculative. We have very recently discovered that a secreted form of invadoplysin is present in vertebrate serum and Drosophila hemolymph. As the gene is essential for life, we hypothesize that the secreted form of invadoplysin may be playing a role crucial to normal physiology. We aim to discover whether invadoplysin is present in a particular serum compartment (such as exosomes or microvesicles) and whether proteolytic activity for invadoplysin can be detected. Should activity be detected, we can begin to ask questions pertaining to the regulation of localization, levels and activity. Importantly, we will be able to address whether this novel form of invadoplysin serves as a potential biomarker for any human disease states.

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Links between microvesicles and cardiac stress
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Introduction: Microvesicle levels are altered in patients with cardiovascular disease. We investigated circulating microvesicle levels during cardiac stress in individuals undergoing stress echocardiography, for the diagnosis of coronary artery disease. Methods: Circulating microvesicles were measured by flow cytometry and a functional coagulation assay before, during and after a stress echocardiogram procedure. Differences in microvesicle were determined between individuals with and without inducible ischaemia, and those with and without vascular disease. Results: Microvesicles derived from platelets, erythrocytes and endothelial cells were elevated immediately following the stress echocardiogram. These elevated levels, in most cases, were cleared within 1 h. Those patients who developed stress-induced myocardial ischemia, had similar baseline microvesicle levels to other participants but, interestingly, their microvesicle levels did not alter during stress. No stress-induced increase in microvesicles was seen in those with a history of vascular disease or in patients with a confirmed diagnosis by angiography. Conclusions: Microvesicles are released into the circulation during cardiac stress and are subsequently cleared quickly from the circulation, suggesting effective clearance mechanisms. This cardiac stress-induced release appears to be a normal response, which is diminished in those with vascular disease. Future work will examine the protein and miRNA content of these elevated microvesicles. We are extending this project into a multicentre study with a minimum of 400 participants to determine the clinical utility of measuring microvesicles during stress echocardiogram. We are also determining the impact of exercise-induced cardiac stress on circulating microvesicles in healthy individuals and in those with hypertension.

Part of this work has previously been presented at the ISEV meeting 2014 and in Circulation Research 2014;114:109–113.

Inflammasomes and sterile inflammation
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We are interested in investigating the regulatory mechanism of the molecular and cellular control of inflammasome activation during cardiovascular disease development. Recent understanding of the pathophysiology of cardiovascular diseases such as atherosclerosis and post-ischemic injury, suggests a major contribution of pathogen-free sterile inflammation in general, and inflammasome activation in particular, to disease development and complications. The inflammasome is a large multiprotein complex, which plays a key role in innate immunity through the production of the pro-inflammatory cytokines IL-1β and IL-18. Activation of inflammasome pathway induces the release of extracellular vesicles. Delineating inflammasome pathways will provide insight into cardiovascular disease pathogenesis and might lead to identification of potential targets for therapeutic intervention. By identifying novel players, we further validated their roles both in fundamental cellular pathways and in our established disease models, including atherosclerosis and post-ischemic injury.

The role of the cytoskeleton in exosome secretion

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Introduction: Vascular calcification is a common complication in patients with renal failure or diabetes type 2. Our previous work has shown that exosomes secreted by vascular smooth muscle cells (VSMCs) play a role in mediating vascular calcification by forming the first nidus for mineral deposition. Importantly, the mechanisms regulating exosome secretion and cargo loading are still largely unknown. In the present study, we tested if the cell cytoskeleton is implicated in exosome secretion by VSMCs. Methods: Exosomes were isolated from VSMCs using differential centrifugation and analyzed by western blotting. Late endosomes/multivesicular bodies and actin filaments were visualized in live VSMCs by cell transfection with DNA vectors encoding CD63-GFP and Tractin-RFP, respectively. VSMCs were imaged by time-lapse acquisition of optically-sectioned 3D volumes captured using spinning disk confocal microscopy (Nikon) and by scanning electron microscopy (SEMI). Results: Together these data suggest that actin cytoskeletal dynamics is important for exosome secretion and may represent a novel target for preventing vascular calcification.

Identification of glycosylphosphatidylinositol-anchored protein T-cadherin on human platelet-derived extracellular vesicles

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Introduction: Low-density lipoproteins (LDL) induce platelet hyper-sensitivity to agonists that initiate platelet activation, degranulation, vesiculation and aggregation. LDL increase free cytoplasmic calcium concentration ([Ca²⁺]i) in platelets, but receptor mediating this Ca²⁺ signalling is not yet known. LDL-induced Ca²⁺ signalling in smooth muscle cells was shown to be mediated by T-cadherin (T-Cad). An increase in the Ca²⁺ concentration triggers the release of extracellular vesicles. Platelet-derived microvesicles (PMV) constitute the major fraction of MV in the circulating plasma and contribute to cardiovascular diseases. The aim of this study was to analyze T-cad on platelets and PMV. Methods: Identification and characterization of T-cad on platelets, we used flow cytometry and immunoblotting with different antibodies. Localization T-cad on resting, activated platelets and PMV was analyzed by confocal microscopy. Lysates from thrombin-stimulated platelets (0, 2, 4 min) and vesicle fraction were analyzed by confocal microscopy. Localization T-cad on resting, activated platelets and PMV was analyzed by confocal microscopy. Lysates from thrombin-stimulated platelets (0, 2, 4 min) and vesicle fraction were analyzed by confocal microscopy. Localization T-cad on resting, activated platelets and PMV was analyzed by confocal microscopy.

Results: Analysis of isolated exosomes revealed enrichment of actin but not tubulin or vimentin indicating that exosomes may be formed in the vicinity of the actin cytoskeleton. Moreover, we observed colocalization between CD63-positive organelles and F-actin filaments. Disassembly of the actin cytoskeleton triggered exosome secretion by VSMCs whilst inhibition of ROCK kinase using Y27632 inhibited exosome secretion. Importantly, we observed the transport of CD63-positive organelles along F-actin filaments. Additionally, we revealed outward budding vesicular structures at the VSMCs surface aligning with cytoskeletal filaments. Summary/Conclusions: Together these data suggest that actin cytoskeletal dynamics is important for exosome secretion and may represent a novel target for preventing vascular calcification.

Summary/Conclusions: Platelets and PMV express T-cad and can mediate vesicle formation via Ca²⁺ mobilization.

Funding

This work was supported by the Russian Science Foundation 14-24-00086.
Introduction: In this preliminary study, we aimed to test applicability of nanoparticle tracking analysis (NTA) to estimate production of vesicles by tumour cells of different origin. Methods: For in vitro experiments, we used a panel of human tumour cells of different origin: MIA PaCa (pancreatic carcinoma), LN229 (glioblastoma), U87 (glioblastoma), A375 (melanoma), HT1080 (fibrosarcoma). Prior to medium collection, cells were washed twice with serum-free medium and cultivated for 48 h in serum-free medium with the addition of recombinant epithelial growth factor. To estimate extracellular vesicles (EVs) production by tumour cells in vivo, we compared blood (plasma and serum) samples before and after removal of tumour, in patients diagnosed with lung adenocarcinoma. NTA assay was performed at Nanosight LM10 instrument with 405 nm laser and EMCCD camera. MicroRNA were isolated using NucleoSpin miRNA columns and quantified with RiboGreen reagent. Results: We found that different cell lines are characterized by significantly different levels of nanoparticles production. While A375 melanoma cells displayed the lowest concentration of nanoparticles in conditioned medium $(1.2 \times 10^{16}$ part./ml), U87 glioblastoma cells production levels were an order of magnitude higher $(1.2 \times 10^{18}$ part./ml). These results are in good agreement with literature data about exosomes production in given cell lines. In case of blood samples, we discovered significant decrease of nanoparticles concentration in both plasma and serum fractions after removal of the tumour: from $1.6 \times 10^{12}$ part./ml in pre-operative samples to $6 \times 10^{10}$ part./ml in post-operative samples. Importantly, nanoparticles concentration correlated to the amount of microRNA extracted from those samples, which confirms that major part of nanoparticles correspond indeed to EVs such as exosomes. Conclusions: Our results support the concept of using NTA as simple direct approach to estimate cell production of EVs both in vitro and in vivo. However, additional assays using specific markers are required to demonstrate contribution of different types of vesicles in the mixed population of nanoparticles measured.

Cell-surface-bound and cell-free exosomes in blood of healthy and breast cancer women
Svetlana Tamkovich1,2, Oleg Tutanov1,2, Alina Grigor’eva1, Tatyana Duzhak3, Vasily N. Aushev1, Evgeniy G. Evtushenko2 and Elena M. Tchevkina1
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Abstracts

Oral session 2: Extracellular vesicles in oncology
Chair: Professor D. Zorov
14:00-15:00

Using nanoparticle tracking analysis for estimation of extracellular vesicles production by tumour cells in vitro and in vivo
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Introduction: In this preliminary study, we aimed to test applicability of nanoparticle tracking analysis (NTA) to estimate production of vesicles by tumour cells of different origin. Methods: For in vitro experiments, we used a panel of human tumour cells of different origin: MIA PaCa (pancreatic carcinoma), LN229 (glioblastoma), U87 (glioblastoma), A375 (melanoma), HT1080 (fibrosarcoma). Prior to medium collection, cells were washed twice with serum-free medium and cultivated for 48 h in serum-free medium with the addition of recombinant epithelial growth factor. To estimate extracellular vesicles (EVs) production by tumour cells in vivo, we compared blood (plasma and serum) samples before and after removal of tumour, in patients diagnosed with lung adenocarcinoma. NTA assay was performed at Nanosight LM10 instrument with 405 nm laser and EMCCD camera. MicroRNA were isolated using NucleoSpin miRNA columns and quantified with RiboGreen reagent. Results: We found that different cell lines are characterized by significantly different levels of nanoparticles production. While A375 melanoma cells displayed the lowest concentration of nanoparticles in conditioned medium $(1.2 \times 10^{16}$ part./ml), U87 glioblastoma cells production levels were an order of magnitude higher $(1.2 \times 10^{18}$ part./ml). These results are in good agreement with literature data about exosomes production in given cell lines. In case of blood samples, we discovered significant decrease of nanoparticles concentration in both plasma and serum fractions after removal of the tumour: from $1.6 \times 10^{12}$ part./ml in pre-operative samples to $6 \times 10^{10}$ part./ml in post-operative samples. Importantly, nanoparticles concentration correlated to the amount of microRNA extracted from those samples, which confirms that major part of nanoparticles correspond indeed to EVs such as exosomes. Conclusions: Our results support the concept of using NTA as simple direct approach to estimate cell production of EVs both in vitro and in vivo. However, additional assays using specific markers are required to demonstrate contribution of different types of vesicles in the mixed population of nanoparticles measured.

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Exosomes, proteases and proteins associated with cellular motility in ovarian cancer metastasis
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Introduction: The NK-cells and asces-derived exosomes as regulators of NK-cells activity in ovarian cancer may be important for peritoneal metastases formation. The role of tumour cell motility in invasion and metastasis is of great importance. The remodelling of actin cytoskeleton plays a central role in tumour invasion and is regulated by actin binding proteins and p45-Ser/β-catenin. The purpose of our investigation was to study the some aspects of ovarian cancer peritoneal canceromatosis related with exosomes and motility-associated proteins. Methods: The study included 30 patients with early and advanced high Grade serous ovarian carcinomas. Samples of primary tissue, peritoneal metastases, ascites and blood were obtained during surgery. We evaluated expression of act binding proteins and p45 Ser beta-catenin in primary tumour and tumour metastases by Western blotting and flow cytometry. Flow cytometric quantitation of NK-cells in blood and ascites was performed. Results: We revealed decrease of p45 Ser β-catenin expression in tumour metastases compared to primary tumour. During ovarian cancer metastasis changes in expression of act binding proteins were various: Arp3 level increased in series: early ovarian cancer – advanced ovarian cancer (primary tumour) – peritoneal metastases. The gelsolin and cofilin-1 levels were decreased in tumour metastases compared to primary tumour. The level of thymosin β-4 was reduced in primary tumour tissues and tumour metastases tissues compared to non-transformed tissue. Several cancer models at ~80°C. Size distribution of the microparticles was characterized by TEM (Jeol, Japan), anti CD-63, CD-24, CD-9 antibodies (BD Biosciences, USA) were used as exosomes markers, protein concentration was measured by NanoOrange Protein Quantitation kit (Molecular Probes, USA), total RNA and miRNA were isolated by mirVana kit, 28S rRNA were quantified by RT-qPCR after reverse transcription with 6-mer random primers, miRNA using TaqMan Small RNA Assays kit (Applied Biosystems, USA). DNA was isolated using “DNA Isolation Kit” (BioSilicatld, Russia) and measured by TaqMan multiplex real-time PCR for LINE1 and α-satellite repeats (2). Results: TEM with immunogold labelling demonstrates presence of exosomes in 30–100 nm membrane-wrapped particles isolated from both plasma and CSB eluates. TEM, NanoOrange and 28S rRNA RT-qPCR data demonstrate that CSB exosomes constitutes 2/3 of total blood exosomes. Exosomes ranged 50–70 nm prevail in blood of BCP, whereas 30–50 nm exosomes in blood of HW. Exosomal DNA is less than 0.3% of cell-free blood DNA. RNA integrity and specific quantity checked by Bioanalyzer (Agilent Technologies, USA) do not differ in cell-free and CSB exosomes. Preliminary data demonstrate overpresentation of cancer-specific miRNA (miR-103, miR-191, miR-195) in exosomes bound with erythrocyte’s as compared with exosomes bound with leukocytes or circulating in plasma. Summary: Exosomal DNA obviously do not have any diagnostic value in contrast to RNA. CSB exosomes represent valuable source of material for small-invasive cancer diagnostics.

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demonstrate the implication of tumour-derived exosomes in cancer immunity including function of NK-cells. In our study in blood of most ovarian cancer patients, NK-cells were not found, or the number of NK-cells was low (from 0 to 5%). In ovarian cancer patients, nearly all NK-cells from peripheral blood were CD107+ but they did not express granzyme B and perforin. In ascitic fluid 20–45% of all CD45+ cells were NK-cells, they were mainly activated (65% – CD107+) and expressed perforin and granzyme B in most cases. Conclusions: Perspective study of exosomes in ovarian cancer will clarify the molecular aspects of regulation NK-cell activity by exosomes in blood and ascites.

This work was supported by the Russian Foundation for Basic Research (grant number 13-04-00169a).
Abstracts

Oral session 3: Extracellular vesicles in tissue regeneration
Chairs: Professor V. Tkachuk and Dr. S. Skaalu

9:30-11:40

Extracellular vesicles secreted by neural stem cells as a novel mechanism of cell-to-cell communication
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Introduction: The development of stem cell-based therapies to repair the central nervous system (CNS) represents a great challenge in the field of regenerative medicine. Neural stem/precursor cells (NPCs) were shown to protect the CNS from chronic degeneration induced by inflammation. While it was first assumed that NPCs directly replace damaged cells, it is now becoming evident that they are able to protect the CNS via a number of bystander mechanisms, possibly involving the exchange of EVs with the microenvironment. Methods and Results: Applying RNA-seq and MS/MS combined with SILAC, we provide a comprehensive characterization of molecules trafficked by EVs. Interestingly, we found highly specific induction of IFN-γ pathway in NPCs exposed to Th1 cytokines that is mirrored in EVs. To understand the functional potential of these vesicles, we employed a combination of microarrays and MS/MS/SILAC on target cells exposed to EVs. Interestingly, we found that the Th1 EVs induced the same changes found in NPCs, resulting in the activation of the IFN-γ pathway in target cells. To better define the relative contribution of the different IFN-γ pathway components, we generated NPCs from mice lacking Stat1, Ifngr1 or Ifng2. We found that only Ifng1 on EVs is indispensable to transfer IFN-γ to target cells. Also we demonstrated that endogenous Stat1 and Ifng1 in target cells are required to sustain the activation of IFN-γ pathway by EV-associated IFN-γ/Ifng1 complexes. Conclusions: Taken together, these results demonstrated that NPC-derived EVs use Ifng1 to recycle and deliver IFN-γ only to fully equipped target cells, highlighting their innate selectivity potential. Our study identifies a novel mechanism of cell-to-cell communication by which NPCs may signal with the microenvironment. This is potentially relevant both in physiological conditions and in the context of degenerative diseases, where grafted stem cells may use EVs to communicate with the host immune system.

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Extracellular vesicles and paracrine activity of mesenchymal stromal cells
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Introduction: Multipotent mesenchymal stromal cells, isolated from bone marrow or adipose tissue (ADSC), enhance tissue regeneration upon transplantation, by guiding resident stem cells amplification and differentiation as well as by stimulating the growth of blood vessels and nerves. Transplantation of human ADSCs to immunodeficient nude mice stimulated the growth of recipient-derived (mouse) vessels without any evidences for incorporation of transplanted cells into growing vasculature. This allowed us to suggest that ADSCs effects on regeneration are mostly mediated by their secretory potential. This study was aimed at elucidating the role of extracellular vesicles (EVs) in ADSC effects on tissue regeneration. Methods: Human ADSCs were isolated from healthy donors and cultured for 3-4 passages. Cells were serum deprived, and cell purity was assessed using multiple cell surface markers. Production of EVs by ADSCs in response to growth factors was assessed by NTA. The content of proteins and microRNAs in EVs was evaluated by angiogenic proteins array and microRNA PCR array. The angiogenic efficiency of ADSC-derived EVs was assessed using in vitro and in vivo models of vasculature formation by HMEC. Results: Secretome of ADSCs defined as CD90+ / CD73+ / CD105+ / CD45 – / CD31 – / CD146+ cells is enriched in EVs-associated proteins, including tetraspanins and annexins. We also detected EVs in ADSC secretome, the majority of these is represented by exosomes. Treatment by growth factors affected the number and content of EVs produced by ADSCs as well as their ability to affect angiogenic behaviour of endothelial cells. PDGF-BB has elevated the content of MMPs in ADSC-derived EVs, and this increased the invasiveness of HMEC. bFGF has down-regulated angio-miRs in ADSC-derived EVs and increased the content of microRNA with known anti-angiogenic activity. The transfer of these microRNAs inhibited angiogenic behaviour of HMEC. Conclusions: ADSCs utilize EVs for controlling angiogenic behaviour of endothelial cells as a part of regeneration program, driven by these cells.

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**Oral session 4: Extracellular vesicles in immunology and pathogens**

**Chairs: Dr. L. Smyth and Dr. A. Kapustin 12:10-13:35**

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**Intercellular transfer of microRNA between regulatory T cells and dendritic cells: a possible regulation mechanism of dendritic cell function**

Lesley A. Smyth, Sim Tung, Marilena Letizia, Dominic Boardman, Laura Dionis, Valentina Bollati, Robert Lechler and Giovanna Lombardi

**Introduction:** Regulatory T cells (Tregs) are a subpopulation of CD4⁺ T cells which function to suppress target immune cells including effector T cells (Teffs) and antigen presenting cells such as dendritic cells (DCs). Tregs employ numerous mechanisms to suppress the action of target cells. One such mechanism, which we recently described, is via the production of secreted membrane vesicles, called exosomes. Recent research has demonstrated that microRNAs (miRNA) are intercellularly transferred via exosomes between immune cells and once inside the target cell they affect their functions. MiRNAs function to regulate gene expression through inhibiting translation of target mRNAs. One example, recently published, is that specific miRNA are transferred by Tregs to Teffs via exosomes, leading to gene regulation in the latter cell type. Whether Treg cells regulate DC function via exosomes-containing miRNA has yet to be elucidated.

**Methods and Results:** To address this, a miRNA array was conducted on both cell types and several murine Treg miRNA specific for each cell type were identified. Interestingly, following coculture of Tregs and DCs, expression of several Treg-specific miRNA increased in DCs. To confirm that this was mediated via the release of and the acquisition of exosomes released by Tregs, we profiled the miRNA expression in Treg-derived exosomes using a QuantStudio 12K Flex Real time PCR system. Several miRNA were identified as being highly expressed in Treg-derived exosomes, and some of these were found to be increase in DCs following co-culture with Tregs.

**Conclusions:** Our data suggest that intercellular transfer of miRNA via exosomes may be a novel mechanism of Treg regulation of DC function.

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**Circulating miR-155, miR-146a, miR-199, miR-93* and miR-423 as biomarkers for graft versus host disease**

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**Introduction:** Despite graft-versus-host disease (GvHD) being a major complication of allogeneic hematopoietic stem cell transplantation (HSCT), no biomarkers are routinely used in the clinic to aid early detection. MicroRNAs are small RNAs that repress translation. They are present in body fluids, including within exosomes, and have been associated with immune function. **Methods:** Six microRNAs (miR-146a, miR-155, miR-222, miR-199a-3p, miR-93* and miR-377) were investigated in total serum (n = 34) and serum exosome (n = 15) samples from HSCT patients every 7 days pre to post-HSCT (day-7 to D28) by TaqMan® qRT-PCR. Results: The training cohort comprised 72(21%) sibling and 27(79%) matched unrelated donor allogeneic peripheral blood stem cell transplants. Nineteen (56%) patients developed acute GvHD (aGvHD). MiR-146a (p = 0.02), miR-199 (p = 0.02) and miR-423 (p = 0.03) were expressed at a higher level at D14 in patients who developed aGvHD versus no GvHD. Higher expression at D14 was validated in an independent cohort (n = 47) (miR-146a p = 0.05, miR-199 p = 0.01 and miR-423 p = 0.02) and was significantly associated with aGvHD development by ROC (miR-146a p = 0.05, AUC = 0.67; miR-199 p = 0.02, AUC = 0.70; miR-423 p = 0.02, AUC = 0.70). MiR-146a (p = 0.003), miR-199 (p = 0.004) and miR-423 (p = 0.03) were expressed at higher levels in patients who did not receive reduced intensity conditioning. A subset of patients (n = 15) were assessed for microRNA expression within serum exosomes. MiR-155 (p = 0.06), miR-146a (p = 0.06) and miR-93* (p = 0.01) expression at D14 was lower in patients who developed aGvHD versus no GvHD. MiR-155 and miR-146a results were validated in an independent cohort (n = 47) (miR-155 p = 0.01 and miR-146a p = 0.02) and associated with aGvHD development by ROC (miR-155 p = 0.09, AUC = 0.64; miR-146a p = 0.05, AUC = 0.69). At D14, miR-155 (p = 0.008), miR-146a (p = 0.03) and miR-93* (p = 0.007) expression was higher in exosomes compared to whole serum for patients who did not develop aGvHD.

**Conclusions:** This study indicates the potential of serum and exosome microRNAs as biomarkers for GvHD. MicroRNAs expression was higher in exosomes compared to total serum, suggesting potential biological activity during aGvHD development. Identifying the function of these microRNAs, as well as their role within exosomes, may advance understanding of GvHD pathobiology.

Part of this abstract was presented at the 41st European Bone Marrow Transplantation Annual meeting and published in a supplementary volume of Bone Marrow Transplantation (Volume 50, Supplement 1, March 2015).

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**The study of biogenesis features of outer membrane vesicles produced by Lysobacter sp. XL1**

Irina Kudryakova, Natalia Suzina, Irina Tefasman and Natalia Vasilyeva

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**Introduction:** Gram-negative bacteria. OMVs play different biological roles: secretion proteins, inter-species communication, inter-kingdom communication. Despite of extensive information about OMVs biogenesis available, this process is still not enough clear. Gram-negative bacterium Lysobacter sp. XL1 forms OMVs containing bacteriolytic endopeptidase L5, – 1 of 5 bacteriolytic enzymes secreted by this microorganism. Knowledge about OMVs biogenesis by bacteria of Lysobacter genus is rather restricted. Methods were used: fractionation of OMVs using sucrose density gradient centrifugation, electronic microscopy including immunocytochemistry with the protein A-gold, SDS-PAG electrophoresis, Western blotting assay, protein and 2-keto-3-deoxyoctonate assays, thin-layer chromatography, determination of lytic OMVs action. Fractionation OMVs enabled the 4 fractions to be obtained. Enzyme L5 was found to be localized inside of the lightest OMVs fraction. This fraction differed in size and protein composition from the other ones. Furthermore, protein L5 molecules were collected in certain loci of bacterial outer membrane. The OMVs were produced from these loci. In this way, secreted enzyme L5 involves biogenesis of OMVs produced by Lysobacter sp. XL1. The phospholipid assay showed that OM contained cardiolipin, phosphatidylethanolamine, phosphatidylglycerol. Interesting result was obtained for OMVs that contained only one major phospholipid, – cardiolipin. Thereby, OMVs of Lysobacter sp. XL1 were formed from loci enriched with cardiolipin. Furthermore, OMVs containing protein L5 were shown to lyze broad range of opportunistic and pathogenic bacteria including strains with multiple resistances to antibiotics. Thus, in this work was established the influence of secreted protein L5 and acidic phospholipid, – cardiolipin, on biogenesis of OMVs produced by Lysobacter sp. XL1. These data will supplement the understanding of OMVs biogenesis produced by Gram-negative bacteria. The results obtained from the study of bacteriolytic effect of OMVs are the basis for development of artificial vesicular structures – liposomes – containing bacteriolytic enzymes of Lysobacter sp. XL1 as a new effective antimicrobial preparation.

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Two types of extracellular vesicles released by Gram-negative and Gram-positive bacteria
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Two types of extracellular vesicles (EVs) released by Gram-negative and Gram-positive bacteria are described. Two bacterial strains with the ability to release membrane vesicles with different genesis into the extracellular space are isolated from several natural biotops: Gram-positive bacteria *Micrococcus yuannanensis* strain JS1 with the pronounced lipolytic activity and Gram-negative bacteria *Stenotrophomonas acidaminiphila* strain FM3 with antimicrobial activity against a wide spectrum of bacteria. The objective of this work was to carry out a complex electron microscopic study in combination with electron-microscopic cytochemistry of the nature, mechanisms and peculiarities of vesicle formation, and simulate vesicle involvement in the processes of intermicrobial antagonistic interaction. The cells of strain JS1 formed the cytoplasmic membrane vesicles (CMV) in local zones adjacent to the cytoplasmic membrane being of narrow “pocket” shape, which penetrate stepwise (presumably through passive diffusion) via a thick murein (peptidoglycan) layer through the cell wall and immobilizing after that on the surface of the fibrils of a loose fibriller capsule. It is found out that strain JS1 has a strongly pronounced lipolytic activity, using effectively Twin 20, 40, 60 and 80 as a carbon source. We hypothesize that lipolytic enzymes can be encapsulated in the extracellular CMV. The envelope of the cells of strain FM3 is characterized by the presence of S-layer with tetragonal symmetry. The cells strain FM3 formed the outer membrane vesicle (OMV), which are incorporated into the peculiar S-layer’s tubular structures. It was shown that strain FM3 is able to suppress growth of a series of Gram-negative and Gram-positive bacteria. The structures which are formed with these microorganisms may play a role of a “bridge,” those directly transporting antimicrobial agents of unknown nature in the vesicle composition from the producer cell to the victim cell. These pieces of evidence point to the possible use of strains JS1 and FM3 for further exploration of various types of biogenesis of EVs in the bacteria-producers of biologically active substances and subsequent estimation of their biotechnological potential.
Oral session 5: Extracellular vesicles in systemic disorders and nephrology

Chairs: Dr. R. Dragovic and Dr. Tannia Gracia

14:15-16:00

Isolation and characterization of syncytiotrophoblast extracellular vesicles
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Introduction: The syncytiotrophoblast (STB) layer of the placenta releases vesicular material, including extracellular vesicles (EVs; microvesicles and exosomes) into the maternal blood. STBEV have many different functional properties and interact with maternal endothelial cells (ECs), platelets and immune cells. Evidence suggests that STB microvesicles (STBMV) and STB exosomes (STBEX) may functionally differ. Using the placental perfusion technique, we aimed to fractionate and purify these 2 populations with the future aim of examining their role in normal and pathological pregnancies.

Methods: STBEV were obtained using a dual placental lobe perfusion model, prepared from normal term placentas (n = 8). These preparations contain STBEV and other EV derived from blood (i.e. red blood cells (RBC), platelets, leukocytes and ECs). We developed a protocol consisting of sequential centrifugation and filtration, to obtain highly pure STBEV preparations that were enriched for either STBMV (10,000 × g pellet – 10 KP) or STBEX (150,000 × g pellet – 150 KP). To assess EV purity and size distribution, a combination of 5-colour flow cytometry, Nanoparticle Tracking Analysis (NTA), transmission electron microscopy (TEM) and western blotting was performed. Results: Flow cytometry (measures EV > 300 nm) of placental perfusate showed that differential centrifugation and filtration effectively removed contaminating RBC, and the pelleted 10 KP samples were highly enriched for the STB marker placental alkaline phosphatase (PLAP) (86.3 ± 0.2%) with very low levels of contaminating RBC EV (2.2 ± 0.5%), platelet EV (5.4 ± 1.7%) and leukocyte/endothelial EV (0.2 ± 0.1%). Analysis of the 10 KP samples (STBMV) using TEM and NTA showed a highly polydisperse distribution of EV (NTA modal size 395 ± 12 nm), whereas the 150 KP samples (STBEX) showed a smaller homogenous population of EV (NTA modal size 147 ± 6 nm). Western blotting showed that the 10 and 150 KP samples were PLAP positive and showed little to no signal for markers of platelets, RBC and leukocytes. Only, the 150 KP samples showed an enrichment of Alix and CD63. Conclusions: Using a combination of methodologies in parallel has enabled the development of a robust protocol to successfully fractionate and purify STBMV and STBEX from placental lobe perfusate.

Extracellular vesicle signalling in the kidney
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Introduction: Extracellular vesicles (ECVs) are found in the urine and originate from all regions of the nephron (1). The method, selectivity and pathological role of ECV internalization is unclear at the moment. We hypothesize ECVs can transfer functional RNA and protein along the nephron, contributing to the pathogenesis of nephrotic disease. Methods: Using nanoparticle tracking analysis (NTA), we developed an antibody-specific fluorescent labelling method allowing rapid quantification of particles, bypassing ultra-centrifugation. This method has been adapted to allow us to characterize the uptake and secretion of ECVs. In vitro functional readouts are used to investigate urinary ECVs pathological role. Results: Using antibody-specific fluorescent labelling, the presence of exosome-sized particles with known urinary exosomal markers confirms that NTA can be utilized to rapidly quantify ECVs without time-consuming sample processing (2). Previous work in our group has shown vasopressin modulates the AQP2 water membrane channel content of ECVs (3). By employing fluorescent labelling, we were able to demonstrate vasopressin regulation of AQP2 expression in vitro and in vivo. To further elucidate the regulatory mechanisms involved of ECV signalling in the kidney, we have now shown that vasopressin-dependent ECV uptake is a cyclic-AMP, clathrin-dependent, receptor-mediated response. ECVs isolated from apoptotic cells protect recipient cells against subsequent drug-induced apoptosis. Interestingly, ECVs from severely injured cells increase apoptosis of healthy cells. Prior inhibition of dynamin-dependent endocytosis nullifies both these responses. Summary: ECVs can transfer functional RNA and protein along the length of the nephron modulated by hormonal stimulation, and its pathological role is multifaceted. These findings raise the possibility of urinary ECVs as potential therapeutic delivery agents.

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Study of the functionality of human urinary exosomes
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Introduction: Exosomes derived from all nephron segments are present in human urine, where their function is unknown. Although one report suggested in vitro uptake of exosomes by renal cortical collecting duct cells, most studies of human urinary exosomes have focused on biomarker discovery rather than exosome function. Methods: Urinary exosomes were isolated from healthy volunteers. In-depth proteomic analysis was performed on each individual sample. For bacterial growth analysis luciferase-expressing commensal and pathogenic E. coli strains were exposed to urinary exosomes, and sequential luminescence readings were obtained. The effect of exosomes on bacterial integrity was evaluated by scanning EM. The microRNA repertoire of urinary exosomes was identified using MiSeq analysis from miRNA enriched samples obtained from 3 different pools of healthy volunteers. Results: In-depth proteomic and electron-microscopic analysis showed that normal human urinary exosomes are significantly enriched for innate immune proteins that include antimicrobial proteins and peptides, and a variety of bacterial and viral receptors. Urinary exosomes potently inhibited growth of uropathogenic and commensal E. coli, and induced bacterial lysis. Bacterial killing was maximal with preserved exosomal structural integrity, and occurred optimally at the acidic pH typical of urine from omnivorous humans. EM showed clear evidence of exosome-induced bacterial lysis. Organisms incubated with secretory exosomes for 15 min showed an increased proportion of lysed phenotypes to 56% versus 2% compared with control samples. A list of 289 mature miRNAs and precursors was identified in urinary exosomes. Conclusions: Human urinary exosomes are innate immune effectors that contribute to host defence within the urinary tract. This fact makes identifying factors that modulate exosomal release and constitution key priorities for future work and may reveal potential therapeutic targets for the treatment
of UTIs. The presence of a significant number of miRNAs suggests potential effects on kidney function.

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Hypophosphatemia signalling and pro-coagulant microparticle release in human vascular endothelial cells

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The incidence of blood vessel disease and heart disease is common in patients in chronic kidney disease (CKD). This is partly mediated by an increase in serum inorganic phosphate (Pi) (hypophosphatemia) which is a universal finding in CKD. Microparticles (MPs) are sub-micron membrane sized particles released from cells upon cell activation or apoptosis. We hypothesized that hypophosphatemia results in an increase in intracellular Pi concentration in endothelial cells (ECs) which inhibits phosphoprotein phosphatases and enhances global protein-(Tyr,Ser/Thr)-phosphorylation. The increase in protein phosphorylation results in cell activation and release of pro-coagulant MPs from ECs. Scanning electron microscopy (SEM) demonstrated that hypophosphatemia enhances cell surface membrane blebbing. Transmission electron microscopy (TEM) indicated that these MPs are between 100 and 200 nm in diameter. NanoSight nanoparticle tracking analysis (NTA) detected a significant sub-micron particle release from ECs under hyperphosphatemic conditions. Flow Cytometry demonstrated the expression of phosphatidylserine and VE-CD144 on Pi-derived MPs. Using a selective colorimetric assay (based on phosphomolybdate complex formation) cell layer Pi demonstrated an increase under hyperphosphatemic conditions. In vitro measurements of the catalytic activity of Phospho-Tyrosine-Protein-Phosphatases indicated that Pi inhibits the catalytic activity of these enzymes. Western blotting demonstrated that there is a significant increase in net cellular protein phosphorylation in ECs under hyperphosphatemic conditions. Using an Automated Thrombin Generation Assay (TGA), high-Pi-derived MPs have been shown to be significantly more pro-coagulant comparing to matched controls. It is concluded that in ECs under Pi-stress there is an elevated level of pro-coagulant MP release. This is important as this provides a link between hyperphosphatemia, MP formation and thrombosis in uremic cardiovascular disease.

Receptor clustering: implication for microvesicles biogenesis and signalling

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Introduction: High local density of a ligand on a microvesicle surface stimulates clustering of corresponding receptor on a cell surface. Receptor clustering has diverse effects on its signalling, and reasons for this diversity are poorly understood. Generation of extracellular microvesicles either from exocytosis or from plasma membrane (PM) shedding is a result of cytoskeleton rearrangement that is likely to be dependent on receptor clustering. Methods: We have analyzed effects of receptor cluster size on its signalling for EphA2 receptor and GPI-anchored receptors (GARs) basing on our studies of EphA2 and T-cadherin (T-cad) and data from other researchers. Results: Kusumi et al. have shown that relatively small clusters of 3–9 GARs are immobilized on PM and stabilize underlying lipid raft that in turn serves as a platform for assembly of intracellular signalling complex. Such stabilization of lipid rafts by T-cad bound to LDL particle may account for Ca²⁺ signalling induced by LDL. EphA2 and ephrin form virtually unlimited clusters that are being rapidly internalized. Unrestricted crosslinking of T-cad by antibodies also leads to its internalization. By blocking of EphA2/ephrin internalization, Salita et al. have demonstrated that these clusters are pulled by actomyosin contraction. In the work of Seiradake et al., EphA2 cluster size have been restricted to hexamer, and this induced a strong contraction but with minimal internalization. We have shown that formation of intermediate clusters of T-cad by adiponectin induce cytoskeleton contraction and membrane blebbing without internalization. Summary: Effects of receptor clustering are dependent on the cluster size. Small clusters facilitate signal transduction, large clusters are internalized and intermediate clusters serve for anchorage of actomyosin fibres to PM needed for change of PM shape during vesicles budding.

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Optimization of exosome isolation protocol: how to improve the resolution of the vesicles by size. Rate zonal centrifugation versus differential centrifugation

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Introduction: Differential centrifugation recognized as “gold standard” for exosome isolation is ineffective in obtaining pure populations of exosomes with sufficiently high yield. Moreover, the vesicle size distribution in final pellet is very sensitive to the variation of centrifugation conditions. In contrast, rate zonal centrifugation is not commonly used for exosome isolation though it provides better resolution of particles by size. We performed theoretical analysis of vesicles sedimentation by differential centrifugation and by rate zonal centrifugation. The experimental data on EV sedimentation illustrates the theoretical conclusions. Methods: HT29 and PC3 cell culture supernatants were subjected either to differential centrifugation (300 g for 5 min, 2,000 g for 10 min, 10,000 g for 30 min and 100,000 g for 70 min) or to rate zonal centrifugation in sucrose. Measurements of vesicles sizes and concentrations were done by NTA. TEM was used to determine the morphology of the particles. Results: The theory predicts that up to 50% of the 70–100 nm population may be cosedimented at 10,000 g centrifugation step. Such a loss is greater in FA rotors, even in case of centrifugation improving the yield and the purity of exosome population. The protocol for rate zonal centrifugation ensuring separation of exosomes and shedding vesicles can be adjusted for any SW rotor using specially designed web-calculator. The work was supported by Ministry of Education and Science of the Russian Federation (grant No. 14.607.21.0068, ID RFMEFI60714X0068).

Characterization of the RNA repertoire in extracellular vesicles

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Introduction: Exosomes contain a variety of different RNAs including both coding and non-coding RNAs. However, the full RNA repertoire in exosomes and how it compares to that of the donor cell remains largely unknown. Furthermore, how much of this RNA is transferable to other cells also remains unknown. Methods: We used high throughput sequencing (RNA-Seq) to characterize the RNA in exosomes. We isolated exosomes by differential ultracentrifugation from the K562 leukemia cell line. We then sequenced the RNA from the exosomes and the corresponding donor cells. We have also set up a system to detect potential transfer of RNA (from K562 donor cells to other acceptor cells). Results: We report RNA-Seq data from K562 cells and their corresponding exosomes. We find a strong correlation between the level of mRNA expression in cells and exosomes. We also present results studies investigating transfer of RNA from K562 cells to macrophages. Conclusions: High-throughput sequencing allows for the characterization of the total transcriptome in exosomes in an unbiased manner. We used RNA-Seq to characterize the full RNA repertoire in K562 cells and their exosomes, as well as investigate potential RNA transfer.

Isolation and proteomic analysis of exosomes secreted by human cells in vitro

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Introduction: Exosomes are endocytic origin 20–100 nm membranous vesicles secreted by many cell types into the extracellular compartment. Secretory exosomes contain a characteristic composition of proteins, mRNAs and microRNAs which can be transferred to recipient cells. As exosomes naturally carry RNA between cells, these particles might be useful in gene cancer therapy to deliver therapeutic short interfering RNA (siRNA) to the target cells. Despite the promise of RNA interference (RNAi) for use in therapy, several technical obstacles must be overcome. Exogenous siRNA is prone to degradation, has a limited ability to cross cell membranes and may induce an immune response. Naturally occurring RNA carriers, such as exosomes, might provide an untapped source of effective delivery strategies. Methods: In the current study, the exosomes of human glioblastoma cell lines were examined in vitro by mass spectrometry (MS), 1- and 2-dimensional electrophoresis, immunoblotting, atomic force microscopy and light-scattering methods. The current study employed several original approaches as isolating exosomes from the cultural media by causing exosomes aggregation with phytohemagglutinin. Next, we investigated the possibility of loading exosomes with exogenous cargos using chemical treatment. Results: By using these methods, we isolated exosomes that were confirmed by dot-blot analysis for various exosomal proteins including CD63, TGS101 and HLA-ABC. Protein profiles were further analyzed by MS that allowed identifying more than 50 proteins. Also, we demonstrate that exosomes can be loaded with siRNA by chemical treatment and deliver siRNA to recipient cells in vitro. The delivery of fluorescently labelled siRNA via exosomes to cells was confirmed using flow cytometry. Two different siRNAs against RAD51 and RAD52 were used to transfet into the exosomes for therapeutic delivery into target cells. The exosome-delivered siRNAs were effective at causing post-transcriptional gene silencing in recipient cells. Conclusions: We identified by MS and Western blot analysis a novel category of exosomal proteins related to apoptosis: PCNA, p53 and 13-3-3. These proteins were obtain in 5 gilal exosome samples but were not detected in exosomes from normal non-cancerous cells. Overall, this study suggests the panel of specific brain tumour exosomal markers to help create non-invasive techniques to diagnose disease. As for loading of exosome, the results suggest that exosomes can deliver the siRNA into the target cells, giving an additional evidence of the ability to use human exosomes as vectors in cancer therapy, including RNAi-based gene therapy.

Part of this abstract has previously been presented at the FEBS congress 2013 “Proteomic analysis of exosomes secreted by human glioblastoma cells hold promise for identifying markers of brain cancer" V.S. Burdakov, T.A. Shtam, S.N. Narzychny, S.B. Landa, N.L. Ronzina, M.V. Filatov and published in Cell Commum Signalling 2013 (11:88) “Exosomes are natural carriers of exogenous siRNA to human cells in vitro" T.A. Shtam T.A., Kovalev R.A., Varfolomeeva E.Y., Makarov E.M., Ii V.V., Filatov M.V.

Nanoparticle tracking analysis: theory, applications for exosomes research and tips and tricks

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Introduction: In this report, we summarize our experience of nanoparticle tracking analysis (NTA) measurements of various samples including inorganic nanoparticles, liposomes and extracellular vesicles to get the quantitative and reproducible results for particle sizes and concentrations. Methods: Measurements were made with Nanosight LM10 instrument (405 nm laser and EMCCD camera) and Nanosight NS500 instrument (532 nm laser and EMCCD camera). Results: The general idea of proper work with NTA is that all NTA samples are extremely diluted solutions. Typical concentration for NTA of $10^{10}$ particles/ml corresponds to 1.7 pM (picomoles per litre). Two main concerns should be kept in mind: avoiding losing the substance and avoiding contamination. Particle free water is a key factor for good NTA measurement. Water of a good and sustained quality could be prepared with well-maintained MilliQ (or analogous) systems fed with purified water. Large amounts of particle free buffers could be prepared by slow filtration of 10 $\mu$m buffer concentrate via sterile 0.22 $\mu$m syringe filter with Nylon membrane (which not only retain particles $>220$ nm, but also adsorbs smaller ones) followed by dilution of this concentrate by particle free water. Intense shaking/vortexing of dilute solutions should be avoided as it causes the release of particles from test tube/vial walls with sizes 30–600 nm and concentrations up to $10^9$ part./ml. Both polypropylene (PP) and glass test tube are affected. The effect is batch dependent: 2 different batches of the same PP test tubes release different concentrations of particles during intense shaking/vortexing. Thus samples for NTA should be mixed gently. If shaking/vortexing is unavoidable, it should be done with concentrated solutions ($>10^7$ part./ml) with a blank buffer control to estimate contamination level. The last frequent mistake is tips/tubes handling. Touching tips with gloves or opening the test tubes in such a way that the gloved thumb touches the inner part of the tube cap causes the contamination as well. Conclusions: A proper design of the experiment is vital for correct and reproducible NTA measurements. Wrong sample preparation and handling could lead to highly distorted size distribution and concentration of particles being studied.

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Reversible optimization of commercial flow cytometers for submicron particle analysis
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Introduction: As microvesicle (MV) analysis becomes of increasing interest, better optimization of quantification techniques, namely flow cytometry, is required to improve analysis of the 0.1–1.0 $\mu$m MV population, particularly the smaller MVs. Dedicated flow cytometers are becoming more common and provide greater resolution than commercial flow cytometers for MV analysis. Building a dedicated flow cytometer however is not a viable option for all research groups. Here we describe reversible steps that can be taken to improve the resolution of a FACSaria flow cytometer for submicron particle analysis. Methods: It is important that cytometer changes are easily implemented and reversible for communal machines. Parameter modifications tested were: neutral density filter (NDF), nozzles (100 $\mu$m, 70 $\mu$m, 35 $\mu$m, 20 $\mu$m, 10$\mu$m) and core stream pressure (low, medium, high). Megamix FSC and SSC beads were used to track the effects on resolution. Resolution was determined using separation index units, calculated by subtracting population medians and dividing by the sum of standard deviations. Results: SSC resolution can consistently and reproducibly be improved by reducing from a high to low flow rate (mean 53% increase), regardless of sheath pressure or nozzles. Increasing sheath pressure to 70 psi increased resolution with both 100 $\mu$m (25% increase) and 70 $\mu$m (91% increase) nozzles. Removal of the NDF increased FSC resolution by as much as 1,143%. Whilst the 100 $\mu$m nozzle showed higher resolution than the 70 $\mu$m nozzle at 35 psi, at 70 psi, the 70 $\mu$m nozzle showed a higher resolution (48%) than 100 $\mu$m nozzle at 70 psi, and this is thought to be because of the Venturi effect. Conclusions: This work shows reversible alterations on commercial machines can improve submicron particle resolution and therefore improve MV analysis. Although not all changes are viable on all machines, understanding potentially adjustable parameters can aid selecting an appropriate machine for MV analysis.

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Magnetic nanoparticles for drug delivery, smart biomaterials and other applications
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Introduction: “Smart” biomaterials that can release cargos upon receipt of an alternating magnetic field (AMF) have been developed (1). These biomaterials, MNPs, are created by crosslinking magnetic nanoparticles (MNPs) and phospholipid vesicles using avidin-biotin recognition. Mixing MNPs with cells within a hydrogel can allow spatiotemporal control over cellular responses (2–4). We have now explored the use of reversible covalent links to create MNPs that are able to first release bioactive compounds then “self-destruct”, dissociation of both the aggregates and the hydrogel. Methods: A novel series of reactive MNPs and lipids have been synthesized. MNPs labelled with either thiol groups or activated disulphide bonds have been shown to react with disulphide-capped or thiol-capped lipids embedded in vesicle membranes. The resulting MNPs were tested for their ability to release 5/6-carboxyfluorescein in solution. Results: Thiol-disulphide exchange was a successful method for the creation of a new class of MNPs that were designed to release their contents upon application of an AMF. Self-destruction of MNPs will be tested in the future after the composition of the vesicles, and the conditions required has been tuned. Conclusions: MNPs containing disulphide crosslinks are exciting candidates for the preparation of remotely controlled smart biomaterials. Furthermore, these reversible covalent interactions between phospholipid vesicles and MNPs may provide insight into how thermoanisotropic MNPs could be conjugated to extracellular vesicles.

Acknowledgements
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Cell invitation and photodynamic quality mixed micelles of lipophilic derivatives of natural chlorines with phospholipids in MCF-7
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Introduction: Due to the increasingly expanding usage of photodynamic therapy (PDT), it is becoming particularly urgent to design new photosensitizers (PS), with high efficiency and low frequency of side effects. It is assumed, that saline-soluble chlorine derivatives, encapsulated in liposomes, can be used as more effective PDT agent. The aim of the study was to identify possible ways of internalization and localization of new lipophilic complexes of a
phosphatidylcholine-based conjugate in MCF-7 cells and assess their application in PDT. **Methods** We have synthesized 6 lipophilic conjugates of chlorines, in which the lipophilic fragment (either hexadecyl- or cholest-5-en-3b-yloxyethyl-) was linked to $13^{1'}$, $15^{2'}$, $17^{3'}$-positions of the macrocycle with formation of corresponding carboxamides. Mixed micelles conjugates, with phosphatidylcholine differing in stoichiometric compositions, were prepared and characterized by absorption spectra, electron microscopy and laser scattering. These micelles were bond to and internalized by human breast carcinoma MCF-7 cells. For further experiments mixed micelles of $17^{3'}$-(hexadecylcarbamoyl) pyropheophorbide conjugate and PC with molar ratio conjugate/PC equal to 1:7 (12.5 mol % conjugate) were used, because these micelles were not accumulated inside the cells. **Results** We found that micelles initially localized in the endoplasmic reticulum vesicles but were then exported outside the cells without damage. They were also found in lysosomes. Their internalization involves several mechanisms (clathrin-dependent endocytosis, caveolin-dependent endocytosis and diffusion) prevalence of each of these mechanisms depends on the liposome size and on the conjugate structure. The PDT effect in the cultured cells depended on the compound of the active compound, intensity and time of laser irradiation. **Conclusions** The presented data reveal that modification of the chlorine macrocycle with lipophilic substituents, solubilization of resultant conjugates in an aqueous medium as mixed micelles with phospholipids, and transfer of mixed micelles to the cells is a perspective approach for targeted delivery of chlorine derivatives, which apparently may be used in PDT.

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