Methods 97 (2016) 35–43

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

How to measure the affinity of aptamers for membrane proteins expressed on the surface of living adherent cells

Nam Nguyen Quang [a,b], Carine Pestourie [c,d], Agnès Cibiel [e], Frédéric Ducongé [a,b,*]

[a] CEA, DSV, I2BM, Molecular Imaging Research Center (MIRCen), Fontenay-aux-Roses, France
[b] Neurodegenerative Diseases Laboratory, Centre National de la Recherche Scientifique (CNRS), Université Paris-Saclay, Université Paris-Sud, UMR 9199, Fontenay-aux-Roses, France
[c] Inserm, UMS006/CREFRE, Service Exploration Non-Invasive, Toulouse, France
[d] Université Fédérale Toulouse Midi-Pyrénées, Université Toulouse III-Paul Sabatier, UMS006/CREFRE, Toulouse, France
[e] Genomic Vision, Bagneux, France

ARTICLE INFO

Article history:
Received 11 September 2015
Accepted 10 November 2015
Available online 14 November 2015

Keywords:
Aptamers
Cell-SELEX
Binding
K_d
Liquid handling robot

ABSTRACT

Recently, an increasing number of aptamers have been selected against biomarkers that are expressed at the surface of cells. This class of targets, mostly membrane proteins, is in close contact with the intra- and extra-cellular matrixes and their three-dimensional structures are inextricably linked to their inclusion in lipid bilayers. Therefore, although binding studies can be performed on the isolated form of these proteins, it remains crucial to measure the affinity of these aptamers in a more physiological environment, i.e., directly on living cells. Here, we describe a procedure for radioactive binding assays that can be adapted for measuring the affinity of aptamers against different cell lines. This method has been semi-automated using a liquid handling robot in order to reproducibly measure the apparent dissociation constant K_d and the apparent number of targets per cell. Relevant issues are discussed including the labeling of aptamers, the cells preparation, the incubation, the washings, the use of non-specific competitors, the data analysis and finally the reporting.

1. Introduction

Membrane proteins are a major source of biomarkers for the diagnosis and over half of the currently approved drugs target this type of protein. In addition to small molecule drugs and antibodies, an increasing number of nucleic acid aptamers have been being selected against membrane proteins since the past 20 years (see for a review [1–5]). Aptamers are nucleic acid-based ligands identified through a process of molecular evolution usually named SELEX (Systematic Evolution of Ligands by Exponential enrichment) [6,7]. They are sometimes compared as chemical antibodies, since, like them, they can be used to inhibit or activate their targets upon binding [3,8] but also to detect them using microscopy or flow cytometry [9–11]. Furthermore, several aptamers selected against membrane proteins have been promisingly used for the vectorization of drugs or contrast agents [3,12–14].

An important step for the identification and the characterization of aptamers is to measure their affinity for their target. Several methods can be used including gel and capillary electrophoresis, ultrafiltration, dialysis, fluorescence anisotropy, surface plasmon resonance, circular dichroism, thermophoresis or isothermal titration calorimetry (see for a review [15,16]). Most of these methods require an almost purified form of the target and no one can measure the affinity of aptamers on membrane proteins present at the surface of living cells. However, affinity measurements can be very different if they are performed with the purified form of a membrane protein or with the same protein present at the surface of a cell. Indeed, the three dimensional conformations of membrane proteins are most of the time dependent on their inclusion in lipid bi-layers but also on their interaction with other proteins to form different complexes at the cell surface. It is also well known that the plasma membrane is a dynamic system where membrane proteins can fold in different conformations depending on their environment.

To solve this drawback, flow cytometry has been used to measure the affinity of fluorescently labeled aptamers directly on living cells [17–19]. In addition to measuring the affinity of aptamers, this method can measure whether the distribution of

Abbreviations: CO-RE Gripper, compressed O-ring expansion gripper; cpm, counts per minute; SELEX, Systematic Evolution of Ligands by Exponential enrichment.

* Corresponding author at: CEA, DSV, I2BM, Molecular Imaging Research Center (MIRCen), Fontenay-aux-Roses, France.
E-mail address: frederic.duconge@cea.fr (F. Ducongé).

http://dx.doi.org/10.1016/j.ymeth.2015.11.011
1046-2023/

the membrane protein is uniform within a cell population. However, the conjugation of aptamers with a fluorophore may have an impact on its affinity. Furthermore, this technique is ideal for non-adherent cells such as blood cells, but is less relevant to use with adherent cells for which the distribution of membrane proteins can change when they are detached. This limit has been demonstrated by Li et al. who observed that the binding of an anti-Tenascin C aptamer was not detected by flow cytometry on detached U251 glioblastoma cells whereas the aptamer showed a very strong binding by microscopy on the same cells once adhered [20].

Radioactive binding assay on adherent living cells was first introduced in the 1970s [21] and is still a very popular method for measuring the affinity of small compounds, natural ligands or antibodies on adherent cells [22,23]. Here, we present an adaptation of this method for nucleic acid aptamers focusing on different key steps which need to be optimized, as well as describing a semi-automated protocol that we use to measure the apparent K_d and the apparent number of targets per cell. Finally, we discuss the necessity to provide an accurate reporting on the experimental conditions that are used to ensure as much as possible the reproducibility of the measurements between different laboratories.

2. Calculation

In the following section, we summarize the fundamental theory of ligand binding studies. More complete explanation can be found elsewhere [24–27].

The simplest type of binding involves a 1:1 association of an aptamer with a target to form a complex that can be represented by the application of the Law of Mass Action as follows:

$$\text{aptamer} + \text{target} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{aptamer—target complex}$$

The rate of association is dependent upon the association rate constant (k_{on}), the aptamer concentration and the target concentration. The rate of dissociation is dependent upon the dissociation rate constant (k_{off}) and the concentration of aptamer–target complex. At equilibrium, the forward and reverse reactions of binding are equal and the equilibrium dissociation constant (K_d) can be defined as:

$$K_d = \frac{k_{off}}{k_{on}} \tag{1}$$

If we assume that, during the binding studies, the concentration of aptamer is very much higher than that of the target, changes in the free aptamer concentration due to binding can be ignored but changes in the free (unbound) target concentration cannot. Therefore at equilibrium:

$$k_{on} \times ([T_{total}] - [TA]) \times [A] = k_{off}[TA] \tag{2}$$

where $[T_{total}]$ is the apparent total concentration of target i.e., the concentration of the maximum amount of aptamer–target complex that can be formed in the binding condition. [TA] is the measured concentration of aptamer–target complex at a given concentration of aptamer $[A]$.

According to Eqs. (1) and (2) can be transformed in:

$$\frac{k_{off}}{k_{on}} = K_d = ([T_{total}] - [TA]) \times \frac{[A]}{[TA]} \tag{3}$$

and

$$[TA] = [T_{total}] \times \frac{[A]}{[A] + K_d} \tag{4}$$

From the Eq. (4), measuring [TA] as a function of $[A]$ provides a hyperbolic plot that could be fit to yield a value for K_d and $[T_{total}]$. The K_d can also be roughly estimated from this curve considering that $K_d = [A]$ at $[TA] = 1/2\ [T_{total}]$. The lower is the K_d, the better is the affinity of the aptamer. Furthermore, $[T_{total}]$ can be used to estimate the number of targets per cell using the equation:

$$\text{Number of target per cell} = \frac{[T_{total}] \times \text{Volume of incubation}}{N_A \times \text{number of cells}} \tag{5}$$

where N_A is the Avogadro constant ($6.022 \times 10^{23}\ \text{mol}^{-1}$).

K_d and $[T_{total}]$ can also be calculated using linear regression from the mathematically equivalent equations of Scatchard (6), Lineweaver–Burk (7) or Hanes (8):

$$\frac{[TA]}{[A]} = \frac{[T_{total}] - [TA]}{K_d} \tag{6}$$

$$\frac{1}{[TA]} = \frac{1}{[T_{total}]} + \frac{K_d}{[T_{total}] \times [A]} \tag{7}$$

$$\frac{[A]}{[TA]} = \frac{K_d + [A]}{[T_{total}]} \tag{8}$$

When a radioactive binding assay is performed, it is important to collect data at concentrations of the aptamer that are below and above the K_d in order to obtain all parts of the hyperbolic curve and get a reliable fit of the equations. Furthermore, it is also important to use a $[A]$ that is many times higher than the K_d (at least ten times) to approach the saturation of the target that will conduct to better estimation of the $[T_{total}]$. If these recommendations are followed, all the equations from (4) to (8) should provide similar values of K_d and $[T_{total}]$. Otherwise, it suggests that the binding does not fit a 1:1 binding model and a different model would be more appropriate. Hence, it should be considered that there are two or more distinct populations of target with different conformations and with different binding constants for each. Another possibility is a positive or negative cooperativity in binding with a single population of target. If these cases are suspected, the hyperbolic binding curve can be fit with the Hill equation:

$$[TA] = \frac{[T_{total}] \times [A]^h}{K_d^h + [A]^h} \tag{9}$$

where h is the Hill slope. If h equals 1, the binding fit a 1:1 binding model. If h is higher than 1, it suggests multiple binding sites with positive cooperativity. If h is less than 1, it suggests multiple binding sites with different affinities for the aptamers or multiple binding sites with negative cooperativity.

Two types of radioactive binding experiments can be performed: saturation and competition. In this paper, only the saturation method is presented since it uses less aptamer than competition studies. However, the competition studies can be useful if the binding do not fit a 1:1 binding model or to measure the affinity against several subtypes of a membrane protein. In a competition experiment, various concentrations of an unlabeled aptamer can be used to compete for binding with a fixed concentration of a radiolabeled ligand. Increasing the concentration of the aptamer decreases the amount of bound radiolabeled ligand. From this experiment IC_{50} value can be obtained and corresponds to the concentration of unlabeled aptamer that inhibits the binding of the radioactive ligand by 50%. Therefore, the dissociation constant for the aptamer is often referenced as the K_i rather than K_d because it is obtained from an inhibition experiment. In practice these experiments can only be used if a radiolabeled ligands with a known K_d is available for a subtype of a membrane protein. The K_i value for the unlabeled aptamer can be obtained from the IC_{50} value using the Cheng–Prusoff equation: