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Predicting the in vivo release from a liposomal formulation by IVIVC and non-invasive positron emission tomography imaging

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ABSTRACT

This study aimed to predict the in vivo performance from the in vitro release of a low-molecular weight model compound, [^{18}F]-2-fluoro-2-deoxy-D-glucose ([^{18}F]FDG), from liposomes and by means of positron emission tomography (PET). Liposomes composed of hydrogenated phosphatidylcholine (HPC) were prepared by a freeze–thaw method. Particle size distribution was measured by dynamic light scattering (DLS). In vitro release was examined with a dispersion method detecting the radioactivity of [^{18}F]FDG. In vivo release of [^{18}F]FDG, following i.p. injection of the liposomes in rats, was determined by using a Micro-PET scanner. Convolution was performed to predict the in vivo profiles from the in vitro data and to establish an in vitro–in vivo correlation (IVIVC). The in vivo predictions slightly underestimated the experimentally determined values. The magnitude of the prediction errors (13% and 19%) displayed a satisfactory IVIV relationship leaving yet room for further improvement. This study demonstrated for the first time the use of PET in attaining an IVIVC for a parenterally administered modified release dosage form. It is therefore possible to predict target tissue concentrations, e.g., in the brain, from in vitro release experiments. IVIVC using non-invasive PET imaging could thus be a valuable tool in drug formulation development, resulting in reduced animal testing.

1. Introduction

In the last 15 years liposomes have frequently been used as drug carriers. They may reduce toxicity and deliver the drug to its sites of action. Other advantages include reduced dosage, sustained drug release and, consequently, reduced frequency of administration. An important factor with significant impact is the rate of release of the drug from the carrier, since only the free fraction of the drug extends the therapeutic effect.

In the pharmaceutical industry, in vitro release tests are an essential tool in (i) taking indirect measurements of drug availability, especially in preliminary stages of product development, (ii) assuring stable release characteristics of the product over time, (iii) assessing the effect of manufacturing process changes on product performance and (iv) quality control and assurance to support batch release.

The rate of release of an agent from liposomes is governed by several factors (Drummond et al., 2008): the lipid composition, the active's physico-chemical properties and its drug–lipid interactions and the liposome size. The lipid type determines the stability and fluidity and, consequently, the permeability of the liposomal membrane.

The principal mechanisms of drug release are diffusion and in vivo disintegration of the liposomal membrane. The latter could lead to the immediate release of most of the entrapped drug from a disrupted liposome. Both mechanisms can appear simultaneously.

In contrast to oral modified release formulations today exist no regulatory standards for dissolution tests for colloidal systems. Due to the considerable differences in formulation and hence in physico-chemical and release characteristics, it is hard to establish a generally applicable method. Thus, different techniques are applied. The most widely used methods for determination of release profiles from disperse systems can be divided into four groups: membrane diffusion, sample and separate techniques, in situ and continuous flow methods. All these methods have been comprehensively reviewed by Washington (1990) and represent their advantages and disadvantages.

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In vivo release is mainly determined by measuring the concentration of free drug in blood, plasma and/or serum. It is also possible to ascertain in vivo release with imaging methods such as magnetic resonance imaging (MRI) or positron emission tomography (PET). PET is an imaging technique with a high temporal and spatial resolution that produces three-dimensional images of the human or animal body using short-lived radioactive substances. One of the ultimate goals of biopharmaceutical research is to establish a suitable approach to describe the relationship between an in vitro property of a dosage form (e.g., the rate or extent of drug release) and a relevant in vivo response (e.g., plasma drug concentration or amount of drug absorbed). Guidances both by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) deal with the development and role of in vitro–in vivo correlations (IVIVC) in oral solid dosage forms (EMEA, 1999; FDA, 1997). As modified release parenteral dosage forms become more and more important, recent developments investigate the role of IVIVC for these dosage forms as well (Burgess et al., 2004; Martinez et al., 2008).

The aim of this study is to establish a quantitative relationship between the in vitro and in vivo release-rate of an active from such a liposomal dosage form with the aid of PET.

2. Materials and methods

2.1. Materials

Phospholipon® 90 H (Ph90H) was a generous gift by Phopholipid GmbH (Cologne, Germany). Isotonic saline solution was purchased from Fresenius Kabi Deutschland GmbH (Bad Homburg, Germany). [^{18}F]-2-Fluoro-2-deoxy-D-glucose ([^{18}F]FDG) was procured from PET Net GmbH (Erlangen, Germany). Rats were anaesthetized with Narcoren (Merial, Hallbergmoos, Germany). Sephadex™ G-25 (medium) was obtained from GE Healthcare Bio-Sciences AB (Uppsala, Sweden) and Penefsky columns from HWS Labortechnik (Mainz, Germany). LiposoFast-Basic extruder and polycarbonate membranes (pore diameter 100 nm) were from Avestin Europe GmbH (Mannheim, Germany). A rotator was purchased from neoLab Migge GmbH (Heidelberg, Germany) and a dose calibrator from Veenstra Instruments (Ahlerstedt, Germany). DLS measurements were performed on a ProSpecD 501 photon correlation spectrometer with ALV-correlator and ALV DLS-software 3.0 (Nanovel, Langenlonsheim, Germany).

2.2. Preparation of liposomes and characterization

2.2.1. Preparation of [^{18}F]FDG-containing multilamellar vesicles (MLV)

The MLV were prepared by the freeze–thaw method (Mayer et al., 1985). Ph90H (100 mg) was dispersed in isotonic saline solution (1 ml). The dispersion was vortexed for 2 min at the highest level and thereafter it was heated for 3 min in a water bath at 68 °C; this procedure was repeated five times. Afterwards [^{18}F]FDG was added to the dispersion and the volume was replenished up to 2 ml with isotonic saline solution. The pH was adjusted to 7.4. The MLV-dispersion was immersed in liquid nitrogen for 3 min, then it was thawed using a water bath at 68 °C for 6 min; this freeze–thaw cycle was repeated three times.

The MLV were separated from the free [^{18}F]FDG by centrifugation gel permeation chromatography (cGPC) using Penefsky columns packed with Sephadex™ G-25 (medium) (Penefsky, 1977). The gel, pre-swollen in isotonic saline solution, was put into Penefsky columns, these were inserted into centrifuge tubes and the gel was compressed via centrifugation. After washing five times with isotonic saline solution the MLV (300 μl) were loaded on the top of the dehydrated gel and centrifuged for exactly 1 min at 3200 rpm. The elute containing the final liposomes was used for the following experiments.

2.2.2. Preparation of [^{18}F]FDG-containing extruded MLV (eMLV)

MLV were prepared as described above by the freeze–thaw method except for taking 70 mg instead of 100 mg Ph90H. Thereafter, the MLV (still containing free [^{18}F]FDG) were extruded through a polycarbonate membrane (pore diameter 100 nm) 11 times using a LiposoFast-Basic extruder at 68 °C. The resulting eMLV were cleared from the free [^{18}F]FDG by cGPC.

2.2.3. Particle size

Size distribution was determined by dynamic light scattering (DLS).

2.3. In vitro release of [^{18}F]FDG

2.3.1. Dispersion method

1 ml liposome-dispersion was diluted to 2 ml with isotonic saline solution in a 2.2 ml MicroCentrifuge Tube. The tubes were rotated using a rotator at 25 rpm at 37 ± 0.5 °C. At 15, 30, 45, 60, 75 and 90 min, 0.2 ml of the dispersion were withdrawn and replaced with 0.2 ml of fresh isotonic saline solution. The sample was separated from the free [^{18}F]FDG by cGPC. The radioactivity was measured before (act1) and after (act2) the cGPC by a VDC-405 dose calibrator.

2.4. In vivo release of [^{18}F]FDG

In order to assess the in vivo release of encapsulated [^{18}F]FDG, 3 rats per different formulations were investigated in a small animal PET scanner. The model used was as follows: The [^{18}F]FDG-containing liposomes were injected i.p. It is assumed that these liposomes stay in the peritoneum for several hours. The [^{18}F]FDG released from the liposomes is circulating in the blood pool. The dominant part of this "free" [^{18}F]FDG will be transported to and trapped in brain cells because of the high-glucose consumption rate of brain cells. The trapping mechanism of [^{18}F]FDG reflects phosphorylation of [^{18}F]FDG by intracellular hexokinase. As [^{18}F]FDG-6-phosphate is not a substrate of glucose-6-phosphate isomerase, this [^{18}F]-labelled compound is retained inside the brain cells. For comparison and to test our model, free [^{18}F]FDG solution was directly injected i.p. and the brain uptake was measured.

The rats were anaesthetized with pentobarbital (40 mg/kg, i.p.) before PET imaging. The animals breathed room air spontaneously through a tracheal tube. For brain imaging they were fixed on the scanner's bed in supine position. 19.2 (±3.2) MBq of [^{18}F]FDG-labelled liposomes or 22.4 (±2.7) MBq free [^{18}F]FDG were injected into the peritoneum. Listmode acquisition lasted for 90 min and was started simultaneously with the injection. The first dynamic was histogrammed into 18 frames of 5 min and images were reconstructed iteratively with OSEM2D algorithm. Each frame consisted of 95 slices with a slice thickness of 0.8 mm and a pixel size of 0.87 mm × 0.87 mm in an 128 × 128 image matrix. A large volume of interest (VOI) for the whole brain was defined and a time activity curve (TAC) was derived using PMOD software package. The standardized uptake value (SUV) was used for quantitative analysis of the VOI. The SUV describes the accumulation of nuclides considering the nuclide-decay, the dose administered and the rat's weight and is used to obtain a standardized, time- and weight-independent activity distribution.

Additionally, a whole body scan was performed 120 min after the i.p. injection.

2.5. In vitro–in vivo correlation (IVIVC) by convolution

Convolution is a model independent method based on the superposition principle. Therefore, it is a precondition that the dynamics of the system's behaviour can be presumed to be linear. This means that the concentration $c(t)$ of a drug at any given time is proportional to the dose $d(t)$ of the drug administered ($d(t) \rightarrow c(t)$, $x \cdot d(t) \rightarrow x \cdot c(t)$) and that the system is time invariant (an input given at a time $t > 0$ produces the same response but shifted by t). The superposition principle states that the total response caused by several inputs applied at various times is the sum of all partial responses considering the time shifts.

In IVIVC the body system reacts to a drug release $I(t)$ by a response $R(t)$, e.g., plasma concentration or a pharmacological effect. The weighting function $W(t)$ determines the system's response behaviour. $W(t)$ is the fastest possible body response produced by the fastest possible 'bolus' input (all of the drug substance is available instantaneously). Any response to a 'delayed' input is obtained by convolution of the input with the weighting function. Mathematically, the relationship between them is given by the convolution integral:

$$R(t) = \int_0^t I(\tau) W(t-\tau) d\tau$$

The cumulative in vitro release profiles of the MLV/eMLV were used for generating the input function $I(t)$ and the standardized uptake values (SUV) of the free [^{18}F]FDG in the rat brain for calculating the weighting function $W(t)$.

Initially all data were fitted in Excel with nonlinear least squares regression to the form of a Weibull distribution to compute additional time points (Langenbucher, 2002, 2003a):

$$F(t) = 1 - e^{-(t/\beta)^{\alpha}}$$

The generalized numerical algorithm for convolution can be written in a triangular arrangement of terms such as:

$$\begin{aligned}
R_0 &= 0 \\
R_1 &= I|_0^1 \bar{W}|_0^1 \\
R_2 &= I|_0^1 \bar{W}|_1^2 + I|_1^2 \bar{W}|_0^1 \\
R_3 &= I|_0^1 \bar{W}|_2^3 + I|_1^2 \bar{W}|_1^2 + I|_2^3 \bar{W}|_0^1 \\
&\cdots \\
R_k &= I|_0^1 \bar{W}|_{k-1}^k + I|_1^2 \bar{W}|_{k-2}^{k-1} + \cdots + I|_{k-1}^k \bar{W}|_0^1
\end{aligned}$$

The index $k = 0, 1, 2\ldots$ denominates discrete interpolation time points standing for either observed time points or values computed from the appropriate function. For $k = 0$ by definition $R_0 = 0$. Each row calculates a time point R_k as the sum of all inputs prior to k multiplied by a corresponding (in reversed sequence) interval of $W(t)$.

According to the point-area method which interprets $I(t)$ and $W(t)$ as trapezoidal areas (and which is numerically equivalent to the interpretation of means), relevant terms are defined as follows:

$$\begin{aligned}
\overline{W}\,| &= \frac{(W_{k-1} + W_k)}{2} \\
\Delta I\,| &= I(t_l) - I(t_{l-1})
\end{aligned}$$

The convolution calculation was performed in Excel with a triangular matrix (Langenbucher, 2003b, 2009). All relevant time values are table headings both in the row above and in the column left of the matrix table. All cells contain the identical formula $\overline{W} \cdot \Delta I$ which computes the product of the interval average of $W(t)$ and the cumulative input for the corresponding time interval. Column sums compute the calculated response.

3. Results

3.1. Characterization of liposomes

3.1.1. Size

The size distribution of the liposomes is depicted in Fig. 1. The weighted diameter of the MLV is 3535 nm and the mean size is 4034 nm. The weighted diameter of the eMLV is 2991 nm and the mean size is 3169 nm. The size distribution of the eMLV is narrower than of the MLV.

3.2. In vitro release of [^{18}F]FDG

The in vitro release of the liposomes is shown in Fig. 2.

As supposed due to the liposome size, the in vitro release of the MLV and eMLV appears similar. Both reach a plateau at approximately 40% of the encapsulated [^{18}F]FDG after 90 min.

3.3. In vivo release of [^{18}F]FDG

TACs and PET images, showing the brain uptake of free [^{18}F]FDG and of [^{18}F]FDG released from the two different liposomal preparations, are illustrated in Figs. 3 and 4. Both display a sustained and reduced brain accumulation of [^{18}F]FDG released from liposomes compared to free [^{18}F]FDG.

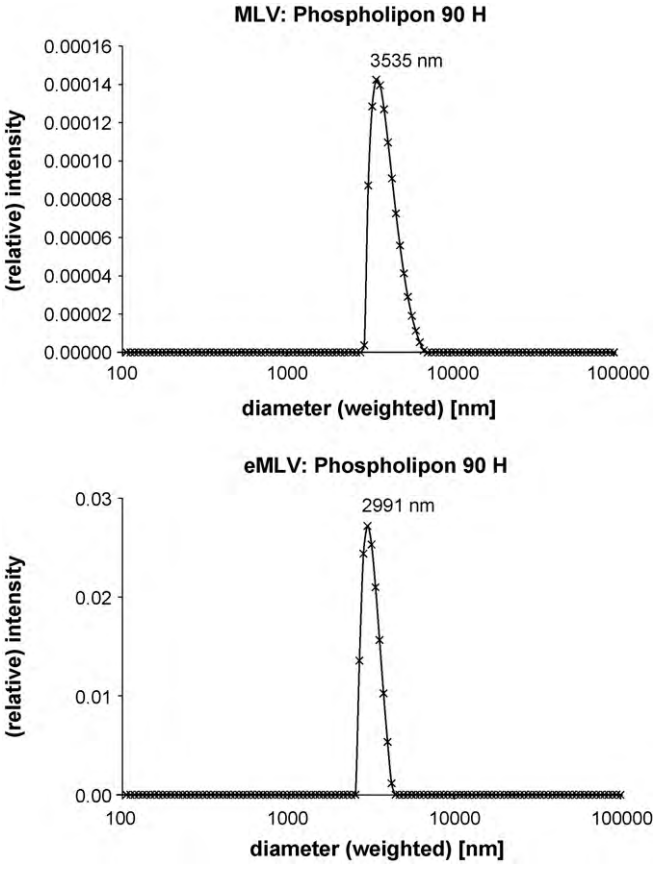

Fig. 1. Size distribution of MLV and eMLV measured by dynamic light scattering. Curve peaks show weighted hydrodynamic diameters of the liposomes.

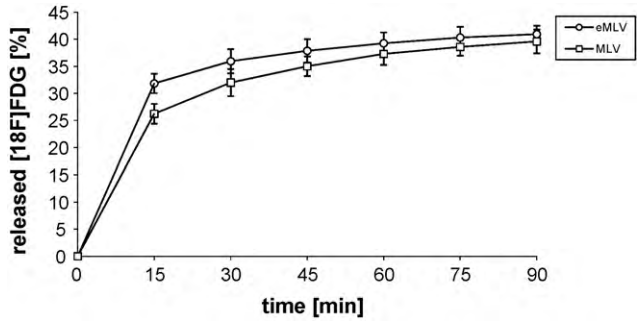

Fig. 2. In vitro release of [^{18}F]FDG from MLV and eMLV determined by a dispersion method.

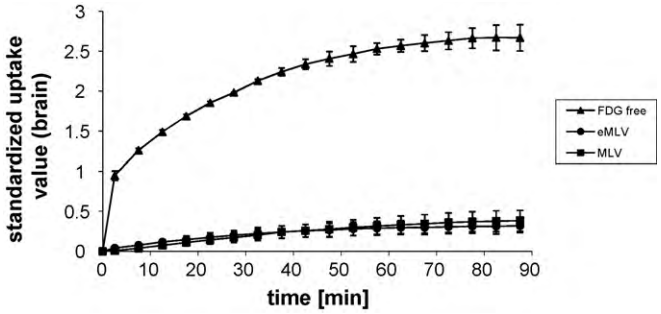

Fig. 3. Time activity curves showing quantitative brain uptake of free/released [^{18}F]FDG.

Table 1
Percent prediction error (% PE) of MLV and eMLV.

Time (min)	% PE	
	MLV	eMLV
15	15.10	44.34
30	5.66	18.42
45	12.92	15.93
60	15.60	14.21
75	15.54	12.44
90	14.17	10.72
Ø	13.17	19.34

% PE = [(observed value − predicted value)/observed value] × 100.

3.4. IVIVC

The fitted cumulative in vitro release data of the MLV/eMLV (input functions) were convolved with the fitted SUVs of the free [^{18}F]FDG in the rat brain (weighting function) with a numerical algorithm in Excel. The comparison of the calculated with the observed response is depicted in Figs. 5 and 6.

To estimate the magnitude of error in predicting the in vivo performance from the in vitro release data, the calculated data (calc) are compared with the observed data (obs) according to the equation for the percent prediction error (% PE):

$$\%\ \mathrm{PE} = \frac{(\mathrm{obs}-\mathrm{calc})}{\mathrm{obs}} \times 100$$

The calculated % PE of both formulations at each time point is shown in Table 1.

4. Discussion

Surprisingly, the MLV and eMLV prepared in this study had almost the same size. This may be due to the composition of Phospholipon® 90 H. It consists of hydrogenated phosphatidyl-

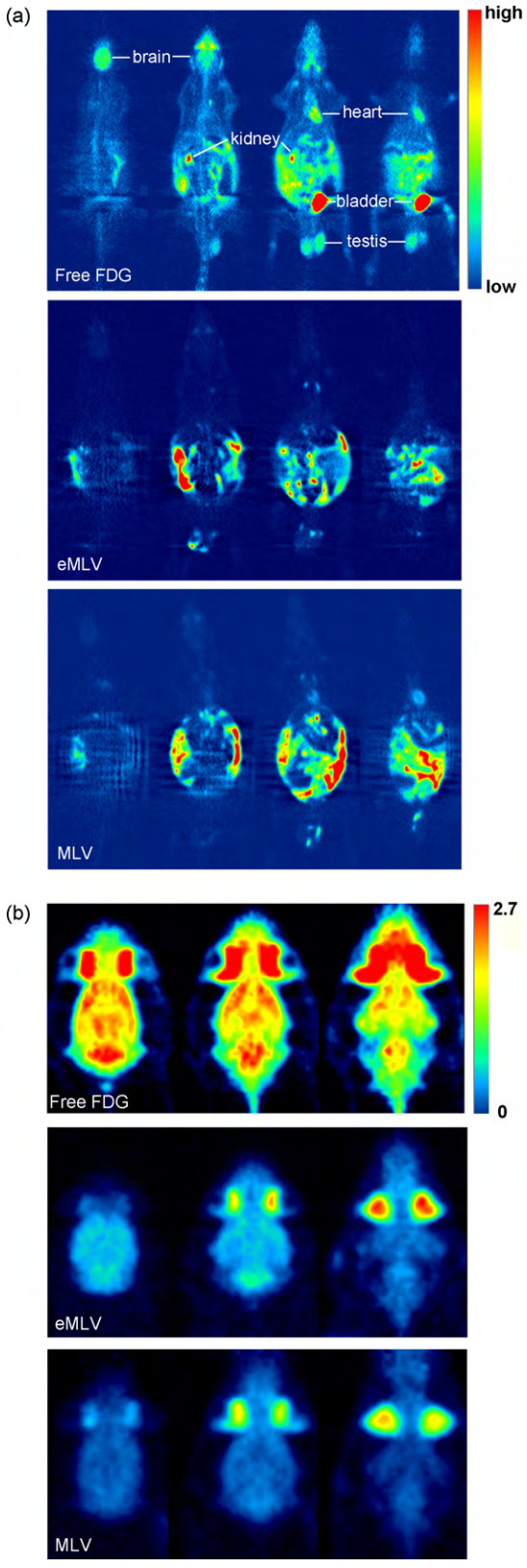

Fig. 4. (a) Whole body images 2 h after i.p. injection (horizontal slices, thickness 7 mm). Free [^{18}F]FDG is taken up by high-glucose using cells such as brain, heart and testis and excreted via kidney and bladder. Liposomal encapsulated [^{18}F]FDG accumulates in the abdomen of the rat. Only released [^{18}F]FDG can be taken up into organs. (b) [^{18}F]FDG uptake of rat brain between 60 and 90 min after i.p. injection (horizontal slices, thickness 3.5 mm). Images are scaled to the same SUV.

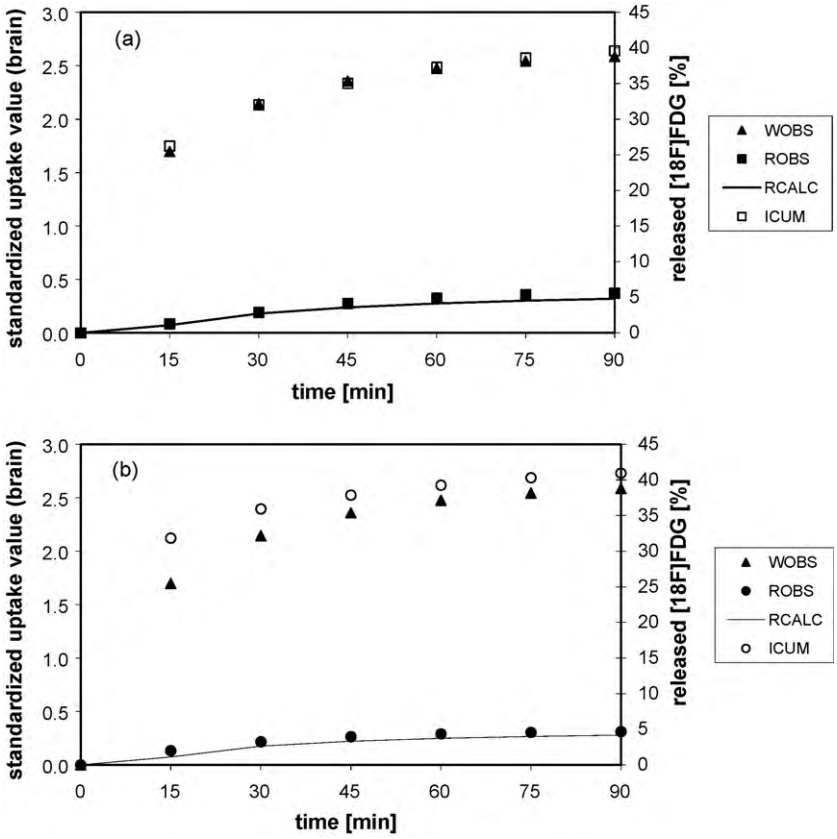

Fig. 5. (a) IVIVC of MLV and (b) IVIVC of eMLV. Both display a relatively good correlation. WOBS = observed weighting function, RCALC = calculated/predicted response, ROBS = observed response, ICUM = cumulative input function (applies to *y*-axis on the right).

choline (HPC) with a fatty acid composition of approx. 85% stearic acid and 15% palmitic acid (min. 98%), unsaturated fatty acids (oleic, linoleic, linolenic acids) (max. 2%) and lysophosphatidylcholine (LPC) (max. 4%). Phosphatidylcholine (PC) has a cylindrical shape and is a bilayer-forming lipid. LPC, however, has only one acyl chain, is therefore cone-shaped and tends to form micelles (Patel et al., 2001). LPC inserted into a bilayer will induce structural changes (Garidel et al., 2007). The liposomes become more elastic and adaptable (El Maghraby et al., 2008), so they can pass through the membrane pores of the extruder forming tubes due to stress. Once they have passed the membrane they will assume their normal shape again (Fig. 7).

The in vivo response calculated from in vitro release and assuming an IVIVC slightly underestimates the in vivo values. The percent prediction error of our model appears useful for formulation development—nevertheless, according to the current EMA and FDA guidance for extended release oral drug products, would not be acceptable from a regulatory point of view in order to obtain a biowaiver. The regulatory agencies have defined valid correlations in that respect at prediction errors <15%. In our study, this is only the case for the MLV, demonstrating the necessity of further improvement.

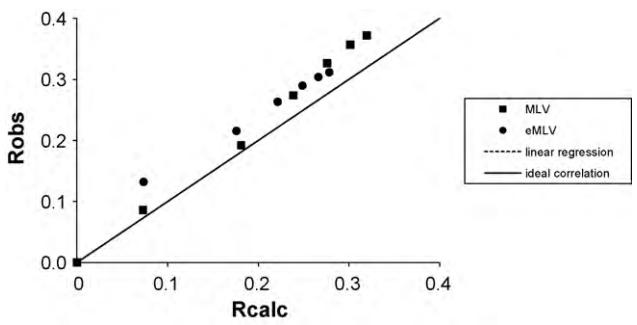

Fig. 6. IVIVC. The calculated response (Rcalc) is plotted against the observed response (Robs). The dashed line represents the linear regression through the origin and all points with the slope 1.1663 and the correlation coefficient $r^2 = 0.9831$. The line of identity is also drawn.

The reason for the underestimation of the in vivo results might lie in an interaction with serum or plasma components in vivo which could result in an increased permeability of the liposomal membrane and an accelerated release of the encapsulated material. The interaction of liposomes with lipoproteins, for example, may affect the retention of encapsulated substances. High-density lipoprotein (HDL) induces liposomal lipids to be transferred to serum lipoproteins, hence destabilizes the liposomes and could even lead to their breakdown and total release of liposomal contents (Allen and Cleland, 1980; Damen et al., 1981; Scherphof et al., 1978). In addition to lipoproteins, the interaction with the complement system is known to promote the leakage of encapsulated material. Drug release may be facilitated by the activation of the complement cascade and following lysis of the liposomes (Funato et al., 1992). Another reason for the underestimation could be the high density of the liposomes in the in vitro dissolution medium (Shazly et al., 2008). In order to adapt the release conditions to the special properties of colloidal systems, the volume of the medium was rather small compared to compendial methods. Due to the supposably high vesicle-to-vesicle contacts the surface area of the liposomes available for contact with the dissolution medium was also limited, so that the release of the encapsulated material might have been hindered.

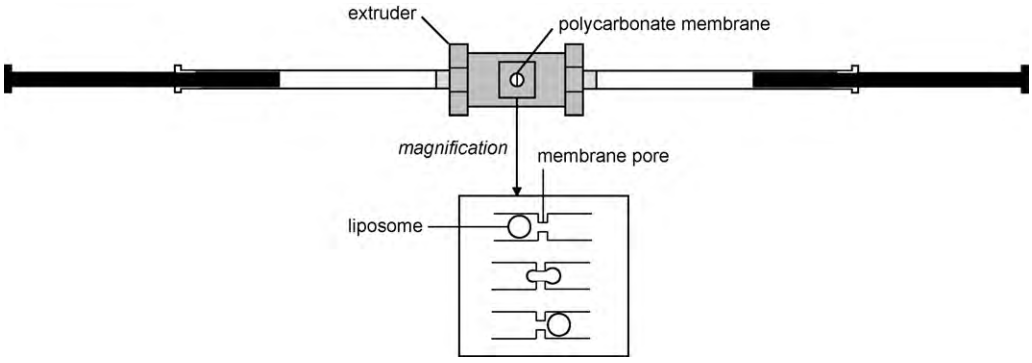

Fig. 7. Extrusion of "flexible" liposomes.

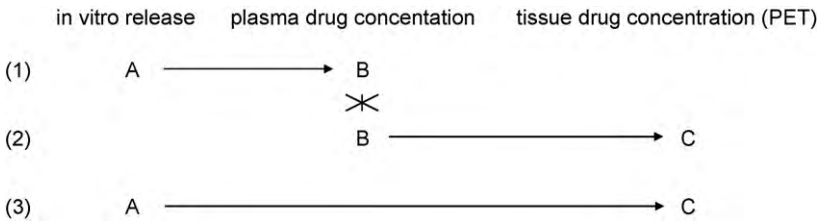

Fig. 8. Approaches used for predicting plasma (1) and tissue drug concentrations (2) so far and our new approach (3) for directly predicting tissue drug concentrations from in vitro data using PET. ⋇, independent of each other.

The relevance of PET in drug development is increasing through consistently new labelling methods which allow a multitude of substances to be labelled with positron emitting radionuclides. The only technical drawback arises from the short half-lives of most radioisotopes which lead to limited follow-up times.

Up to present, PET studies have predominantly explored the pharmacodynamics of a drug, i.e. the drug's effects on the body especially at a target site such as drug–receptor interactions or transporter activities. But PET also allows direct measurement of the labelled drug's biodistribution. Thus it is an excellent tool for pharmacokinetic studies in early drug development. Brain kinetics, as in our case, or that of other organs can be determined quite easily. Contrary to the older technique of killing and dissecting animals to obtain the same information, this method is far quicker. Furthermore, PET is a non-invasive method, so it permits repeated investigations of the same animals and can reduce the numbers of animals required for pre-clinical studies.

Other scientists have developed a convolution-based approach either for IVIVC using plasma drug concentrations as in vivo data (Gillespie, 1997; O'Hara et al., 2001; Veng-Pedersen et al., 2000) or

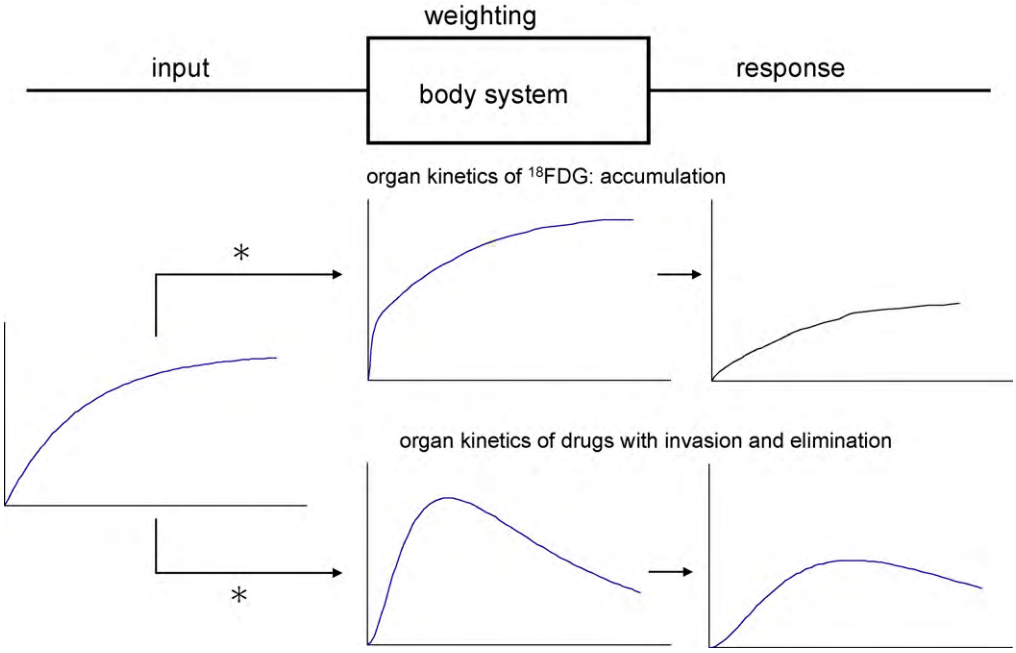

Fig. 9. Only the ^{18}FDG/drug released from the administered liposomes can enter organs from the blood circuit. For IVIVC it is irrelevant whether only input into organs takes place (^{18}FDG) or both input into and elimination out of organs occur, as is the case for many drugs. *, convolution sign.

for predicting drug distribution in tissues from plasma drug concentrations by means of PET (Bergstrom et al., 2006; Rosso et al., 2009). We combined these two approaches to establish an IVIVC using convolution with a weighting function on the basis of PET data (Fig. 8). Hence we can predict the in vivo performance of a compound in tissues from in vitro data. We have chosen ^{18}FDG as a model compound due to the fact that ^{18}FDG is the most commonly used and therefore most well-established PET tracer. But compared to many other drugs ^{18}FDG is an exceptional compound since it is trapped inside cells after phosphorylation. So, ^{18}FDG accumulates in organs. Other drugs normally show both input into and elimination out of cells. Even in this case the development of an IVIVC is feasible. Only the shape of the weighting and response functions will be modified (Fig. 9).

A promising novel approach for establishing an IVIVC using PET is described. This can be used to optimize methods for in vitro release measurement and ultimately aid in pharmaceutical formulation development.

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