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Assessing potential peptide targeting ligands by quantification of cellular adhesion of model nanoparticles under flow conditions

Ellen Broda [a], Frauke Martina Mickler [a], Ulrich Lächelt [b], Stephan Morys [b], Ernst Wagner [b], Christoph Bräuchle [a,*]

[a] Department of Chemistry and Center for NanoScience (CeNS), Ludwig-Maximilians-Universität München, Butenandtstr. 5-13, D-81377 München, Germany
[b] Department of Pharmacy and Center for NanoScience (CeNS), Ludwig-Maximilians-Universität München, Butenandtstr. 5-13, D-81377 München, Germany

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ABSTRACT

Sophisticated drug delivery systems are coated with targeting ligands to improve the specific adhesion to surface receptors on diseased cells. In our study, we developed a method with which we assessed the potential of peptide ligands to specifically bind to receptor overexpressing target cells. Therefore, a microfluidic setup was used where the cellular adhesion of nanoparticles with ligand and of control nanoparticles was observed in parallel under the same experimental conditions. The effect of the ligand on cellular binding was quantified by counting the number of adhered nanoparticles with ligand and differently labeled control nanoparticles on single cells after incubation under flow conditions. To provide easy-to-synthesize, stable and reproducible nanoparticles which mimic the surface characteristics of drug delivery systems and meet the requirements for quantitative analysis, latex beads based on amine-modified polystyrene were used as model nanoparticles. Two short peptides were tested to serve as targeting ligand on the beads by increasing the specific binding to HuH7 cells. The c-Met binding peptide cMBP2 was used for hepatocyte growth factor receptor (c-Met) targeting and the peptide B6 for transferrin receptor (TfR) targeting. The impact of the targeting peptide on binding was investigated by comparing the beads with ligand to different internal control beads: 1) without ligand and tailored surface charge (electrostatic control) and 2) with scrambled peptide and similar surface charge, but a different amino acid sequence (specificity control). Our results demonstrate that the method is very useful to select suitable targeting ligands for specific nanoparticle binding to receptor overexpressing tumor cells. We show that the cMBP2 ligand specifically enhances nanoparticle adhesion to target cells, whereas the B6 peptide mediates binding to tumor cells mainly by nonspecific interactions. All together, we suggest that cMBP2 is a suitable choice for specific receptor targeting whereas the peptide B6 should not be considered as specific targeting moiety.

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1. Introduction

Nanoparticle based strategies for diagnostics [1] and therapy [2,3] are powerful tools in an evolving field to detect, image, target, or treat diseased tissue and cells [4,5]. Functional targeting groups on nanoparticles are designed to improve specific recognition of and attachment to receptors which are upregulated and exposed on the target cell surface [4,6]. Furthermore, certain receptor–ligand interactions can facilitate the uptake of such nano therapeutics by receptor-mediated endocytosis and thus the accumulation within the diseased tissue.

In the case of cancer therapy, therapeutic nanoparticles can address circulating tumor cells directly within the blood flow as well as tumor endothelial cells at the vessel walls. On the other hand, primary tumors are reached after extravasation of leaky blood vessels [7].

Whether the nanoparticles bind to target cells depends on the following steps: 1) the nanoparticles have to reach the proximity of the cell surface, 2) avoiding nonspecific interactions with extracellular components and non-target cells and 3) recognizing of and attaching to receptors on target cells. First, the distribution of the particles within the tumor region depends on the flow velocity of the fluid and the interstitial fluid pressure which is increased in solid tumors [8]. The diffusion of the particles within the tumor surrounding area is affected by their size and shape [9]. Second, functional groups on the particle surface contribute to nonspecific interactions such as electrostatic and van der Waals interactions with extracellular matrix components. Typically, positively charged nanoparticles exhibit a high affinity to cell surfaces as they can interact with negatively charged membrane glycans [10]. Adsorption of serum proteins on the nanoparticle surface further mediates cell binding and subsequent particle endocytosis [11]. To reduce undesired cellular uptake and improve the biodistribution, nanoparticles can be shielded by neutral molecules like polyethylene glycol (PEG) [12]. Third, a targeting ligand can enhance specific binding to the receptors on target cells. The binding affinity of the targeting ligand, the flexibility of the attached linker [13] as well as the number of ligands [14] on the particle surface influence targeting effectivity.

The amount of cellular associated nanoparticles is either appraised qualitatively by visualization or quantified by various methods

* Corresponding author.
E-mail address: christoph.braeuchle@cup.uni-muenchen.de (C. Bräuchle).

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including light and electron microscopy, radioactive labeling and fluorescence spectroscopy [14]. A commonly used fluorescence technique is flow cytometry which allows for measuring large numbers of cells within a short time range resulting in good statistics [15]. Nonetheless, particle localization cannot be determined. Furthermore, the obtained particle numbers are rather relative instead of absolute values without additional elaborate calibration [14]. In general, quantification techniques which are based on fluorescence intensity require many optimized conditions e.g. concerning the illumination, autofluorescence, crosstalk and potential controls [16]. Additionally, such techniques face problems like photo bleaching and quenching effects.

Therefore, we quantified cellular adhesion by counting the number of fluorescent spots of nanoparticles bound to single cells. Thus, we were able to monitor particle localization and distribution on the cells simultaneously. To this purpose, we synthesized model nanoparticles as it was essential to provide single, stable, preferably non-aggregating labeled nanoparticles which mimic the surface characteristics of therapeutic nanoparticles.

Traditionally, *in vitro* studies are performed under static conditions, where nanoparticles approach the cell surface by gravitational sedimentation and can agglomerate during this process [17]. A microfluidic setup ensured reduced sedimentation effects and provided constant particle concentrations as well as cell environmental conditions. Additionally, we compared the adhesion of nanoparticles with targeting ligand to nanoparticles without, which served as an internal control. Both nanoparticle types were passed simultaneously over an adhesive cell monolayer in a microfluidic channel. Absolute and relative particle numbers could be determined. We tested two short peptide ligands, the c-Met binding peptide (cMBP2) and the transferrin receptor (TfR) targeted peptide B6 for their potential to serve as a targeting molecule.

Short peptide ligands possess several advantages such as small size, easy and cheap synthesis, good biocompatibility and low immunogenicity [18,19] compared to large protein ligands and monoclonal antibodies. The tyrosine kinase c-Met (receptor for the hepatocyte growth factor) has gained increasing attention recently due to its role in sustaining tumor progression [20]. The epitope-mimicking cMBP2 (12 amino acids) has been selected by Kim et al. from a combinatorial peptide library [21] to bind c-Met and was recently tested for non-viral gene delivery applications as a potent targeting ligand [22]. The B6 peptide (9 amino acids) was chosen by Xia et al. from a phage display assay to bind the human TfR [23], which is overexpressed in various human cancer cell lines and enhances their metastatic potential [24]. So far, the peptide ligand has been applied for brain delivery of PEG-PLA nanoparticles [25] and dual targeting approaches with polyplexes [26].

With our experiments we aimed to provide a useful technique which allows for selecting suitable targeting ligands which enhance the specific binding of nanoparticles to receptor overexpressing target cells.

2. Results and discussion

2.1. Microfluidic setup for quantitative analyses of cellular nanoparticle adhesion at the single cell level

We developed and optimized an experimental flow setup with internal control for quantitative adhesion studies on a single cell level. With this approach we were able to quantify the effect of targeting ligands on cellular adhesion of nanoparticles.

Fig. 1A shows the flow system where homogenously suspended nanoparticles within a warmed reservoir (T = 37 °C) are passed through a warmed flow channel (T = 37 °C), containing a cell monolayer, with a tunable laminar shear flow which is generated by a syringe pump. With the use of microfluidics, constant concentrations, similar environmental conditions and reduced influence of sedimentation of larger particles/aggregates are provided [27,28]. Moreover, by adapting the experimental conditions, studies could be performed to target the vascular endothelium [29] for treatment of infectious or cardiovascular diseases, or components of or within the blood flow such as metastatic cancer cells [30] could be addressed. These approaches represent a bridge to clinical transfer of nanoparticles [31].

In a flow channel, beads equipped with ligand were directly compared with control beads by counting the differently fluorescent labeled beads associated to single cells (Fig. 1B). Shear flow was applied for 40 min. At this optimized time point, the number of bound particles per cell was high enough to result in good statistics, but still low enough to count single particles. The cells were fixed afterwards and the fluorescence of bound beads was measured by alternating excitation widefield microscopy. The numbers of beads per cell were counted for both, targeted and control beads, and in a third step a factor was calculated which defines the binding affinity of beads with ligand compared to control beads.

Using the internal control, the influence of experimental heterogeneity (including cell density, cell surface environment and receptor density) on the result could be minimized. The calculation of factors (the number of beads with ligand divided by the number of control beads) furthermore enabled that the varying distribution of both types of nanoparticles (with ligand and control) on single cells could be illustrated. A number of experimental parameters have been reported which influence the adhesion and uptake mechanisms of nanoparticles on and into cells such as particle size [27,32], shape [33] and functionalization [30,34,35], cell type and shear rate [30,34,36]. By keeping the particle size and shape constant and by varying the particle functionalization, we were able to investigate the effect of two different receptor ligands on cellular adhesion.

Furthermore we validated the influence of two different shear forces. A relatively low shear force (26 dyn/cm^2) was chosen at which a laminar flow of the beads within the flow channels could be observed and the beads did not tumble around. Furthermore, the shear force is within a range of increased interstitial fluid pressure to which cancer cells might be exposed to [8,37]. The influence of shear flow on particle adhesion was tested by increasing the shear flow by one order of magnitude (shear force: 263 dyn/cm^2).

Applying highly sensitive fluorescence microscopy, we were able to determine the precise localization of the beads on, within or next to the cells. Furthermore, we monitored the integrity of the applied particles and cells during the measurement. With our analysis we were able to detect rare events resulting in higher accuracy and to control which experimental factors influence the results. We determined that following factors have a considerable effect on the number of beads per cell: cell density, cell size, receptor expression level, particle aggregation and degradation.

2.2. Synthesis of model nanoparticles for receptor targeting and adhesion studies

Model nanoparticles (Fig. 2A) were established to cope with two purposes: first, to investigate the effect of targeting ligands, comparable and easy-to-synthesize nanoparticles were required which mimic the surface characteristics of common drug and gene delivery devices. Second, the model delivery system should meet the requirements for quantitative analysis. Desired parameters are low aggregation behavior, good detectability, reproducibility and stability over several months. These goals were accomplished by providing labeled, PEGylated polystyrene beads to which peptide ligands or control molecules were coupled.

Commercially available polystyrene beads (200 nm diameter) with surface amino groups were labeled with fluorescent dyes (5% Cy5 or 8% ATTO 488) via *N*-hydroxysuccinimide (NHS) ester chemistry. Using the same coupling method, remaining surface amino groups were linked to NHS-PEG5-OPSS which contained 5 kDa PEG for surface shielding and *ortho*-pyridyldisulfide (OPSS) for conjugation with thiols.

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