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A Quantitative Relaxometric Version of the ELISA Test for the Measurement of Cell Surface Biomarkers**

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Abstract: *Quantitative measurement of marker expression in diseased cells is still a topic of considerable interest and different methodologies are currently under intense scrutiny. This work aims at developing an in vitro diagnostic method based on the release of paramagnetic species from relaxometrically "silent" liposomes operated by the action of a phospholipase A_2 (PLA_2) previously targeted to the epitope of interest. The released paramagnetic species causes an increase of the longitudinal water proton relaxation rate proportional to the number of PLA_2 bound to the cell outer surface. The sensitivity of the herein proposed method, named R-ELISA, was attempted in the detection of folate receptor expression on human ovarian cancer cells by functionalizing PLA_2 with folic acid. Receptor/cell number of 8.3×10^5 has been measured on IGROV-1 cells. The R-ELISA assay can detect nanomolar cell suspension receptor concentrations and has been validated by well-established spectrofluorimetric procedures.*

In recent years the term "personalized medicine" has become increasingly popular in oncology. It deals with the stratification of patients according to their responsiveness to a given therapeutic treatment.[1a,b] To this purpose patients have to be phenotyped with proper diagnostic tools and, currently, many efforts are devoted to identify new procedures and markers to tackle this task.[2a,b] In principle, the selected diagnostic tool should allow to assess the peculiar molecular characteristics of the tumor lesion providing the physician with a fundamental piece of information for an effective treatment. Thus personalized medicine approaches require the development of fast, sensitive, and quantitative methods for the measurements of clinically relevant markers in order to propose the best treatment for each patient. In this contest the quantification of the receptor density on tumor cells is still a topic of interest and different methodologies are currently under intense scrutiny. In principle, detection methods should provide a high sensitivity and specificity, and enable rapid measurements with minimal sample processing.

To achieve this goal a number of probes have been developed based on optical, electronic, and magnetic detection.[3a–d] However, the clinical translation of these systems is still difficult because they often require lengthy sample purification or long assay time. Furthermore, immune-hystochemistry and cytofluorimetry protocols allow the determination of only a few percentage of the positive cells with respect the entire population without an accurate determination of the number of receptors/markers expressed by each cell. Biosensing strategies based on NMR detection of properly functionalized iron oxide nanoparticles have recently received considerable attention, since they offer unique advantages over other detection methods.[4a,b] Specifically, since biological samples have a negligible magnetic background, magnetic nanoparticles can be used to obtain highly sensitive measurements in minimally processed samples. In general, the high payload of paramagnetic ions endows these systems a good T_2* susceptibility and causes the NMR proton signals of solvent water to broaden as a function of proton concentration.[5] However, T_2* may be affected by a number of uncontrolled conditions associated to local inhomogeneities in the specimen and in the magnetic field. Conversely, the longitudinal relaxation time (T_1) is considered the relaxation parameter that can be measured more accurately and its value is less prone to be affected by uncontrolled experimental conditions. Gd- and Mn-based contrast agents decrease T_1 of the solvent water protons to an extent that is directly proportional to their concentration.[6a,b] The disadvantage of the use of these agents is their relatively low sensitivity that forces to find suitable strategies to increase their concentration at the target site. Nanosized carriers such as liposomes have been considered to this purpose as they can be loaded with large payloads of Gd/Mn-complexes or ions.[7] Even thought for the application tackled in this work, the major limitation in the use of targeted liposomes (i.e. functionalized with the specific ligand) deals with the fact that their large size (100–150 nm) limits the maximum number of units that can be bound at the external cell surface. One can calculate a threshold of less than 20000 liposomes for a 20 μm diameter cell. Furthermore, liposome multivalency prevents the determination of the exact stoichiometry of the liposome/receptor binding sites. On this basis an alternative strategy to determine the target receptor density using enzyme-sensitive liposomes has been designed. It relies on the indirect detection of the target epitopes in

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a way that is comparable to techniques like ELISA. In the first step, the specific ligand (able to bind selectively to the receptor of interest) conjugated to an enzyme is added to the cell suspension. This leads to targeting the selected receptor by the modified enzyme. In the second step, "silent" paramagnetic liposomes (SPLs) are added. The entrapped contrast agent shows little relaxation enhancement, because of the low water exchange between the inner cavity of the liposome and the bulk water.[8a,b] However, if the enzyme is able to destabilize the liposome membrane, it results in the release of the entrapped paramagnetic payload with the consequent increase in relaxivity (which is the ability of the contrast agent to decrease the water proton relaxation rate). As the liposome stability will only be affected by the enzyme, the relaxivity enhancement is a direct measure for the enzyme activity and, in turn, of the targeted receptor concentration. As a proof of concept the herein proposed method, named R-ELISA (relaxometric enzyme linked in cells suspension assay), has been used to detect folate receptor (FR) expression on human ovarian cancer cells using a phospholipase A_2 enzyme (PLA_2) functionalized with folic acid (PLA_2-FOL). Overexpression of FR on many cancer cells (ovary, brain, kidney, breast, and lung) identifies this receptor as a potential target for many types of ligand-directed cancer therapeutics.[9a–d] The selected PLA_2 catalyzes the cleavage of the phospholipid acyl chain at the sn2 position.[10a–c] A phospholipid head group enters the active site, exposing its acyl chain which can subsequently be cleaved. The consequent destabilization of the phospholipid bilayer causes an increase in the observed relaxation rate ($R_{1obs} = 1/T_1$) directly proportional to the number of PLA_2 bound to the cell outer surface.

Thus the quantification of the epitopes of interest relies on the amplification effect associated to the release of the paramagnetic payload from the liposomes operated by the action of the targeted phospholipase. The measure of R_{1obs} is a well-established methodology and can be carried out on any NMR instrument. Multiple samples can be simultaneously measured by acquiring T_1-weighted NMR images by extrapolating T_1 values from the observed signal intensity values.

SPLs used in R-ELISA assay are made of a mixture of saturated phospholipids (DSPC:DSPE-PEG2000 95:5) that yield a compact bilayer responsible for a slow water exchange rate between the inner and outer compartments. This formulation yields to SPLs with a millimolar relaxivity (r_{1p}) of about 0.5 $mM^{-1}s^{-1}$, that can be switched on only upon the action of the proper stimulus that allows the "bulk" water protons to enter in contact with the paramagnetic metal complexes. In order to increase the stability and limit cellular phagocytosis, SPL membrane composition contains a 5% of peghilated phospholipids.[11] For the preparation of SPLs the lipid film was hydrated with a 200 mM solution of Gd-HPDO3A, a commercially available, MRI contrast agent largely used in clinical practice.[12] SPLs thus formed were extruded through progressively smaller pore sizes to select a population with an average diameter of 120 nm. The r_{1p} of entrapped Gd-HPDO3A was 0.5 $mM^{-1}s^{-1}$. This value was about eight times lower than that reported for the free complex at 25 °C. The SPL stability was tested at 37 °C in isotonic Hepes/NaCl buffer (HBS), by measuring R_{1obs} at 0.5 T as a function of the time. Figure 1 shows that R_{1obs} remained constant for 24 h with a slight increase at longer times. Upon the addition of PLA_2, a marked R_{1obs} increase proportional to the enzyme concentration is observed as a consequence of the progressive cleavage of the phospholipid acyl chain at the sn2 position. The R_{1obs} plateau reached after 24 h incubation corresponds to the relaxivity of the free complex thus indicating the complete release of the relaxation agents from the particle. This conclusion is fully supported by measuring the ^1H nuclear magnetic resonance dispersion (NMRD) profiles (see the Supporting Information). Mass spectrometry analysis of the enzyme-treated and control liposome showed a decrease in DSPC (molecular weight, Mw = 790.46 Da) and an increase in cleaved DSPC (Mw = 523.68 Da) mass peak (see Figure S1).

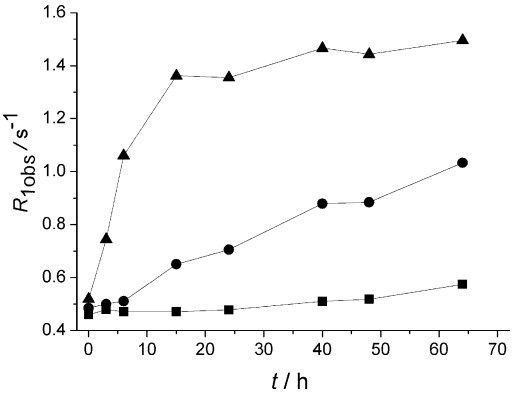

Figure 1. SPL stability has been followed by measuring the relaxation rates at 21.5 MHz in HBS in the absence (■) and in the presence of two different PLA2 concentrations (0.06 μM (●) and 1 μM (▲), respectively). The Gd concentration was 0.22 mM in all the solutions.

The PLA_2 conjugation to folic acid was carried out coupling folate-γ-ethylenediamine[13] to a PEG-3000 spacer through the γ carboxy group to maintain the high affinity for the FR. Then the FolatePEG_{3000} was coupled to PLA_2 using HydraLink chemistry that is based on the conjugation of a 2-hydrazinopyridyl moiety with a benzaldehyde to yield a stable bis-aromatic hydrazone. Therefore, the 2-hydrazinopyridine was linked to FolatePEG_{3000} by reaction with Boc-6-hydrazinonicotinic acid followed by Boc deprotection (Boc = *tert*-butoxycarbonyl). PLA_2-benzaldehyde was prepared through coupling of succinimidyl 4-formyl benzoate with lysine γ-NH_2 exposed on the surface (see the Supporting Information). The number of folate conjugated to each PLA_2 was 1.2 and this number was quantified spectrophotometrically by measuring the absorbance at $\lambda = 350$ nm. Figure 2 shows that there is not any significant difference in the degradation activity of the native and folate conjugated enzymes.

The FRα expression was determined by flow cytometry carried out by incubating cells (45′) with a monoclonal antibody (mAb) specific for FRα and post-incubating with a secondary FITC-labeled Ab (FITC = fluorescein isothiocyanate). The aspecific binding of the FITC-labeled Ab was evaluated by incubating cells with the same amount of secondary Ab without pre-incubation with the primary Ab. Cells were grown in folic-acid-free media and incubated with

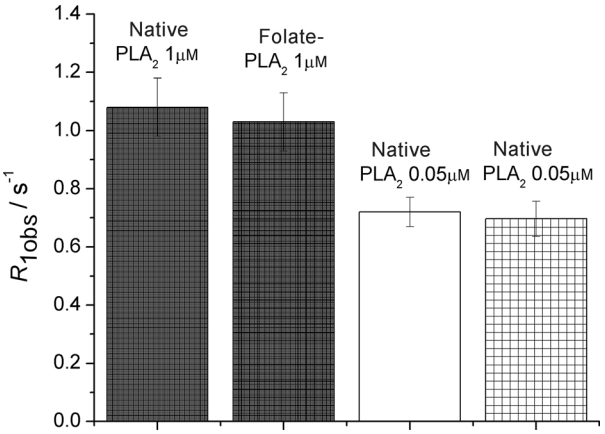

Figure 2. PLA_2 and PLA_2-FOL enzymatic activity comparison. The enzymatic degradation was followed by measuring R_{1obs} at 25 °C, 21.5 MHz, and pH 7 in HBS, of SPL incubated in the absence or in the presence of different concentrations of native and folate-conjugated 1 and 0.05 μM PLA_2, respectively.

serum to block any aspecific interactions. From fluorescence-activated cell sorting (FACS) analysis (Figure 3) it was possible to evaluate that more than 90% of the IGROV-1 cells express a significant level of FR whereas Hela FR expression results are significantly lower (< 11%). In order to quantify the FR density, cells incubated with FRα-specific mAb (using the protocol described above) have been analyzed by a spectrofluorimetric assay. Fluorescence intensity was measured using an excitation and emission wavelength of 492 and 517 nm, respectively. The calibration curve ($y = 116023x$-35427) has been obtained using a standard FITC solution. Using this method 900000 ± 70000 and 40000 ± 10000 receptors per cell were measured for IGROV-1 and Hela cells, respectively.

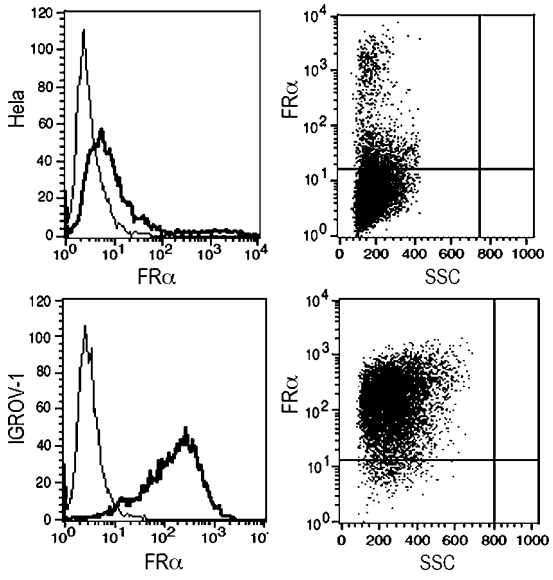

Figure 3. FACS analysis of the FRα density on the IGROV-1 and Hela cell lines. The positivity of cell lines to FRα receptors are analyzed using the CELLQUES PRO program.

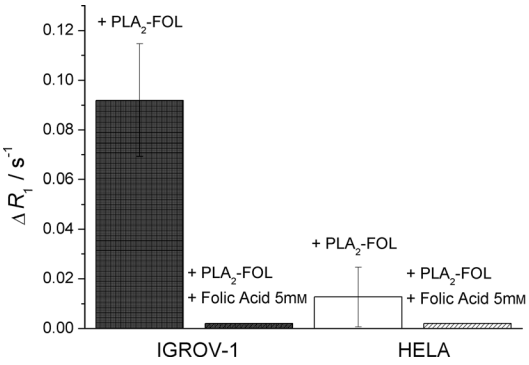

Figure 4. Comparison between IGROV-1 and Hela cell lines incubated for 15 h at 37 °C with PLA_2-FOL in the absence or in the presence of free folic acid as a competitor. ΔR_{1obs} (21.5 MHz, 25 °C) have been calculated subtracting R_{1obs} of SPL incubated in the presence or in the absence of cells pre-incubated with PLA_2-FOL

In order to set up the R-ELISA assay, the SPL stability has been verified in the presence of IGROV-1 cells by measuring the relaxation rate of their suspension that remained 0.5 s^{-1} for 24 h. Then IGROV-1 and Hela cells have been incubated in the presence of an excess of PLA_2-FOL for 1 h at 37 °C. Figure 4 shows that the enhancement of R_{1obs} measured on IGROV-1 incubated with SPL (for 15 h) is significantly higher than that observed on Hela cells that underwent the same treatment. In the presence of an excess of free folic acid the liposome remains intact indicating the absence of enzyme-aspecific binding. Cell viabilities of 95 and 93% were measured in the absence and in the presence of PLA_2-folate, respectively. This indicates that the enzyme does not cause a significant change of cell viability during the incubation period.

R-ELISA assay was carried out on IGROV-1 and Hela cells by applying the following five steps:

1) Detaching cells from growing flasks obtaining a suspension of a about three millions cells in 0.3 mL medium.
2) Incubating cells in the presence of an excess of PLA_2-FOL (0.1 μM)) for 1 h at 37 °C, to obtain the selective targeting of the enzyme to the FRs.
3) After washing, addition to the cell suspension of an aliquot of SPL (0.22 mM in Gd) and transfer in NMR tubes (0.1 mL) for the R_1 measurement at $t = 0$ and $t = 15$ h. Only R_{1obs} differences (ΔR_1) between free and PLA_2 incubated SPL greater than 4% have been considered significant for the receptor density determination.
4) Assessment of the enzyme concentration using the calibration curve obtained by measuring R_{1obs} of the SPL solution in Roswell Park Memorial Institute in the presence of increasing concentrations of PLA_2. Figure 5 shows a linear dependence between the enzyme concentration (10–70 nM) and R_{1obs}. The calibration curves obtained in the absence and in the presence of cell suspension are not significantly different. Determination of the number of receptors normalized to the cell number (determined by measuring the total protein concentration) using the following equation. The number of receptors per cell is given by ([enzyme (M)]·V(L)·6.022 e23) divided per cell number.

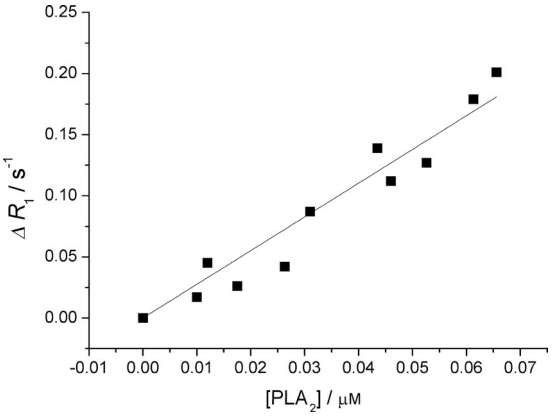

Figure 5. Calibration curve obtained by measuring ΔR_1 versus [PLA2] concentration.

Finally, the differences between FR densities determined on IGROV-1 using the R-ELISA assay and those determined by cytofluorimetry were statistically analyzed using the Student's two-tailed t test. The calculated p value was 0.7 thus indicating that the two data sets are not significantly different (Table 1). ΔR_1 measured in the presence of Hela (2%) was below the detection limit.

Table 1: FR density. FRα densities were determined on IGROV-1 and Hela cells using R-ELISA assay and spectrofluorimetry, respectively. Data were expressed as mean values ± SEM (standard error of the mean value).

Cell line	FRα density (R-ELISA)	FRα density (spectrofluorimetry)
IGROV-1	830000 ± 140000	900000 ± 70000
Hela	< 150000	40000 ± 10000

In summary, the herein reported results definitely support the working hypothesis that the incubation of cells with modified enzymes yields a FR-α-dependent increase in the observed relaxation rate indicating that the modified enzyme binds to the FR-α and is still enzymatically active. The R-ELISA method has been validated by the well-established cytofluorimetric procedure. The sensitivity of R-ELISA can be further improved by loading the liposomes with paramagnetic systems endowed with higher relaxivity as well as by replacing liposomes with other types of nanoparticles able to carry a larger payload. Different phospholipids with enhanced responsiveness to the selected enzyme will be tested in order to reduce the incubation time from 15 to 2–4 h.

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