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Investigations on Surface Modified Dendrimers: Enzymatic Hydrolysis and Uptake into MCF-7 Breast Cancer Cells

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A series of G_0 and G_1 generation 1,3,5-tris(3-aminopropyl)benzene dendrimers surface modified with the amino acids phenylalanine (Phe), methionine (Met), aspartic acid (Asp), and diaminopropionic acid (Dap) was investigated with regard to their stability against enzymatic hydrolysis with the model enzymes papain, chymotrypsin, trypsin, and pepsin. Additionally, the cytosol of MCF-7 cells was used to get an insight into the possible degradation in intracellular medium. The cytosol, chymotrypsin, and papain had similar activities on the tested dendrimers and efficiently cleaved methionine and phenylalanine from the surface of the dendrimers. The cellular uptake of the dendrimers into the MCF-7 cells depended on the surface modification (Phe > Met unmodified ≫ Dap = Asp) and the generations ($G_0 > G_1$).

Introduction

Macromolecular carriers are attractive and promising candidates for the delivery of cytostatics because of their strong influence on the pharmacological properties. Two advantages over other drug delivery systems are noticed: 1) the solubility of the conjugate can be increased by use of polar macromolecules[1] and 2) the renal excretion is reduced when the conjugate exceeded a molecular mass of 40 kD resulting in a long circulation duration in the body. The high-molecular mass of a macromolecule addresses the cytostatics through the so-called enhanced penetration and retention (EPR) effect, predominantly in tumor tissue, lowering the side effects of the cytostatics in nontarget tissues.[2–5]

Polymer-bound cytostatics are usually not effective.[6,7] They have to be selectively released in tumor tissue or in tumor cells. A very elegant method to achieve the drug release is to use the well-known elevation of proteolytic enzymes in tumor cells. Therefore, polypeptides were synthesized as a cleavable spacer between the drug and the polymer.[8,9] These basic studies were performed with linear macromolecules predominantly the *N*-(2-hydroxypropyl)methacrylamide (HPMA) polymers. The polypeptides bound to these macromolecules were digested when the length of the peptide exceeds three amino acids.[8]

However, it is impossible to obtain a well-defined drug delivery system. On the one hand the synthetic routes used allow only a statistical drug distribution in the molecule and on the other hand linear macromolecules form coiled structures including a significant part of the bound cytostatics then not available for enzymatic cleaving.

Dendrimers are suggested to be better drug carriers because of their globular structure and their defined synthesis.[10] Therefore, we determined in this initial study the stability of dendrimers surface modified with the amino acids phenylalanine (Phe), methionine (Met), aspartic acid (Asp), and diaminopropionic acid (Dap) against the model enzymes papain, chymotrypsin, trypsin, and pepsin. To get an insight into possible intracellular enzymatic degradation we also used the cytosol of MCF-7 breast cancer cells. For the quantification of degradation products we developed an analytical method using separation and capillary electrophoresis (SPE).

Results and Discussion

Cleavage of amino acids from the dendrimers by enzymes

Overexpression of proteolytic enzymes is characteristic for extroversive cancers. Therefore, many tumor cells possess high levels of cysteine proteases such as cathepsine B and L,[11,12] aspartate protease cathepsine D,[11–13] and serine proteases including the plasminogen activators.[11,14–16] To screen the amino acid terminated dendrimers[17] (see Figure 1) for stability against proteolytic degradation, in a first experiment we used commercially available enzymes with similar selectivity which were easy to handle: papain, pepsin, chymotrypsin, and trypsin.

The stability against enzymatic degradation depended on the terminal amino acid and the enzyme used. All dendrimers were stable against pepsin and trypsin, whereas papain and chymotrypsin cleaved the bond to methionine and phenylalanine but not to aspartic acid and diaminopropionic acid.

The digestion could be followed by capillary electrophoresis and made a kinetical analysis of the reaction possible. Figure 2 shows the typical reaction curve of a degradation on the example of $G_0(Phe)_3$ and chymotrypsin. It followed a pseudo first-

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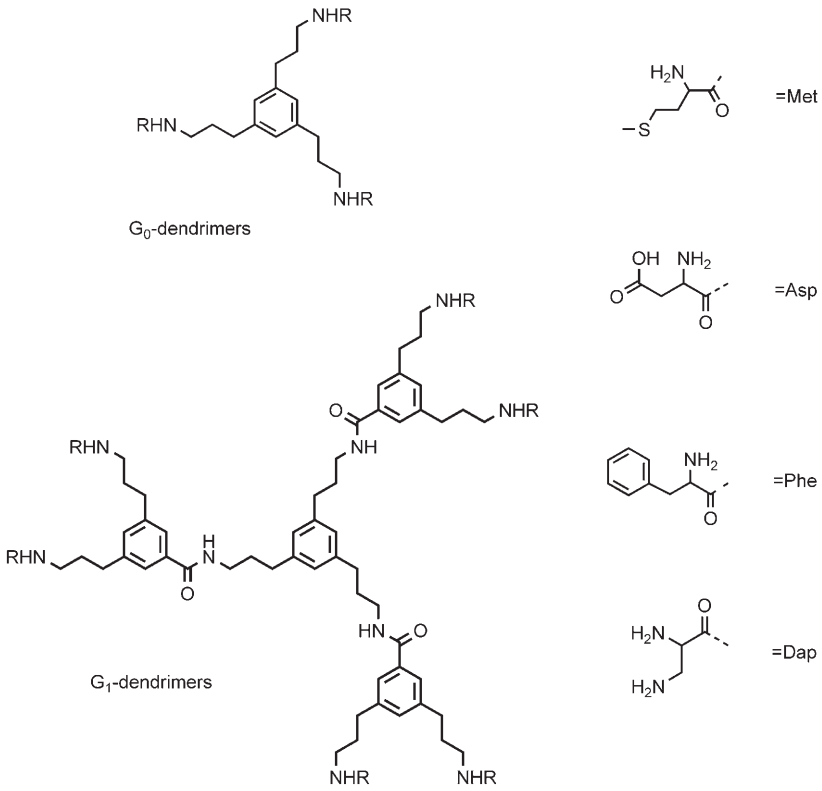

compd.	R	compd.	R
G_0	H	G_1	H
$G_0(Met)_3$	Met	$G_1(Met)_6$	Met
$G_0(Phe)_3$	Phe	$G_1(Phe)_6$	Phe
$G_0(Asp)_3$	Asp	$G_1(Asp)_6$	Asp
$G_0(Dap)_3$	Dap	$G_1(Dap)_6$	Dap

Figure 1. Structures of the investigated dendrimers.

order reaction with a rate constant of $-0.0647\ h^{-1}$ for the first loss of phenylalanine (Table 1). Papain cleaved $G_0(Phe)_3$ with the same efficacy ($-0.0604\ h^{-1}$). In contrast, $G_0(Met)_3$ showed a clear preference for papain ($-0.0763\ h^{-1}$), chymotrypsin cleaved the dendrimer methionine bond with a distinctly lower rate constant ($-0.0054\ h^{-1}$).

The influence of the generation on degradation was studied on methionine terminated dendrimers. $G_1(Met)_6$ ($-0.1646\ h^{-1}$) was converted two times faster by papain than $G_0(Met)_3$ ($-0.0763\ h^{-1}$), whereas chymotrypsin degraded $G_1(Met)_6$ ($-0.072\ h^{-1}$) 13 times faster than $G_0(Met)_3$ ($-0.0054\ h^{-1}$). Larger dendrimer size might provide a better fit for the active site of the enzyme leading to a faster degradation. This might also be the explanation of the inertness of Asp and Dap bearing dendrimers. Theoretical evaluation of the three-dimensional structures of the $G_x(Asp)_{(x+1)*3}$ and $G_x(Dap)_{(x+1)*3}$ type revealed a condensed structure, whereas degradable Phe- and Met-terminated dendrimers present their surface modifications well accessible to the environment.[18]

The investigations on $G_1(Phe)_6$ remain quite difficult, because it was soluble in water but showed a strong tendency to precipitate in buffer. This effect was already described for other dendrimers with neighboring lipophilic and cationic groups at the surface.[19,20] Reduction of the lipophilic character of $G_1(Phe)_6$ by cleavage of terminal phenylalanine groups allowed a detection of the degradation products by capillary electrophoresis but no calculation of the rate constant.

To exclude that the inert dendrimers of the $G_x(Asp)_{(x+1)*3}$ and $G_x(Dap)_{(x+1)*3}$ type caused an inhibitory effect on the enzymes or that the enzymes did not work, we performed control experiments with $G_1(Dap)_6$. We added casein to a preincubated (24 h) dendrimer/enzyme mixture and monitored the product profile by capillary electrophoresis. The electropherogram was identical to that obtained with a casein/trypsin mixture without dendrimer (see Figure 3). This finding clearly demonstrated that $G_1(Dap)_6$ had no effect on the activity of the enzyme.

Furthermore, it has to be mentioned that the enzymes were necessary to cleave the dendrimers. Hydrolytic processes were not observed during the incubation (72 h) of aqueous solutions in a pH range of 2.7 to 8.1 without enzyme.

Digestion with cytosol of MCF-7 breast cancer cells

In the next experiment we tried to evaluate if the above described results obtained with isolated enzymes can be correlated with the proteolytic behavior in breast cancer cells.[9] Therefore, the cytosol of MCF-7 cancer cells was prepared, lyophilized, and dissolved in a buffer designed to mimic late endosomal and lysosomal conditions.[8]

This cytosolic preparation successively cleaved the amino acids from the dendrimers (Figure 4) with specificity comparable to papain, however, with 7–8 times lower rate constants (see Table 1). This may be due to the presence of cytosolic inhibitors of the lysosomal proteases, for example, stefines. Inter-

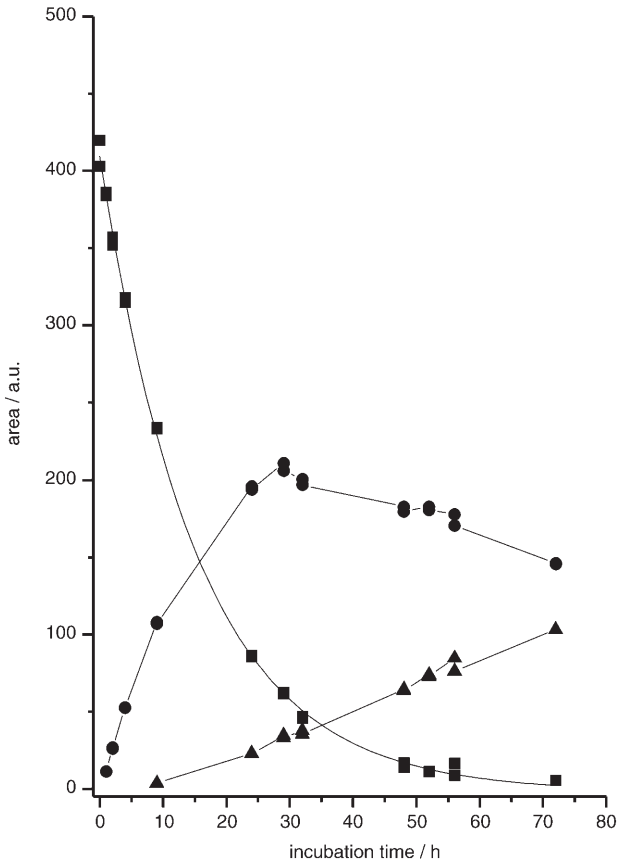

Figure 2. A represent kinetic for the digest of $G_0(Phe)_3$ by chymotrypsin (▲($G_0(Phe)_3$; ● ($G_0(Phe)_2$; ■ ($G_0(Phe)_1$)

estingly, the high protein content prevented the precipitation of the $G_1(Phe)_6$ and allowed a calculation of the rate constant for the degradation. In contrast to the methionine bearing dendrimers, the rate constant did not increase with generation ($G_0(Phe)_3 \rightarrow G_1(Phe)_6$). A possible explanation of the low value of $-0.0024\ h^{-1}$ obtained with $G_1(Phe)_6$ might be a strong binding to cytosolic proteins.

Table 1. Velocity constants for the first step of the degradation of the dendrimers.[a]

compd.	papain	chymotrypsin	MCF-7 cytosol
$G_0(Met)_3$	−0.0763	−0.0054	−0.0112
$G_0(Phe)_3$	−0.0604	−0.0647	−0.0071
$G_1(Met)_6$	−0.1646	−0.072	−0.0208
$G_1(Phe)_6$	n.d.[b]	n.d.[a]	−0.0024

[a] Velocity contants give per h. [b] not determinable.

Digestion under cell culture conditions

The interpretation of experiments on intact cells is much more difficult because various parameters have to be considered. The cell culture medium contains in addition, anorganic salt enzymes and proteins which can bind or digest the dendrimers. Furthermore, prior to a possible intracellular degradation the dendrimers have to cross the cell membrane.

MCF-7 cells were incubated with the respective dendrimers for 24 h. Subsequently, the cells were lysed and the dendrimers were separated at a basic pH value of 9.0 using SPE materials. Under the basic conditions used a stable bondage via their amino groups on the polymeric adsorption material took place. Other components could be eluted with neutral or basic solutions. Desorption of the dendrimers from the polymer is then conveniently possible with the phosphate buffer (pH 2.7) used for CE analyses supplemented with methanol (see Table 2).

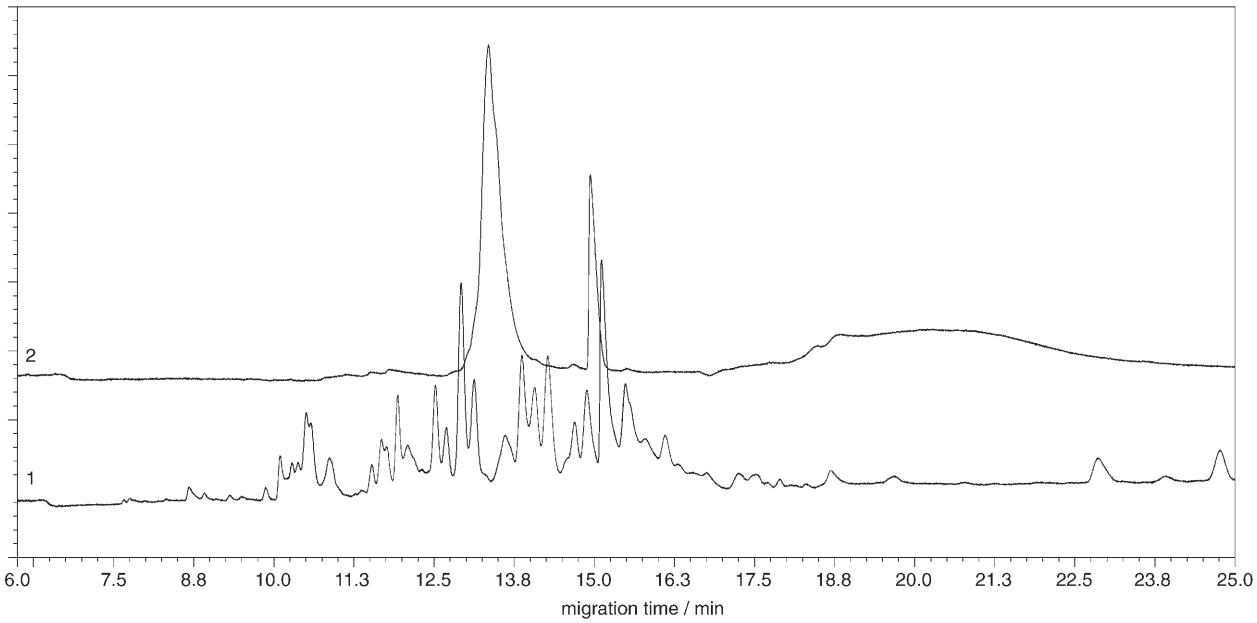

Figure 3. Electrophoretical control of activity for trypsin after a 24 h incubation with $G_1(Dap)_6$ by the digestion of casein: Lane 1 product profile, lane 2 undigested casein.

Table 2. Mixtures necessary for elution and the recovery of dendrimers from the SPE cartridges.[a]

compd.	recovery without cell lysate [%]	recovery in cell lysate [%]	elution mixture[b]
G_0	104±3	90±1	100:0
$G_0(Met)_3$	87±8	94±15	50:50
$G_0(Phe)_3$	91±4	81±3	50:50
$G_0(Asp)_3$	72±11	65±4	50:50
$G_0(Dap)_3$	87±8	72±2	100:0
G_1	88±6	n.d.[c]	50:50
$G_1(Met)_6$	59±3	n.d.[c]	10:90
$G_1(Phe)_6$	64±7	n.d.[c]	10:90
$G_1(Asp)_6$	81±3	n.d.[c]	50:50
G_2	71±5	n.d.[c]	50:50

[a] Dendrimers applied as 0.26 mg in 4 mL to the cartridge, $n=3$. [b] *v/v* phoshate buffer pH 2.7:methanol. [c] not determinable.

In a first experiment an aqueous solution of the dendrimers were examined. The recovery rate was nearly independent of the attached amino acid (for example, recovery of $G_0(Met)_3$, $G_0(Phe)_3$, and $G_0(Dap)_3 \approx 90\%$). With increased generation, however, the recovery was lowered (for example, G_0 (104%) > G_1 (88%) > G_2 (71%)) and could not be abolished by higher methanol portions in the elution mixture. These trends contradicted the Asp-modified dendrimers $G_0(Asp)_3$ and $G_1(Asp)_6$ with a recovery rate of 72% and 81%. It is worthy to mention that the addition of cell lysate to the dendrimer solution had only low effects on the recovery rate (see Table 2). Therefore, it is possible to study the accumulation and the degradation of the dendrimers in intact cells.

The cells were incubated for 24 h with G_0, $G_0(Met)_3$, or $G_0(Phe)_3$. In the SPE eluates obtained from the lysates only one species was detected which migrated identically to the **G_0** molecule. ($t_m = 6.1$ min, see Figure 5), indicating a complete degradation of the dendrimers. Under the conditions used none of the possible degradation products could be identified. Therefore, it was tried to lower the detection limit for these compounds by post-column derivation in the SPE eluates. After cleavage of amino acids from the dendrimer, the resulting primary amines were reacted with fluorescamine which enlarged the chromophore improving DAD-detection and additionally led to fluorescent agents. But again the electropherograms exhibited only the signal for the G_0 dendrimer and gave no hints for possible breakdown products.

To confirm these electrophoretical findings the SPE eluates were analyzed by MALDI-TOF-MS. Although the salt content in the samples was relatively high it was possible to identify the G_0 dendrimer with high intensity in the lysates of cells incubated with $G_0(Met)_3$, $G_0(Phe)_3$, $G_0(Asp)_3$, and $G_0(Dap)_3$. With the exception of $G_0(Phe)_3$ and $G_0(Asp)_3$ which were found in traces, no further dendrimers were present. Apparently, the proteolytic activity in intact cells was very high and the amino acids were completely cleaved from the educts.

The results obtained with dendrimers of the G_1 generation were not as clear. If at all, the electropherograms showed only signals for the G_1 molecule. In the MALDI-TOF mass spectra of $G_1(Asp)_6$ and $G_1(Dap)_6$ no mass originating from the dendrimer could be detected. $G_1(Phe)_6$ and $G_1(Met)_6$ nearly completely lost their surface bound amino acids. G_1 and the sodium adduct of G_1 were detected with high intensities (see Figure 6). $G_1(Phe)_1$ and $G_1(Met)_1$ were present in traces.

To verify whether the dendrimers were extra- or intracellularly digested we also electrophoretically examined the cell culture media. In the media the intact molecules and in distinctly lower concentrations the first digestion products (cleavage of one amino acid) were found. As no G_0 dendrimer could be detected, it can be assumed that the molecules reached the cytosol and were digested there.

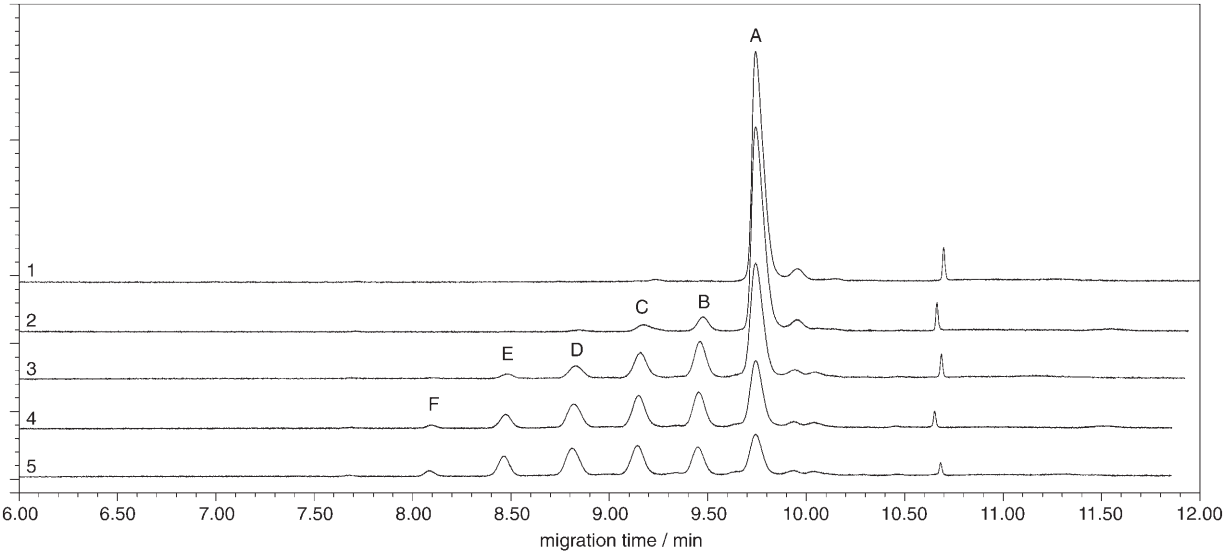

Figure 4. Overlay of electropherograms of the different time points of the digest of $G_1(Met)_6$ (Peak A) by MCF-7 cytosol. The methionine content in the dendrimer decreases with rising alphabet. Time points: $t=0$ h (lane 1), $t=4$ h (lane 2), $t=25$ h (lane 3), $t=50$ h (lane 4), and $t=70$ h (lane 5).

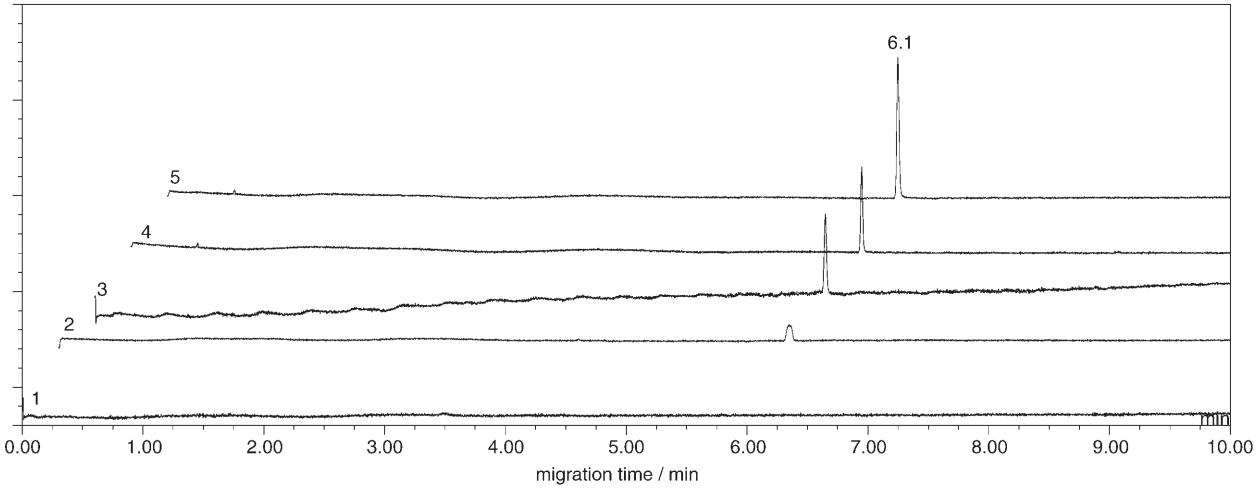

Figure 5. Electropherograms of SPE eluates of the cellular uptake studies for G_0 (lane 2), $G_0(Met)_3$ (lane 3), $G_0(Phe)_3$ (lane 4). For comparison a blank (lane 1) and a standard of compound G_0 (20 μg mL^{-1}) are depicted.

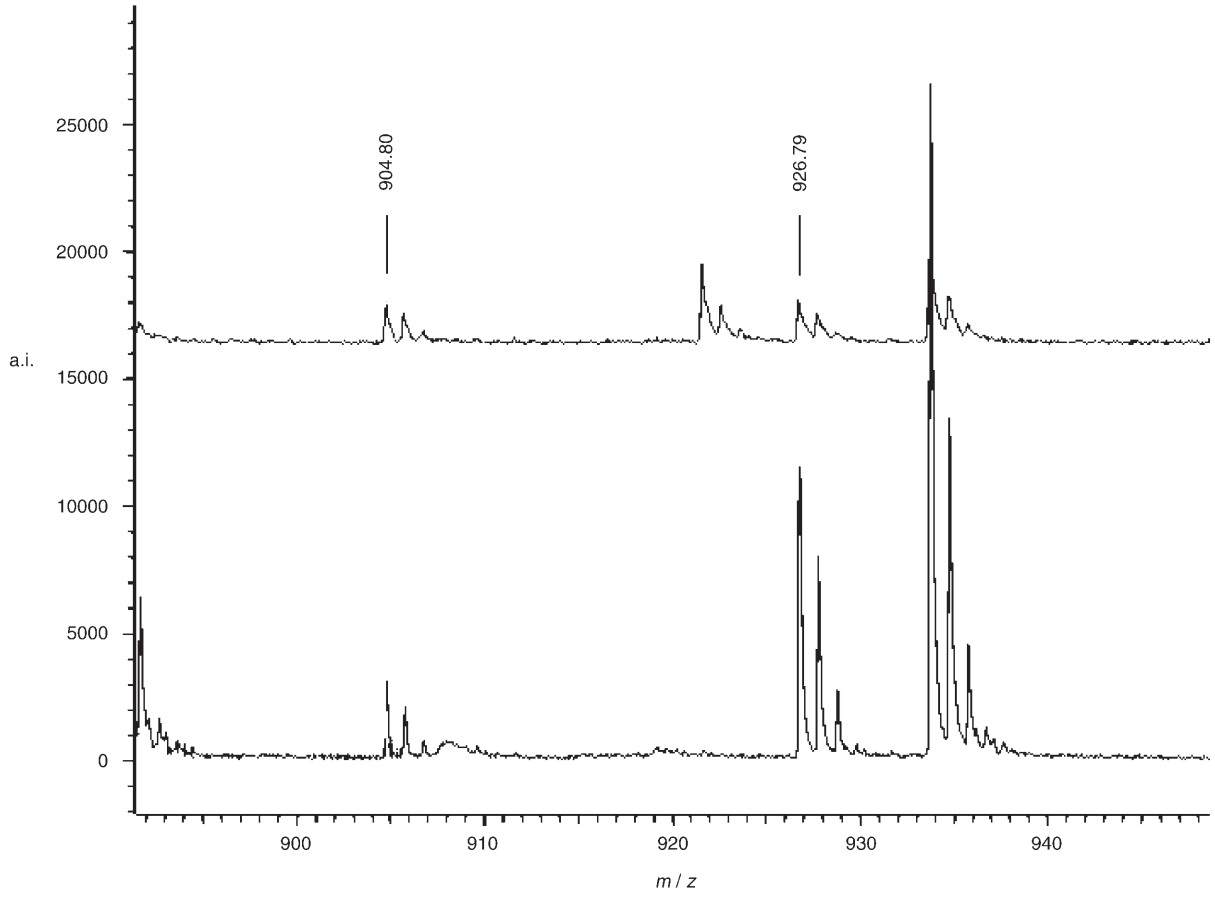

Figure 6. MALDI-TOF spectra from eluates of the cellular uptake of G_1 (top) and $G_1(Phe)_6$ (bottom). ($[G_1+H]^+ = m/z$ 904.8 and $[G_1+Na]^+ = m/z$ 926.79).

Due to the complete degradation of the dendrimers it is possible to calculate the intracellular accumulation for G_0, $G_0(Met)_3$, and $G_0(Phe)_3$ on the basis of the G_0 content. Whereas G_0 was 1.2 ± 0.1 times accumulated in the cells, $G_0(Met)_3$ and $G_0(Phe)_3$ caused a cellular accumulation grade of about 3.6 ± 0.2 and 9.6 ± 0.7, respectively.

The results obtained in this study are very helpful for the interpretation of the in vitro effects[17] for example, of G_0, $G_0(Met)_3$, and $G_0(Phe)_3$. In the in vitro assay for cytotoxicity G_0 and $G_0(Met)_3$ reduced the proliferation of MCF-7 cells only marginally. This might be the consequence of a fast digestion of $G_0(Met)_3$ giving at the end the nontoxic G_0. $G_0(Phe)_3$ showed a

considerable higher cytotoxic activity in this assay although a fast degradation was also observed. However, because of its distinctly higher accumulation rate a toxic level of surface modified G_0 molecules was reached. Nevertheless, it will be necessary to study the kinetics of digestion of the dendrimers, which will be part of a further study.

Besides intracellular effects, cell death can also be achieved by membrane effects. Dendrimers of higher generations accumulated less in tumor cells but caused, as already earlier reported enormously increased efficacy for membrane rupture with increasing dendrimer generation.[21,22]

Conclusions

In this study we showed the accessibility for proteolytic enzymes of amino acids bound to dendrimers, making them attractive delivery systems for drugs. Electrophoretical methods for the detection and quantification of dendrimers in tumor cells were presented. With these tools the efficient degradation in MCF-7 cancer cells of digestible dendrimers was demonstrated. In future we hope to lower the detection level for dendrimers using fluorescence detection to quantify the break down of amino acid modified dendrimers as well as to study their degradation up to 24 h. This would give a more detailed insight into their mode of action.

Experimental Section

Capillary electrophoresis: All investigations were performed on an Agilent 3D capillary electrophoresis system equipped with a diode array detector (capillary: uncoated, 56 cm length/50 μm i.d; running buffer: 0.02 M NaH_2PO_4 adjusted to pH 2.7 with H_3PO_4; applied voltage: 30 kV; cassette temperature: 20 °C). For the enzyme activity control an uncoated capillary (76 cm length/50 μm i.d.) was used. The separation was performed in $Na_2B_4O_7$ buffer (0.1 M; pH 9.6) with 30 kV at 20 °C. Samples were injected by pressure (50 mbar for 4 s). At the beginning of a sequence, the capillary was consecutively flushed with 1 M NaOH for 5 min, double distilled water, and running buffer. Between each run the capillary was flushed with running buffer for 5 min.

Nonenzymatic and enzymatic hydrolysis of dendrimers: For the investigation of the nonenzymatic hydrolysis the dendrimers were incubated in the respective buffers (pH 2.7 to 8.1) for 72 h at 37 °C. Aliquots were acidified to pH 2.7 and analyzed by capillary electrophoresis. The incubations with standard enzymes were performed with 1 mg mL^{-1} dendrimer and 10U enzyme. Tris-buffer (0.1 M, pH 8.1) was used as digestion buffer for trypsin, NaH_2PO_4 (0.1 M adjusted with NaOH to pH 6.6) for chymotrypsin and papain. Papain was activated prior to use by short incubation with 10 mM L-cysteine and 1 mM ETDA in the digestion buffer at 37 °C. For the digestions with pepsin the running buffer was used. After appropriate time points an aliquot was taken and acidified with 0.1 M phosphoric acid to pH 2.7 and immediately analyzed by capillary electrophoresis. The enzyme activity was tested prior to incubation with the dendrimers using standard proteins: BSA (treated with 10 mM cysteine at 70 °C for 10 min) was appropiate for pepsin, chymotrypsin, and papain, whereas casein was used for trypsin. The cytosolic fraction used for digestion was isolated as follows: MCF-7 cells were harvested and washed with ice-cold PBS. Postnuclear supernatent containing all cytoplasmic compounds were yielded by swelling the cells in ice-cold hypotonic buffer (10 mM Tris, 10 mM NaCl, 1.5 mM $MgCl_2$ (x 6 H_2O), adjusted to pH 7.4) and rupturing the cells by vortexing. After centrifugation (2000 g, 5 min^{-1}, 4 °C) the supernatant was lyophilized. The lyophilisate was resolved in the digestion buffer (0.2% *w/v* Triton-X-100, 10 mM cystein-hydrochloride, 2 mM EDTA, 0.1 M KH_2PO_4 adjusted with KOH to pH 5.5). After a short time of activation the dendrimers were added resulting in a concentration of 1 mg mL^{-1}.

Cell culture conditions: The human MCF-7 breast cancer cell line was obtained from the American Culture Collection (ATCC, Rockville, Md.). The MCF-7 cells were maintained in MEM Eagle's medium containing L-glutamine, supplemented with $NaHCO_3$ (2.2 g L^{-1}), sodium pyruvate (110 mg L^{-1}), gentamycin (50 mg L^{-1}), and 10% fetal calf serum (FCS; Gibco Eggenheim, Germany) using 75 cm^2 culture flasks (Nunc) in a water-saturated atmosphere (5% CO_2) at 37 °C. The cells were serially passaged weekly following trypsinization using 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA). Mycoplasma contamination was routinely monitored, and only mycoplasma-free cultures were used.

Cell accumulation studies: MCF-7 cells were seeded in 75 cm^2 culture flasks (Nunc). When the cells reached 50–60% confluence (approximately five days after incubation) the medium was exchanged by serum free EMEM containing the dendrimers. After 24 h the media was removed and stored for later analysis. The cells were harvested by trypsination and washed with ice-cold PBS. The resulting cell pellets were stored till analysis at −18 °C. The cells were sonificated in adsorption buffer (0.02 M $MgCl_2$, 0.1 M $Na_2B_4O_7$, 0.1 M NaCl, und 0.1% *w/v* Triton-X-100 in double distilled water). An aliquot was taken for protein measurement according the method of Bradford.[23] The cell lysate was put on a solid phase extraction column (strata-X 30 mg, Phenomenex, conditioned by 1 mL methanol and 1 mL water) and after washing with $Na_2B_4O_7$ buffer (0.01 M; pH 9.6) the compounds were eluted with running buffer/methanol as mentioned in Table 2. Calibration was performed with external standards. The cellular concentration of the dendrimers in the MCF-7 cells was determined as previously published.[24] The accumulation grade was the ratio of cell associated dendrimer and the compound concentration in the medium (100 μM). For recovery experiments adsorption buffer or cell pellets were spiked with a known amount of compound (0.26 mg applied in 4 mL). For post column derivatization 238 μL eluate was brought to pH 8 by adding 12 μL of 1 M NaOH and 50 μL of a fluorescamine solution (1 mg mL^{-1} in acetonitrile) was added. Then the samples were shaken and were immediately analyzed by capillary electrophoresis. MALDI-TOF-MS of the SPE eluates was performed with a Bruker Reflex with a delayed extraction source. For all samples the matrix used was CCA.

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Keywords: capillary electrophoresis · cellular uptake · dendrimers · enzymatic hydrolysis · MCF-7 breast cancer cell line

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