5

The cell viability effects of layer-by-layer cell encapsulation

Abstract

Layer-by-layer functionalization of the cell membrane of mammalian cells might prove to be a novel method for all sorts of cell therapy. If cell encapsulation can be tuned to encapsulate only cells of a specific type, this method may be useful the fight against circulating malignant cells, e.g. in leukemia or metastatic tumor cells. For tumor therapy by cytotoxic cell encapsulation, it is necessary to know whether the cell encapsulation has cytotoxic effects on both the tumor cells and on non-malignant cells. In this chapter, the CXCR4-specific cell surface modifications of the previous chapter have been expanded to layer-by-layer cell encapsulation. The interactions between the layers were provided by multivalent β-cyclodextin–adamantane non-covalent interactions. All the building blocks for the encapsulation were shown to have no negative effect on the cell viability. After functionalizing the cell membrane of CXCR4-overexpressing cells with adamantane-groups, up to 5 layers of polymers were added to the cells, where each subsequent layer decreased the viability of the cells. This effect was only observed when cells were kept in suspension, and not when this cell-encapsulation strategy was applied to adhered cells. Control studies with a cell line also showed no negative effects of the subsequent exposure of the polymers. After encapsulating cells in 5 layers of polymers, the polymer layers could still be penetrated by the chemotherapeutic drug doxorubicin, opening the way for combined therapy using chemotherapeutics and cell encapsulation strategies.

5.1. Introduction

Layer-by-layer (LbL) functionalization is a supramolecular technique used to chemically alter surfaces.[1] In this approach each layer is designed to interact with the previous layer, thereby changing the surface composition. When applied to cells this technique is called cell encapsulation and can be used to influence the interactions with the environment.[2] For instance, therapeutic cells or transplants can be shielded from the immune system, leading to a lower chance of repulsion or physical stress.[3, 4] On the other hand, if diseased e.g. tumor cells can be isolated, metastasis and/or growth of the disease could be halted, thereby improving the therapeutic efficacy.

Historically, the first layer of cell encapsulation was always a positively charged polymer that interacts with the negatively charged cell surface through electrostatic interactions.[5, 6] Subsequently, a layer with negatively charged substituents can bind to this modified surface. This process can be repeated by alternating between positively and negatively charged polymers. A large disadvantage in this method is that most cationic polymers used in the electrostatic LbL method, such as poly-L-lysine and polyethyleneimine, are considered toxic to eukaryotic cells.[7, 8] To circumvent cytotoxic effects of the polycations, alternative methods based on hydrogen bonds between the layers were applied which did not influence the viability or function cells.[9, 10]

An ideal cell encapsulation method would include a selection mechanism for which cells are encapsulated and which cells are not. For the selection of certain cells, surface bound receptors are very suitable. In the previous chapter, a method was described to alter the exterior of one part of the cells in a population, based on the presence or absence of the

chemokine receptor 4 (CXCR4), a receptor that is abundant on both tumor cells and stem cells.

In the current study, the feasibility of a receptor-specific LbL encapsulation method based on cyclodextrin (CD)—adamantane (Ad) interactions and its impact on cell viability was investigated. For fluorescence measurements, CD- and Ad-polymers were grafted with two different fluorescent dyes, Cy3 and Cy5. These fluorophores were chosen because of their compatibility with each other and GFP.

The amount of surface that can be used for cell encapsulation was presumed to be dependent on the state of the cells. Cells that are kept in suspension can be covered all-around, while adhered cells cannot be functionalized on the bottom-side (Figure 1). The viability effect of cell encapsulation was assessed for both cells in suspension and adhered cells. Finally, the penetration of the chemotherapeutic agent doxorubicin through a 5 layer encapsulation construct was investigated.

5.2. Experimental section

5.2.1. General

All starting chemicals were obtained from commercial sources and used without further purification. ISOBAM-04 was kindly supplied by Kuraray Europe GmbH. NMR spectra for compound analysis were taken by using a Bruker DPX 300 spectrometer (for **3**) or a Bruker AMX 500-MHz with a TXI gradient probe (for polymers).

The synthesis of Ac-TZ14011-Ad (**1**) and CD_{74}-Cy3-polymer (**2**) are described in the previous chapter.

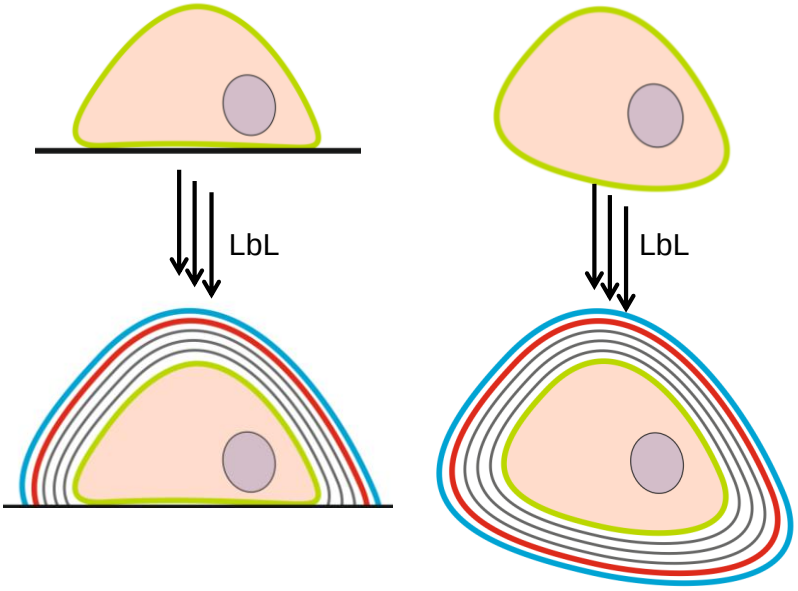
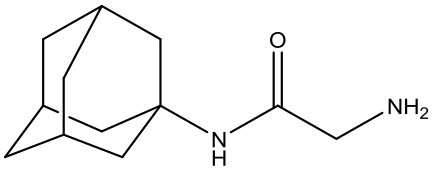


Figure 1: Schematic drawing of layer-by-layer encapsulation of cells, showing possible differences between encapsulation of adhered cells (left-hand side) and cells in suspension (right-hand side).

5.2.2. Synthesis

Synthesis of adamantan-1-yl-2-aminoacetamide (3)



Synthesis of **3** was performed according to a previously published procedure.[11] In short, N-(tert-butoxycarbonyl)-glycine (0.35 g, 2.0 mmol), PyBOP (1.3 g, 2.5 mmol) and DiPEA (0.55 mL, 3.0 mmol) were added to 15 mL dry THF. The reaction was stirred for 1 h at RT, after which amantadine hydrochloride (0.38 g, 2.0 mmol) and DiPEA (0.55 mL, 3.0 mmol) were added and the reaction was stirred for 24 h at RT. After removal of the solvents *in vacuo*, the product was purified by silica column

chromatography (eluens 2 % MeOH in $CHCl_3$). After removal of the solvents *in vacuo*, the product was dissolved in CH_2Cl_2 and 4 ml trifluoroacetic acid was added. The reaction was stirred for 1 h at RT. The solvents were removed *in vacuo*, and the product was washed three times with Et_2O, yielding 0.25 g adamantan-1-yl-2-aminoacetamide (34 % over 2 steps).
^{1}H-NMR (DMSO-d6, 300 MHz): 8.00 (s, 3H), 3.48 (s, 2H), 2.04 (s, 3H), 1.96 (s, 6H), 1.64 (s, 6H). ^{13}C-NMR (DMSO-d6, 75 MHz): 165.2, 51.8, 41.5, 40.9, 36.4, 29.3.

Synthesis of CD_{82}-Polymer (4)

Synthesis of **4** was largely similar to the previously described synthesis of **2** (described in the previous chapter). ISOBAM-04 (12 mg, 0.2 µmol) was dissolved in 3 ml dry DMSO. DIPEA (20 µL, 100 µmol) and 160 equivalents of 6-monodeoxy-6-monoamino-β-cyclodextrin (37.5 mg, 32 µmol) were added and the solution was stirred overnight at 80 °C. After cooling to RT, the polymer was dialyzed against H_2O for 1 day. The solution was dialyzed against $NaHCO_3$ buffer pH 9.0 for one day and dialyzed against pure H_2O for 5 days while daily refreshing the dialysis medium. The solution was lyophilized to give a white powder (19.1 mg).

The grafting efficiency of the cyclodextrin groups onto the polymers was determined using ^{1}H-NMR measurements. Comparing the integrals of CD peaks (5.2-3.4 ppm) with those of the polymer backbone (3.0-0.8 ppm) revealed that the polymer was functionalized with on average 82 CD groups, resulting in an estimated molecular weight of 154 kDa and an estimated reaction yield of 62 %.

Synthesis of Ad_{120}-Polymer (5)

ISOBAM-04 (50 mg, 0.83 µmol), 100 equivalents of adamantan-1-yl-2-aminoacetamide (**3**) (30 mg, 83 µmol), and DIPEA (83 µL, 0.4 mmol) were added to 5 ml dry DMSO and the reaction was stirred overnight at 80 °C. After cooling to RT, and dialysis in a manner identical to that described above, the solution was lyophilized to give 49.3 mg of a white solid.

The grafting efficiency of the adamantane groups on the polymer backbone was determined by ^{1}H-NMR measurements. Comparing the integral of the glycine spacer peak (3.7 ppm) with those of the polymer backbone (3.0-0.8 ppm) revealed that the polymer was functionalized with on average 120 Ad groups, resulting in an estimated molecular weight of 90 kDa and an estimated reaction yield of 66 %.

Synthesis of Ad_{150}-$Cy5_{0.5}$-Polymer (6)

ISOBAM-04 (50 mg, 0.83 µmol), 1.2 equivalents of Cy5-NH_2 (1.0 µmol) and DiPEA (83 µL, 0.4 mmol) were added to 5 ml dry DMSO and the reaction was stirred 80 °C overnight. Adamantan-1-yl-2-aminoacetamide (**3**) (30 mg, 83 µmol) was added, and the reaction was stirred again at 80 °C overnight. After cooling to RT and dialysis in a manner identical to that described above, the solution was lyophilized to give a blue solid (46 mg).

The grafting efficiency of the adamantane groups was determined similar to **5**, revealing that **6** was functionalized with on average 150 Ad groups. The grafting efficiency of the fluorophore groups was determined by UV/Vis spectroscopy. A fraction of the polymer was weighted and dissolved in H_2O to a concentration of 1 mg/ml. UV/Vis measurements of this solution revealed that on average 0.5 Cy5 groups (ϵ = 250,000 M^{-1} cm^{-1})

were grafted to each polymer. Combining the NMR and UV/Vis data resulted in a final estimated molecular weight of 92 kDa and an estimated reaction yield of 57 %.

DOSY analysis

Diffusion Ordered Spectroscopy (DOSY) measurements were performed to verify the purity of the polymers. None of the polymer solutions showed presence of any small molecules (Figure 2). The hydrodynamic radii of the polymers could be extracted from the diffusion constants using the Stokes-Einstein equation (Eq 1):

$$D = \frac{K_B * T}{6\pi * \eta * r} \qquad (1)$$

D = Diffusion constant, K_B = Boltzmann constant, T = temperature, η = the viscosity, and r = radius

Fluorescence quantum yield measurement

For the fluorescently labeled polymers (**2** and **6**), a dilution series was made, and the fluorescence intensity was plotted against the measured absorption (A < 0.1 to prevent inner filter effects). The slopes were compared to the slopes of unconjugated fluorophores, of which the fluorescence quantum yield are reported as QY(Cy3) = 4 % and QY(Cy5) = 27 %.[12]

5.2.3. Cell culture

Human MDAMB231 cells, transfected with human CXCR4 conjugated to GFP (MDAMB231-X4), were kindly provided by Dr. Gary Luker (Center for

Molecular Imaging, University of Michigan, USA). Native MDAMB231 cells with basal CXCR4 expression were used as a negative control. Cells were maintained in Dulbecco's minimum essential medium (DMEM) enriched with 10% fetal bovine serum and 5 mL Penicillin/Streptomycin (10k units/mL Penicillin; 10 mg/mL Streptomycin) (all Life Technologies Inc., Breda, The Netherlands). Cells were kept under standard culture conditions.

Building of layers upon the cells, first of all the cells were incubated with Ac-TZ14011-Ad (**1**, 0 °C, 15 min). After that they were incubated alternately with CD- and Ad-polymers in that order (0 °C, 15 min). After each incubation with CD-polymer, cells were washed with PBS. The outer two layers always consisted of polymers with a fluorescent label (**4** or **6**). This is combined in the following incubation scheme:

Table 1: Scheme for the LbL of cells with 1-5 layers of polymers

t (min)	0	15		30	45		60	75	
1	**1**	**2**	Wash						
2	**1**	**2**	Wash	**6**	Wash				
3	**1**	**4**	Wash	**6**	**2**	Wash			
4	**1**	**4**	Wash	**5**	**2**	Wash	**6**	Wash	
5	**1**	**4**	Wash	**5**	**4**	Wash	**6**	**2**	Wash

For the confocal experiments cells were seeded on glass-bottom wells containing 2 mL of standard culture medium (confluence 70-80%). Solutions of **1**, **2**, **4, 5** or **6** were added to the medium according to Table 1 to obtain final [Ad] or [CD] of 10 µM. Washing steps were performed using PBS at 0 °C. The [fluorophore] in the two last incubation steps was 50-150 nM.

Live cell images were taken on a Leica SP5 confocal microscope under 63x magnification. GFP luminescence (CXCR4 receptor) was

measured using 488 nm excitation and emission was collected at 500-525 nm. Cy3 fluorescence (**2**) was measured using 514 nm excitation and emission was collected at 570-600 nm. Cy5 fluorescence (**6**) was measured using excitation at 633 nm and emission was collected at 650-700 nm.

5.2.4. Cell viability assays

MTT assay

An MTT test was used to determine the cell viability after the different incubation steps according to reported procedures.[13, 14] MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was dissolved in PBS to a concentration of 5 mg/ml. The total incubation volume in each well was adjusted to 200 μL using PBS. Cells were optically checked under a light microscope for blebbing and 20 μL MTT solution was added, resulting a total incubation volume of 220 μL, and the plate was incubated for 4 h at 37 °C. The medium was removed and 100 μL DMSO was added to each well to extract the insoluble formazan product. After 10-15 minutes at 37 °C, absorbance at 545 nm was measured in a plate reader (1420 Multilabel Counter, Perkin Elmer) to determine the uptake of MTT. Hereby a higher absorbance related to a higher metabolic rate, thus a higher degree of live cells. Measurements were corrected for background and normalized to untreated cells. Normalized values were fitted with equations in the GraphPad Prism 6 software. The K_D values were calculated using the "Binding-Competitive, One site-Fit LogIC50" nonlinear regression equation (Eq 2).

$$y = \frac{Top - Bottom}{1 + 10^{x - \log LD_{50}}} + Bottom \qquad (2)$$

Viability effects of the individual compounds

Adherent cells: MDAMB231-X4 cells were harvested, counted and diluted to 40,000 cells/mL using medium. To each well of a transparent 96-well plate (Cellstar®, Greiner Bio-One, Alphen a/d Rijn, The Netherlands) was added 100 µL (4,000 cells) and the cells were allowed to adhere for 24 h at 37 °C. A solution of the compound of interest (**1, 2** or **6**, 50 µl/well, n=2 for each concentration) was added to the cells (final concentration 1nM - 90 µM). To determine the cytotoxic effect of the compounds added, the cells were incubated for 24 h at 37 °C (70-80 % confluence was reached in the untreated wells). After incubation, an MTT assay was performed as described above.

Cells in suspension: MDAMB231-X4 cells were harvested, counted and diluted to 80,000 cells/mL using medium. Of this solution, 200 µl (16,000 cells) was added to each polystyrene tube. A solution of the compound of interest (**1, 2** or **6**, 100 µl/tube) was added (final concentration 1nM - 90 µM). The tubes were shortly vortexed, and the contents of each tube was divided over two wells in a 96-wells plate (8,000 cells/well) and incubated for 24h at 37 °C (70-80% confluence), after which an MTT assay was performed.

Control experiments: In each experiment, control cells (only incubated with PBS; same cooling, washing and incubation steps) were included to correct for the possible loss of viability due to different incubation times on ice.

Viability effects of LbL encapsulation

Adherent cells: MDAMB231-X4 cells were harvested and seeded similar to the procedure for the individual compounds. Before incubations, cells were

cooled on ice. In each well, cells were incubated according the LbL functionalization scheme depicted in Table 1, where polymers **2** and **6** were used instead of polymers **4** and **5**. In the first step, 50 µl of a Ac-TZ14011-Ad (**1**) solution was added (incubation concentration 5 nM – 90 µM; n=2 for each concentration). In all consecutive steps, the amount of CD or Ad was kept equal to the previous step. Due to the volume increase when **2** was added, incubation concentrations of **2** were always 75 % of the concentrations of **1** and **6**. After the final compounds, the 96-wells plates were put in a cell incubator at 37 °C for 24 h and an MTT assay was performed.

Cells in suspension: Functionalization with Ac-TZ14011-Ad (**1**) and the LbL building of polymer layers was very similar to adhered cells. The differences were that freshly harvested cells were used in suspension, that incubations (100 µl in each step) were performed in polystyrene tubes (16,000 cells/tube) and that the tubes were shortly vortexed after each incubation to keep the cells in suspension and allow complete coverage. After the final compounds, to determine the viability influence of the different incubations, cell suspensions were transferred into 2 wells on a 96-wells plate and the 96-wells plates were put in a cell incubator at 37 °C for 24 h so the viable cells could adhere for the MTT assay.

Cell-line specific cytotoxicity: In one experiment where 5 layers of polymers were added to cells in suspension, the CXCR4-overexpressing MDA-MB-231-X4 cells were replaced by MDAMB231 cells with a basal CXCR4 expression.

The effect of LbL encapsulation on the toxicity of doxorubicin

MDAMB231-X4 cells in suspension were encapsulated with 5 layers following the procedure described above with [CD] = [Ad] = 1.0 µM to

prevent cytotoxicity from the encapsulation. The medium was removed by centrifugation (3 min, 4 °C, 300 x g) and decantation. After that, to each tube, 100 µl of a doxorubicin dilution (0.5 nM – 60 µM) was added. The cells were transferred to a 96-wells plate and put in a cell incubator at 37 °C for 24 h.

As a control, the same dilutions of doxorubicin were added to tubes in which the cells were incubated only with PBS, with all other steps (cooling, washing, etc) the same.

Statistical analysis

Differences between found LD_{50} values were compared using One-Way ANOVA with post-hoc Tukey tests. In the study comparing the toxicity of doxorubicin, a Student's t-test was applied.

5.3. Results and discussion

5.3.1. Polymer synthesis and analysis

To prevent competition between the fluorescent label and the Ad or CD, the fluorescent label was conjugated first. The CD-polymers were synthesized as described previously.[15, 16] The different polymers were analyzed using ^{1}H-NMR, DOSY, UV/Vis and fluorescence measurements (see methods section). The grafting ratios of the different ISOBAM-04-based polymers are summarized in Table 2.

Table 2: Grafting parameters of the different polymers used in this study

	CD or Ad/polymer	Fluorophore	QY	MW (kDa)[a]	r (DOSY) (nm)
2	72 CD	1.5 Cy3	9 %	142	7.4
4	82 CD			154	6.2
5	120 Ad			90	5.7
6	150 Ad	0.5 Cy5	46 %	97	4.9

a: Molecular weight was estimated using the grafting ratios found by ^{1}H-NMR

Although ^{1}H-NMR was successfully used to determine grafting ratios of the CD-groups, determination of grafting ratios of the adamantane groups required the introduction of a short glycine spacer, which was introduced between the adamantane moiety and the polymer backbone. Other than the Ad-peaks, the ^{1}H-NMR-peaks of this glycine spacer (3.7 ppm) did not overlap with the peaks of the polymer backbone and could thus be used to determine the degree of functionalization. In the polymer grafting the polymers Ad polymers show a slightly larger grafting ratio compared to the CD polymers (Table 2).

5.3.2. LbL encapsulation method based on supramolecular interactions

To study the LbL functionalization, confocal images were taken of adhered cells with fluorescent polymers (**4** or **6**). The final two layers always consisted of fluorescent polymers, while the other layers consisted of non-fluorescent polymers (**3** or **5**). A schematic representation of the layer construction is given in Figure 2A. With up to five layers of polymers, the fluorescence signals of Cy3 and Cy5 showed co-localization with the previous layer and the fluorescence signal of the CXCR4 receptors containing an GFP-tag (Figure 2B).

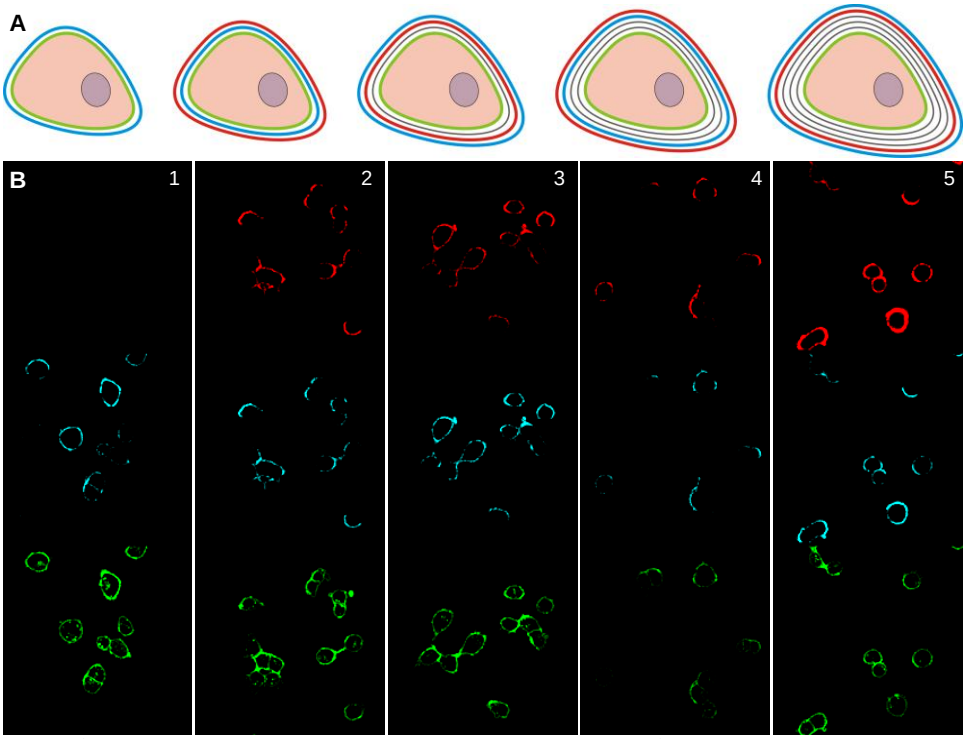


Figure 2: A) Schematic representation of the layers of polymers around the cells. B) Confocal images of cells with increasing number of layers, in which the two outermost layers were fluorescent. Blue and red layer in A) correspond to the cyan and red images in B) below. Cy5 in red, Cy3 in cyan, GFP in green.

To determine the exact structure of the LbL construct, the resolution of confocal microscopy (± 50 nm)[17] is much too low compared to the size of the layers (± 5 nm). However, co-localization on confocal microscopy of each layer with the previous one shows that all layers are attached to each other. The uneven distribution of polymers around the cells seems to follow the unevenly distributed receptor. All polymers are negatively charged due to the high abundance of carboxylates, this provides a repulsive electrostatic interaction between the layers (except for the interaction of the first polymer layer with Ac-TZ14011-Ad, which is described in the previous chapter). The host-guest interactions between the

polymer layers apparently overcome the repulsive electrostatic forces, similar to the system described by Grana-Suarez et al.[15]

5.3.3. Viability effects of the individual components

Building multiple layers around a cell membrane could impede with cell function and influence the cell viability. This effect was assessed in both adhered cells and cells in suspension using a MTT-based cytotoxicity assay.

Incubation of adhered cells with compounds of interest is the standard method to determine cell viability and cytotoxicity,[13] allowing a simple follow-up by an MTT assay, for which the cells need to be adhered. In cell encapsulation, adhered cells would only represent partial coverage of the cell surface. While a complete encapsulation may be achieved on cells in suspension (Figure 1), this difference in encapsulation coverage could influence the cell viability. Therefore, next to the standard method, the incubation steps were performed in solution, then viable cells were allowed to adhere, after which an MTT assay could be performed.

In order to correctly assess the cytotoxicity of the LbL construct, it is prerequisite that the individual components are non-toxic in these conditions. Cyclodextrin and amantadine are not considered cytotoxic at the used concentrations,[18, 19] and when applied separately, none of the individual components showed any loss of viability. This was tested up to at least 90 µM (Figure 3A,B). This outcome was identical for both incubation methods.

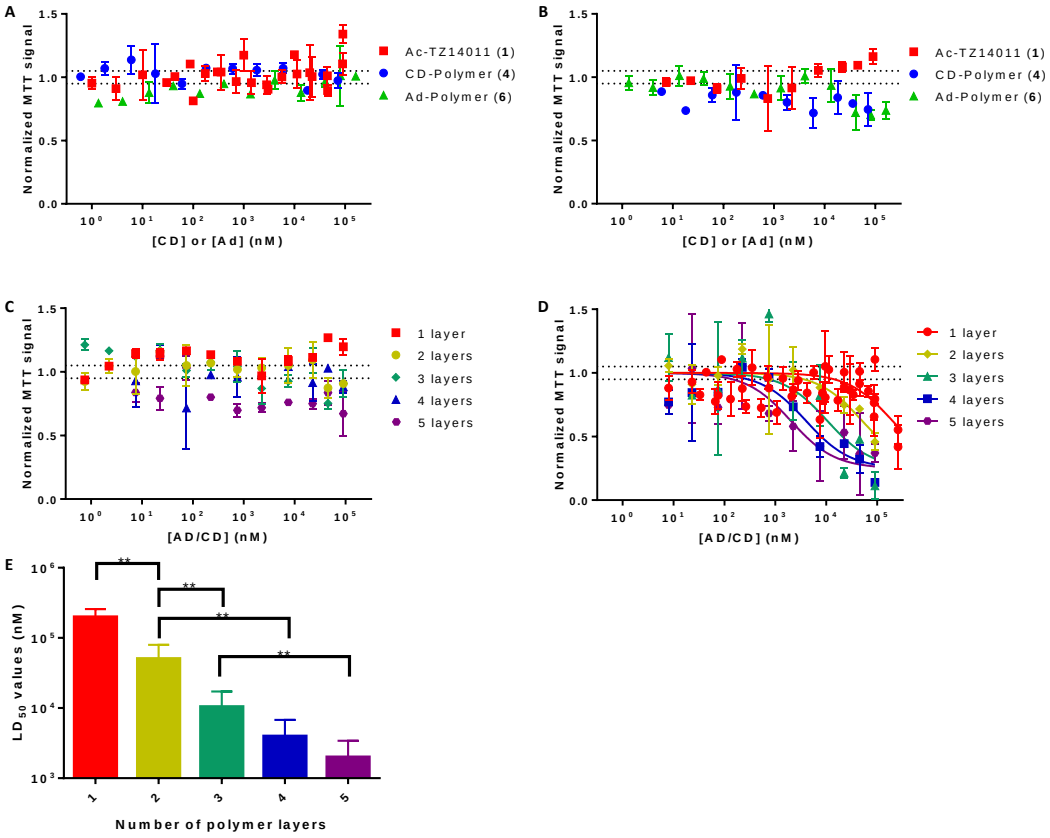


Figure 3: Normalized MTT assay values of cell viability assays based on MTT absorption. Effects of individual components on A) adhered cells and B) cells in suspension. Effects of LbL functionalization on C) adhered cells and D) cells in suspension. Dotted lines are eye-guides at 95% and 105% viability. E) Fitted LD_{50} values of LbL construction in suspension, showing the statistical differences ** p<0.01

5.3.4. Viability effects of LbL encapsulation

Up to five layers, the layer-by-layer encapsulation yielded no measurable effects on adhered cells (Figure 3C), including the concentrations used during confocal imaging (Figure 2). When the same encapsulation occurred in suspension, cytotoxic effects were observed at higher concentrations, We observed a correlation between the number of polymer layers around the cells leads and cell viability (Figure 3D,E). Control experiments

undergoing the same procedure did not show these effects. MTT assays showed that first layer did not seriously affect cell viability, with an LD_{50} (the concentration at which only 50 % the MTT signal of control cells was obtained) of 200 µM, higher than the highest concentration used. For each additional layer, the LD_{50} decreased significantly, going to 50 µM (2 layers), 10 µM (3 layers), 4.0 µM (4 layers), up to at 2.0 µM for 5 layers. In the light microscopy checks a decrease in cell number was observed.

The amount of the cell surface that is encapsulated seems to have an effect on MTT read-out, where complete encapsulation is required to decrease the MTT signal. The difference in MTT read-out between incubation conditions can be explained by different mechanisms, since MTT only measures the amount of adhered metabolically active cells in each well.[20] Cells can be directly affected by the encapsulation, leading to prohibition of cell division or lack of nutrients. Cell encapsulation may also disturb the adherence of cells to the surface, leading to cells being washed away before the MTT assay. Another explanation, independent of encapsulation coverage, is that keeping the cells in suspension induces stress and enhances the negative influence of external stimuli.

5.3.5. Cell line specific viability effects

To examine the effect of encapsulation on different cell lines, a comparison was made using both the CXCR4-overexpressing MDAMB231-X4 cells and MDAMB231 cells with basal CXCR4 expression (Figure 4). These cells only differ in the higher expression of CXCR4, which is often used as a tumor marker.[21] The cell line with lower CXCR4 expression showed a large

decrease in the cytotoxicity of the LbL encapsulation, with the LD_{50} increasing 36-fold from 2.0 to 72 µM ($p < 0.001$).

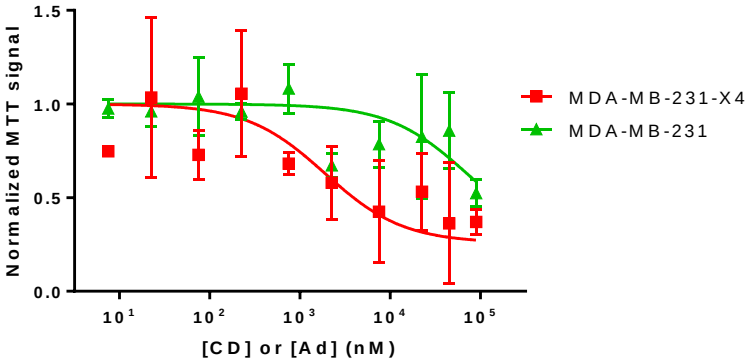


Figure 4: MTT assays comparing the viability effects in suspension of the amount of 5 layers of polymers (red squares) with a control cell line with lower amount of CXCR4 expression (green triangles).

The difference between the two cell lines indicates that this approach may be used for selective encapsulation of a certain cell type. The current system only works *in vitro* at low temperatures to prevent internalization of CXCR4 and Ac-TZ14011-Ad.[22] For future applications, it will be necessary to use a target receptor which does not internalize as fast. Since this LbL method is generic after the first step, this LbL encapsulation method may be expanded to different target receptors and different target cells.

5.3.6. The effect of encapsulation on chemotherapeutics

When cancer cells such as the MDAMB231-X4 cells are encapsulated by multiple layers, an impenetrable capsule around the cell could be formed that would render them insensitive to chemotherapeutics. To study if this is the case, the ability of a doxorubicin, a commonly used chemotherapeutic

agent, to penetrate through a 5-layer construct was investigated. Cells were encapsulated with five polymer layers in suspension to achieve optimal coverage, and after that doxorubicin (0.5 nM – 60 µM) was added. The response of encapsulated cells against increasing concentrations of doxorubicin were similar to non-encapsulated cells, with LD_{50} values of 1.6 and 1.2 µM after 24 h, respectively (Figure 5). Since doxorubicin is only toxic when internalized, this finding suggests that this encapsulation method still allows for relatively small molecules to enter the cell.

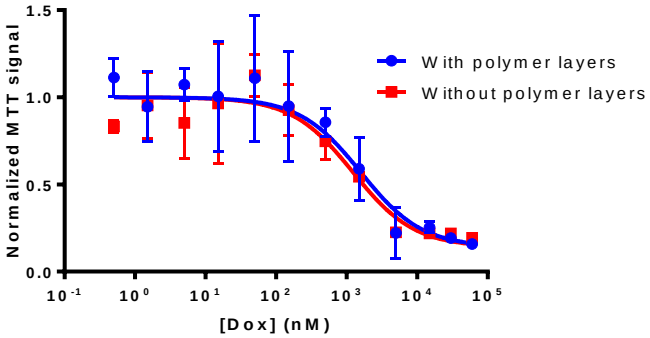


Figure 5: MTT assays comparing the viability effects of the chemotherapeutic drug doxorubicin in the presence (blue circles) or absence (red squares) of 5 layers of polymer

The fact that the cell encapsulation did not influence the efficiency of doxorubicin, opens the way for combined therapy strategies. The combination of cell encapsulation and chemotherapeutics may be of special interest in the fight against circulating malignant cells, e.g. in leukemia or metastatic tumor cells, where prevention of adherence and direct cytotoxicity can be combined to fight tumor cells.

5.4. Conclusions

Cell encapsulation could be realized using supramolecular interactions between cyclodextrin and adamantane. The individual components were non-cytotoxic, but a combination led to a decrease in *in vitro* cell viability for cells in suspension. This induced cytotoxicity was dependent on the cell line used. Although the cell surface could be efficiently covered, the functionalized surface could still be penetrated by the therapeutic agent doxorubicin.

1. Richardson, J.J., M. Björnmalm, and F. Caruso, Technology-driven layer-by-layer assembly of nanofilms. Science, 2015. 348(6233).
2. Fakhrullin, R.F., I.S. Choi, and Y.M. Lvov, Cell Surface Engineering. RSC Smart Materials. 2014: The Royal Society of Chemistry.
3. Orive, G., et al., Cell encapsulation: promise and progress. Nat Med, 2003. 9(1): p. 104-7.
4. Matsuzawa, A., M. Matsusaki, and M. Akashi, Effectiveness of Nanometer-Sized Extracellular Matrix Layer-by-Layer Assembled Films for a Cell Membrane Coating Protecting Cells from Physical Stress. Langmuir, 2013. 29(24): p. 7362-7368.
5. Fakhrullin, R.F., et al., Living fungi cells encapsulated in polyelectrolyte shells doped with metal nanoparticles. Langmuir, 2009. 25(8): p. 4628-34.
6. Thomas, M.B., et al., Enhanced viability of probiotic Saccharomyces boulardii encapsulated by layer-by-layer approach in pH responsive chitosan–dextran sulfate polyelectrolytes. J Food Eng, 2014. 136: p. 1-8.
7. S. De Koker, B.G.D.G., C. Cuvelier, L. Ferdinande, W. Deckers, Cellular Uptake, Degradation and Biocompatibility of Polyelectrolyte Microcapsules. 2007.
8. Wilson, J.T., W. Cui, and E.L. Chaikof, Layer-by-layer assembly of a conformal nanothin PEG coating for intraportal islet transplantation. Nano Lett, 2008. 8(7): p. 1940-8.
9. Kozlovskaya, V., et al., Ultrathin polymeric coatings based on hydrogen-bonded polyphenol for protection of pancreatic islet cells. Adv Funct Mater, 2012. 22(16): p. 3389-3398.
10. Kozlovskaya, V., et al., Hydrogen-bonded LbL shells for living cell surface engineering. Soft Matter, 2011. 7(6): p. 2364-2372.
11. Wang, Y., et al., Organocatalytic asymmetric Michael addition of aldehydes and ketones to nitroalkenes catalyzed by adamantoyl l-prolinamide. RSC Advances, 2015. 5(8): p. 5863-5874.
12. Mujumdar, R.B., et al., Cyanine dye labeling reagents - sulfoindocyanine succinimidyl esters. Bioconjugate Chem, 1993. 4(2): p. 105-111.
13. Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods, 1983. 65(1-2): p. 55-63.
14. Gerlier, D. and N. Thomasset, Use of MTT colorimetric assay to measure cell activation. J Immunol Methods, 1986. 94(1-2): p. 57-63.

15. Grana-Suarez, L., W. Verboom, and J. Huskens, Cyclodextrin-based supramolecular nanoparticles stabilized by balancing attractive host-guest and repulsive electrostatic interactions. Chem Commun, 2014. 50(55): p. 7280-7282.
16. Weickenmeier, M., G. Wenz, and J. Huff, Association thickener by host guest interaction of a beta-cyclodextrin polymer and a polymer with hydrophobic side-groups. Macromol Rapid Comm, 1997. 18(12): p. 1117-1123.
17. Schrader, M., S.W. Hell, and H.T.M. van der Voort, Potential of confocal microscopes to resolve in the 50–100 nm range. Appl Phys Lett, 1996. 69(24): p. 3644-3646.
18. Vernier, V.G., et al., The toxicologic and pharmacologic properties of amantadine hydrochloride. Toxicol Appl Pharmacol, 1969. 15(3): p. 642-65.
19. Matilainen, L., et al., In vitro toxicity and permeation of cyclodextrins in Calu-3 cells. J Control Release, 2008. 126(1): p. 10-6.
20. Berridge, M.V. and A.S. Tan, Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. Arch Biochem Biophys, 1993. 303(2): p. 474-82.
21. Kuil, J., T. Buckle, and F.W.B. van Leeuwen, Imaging agents for the chemokine receptor 4 (CXCR4). Chem Soc Rev, 2012. 41(15): p. 5239-5261.
22. Kuil, J., et al., Hybrid peptide dendrimers for imaging of chemokine receptor 4 (CXCR4) expression. Mol Pharm, 2011. 8(6): p. 2444-53.