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Technical note

Tumor tissue slice cultures as a platform for analyzing tissue-penetration and biological activities of nanoparticles

Lea Merz [a,2], Sabrina Höbel [b,2], Sonja Kallendrusch [a], Alexander Ewe [b], Ingo Bechmann [a], Heike Franke [c], Felicitas Merz [a,1], Achim Aigner [b,*]

[a] Institute of Anatomy, Medical Faculty, University of Leipzig, Germany
[b] Rudolf-Boehm-Institute for Pharmacology and Toxicology, Clinical Pharmacology, Medical Faculty, University of Leipzig, Germany
[c] Rudolf-Boehm-Institute for Pharmacology and Toxicology, Medical Faculty, University of Leipzig, Germany

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ABSTRACT

The success of therapeutic nanoparticles depends, among others, on their ability to penetrate a tissue for actually reaching the target cells, and their efficient cellular uptake in the context of intact tissue and stroma. Various nanoparticle modifications have been implemented for altering physicochemical and biological properties. Their analysis, however, so far mainly relies on cell culture experiments which only poorly reflect the *in vivo* situation, or is based on *in vivo* experiments that are often complicated by whole-body pharmacokinetics and are rather tedious especially when analyzing larger nanoparticle sets. For the more precise analysis of nanoparticle properties at their desired site of action, efficient *ex vivo* systems closely mimicking *in vivo* tissue properties are needed.

In this paper, we describe the setup of organotypic tumor tissue slice cultures for the analysis of tissue-penetrating properties and biological activities of nanoparticles. As a model system, we employ 350 µm thick slice cultures from different tumor xenograft tissues, and analyze modified or non-modified polyethylenimine (PEI) complexes as well as their lipopolyplex derivatives for siRNA delivery.

The described conditions for tissue slice preparation and culture ensure excellent tissue preservation for at least 14 days, thus allowing for prolonged experimentation and analysis. When using fluorescently labeled siRNA for complex visualization, fluorescence microscopy of cryo-sectioned tissue slices reveals different degrees of nanoparticle tissue penetration, dependent on their surface charge. More importantly, the determination of siRNA-mediated knockdown efficacies of an endogenous target gene, the oncogenic survival factor Survivin, reveals the possibility to accurately assess biological nanoparticle activities *in situ*, i.e. in living cells in their original environment.

Taken together, we establish tumor (xenograft) tissue slices for the accurate and facile *ex vivo* assessment of important biological nanoparticle properties. Beyond the quantitative analysis of nanoparticle tissue-penetration, the excellent tissue preservation and cell viability also allows for the evaluation of biological activities.

1. Introduction

Nanoparticles are extensively explored for the efficient delivery of therapeutic candidate molecules like nucleic acids. Their success strongly depends on their potential to overcome biological barriers, to mediate tissue penetration for actually reaching their target cells and to facilitate cellular internalization (see e.g. [1] for review). While *in vitro* monolayer cell culture experiments monitor these requirements only poorly, animal models are barely suitable as a screening platform due to high cost, complicated whole-body pharmacokinetics, species differences and the evolving goal of reducing animal experiments (3R concept).

Among nanoparticles for nucleic acid delivery, polyethylenimine-based complexes have been well established for *in vitro* and *in vivo* applications [2]. For altering physicochemical and, concomitantly, biological properties, various modifications including PEGylation [3] or liposome-coating [4] have been intro-

Abbreviations: dH_2O, distilled water; PEG, polyethylene glycol; PEI, polyethylenimine; RNAi, RNA interference; siRNA, small interfering RNA.

* Corresponding author at: Rudolf-Boehm-Institute for Pharmacology and Toxicology, Clinical Pharmacology, University of Leipzig, Haertelstrasse 16 – 18, D – 04107 Leipzig, Germany.
E-mail address: achim.aigner@medizin.uni-leipzig.de (A. Aigner).
[1] Present address: GSI Helmholtz Center for Heavy Ion Research, Department of Biophysics, Darmstadt.
[2] These authors contributed equally to the work.

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duced, with the aim of shielding the otherwise positive surface charge in order to improve efficacy, blood circulation half-life, uptake specificity and/or biocompatibility. In many cases, however, *in vivo* results considerably differ from cell culture data [5], thus emphasizing the need for powerful *ex vivo* test systems for establishing structure-function relationships.

Organotypic slice cultures derived from rodent brain are widely used in neuroscience due to their straightforward access for pharmacological intervention. This approach has recently also been extended towards tumor-derived slice cultures. Due to the possibility to study tumor cells in their intact environment including extracellular matrix and to directly apply test substances, they provide a unique tool to determine tissue responses to various stimuli [6,7]. In sharp contrast, only few studies have employed nanoparticles in tissue slice cultures so far. PLGA nanoparticles were used for the specific MMP-9 inhibition through MMP-1 delivery in hippocampal slice cultures [8] or for antisense inhibition of telomerase in human lung cancer tissue slices [9].

As analysis platform for the systematic assessment of nanoparticles and nanoparticle modifications with regard to their biological efficacies and other properties like tissue penetration, tissue slices have not been explored.

In this proof-of-principle study, we establish slice cultures from different tumor xenograft tissues as a model system for nanoparticle analysis. As representative examples, we analyze biological properties, namely tissue penetration and siRNA-mediated knockdown efficacy, of PEI-based complexes.

2. Materials and methods

2.1. Materials

The tumor cell lines PC3 (prostate carcinoma) and U87 (glioblastoma) were obtained from the American type culture collection (ATCC; Manassas, VA) and cultivated under standard conditions (37 °C, 5% CO_2) in Iscove's Modified Dulbecco's Medium (IMDM; Sigma, Taufkirchen, Germany), supplemented with 10% fetal calf serum (FCS; Life Technologies, Darmstadt, Germany). The identities of the cell lines were authenticated by DNA (STR) profiling. The branched PEI F25-LMW (4–12 kDa) was prepared as previously described [10]. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was purchased from Avanti Polar Lipids (Alabaster, AL). PEG-PEI was synthesized by PEGylation of PEI F25-LMW using $MS(PEG)_{24}$ as described previously [11]. Alexa647-labeled siRNA was purchased from Qiagen (Hilden, Germany), unmodified siRNAs were from Biospring (Frankfurt, Germany) or Thermo Fisher (Waltham, MA) with sequences as detailed in the Suppl. Information.

2.2. Tumor cell xenografting, tissue slice preparation and cultivation

For the generation of subcutaneous tumor xenografts, 5×10^6 PC3 or U87 cells in 150 µl PBS were injected into both flanks of 6–8 weeks old athymic nude mice (Crl:NU-Foxn1nu, Charles River Laboratories, Sulzfeld, Germany). Animal studies were conducted according to the national regulations of animal welfare and approved by the local authorities (Landesdirektion Sachsen, Germany). When the xenografts reached ~8 × 8 mm (length × width) in size, mice were sacrificed, tumors were excised and directly subjected to sectioning as described previously for head and neck squamous cell carcinoma samples [6], with minor modifications. In brief, tumor tissue was cut into cubes of ~5 × 5 × 5 mm side length using an autoclaved standard razor blade. The cubes were placed on a pile of sterile filter membranes soaked with the preparation medium. To facilitate the cutting procedure and to obtain appropriate slices, each cube was fixated by Histoacryl glue (Braun, Melsungen, Germany). Slices of 350 µm thickness were prepared by a tissue chopper (McIlwain TC752, Campden Instruments, Loughborough, UK, or Saur, Reutlingen, Germany). After cutting, the slices were placed in a container with preparation medium composed of MEM (Gibco Thermo Fisher) + 1% penicillin/streptomycin. The adherent glue was removed and slices sticking together were carefully separated by using two scalpels under a stereomicroscope. Slices were placed on membrane culture inserts using the wide opening of a glass pipette with the fine tip broken off, and residual preparation medium was removed with a normal pipette. Slices were placed in groups of three per membrane and cultivated in 6-well plates, each filled with 1 ml cultivation medium. The cultivation medium was composed of MEM, 25% Hank's Balanced Salt Solution (with Ca and Mg; Gibco), 10% Normal horse serum (Gibco), 1% penicillin/streptomycin, 1% L-glutamine, and 0.45% glucose. For cultivation, plates were kept in a humidified incubator at 37 °C and 5% CO_2. The cultivation medium was replaced 24 h after preparation and then every other day.

2.3. Complex preparation and analysis

PEI/siRNA and PEG-PEI/siRNA complexes were prepared by mixing 15 µg siRNA with 112.5 µg PEI F25-LMW or 75 µg PEG-PEI, each dissolved in 37.5 µl 10 mM HEPES/150 mM NaCl, pH 7.4 and incubated for 10 min. After mixing and vortexing, the complexation was allowed to proceed for 30 min at room temperature. Lipopolyplexes were generated as described previously [4]. Briefly, DPPC liposomes were prepared by hydration of a dried lipid film in an ultrasound bath sonicator and extrusion, and then mixed with preformed PEI/siRNA complexes at a lipid/PEI mass ratio = 1.7. After vigorous pipetting, the mixture was vortexed and incubated for 60 min at room temperature prior to use.

Zeta potentials and particle sizes were determined by phase analysis light scattering (PALS) and photon correlation spectroscopy (PCS) with a Brookhaven ZetaPALS system (Brookhaven Instruments, Holtsville, USA), upon diluting the complexes to 1.5 ml in pure water. The manufacturer's software was used for data analysis, with applying a viscosity and refractive index of pure water at 25 °C. For size measurement, the complexes were analyzed in five runs with a run duration of 1 min, and results are expressed as intensity weighted mean diameter. Zeta potentials were determined in ten runs, with each run containing ten cycles, and applying the Smoluchowski model. Bars represent mean ±SD of the individual readings.

2.4. Tissue slice treatment, tissue slice analysis and determination of tissue penetration

Slices were cultivated in the incubator as described above for 24 h prior to treatment with PEI-based complexes. 75 µl of the complexes, i.e. approximately 3 drops per slice, were applied using a 100 µl-pipette. The slices were then further cultivated for 24 h, unless indicated otherwise.

To obtain a time series for the evaluation of histology, the slices were fixed for 24 h in 4% paraformaldehyde prior to embedding in paraffin. 7 µm sections were dewaxed and rehydrated in decreasing alcohol series, stained with hematoxylin/eosin (H&E) and visualized with a Zeiss Axioplan 2 microscope using 20× and 40× objectives to examine the morphology of the cultivated tissue. For immunohistochemistry, the deparaffinized sections were washed 3 times for 10 min in phosphate buffered saline (PBS), treated with 1.5% Triton X-100/PBS for 10 min for permeabilization and blocked with 10% normal goat serum in 1.5% Triton X-100/PBS for at least 1 h. Primary antibodies against the proliferation marker Ki67 (rabbit, 1:100; DCS, Hamburg, Germany) or cleaved caspase-3 (rabbit, 1:400; Cell