ABSTRACT

Alzheimer’s disease (AD) is characterized by cerebrovascular and neuronal dysfunctions. AD is a very difficult disease to identify with no evident and no efficient treatment available until today. Biologically, the reasons underlying the difficulty to diagnose are several, there is not a biomarker that alone tell us with no doubt that its presence is an indication for Alzheimer’s, but several that are strongly related with that possibility. In addition, any biomarker or compound created for therapeutics or diagnose must be able to cross the blood-brain barrier (BBB). Based on previous results, a single domain antibody developed by TechnoPhage, appears as a possible novel approach to tackle this problem. Here are shown some of the preliminary results for the pharmacokinetics of this antibody (sdAb#Q), using nuclear and fluorescent techniques. In vivo nuclear biodistribution studies, using $^{99m}$Tc conjugated with sdAb#Q, the injected activity per gram (%I.A./g) in the brain was $0.55 \pm 0.13$, 2 minutes post injection and $0.09 \pm 0.01$, 60 minutes post injection. These preliminary results show that antibody sdAb#Q possess all the desired features, namely the ability to cross the BBB, but also a fast clearance profile, preventing neurotoxicity. Using the fluorescent spectroscopy for biodistribution, the results were not conclusive and need further optimization in the future. Nowadays, there are several biomarkers used in association with brain imaging techniques however they are not accurate enough. Therefore, the creation of a widely accepted biomarker continues.

**Keywords:** Alzheimer’s Disease, Brain Imaging, Biomarkers, Single-Domain Antibodies, Brain-Blood Barrier.

1. INTRODUCTION

1.1 Alzheimer’s Disease

In the United States, Alzheimer’s Disease (AD) is the most common cause of dementia in elderly, affecting more than 5 million people. It is estimated that the total number of people with dementia worldwide will reach 115.4 million by 2050, with AD patients contributing 60 to 70% to this value [1,2]. The costs associated with the disease tend to increase every year, being estimated that by 2050 they will reach a trillion dollars unless some efficient disease modifying treatments are developed [3]. Alzheimer’s Disease is a type of dementia characterized by cerebrovascular and neuronal dysfunctions leading to a progressive loss in cognitive functions such as thinking, memory loss, among others.
AD has been considered as a protein disease, caused by the accumulation of incorrectly folded amyloid-beta protein (Aβ) and tau protein (a microtubule associated protein) in the brain, leading to the formation of structures called plaques and tangles, respectively. Amyloid plaques in AD brains are formed by amyloid-beta (Aβ) peptide. It is originated by sequential proteolytic cleavage of the amyloid precursor protein (APP) by the β-secretase and γ-secretase enzymes. APP is a transmembrane protein that penetrates through the neuron's membrane and is critical to neuron growth, survival, and post-injury repair [4]. Amyloid-beta peptide, that has the ability to enter in the brain, may be in the origin of Alzheimer’s Disease. The blood–brain barrier (BBB) is a diffusion barrier, which prevents influx of most compounds from blood to brain. It is not known exactly how changes in the production and aggregation of the Aβ peptide give rise to AD [5], however the deposition of Aβ peptides, within neurons and amyloid plaques in brain tissue appears early in the course of the disease and is a well-known hallmark of AD [6]. The BBB is the main hurdle in brain drug development and is the most important factor limiting the future growth of neurotherapeutics.

1.2 Brain Imaging

AD is a very difficult disease to identify, with no evident and no efficient treatment available until today. The problem starts in diagnosis, because in the beginning symptoms are very mild and not taken into account by patients. Doctors normally attribute them to other conditions, mainly aging, but also with other dementias and diseases, until other exams are required [7,8]. Thus, there is an urgent demand to deeply understand the disease mechanisms, the early biomarkers that are disease related and how can we target and monitor their presence. For these, techniques that allow us to monitor the brain structure and function are crucial to make diagnosis more accurate [9]. Techniques like functional magnetic resonance imaging (MRI), single photon emission computed tomography (SPECT) and positron emission tomography (PET) give information about underlying physiology, the brain chemistry and therefore complement the data from the structural imaging in providing insights into the brain’s performance [10,11]. Antibodies can be a useful tool in nuclear medicine, since they bring new possible approaches to treat diseases, complementing the world of radionuclides in medicine.

1.3 Antibodies and Clinical Uses

Production of monoclonal antibodies was a breakthrough that revolutionized different fields from academic research to therapeutic applications, not all problems were solved specially compared with the solutions that small molecules offer. Some disadvantages include their large size (approximately 150 kDa) and relative instability which has generally limited them to injectable routes of administration, as well as the relative expense of manufacturing [12]. In the early 1990’s, it was discovered that in addition to the conventional antibodies, camelids and sharks also produce unusual antibodies composed only of heavy chains. These peculiar heavy chain antibodies (hcAbs) lack light chains. Therefore, the antigen-binding site of hcAbs is only formed by a single domain that is linked directly via a hinge region to the Fc-domain. The variable domain is designated as variable domain of camelid
heavy chain antibody (VHH) for camelid hcAbs, and more generally as single domain antibody (sdAb). The molecular weight of this heavy chain variable domain, varies approximately between 12 and 15 kDa. Using protein engineering techniques, it is possible to change, for example, amino acids in order to overcome a problem such as solubility, or to improve the binding with a certain target. Considering the statistics presented above and all the difficulties associated with the disease, the work here present tries to overcome all this. The development of a biomarker that acts in an early stage, allowing the use of the tools for the treatment, or at least preventing disease progression. Either way, the benefit for the patient is already enormous, and the costs associated with the disease can decrease dramatically. Although the results here present are preliminary and need further validation, they are a good indicator that this goal can be achieved.

2. METHODS

2.1 Protein Production and Purification

*Escherichia Coli* BL21 cells expressing the single domain antibody sdAb#Q were cultured in SB media containing 100 µg/mL of ampicillin and incubated overnight at 37 °C. Cells were induced using 0.6 mM of IPTG (isopropyl-beta-D-thiogalactopyranoside) and lysed by sonication. Soluble protein was separated from cell debris and sdAb#Q was recovered using a chromatography system (ÄKTApRime plus, GE Healthcare Life Sciences) coupled with a prepacked Ni²⁺ sepharose resin (HisTrap HP, GE Healthcare Life Sciences). To assess protein purification a 15 % SDS-PAGE gel was stained with Coomassie Blue. A western blot using anti-HA-HRP (high affinity, 3F10, Roche) was done to detect sdAb#Q (data not shown).

2.2 Antibody Radiolabeling with ⁹⁹ᵐTc

The sdAb was conjugated with technetium-⁹⁹ (⁹⁹ᵐTc). The ⁹⁹ᵐTc was eluted from a ⁹⁹Mo/⁹⁹ᵐTc generator, in the form of pertechnetate [⁹⁹ᵐTcO₄⁻]. From [⁹⁹ᵐTcO₄⁻], it was generated the organometallic aqua complex, technetium-tricarbonyl [⁹⁹ᵐTc(CO)₃(H₂O)₃⁺]. In a reaction tube, it was added the amount of protein required to have 2.5 mg/mL of final concentration for a final volume of 500 µL. The eppendorf containing the protein was purged with nitrogen, before adding the technetium-tricarbonyl to the reaction tube, to prevent the bond formation between atmospheric oxygen (O₂) and technetium. The ⁹⁹ᵐTc-tricarbonyl was added until 500 µL. The mixture was incubated in a water bath at 37 °C for 30 minutes. Using instant thin-layer chromatography (iTLC) developed with 95 % methanol + 5 % HCl 6 N, it was measured the labeling efficiency. Unconjugated free ⁹⁹ᵐTc-tricarbonyl will migrate to the solvent front. Using a γ-radioactivity-TLC-scanner (raytest γ-MiniGITA) it was verified if the ⁹⁹ᵐTc-tricarbonyl was bound to the protein. The reaction was transferred to an Amicon® Ultra-0.5 mL Centrifugal Filters 3K (Millipore) and centrifuged at 14000 g for 10 minutes to remove free ⁹⁹ᵐTc-tricarbonyl by ultrafiltration. A gamma detector was used to determine radioactivity. It was added 400 µl of PBS 1x in Amicon® to remove the free ⁹⁹ᵐTc-tricarbonyl and centrifuged at 14000 g for 5 minutes. It was verified if the ⁹⁹ᵐTc-tricarbonyl free in the mixture has reduced, use a γ-radioactivity-TLC-scanner. To finalize, the radioactivity was checked in a gamma detector. The desired radioactivity at end of the reaction should be between 1.9 - 2.5 mCi (for biodistribution using 6 animals).
2.3 In vivo Biodistribution study

All animal experiments were performed in accordance with the guide-lines for animal care and ethics for animal experiments outlined in the National and European Law. Caesarean Derived-1 (CD1) mice were injected intravenously into the tail vein with 100 μg of the antibody-99mTc conjugate. All mice were sacrificed by cervical dislocation, at 2 and 60 minutes post injection. Measure the activity administered to the animal using a dose calibrator (Capintec, Inc). The tissues of interest were dissected, rinsed with PBS 1x to remove blood excess, weighted, and their radioactivity was measured using a gamma counter. Blood and urine samples were collected at the sacrifice time and analyzed by iTLC. The biodistribution results were expressed in percentage of the injected activity per gram of tissue (% I.A./g).

2.4 Single Photon Emission Computed Tomography (SPECT) Imaging

For in vivo imaging studies it was used the same protocol as in the biodistribution study. The animals were injected intravenously in the tail vein with 100 μg of the antibody-99mTc conjugate and sacrificed by cervical dislocation. Images were acquired using a SPECT equipment, at 2 and 60 minutes post injection.

2.5 Fluorescence Imaging

This method requires the use of IVIS Lumina (In Vivo Imaging System, PerkinElmer) equipment and of a fluorophore to conjugate with the sdAb#Q, in this case Vivo Tag *800 (PerkinElmer). Antibody was labeled by adding 1 mL of antibody at 1 mg/mL to 10 μL of fluorophore solution and mixed well for 1 hour at room temperature. Free fluorophore molecules were removed from the solution, using a PD-10 size exclusion column (GE, Healthcare Life Sciences) according to the manufacturers indications. Fractions were collected and stored at 4 °C until further use. For the image acquisition, the animals (CD1 mice) were anesthetized before acquisition in an In Vivo Imaging System (IVIS Lumina, PerkinElmer). Mice were carefully shaved and cleaned using 70 % ethanol. Animals were injected with 100 μL of protein-fluorophore conjugate. The settings were adjusted until good fluorophore visualization has been obtained. The excitation and emission filter used was the one for Cy 5.5. The parameters adjust for the different settings presented in the Results are: exposure time, binning and f/stop. After acquiring the images, the colors can be altered to improve fluorophore detection.

3. RESULTS

Based on previous results (data not shown), a single-domain antibody, sdAb#Q, was selected and successfully large-scale produced in E.coli cells. To determine the antibody profile inside the organism different experiments were performed.

3.1 In vivo Biodistribution Study

In collaboration with Dr. João Galamba Correia, biodistribution assays using 99mTc radioisotope were performed at Nuclear Technologies and Science Center (C2TN). In the experiment presented, CD1 mice were sacrificed at two different time points: 2 and 60 minutes, and the organs collected are listed in Table 1. All mice received the injection in the tail and were injected with 100 μg of antibody conjugated with 99mTc. The organs collected in both experiments are listed. At 2 minutes post injection, antibody is mainly present in circulation,
in the kidneys and in the liver. Although the percentage of antibody in the brain is low in comparison with other organs, it must be taken in consideration that the brain is highly protected by the blood-brain barrier. As expected, 60 minutes post injection the antibody is concentrated in the liver to be metabolized, in the kidney to be excreted and in the urine, indicating that the organism has the capacity to metabolize and excrete the antibody. The clearance of the antibody, over time, from the brain is also clear, which is a desirable feature for a biomarker because it prevents toxicity.

Table 1: Biodistribution results of the single domain antibody sdAb#Q conjugated with $^{99m}$Tc. The CD1 mice were sacrificed by spinal cord dislocation, at 2 and 60 minutes post injection. After the sacrifice, the organs were collected, weighted and gamma radiation was measured. The n represents the number of animals used for each experiment. Results are presented in percentage of injected activity per gram of organ (%I.A./g), except for excretion that is presented in percentage of injected activity (%I.A.).

<table>
<thead>
<tr>
<th>Organ</th>
<th>Single Domain sdAb#Q -$^{99m}$Tc (%I.A./g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy Model (CD1)</td>
</tr>
<tr>
<td></td>
<td>2 min</td>
</tr>
<tr>
<td></td>
<td>(n=3)</td>
</tr>
<tr>
<td>Blood</td>
<td>15.2 ± 5.0</td>
</tr>
<tr>
<td>Liver</td>
<td>10.3 ± 1.2</td>
</tr>
<tr>
<td>Intestine</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>Spleen</td>
<td>3.9 ± 0.6</td>
</tr>
<tr>
<td>Heart</td>
<td>4.0 ± 0.9</td>
</tr>
<tr>
<td>Lung</td>
<td>6.5 ± 0.5</td>
</tr>
<tr>
<td>Kidney</td>
<td>33.1 ± 6.6</td>
</tr>
<tr>
<td>Muscle</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Bone</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>Brain</td>
<td>0.55 ± 0.13</td>
</tr>
<tr>
<td>Excretion (%I.A.)</td>
<td>-</td>
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</tbody>
</table>

3.2 Stability Assay

In the stability assays, urine and blood were analyzed to confirm antibody stability. Mice were sacrificed according to standardized protocols via cervical dislocation, at 2 and 60 minutes post injection. The blood and urine samples that were used in this experiment, are the same that were collected in the previous experiment of the biodistribution assay. From the previous experiment, only one sample for each time-point was used. In Figure 1 a), in the urine analyzed of the mouse sacrificed 2 minutes post injection, there is
no evident peak because the level of gamma radiation detected is low, meaning that there is not a traceable amount of sdAb#Q present in the urine since the antibody did not have time to be excreted. In Figure 1b), in the urine analyzed of the mouse sacrificed 60 minutes post injection, there is gamma radiation detection, represented by the peak near the solvent front. The peak probably represents the antibody that has been metabolized and excreted. The peak deviation from the origin to the solvent can also indicate that the antibody might have suffered some modifications during metabolization, in order to be excreted. In Figure 1 c), in the blood analyzed of the mouse sacrificed 2 minutes post injection, there is a peak that represents the antibody present in circulation. In Figure 1 d), in the blood analyzed of the mouse sacrificed 60 minutes post injection, there is a not clear peak at the origin, that probably corresponds to the antibody, meaning that there are still traces of antibody in circulation, however the levels are low, due to metabolism and excretion. Therefore, the organism has the capacity to metabolize and excrete the antibody.

3.3 Single Photon Emission Computed Tomography (SPECT) Imaging

The antibody was conjugated with radioisotope $^{99m}$Tc. The CD1 mice were sacrificed according to standardized protocols via cervical dislocation, at 2 and 60 minutes post injection. Gamma radiation was detected using a SPECT equipment. Both mice were
injected in the tail with approximately 100 µg of antibody. In Figure 2 a), it is possible to see that 2 minutes post injection antibody is mainly concentrated in the liver and in the kidneys but also in the brain, however in less amount than in the remaining organs.

In Figure 2 b), after 60 minutes post injection the antibody is mainly concentrated in the kidneys and some in the liver, ready from excretion. Antibody is no longer observed in the brain. The other high intensity spot in Figure 2 b) represents the injection site. Even though the presence of the antibody in the brain is clear in Figure 2 a), it needs further confirmation because antibody can be “trapped” in BBB, and not actually in the brain. Since it was a preliminary imaging test, quantification of radioactivity per organ was not done, but these images can be complemented with biodistribution data, present in Table 1.

3.4 Fluorescence Imaging

Fluorescence imaging was also done, a first experiment (data not shown) was performed to test and to obtain the best acquisition conditions before trying the acquisition using two mice, one with the protein unconjugated (control) and other with the protein conjugated with the fluorophore Vivo Tag® 800. The excitation and emission wavelength are 785 nm and 810 nm, respectively. In this first experiment, only one mouse was used and different acquisition settings were tested, namely exposure time, the binning, f/stop and image color vary to improve the visualization of the fluorophore. The different settings tried did not show clear changes in antibody distribution over time.

![Figure 2](image)

Figure 2: Single photon emission computed tomography scan images of CD1 mice injected with 100 µg of antibody conjugated with 99mTc. Mice received tail injection. a) CD1 mouse was sacrificed 2 minutes post injection. b) CD1 mouse was sacrificed 60 minutes post injection. Legend: B - brain; L – liver; K - kidneys; IS - injection site.

![Figure 3](image)

Figure 3: Fluorescence images were acquired using IVIS Lumina (PerkinElmer) equipment. sdAb#Q was conjugated with fluorophore Vivo Tag 800 (PerkinElmer). Images a), b) and c) were acquired at different time points. The top mouse is the control mouse that has been injected with protein unconjugated, and the lower mouse is the mouse injected with protein conjugated with VivoTag 800. Legend: p.i – post injection.
However, the best selected settings were used in the experiment in Figure 3. Before the acquisition, CD1 mice were anaesthetized using isoflurane following the standardized protocols, were also shaved in the back and cleaned with ethanol 70 % before injection. Both mice were injected in the tail with around 100 μg of protein. The control mouse is located in the top of the images of Figure 3 and was only injected with protein without fluorophore, the lower mouse was injected with protein conjugated with the fluorophore VivoTag® 800. In Figure 3 are represented different acquired images over time, at 1, 8 and 15 minutes post injection. It is possible to observe a color change in the brain area, that tends to decrease over time. There is no evidence that guarantees that the color change is due to antibody biodistribution, because there is also changes in the control mice, even though they are less pronounced. There is no evidence of antibody in the excretory organs, at 15 minutes post injection.

4. DISCUSSION

In previous work, single domain antibody sdAb#Q was selected due to its high yields production in E.coli cells (data not shown). With antibody produced in large scale and purified, biodistribution assays were done using sdAb#Q conjugated with Tc radionuclide. The antibody biodistribution was assessed at two different time points. At 2 minutes post injection, the antibody is mainly present in blood circulation, in the liver and in the kidneys (normal for these sdAbs as these are described as their excretion organs). A small amount of antibody is also present in the brain which is very good, since the brain is a highly protected organ and every molecule or biomolecule to reach the brain must be able to translocate the blood-brain barrier, efficiently. As mentioned before, although the results point in the direction that the antibody can target the brain, in vitro and in vivo assays can be done to corroborate these results. In vitro assays include models that mimic the blood-brain barrier and in vivo assays comprise immunoblotting techniques to detect the presence of the antibody in the brain of animals that have been administered with sdAb#Q. Although further confirmation is needed, considering that only 5 % of the molecules created today are able to translocate the BBB, this is an outstanding result. After 60 minutes post injection, the antibody is present in the organs where is metabolized and excreted. These results are expectable, since the antibody primarily targets the brain, where in AD patients amyloid-beta is present, and then is cleared to the excretion organs. Due to its reduced size (∼15 kDa) the antibody has a short-half live in the organism and is excreted fast. This is an important feature because if the antibody had a size superior to the kidney filtration cut-off (∼70 kDa), it would remain in the organism longer and since it is conjugated with a radionuclide, it would expose the organism to higher doses of radiation (99mTc half-life is 6h).

The stability assays complement the information previously obtained from the biodistribution assays. Stability was tested in blood and in urine. Comparing the data of Table 1 and Figure 1, the results are similar. At 2 minutes post injection, the antibody is not presence in urine and clearly present in the blood. At 60 minutes post injection, the opposite happens, antibody was metabolized and is not present in the
blood (Figure 1) and present in urine. The graph of the urine 60 minutes post injection in Figure 1 b), has a peak closer to the solvent front than to the origin, meaning that antibody is not stable in urine and that it has been degraded for excretion.

The SPECT scan images presented in Figure 2, are also complementary to the biodistribution assay results present in Table 1, the same time points were used. Unequivocally, 2 minutes post injection the antibody is located in the liver, kidneys and some in the brain, as the results of the biodistribution assay have demonstrated. And 60 minutes post injection, the antibody is concentrated in the kidneys for excretion. There is no evident presence of antibody in the brain. After targeting the brain, the antibody demonstrated fast clearance, confirming its capacity to cross the BBB. If this was not observed, and the antibody tend to accumulate in the brain it could generate neurotoxicity and it could not be used as a diagnostic tool. Despite the excellent results, this experiment needs to be repeated using a higher number of animals.

In the fluorescence imaging assay present Figure 3, antibody sdAb#Q was conjugated with the fluorophore VivoTag® 800. Two mice were used, a control mice injected with unconjugated protein. The lower mouse was injected with protein conjugated with the fluorophore. Over time, there is a decrease in the color intensity, especially in the brain area. In the control mouse there is also some changes. However, in both mice no other organs can be detected. These mice have been shaved to reduce the fur fluorescence. Different reasons can help to explain these results. Fluorescence signals suffer from several signal reducing factors like severe tissue attenuation. This factor depends on the wavelength of the emitted light. Theoretically, near-infrared (NIR) excitation and emission fit best to the optical transparency window of tissue (650–1200 nm). Due to tissue heterogeneity emitted light will suffer from scattering, shorter wavelengths will yield stronger scattering compared to the longer wavelengths. Combined with surface reflections these factors result in a decrease in signal strength and imaging resolution with increasing tissue depth. When excitation light is required, autofluorescence (endogenous fluorescence of tissue) may generate a background signal that is not related to the imaging agent [13]. In future experiments, acquisition of the ventral plane can be attempted to try to visualize some organs. By acquiring in this position we are reducing the amount of tissue that photons need to travel before being acquired. However, it must not be forgotten that the plane used in Figure 3 is the best plane for detecting the brain. Another possible optimization of this assay, is to use nude mice (mice homozygous for the nude spontaneous mutation), that are hairless, allowing a possible improvement in the organs visualization [14]. This assay needs further optimization before being validated. Considering all the results here present, the single domain antibody sdAb#Q has demonstrated ability to cross the BBB and to be cleared fast from the organism. Both features are outstanding for the development of a diagnostic tool, that over the years have considered essential. The necessity of creating an antibody with these characteristics is a demanding need since Alzheimer’s and dementia are considered a tremendous problem that are considered to affect more and more people over the years.
5. CONCLUSION

Alzheimer’s disease is in fact a problem since there is an increasing number of people suffering from this disease. It is estimated that the total number of people with dementia worldwide will reach 115.4 million by 2050, with Alzheimer’s patients contributing 60 to 70% to this value. The work here present demonstrates that single domain antibodies can be successfully produced. With the different molecular imaging modalities tested in this work, it is clear that the antibody is targeting the brain. This confirmation is more evident when using the nuclear conjugation with $^{99m}$Tc, not only by the SPECT scan images but also by the biodistribution assays performed. This radioisotope is a widely used radioactive tracer in nuclear medicine, that emits gamma radiation suitable for detection. The results from the biodistribution assays corroborates what is already known, that this radioisotope has a low physical and biological half-life (6h and 24h, respectively) and that is rapidly cleared from the organism. Fluorescent imaging using a fluorophore did not produce as evident results as the nuclear imaging, however there is a small evidence that fluorescent imaging will confirm those results. To obtain similar results several optimizations will have to be done to the acquisition settings and different time points used, so a clear biodistribution of the antibody can be visualized.

REFERENCES