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DIPLOMARBEIT

Titel der Diplomarbeit

„A novel drug delivery system based on lectin-mediated binding of PLGA nanoparticles to urinary bladder cells“

Verfasserin

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Inhalt

ABSTRACT

The insufficient delivery of drugs in the therapy of various urinary bladder diseases, most prominently cancer, is considered a primary cause for shortcomings in the contemporary treatment schedules. This work offers a novel strategy of drug delivery to bladder cancer, based on biorecognition with two lectins (PNA & WGA) in order to gain a stronger cell adhesion and more selective targeting of malignant tissue.

Biocompatible PLGA micro- and nanoparticles were surface-modified with PNA and WGA, and characterized with regard to their binding capacity on three human urothelial cell lines and donor cells, corresponding to healthy tissue and low grade or high grade carcinoma, respectively.

Flow cytometry was used to determine binding capacity and specificity on single cells and fluorescence microscopy was used to investigate particle binding on cell monolayers. Basic experiments featured a co-culture of SV-HUC and 5637 cells, a time lapse study of binding and an investigation on the influence of Pluronic-F68® on the binding capacity. Investigations for WGA binding capacity featured N, N`, N``-triacetylchitotriose, to proof the benefit obtained by a surface modification with WGA, whereas neuraminidase was used to investigate the causative principle for increased PNA-binding to different tumor stages.

The surface modification with WGA significantly increased particle binding rates compared to HSA- and non-surface modified particles to all cell lines and binding maxima were reached within 30min. Considering the intended form of application as an instillation, the short time required to reach maximum binding could be of great benefit, and furthermore the PLGA particles may probably be washed out to a lesser extent after the termination of the instillation, which would increase residence time and drug exposure.

This suggests promising potential for a use in intravesical drug delivery systems, and provides novel perspectives for the regionalized therapy of diseases of the human urinary bladder.

EINLEITUNG UND ZIELSETZUNG

Blasenkrebs steht nach Colon-, Lungen und Prostatakrebs an vierter Stelle der häufigsten Krebserkrankungen in der westlichen Welt. Meist ist nur das Urothel betroffen und der Tumor kann durch einen chirurgischen Eingriff entfernt werden. Eine anschließende adjuvante Chemotherapie minimiert das Risiko eines weiteren Tumors.

Der Arzneistoff wird dazu in flüssiger Form als Instillation verabreicht. Hierbei wird ein Katheter in die Blase eingeführt, durch den man eine Lösung, Emulsion oder Suspension der Zytostatika direkt an den beabsichtigten Wirkort bringt. Instillationen mit lokal wirksamen Substanzen werden ebenso bei anderen Erkrankungen der Harnblase, wie beispielsweise interstitieller Zystitis, angewandt. Diese Art der Therapie versucht den Vorteil einer hohen lokalen Wirkstoffkonzentration zu nutzen und minimiert gleichzeitig die Wahrscheinlichkeit für das Auftreten von systemischen Nebenwirkungen.

Infolge der natürlichen Barrierefunktion des Urothels stellt die geringe Resorption des Arzneistoffes die größte Herausforderung für eine erfolgreiche instillative Behandlung dar. Außerdem wird der überwiegende Anteil des eingebrachten Medikamentes bei der Entleerung der Harnblase ausgespült, wodurch die effektive Diffusionsdauer nur ein bis zwei Stunden beträgt. Aufgrund der schlechten Resorption und der kurzen Verweildauer sollte also bald darauf die nächste Dosis verabreicht werden. Eine Instillation wird vom Patienten jedoch zumeist als unangenehm bis schmerzhaft empfunden und sollte daher möglichst weniger Wiederholungen bedürfen.

Eine Verarbeitung des Arzneistoffes in Nanopartikeln aus biodegradierbaren Materialien könnte hierbei eine Verbesserung bieten, wobei die Arzneistoffe auch vor den widrigen Bedingungen im Harn geschützt wären. Durch Modifikation der Partikeloberfläche mit einem Lektin erhält man zudem eine Art „Adapter“, welcher es den Partikeln erlaubt, an der Glykokalyx der Zielzellen haften zu bleiben. Falls durch bestimmte Krankheiten die strukturelle Zusammensetzung der Glykokalyx verändert würde, wäre zusätzlich eine selektive Adhäsion an erkrankte Areale (Targeting Effekt) möglich. Darüber hinaus könnten derart modifizierte Nanopartikel aufgrund ihrer geringen Größe auch von den Urothelzellen aufgenommen werden und somit ein Wirkstoffreservoir bilden. Außerdem würde der Arzneistoff bei der Drainage so auch nicht ausgeschwemmt. In weiterer Folge sollte sich dadurch der Effekt der Behandlung steigern lassen und die Zahl der pro Patient

notwendigen Instillationen ließe sich senken. Dies wiederum erhöht den Komfort der Behandlung und führt zu einer gesteigerten Compliance.

Unter Einsatz gut charakterisierter Zellkulturmodelle sollte deshalb in der vorliegenden Diplomarbeit das Konzept für ein Delivery System entwickelt werden, welches intravesikal eingesetzten Arzneistoffen zukünftig die Möglichkeit bieten soll, ihre volle Wirkung zu entfalten.

INTRODUCTION

Bladder cancer is the fourth most common malignancy among men in the Western world, following prostate, lung and colon cancer, but the high recurrence rates makes it probably the most prevalent malignancy of these four, and certainly the most expensive when calculated per patient treated (1). Unfortunately, the National Cancer Institute funding for bladder cancer is quite low when compared to other common malignancies (2).

In most cases only the urothelial tissue is affected and the tumor is non-muscle-invasive, so the first line treatment is transurethral resection (TURBT). To minimize tumor recurrence rates, adjuvant instillation of a chemotherapeutic or immunomodulatory agent is performed immediately after surgery. These instillations include Bacillus Calmette-Guérin (BCG) as the only agent to also decrease progression rates, but with severe side effects in many cases. Chemotherapeutic drugs often have difficulties to withstand the rather harsh environment of the urine, and suffer from rapid inactivation. Oral application of sodium bicarbonate provides a possibility to alkalize the urine and therefore prevent this to a certain extent, though with higher additional effort in treatment preparation.

The biggest challenge, however, is the natural barrier presented by the urothelial tissue, so only a small portion of the instilled drug is actually absorbed. Penetration rates into the tissue are thus usually very low and considerable effort was made to increase bioavailability. Electromotive drug administration (EMDA) and local microwave induced hyperthermia are only two examples to increase bladder wall penetration, though with additional devices required for application. Treatment efficacy of intravesical therapy is also restricted by a very short residence time, which is typically limited to 120min at best due to patient compliance. As a consequence, several repetitions are necessary in order to have an impact on recurrence rates. However, instillations are not only uncomfortable but sometimes also painful for the patient and should therefore be optimized for an effective cancer therapy.

Nanoparticles may provide a new approach towards protecting the drug from the harsh environment and to create a bioadhesive delivery system. Among the potential carriers for targeted delivery, micro- and submicrometer particles, based on biocompatible and biodegradable polymers such as polylactide, polyglycolide, and poly (D,L-lactide-co-glycolide) (PLGA), play a major role (3).

PLGA is already successfully used in clinical practice for implants with controlled sustained release and targeted delivery systems (4). In addition, the surface of such particles could be modified with lectins in order to increase adherence to the glycocalyx of the urothelial cells. Lectins are relatively cheap as compared to antibodies and are very likely to durably sustain the environment of the urine due to their stable molecular structure. Moreover, their binding to the glycocalyx is highly specific. Therefore, they might serve as an adapter for the drug particle – cell interaction. Particles bound to urothelial cells via lectins would probably be washed away to a lesser extent, when the bladder is drained after instillation. Thus, exposure time might be significantly increased and due to their small size the particles could also be absorbed via endocytosis. Since many diseases such as cancer and inflammations cause characteristic changes in the structure of the glycocalyx, even a targeting effect may be achieved by a combination of appropriate lectins and surface-modifiable nanoparticles.

The herein presented investigations were carried out on three different cell lines representing a test system with fewer variable factors than studies in vivo. Moreover, cell lines can provide models for healthy and cancerous urothelial tissue, which makes them ideal ex vivo models for testing new technologies. Primary cells were obtained in cooperation with the Wilhelminen Spital, Vienna, to compare the results with a tissue of more “in vivo-like” character, featuring a variety of cells from different patients.

MATERIALS & METHODS

Materials:

Resomer RG503H (PLGA) was obtained from Boehringer Ingelheim (Ingelheim, Germany). 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4adiaza-s-indacene (BODIPY 493/503) was obtained from Molecular Probes (Invitrogen Corp., Carlsbad, CA, USA).
Wheat germ agglutinin (WGA) from Triticum vulgare and Peanut agglutinin from Arachis hypogea were acquired from Vector laboratories (Burlingham, USA). Human serum albumin (HSA), 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDAC), N-hydroxysuccinimide (NHS), Pluronic® F-68 and neuraminidase were bought from Sigma Aldrich (Vienna, Austria). N, N`,N``-triacetylchitotriose was obtained from Fluka (Buchs, Switzerland).
All other chemicals used for the experiments were of analytical purity.

Surface modification of PLGA microparticles with F-PNA and F-WGA:

The microparticles used for surface modification were produced by U. Länger during her work (3). Briefly, 100mg of microparticles were suspended in 10ml double distilled water with 0.1% Pluronic. Sufficient dispersion was achieved by stirring for several hours and gentle sonification under cooling. Centrifugal force was used with 1300G for 10min to gain a pellet, which was resuspended in 700µl 20mM Hepes/NaOH pH 7.0. For modification 37.5µl 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDAC) solution (80mg EDAC per 250µl 20mM Hepes/NaOH pH7.0) and 25µl N-hydroxysuccinimide (NHS) solution (2% solution in 20mM Hepes/NaOH pH 7.0) were added to the particle suspension.
The suspension was incubated end-over-end for 3 hours at room temperature and then washed twice with 700µl of 20mM Hepes/NaOH pH 7.4 by centrifugation. The sample was divided into two aliquots. Per aliquote 500µl containing 0.5nmol F-PNA and F-WGA, respectively, were added. End-over-end incubation time was set to 48 hours.

To maintain saturation of unreacted binding sites, the particle suspension was finally incubated with 200µl of a 5% glycine solution in water for 30min.
To remove unbound lectines and side products, 4 washing steps via centrifugation were carried out.

Nanoparticle preparation:

A solvent evaporation technique was used to obtain nanoparticle suspensions in aqueous buffer. All steps of the manufacturing process require protection from light to prevent photobleaching of the applied fluorophore. 400mg PLGA and 0.25mg BODIPY 493/503 were dissolved in 2g ethyl acetate by magnetic stirring at 4°C to avoid evaporation. 6g of a 10% aqueous Pluronic® F-68 solution were prepared separately and 100g of a 1% aqueous solution of Pluronic® F-68 solution were kept on a magnetic stirrer at 600rpm.
After complete dissolution, the 10% Pluronic® F-68 solution was poured into the PLGA/BODIPY/ethyl acetate solution. The two phases were emulsified by sonification for 50s under cooling to break down the droplet size to nanometer range and then poured into the 1% Pluronic® F-68 solution under constant stirring.
The emulsion was left under stirring for another hour to evaporate remaining ethyl acetate. Last traces of ethyl acetate were then removed under reduced pressure. Potential PLGA aggregates were removed by filtering the final suspension through a syringe filter of 1µm pore size.

Nanoparticle characterization:

The mean zeta potential, apparent size and PDI of the particles was investigated after preparation and surface modification to detect any alternation or instabilities.
To measure the mean particle size and PDI the nanoparticle suspensions were diluted 1:100 with double-distilled water and characterized via dynamic light scattering on a Zetasizer Nano ZS (Malvern Instruments Ltd., UK). The zeta potential was determined after 1:20 dilution with the respective buffer using disposable folded capillary cells. Both analyses were carried out at 25 °C.

Surface modification of the nanoparticles:

20ml nanoparticle suspension were washed with 40ml 20 mM Hepes buffer pH 7,4 containing 0,5% Pluronic® F-68 on the Vivaflow filtration system (MWC 100.000; Vivasience AG, Sartorius Group, Hannover, Germany). The 20ml aliquots were then transferred into 50ml Greiner tubes and each mixed with 240mg EDAC dissolved in 750µl and 10mg NHS dissolved in 500µl of the same buffer. The tubes were incubated end-over-end for 4 hours. Following this activation step of the free carboxyl groups at the particle surface the aliquots were washed twice with 40 ml of the same buffer to remove excess reagent.

For coupling 0.5nMol of lectin were used. For better handling the proteins were added as stock solutions containing 1mg protein per ml. So either 666µl WGA-, 2037µl PNA- or 1241µl human serum albumin (HSA)-stock solution were added to each tube of activated particles, each equivalent to 0.5nMol. To support the stability of the lectins, 200µl of a 100mM $MnCl_2$-, $CaCl_2$- and $MgCl_2$-stock solution was included in the reaction mix. The particles were incubated end-over-end for 12 hours. Potentially unreacted sites were then saturated with 300mg glycine dissolved in 3ml PBS.

After a further hour of end-over-end incubation, the suspensions were ready for the final purification. In dependence of the molecular weight of the protein coupled, two different methods were applied, either based on centrifugal force or the Vivaflow system as mentioned above.

Centrifugation was used to wash the particles modified with HSA. In this case the nanoparticle suspension was transferred into eppendorf vials containing 400µl of a glycerine/ Hepes/NaOH buffer mixture (7+3) at the bottom to support particle retrieval and to prevent aggregation. After 15 minutes of centrifugation at 14.000rpm the upper 1500µl were discarded and the pellet obtained was resuspended with 750µl of Hepes pH 7,4. An advantage of this technique is the possibility of concentrating the particle suspension if needed. The particles modified with lectins were washed twice with the Vivaflow system. After the final purification all particles were stored at -80°C in aliquots of 500µl.

Cell culture:

The cell lines SV-HUC-1, HT 1376 and 5637 were obtained from the American Type Culture Collection (Rockville, MD, USA). Tissue culture reagents were obtained from Sigma (St. Louis, USA) and Gibco Life Technologies Ltd. (Invitrogen Corp., Carlsbad, CA, USA). All cell lines were cultivated in sterile, humidified 5% CO_2/95% air atmosphere at 37°C in 75cm² tissue culture flasks from Greiner.

SV-HUC-1 cells were cultivated in Ham`s F 12 medium with 1% Penicillin/Streptomycin which contained 500ml Ham`s F12 from Gibco, 50ml fetal calf serum (FCS) and 0.073 g L-Glutamine. This cell line was propagated approximately once a week. For experiments cells between passage 34 and 60 were used.

The cell line 5637 was cultivated in a medium containing 1000ml RPMI, 100ml fetal calf serum (FCS), 20ml L-Glutamine (8.5mg/ml) and 2ml Gentamycin (75µMol/ml). Due to their vivid growth the cells were passaged every three days. Passages used for experiments included cells between passage 31 and 94.

HT-1376 cell cultivation was performed in a medium containing 500ml DMEM from Gibco, 90ml fetal calf serum (FCS), 0.17g L-Glutamine and 1% Penicillin/Streptomycin. Experiments were carried out with cells from passage 13 to 25.

5637 and SV-HUC-1 (1:10) co- culture:

Single cells of different malignant origin (5637 and SV-HUC-1) were mixed to generate a co-culture tissue model, featuring both cancerous and healthy areas. The varying growth rate required a tenfold excess of the healthy SV-HUC-1 to obtain an equally balanced tissue.

Cultivation was carried out on glass slides (10mm diameter) in 24 well plates with 50 000 cells per well for seeding. Preliminary experiments proved RPMI to be a suitable medium for both cell lines. After 10 days of cultivation the cells were almost confluent and thus stained with F-PNA and propidium iodide at 4°C, where cells are in a metabolically quiescent state and internalization is reduced to a minimum. Each well was incubated with 66pmol F-PNA for 30min, and then washed twice with PBS Ca^{2+}/Mg^{2+}. Cells were fixed with 2% paraformaldehyde solution for 15min and subsequent incubation with 50mM NH_4Cl solution to block unreacted sites.

In order to permeabilize the cell membrane Triton X-100 in PBS was applied at a concentration of 0.1% for 15min. Cell nuclei were then stained with a 0.01% solution of propidium iodide in PBS by incubation for 30min.

Primary cells:

Primary cells were obtained from the Department of Urology and the Department of Pathology and Microbiology of the Wilhelminenspital Vienna at informed patient consent. Immediately after surgical extraction, the tissue samples were transferred in a tube with a special sterile transport medium (15ml). This transport medium contained an antibiotic, 10mM Hepes, 2.35 mg aprotinin and 500ml HBSS with calcium and magnesium. Due to the instability of gentamycin in solution, the antibiotic was added to the solution immediately before delivering the tubes to the hospital. The gentamycin concentration varied from 0.2 to 0.1%. All solutions were stored for a maximum of 14 days. The protocol used to cultivate the primary cells was based on the publication "Culture of Human Urothelium" by J. Southgate et al. (5).

Tissue samples were transferred to the Department of Pharmaceutical Technology and Biopharmaceutics at 4°C and processed as soon as possible.

For separating the urothelial cells from the stroma, the samples were treated according to their size with 5-10ml stripping solution. This stripping solution contained 10mM Hepes, 2.35 mg aprotinin, 500ml HBSS with calcium and magnesium and 50ml 1% EDTA solution.

After incubation over night at 4°C, the vials were warmed to 37°C for 20 minutes and carefully shaken by hand. The physical force was necessarily required to quantitatively separate the urothelium from the underlying tissue, which was then disposed. The hereby obtained cells were then isolated from the stripping solution by centrifugation for 5 minutes at 1000rpm and, depending on the size of the cell pellet, resuspended in 1-2 ml of collagenase solution.

The collagenase solution contained 11.47mg collagenase IV, 10mM Hepes and 60ml HBSS with calcium and magnesium. After sterile filtration, the collagenase solution was stored at -20°C in aliquots of 2ml. The primary cells were incubated with the collagenase for 20 minutes at 37°C in order to digest the urothelial sheet and obtain a single cell suspension. Then, 3ml cKSFM (complete keratinocyte serum free medium) were added

and eventually remaining tissue sheets were disaggregated with a glass pipette. The cells were then collected by centrifugation as before.

cKSFM was bought from Gibco and also used for cultivation. Apart from the standard supplements such as bovine pituitary extract (BPE) and human growth factor (EGF) it contained 500µl of cholera toxin solution per 500ml medium in order to support cell binding to the growth support.

Cholera toxin solution was prepared from a stock solution containing 1mg cholera toxin per ml sterile water for cell culture purpose. 150µl of this stock solution were mixed with 5ml cKSFM to prepare the final cholera toxin solution.

Different concentrations of the antibiotics gentamycin, penicillin and streptomycin were used in course of this work to achieve a compromise between optimized growth and as little risk of infections as possible.

To subcultivate the cells, a solution of 0.1% EDTA in PBS was used to support detachment before trypsination. In order to prevent excessive loss of cells by this treatment, it was always performed under microscopic control. To subcultivate the cells, 1ml of trypsin was used per 25cm² tissue culture flask. Within approximately two minutes the cells could be removed from the surface of the flask, but it was always decided individually according to visual observation when to add the trypsin inhibitor with 5ml medium to terminate the reaction.

For the preparation of the trypsin inhibitor 250 mg of soybean trypsin inhibitor were dissolved in 5ml PBS + Ca^{2+}/Mg^{2+}. Aliquots of 50µl each were stored at -20°C to give a sufficient concentration for the inhibition of 1ml of trypsin.

The primary cells were cultivated in gelatin coated 25cm² and 75cm² tissue culture flasks from Greiner. During this work cells of the samples 57 to 95 were cultivated and four of them (sample “81”, “83”, “89” and “92”) were used for experiments.

Interaction of surface modified nanoparticles with bladder single cells:

Flow cytometry was used to investigate the interaction between surface modified BODIPY nanoparticles and bladder single cells. Briefly, 100µl of cell suspension with a concentration of 400 000 cells per ml were mixed with 100µl nanoparticle suspension and incubated at 4°C for various periods of time.

Standardized dilutions in regard to relative fluorescence intensities of both, modified and plain particles were applied to guarantee comparability between the individual batches. These dilutions were freshly prepared prior to each experiment using 20mM isotonic Hepes/NaOH pH 7,4 with 1% Pluronic and characterized on a fluorescence microplate reader (e/e: 480/525 nm; Infinite 200, Tecan Group Ltd., Grödig, Austria).
After incubation the cells were washed twice with 20mM isotonic Hepes/NaOH pH 7.4 to remove unbound or loosely bound particles. Hereby, it was obligatory to keep the temperature at 4°C and ensure protection from light in order to prevent photobleaching. For flow cytometry every sample was resuspended in 1ml PBS and analyzed on an EPICS XL-MCL analytical flow cytometer (Coulter, Miami, USA) using a forward-versus-side scatter gate for inclusion of the single-cell population and exclusion of cell aggregates. Fluorescence was detected at 525 nm (10-nm bandwidth).

Binding specificity of WGA-BODIPY-PLGA nanoparticles:

Binding specificity of the carbohydrate–lectin interaction between surface modified WGA-BODIPY-PLGA nanoparticles (WGA-BOD-NP) and the glycocalyx of the cell lines SV-HUC-1 and 5637 was verified by a competitive inhibition assay using N,N`,N``-triacetylchitotriose. For the experiment 50µl cell suspension, 100µl WGA modified nanoparticle suspension and 50µl N,N`,N``-triacetylchitotriose solution (0.0625; 0.03125; 0.01563; 0.00781; 0.00391 and 0.00195 µmol/50µl) in PBS + Ca^{2+}/Mg^{2+} were mixed and incubated for 30min at 4 °C. After washing with cold PBS the mean cell associated fluorescence intensity was determined by flow cytometry.
The blank consisted of 50µl cell suspension and 150µl PBS + Ca^{2+}/Mg^{2+} to assess cellular autofluorescence. The positive control was a mixture of 50µl cell suspension, 100µl nanoparticle suspension and 50µl of PBS + Ca^{2+}/Mg^{2+} instead of the sugar solution representing the value for maximum binding without inhibition. Each concentration was analyzed in triplicate.

Treatment with Neuraminidase to investigate the increase of F-PNA binding on malignant bladder cell lines:

The aim of these experiments was to quantify the F-PNA-cell interaction in order to gain more information about the underlying principle of PNA selectivity. The ability of neuramininidase to cleave sialic acid groups in the glycocalyx and reveal underlying binding sites was assumed as a suitable model for these investigations. Thereby, the process of incomplete glycosylation should be mimicked, which is known to increasingly occur with malignant transformation in the human urothelium. The surface obtained should thus resemble a cancerous cell membrane with a higher quantity of binding sites for F-PNA, which might be determined via flow cytometry.

To yield a suitable working environment and stability for the enzyme according to the provider's instructions (Sigma Aldrich – Vienna) one unit lyophilized neuraminidase was dissolved in 1ml 0.2M sodium acetate buffer pH 5.5.

Briefly, 50µl cell suspension containing 300 000 cells were mixed with 50µl neuraminidase solution containing 0.05 or 0.1 units, respectively. The samples were incubated at 37°C for one hour to use the full potential of the enzyme.

After washing the cells three times with PBS to remove the neuraminidase, the cells were kept in 100µl PBS and the temperature was lowered to 4°C.

Subsequent incubation for 30 minutes with 100µl of F-PNA solution (containing 25pmol and 50pmol, respectively) was carried out at 4°C under protection from light to prevent photobleaching of the fluorophor.

After washing with PBS the mean cell associated fluorescence intensity was determined by flow cytometry.

RESULTS

5637 and SV-HUC (1:10) co- culture:

According to the results of the current diploma thesis, F-PNA can be used for selective tumor staining to mark cancerous cells. In this experiment two different cell lines were used: SV-HUC representing healthy cells and 5637 for cancerous cells.

The nature of these two cell lines is also reflected by the differences in the structure of their glycocalices, which can be used for differentiation. To provide a suitable co-culture tissue model featuring both, cancerous and healthy areas, SV-HUC and 5637 single cells were mixed. Varying rapidity of growth required a tenfold excess of SV-HUC cells at the point of seeding to obtain an equally balanced tissue after four to five days of culture. The resulting co-culture samples were first stained with F-PNA, which only binds to cancerous cells. To stain the cell nuclei, propidium iodide was used after fixation with 2% paraformaldehyde solution and permeabilisation using Triton X 100.

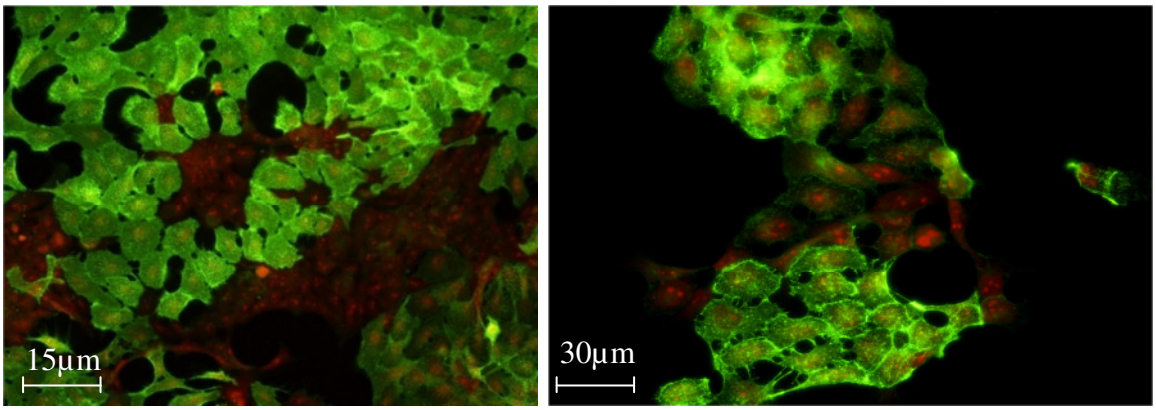

Figure 1.a: A co culture of urinary bladder cell lines (5637 and SV-HUC, representing cancerous and healthy tissue) showing characteristic staining with F-PNA (green) and probidium iodide (red). Due to differences in the structure of the glycocalyx F-PNA is only able to stain 5637 cells, whereas probidium iodide affects all cells. These pictures illustrate the potential of PNA as a selective targeter in cancer therapy or enhanced diagnostics.

As a consequence of this treatment some cells revealed more F-PNA binding on their surface than others, were only the propidium iodide staining was visible.

In figure 1.b F-PNA binding on a 5637 culture is demonstrated. As this was a homogenous culture its staining showed little variation, whereas in figure 1.c F-PNA staining of the co-culture was highly variable from region to region.

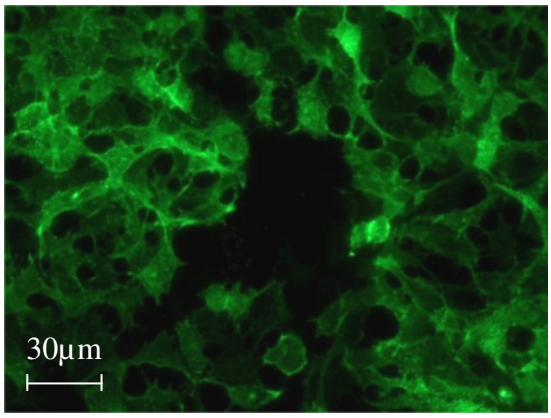

Figure 1.b: 5637 cells stained with F-PNA

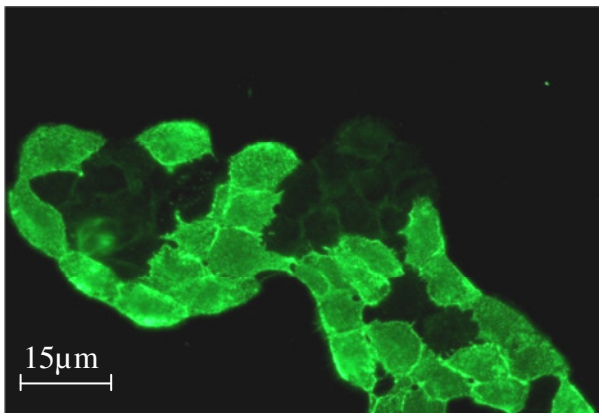

Figure 1.c: Co-culture stained with F-PNA

In figure 1b the effect of F-PNA on a 5637 culture is shown. As this was a homogenous culture of one cell line its staining showed little variation, whereas in figure 1c the co-culture was stained with F-PNA and therefore differently intense staining occurred.

The images demonstrate that F-PNA binding at the surface of 5637 cells was stronger than on the healthy cells. The SV-HUC areas present only moderate F-PNA staining. Thus mainly the cell nuclei are noticeable. In figure 1c the co-culture was incubated only with F-PNA in order to visualize the difference in staining behavior once again.

The results illustrate the potential of PNA as a selective targeter in cancer therapy or enhanced diagnostics.

F-PNA and F-WGA surface-modified microparticles:

Since F-PNA can be used for differentiation of healthy and cancerous cells it may also be a key element in drug targeting with the aim to bind particles via lectins to specific cells. Immobilized F-PNA at the surface of particles may thus create a shuttle system to deliver drugs precisely to their destination.

The surface modification of microparticles was a first approach to investigate the modification procedure in principle, prior to experiments with nanoparticles. The microparticles, which were used for modification, did not contain any fluorophore or color and therefore did not show any fluorescence. After the modification process with F-PNA and F-WGA, respectively, which included extensive washing as the final step, the particles were visibly stained with the respective fluorescence-labeled lectins (Fig. 2).

The images obtained via fluorescence microscopy thus proved successful modification with F-PNA and F-WGA.

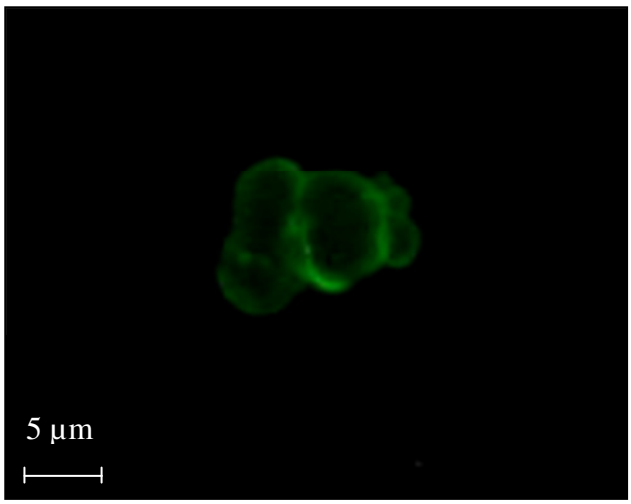

Figure 2.a: Modification with F-PNA

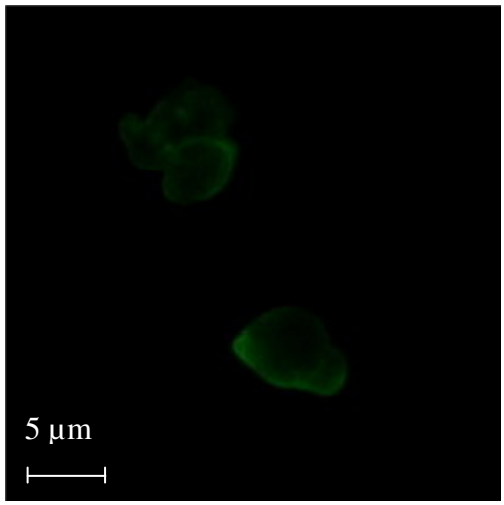

Figure 2.b: Modification with F-WGA

PLGA microparticles (focus set to the middle of the particles for both figures) showing significant fluorescent staining after surface modification with both lectins: F-PNA and F-WGA, respectively.

Interaction of surface modified nanoparticles with bladder cells: Influence of incubation time and Pluronic-F68® concentration:

To investigate the influence of the incubation time and Pluronic-F68® concentration a series of experiments was carried out. The necessity to clarify the consequences of basic conditions is crucial and requests to be examined before deeper research.

For surface modified nanoparticles the use of Pluronic-F68® was obligatory to prevent agglomeration during modification. The nanoparticles were also stored in a suspension containing Pluronic-F68® to rule out instabilities, so the omnipresence of Pluronic-F68® required an investigation of its influence on the particle binding parameters.

In order to assess time dependency of the binding of WGA-BOD-NP to SV-HUC and 5637 single cells, incubation with particles was performed for 15, 30, 60 and 120 min at 4 °C. Particle dilutions were used at a fluorescence intensity of 1000 and samples were analyzed in triplicates.

As illustrated in figure 3a, the rapid binding of surface-modified nanoparticles had reached its maximum within 30min for both cell lines. As compared to 15min, the fluorescence intensity increased by 54.2% with a standard deviation of ±1.8% after 30min in case of 5637 cells. For SV-HUC, an increase of only 22.8% with a standard deviation of ±6.3% was observable for the 30min incubation period, as compared to the first measurement. The effect reached after a prolonged incubation time is limited for both cell lines; the mean cell associated fluorescence intensity is even slightly reduced after a

maximum at 30min. Due to these findings the incubation time for all further experiments was set to 30min, in order to compare always maximal binding capacities under the various conditions.

As the use of Pluronic-F68® was obligatory to assure the stability of the nanoparticle suspension, it was crucial to investigate the influence of Pluronic-F68® concentration on lectin mediated binding. Different dilutions of Pluronic-F68® were used, while the same amount of particles was applied. The highest concentration of Pluronic-F68® tested was 2% and the lowest concentration contained 0,111% of Pluronic-F68® in Hepes/NaOH pH 7.4. In order to ensure comparable conditions, particle concentrations were adjusted to a fluorescence intensity of 1000. After incubation for 30min at 4°C, all samples were investigated in triplicate via flow cytometry in order to quantify the mean cell-associated fluorescence intensity.

As visualized by the graph in figure 3.b, Pluronic-F68® concentration does not seem to affect the lectin-mediated binding of BOD-NP to the glycocalyx of 5637 cells. Only insignificant variations could be observed. Though even higher concentrations of Pluronic did not seem to influence the binding capacity, all experiments were carried out with 0.25% Pluronic-F68® for better comparability.

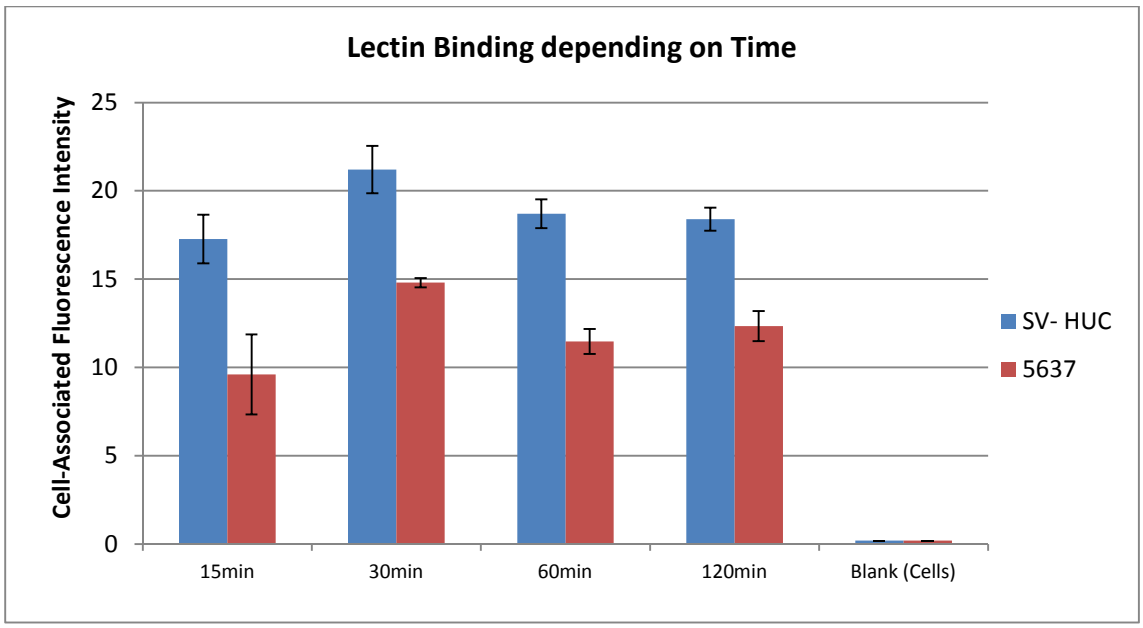

Figure 3.a: Investigation of incubation time

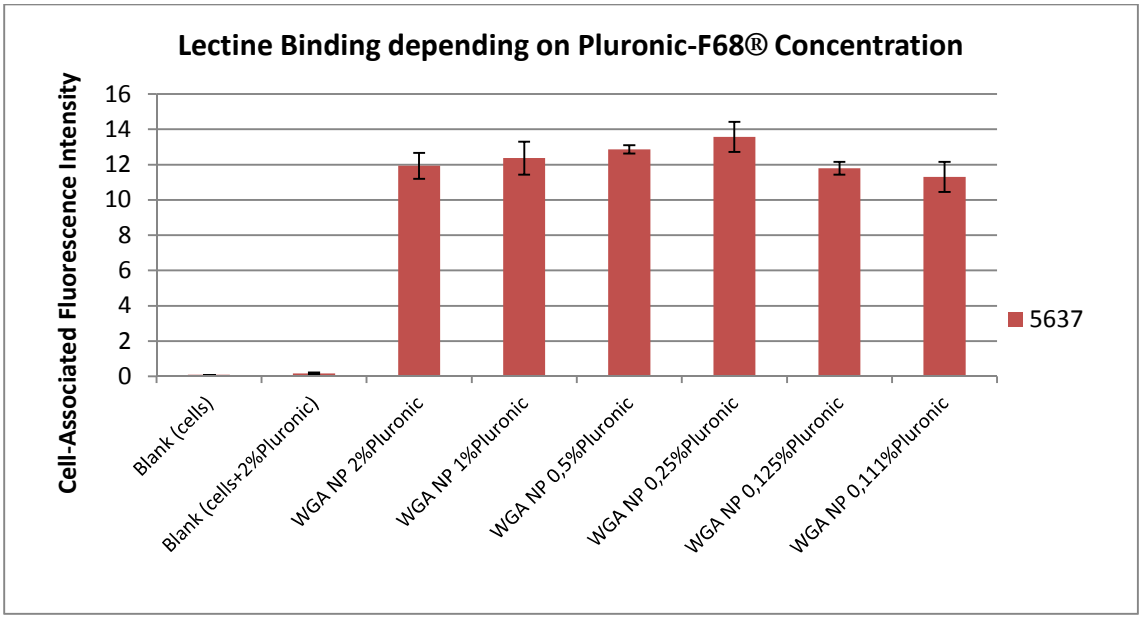

Figure 3.b: Influence of Pluronic-F68®
Figure 3.a illustrates the steep onset of the rapid binding of surface-modified nanoparticles, which had reached its maximum within 30min for both cell lines. As visualized by the graph in figure 3.b, Pluronic-F68® concentration does not seem to affect the lectin-mediated binding of BOD-NP to the glycocalyx of 5637 cells.

Interaction of nanoparticles with bladder cells: Impact of the surface modification with WGA and HSA as compared to non-surface-modified BOD-NP

The aim of this study was to investigate binding intensities of differently surface-modified nanoparticles to bladder single cells and to examine the influence of the lectin-modification on the interaction with the cells.

In this experiment, the surface of the particles varied from non-modified, HSA-modified to WGA-modified. For each kind of particles three dilutions were used at fluorescence intensities of 500, 1000 and 2000.

To guarantee comparability, incubation time was fixed to 30min for every sample while internalization was reduced to a minimum by keeping the temperature at 4°C.

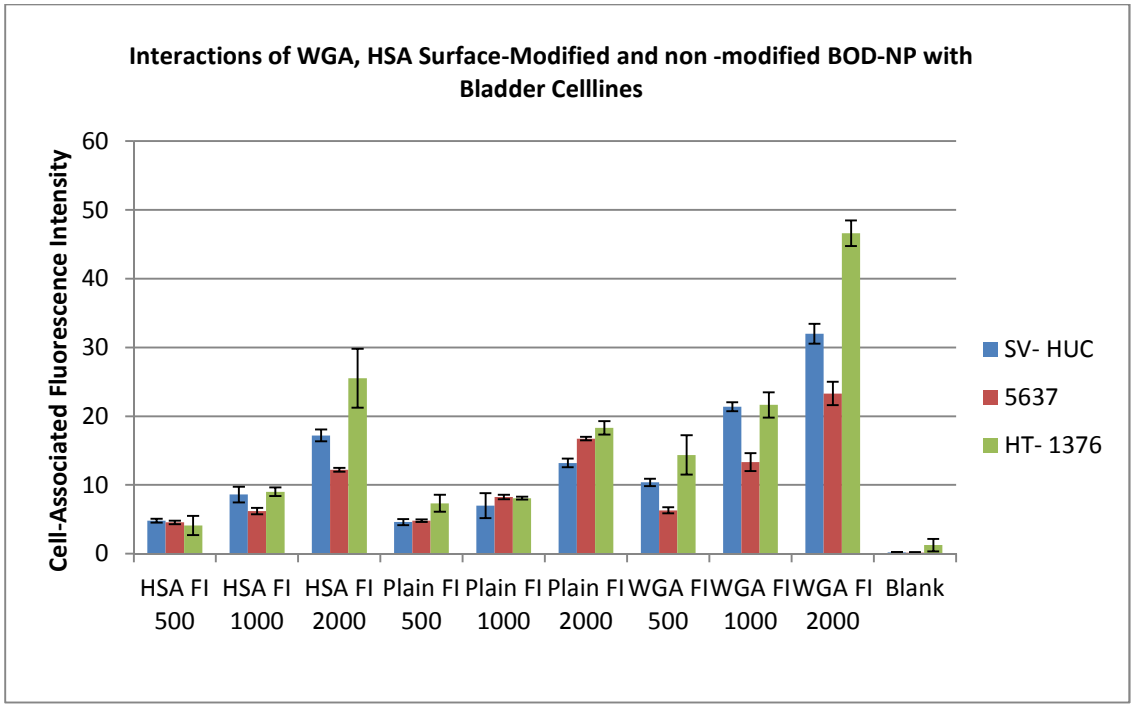

Figure 4: Survey of all urinary bladder cell lines, incubated with various concentrations of WGA-BOD-NP, HSA-BOD-NP and non-modified particles (Plain) for 30min at 4°C, showing the benefit of surface modification with WGA concerning particle – cell interactions.

The results indicate, that WGA-BOD-NP generally possess a higher interaction potential for all cell lines.

SV-HUC cells bound WGA-modified particles almost three times more efficient than non-surface-modified BOD-NP. This tendency was also evident when compared to HSA-BOD-NP particles. In this case, the cells still bound twice as much WGA-BOD-NP as HSA-modified particles.

For HT-1376 cells, particle binding was increased about 2.5-fold by the use of WGA-modification as compared to plain particles. This cell line also exhibited a slightly higher affinity to HSA-surface modified particles than to non-modified particles. As compared with the other two cell lines, HT-1376 cells generally possessed the highest auto fluorescence intensity, but also showed significantly higher interaction potential with all particle types.

The dependence of particle interaction and surface modification was slightly lower for 5637 cells, but still noticeable. WGA merely doubled the mean cell-associated fluorescence intensity when compared to HSA, which induced the lowest binding rate on 5637 cells. This is accompanied by a rather high affinity to plain particles, whereas both, SV-HUC and HT-1376, showed more HSA-dependent binding activity than for plain surfaces.

Binding Specificity of WGA-BOD-NP:

In order to estimate the extent to which the binding of WGA-BOD-NP to SV-HUC and 5637 cells is mediated via the specific interaction of immobilized lectins with carbohydrate moieties of the cells' glycocalyx, a competitive inhibition assay was performed.

Since WGA specifically binds to N-acetyl-neuraminic acid and N-acetyl-D-glucosamine structures, N, N`, N``-triacetylchitotriose was used as the complementary carbohydrate of choice (6). That way, the lectins' binding sites should be covered in order to reduce specific binding to a minimum and any remaining binding might only take place via unspecific interactions.

With higher concentrations of the carbohydrate the increasing viscosity of the sample suspension sets a natural limit for testing. Measurements were thus carried out between 0.0625 and 0.0019µmol N, N`, N``-triacetylchitotriose per 50µl.

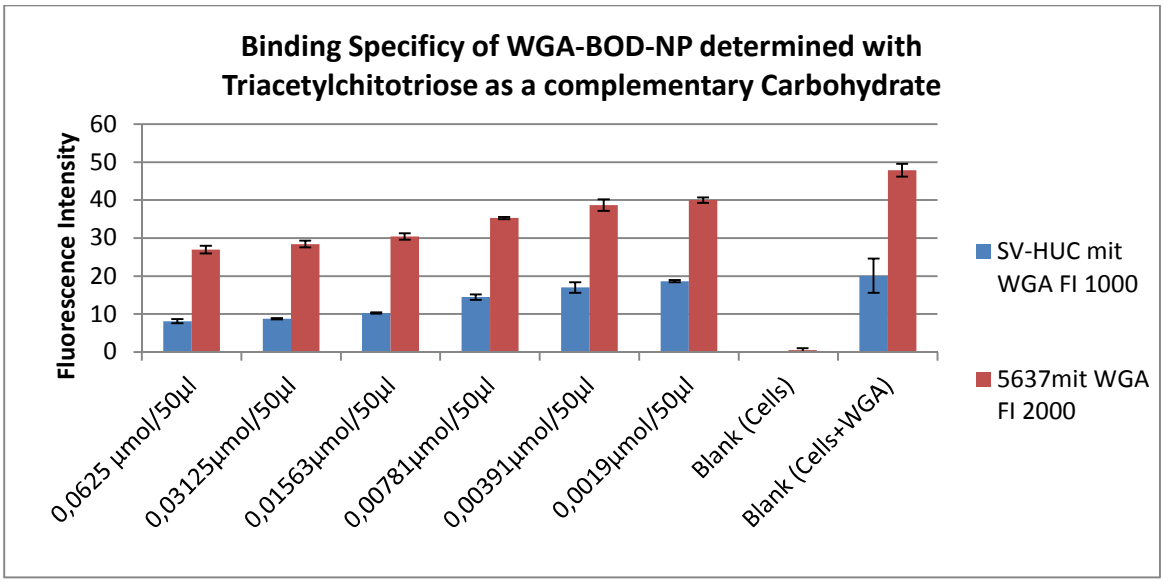

Figure 5: Mean cell-associated fluorescence intensity in course of competitive inhibition of WGA-BOD-NP binding to SV-HUC and 5637 cells by addition of increasing amounts of the complementary carbohydrate N, N`, N``-triacetylchitotriose at 4 °C..

As illustrated by figure 5, increasing amounts of N, N`, N``-triacetylchitotriose led to a corresponding decrease of the mean cell-associated fluorescence intensity. At the maximum inhibitor concentration, particle binding to SV-HUC was reduced to less than one half. Minor concentrations of inhibitor on the contrary revealed incremental higher amounts of particles bound to the glycocalyx.

For 5637 cells only a minor effect could be observed, but the mean cell-associated fluorescence intensity was also significantly reduced by the use of the complementary carbohydrate. In this case, the interdependence of inhibitor concentration and interaction potential of the immobilized lectin is significantly less pronounced when compared to SV-HUC cells, but still noticeable.
Since higher concentration of triacetylchitotriose also lead to higher viscosity, the maximum concentration for testing was set to 0.0625µmol per 50µl to guarantee equal conditions.

Neuraminidase treatment to investigate PNA binding:

Via the co-culture model of SV-HUC and 5637 cells, a highly selective binding of F-PNA to cancerous cells could be demonstrated. However, the selectivity was proved by staining, a procedure that delivers only qualitative information. The aim of this experiment was to quantify the F-PNA-cell interaction and gain more information about the underlying principle for PNA selectivity.
A suitable model for these investigations was found in the ability of neuramininidase to cleave sialic acid groups in the glycocalyx and reveal the underlying binding sites. This mimics also the process of incomplete glycosylation, which is known to increasingly occur with malignant transformation in the human urothelium. The surface obtained thus resembles a cancerous cell membrane with a higher quantity of binding sites for F-PNA, which can be determined via flow cytometry.
All three cell lines were treated with different concentrations of the enzyme, to obtain comparable data across the full range of tumor development. Each sample was analyzed in duplicate, and two concentrations of F-PNA were applied to cells without prior neuraminidase treatment to serve as a blank.

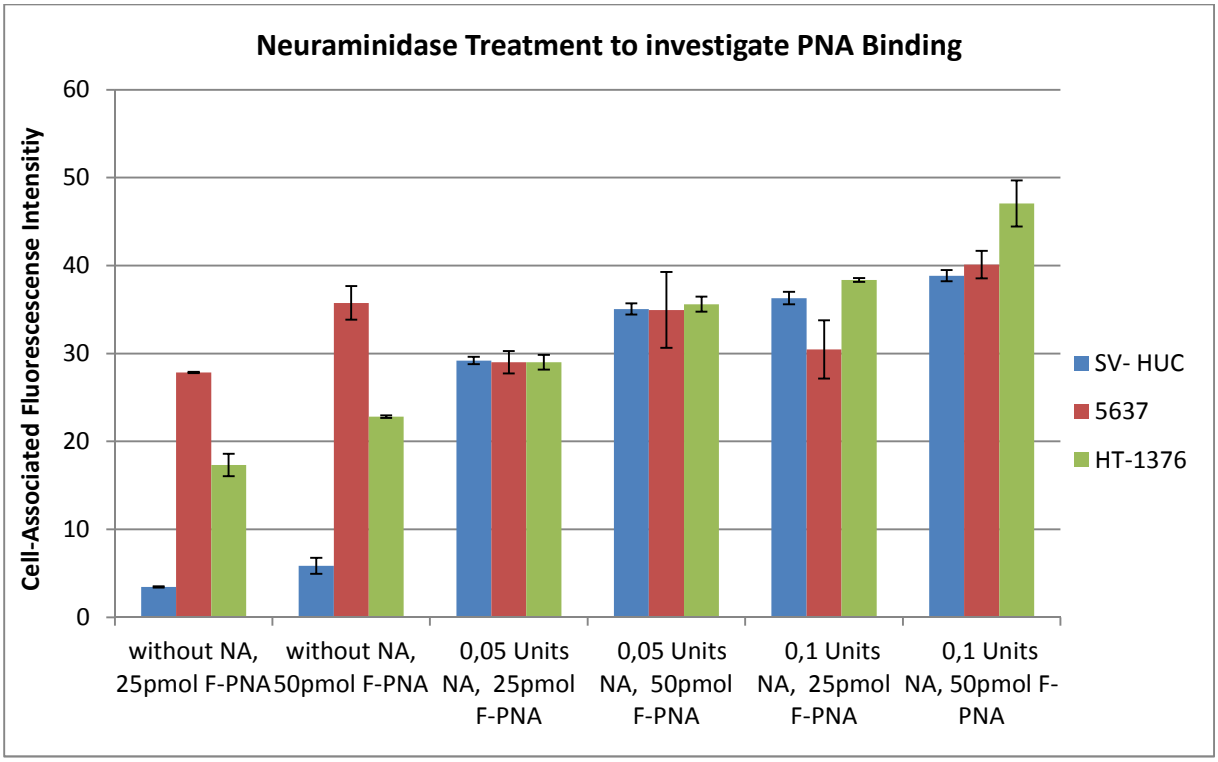

Figure 6: Mean cell-associated fluorescence intensity in course of different concentrations of F-PNA bound to SV-HUC, 5637 and HT-1376 cells under the influence of neuraminidase (NA). The binding of F-PNA to SV-HUC and HT-1376 was increased and both cell lines showed a proportional increase of lectin binding by raising the concentration of neuraminidase, whereas 5637 cells only showed a minor increase of the mean cell-associated fluorescence intensity.

The cell lines HT-1376 and SV-HUC exhibited a distinct relation between addition of increasing amounts of neuraminidase and a corresponding increase of the mean cell-associated fluorescence intensity.

The binding of F-PNA to SV-HUC cells was increased tenfold with the use of 0.1 units of neuraminidase per 100µl. For HT-1376 cells also increasing binding rates of F-PNA were found. Both cell lines showed a proportional increase of lectin binding by raising the concentration of neuraminidase. For 5637 cells such an impressive potential could not be observed. When compared to the results without neuraminidase 5637 cells revealed only a minor increase of the mean cell-associated fluorescence intensity.

Visualization of the particle binding to bladder cells:

The experiments above featured quantitative measurement methods to determine BOD-NP binding to bladder cell lines. In this investigation the goal was to visualize the fluorescent particle binding to the cells. SV-HUC single cells were incubated at 4°C for

30min. Then the cells were washed three times to remove any surplus of particles. Images were taken immediately after incubation in order to prevent internalization.

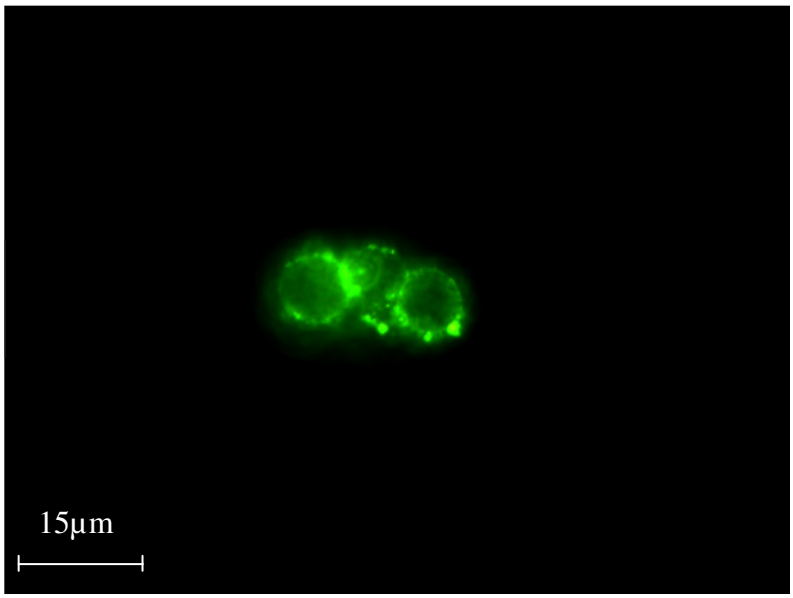

Figure 7: SV-HUC single cells incubated with WGA-BOD-NP (focus was set to the middle of the cell).
Figure 7 shows an image of the interaction between particles and single cells. The focus was set to the middle of the cell in order to demonstrate particle binding to the cell surface.

In this image clusters of particles that are bound to the cells are visible. The picture shows different particle clusterings at the cell surface with slightly varying diameter. Fluorescence intensity naturally increases with the size of the particle clusterings. Single nano particles are not directly visibly, but contribute to the staining effect of the cell surface. In the area of the left two cells overlapping, fluorescence intensity naturally increases.

Studies with primary cells:

Cell lines provide models that are characterized by their comparability, which is crucial for scientific investigations. Isolation took place often years ago and usually the cells can be cultured continuously for several months as well as deep frozen for storage. Moreover, conditions for cultivation and splitting were studied in detail, so no major problems should occur. Cell lines are thus a perfect model for broad studies. However, one

drawback might be found in some differences as compared to in vivo accompanied by a lack of significance.

Primary cells on the other hand might reflect more realistic the in vivo situation due to the recent isolation. However, the cells originate from various -often multi morbid- patients of different age and gender. This comes with the challenge of knowing very little about their preferences and needs with respect to cultivation, since each sample is rather unique. The donor cells used in this study derived from cooperation with the Department of Urology and the Department of Pathology and Microbiology of the Wilhelminenspital Vienna.

Concentration dependency of WGA-BOD-NP to primary cells:

In order to elucidate the interaction potential of particles with primary cells, an experiment was carried out, featuring cells of three different tissue samples. Herein, the concentration dependency of the WGA-BOD-NP binding was investigated with passage 2 of the samples 89 and 92 (both peritumoral). Due to the moderate amount of cells of sample 92 measurements were analyzed only in duplicates in this case.

Unfortunately the cells of sample 83, which derived from a healthy area, had to be investigated at passage 1 due to culturing reasons. For this sample the amount of available cells was also rather limited. Thus, only two particle dilutions could be tested, but these were analyzed in triplicate. Incubation time was set to 30min at 4°C as above.

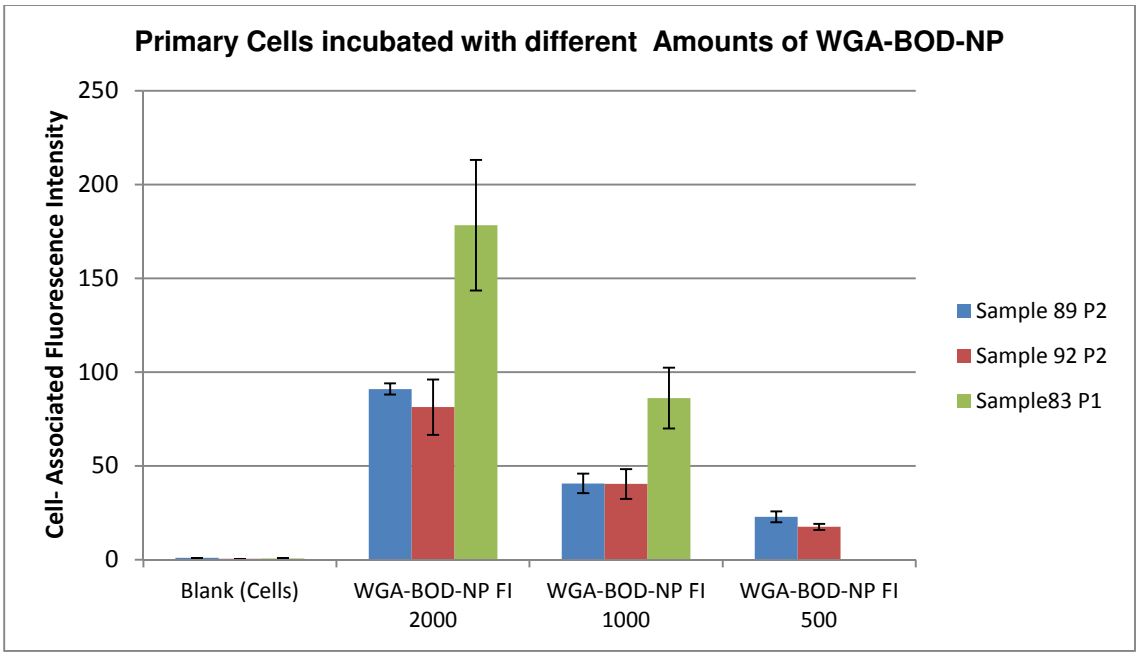

Figure 8: Interaction of WGA-BOD-NP with primary cells of peritumoral and healthy origin (Sample 89 & 92 peritumoral, Sample 83 healthy).

According to their origin the samples showed different binding activity for WGA-BOD-NP. Nevertheless within each sample a proportionality between particle quantity and mean cell-associated fluorescence intensity could be observed. For sample 89 P2 e.g. the fluorescence intensity was almost doubled when increasing the amount of particles from 500FI to 1000FI. Increasing the particle concentration to 2000FI resulted in an increase of 99.6%. For sample 92 P2 similar results could be determined. Sample 83 P1 was only tested at two concentrations of particles. Interestingly, these cells revealed a considerably higher tendency for particle binding at the individual particle concentrations.

Binding activity of WGA-BOD-NP vs. HSA-BOD-NP:

Experiments with cell lines showed higher binding activity for lectin modified particles. Thus the benefit of surface modification with WGA as compared to HSA should be investigated also for primary cells. The experiment included the respective particles at two concentrations, 2500 and 5000 fluorescence intensity (FI). Incubation time was set to 30min at 4°C and samples were analyzed in triplicates.

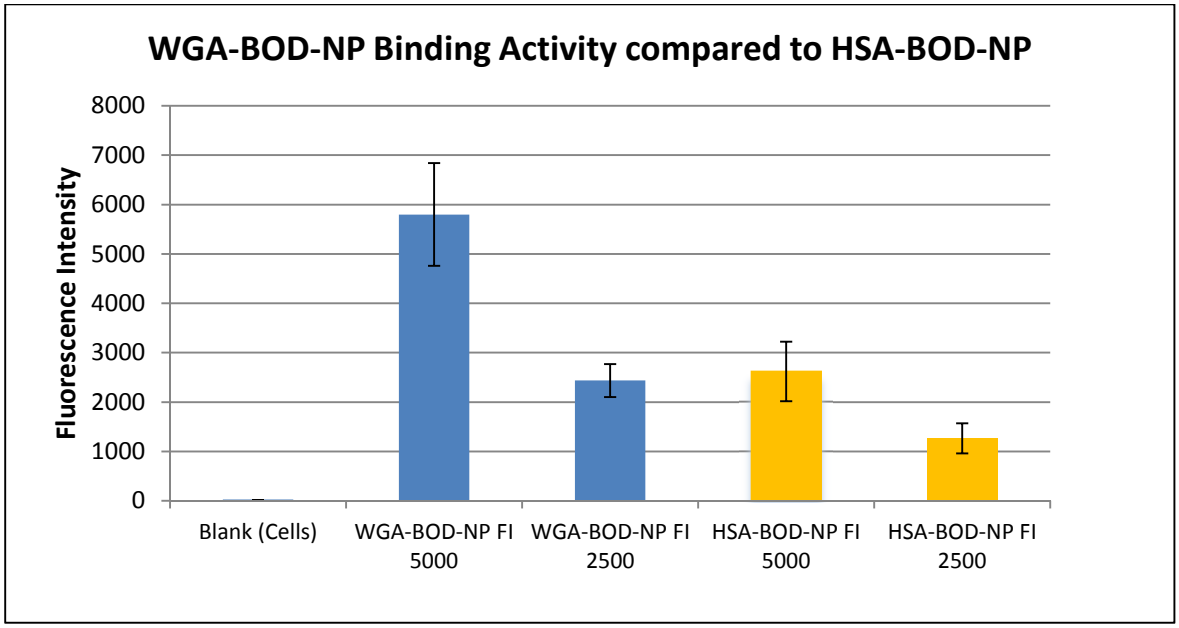

Figure 9: WGA-BOD-NP binding activity as compared to HSA-BOD-NP for sample 81.

As to the results obtained with cell lines, this sample of primary cells confirmed the trend of increased binding of WGA-BOD-NP in comparison to HSA-BOD-NP. In the case of

sample 81, which derived from a healthy area, lectin modification doubled the amount of particles bound to single cells.

DISCUSSION

Targeted delivery of active chemotherapeutic agents to the site of disease is a promising strategy for the future improvement of cancer treatment, and has therefore attracted attention in multiple fields of experimental and clinical oncology.

However, comparatively little research has addressed the application of refined delivery strategies for the therapy of superficial bladder cancer, where a straightforward local accessibility would greatly facilitate the use of bioadhesive or targeted formulations. Previous studies regarding binding and uptake of plant lectins in human urothelial cell lines (7) have shown the potential of using specific interactions with luminally exposed glycoproteins for an improved and more targeted tissue delivery. In particular, wheat germ agglutinin (WGA) exhibited a very high bioadhesive potential, while peanut agglutinin (PNA) emerged as the most potent discriminator between healthy and cancerous tissue. These findings might be essential for developing more potent local therapy schedules for urothelial cancer in future.

PNA and WGA were thus subjected to a more detailed screening with regard to their ability of serving as targeting/cytoadhesion-mediating ligands in particle-based drug delivery systems. The initial step in this study was to establish a visual proof of the ability of PNA to effectively discriminate healthy from diseased tissue. This could be achieved via fluorescence microscopy in a co-culture tissue model featuring healthy (SV-HUC) and cancerous (5637) cells, which indicated that also cells growing in close proximity will be selectively affected by a PNA-based targeting strategy.

F-PNA and F-WGA modified microparticles:

As a next critical step for the development of lectin-mediated treatment strategies, it was necessary to advance from the level of the single, fluorescence-tagged targeting molecule to a delivery system that is able to carry pharmaceutically active ingredients as a payload while still maintaining the functional recognition principle of the targeting ligand.

In this study, particulate carriers made of PLGA where chosen as a delivery system. This biodegradable matrix not only fulfils the criteria mentioned above, but is already successfully used in biomedical scaffolds with controlled, sustained release and other

delivery systems (4). PLGA is approved by the Federal Drug Administration (FDA) and established protocols for surface modification using NHS and EDAC are already available (8). The next step towards the use of lectins in bladder cancer therapy was to evaluate the possibility of performing surface modification of PLGA particles with the two most promising lectins, WGA and PNA, and to characterize the thereby induced effect upon particle-cell interaction. For ease of handling and to enable straightforward analysis of the results, preliminary experiments were carried out on particles in the size range of 1– 10µm. Since the microparticle matrix does not show any autofluorescence in the respective wavelength, it was possible to use fluorescence-labeled lectins (F-WGA and F-PNA) to demonstrate successful surface modification. To remove any unbound lectin and side products, the surface modification protocol was completed by washing the particles four times via centrifugation. As illustrated in figure 2, the microparticles showed a characteristic peripheral staining after the coupling procedure, indicating successful surface modification.

WGA-, HSA- and non-surface-modified BOD-NP:

Successful surface modification of microparticles with both lectins, however, does not automatically guarantee appropriate results for particles in the nanometer size range. PNA and WGA distinguish themselves not only in their characteristics concerning the future field of application; the two lectins also possess a significantly different molecular weight and differ in other physicochemical parameters. With a molecular weight of 110 kDa, PNA requires an individual washing procedure during and after surface modification as compared to WGA. The dimer of WGA, which is usually formed in physiological environment, has a molecular weight of 36 kDa and is therefore less complicate to remove. This work thus primarily focused on BOD-NP modified with WGA.

As expected, no major alterations were found when using WGA for the surface modification of NP in comparison to the microparticle preparation. The particle suspensions could be stored in small aliquots at -70°C to provide an easy handling for experiments.

Influence of incubation time and Pluronic-F68® concentration on particle-cell interaction:

To give a proof of concept, the time-dependent binding of WGA-BOD-NP to urothelial cells was to be examined as one of the key parameters. When WGA-BOD-NP were incubated with human bladder cells, the maximum binding capacity was reached within 30min irrespective of the cells histological origin (healthy or cancerous tissue). The short time required to reach maximum binding might be advantageous considering the potential application in form of an instillation, which typically lasts no longer than 60 minutes.

Prolonged incubation did not lead to higher cell-associated fluorescence intensities. It seemed, on the contrary, that incubation for more than one hour resulted in a tendency for lower fluorescence signals. Since internalization was unlikely due to a constant temperature of 4°C and photo bleaching was prevented by protection from light, this effect might be based on quenching effects between adjacent fluorophores at the cell surface.

Besides time dependency, the influence of the surfactant Pluronic-F68® had to be examined. This nonionic tenside is – like PLGA – approved by the FDA, and generally considered as not harmful. In NP preparations, the use of Pluronic-F68® often is obligatory to assure the stability of the suspension during modification and storage. However, since Pluronic-F68® is an amphoteric molecule, it might influence the particle interaction with the cell membrane to a certain extent. The necessity to clarify its effect on binding capacities was thus evident.

Preliminary experiments were carried out comprising different dilutions of Pluronic-F68® and a constant amount of WGA-BOD-NP. The results showed only negligible influence of Pluronic-F68® on particle binding for surfactant concentrations of 0.11% to 2.00%. Nevertheless, care was taken to carry out all experiments at a constant level of 0.25% Pluronic-F68® to guarantee direct comparability.

During surface modification, the Hepes/NaOH buffer pH 7.4 contained 0.5% Pluronic-F68®. The potential influence of this concentration on the quantity of lectin coupled to the PLGA matrix was not determined, but successful immobilization of WGA was achieved in all cases.

Interaction of WGA-, HSA- and non-surface-modified BOD-NP with bladder cells:

After evaluating the optimum parameters for cell interaction studies with regard to time and stabilizer concentration, it was of great interest to determine the differences in binding capacities of WGA-BOD-NP, HSA-BOD-NP and non-surface-modified particles. This experiment comprised a comparative analysis of all three cell lines incubated with different amounts of WGA-, HSA- and non-surface-modified BOD-NP. As expected, the results showed a clear concentration dependency. Moreover, it could be confirmed that modification with WGA resulted in higher binding rates. WGA-modification approximately tripled the amount of particles bound to SV-HUC, for 5637 cells the binding rates were doubled and for HT-1376 binding rates increased 2.5 fold, respectively, in comparison to non-surface- modified- BOD-NP.

HSA-BOD-NP showed notedly lower binding rates as compared to WGA-BOD-NP. The fact that SV-HUC bound more HSA- and WGA-BOD-NP than 5637 was unexpected, since a recent study (7) revealed higher binding for the pure WGA to 5637 cells. Thus, it might be concluded that the binding rate of a free lectin in solution cannot be directly compared to the binding behavior of a particle decorated with the same lectin.

Interestingly, HSA-BOD-NP generally showed higher binding rates than non surface-modified particles. This might be explained by the more hydrophilic surface, caused by the process of modification with a protein that is rather hydrophilic as compared to the pristine PLGA matrix. However, since the physicochemical characteristics of the PLGA matrix may change during modification, particle-cell interaction naturally might also be affected. The unspecific binding of non surface-modified particles to urothelial cells can thus not be directly compared to particles that were subjected to the process of modification.

Binding rates for non-surface-modified NP correlated to the applied concentration, but showed a different relative distribution between the individual cell lines, as compared to lectin-modified NP. The highest affinity was found for HT-1376 and the lowest for SV-HUC, whereas in case of modified particles the lowest binding rates were observed for 5637 cells.

Binding Specificity of WGA-BOD-NP:

For targeted delivery applications, it is crucial to quantify the degree of specificity in the particle-cell interaction. In order to investigate the extent to which the binding of WGA-BOD-NP to SV-HUC and 5637 cells is mediated by the specific interaction of the immobilized lectin with carbohydrate moieties of the cells' glycocalyx, a competitive inhibition assay was performed. Since N, N`, N``-triacetylchitotriose is the carbohydrate that shows the highest affinity for the active binding site of WGA, it was used to antagonize the lectin–cell interaction.

As expected, increasing amounts of the sugar component generally led to a corresponding decrease of the mean cell-associated fluorescence intensity caused by WGA-BOD-NP.

With the maximum inhibitor concentration, specific particle binding to SV-HUC was reduced by 56%, indicating that up to 44% of the total binding capacity might be mediated via unspecific interaction. This ratio could not be reached with 5637 cells, where the decrease of the mean cell-associated fluorescence intensity amounted up to 31% in presence of the complementary carbohydrate. Higher concentrations of N, N`, N``-triacetylchitotriose led to higher medium viscosity; to guarantee relatively equal conditions, the maximum concentration for testing was thus set to 0.0625µmol per 50µl. Due to the limit given by the increase in viscosity, the maximum possible inhibition was probably not reached for 5637 cells. The amount of specific binding may thus be higher than determined via the given setup. However, considering the results obtained with non-surface-modified particles, 5637 are likely to hold a generally higher degree of unspecific binding.

The influence of neuraminidase treatment on PNA binding:

Since the benefit resulting from a predominant targeting of cancerous cells is obvious, various assays focused on PNA as the targeting ligand. The results obtained in the co-culture staining of SV-HUC and 5637 cells confirmed a highly selective binding of F-PNA to cancerous cells under completely equal conditions.

The next step was to gain more information about the underlying mechanism of the F-PNA-cell interaction and its selectivity for cancerous cells. Tumor cells develop different glycosylation patterns as compared to normal ones, which might allow PNA to more

directly access its corresponding binding site. In healthy cells, these binding sites are shielded by sialic acid, hindering PNA from reaching the reactive carbohydrate moiety. Due to the ability of neuraminidase to cleave terminal sialic acid groups in the glycocalyx and thereby reveal the underlying binding sites, its application could present a suitable model for investigating the selectivity of PNA for cancerous cells. That way the process of incomplete glycosylation, which is known to increasingly occur with malignant transformation in the human urothelium, might be mimicked. All three cell lines were treated with different concentrations of the enzyme followed by a F-PNA binding assay analyzed via flow cytometry to determine any differences that might be associated with the process of tumor development.
The binding of 25 pmol F-PNA per 100µl to SV-HUC increased ten-fold after treatment with 0.1 units of neuraminidase per 100µl. Since this cell line represents healthy urothelial cells with presumably complete glycosylation, these cells were characterized by the most significant alteration due to the enzyme treatment.

Because of their more incomplete glycosylation, 5637 cells did not show such a strong increase in F-PNA binding. When compared to the assays without neuraminidase, 5637 cells showed only little increase of the mean cell-associated fluorescence. This is most probably due to the fact that sialic acid groups are already lacking in this stage of tumor development, so that the effect caused by the enzyme treatment is reduced to a minimum.
For HT-1376 cells, two-fold higher binding rates of F-PNA were found even without neuraminidase treatment. Interestingly, HT-1376 revealed an additional increase of lectin binding by raising the concentration of neuraminidase, even though these cells derived from a high grade carcinoma. However, the HT-1376 cell line represents an isolated example for a single cancer, and might not necessarily be regarded as a general representative for high grade carcinoma. Incomplete glycosylation also is only one out of various characteristic differences that distinguish healthy from cancerous cells. Regardless of the unexpected result for this cell line, neuraminidase generally increased PNA binding rates, indicating an important role of incomplete glycosylation associated with higher binding to terminal cancer.

The experiments discussed above focused on the quantitative evaluation of BOD-NP binding to bladder cell lines. In addition, time lapse imaging was used to visualize the process of particle-cell binding and confirm the results of the fluorometric analysis.

Single cells were incubated with WGA-BOD-NP at 4°C for 30min and then washed three times to remove any unbound particles. Images were taken instantly to prevent internalization.
Images depicted particles bound to the surface of respective single cells, which were not removed during the three washing steps after incubation. The focus was set to a middle plane, so the silhouette of the cells appeared dominant. Moreover, not only cells but also particles clustering in aggregates of slightly varying diameter were visible.

Studies with primary cells:

The experiments described above were carried out with cell lines representing healthy tissue or low and high grade carcinoma, respectively. Cell lines provide models that are highly valuable in regard to their comparability, which is crucial for scientific investigations. In principle, they are a perfect model for testing, but may lack in significance for the *in vivo* state. Primary cells, on the other hand, are closer to the in vivo state, but entail a higher maintenance effort and may cause problems in regard to inter- and intraexperimental comparability. Recent isolation from various - often multi-morbid - patients of different age and gender are only a few issues to be mentioned in this respect.
In order to show the characteristics of particle binding on primary cells and gain first insights into the more in vivo-like state, an experiment was carried out featuring donor cells of three different tissue samples. The samples designated “89” and “92” derived from peritumoral tissue; samples “81” and “83” were cultivated from a morphologically healthy region of the bladder wall.
According to their origin, the samples showed different binding activity for WGA-BOD-NP. Yet, each sample demonstrated proportional variations of the mean cell associated fluorescence intensity with regard to the particle quantity used for incubation. So within each sample, a clear dependency between binding rates and the concentration of WGA-BOD-NP could be established.
Since the experiments with cell lines showed higher binding activity for lectin-modified particles, the purpose of the experiments with primary cells was to investigate the benefit of surface modification with WGA as compared to HSA also for *ex vivo* samples. For this experiment the respective particles were applied in two concentrations, (2500 and 5000 fluorescence intensity) and incubated with cells of sample “81” that derived from a

healthy urothelium region. As observed previously for the cell lines, this sample showed distinctively higher mean-cell associated fluorescence intensities for the particles modified with WGA, proving the benefit of surface modification with lectins.

CONCLUSIONS

Various assays featuring three bladder cell lines (representing healthy and differently staged malignant tissue) indicated a higher affinity for PLGA nanoparticles after surface modification with wheat germ agglutinin as compared to human serum albumin- and non-surface-modified particles.

Whereas recent studies with free plant lectins (7) showed an increased affinity of WGA for malignant cells as compared to healthy ones (HT-1376 > 5637 > SV-HUC), the results found for lectin-modified particles could not confirm this trend (HT-1376 > SV-HUC > 5637). Since SV-HUC bound more WGA-BOD-NP than 5637, it seemed that the binding rates of free lectins cannot be directly compared to the binding behavior of a lectin-modified particle.

For comparison, the results obtained for WGA-BOD-NP were related to particles modified with human serum albumin and non-modified particles. The relatively high binding rates for HSA-BOD-NP as compared to non-surface-modified particles can be explained by the more hydrophilic surface caused by the process of modification with a protein. For HSA-BOD-NP, binding rates increased exactly as for WGA-BOD-NP: 5637 < SV-HUC < HT-1376. In contrast, non surface-modified particles showed the trend to bind more particles with increasing degree of malignancy.

First experiments using primary cells from healthy and peritumoral regions incubated with surface-modified particles matched the results obtained with the cell lines. However, more data are required to definitely conclude on the binding pattern.

Since lectins are a potential tool to deliver drug-entrapping PLGA particles to their target, it might be a highly useful approach for future intravesical therapy. Lectins coupled to nanoparticles may thus provide a versatile delivery system for various urothelial diseases including bladder cancer, since particle suspensions could be easily applied in form of a conventional instillation. All particles (surface-modified and non-modified) reached their maximum binding rates within 30min which is a reasonable incubation time for this application. Based on the current results of this work, further research to improve the targeted therapy of urinary bladder cancer would be highly warranted.

ZUSAMMENFASSUNG

In der vorliegenden Arbeit wurden Untersuchungen mit PLGA-Nanopartikeln an drei unterschiedlichen Urothelzelllinien durchgeführt, mit dem Ziel, die Therapie von Erkrankungen der Harnblase, allen voran Blasenkrebs, zu verbessern. Die drei dafür eingesetzten Zelllinien bestanden aus SV-HUC als Modell für gesundes Gewebe und den zwei malignen Zelllinien 5637 und HT-1376, die von verschieden differenziertem Tumor-Urothel stammen.

Lektine stellen einen neuartigen Ansatz zur Entwicklung von biorekognitiv getargeteten Delivery-Systemen dar, die auch an der Oberfläche arzneistoffbeladener Polymer-Partikel immobilisiert werden können. Durch Wechselwirkung mit an der Zellmembran exprimierten Zuckerketten könnte so die Partikel-Gewebe-Interaktion verbessert und eine effizientere Anreicherung des Wirkstoffes am Zielort erreicht werden. Bei der Anwendung in der instillativen Therapie könnte ein derartiges Delivery-System nicht nur von der einfachen Zugänglichkeit profitieren, sondern darüber hinaus den verkapselten Wirkstoff vor äußeren Einflüssen schützen und durch Bioadäsion zu einer längerdauernden Exposition führen, wodurch die Frequenz der notwendigen Behandlungen verringert werden könnte. Im hier vorgestellten Ansatz wurde aufgrund seiner ausgeprägten Zellaffinität am urothelialen Gewebe WGA als Lektin ausgewählt.

Mittels Flowzytometrie konnte festgestellt werden, dass eine Oberflächenmodifikation der Nanopartikel mit WGA generell zu einer erhöhten Bindungsaffinität führt. Als Vergleich dienten sowohl nicht modifizierte Nanopartikel, als auch Nanopartikel, die mit HSA modifiziert worden waren.

Eine kürzlich veröffentlichte Studie (7), beschreibt die mit unmodifizierter Lektinlösung gefundenen Zusammenhänge zwischen Bindungsaffinität und Malignizitätsgrad (SV-HUC < 5637 < HT-1376) für WGA. Für WGA-modifizierte Partikel konnte diese Reihenfolge jedoch nicht bestätigt werden. In der vorliegenden Arbeit zeigte sich folgende Abfolge in der Bindungsaffinität: 5637 < SV-HUC < HT-1376. Da hier „gesunde“ SV-HUC Zellen eine stärkere Partikelinteraktion aufwiesen als die Zelllinie 5637, liegt der Schluss nahe, dass sich das Bindungsverhalten eines Lektins nicht direkt auf das eines Lektin-modifizierten Partikels umlegen lässt.

Interessant war auch, dass eine relativ hohe Bindungsaffinität für HSA-modifizierte Partikel festgestellt werden konnte. Dies könnte sich eventuell dadurch erklären lassen,

dass die Partikeloberfläche durch die Modifikation mit einem Protein im Gegensatz zu der reinen PLGA-Matrix an Hydrophilie gewinnt. Für nicht modifizierte Partikel wurde ein Bindungsmuster ermittelt, das – ähnlich wie für WGA in Lösung beobachtet – einen Bezug zur Malignität nahelegt. In diesem Fall war die Reihenfolge jedoch umgekehrt: SV-HUC > 5638 > HT-1376.

Die Spezifität der Bindung von WGA-BOD-NP wurde mit Hilfe von N, N`, N``-Triacetylchitotriose, das mit den Zuckerstrukturen an der Zelloberfläche um die Bindungsstellen des Lektins konkurriert, nachgewiesen. Da N, N`, N``-Triacetylchitotriose nur die Interaktion zwischen WGA und Glycocalyx inhibiert, sind unspezifische Wechselwirkungen zwischen Partikel und Zelloberfläche immer noch möglich. Deshalb erreicht die Zellbindung ein Plateau, das auch duch weitere Erhöhung der Konzentration von N, N`, N``-Triacetylchitotriose nicht unterschritten werden konnte und somit dem Ausmaß der unspezifischen Partikelinteraktion entsprechen dürfte. Zusatz von N, N`, N``-Triacetylchitotriose führte bei der Zelllinie SV-HUC zu einer Reduktion der Bindung von WGA-BOD-NP auf weniger als die Hälfte. Bei der Zelllinie 5637 war dieser Effekt etwas schwächer ausgeprägt. Der Anteil an unspezifischer Bindung liegt hier bei etwa 60% .

Mit Neuraminidase, die Sialinsäurereste von Zuckerstrukturen der Glykokalyx abspalten kann, wurde eine Zelloberfläche erhalten, die malignen Urothelzellen stark ähnelt, da bei zunehmender Malignität die Glykosylierung inkomplett ist. Eine Vorbehandlung mit diesem Enzym bewirkt bei SV-HUC Zellen, die als gesund einzustufen sind, eine zehnfach gesteigerte Bindung von F-PNA. Da die Zelllinien 5637 und HT-1376 von Tumor-Urothel abstammen und damit bereits malign transformiert sind, war dieser Effekt hier deutlich geringer ausgeprägt. Daraus lässt sich ableiten, dass PNA eine gewisse Selektivität für maligne Zellen aufweisen sollte, und dies ließe sich für eingezieltes targeting ausnützen.

Erste Versuche an Primärzellen aus peritumoralem Gewebe bzw. gesunden Regionen bestätigten die Ergebnisse, die in den Studien an Zelllinien ermittelt wurden. Auf diesem Gebiet sind für eine abschließende Beurteilung allerdings noch umfangreichere Daten notwendig.

In der Praxis könnte ein nanopartikuläres Delivery-System, wie in dieser Arbeit vorgestellt, in Form einer konventionellen Instillation verabreicht werden. Hierbei wäre

von Vorteil, dass die Partikelinteraktion nach WGA-Modifikation sehr rascherfolgt und ihre volle Ausprägung bereits nach 30min erreicht hat.

Allgemein könnte auf diese Weise nicht nur die intravesikale Therapie des Blasenkarzinoms sondern auch die Behandlung anderer lokal zugänglicher Erkrankungen des Urothels, wie beispielsweise bakterielle Harnwegsinfekte, entscheidend vereinfacht werden. Dennoch bleiben für eine abschließende Einschätzung des generellen Potentials umfangreichere Tests in klinischen Studien abzuwarten.

REFERENCES

(1) Avritscher EB, Cookslec CD, Grossman HB, et al. "Clinical model of lifetime cost of treating bladder cancer and associated complications" Urology 2006; 68; 549-53

(2) Yair Lotan, MD et al. "Key Concerns About the Current State of Bladder Cancer" Cancer 2009; 115:4096-103

(3) Ratzinger G., Länger U., Neutsch L., Pittner F., Wirth M. and Franz Gabor, Surface Modification of PLGA Particles: "The Interplay between Stabilizer, Ligand Size, and Hydrophobic Interactions" Langmuir 2010, 26(3), 1855–1859

(4) Astete, C.E. and Sabilov, C.M. (2006) „Synthesis and characterization of PLGA nanoparticles" Journal of Biomaterials Science 17 (§): 247-289J.

(5) Jenifer Southgate, John R.W. Masters and Ludwik K. Trejdosiewicz "Culture of Human Urothelium" Cell Biology Capt. 12; 381-399; 2002

(6) Goldstein, S. Hammarstrom, G. Sundblad, Biochim. Biophys.Acta 1975, 405, 53.

(7) Neutsch L., Plattner V., Polster-Wildhofen S., Zidar A., Chott A., Borchard G., Zechner O., Gabor F., Wirth M. "Lectin-mediated Biorecognition as a Novel Strategy for Targeted Delivery to Bladder Cancer Improving the Efficacy of Adjuvant Intravesical Therapy"

(8) Fillafer C., Friedl D. S., Wirth M., and Gabor F. "Fluorescent Bionanoprobes to Characterize Cytoadhesion and Cytoinvasion" 10.1002/small.200701086

ABKÜRZUNGEN/ ABBREVIATIONS

BCG…….Bacillus Calmette- Guérin

BPE……..Bovine pituitary extract

cKSFM……complete keratinocyte serum free medium

EGF…….. Human growth factor

EDAC……..1-ethyl-3(3-dimethylaminopropyl) carbodiimide

EMDA……..Electromotive drug administration

FDA…….Federal Drug Administration

FIU……..Fluorescence Intensity Units

HSA…….Human serum albumin

MP……Microparticles

NA…….Neuraminidase

NHS……..N-hydroxysuccinimide

NP…….Nanoparticles

PLGA……polyD,L-lactide-co-glycolide

PNA……..Peanut agglutinin

TURBT…….Transurethral resection of the bladder tumor

WGA…….Wheat germ agglutinin

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