http://dx.doi.org/10.4172/2324-8777.S3-001

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International Publisher of Science, Technology and Medicine

Journal of Nanomaterials & Molecular Nanotechnology

Enrichment and Characterization of Thrombocyte-Derived Extracellular Vesicles

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Abstract

Extracellular vesicles (EV) represent a heterogeneous population of nano-sized membrane-limited bodies released by a variety of cells, generated through different mechanisms, and characterized by different size distribution and protein composition. As central players in intercellular communication, EVs are involved in a number of physiological and pathological processes, such as coagulation, inflammation, or tumor progression [1-3]. Elevated levels of EV observed in sepsis or Crohn's disease [3,4] suggest their high potential as biomarkers. Based on their biogenesis, diameter, and membrane markers, they are classified into endosome-derived exosomes (30-150 nm), plasma membrane-derived microvesicles (100-1000 nm; also referred to as microparticles or ectosomes), and apoptotic bodies (1000-3000 nm).

Due to their size overlap with bacteria, viruses, and protein complexes, the isolation, quantification, and characterization of EVs represent a challenge, and standardized protocols are still missing [5]. In this study, we enriched microvesicles (MV) and exosomes (EX) from platelet concentrates and investigated their procoagulant role.

Platelet concentrates were obtained from healthy volunteer donors using a Trima Accel® blood collection system (Version 5.0, Gambro BCT). After removal of platelets by centrifugation (1500 g, 15 min, RT), MVs were obtained by centrifugation at 20000 g (2 x 30 min, 4 °C). To enrich exosomes, the supernatant after MV removal was centrifuged at 100000 g (2 x 60 min, 4 °C). Alternatively, a commercial exosome isolation kit (Invitrogen) was used. Size distribution of isolated EVs was assessed by nanoparticle tracking analysis (Nanosight, Malvern). Flow cytometry was performed using the CytoFLEX flow cytometer (Beckman Coulter, detection limit 100 nm) with Annexin V (AV) as MV marker, CD41 as platelet marker and CD63 as EX marker. EV preparations were standardized with respect to protein content (DC assay, Biorad). Tissue factor (TF) expression was assessed by Western blotting using the monoclonal TF9-10H10 antibody. EV-induced thrombin generation was studied using a thrombin generation assay (Technoclone).

Mean particle sizes detected by NTA were 156 nm for MVs vs. 135 nm for EX (centrifugation) and 80 nm for EX (kit). Flow cytometry of the MV preparation showed 55% $CD41^+AV^+$ and 0.5% $CD63^+$ events in the MV gate. For the EX preparations, the distribution of markers depended on the isolation protocol with 4% $CD41^+AV^+$ and 2% $CD63^+$ events in the MV gate for EX (centrifugation) vs. 0.5% $CD41^+AV^+$ and 10% $CD63^+$ events for EX (kit). Western Blotting revealed the presence of TF in all preparations, with an approximate signal ratio of 1:3:10 for MVs *vs.* EX(centrifugation) *vs.* EX(kit). All EV preparations induced thrombin generation in MV depleted plasma with the strongest effect for MVs.

Our data strongly support the procoagulant role of extracellular vesicles and reveal differences between MV and EX preparations with respect to TF content and thrombin generation. As to the presence of tissue factor in microvesicle and exosome preparations, the extent of its actual association with vesicles remains to be elucidated.

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http://dx.doi.org/10.4172/2324-8777.S3-001

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The Selection of the Polymer-Solvent System and Process Conditions for Electrospinning of Hyaluronic Acid

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Introduction

The scientific and technical progress has increased the interest in available and new techniques for the preparation of various types of materials at nanoscale, due to their unique properties. Starting from the 90s, we are dealing with the development and numerous modifications of electrospinning - the technique of preparation of micro- and nanofibers, which is known since the late nineteenth century. Electrospinning is a process in which nanofibers are produced under the influence of an electric field from a stream of polymer solution or polymer melt supplied through the nozzle holes of certain geometry and received at the collectors, with a horizontal or vertical arrangement. This allows obtaining a predetermined morphology and structure of fibers, depending on the selection of certain parameters [1].

The major obstacle in electrospinning of biopolymers is to determine the proper characteristic of the spinning solution, including molar mass of the polymer, concentration, viscosity and surface tension. All of this supports the appropriate course and stability of the fiber spinning process, yielding a fibrous material of defined characteristics. However, the major problem in electrospinning of hyaluronic acid and its derivatives is very high surface tension and apparent dynamic viscosity values of the HA aqeous solutions, which prevents the fibre spinning process and does not yield in a fibrous material [2,3]. In terms of its final application in regenerative medicine, the best hyaluronic acid solvent is water, yet it is characterized with high surface tension of 72,75 mN/m. In our research we have improved the properties of the spinning solutions by introducing N-methylpyrrolidone and ammonia water, which caused the reduction in the surface tension and enabled to obtain nanofibers [4].

Materials and methods

The hyaluronic acid was purchased as the cosmetic purity sodium hyaluronate in Contipro Biotech (Czech Republic). The solutions were prepared with N-methylpyrrolidone and ammonia water as solvents in ratio 1:2 respectively with concentration 12 % of HA w/w. Surface tension measurements were made by the use of the process tensiometer using the Wilhelmy's plate method. Rheological properties of the polymer solutions were determined using a rotational rheometer Anton Paar Rheolab QC at the controlled temperature 20 °C, by using a thermostatic bath. Shear stress and shear rate has been approximated by exponential dependence, according to the Ostwald de Waele's model.

Results

Rheological characteristics of the solutions, together with the surface tension are shown in Table 1 SEM images of the fibers obtained from the electro spinning are shown in Figure 1.

Conclusions

In the frame of our research, we have successfully obtained hyaluronic acid-based nanofibers. In the frame of the preliminary studies we have selected the optimal composition and concentration of the spinning solution, as well as molar mass of the polymer, enabling to obtain nanometric fibers from the range of 72.9-147.5 nm [1]. An analysis of the flow curves of the spinning solutions has shown that the tested solutions are non-Newtonian shear thinned liquids.

Table 1: Rheological characterization and surface tension of the HA solution

Polymer	Solvent	Concentration	Rheological Parameters		Surface tension [mNm^{-1}]
			k	n	
100-150 kDa	AW/NMP	12	43.86	0.90	60.07

http://dx.doi.org/10.4172/2324-8777.S3-001

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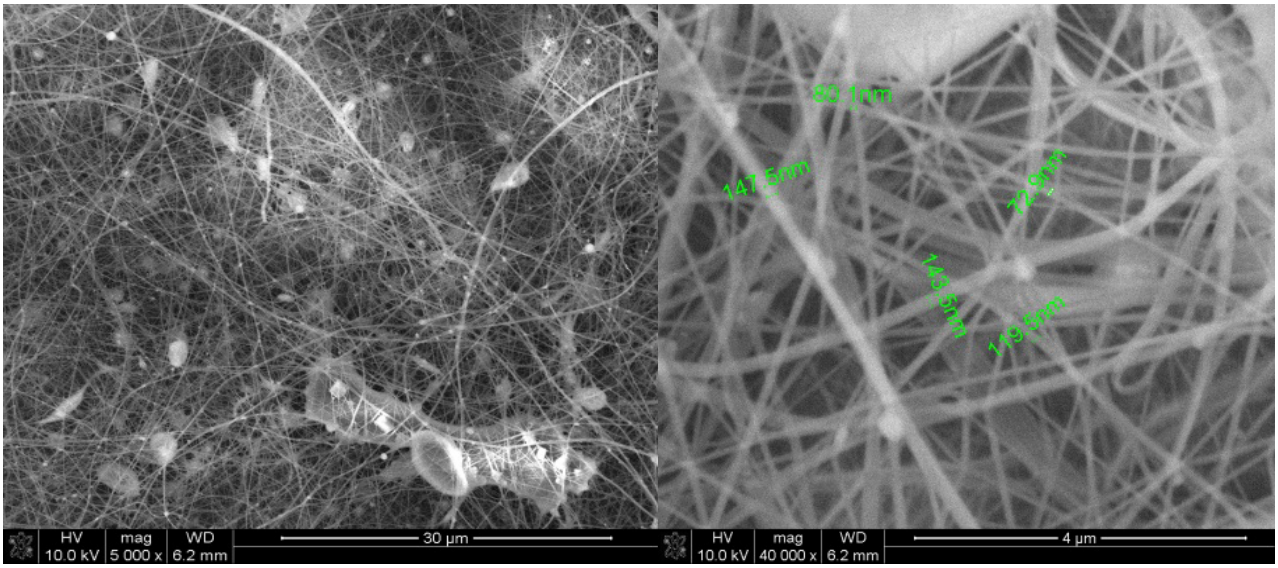

Figure 1: SEM image of fibers obtained from the spinning liquid with the composition: 12% HA in AW-NMP solution systems

Acknowledgements

The presented work is a part of project DEC-2012/07/B/ST8/03378 and was financed by the National Science Center in Poland.

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http://dx.doi.org/10.4172/2324-8777.S3-001

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Journal of Nanomaterials & Molecular Nanotechnology

Suitability of Liposomal Nanocarriers for Pulmonary Drug Administration depends on the applied Nebulizer Device and Particle Surface Characteristics

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Pulmonary drug delivery by inhalation has a couple of advantages (e.g. localized drug deposition, reduced side effects) over other administration routes. However, the aerosolization process might cause stability problems for sensitive drug molecules. In this study we have tested three liposomal formulations with different surface characteristics using commercially available nebulizer systems (air-jet, ultrasound, and vibrating-mesh) [1]. The liposomes were prepared by dry lipid film rehydration and size extrusion to result in homogeneous particles of similar sizes between 140-165 nm and polydispersity indices <0.1. The liposomal stability upon nebulization was determined by size and polydispersity measurements as well as leakage of a hydrophilic fluorophore quencher system by fluorescence spectroscopy. The structural integrity was checked with transmission electron microscopy images before and after nebulization. None of the liposomal formulations had an impact on the aerosol droplet size distribution of the nebulizers. The liposomal transport efficiencies and aerosol output rates were highest for the vibrating-mesh nebulizer. However, the vibrating-mesh nebulizer showed the highest release rate of the encapsulated model drug system independent of the liposomal surface characteristics. The most stable formulation among all nebulizer types was the conventional formulation containing phosphatidylcholine and cholesterol. The nebulization process did not affect size or size distribution. On the contrary, the polymer coated and positively charged formulations were more prone to aggregation and had higher drug release rates. With this study we could show that the most effective nebulizer for the application of liposomal nanocarriers is the vibrating-mesh nebulizer transporting the highest amounts of liposomes to the aerosol. Further we can say that the conventional liposomal formulation showed the best performance throughout all different nebulizer techniques.

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http://dx.doi.org/10.4172/2324-8777.S3-001

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Journal of Nanomaterials & Molecular Nanotechnology

Development of Biodegradable Polymer Facilitating the Local Treatment of Radioisotopes

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Radiation emitted from a radioisotope is tricky to manage. However, it can be used very efficiently depending on the method used. Recently, the application field of medical radioisotope has been diversified. Medical radioisotopes are used for two purposes. Radioisotopes used for diagnostic purposes are administered into the body and must be able to release a signal to the sensor located in vitro, and thus gamma-ray emitting radionuclides are mainly used [1]. Alternatively, radioisotopes used for the treatment have low permeability but a significant effect on the surrounding cells, and thus alpha rays or beta rays emitting radioisotopes, mainly metallic radioisotopes, have been used [2]. This concept is only effective if the target cell has spread in many places. By developing a biodegradable polymer material having a nano-chelator concept can label a metallic radioisotope to maximize this therapeutic efficiency. Polycaprolactone (PCL) is one of the polymeric materials used for the regeneration of living tissues, including the liver. When injected using an injector having a fine pore size, it is possible to produce a nano web. PCL can react with a metal radioisotope such as Ga-68 or Lu-177 through a chelating action of the lactones of PCL. For the treatment of cancer such as in the prostate, direct transplantation in the organ has already been used. A material producing method using the adsorption of radioisotopes with a biodegradable polymer can suppress the growth of the cancer cells and help the tissue regeneration due to the incision site of the cancer. In this study, we use Lu-177 for the medical radioisotope. We labeled Lu-177 on three kinds of PCL fibers given the change in thickness. The ^{177}Lu-labeled macromolecular membrane exhibited high stability in saline. These results suggest that this radio-molecule is possible to apply for a medical device. In the future, we will investigate a stable labeling and injecting method of the radio-molecule for its medical application.

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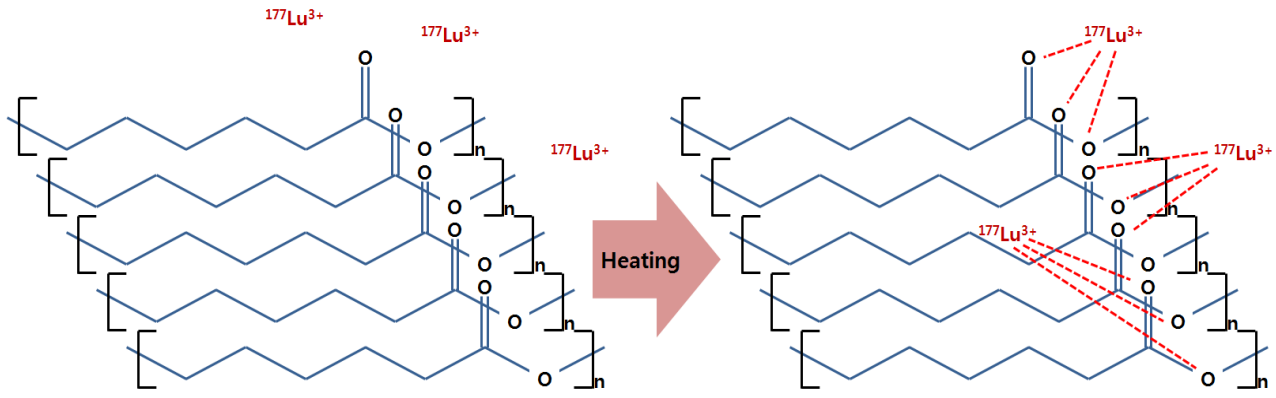

Figure 1: Covalent bonding of $^{177}Lu^{3+}$ with polycaprolactone

http://dx.doi.org/10.4172/2324-8777.S3-001

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Journal of Nanomaterials & Molecular Nanotechnology

Alginate-Chitosan Synthetic Vesicles as a Promising Inhibin-A Delivery System for Acute Kidney Injury therapy

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Acute kidney injury (AKI) is an emergent public health problem that affects millions of patients worldwide. Several pharmacologic therapies that can accelerate recovery and improve survival were successful in experimental models but failed to manifest any significant beneficial effect in clinical practice[1]. Recent studies have indicated that adult renal stem/progenitor cells (ARPCs) are able to repair damaged renal proximal tubular epithelial cells (RPTECs) in AKI induced by toxic agents, via the secretion of both inhibin-A (INHB-A), FGF2 chemokines and specific microvesicle-vehicled mRNA . Our goal was to synthesize a natural polymer-based nanosystem for efficacious delivery of INHB-A.Alginate (AL) and chitosan (CS), due to their promising properties are being exploited for the development of drug delivery systems [2]. INHB-A-loaded Polysaccharides Synthetic Vesicles (INHB-A-PSSV) were synthesized by two steps methods: ionotropic pre-gelation of AL core, followed by CS polyelectrolyte complexation. A microfluidic device was appositely fabricated in order to optimize the INHB-PSSV -at interface-assembly process, in terms of polymers and INHB-A working amount as well as vesicles size distribution. TEM and DLS characterization showed highly mono-disperse spherical PSSV ((157 ± 30) nm in diameter) and ζ-potential measurement ((+56 ± 4) mV) confirmed the CS coating stability. Cellular uptake and INHB-A-PSSV effectiveness were tested in an in vitro model of cisplatin (CisPt) induced cell toxicity. RPTECs were exposed to 2.5 μmol/l CisPt for 6 h and, after drug withdrawal, cell viability was performed at 3 days after treatment. CisPt treated cell viability significantly decreased, compared with healthy control. We showed that addition of INHB-A-PSSV to CisPt -treated RPTECs led to a substantial increase in cell number and viability after 3 days of culture. Remarkably, a very low dosage of functional loaded protein (8 ng/25μl) was sufficient to induce cell regeneration and the percentage of viable cells was similar to that of RPTECs without CisPt treatment.

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http://dx.doi.org/10.4172/2324-8777.S3-001

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Journal of Nanomaterials & Molecular Nanotechnology

The Effect of Nanoparticle Size and Nuclear Localization Sequence Density on Nuclear Targeting Efficiency of Chitosan Nanoparticles

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Many recently discovered therapeutic proteins seem to function in the nucleus [1-3]. Chitosan nanoparticles (NPs) have attracted interest as protein delivery vehicles due to their biocompatibility and ability to escape the endosomes [4] offering high potential for nuclear delivery. Molecular entry into the nucleus occurs through the nuclear pore complexes [5], the efficiency of which is dependent on NP size and the presence of nuclear localization sequence (NLS)[6].

Chitosan nanoparticles of different sizes (S-NPs ≈ 25nm; L-NP ≈ 150 nm) were formulated, and modified with different NLS densities (low, intermediate and high). S-NPs and L-NPs and their NLS modified forms were evaluated with respect to their protein loading capacity, extent of cell association, cell uptake, cell surface binding and finally nuclear delivery efficiency. To avoid errors generated with cell fractionation and nuclear isolation protocols, nuclear delivery for both S-NPs and L-NPs was assessed in intact cells utilizing FRET fluorometry and microcopy.

NLS exerts a size and density dependent effect on nanoparticle uptake and surface binding, with a general reduction in NP cell surface binding and an increase in cell uptake with the increase in NLS density (up to 8.4 folds increase in uptake of High-NLS-L-NPs compared to L-NPs). However for nuclear delivery, unmodified S-NPs show higher nuclear localization rates when compared to NLS modified NPs (up to 5 folds by FRET microscopy). For L-NPs an intermediate NLS density seems to provide highest nuclear localization (3.7 folds increase in nuclear delivery compared to High-NLS-L-NPs). Results indicate that a higher NLS density does not result in maximum protein nuclear localization and that a universal optimal density does not exist with several factors including NP size affecting the optimal NLS density.

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http://dx.doi.org/10.4172/2324-8777.S3-001

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Supramolecular Peptide Architectures: Morphology of a Self-Assembled Designer Peptide Double Helix

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A very active area of research aims to use peptide-based nanostructured materials as biomimetic artificial matrices in nanomedicine [1]. In our studies, self-assembling amphiphilic designer peptides serve as building blocks.To fully exploit the potential of these molecules, it requires a deep understanding of the peptides' individual, as well as their collective morphology, the underlying dynamic assembly mechanisms and how these materials act at the interface of synthetic and biological membranes. By combining Synchrotron small angle X-ray scattering (SAXS), transmission electron microscopy (TEM), infrared (IR) and circular dichroism (CD) spectroscopy we studied the concentration-dependent self-assembly of an 8-residue amphiphilic designer peptide. Above a critical aggregation concentration this peptide forms a variety of structural intermediates and finally develops to unique supramolecular double helices with lengths of several hundreds of nanometers and uniform diameters of about 24 nm.SAXS and IR spectroscopy allowed a detailed look at the internal organization of the structures. We propose a 3-layered model, where monomers are interdigitated and tightly packed, held together by multiple weak noncovalent bonds. The double helices are intertwined to a network, showing hydrogel properties. These double helices remained structurally unaltered for several months [2]. To probe the interaction of peptide-assemblies with artificial membrane mimicks, we performed differential scanning calorimetry (DSC) and SAXS. Even at high concentrations the membrane integrity remained unaffected by the peptides' presence. Based on our results, this peptide has a high potential to meet the needs of a next-generation biomaterial in future medical and technological applications.

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http://dx.doi.org/10.4172/2324-8777.S3-001

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Journal of Nanomaterials & Molecular Nanotechnology

Consumers, Nanotechnology in Medicine and Responsibilities

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Abstract

New and emerging ('modern') technology is getting increasingly more vital in our everyday lives. This to the extent that we become to expect that technology develops to solve more challenges and increasingly complex ones.

Few other areas are the hopes, hypes and expectations on emerging technologies higher than for medicinal purposes. What we found in our very first project on nanotechnology was that people's reflections on risks increased the closer the application was to our skin: few had any qualms over car tyres or tennis rackets, but more so for clothes. When cosmetics were discussed risks and side-effects where definitely on the agenda, not to mention potential applications in food! However, moving "through" our skin, and talking of nanotechnology in medicine, the risks where far less on the agenda. In the face of (the risk of) cancer, it seems many would accept substantial risk levels for a potential cure for cancer (or other deadly diseases).

Developing a Virus Interaction Sensor Platform

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A subset of virus species, e.g. HIV and Influenza, are surrounded by a lipid bilayer, termed the envelope. We hypothesize, that the protein content of the envelope will vary in response to the virus-surrounding medium as proteins or other factors present may be associated, with potential benefit for the virus particle.We want to define the viral envelope proteome in response to external stimuli (ranging from albumin to glycosylphosphatidylinositol-anchored green fluorescent protein to complete serum). In order to control the lipid bilayer more precisely, we have established a platform to investigate association or insertion events by using tethered liposomesfor Quartz Crystal Microbalance (QCM) and Dual Polarization Interferometry (DPI) measurements. By using this platform, we were able to identify differences in association and dissociation behavior depending on the challenge used. We are currently investigating the tethering of virus particles, enabling real-time sensing of association events from the surrounding media with viral envelope bilayers and linking the biophysical with biochemical data. Potential applications for the sensor include the screening for novel targets for antiviral intervention and the evaluation of antivirals themselves, i.e. antibodies or small molecules.

http://dx.doi.org/10.4172/2324-8777.S3-001

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Journal of Nanomaterials & Molecular Nanotechnology

Biopolymer Nanotechnologies Combined with Stem Cell on Tissue Regeneration

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Regenerative medicine associated with nanotechnology is one of the great innovations of this century and the combination of biopolymer nanofibers and nanoparticules with stem cells seems to be the future for organs and tissues recovery. This presentation aimed to demonstrate with this tool the possibilities to regenerate of injured tissue with nanostructured as Burned Tissue and Parkinson Disease as well to tracking the cells after transplantation. **Methods**: At first study**,** nanostructured membranes used were composed of bacterial cellulose and gellan associated with lysozyme (MCG/LZ) and of bacterial cellulose, tamarind xyloglucan and gellan associated with lysozyme (MCGT/LZ). Mesenchymal Stem Cells (MSC) were used. Cellular adhesion and proliferation assays, and electron microscopy scanning of the membranes were performed. The membranes seeded and not seeded with mesenchymal stem cells were used as burn treatment in preclinical model. On the

second study, *in vitro* assays were performed to test for adhesion and proliferation with the human adipose-derived MSC from adipose liposuction. Then, those cells, $1x10^3/cm^2$ were seeded and differentiated to dopaminergic neurons in established medium conditions and time, maintained in an Incubator with 5% CO2 at 37 °C (Patent); thereafter, it was analyzed the dopamine production by ELISA assay for allowing the quantification of their performance. Optical and Electron Microscopies were done in membrane and cells. The Flow Cytometric analysis was done in human adipose-derived MSC fraction and tri-lineage pluripotency test. The immunoassay was done to demonstration the dopaminergic neuron differentiation by ß-tubulin. On third study, *in vivo* studies, curcumin-loaded nanoparticles (Cur-NPs) were injected in the *substantia nigra* of the adult rat by stereotaxic surgery using the followings coordinates from the bregma (anteroposterior -5.0mm, dorsoventral 7.7mm, mediolateral ± 2.1mm). It was injected 2.0 µL of Cur-NP (0.426 mg/mL) using a Hamilton syringe. After 24h, the rat was euthanized by a lethal dose of anesthetic and the brain removed and frozen in liquid nitrogen. Tissue sections were cut using cryostatic microtome and histopathological analyzed by

fluorescence microscopy Zeiss Axio Vert.A1. **Results:** On first study, results, the MSC were able to proliferate and expand on nanostructured membranes The MCGT/LZ membrane presented the best results of tissue regeneration, reaching 90% of epithelization rate ($p= 0.02$) [1,2]. On the second study; the human adipose-derived MSC had differentiated to dopaminergic neurons on the membrane and those cells were able to product dopamine: 0,8 ng/mL. On the third, study, the Cur-NPs showed a drug content of 426 µg/mL and high entrapment efficiency, demonstrating their suitability in the encapsulation of curcumin [3]. The Cur-NP could be observed in host tissue after transplantation. **Conclusions:** The nanostructured membranes seeded with stem cells demonstrate to be efficient alive bioactive dressings, effective for tissue regeneration, particularly in burned tissue. The results allow the utilization the developed implant in preclinical Parkinson Disease Model. Cur-NPs could be used for tracking cellular therapy on histopathological analysis. **Financial support of CAPES (Brazil) and Institute Carnot POLYNAT (France).**

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http://dx.doi.org/10.4172/2324-8777.S3-001

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Journal of Nanomaterials & Molecular Nanotechnology

An Organ-Like Microfluidic Model of the Blood–Brain Barrier for Testing Novel Methods of Nano-Particulate Drug Delivery to the Central Nervous System

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Treatment of diseases of the central nervous system like Alzheimer's or epilepsy remains a major challenge as most available drugs cannot pass the blood–brain barrier (BBB) [1]. Nanotechnology offers novel methods for drug delivery that activate cell transport systems to carry drugs over the BBB [2]. However, testing these drug delivery tools for their efficacy and safety with existing test systems like animal models or static two dimensional cell cultures can be difficult as these models are either difficult to handle or lack comparability to the human *in vivo* situation.

To this end we are developing a novel *in vitro* model of the BBB to mimic the *in vivo* environment [3]. For the first time endothelial cells have been actively arranged by dielectrophoresis (DEP) to facilitate the formation of a tight 3D cell structure in a microfluidic system. The system comprises a pair of electrodes on opposite sides of parallel flow channels with an assembly of insulating micropillars arranged between them (Figure 1A). We use two-phase *in situ* polymerization to form a vertical matrix between the micropillars to support cell adhesion (Figure 1C). We have successfully demonstrated the assembly of cells in the chips' microstructures (Figure 1B).

By providing stimuli of shear forces and cell-cell interactions we expect cells to form a barrier with behavior that closely mimics the *in vivo* situation. The microfluidic system enables the collection of very small sample volumes and superb optical accessibility of the cells to closely study the transport of drugs or nanoparticles across the cell layer.

Acknowledgement

Funding for this research was obtained in part from the Federal Ministry for Education and Research (BMBF) through grant no. 16SV5952

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http://dx.doi.org/10.4172/2324-8777.S3-001

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Journal of Nanomaterials & Molecular Nanotechnology

Nanopinion - Engaging Beyond the Scientific Community

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How do people form an opinion on a rather unknown subject, such as nanotechnologies? How can we launch broad social discussion of nanotechnologies without being influencing? This was one of the main questions the NanOpinion project wanted to answer while offering a broad range of outreach and engagement activities for the general public. These activities allowed at the same time the collection of over 8000 questionnaires across Europe that were analysed together with workshop protocols, interviews and opinion polls. The results reassured the multi-channel approach to provide the necessary elements to support opinion forming: As nanotechnologies mostly cannot be regarded as an area of interest per se, other relevant topics (e.g. food or health) of interest have to be linked with. Engagement activities shall then take place where people follow this interest (e.g. a food festival or health care centres). On the spot, a range of target group tailored activities have to be offered which allow for participation on different levels according to people's interest, time resources and pre-knowledge. The different engagement activities should also motivate for active contribution – in NanOpinion for example, people were invited to take part in opinion polls, participatory workshops, or interactive programmes such as theatrical discussion games etc. Still information has to be provided in a balanced way, so that neither benefits nor risks are biased. Finally, results have to be published and fed back to the participants. Follow up activities for discussing the results would be an interesting next step which could be considered in subsequent projects.

Acknowledgement:

The NanOpinion project (http://nanopinion.eu/) – Monitoring public opinion on Nanotechnology in Europe - was funded within the EC 7th FP, with the contract number: NMP2011.3-4-290575.

http://dx.doi.org/10.4172/2324-8777.S3-001

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Journal of Nanomaterials & Molecular Nanotechnology

Electrostatically Bound Glutathione Reduces Cytotoxicity of Polyethyleneimine Coated Magnetic Nanoparticles

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Cationic additives in nanoparticles, such as polyethyleneimine (PEI), can trigger cell death and toxic responses [1]. PEI induced toxicity is still the major obstacle in its use [2]. The aim of our study was to develop a simple method for reducing toxicity of Cobalt ferrite-polyacrylic acid (PAA) - PEI multilayer nanoparticles (NPs) by additional binding of glutathione (GSH). Possible mechanisms of toxicity reduction are the anti-oxidative effect of GSH by ROS scavenging processes and reduced membrane damage, due to lower surface charge of PEI. Cobalt ferrite-PAA-PEI-FSC(fluorescein modified NPs)were used as a modification control without anti-oxidative properties. All experiments were performed on Chinese hamster ovary (CHO) cell line. We have shown reduced toxicity with propidium iodide viability assay, MTS viability assay, ROS measurements and total intracellular glutathione determination (Figure 1). TEM micrographs showed that internalization was achieved with or without modification and transfection with GFP followed by fluorescence microscopy showed equal efficiency at higher viability using GSH formulation.

Acknowledgement

This work was supported by Slovenian Research Agency within projects J2-6758, J3-6794 (young researchers program) and MRIC UL IP-0510 Infrastructure program.

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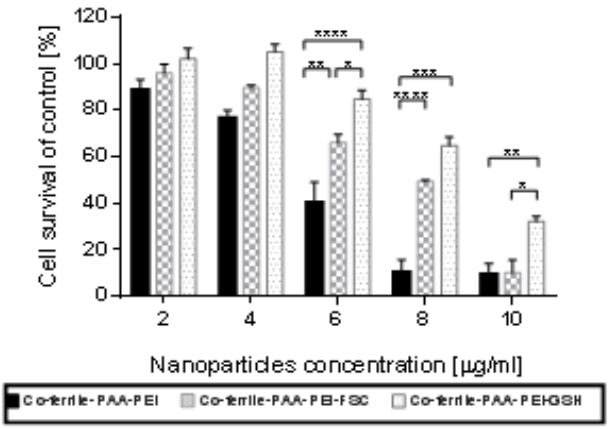

Figure 1: Propidium iodide viability assay on CHO cells after 24h of exposure to Cobalt ferrite-PAA-PEI, Cobalt ferrite-PAA-PEI-GSH and Cobalt ferrite-PAA-PEI-FSC NPs. Mean with SEM (n=3) is shown. *p<0.05, **p<0.001, ***p<0.0001, ****p<0.00001

http://dx.doi.org/10.4172/2324-8777.S3-001

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Journal of Nanomaterials & Molecular Nanotechnology

Calcium Phosphate Scaffolds Combined with Autologous Biomaterials in the Treatment of Various Bone Defects: A Concept for Bone Tissue Engineering in Vivo

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Interest in applications for bone tissue engineering continues to increase as clinically relevant methods alternative to traditional treatments of bone defects.Recent progress in the studies of molecular basis of bone development and regeneration, adult stem cell biology,will provide fundamental knowledge for that. Autologous biomaterials enriched with progenitor/stem cells and growthfactors can be produced from components of bone marrow,peripheralblood,adiposetissue,cancellous bone, and represent a very interesting research field for bone regeneration and suppose a good perspective of future in the clinical practice [1].The adjunctive clinical benefit of the autologous biomaterials preparation can be explained on the basis of tissue engineering, i.e.,tissue engineering generally combines three key elements for regeneration:1) scaffolds or matrices, 2) signaling molecules or growth factors,and 3) cells. Stem cells need a scaffolds that facility their integration, differentiation, matrix synthesis and promote multiple specific interactions between cells.Calcium phosphate scaffolds aresynthetic / artificially designed substitutes which have numerous interconnecting pathways similar to cancellous bone and facilitates bone formation by providing an exceptional osteoconductive scaffolding which results from the retention of the natural porous architecture and trabeculation of human cancellous bone. Calcium phosphate scaffolds showresorbable characters during bone regeneration, and can be completely substituted for the bone tissue after stimulation of bone formation[2].The use of autologous biomaterials combined with calcium phosphate scaffolds is a recent and promising innovation in bone regeneration [3].Bone tissue engineering could not have advanced to the current stage without the incorporation of interdisciplinary skill sets of stem cell biology,bioengineering,polymerchemistry,mechanicaleng ineering,robotics, etc.Our experience with autologous biomaterials combined with calcium phosphate scaffoldsin the treatment of various bone defects is presented. The techniques are based on stimulation of natural events continuously present in living bone, that is,the process of bone remodeling and offering both osteoinduction and osteoconductive features.

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http://dx.doi.org/10.4172/2324-8777.S3-001

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Journal of Nanomaterials & Molecular Nanotechnology

Quantification of Nanowire Uptake by Living Cells

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Abstract

Nanostructures fabricated by different methods have become increasingly important for various applications at the cellular level. In order to understand how these nanostructures "behave" and for studying their internalization kinetics, several attempts have been made at tagging and investigating their interaction with living cells. In this study, magnetic iron nanowires with an iron oxide layer have been coated with (3-Aminopropyl)triethoxysilane (APTES), and subsequently labeled with a fluorogenic pH-dependent dye pHrodo™ Red, covalently bound to the aminosilane surface. Time-lapse live imaging of human colon carcinoma HCT 116 cells interacting with the labeled iron nanowires has been performed for 24 hours. As the pHrodo™ Red conjugated nanowires are non-fluorescent outside the cells but fluoresce brightly inside, internalized nanowires could be distinguished from non-internalized ones and their behavior inside the cells could be tracked for the respective time length. A machine learning-based computational framework dedicated to automatic analysis of live cell imaging data, Cell Cognition, has been adapted and used to classify cells with internalized and non-internalized nanowires and subsequently determine the uptake percentage by cells at different time points. An uptake of 85 % by HCT 116 cells has been observed after 24 hours incubation at NW-to-cell ratios of 200. While the approach of using pHrodo™ Red for internalization studies is not novel in the literature,this study reports for the first time the utilization of a machine-learning based time-resolved automatic analysis pipeline for quantification of nanowire uptake by cells.This pipeline has also been used for comparison studies with nickel nanowires coated with APTES and labeled with pHrodo™ Red, and another cell line derived from the cervix carcinoma, HeLa. It has thus the potential to be used for studying the interaction of different types of nanostructures with potentially any live cell types.

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Journal of Nanomaterials & Molecular Nanotechnology

Microfluidic Structures on Flexible Foils Produced by R2R Imprinting

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The importance of sensors and miniaturized analytical systems to determine chemical and biochemical parameters increases steadily. Such so called labs-on-chip or micro-total-analysis-systems(μTAS)find applications in diagnostics, patient monitoring, biotechnology, and environmental monitoring. Healthcare industry is currently moving toward personalized medicine. More than ever, rapid, accurate tests are needed to increase pharmaceutical research yield and offer single us diagnostics with additional and more complex functionalities. By offering innovative solutions, microfluidic technologies have partially successfully filled this gap.

For high volume applications like diagnostics and life science consumables, requirements such as simple operation and low-cost production become crucial factors. A total adoption of the technology by industry is massively dependent on the implementation of new, cost-efficient, high-throughput production technologies. An important step towards the low-cost fabrication of monolithically integrated bioanalytical systems can be expected from the fast progress in roll-to-roll processing technology. Micro- and nanofabrication technologies such as nanoimprint lithography (NIL) are suitable for the production of microfluidic and microoptic structures in flexible polymer foils. In combination with printing of functional materials this opens new opportunities for the integration of e.g. microfluidic structures and sensors on flexible substrates. The current trends to miniaturize, automatize and parallelize assays while simultaneously increasing resolution and accuracy are directly addressed by such approaches.

JOANNEUM RESEARCH operates a roll-to-roll pilot line capable of continuous coating and imprinting processes on flexible foils. Industrial production processes combined with optimized resin chemistry are developed with the focus on the production of microfluidic patterns for biological and medical diagnostics.

Acknowledgement

Financial support of the Austrian Research Promotion Agency (FFG) in the framework of the Research Studio Austria "Modular Microfluidic Systems on Foil" and funding received from the European Union's Horizon 2020 research and innovation programme under grant agreement No 646260"R2R Biofluidics" are gratefully acknowledged.

http://dx.doi.org/10.4172/2324-8777.S3-001

SciTechnol
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Journal of Nanomaterials & Molecular Nanotechnology

Brain-Targeted Piperine-Loaded Chitosan Nanoparticles: A Novel Era in Alzheimer's Therapy

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Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder with no curative therapy. The treatment of central nervous system disorders has been hindered by the presence of the blood-brain barrier (BBB). Consequently, mucoadhesive – nanoformulated -intranasally delivered drugs could be used to bypass such barrier. Piperine (PIP)is a phytopharmaceutical with a reported neuroprotective potential. Its brain delivery is challenging as it suffers from hydrophobicity and first pass metabolism[1,2]. In this context, chitosan nanoparticles(CsNPs), the mucoadhesive polymer known for its biocompatibility, biodegradability, safety and its tight junction opening potentials, has been proposed as a drug delivery system for PIP.

Aims

The objectives of this study are to evaluate the brain targeting efficacy and potential neurotoxicity of the intranasal Cs NPs for PIP delivery in sporadic dementia of Alzheimer type (SDAT) animal model.

Materials and Methods

Twenty one Cs formulations were screened based on particle size (PS), zetapotential (ZP), polydispersity index (PDI), % entrapment efficiency (EE), solubility and release studies, and transmission electron microscopy. SDAT was induced in 48 male Wistar rats on which behavioral (Morris water maze test) and biochemical (brain oxidative stress parameters and acetylcholine esterase activity) testing was conducted. Moreover, brain caspase-3 activity and TNF-α levels were assayed to check the potential neurotoxic effect of the studied formulations.

Results

Optimized CS-NPs exhibited spherical morphology and a sustained invitrorelease pattern for PIP. In contrast to plain drug,PIP loaded CS-NPs could significantly improve cognitive functions as efficient as the standard drug(donepezil) with additional privileges of dual mechanism(acetylcholine esterase inhibition and antioxidant effect), non-invasive route and lower dose. Additionally, the studied formulations showed no neurotoxic (apoptotic and inflammatory) effect compared to the positive control group. In contrast, PIP-loaded Cs NPs (0.25 mg/kg/day) showed a significant decrease in caspase 3 activity and TNF-α levels compared to the positive control group suggesting the possible anti-apoptotic and anti-inflammatory activity of PIP.

Conclusion

The primary results show that intranasal chitosan nanoformulation is a safe, highly promising delivery system for PIP delivery to the brain and will be further investigated to optimize its therapeutic potential.

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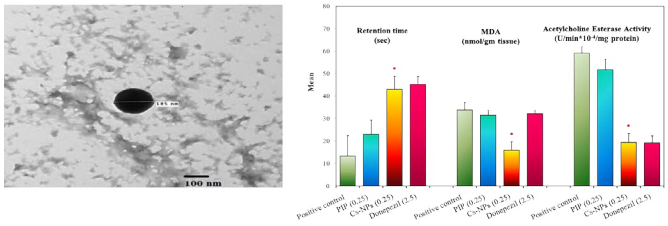

Figure 1: Left:TEM analysis of PIP-loaded Cs NPs (10000x magnification) which revealed spherical particle morphology and denied any aggregation. Right: In vivobioactivity.The treatment with PIP-loaded Cs NPs (0.25 mg/kg/day) led to pronounced improvement in the retention time, oxidative stress (MDA) and acetylcholine esterase activity as compared to the positive control and free PIP (0.25 mg/kg/day) groups

http://dx.doi.org/10.4172/2324-8777.S3-001

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Journal of Nanomaterials & Molecular Nanotechnology

Differential Killing Effect of a HyperbranchedPolyglycerol Nanoparticle Drug Formulation is Associated with Different Uptake Mechanisms Utilizedby Cancer and Normal Cells

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We have synthesized a nanoparticle (NP) based on hyperbranchedpolyglycerol (HPG) for systemic delivery of anticancer drugs. Thisunimolecular NP contains a hydrophobic pocket made up of C_{10} alkyl chains and a hydrophilic HPG shell.This NP was capable of holding a hydrophobic drug docetaxel (DTX) and releasing it in a sustained manner.The NP formulation of DTX was found to skew the sensitivities of normal and cancer cells to the drug, killing more of the cancer cells while sparing the normal cells.We hypothesized that the differential killing effect is attributed to different uptake rate and/or mechanisms of the HPG NP by these cells.To test this hypothesis, HPG NP was labeled with Alexa 488 and the rate of uptake of the NP in normal and cancer cells was assessed by flow cytometry.We found that cancer cells take up the HPG NP faster than the non-transformed cells. Using various specific inhibitors of endocytic pathways, we found that non-transformed cells took up HPG NP only throughmacropinocytosis while the cancer cells utilizebothclathrin-mediatedendocytosis andmacropinocytosis to take up HPG NP.These observations are consistent with our hypothesis and suggest that HPG NP can be designed to achieve anticancer drug delivery with cellular specificity.

Human Derived Biomaterials and Cells for Tissue Regeneration

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Plasma derived fibrin matrix is one of the most versatile biomaterials for tissue engineering and regenerative medicine.Being involved in a > 30 year development we can demonstrate advantages and limitations, application techniques and its special use for growth factor and cell delivery as well as a gene activated matrix.

Another goal in our group is to use „medical garbage “for regenerative purposes.Therefore we use placenta derived materials (PD) – substances (e.g. collagen) and PD structures (e.g. amnion decellularized vasculature) as well as PD cells (e.g. amnion MSC). “Living” Amnion is used either directly (cryopreserved) using a clinically approved process, e.g. for wound healing and antifibrosis or in a new process that the stem cells residing on and in amnion (“sessile” cells) are differentiated in toto (osteo, chondrogenic direction). In addition we use cells from liposuction and from umbilical cord.

Isolated stem cells are cultured with platelet derived factors (from outdated platelets) to avoid animal products and used directly or pre-differentiated, in autologous or allogeneic fashion.

This talk should give an overview about the use of above mentioned procedures within the Austrian Cluster for Tissue Regeneration.