Meta-Engineering in Drug Design: A Roadmap to Implement a Novel Liposome-Based Testbed

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ABSTRACT

Retroviruses cause a variety of the most serious diseases of man and animals. A new class of antiretroviral drugs, so-called 'entry inhibitors', block virus entry into the host cell. Until now, antiretroviral drug have been designed and evaluated in complex natural virus-cell systems, but the complexity impedes detailed insights into the underlying molecular mechanisms of virus-cell interaction. Therefore, we propose to engineer a novel model system which reduces the complexity of the components involved in virus-cell interaction. The proposed artificial model system will provide an *in vitro* testbed for antiretroviral drug design and validation. The system will combine the advantages of natural and artificial models by consisting of artificial liposomes equipped with a minimal cellular machinery providing nothing but the components needed for the molecular processes in virus-cell interaction. We are able to refer to data of several 'entry inhibitors' tested in a natural virus-cell system, and we already established liposome containers separated from the surrounding by a lipid membrane that enclose sugars, and amino acids. Here, we present results of encapsulating nucleotides and organic and inorganic ions. Further, we discuss how to proceed on the way towards a novel liposome-based testbed for antiretroviral drug design and validation.

Keywords: Inhibitory peptides, Liposomes, Cell-free expression system, Living Technology

INTRODUCTION

Retroviruses (e.g. HIV) incorporate their genes into the host's genome and thus establish life-long infections that frequently terminate in fatal diseases (e.g. AIDS). Since xenotransplantations [1] will gain in importance, risks concerning the transfer of animal retroviruses to humans have to be assessed beforehand. Feline leukemia virus [2], a naturally occurring gammaretrovirus of domestic cats and some related small felids [3, 4], serves both as a model of the multifaceted pathogenesis of retroviruses (i.e. in tumor and AIDS research) [3] and as a testbed for the risk assessment of xenotransplantation. For most retroviral diseases, therapies are either absent or have the disadvantage of developing drug resistance and/or having high toxicity profile. Recently, a novel promising antiretroviral drug class was developed: Small synthetic peptides, termed 'entry inhibitors' or 'fusion inhibitors'. Operating very early in the viral life cycle, they interfere with binding onto the surface of the host cell, virus and host cell membrane fusion, and virus entry into the host cell [5-7]. In contrast to other antiretroviral drugs, fusion inhibitors have a low toxicity profile [8, 9].

In the development of antiretroviral therapies one attempts to exploit the retroviral vulnerability of a general structure and a simple genomic organization. The viral gene *env* [10] encodes for an envelope protein inducing viral cell entry: Interaction of the envelope protein and a specific host cell surface receptor induces a fusion of the virus and host cell membranes [11]. Virus subgroups differ in the characteristics of the envelope protein resulting in differences in cell targeting and specific disease capacity [12-16]. The virus host cell receptors for all FeLV subgroups have been defined recently [13-15, 17-24]. As point of vantage the interaction of virus envelope protein and specific host cell receptors has been studied extensively in *in vitro* systems that use natural cells [25]. Since in natural cells a tremendous number of various processes take place simultaneously, such systems often fail to elucidate elementary molecular mechanisms of the system. Thus, in the last years, serious efforts were made to replace the natural virus-cell system by an artificial virus-liposome model. Liposomes feature an aqueous compartment partitioned off the surrounding by an impermeable lipid membrane. The simplicity in structure reduces the complexity of the system [26-28]. On the other hand, the complete absence of cellular components in the artificial virus-liposome systems has unfavorable effects on validity and comparability with the virus-cell and the biological (*in vivo*) system. Therefore, we propose to engineer a novel virus-liposome model system which is simple to understand but complex enough to draw conclusions on the natural system. Only recently, wet-laboratory approaches in engineering and meta-engineering spanning a wide variety of research disciplines have been unified in the concept of Living Technology [29]. The growing field of Living Technology is likely to gain in importance in new engineering disciplines with multiple applications in the medical, material, information, energy, and environmental sciences [30].

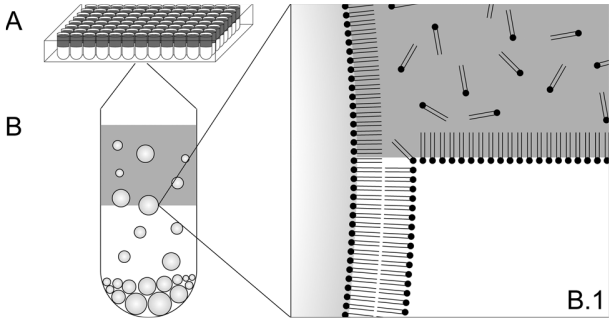


Figure 1. Schematic representation of the parallel liposome formation. (A) Liposomes are produced in 96-well microtiter plates, providing parallel formation of up to 96 distinct liposome populations. (B) The sample is composed of two parts: water droplets (light gray) in the oil phase (dark gray), hosting nucleic acids (cp. Fig.2) or (in)organic ions (cp. Table 1) and the bottom aqueous phase (white), which finally hosts the liposomes. (B.1) Due to their amphiphilic character, phospholipids (black), solved in mineral oil, stabilize water-oil interfaces by forming two monolayers. These two monolayers form a bilayer when a water droplet, induced by centrifugation, passes the interface. Due to both the density difference of the *inter*- and *intra*-liposomal fluid and the geometry of the microplate bottom, vesicles pelletize in the centre of the well (cp. B).

In this paper, we characterize the basic specifications, summarize what has been achieved so far, present our current results, and discuss how to realize the missing components to engineer the novel virus-liposome model system that will provide a testbed for antiretroviral drug design and validation. The system will combine the advantages of the virus-cell and the virus-liposome models by consisting of liposomes equipped with a cellular machinery composed of a minimal number of components and providing nothing but the molecular processes of the virus-cell interaction.

For the implementation of the testbed, the following components need to be realized: (i) A cell-like container (ii) encapsulating the minimal cellular machinery providing protein synthesis and (iii) data of 'entry inhibitors' collected in a natural system.

(i) A cell-like container: Liposomes are the most studied systems among biomimetic structures [31] and have frequently been used as models for living cellular structures [32], since they make an ideal tool for investigating enclosed systems containing ongoing biochemical processes, including the replication of RNAs [33], the PCR [34], and polypeptide synthesis [35]. Over the last decades, several *in vitro* liposome formation and analytical characterization procedures were developed (for a review see [36]).

(ii) Encapsulation of the minimal cellular machinery: Commercially available cell-free expression systems provide the minimal cellular machinery to synthesize proteins in vitro [37, 38]. Usually cell-free expression systems that originate from wheat germ or *Escherichia coli* (*E. coli*) are used. Though synthesis of soluble proteins in liposomes is well established [32, 39-42], expression of membrane-associated proteins is restricted to spontaneously inserting hemolytic proteins that do not rely on cellular machinery providing appropriate protein localization [43, 44]. Only recently, the appropriate protein localization of integral membrane proteins was reported for a novel expression system consisting of a fusion between *E. coli* inner membrane protein (GlpF) and eukaryotic integral membrane proteins [45]. This system allows for the synthesis of eukaryotic integral membrane proteins in *E. coli*.

(iii) Data of 'entry inhibitors': Inhibitory peptides were already designed and analyzed *in vitro* in a natural virus-cell system [46]. These results providing data to validate the quality of a natural system (virus-cell) are necessary to verify and validate an artificial virus-liposome testbed.

By introducing microtiter plates in liposome formation and increasing the versatility [47, 48] of an established liposome formation procedure [44, 49, 50], we provide parallel and high-throughput analyses [47]. In addition, we implemented and evaluated a DNA-mediated self-assembly procedure to generate multi-compartment aggregates of programmable and predefined composition [47, 51] and discussed their application in personalized medicine [52].

Introducing asymmetry in the *inter*- and *intra*-liposomal fluid results both in an independent composition control of the inner and outer medium, and in an increased liposome manageability [47, 48, 52]. Broadly speaking, natural cells contain sugars that provide energy for cells, lipids that build up the cell membranes, amino acids that are the subunits of proteins, and nucleotides that code for the development and functioning of all known living organisms. Artificial liposome membranes are made of lipids as well. From the remaining three major families of small organic molecules we already tested the encapsulation of sugars [47, 48] and amino acids. Here, we therefore analyzed liposome formation and liposome stability in dependence of incorporation of nucleic acids (DNA strands). Moreover, organic and inorganic ions are essential to all cellular processes. Thus, to set up artificial liposomes hosting the cellular machinery needed to express proteins, one has to provide not only the genetic blueprint (DNA strands) but also a cell-like composition of the *intra*-liposomal fluid. We therefore analyzed liposome formation and liposome stability in dependence of incorporation of both organic and inorganic ions in this study.

By engineering a simple but more cell-like virus-liposome model system, we expect to provide a high-throughput testbed for the design and validation of novel antiretroviral drugs.

MATERIALS AND METHODS

The established liposome formation protocol [49] was technically modified as follows: introduction of (i) 96-well microtiter plates U96 to increase procedural manageability in laboratory experimentation and (ii) a density difference between *inter*- and *intra*-liposomal solution to induce liposome pelletization (Fig.1). Solutions of the liposome lumen and the surrounding medium were equal in osmolarity but differed in the degree of polymerization of dissolved saccharides (*intra*-liposomal: disaccharides, *inter*-liposomal: monosaccharides) providing density differences between the lumen and the environment. The sample was composed of two parts: (i) an oil phase hosting water droplets with sucrose (disaccharide) and either fluorescently labeled (Alexa Fluor 488) DNA strands (10 micromolar) or (in)organic ions (for a list of tested ions and concentrations, see captions of Table 1) as additives and (ii) the bottom aqueous phase, which finally receives the liposomes. Due to their amphiphilic character, phospholipids (dissolved in mineral oil) stabilize water-oil interfaces by forming monolayers. Two monolayers form a bilayer when a water droplet, induced by centrifugation, passes the interface. Liposome membranes were exclusively made of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine. Due to both the density difference of the *inter*- and *intra*-liposomal fluid and the geometry of the microplate bottom, liposomes pelletize in the centre of the well. Liposome formation was performed in duplicates. Length of circumference of the liposome pellet is used as a measure of liposome yield (cp. methodology in [47]). The liposome yield was compared to the control (without addition of ions) providing values of relative liposome yield. Light-microscopy was performed using a Wild M40 inverted microscope equipped with a MikoOkular microscope camera. All camera settings were identic-

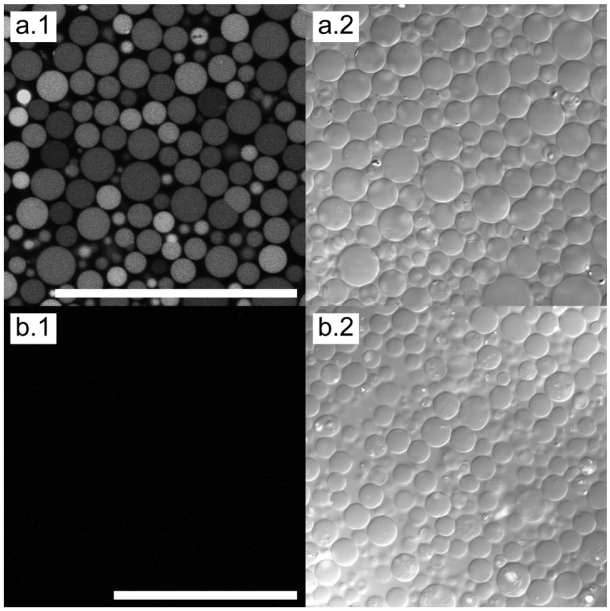


Figure 2. Encapsulation of nucleic acids. (a.1, b.1) Confocal laser scanning microscope and (a.2, b.2) differential interference contrast micrographs of two liposome populations. Only liposome population (a) incorporates fluorescently labeled DNA strands. Liposome population (b) does not hold DNA strands and is used as control. Scale bar represents 100μm.

al for the recordings. Confocal laser scanning microscopy was performed using an inverted Leica Confocal DMR IRE2 SP2 confocal laser scanning microscope.

RESULTS AND DISCUSSION

Liposomes were found to sediment and hence to be easily available for inverse microscopy (Fig.2). The fluorescence signal was found exclusively in the lumen of the liposomes and only if fluorescently labeled DNA strands were present during liposome formation (Fig.2.a.1). Autofluorescence was absent (Fig.2.b.1). Thus, one can conclude that nucleic acids are incorporated efficiently into the liposomal lumen if present during liposome formation.

The (in)organic molecular sodium salts tested (Table 1) only differed in the anionic component. The relative liposome yield differed in dependence of the concentration of the sodium salt. At a concentration of 10 millimolar the liposome yield is either the same or higher than in the control experiment except for sodium dihydrogenphosphate. Sodium dihydrogenphosphate was chosen as a negative control, due to its capacity to prevent oil separation. Sodium phosphates are therefore used as emulsifiers and detergents. Thus, as expected liposome formation is inhibited completely in the presence of sodium dihydrogenphosphate. At 50 millimolar sodium formate and sodium N-lauroylsarcosinate prevent liposome formation as well. Both are amphiphilic, therefore probably destabilizing the phospholipid monolayers needed for the liposome production. The negative effect on liposome formation observed for most halogen sodium salts (e.g. sodium chloride; data not shown), is absent for most of the (in)organic molecular sodium salts tested in this study. (In)Organic molecular sodium salts may therefore provide viable alternatives to halogen sodium salts when it comes up to providing cell-like composition of the *intra*-liposomal fluid.

Table 1. Relative liposome yield in dependence of (in)organic sodium salts. The liposome yield is expressed as a percentage of the control (*intra*-liposomal fluid without the addition of salt).

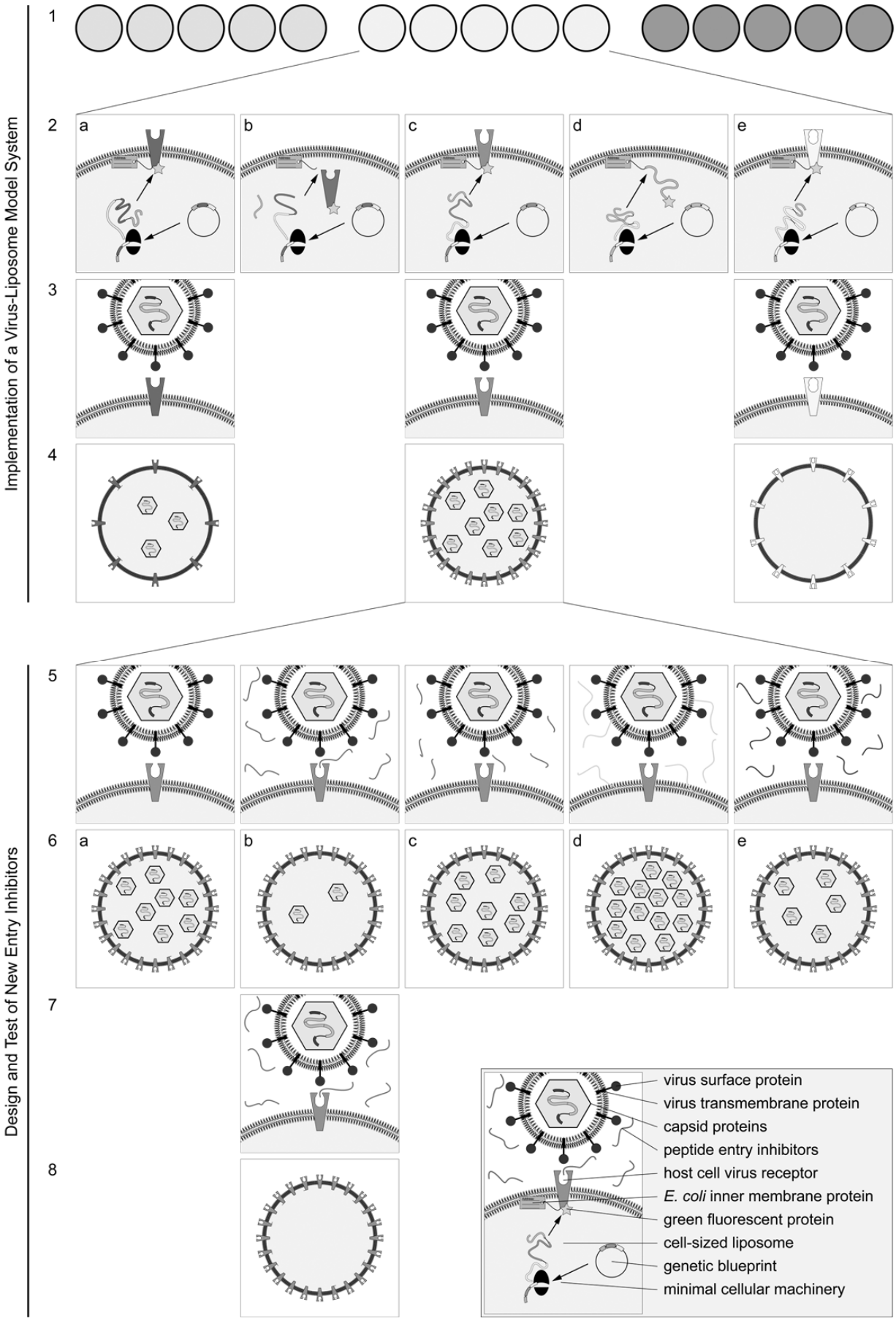
	50 millimolar	10 millimolar
Positive control	100.000 ± 1.779	100.000 ± 3.581
Negative control, Sodium dihydrogenphosphate, $H_2NaOP \cdot 2\ H_2O$,	0	0
Sodium formate, $CHNaO_2$	0	97.401 ± 5.100
Sodium N-lauroylsarcosinate, $C_{15}H_{23}NNaO_3$	0	105.924 ± 3.910
Sodium acetate trihydrate, $CH_3COONa \cdot 3\ H_2O$	88.893 ± 6.350	100.793 ± 8.964
Sodium hydrogen carbonate, $NaHCO_3$	97.185 ± 1.754	105.291 ± 3.899
Sodium carbonate, Na_2CO_3	99.426 ± 6.221	103.075 ± 3.859
Sodium bisulfate, $Na_2S_2O_6$	113.392 ± 3.010	118.579 ± 3.689

We implemented containers partitioned off the surrounding by a lipid membrane, we were able to enclose sugars, amino acids, nucleotides, organic and inorganic ions, and we will be able to refer to data of several 'entry inhibitors' already tested in an established virus-cell model system. In the remaining paragraphs we point out how to proceed on the road towards a novel virus-liposome model system that may provide an effective testbed for antiretroviral drug design and validation (Fig.3).

Different cell-free expression systems are commercially available. Encapsulation of the cell-free expression systems may be performed using established procedures [44]. The genetic blueprint to produce host cell virus receptor proteins is composed of three different parts (Fig.3.2): i) *E. coli* inner membrane protein GlpF [45], providing appropriate protein localization of integral membrane host cell virus receptor proteins, ii) one of the host cell virus receptor candidates that are assumed to enable virus-liposome interaction and membrane fusion, and iii) a green fluorescent protein (GFP) providing information about the protein localization. The three genetic components may be cloned and expressed in cell-free expression systems available.

Routine procedures for cloning [53] are available and the sequence of the constructs can be amplified by polymerase chain reaction (PCR) and inserted into the vector pI VEX 2.3d (cp. [54]). All constructs may be verified by sequencing and gene expression, size, native conformation, and the proper membrane localization of the proteins may be analyzed for each combination of cell-free expression system and fusion protein by using RT(reverse transcriptase)-PCR, western blotting, fluorescence and confocal microscopy, and ELISA (enzyme-linked-

Figure 3. (opposite). Schematic representation of the methodological procedure to engineer a novel virus-liposome model system that provides an effective testbed for antiretroviral drug design and validation. (1) Three different commercially available cell-free expression systems are depicted, hereafter details are provided for one of these. (2) In each transcription-translation cell-free expression system a distinct host cell protein is expressed that is assumed to enable virus cell entry. Concerning protein expression and localization different results are conceivable: (a, c, e) gene expression, size, native conformation and the membrane localization of the fusion proteins are adequate; (b) mislocalization or (d) misfolding of the host cell receptor. (3) The number of virus-liposome model systems is reduced – only 'convenient' (see text) combinations of liposome stability, cell-free expression system, fusion protein expression and localization are further used and incubated with native virus. (4) The virus load differs in dependence of host cell surface receptor density and its accuracy in respect to the virus envelope protein. The most promising virus-liposome model system is selected for the further procedure. (5) Peptide entry inhibitors that differ in length and/or sequence either decrease (6.b, 6.e), not affect (6.c), or increase (6.d) the virus load compared to the control (6.a). (7) The inhibitory potential of the most promising antiretroviral peptide is increased using an iterative *in silico* optimization procedure, finally resulting in a potent antiretroviral drug (8).


Implementation of a Virus-Liposome Model System
Design and Test of New Entry Inhibitors
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7
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virus surface protein
virus transmembrane protein
capsid proteins
peptide entry inhibitors
host cell virus receptor
E. coli inner membrane protein
green fluorescent protein
cell-sized liposome
genetic blueprint
minimal cellular machinery

immunosorbent assay) techniques. The aim should be to establish 'viable' combinations of liposome stability, cell-free expression system, fusion protein expression and localization.

Having appropriately equipped liposomes, the capacity of FeLV to enter the liposome mediated by the host cell receptor on the surface will be analyzed by co-incubating liposomes and virus (Fig.3.3), removing of excess virus, lysis of liposomes, and quantification of virus load using RT-PCR (Fig.3.4). Precautions have to be taken to prevent that the selection of the most promising virus-liposome model system (Fig.4.4) not only depends on the receptor surface density (as implied in Fig.4) but also on the quality of attachment of receptor and virus envelope protein (cp. geometrical match of receptor and virus envelope protein is better for the virus-liposome model system (a) of Fig.4.4 but the larger number of receptors is sufficient that the virus-liposome model system (c) outperforms (a) that would actually be better suited). To prevent such a problem, virus load will have to be correlated with the receptor surface density quantified for example by using the GFP-fluorescence signal.

To review the appropriateness of the novel model system liposomes and virus are co-incubated either in the presence or absence of peptide entry inhibitors (Fig.3.5) whose antiretroviral activity have already been tested [46]. The liposome virus load is quantified (Fig.3.6) and compared to the virus-cell system resulting in a qualitative comparison of the two model systems. Based on these data, a genetic algorithm to evolutionary design experiments in wetware established by Poli and coworkers [55] will be used to design new antiviral peptides, tested, and iteratively optimized (Fig.3.7), resulting in potent peptide entry inhibitors (Fig.3.8).

CONCLUSIONS

In previous work, we implemented cell-like containers separated from the surrounding by a lipid membrane that enclose sugars and amino acids. In the present study, we enlarged the range of substances enclosed by nucleotides and organic and inorganic ions. Thus, all substances are incorporated that are required to implement a basic metabolism within liposomes. The simplicity of these elementary cell-like containers providing a basic metabolism may be exploited in the design of an effective liposome-based testbed for antiretroviral drug design and validation. Since the inhibitory potentials of some instances of a new class of antiretroviral drugs are already tested using common *in vitro* systems, the effectiveness of the new liposome-based testbed in validation of antiretroviral drugs may be evaluated. The new liposome-based testbed potentially offers high-throughput analyses of antiviral drugs and may optimize or open up bottlenecks inherent to current technologies applied in drug design.

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