Universidade do Minho
Escola de Ciências

Pedro Filipe Ferreira de Sousa Moreira

Zebrafish embryos as *in vivo* model for toxicity evaluation: screening of nanoparticle formulations DODAB:MO and DODAC:MO

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Zebrafish embryos as *in vivo* model for toxicity evaluation: screening of nanoparticle formulations DODAB:MO and DODAC:MO

Master in Molecular Genetics

Work developed under supervision of
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Declaração

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Zebrafish embryos as in vivo model for toxicity evaluation: screening of nanoparticles formulations DODAB:MO and DODAC:MO

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“The ones who are crazy enough to think that they can change the world, are the ones who do”

Steve Jobs

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ii) Abstract

Zebrafish (*Danio rerio,* Hamilton 1822) has emerged as a viable alternative in biomedical trials in relation to other organisms such as small mammals, also used for *in vivo* toxicity tests, given their morphological characteristics and well characterized biology.

The increasing application of nanoscale particles on Medicine, Pharmacy and Cosmetics, particularly as nanovehicles for targeted delivery of therapeutical agents, leads to an urgent requirement of evaluation of these nanoparticles toxicological profile, not only at the cell level, but also at the all-organism level.

The ZET (zebrafish embryo toxicity) protocol has been widely considered on environmental sciences, to evaluate the potential toxic effects of chemicals and pharmacological compounds that reach the natural ecosystems. This test is already approved by the OECD (Organization for Economic Co-Operative Development) for acute aquatic toxicity testing. The ZET assay gained particular wide notice for assessment of the toxicity and bioactivity of metallic nanoparticles, carbon-based nanostructures and polymers, screening ecological benchmarks. On the other hand, investigation of the nanotoxicity profile of non-metallic nanoparticles, such as liposomes, based on zebrafish embryogenesis is particularly underexplored.

The work of this Msc thesis focus on validation of the ZET test as a fast-track and reliable *in vivo* tool to compose the toxicological profile of dioctadecyldimethylammonium bromide or chloride (DODAB/DODAC):monoolein (MO) liposomes, developed by our research group for gene therapy, in order to anticipate possible risks at different levels of biological organization. The nanoformulations tested include different ratios of the cationic lipid components - DODAC or DODAB - with MO (4:1, 2:1, 1:1. 1:2 and 1:4). These ratios are related with the interesting capacity of MO, present in different proportions in the mixtures, in promoting cell transfection for further use in gene therapy as non-viral vectors.

iii) Resumo

O peixe-zebra (*Danio rerio,* Hamilton 1822) tem surgido como uma alternativa viável em ensaios biomédicos em relação a outros organismos, como roedores, também usados para testes de toxicidade, pelas suas características morfológicas e a sua biologia muito bem caracterizada.

O aumento do uso de partículas à escala nano em Medicina, Farmácia e Cosmética, em particular os nano-veículos para a entrega de fármacos a alvos específicos, leva a uma urgência na avaliação destas partículas em termos toxicológicos, não só a nível da célula mas também para o organismo como um todo.

O protocolo ZET (zebrafish embryo toxicity) tem sido vastamente considerando em ciências ambientais para avaliar os potenciais efeitos tóxicos nos embriões de peixe-zebra perante a exposição a compostos químicos e farmacológicos. Este ensaio esta aprovado pela OCDE (Organization for Economic Co-Operative Development) para análises de toxicidade em ambiente aquático. O ensaio ZET já se encontra bem validado para avaliação da bioatividade de nanopartículas metálicas nano-estruturas baseadas em carbono e polímeros, no rastreio de pontos de referência ecológico. Por outro lado, a investigação de um perfil de nano-toxicidade para partículas não metálicas, como lipossomas, baseados na embriogénese de peixe-zebra, é particularmente inexplorada.

O trabalho da presente tese de mestrado irá focar-se na validação do ensaio ZET como um meio fiável e rápido de avaliar os efeitos da exposição a formulações de lipossomas à base de brometo ou cloreto de dioctadecildimetilamonia (DODAB/DODAC):monooleína (MO) desenvolvidos pelo nosso grupo de trabalho, de forma a prever possíveis riscos a nível celular e tecidular, bem como no que diz respeito ao desenvolvimento embrionário.

As formulações testadas incluem diferentes frações de lípido catiónico - DODAB ou DODAC - com MO (4:1, 2:1, 1:1, 1:2 e 1:4), interessando, também, estimar a capacidade da MO, presente em diferente proporção nas misturas, na promoção da transfeção celular para aplicação destes sistemas em terapia génica como vetores não virais.

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Introduction

1. General introduction

This work is based on the validation of the zebrafish as a reliable tool for toxicological testing of non-metallic nanosystems.

This organism, zebrafish, is a widely used organism in biology as a way to test toxicological effects of various compounds. The biology and morphology of the zebrafish, as well as their development from embryo to adult, is very well known, as such its use as a tool for toxicological testing is all justifiable.

Being easy to keep these animals in an animal facility, at a low cost and maintenance index, zebrafish became a desirable organism for laboratory tests. Furthermore, displaying external fertilization and resistance to some extent, facilitates the collection of reliable results in a relatively short time.

Within a period of 80 hours after fertilization, it is possible to preview the results of test compounds, allowing continuous monitoring of the development and, where necessary, adjustments on modes and administration concentrations without spending too much time in adjustments, being possible to repeat the tests every week.

This organism can be used as an intermediate form between animal cell culture assays, *in vitro*, and small mammal models, *in vivo* long-term experiments, therefore results obtained in cell lines can be confirmed in these animal embryos before going on to more complex animals. Zebrafish allow easy adjustment of concentrations and proportions of nanosystems conjugations and, also, assess where these compounds may become harmful to a complex organism before being administered to long-term animal models.

Another important point in zebrafish use in toxicological tests relies on the available legislation regarding use of animals in laboratory experiments. Faced with this legislation, embryonic forms are not "animals" and are, therefore, not covered by ethical issues, suggesting, among other considerations, using animal models in assays or experiments only if all other assumptions are exhausted. Therefore, using the zebrafish embryo is legally distinct from directly using animal

models and we are thus ethically respecting the legislation in force in avoid animal experimentation.

Zebrafish embryos has been used, as expected, in assays for toxicological tests on metallic nanosystems with some positive results. With this animal model some preliminary results of toxicological impact may be obtained, not only at the level of the organism, but also organ-specific information and regarding developmental processes.

There are numerous assays with metal nanosystems in zebrafish, which makes this organism a reliable vehicle for testing this type of particles. However, regarding non-metallic systems, such as the systems tested within this thesis work, information is scarce, with a very limited number of non-metallic systems tested. Therefore this work, in addition to assessing the effects of the non-metallic particles described below, also intended to validate this animal model as a reliable vehicle in such toxicological testing of non-metallic particles.

The particles tested during this work which were developed by our work group consist in cationic surfactants, dimethyldioctadecylammonium bromide (DODAB) and dimethyldioctadecylammonium chloride (DODAC), in combination with a helper lipid, monoolein (MO), in various ratios, forming the system designated as DODAX:MO.

The ratios used (4:1, 2:1, 1:2 e 1:4) were previously tested in *in vitro* assays, being the same proportions to DODAB and DODAC together with monoolein.

This cationic surfactants was tested alone and together with monoolein, as a way to compare the index of toxicity and where the compound will be act in the embryo when the surfactant act alone and in conjugation with a lipid helper. This analysis is important for the calibration of concentrations, by adjusting the concentration will be easier to develop the proportions of the compounds that will be tested in the form of nanosystems.

In addition, the lipid mixtures with different ratios will be tested as such, that is, as simple combinations of unformulated DODAC or DODAB with MO. With this additional approach, it is possible to compare levels of toxicity when the compounds are simply mixed and when they are in a nanoparticle form.

It has been reported that DODAC is more cytotoxic than DODAB and that liposomes with MO (neutral), in either of the formulations, make the general

cytotoxicity decrease (reduced positive charge) than if only including cationic lipids.

This work arises as a continuation of combined efforts of researchers from the Biology Department and the Physics Department of University of Minho, as well as the spin-off Nanodelivery – I&D em Bionanotecnologia, Lda.

This project was carried out under the orientation of Professor Doctor Andreia Gomes (Biology Department), responsible for biological validation of the lipoplexes and the zebrafish facility and a member of the referenced spin-off, Professor Doctor Maria Elisabete Real Oliveira (Physics Department), responsible for developing formulations of lipoplexes of the family DODAX:MO, and Doctor Marisa Passos, co-responsible for the zebrafish facility and expert in zebrafish development.

1.1. Zebrafish

1.1.1. General description

Zebrafish, is a small fresh water fish, from the Asian continent, which has been used in the research as an important vertebrate model for many biological areas (Feitsma *et al.*, 2008; Hamilton, 2011; A. J. Hill *et all*, 2005; Rizzo *et all*, 2013; Santoriello *et all*, 2012; Spence *et all*, 2008).

Figure 1: Female Zebrafish, photo taken in NanoDanio Laboratory, University of Minho, 2015.

The geographical distribution of zebrafish includes all the Asian continent, having his natural focus on north-eastern India, Bangladesh and Nepal, in the Brahmaputra and Ganges river basins (Engeszer, Patterson, Rao, and Parichy, 2007; Hamilton, 2011; Lawrence, 2007). These fishes have also been found in others river basins from Asia, such as Mahanadi, Godavari, Cauvery, Indus and Pennar, and also in some regions without details regarding correct localization and correct classification about the specimen, such as Rajasthan, Gujarat and Andra Pradesh, from the Arabian Sea and on the Myanmar and Sri Lanka regions (Spence *et al*., 2008).

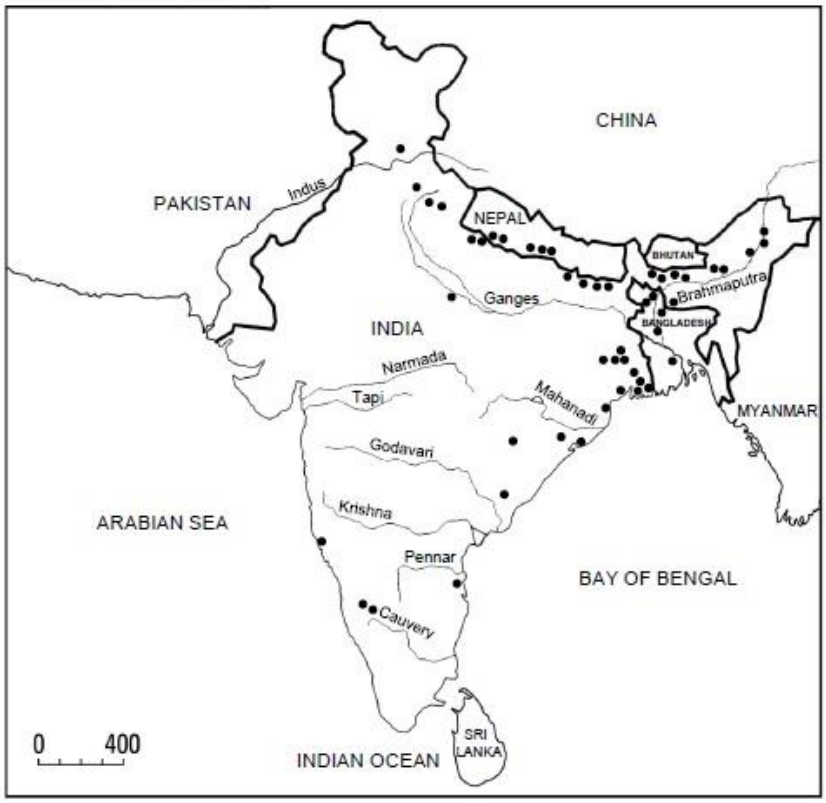

Figure 2: Danio Rerio geographical distribution and localization (Spence et al., 2008).

This continental localization is the perfect habitat for zebrafish due to its characteristic climate. The clime of some regions of the Indian subcontinent include the extension of freshwater habitat affected by the monsoons, a characteristic that is important for the proliferation of this species (Spence et al., 2008). Zebrafish are found in low level water bodies or shallow ponds, commonly in contact with rice fields, where the waters have low-movement (Hamilton, 2011; Spence et al., 2008) and the connection with the main rivers is possible during the monsoon months, when the elevation of the water flow creates blind channels for the fish movement and rice cultivation (Spence et al., 2008).

Zebrafish habitat is essentially characterized by low level water bodies, with approximately 30 cm of depth, with unshaded zones and aquatic vegetation, amidst rice cultivation which removes the threat of large predators (Hamilton, 2011; Spence et al., 2008).

The localization on cultivated fields provide large amounts of subtracts for zebrafish diet. Due to the use of some fertilizers, the growth of zooplankton is high, attracting zebrafish specimens (Spence et al., 2008).

Zebrafish are omnivorous freshwater fishes, whose wild diet contains aquatic insects, or aquatic larval of terrestrial forms of insects, to zooplankton. Its diet may additionally include filamentous algae, vascular plant materials, spores, invertebrate eggs, fish scales, arachnids, detritus, sand and mud. Most of its feeding occurs in the water column, although some of its diet show they can feed from the substrate and on the surface (Spence et al., 2008).

The climate conditions of the Asian subcontinent imply a temperature range between 6º C in the winter and 34º C in the summer (Spence et al., 2008), which translates into thermal tolerance of this organism between 6.7º C and 41.7º C (Lawrence, 2007). However, some expeditions studies reveal that the normal locations where zebrafish are found had still or slow waters with a temperature ranging 24.6º C to 38.6º C (Engeszer et al., 2007).

Some characteristics, which will be described later, and its well-established behavior and biology studies make this organism an important model for scientific research (A. J. Hill et al., 2005; Rizzo et al., 2013) having such extensive information available regarding all stages of development of both sexes, from larvae, juveniles to adult stages (A. J. Hill et al., 2005).

1.1.2. Taxonomic and morphologic characteristics

The knowledge about this organism is more specific and extensive than for other fish species, from physiological to biochemical and biological cues, from earlier stages of development to adult forms (Engeszer et al., 2007; Hamilton, 2011; A. J. Hill et al., 2005; Lawrence, 2007).

Zebrafish is a small freshwater teleost, about 3 cm long, with a characteristic coloration, translucent and exhibit, from head to tail, five longitudinal blue stripes (Hamilton, 2011; Lawrence, 2007; Wixon, 2000). Different colorations and patterns have however been described in several specimens (Hamilton, 2011; Quigley and Parichy, 2002).

As Francis Buchanan-Hamilton described in 1822, the taxonomic characterization of *Danio rerio* includes them in Animail kingdom, Chordata phylum, Actinopterygii class, Cypriniformes order, Cyprinidae family with Danioninae subfamily, and *Danio* genus (on http://eol.org/pages/204011/overview, 05/08/2015).

The description and phylogenetic classification based on morphological characteristics is already currently better understood (Metscher and Ahlberg, 1999). The localization of the zebrafish in the phylogenetic tree promotes its use as an animal model, having in common with other higher vertebrates some processes or structures, as a general pattern development of a few large structures of the animal, similarly to its common ancestor, such as the development of somites, the axe body, branchial skeleton and various organs, such as the senses, and the nervous system (Metscher and Ahlberg, 1999).

All these features make the zebrafish as a reliable species for use in laboratory and animal model in laboratory studies, and can be a reliable tool as an intermediate form amid *in vitro* and *in vivo* assays in larger animals models (Metscher & Ahlberg, 1999).

Zebrafish, as others fishes, might give an idea that the use of these animals in experimentation to apply on human, cannot be right, but wrong is thinking by this way. The genome of zebrafish is complex, this fish have more chromosomes than the human genome, twenty-three pairs, one pair more than the human. In its evolution over the time, occurred a whole-genome duplication, resulting on this extra pair that provides a higher number of duplicate genes, paralogs, whole-duplication that didn't happened in mammals (A. J. Hill et al., 2005). Some of those duplicate genes are found in mammals, having, some of them, new function or, even, expression in other type of tissue as the original genes, orthologs (A. J. Hill et al., 2005).

Back to morphological characteristics, the zebrafish has a characteristic size rounding the 30 mm and rarely exceeding 40 mm in total length, measured from the head to the caudal fin (Hamilton, 2011; Lawrence, 2007; Spence et al., 2008; Wixon, 2000). In terms of appearance, it has a compressed body on both sides of the fusiform shape. Starting the description for the animal's head, its mandible is shown to be non-uniform, that is, the inferior portion from the mandible protrudes to the superior portion, having an oblique mouth, the visual

system is shown central and visible only from the front or laterally when viewed superiorly the eye is not possible to notice (Hamilton, 2011; Spence et al., 2008).

Another visual feature easily identifiable is the longitudinal lines present throughout the body with dark blue color, having an incomplete lateral line, which extends to the base of the pelvic fin. The striped color is visible also in its anal fin having a difference in the dorsal fin, where this appears with a darker coloration on the upper edge with white terminals (Spence et al., 2008).

In a cellular form, this pattern of colors that characterized the zebrafish is defined by several types of pigment cell, melanophores, responsible for the dark blue hue, can be concentrated and scattered in response to stimuli serving also as a form of camouflage and also aggregate and disperse in response to light variations and signaling, providing a dark tone when the fish exhibits an aggressive stance, xanthophores responsible for golden hue and iridophores, which gives it the iridescent hue (Spence et al., 2008).

During the development of the zebrafish patterns, they exhibits a formation of two lists of centrally form in the body, later, with the development of the animals, more lists are added in both of sides, in the superior base to the lower base (Spence et al., 2008).

In the sex distinction in these animals, the tone is the same, however the males have a more yellowish in the anal area (Spence et al., 2008). Zebrafish presents sexual dimorphism.

In the larval stage, a discrimination between genders in these animals is difficult, however during the beginning of the reproductive cycle, females will develop a characteristic belly and makes the identification easier, and the flat abdomen of males becomes yellow (Spence et al., 2008).

1.1.3. Life cycle and embryonic development

Reproduction of zebrafish presents itself differently in wild conditions and biotherium. The information about the reproduction of this species wild population is low, making the information on posture in these conditions scarce (Engeszer et al., 2007; Lawrence, 2007; Spence et al., 2008), therefore, in the laboratory, a major objective of research in zebrafish is the rate of animal posture, that is, when it is intended to obtain a high value of embryos per posture (Engeszer et al., 2007; Spence et al., 2008).

The differentiation and sexual maturity of this animal develops differently in comparison to other model species used in laboratory. Initially, all animals are born with the same type of sexual organs, all have ovaries. Only after five to seven weeks after fertilization, the males begin sexual differentiation, crossing a intersexual phase, and finally around the third month post fertilization these begin to develop male gonads, testes, all depending on external conditions where the animals are located, including the existing feed rate and the consequent growth, animals that exhibit a more rapid growth tend to present themselves as female zebrafish and, on the other hand, animals with a slower rate of development, tend to present themselves as males, and may then, the food getting easily be related to this phenomenon (Engeszer et al., 2007; A. J. Hill et al., 2005; Spence et al., 2008).

The stimulation to the posture of the animals occurs differently on wild condition and under laboratory, tests developed in animals captured in the wild showed the need for a ratio of one female to one male to obtain embryos, but in situations of captivity, this ratio becomes different, thus requiring a greater number of males than females. These data appear to be justified by the frequency of postures and food availability in wild conditions, but not only, the concentration of male pheromones is also of higher relevance. In natural conditions, the prevalence of mating occurs more seasonally, while in laboratory is most common, given a simulation of ideal conditions for the practice of fertilization more frequently. In wild situations, the availability of food will vary according to the seasons, so the presence of abundance transmits the need for propagation

of the species, hence the ratio be lower in the laboratory, where these conditions are elaborated in order to get a higher posture and more systematic and, possibly, this is the justification for the need for a different animal ratio (Engeszer et al., 2007; Lawrence, 2007; Spence et al., 2008).

The maturation to the beginning of sexual life in these animals, appears to be the same in both wild and captivity conditions and tends to be linked to the size and not the age of the animal, while from about 24 mm, animals are ready for the beginning of sexual activity (Spence et al., 2008).

The frequency of postures can be stimulated weekly in the laboratory, depending on the age of the animals, while younger females have an egg maturation cycle rounding 6 days between clutches. Though, as will increasing animal age, the greater is the need of time to complete the ovulation cycle and get ready for an upcoming spawn. Moreover, the older the animal is, the greater the time required for a next spawning, but the number of embryos tends to be higher (Lawrence, 2007; Spence et al., 2008).

The spawning process is generated when specimens of both genders are placed in contact, in conditions of substrate, temperature and food provision that conducts to reproduction. In such optimal conditions, the addition of males to females, will trigger the release of male pheromones that stimulate females spawn, and it is required contact time to generate this reaction, causing the female draw up a spawning, all the eggs in only once time (Spence et al., 2008). The amount of pheromone present on the insulation for reproduction, will lead to the increase or decrease of the number of released eggs, that is, it was proved that females exposed to a higher concentration of male pheromones lead to increase the spawning and provide more resistant embryos, thus justifying the use of more males than females under laboratory conditions (Lawrence, 2007; Spence et al., 2008).

The eggs are released in an uncontrolled form to the substrate, i.e., there is no treatment by the animals with the accommodation objective of the eggs, and they are released to the substrate and enabled when they come into contact with water and, then, fertilized by the sperm from the males. The eggs can, in a situation of no fertilization, for some reason, develop up until the early cleavages, not evolving from there and leading to death of the egg (Lawrence, 2007; Spence et al., 2008).

The embryonic development, as mentioned above, has the beginning when the egg is released and comes into contact with the water, being fertilized by male sperm, the embryo continues its development, relatively quickly, as a characteristic of this species (Lawrence, 2007; Spence et al., 2008).

The phases of this development have been very well described by Kimmel 1995, and his basis of embryonic analysis were taken into account on the preparation for this project.

The several stages of the embryonic development are described in the following table 1 in a simple manner, being described and illustrated later in a more deeply way. The eight stages of embryonic development are internally divided in accordance with the internal phase of the embryo, i.e., within each phase are sub-phases that qualify the embryo in a temporally way according to the embryonic development (Kimmel *et al*., 1995). First, the embryo experiments de zygote phase, having, inside the corion the one-cell stage (Kimmel et al., 1995). At this phase the egg has been fertilized recently, experimenting the first cleavage, the zygote stage, over a time gap around 40 minutes after fertilization, and having a full diameter rounding the 0.7 mm (Kimmel et al., 1995).

Table 1: The several fases of development of zebrafish, with the time-points and description, from (Kimmel et al., 1995).

Phase of development	Hour post fertilization	Description
Zygote	0	The newly fertilized egg through the completion of the first zygotic cell cycle
Cleavage	¾	Cell cycles 2 through 7 occur rapidly and synchronously
Blastula	2 ¼	Rapid, metasynchronous cell cycles (8, 9) give way to lengthened, asynchronous ones at the midblastula transition; epiboly then begins
Gastrula	5 ¼	Morphogenetic movements of involution, convergence, and extension form the epiblast, hypoblast, and embryonic axis; through the end of epiboly
Segmentation	10	Somites, pharyngeal arch primordia, and neuromeres develop; primary organogenesis; earliest movements; the tail appears
Pharyngula	24	Phylotypic-stage embryo; body axis straightens from its early curvature about the yolk sac; circulation, pigmentation, and tins begin development
Hatching	48	Completion of rapid morphogenesis of primary organ systems; cartilage development in head and pectoral tin; hatching occurs asynchronously
Early larva	72	Swim bladder inflates; food-seeking and active avoidance behaviors

During the one-cell stage the embryo experiments some movements to form the first cell, is activated some cytoplasmic movements beginning a transmission of direction to the animal pole, occurring a segregation of the blastodisc from the yolk more clear granule-rich vegetal cytoplasm. The development and segregation takes during the early cleavage stage (Kimmel et al., 1995).

The next stage, after the formation of the one-cell stage, is called the cleavage period, this period is differentiated in six stages, between the two-cell stage and 64-cell stage, having a time period between ¾ of hour and two hours. The phase is characterized by a constant division of cells in a fifteen minutes interval between each cell, having a meroblastic division of the cytoplasm, that is, the cleavage it's partial, having an incomplete uncut of the blastodiscs and blastomeres (Kimmel et al., 1995).

As the divisions are occurring, the cleavages will be more complete, ending the cleave stage with an embryo with 64-cell development (Kimmel et al., 1995).

After de 64-cell formation, the next division relates the next stage of embryonic development, the blastula period, and this period is sub-divided into nine stages, between the 128-cells stage and the 39%-epiboly stage. The blastula stage refers to the stage where the blastodisc change the appearance, beginning to look ball-like. This period is important for the embryo, is here that begins the epiboly, the embryo initiate the midblastula transition, his yolk have a formation as yolk syncytial layer, and starting the epiboly (Kimmel et al., 1995).

The division of cells remains in a synchronizing form, leading to more flattened shape form, ending into 30%-epiboly, starting, so, the next stage of development (Kimmel et al., 1995).

This next stage is called gastrula period, between the 5 ¼ hour and 10 hours post fertilization, sub-divided into six stages, between the 50%-epiboly stage, at 5 ¼ hour, and the bus stage, at 10 hours (Kimmel et al., 1995).

This period is characterized by a development of the complete epibolic arc, here the cell maintain their movement into a convergence, extension and involution form, starting the formation of the primary germ layers and embryonic axes. Between the 50%-epiboly and the 90%-epiboly stages, the epibolic arc going to close the yolk, surrounding this compound, after the complete surrounding, the embryo is close and passed to another stage, bud stage, the last of the gastrula period, defining 100%-epiboly, here, the embryo starts his segmentation mode, pass into other period of development (Kimmel et al., 1995).

This next period is called the segmentation period, and is divided into five stages, classified by the presence of a number of somites. This period it is comprised between 10 $^{1}/_{3}$ hour and 22 hours post fertilization, between the one-somite stage until the 22-somite stage (Kimmel et al., 1995).

This period is the period of differentiation, here the embryo takes a form like a future animal, occurs the somite formation, is develop one kind of rudimental form of the future organs, and his tail becomes to having a normal form (Kimmel et al., 1995).

The major classification of this period is the somites formation, as is classified all the stages of this period, this somites have a development in the

trunk and tail in sequence, having a formation respecting some norms, the anterior somites develop first and after the posterior ones, in the other hand, the time of development from the somites respects some time-lines, so, earlier somites have a rate from approximate three somites per hour, then, after the six first somites, they occur more slowly, two somites per hour (Kimmel et al., 1995).

After the bud stage appended, is initiated the pharyngula period, this period is characterized by the possibility of compere several vertebrates morphologically, in this phase, the embryo have his conformation like a future animal. This period correspond to the second day of embryogenesis, is viewed like an animal formed with a bilateral organization, having a well-developed notochord, the set of somites are well-developed too extending to the end of long post-anal tail, his nervous system is hollow and extended, and his metencephalon have a rapid cerebellar morphogenesis, finally, his brain is well-developed into 5 lobes (Kimmel et al., 1995).

His development have an aperture into his angle, so, the embryo begins to open and form a normal animal, this angle is classified as head-trunk angle (Kimmel et al., 1995).

This period is sub-divided into four stages between the prime-5 stage, at 24 hour post fertilization, and high-pec stage, 42 hours post fertilization, after this stage, the embryo hatched to an early larva mode (Kimmel et al., 1995).

The hatching period talk about the growth until to hatch, so, the embryo keep growth in the normal index about earlier, now with the morphogenesis of most part of the rudiments organs are completed, and their development decrease considerably (Kimmel et al., 1995).

This hatching period is sub-divided into three stages, from the long-pec stage on 48 hours post fertilization, to the pec-fin stage, on 60 hours, where the pectoral fin is now a flat flange, and, finally, protrunding-mouth stage, on 72 hours post fertilization (Kimmel et al., 1995).

After the animal hatched, he pass to the final period of embryonic development, the early larval period, at day three, the larva have almost his development complete, beginning to swim, having swift scape responses and initiate his respiration (Kimmel et al., 1995).

This embryonic development takes about 80 hours, which makes this animal a good animal model to fast assays.

1.2. Zebrafish embryos as a non-animal model for non-environmental toxicological studies

The use of other models for toxicological analysis may take some extra time in comparison with zebrafish embryos, and some models have more restrictions by law, what can make expansive (a. J. Hill, 2005), and that can be resolved with the use of this organism, having some advantages in comparison with others models common used in research (1, 2, 3, 4) (Feitsma & Cuppen, 2008; a. J. Hill, 2005; Rizzo et al., 2013; Santoriello & Zon, 2012).

This organism has a high similarity, in term of genetic characteristics, with the human, about 70% of the genes can cause diseases in human have a functional homologs genes in zebrafish (Santoriello & Zon, 2012), having some molecular processes, genes, receptors and the development in the early stages are gene programed and conserved inside animal phyla (A. J. Hill et al., 2005).

Having twenty-three pairs of chromosomes, with duplicated genes present in mammals, allows a high advantage when use zebrafish as animal model in experimentation. In mammalian, when a mutation occur in a ortholog gene, probably drive to an embryonic mortality, but in zebrafish, having more duplicated genes, the embryo can remain viable, causing a less severe phenotype, allowing the monitoring of the gene function, and this cannot be possible in mammalian organism, due the embryonic mortality (A. J. Hill et al., 2005).

Zebrafish, have more advantages very known in comparison with the others models, as small size, husbandry and his early morphology (Feitsma & Cuppen, 2008; A. J. Hill et al., 2005; Rizzo et al., 2013; Santoriello & Zon, 2012).

His small size is useful in comparison with others fishes used on research, the size about 3 cm, which have an adult, allows the facility of husbandry and his costs, the maintenance is cheap and there are several companies specialized in these organisms and their maintenance (Feitsma & Cuppen, 2008; A. J. Hill et al., 2005).

This small size it’s seen, also, in the larvae stage, this reduced size allows the low volume of solutions and space to proceed the experiment. The use of low

volumes of solutions limited the possible volumes of waste and allows the use of controlled concentration of reagents (A. J. Hill et al., 2005).

In toxicological experimentation, the small size of zebrafish embryos are very important, with this size it's possible test several solutions and concentrations in only one plate and, also, create replications to secure the procedure, testing, all conditions and replications at the same time (A. J. Hill et al., 2005). The zebrafish embryos can survive on the plate several days, his small size and his absorption of yolk allows the monitoring on growth and the impact the solution can cause to the organism (Feitsma & Cuppen, 2008; A. J. Hill et al., 2005).

Despite the small size, this fish has a high fecundity, the zebrafish female can provide from 200 to 300 eggs every 5 – 7 days, and this are a very useful advantage, using several ratios of fishes, this number of embryos can be much higher, allowing amplify the substrates that will be used, and this procedure can be repeat weekly (Feitsma & Cuppen, 2008; A. J. Hill et al., 2005).

These embryos have a special feature, they are transparent, and this are a special advantage for zebrafish to be use on toxicology assays, this allows the monitoring the changes over time by confocal, optical and florescence microscopy, from the embryo stage to larvae mode, when the tissues become more dense and start to appear the pigmentation (Feitsma & Cuppen, 2008; A. J. Hill et al., 2005).

This set of characteristics, including the fast embryonic development and his translucent appearance, makes this animal is a good animal model for fast-track toxicological assays, managing, then, evaluate the necessary morphometric characteristics for completion cytotoxic points of the test compounds on test.

1.2.1. Zebrafish embryo toxicity (ZET) assay for in vivo fast-track validation of nanoparticles

The large knowledge about zebrafish makes this organism a good model for toxicological studies, the well-study of all points of development of this fish, from embryo stage to adult of both genders, shows the potential to explore this animal in fast-track validation of chemical contacts (A. J. Hill et al., 2005; Lawrence, 2007; Rizzo et al., 2013; Santoriello & Zon, 2012).

Before using this fish as an animal model, it's necessary to have some points into consideration. It's necessary to take into account the possibilities and limitations of analysis the zebrafish can provide (A. J. Hill et al., 2005; Rizzo et al., 2013), these possibilities are listed in the following table.

Table 2: List of several types of toxicity investigations using Zebrafish as animal model for toxicity assays.

Types of Toxicity Investigations Using Zebrafish	Reproductive toxicity
	Developmental toxicity
	Acute toxicity
	Neurotoxicity
	Cardiotoxicity
	Ocular toxicity
	Endocrine toxicity
	Neurobehavioral toxicity
	Vascular toxicity
	Carcinogenicity

The use of zebrafish can provide a fast result in terms of toxicology analysis and this organism has been commonly used for this propose due several reasons in comparison with others animal models (A. J. Hill et al., 2005; Rizzo et al., 2013), having some limitations, can transmit some fast results providing information about toxicological points in the development time and long-term development defects and effects (Rizzo et al., 2013).

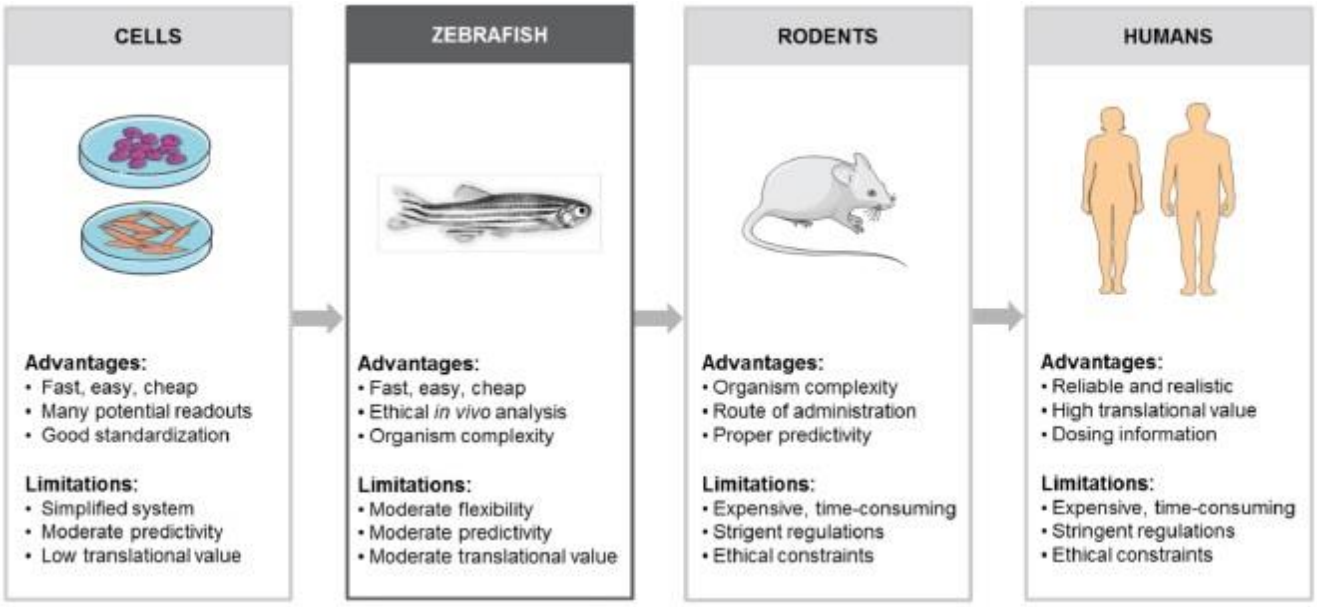

Figure 3: Sequence of testing steps in research, beaded the advantages and disadvantages of each elapsed stage, from in vitro tests to humans trials. From (Rizzo et al., 2013)

Zebrafish as a non-mammalian model for experimentation, have some disadvantages in comparison with mammalian models, mainly for human research in some kinds of organ disorders (Santoriello & Zon, 2012).

The lung and mammary gland are lacked in this fish so the research about disorders in these particular organs are difficult, on the other hand the genetic manipulation in this animal for cancer research can be productive. So, zebrafish, have been applicate in some genetic areas, adopting some technics used in mouse, for example DNA transposon system *Sleeping Beauty*, has successful results in identification of some conserved and novel cancer genes (Santoriello & Zon, 2012).

Other technic now used in zebrafish is about morpholinos, basically they are antisense oligonucleotides that inhibit translation or affect splicing, and having too some good results in cancer research (Santoriello & Zon, 2012).

As zebrafish development are fast and the use of morpholinos have been useful in this organism due this fast development. The morpholinos are insert into zebrafish embryos when they are at 1 to 4 cells stage, remaining with active performance for 6 or 7 days and, in this organism, at this days post fertilization, the most of the organs are already formed allowing a fast and easy detection of *in vivo* gene function. In others models for biological experimentation this assay

is difficult, the development of them are slow and the antisense oligonucleotides are degraded during the development (Santoriello & Zon, 2012).

This organism, by having a rapid development, keeping some purposely altered genes during his development, is used in several studies in the area of human health, as in hematological disorders, simulation of solid human tumors, human disorders as heart, muscle, kidney, central nervous system and ocular (Santoriello & Zon, 2012).

Despite the advantages that this animal model has to a rapid research of toxicity of particles used in biomedicine, the predominant use, in the literature, refers to metallic nanoparticles, having a low information on non-metallic particles.

About metallic nanosystems tested in zebrafish embryos, there is many useful information, the assays of this animal embryos are becoming more common, this is because the easy maintenance in the laboratory, the morphology of the embryos, ease of reproduction and obtaining new embryos weekly and his rapid embryonic development allows to obtain results quickly, makes this animal as a reliable vehicle for toxicological testing compounds, however the most information lies only about metallic particles, as in the case of copper-based nano-particles (Griffitt, Hyndman, Denslow, & Barber, 2009), silver-based nanoparticles (Bar-Ilan, Albrecht, Fako, & Furgeson, 2009; Cowart, Guida, Shah, & Marsh, 2011; Griffitt et al., 2009), based on gold nanoparticles (Bar-Ilan et al., 2009; Powers, Slotkin, Seidler, Badireddy, & Padilla, 2011).

The information of metal nanoparticles tested in zebrafish, is offered in a large number of possibilities, but to non-metallic nanoparticles, such as the lipoplexes are, this information is more restricted, requiring a larger number of tests for validating this animal as a reliable vehicle toxicological tests for non-metallic nanoparticles.

Being DODAx:MO nanosystems as a non-metallic nanosystem, this work will be evaluate, not only the low toxic content of this nanosystems, such as the validation of this animal model for this type of assay.

From the observation and measurement of a number of different toxicological endpoints, can be evaluated where and at the level of nanosystem will act in the embryo and how they will do, always getting a comparison with embryos in normal development.

1.2.2. Collection and analysis of different toxicological endpoints

During the toxicity assays with zebrafish embryos, are made visualizations at the microscope and captured images for future comparison with the control embryos, these views are developed daily between 8 and 80 hours post fertilization, following the embryonic development from initial epiboly and early larvae periods.

According to the OECD standards, embryos are treated according to certain indications for the test to be feasible. To obtain statistically acceptable results, is require four replicates of each condition, which will be alternated by daily views, being analyzed two replicates per day (Braunbeck & Lammer, 2006).

The visualizations are prepared in order to obtain different toxicological endpoints, always fulfilling timetables for this purpose, and, then, developed four views for four days, fulfilling the 8, 32, 56 and 80 hours post fertilization, achieving then evaluate the different toxicological endpoints planned for the ZET assay (Zebrafish embryo toxicity) (Braunbeck & Lammer, 2006; Kimmel et al., 1995).

Due to the morphological characteristics of zebrafish embryos, the views in these time-points allow evaluating the characteristics of the developing embryo within the various periods. The transparency of embryos allows us to verify possible internal changes in the embryo, and to complete the time-point provided it is possible to determine if the development has reached time period for the time in question (Braunbeck & Lammer, 2006; Kimmel et al., 1995; Spence et al., 2008).

For the four visualizations elaborated in the ZET assay, some different toxicological endpoints are evaluated, according the period in question. The several endpoints are summarized in the following table.

Table 3: Endpoints evaluated according the hours post fertilization

Hours Post Fertilization	Endpoints
8	Mortality Epiboly Hatching
32	Mortality Spontaneous movements Cardiac frequency Hatching
56	Mortality Spontaneous movements Cardiac frequency Hatching
80	Mortality Hatching Free swimming

In addition to the parameters described in the table above, the embryos and his several parts, need to be measured for comparison, and monitoring if that development is, effectively, normal for the period of development in which it is (Braunbeck & Lammer, 2006; Kimmel et al., 1995).

The correspondent parameters per hour post fertilization are describe in the next points.

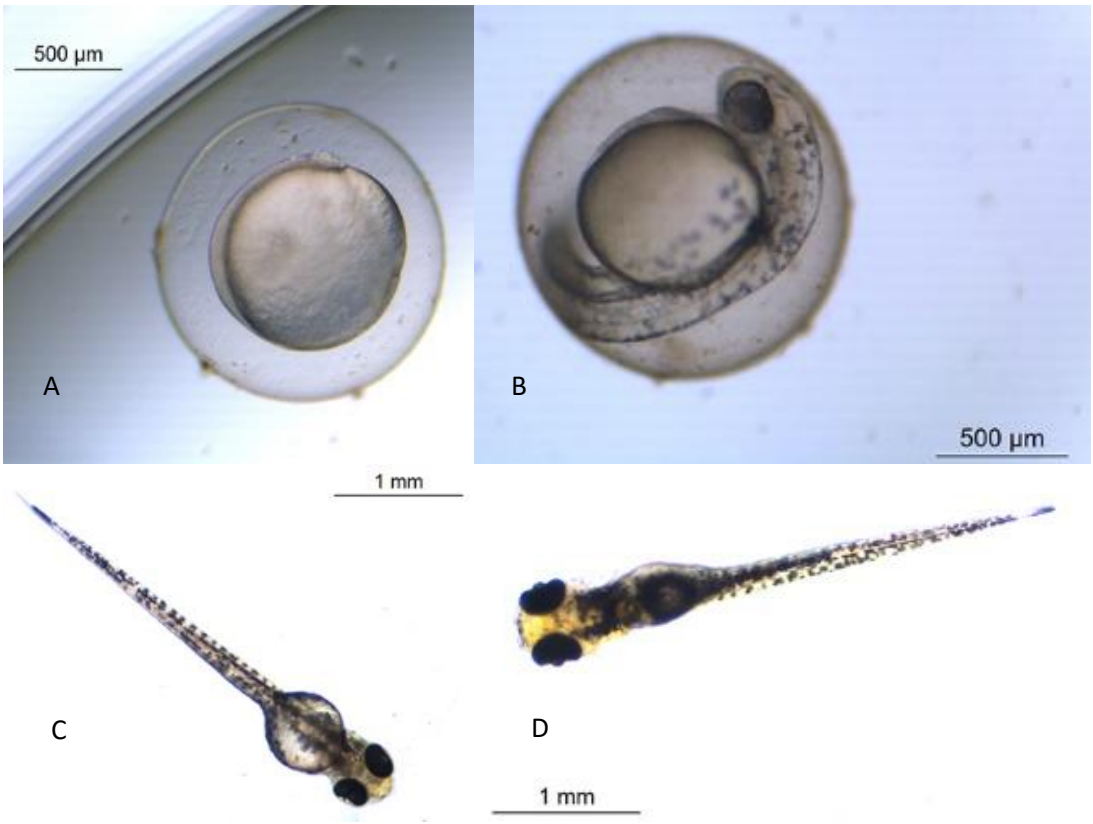

Figure 4: Zebrafish at several time-points of ZET experiment. A: 8 Hours post fertilization; B: 32 Hours post fertilization; C: 56 Hours post fertilization; D: 80 Hours post fertilization.

1.2.2.1. 8 Hours post fertilization

At this time-point, the embryo stay on epiboly period, as is verified in the previous image. During this period is evaluated the epiboly of the embryo, it is expected that, at 8 hours post fertilization, the embryo is found in 75%-epiboly stage, that is, the epibolic arc has not closed yet all around the yolk (Kimmel et al., 1995).

In addition to the epiboly analysis, the internal embryo conditions are examined, that is, if they have delays of embryonic development or, for some reason, may have already exceeded the stage of epiboly and is in bud or within segmentation period (Braunbeck & Lammer, 2006; Kimmel et al., 1995).

On the other hand, hatching is always measured, though at 8 hours post fertilization is expected no hatching, the values should be recorded to reinforce the results (Braunbeck & Lammer, 2006; Kimmel et al., 1995; Spence et al., 2008).

Measurements are prepared with the comparison objective of internal volumes of the various compartments, the yolk and involucres volume are calculated, and the percentage of epibolic arc is calculated too, subtracting the remaining to run all around the yolk of the embryo (Kimmel et al., 1995).

1.2.2.2. 32 Hours post fertilization

At this time-point of visualizations, the embryo is expected to stay on pharyngula period, as is verified in the previous image. During this period is evaluated the several endpoint from the embryo, it is expected that, at 32 hours post fertilization, the embryo is found between the prim-16 and prim-22 stages (Kimmel et al., 1995).

In this time-point, the most part of the organs are developed, is easy to see the eyes and pupils, the hearth is already functional and is easy to evaluated the cardiac frequency, the development in this time-point is evaluated from the head-trunk angle, that angle should be rounding the 95º (Kimmel et al., 1995).

On 32 hours post fertilization, the evaluation elaborated are more specific, the spontaneous movements are recorded, they can indicate possible errors on the system nervous development, the cardiac frequency is now possible to measure, recording the frequency in an interval of 10 seconds (Braunbeck & Lammer, 2006; Kimmel et al., 1995; Spence et al., 2008).

The hatching, mortality and pigmentation are evaluated too, and the measurements registered too, egg volume, yolk volume, eye and pupil volumes, and the head-trunk angle also registered.

1.2.2.3. 56 Hours post fertilization

At 56 hours post fertilization, the embryo is expected to stay on hatching period, more specifically between the long-pec stage and the pec-fin stage (Kimmel et al., 1995).

In this period is expected all embryos hatched, and this evaluation is important to analyze the normal development of this embryos, the measurements are a little different from the previous hours of visualizations (Kimmel et al., 1995).

Here, the animal is already out from the egg, his growth no longer allows its insertion into the egg, getting out by breaking the egg and go out to the external environment. When outside the egg, the animals tend to adhere to the wall of the microplate wells, however it is possible to have a notion if the development of the nervous system is consistent with the expected for the time-point of embryonic development in question, having, too, a small free swimming but not always constant, this free swimming is characterized with a small start-ups but do not remain in swimming for a long time (Kimmel et al., 1995).

During this time-point, in addition to the assessment of mortality, as in all time-points, the hatching is a parameter with greater impact at 56 hours post fertilization. The pupil is not visible at this point of development as such is only possible to measure the eye in its entirety, the pigmentation is sharper and stronger, the total length of the animal is measured, the yolk volume, which over time will tend to decrease, and its yolk extension along the body towards the tail, too, will be consumed over the time, this is because both the yolk such as its extension are compartments of nutritional reserves for this animals, needing to be fed just when these reserves run out (Kimmel et al., 1995).

The cardiac frequency is measured in the same way, in an interval of 10 seconds. If, for some reason, the animals have not reached the goal of hatching, will be evaluated if this animal has spontaneous movements.

1.2.2.4. 80 Hours post fertilization

The 80 hours post fertilization, correspond to the last time-point of views, and the evaluation parameters are lower.

Here it is expected that the animals are in early larvae stage, that is, already have a sufficient size and development to move across the well with self-starting and response to external stimuli (Kimmel et al., 1995).

In this period of embryonic development, the evaluated parameters are mortality, as in every time-point, the hatching, hoping to be 100% hatched animals, the pigmentation tends to be darker and full, and the most important parameter for this time, the free swimming, it is expected that animals are able to respond to standard stimuli swimming (Kimmel et al., 1995).

1.3. NanoDanio Laboratory

For the application of the nanosystems developed by our working group in zebrafish embryos, a new laboratory was created in our department.

NanoDanio Laboratory was created in 2014 for zebrafish experimentation inside de Biology Department, were all aquaria system, as well the animals and equipment for the assays are distributed.

1.3.1. Aquaria system operation

The NanoDanio is constituted by a sequence of aquariums, independent of each other, each of them has an indicated purpose. This sequence of aquariums consists of four units, one of which, the aquarium stock of reproducers, is presented as the largest of the four, having a capacity of 100 liters, the other three, for breeding, hatching and microplates incubator aquariums, are shown smaller, 50 liters, compared to the stock aquarium.

The aquarium responsible for the stock of reproducers is the only one who is in permanent operation. All other aquariums are always in operational mode but on standby until required their use.

Each aquarium has a specific purpose, the stock aquarium of reproducers serves as a holding, where animals are kept and monitored daily, possessing ideal conditions for growth and well-being of the same. Having a constant temperature at 26º and controlled flow of water, the animals are in stable way, simulating an adverse condition for reproducing, maintaining a normal reproductive cycle but without posture and fertilization.

Of the remaining three aquariums, one of which is intended exclusively for the reproduction of zebrafish, breeding aquarium, this one has a sterile internal environment, the microflora and physico-chemical conditions are bound to conclude an environment conducive to a successful reproduction. The temperature is controlled, lying above the stock aquarium temperature, between 28 and 29 degrees.

The other two aquariums work as hatching aquarium and microplates incubator aquarium, respectively.

In relation to the hatching aquarium, is used, as the name suggests, to stabilize and hatching new animals and "live food" for the reproducers and larvae, that is, at the time of reproduction and successful fertilization, the surplus embryos are collected, washed, and placed in aquariums of 5 liters with heated water, typically at the reproduction temperature, and fired aeration, then these small volume aquariums are dipped in the hatching tank equaling the level of

internal water in the 5 liter aquariums at the level of the external water aquarium, hatching aquarium, and thus be in incubation and hatching mode.

Furthermore, this tank have another purpose, the hatching of live food as a nutritional food, both to reproducers, larvae and juveniles. This live food is *Artemia sp.* and is no more than small crustaceans that live in water with salinity environments.

The laboratory production of this crustaceans is elaborated by simulating the environment conducive to their development, *Artemia* cysts are placed into saline conditions containing 35 grams per liter of water, placing the water with salinity content together with an incubation temperature between 26 and 28 degrees with direct oxygenation, cysts begin to hatch, after filtration and separate non hatched cysts, the *Artemia sp.* can be provided to the animals.

In the arrangement of available fixed aquariums, the last aquarium is the microplate incubation aquarium, this aquarium has a key purpose of the tests performed by the NanoDanio team, working as incubation system assay plates, where the harvested embryos are isolated in plates for testing the systems, tested by the group, are kept during the assays.

This aquarium have inside support platforms that only allows the rise of a certain level of water to dip the test plates. So, the plates are in external contact with the aquarium water, maintaining a normal incubation temperature for the proper performance of the assay. This aquarium is always with the standard room temperature between 28 and 30 degrees, temperature which occurred posture and fertilization of the embryos and have to be maintained for normal development of animals.

Regarding the availability of aquariums, another aquarium was purchased later, aquarium that is independent of the entire aquarium system, serving only to juvenile isolation when they reach a high age, not allowing contact with other animals in development and less post fertilization days. There is a risk of competition between animals of different and disparate ages, the most developed are isolated from the larval community, going for a 20 liters aquarium, where they will have more room for development in adult and decrease competition among animals. This aquarium have an isolate supply of water, so it required more maintenance to control the conditions of their water.

This entire system of aquariums, with the exception of juvenile aquarium, has a supply circuit and a common return water circuit, so, all the aquariums are supplied by the same reservoir, being removed later, over a return system water to the waste pipes.

The internal water circuit is based on a continuous system of permanent water supply to the aquariums, this system is powered by network building water circuit, injected into a stabilizing tank for supplying all the aquariums. This reservoir, has the function, in addition to storage, water aeration, climate control and dechlorination the building network water, so, internally, the container has a resistance that puts water at normal temperature for animals , 26 degrees, thus avoiding thermal shock when the water entering the stock reproducers aquarium, since it is the aquarium in continuous operation, has also a direct aeration, the air flow injected in the reservoir is maximal, so the water is transferred to all aquarium, as well as heated, is also well dissolved oxygen content. Finally inserted in the reservoir, also found an activated charcoal filter, this filter has the function of reducing the chlorine maximum dissolved in water, the water with the tank is fed comes directly from the water network building system, as such has preventive chlorine levels against microorganisms, this chlorine can be harmful to zebrafish, so the need for chlorine removal from the internal NanoDanio water circuit is existent, and these chlorine values, in conjunction with other physical-chemical parameters, are measured in the fed reservoir and in the aquariums regularly.

This water circuit has also a return component, all the aquariums have a normal internal water level, above that level the water is excreted by the pipework fixed on the side of the aquariums.

This return circuit allows the water to circulate inside the aquariums, making the aquarium maintenance of the internal environment in terms of organic load and levels of chemical compounds existing dissolved in water. The water outflow is similar to the input, so the volume of water injected into each aquarium is the same excreted, being regulated according to the needs of each aquarium, in the case of aquariums in which the use is not permanent; the water input is recorded drop by drop, just not to keep the volume of water in stagnant way, the fact that it has water in circulation with a minimum volume enables good internal conditions of the aquariums to be used when needed, in terms of the stock

reproducers aquarium, the injected water amount is regulated according to the number of animals, so the greater the number of animals present in the aquarium, the greater the organic load present in the internal water of the aquarium and as such the greater change of the internal physical and chemical internal characteristics, thus the input volume of water will have to compensate for the increase in organic load, the larger the number of animals, higher the input of volume of water, greater renewal, best internal conditions for the animals.

The return system is connected directly to the building's sewer system, such the aquariums do not have any kind of chemical injection, and the circuit can drive, without problem, directly to the normal network usage sewers.

In addition to the water aquariums systems, the NanoDanio laboratory also has climate control and its own lighting system, which are two important factors in the regulation of laboratory and maintenance of the welfare of animals.

In terms of lighting, the laboratory has a photoperiod control, have a direct light to aquariums with a photoperiod controlled to simulate day and night, 12h / 12h, the normal lighting of a room is not activated to avoid disruption of the internal conditions of animals, when it is necessary to work more illuminated shaped, for example, washing and incubation of embryos, we use a desk lighting and not directed to the animals. Before the lighting system turn on and after being turned off, the internal workings of the NanoDanio are ceased, if is the needed to continue work, such as observations and recording embryo images, the work runs in another room.

The general internal environment of the laboratory is also controlled in terms of temperature and humidity. The temperature should remain in the same variance all year, rounding the 25 degrees, with not very sharp moisture, thus avoiding changes in the internal conditions of aquariums.

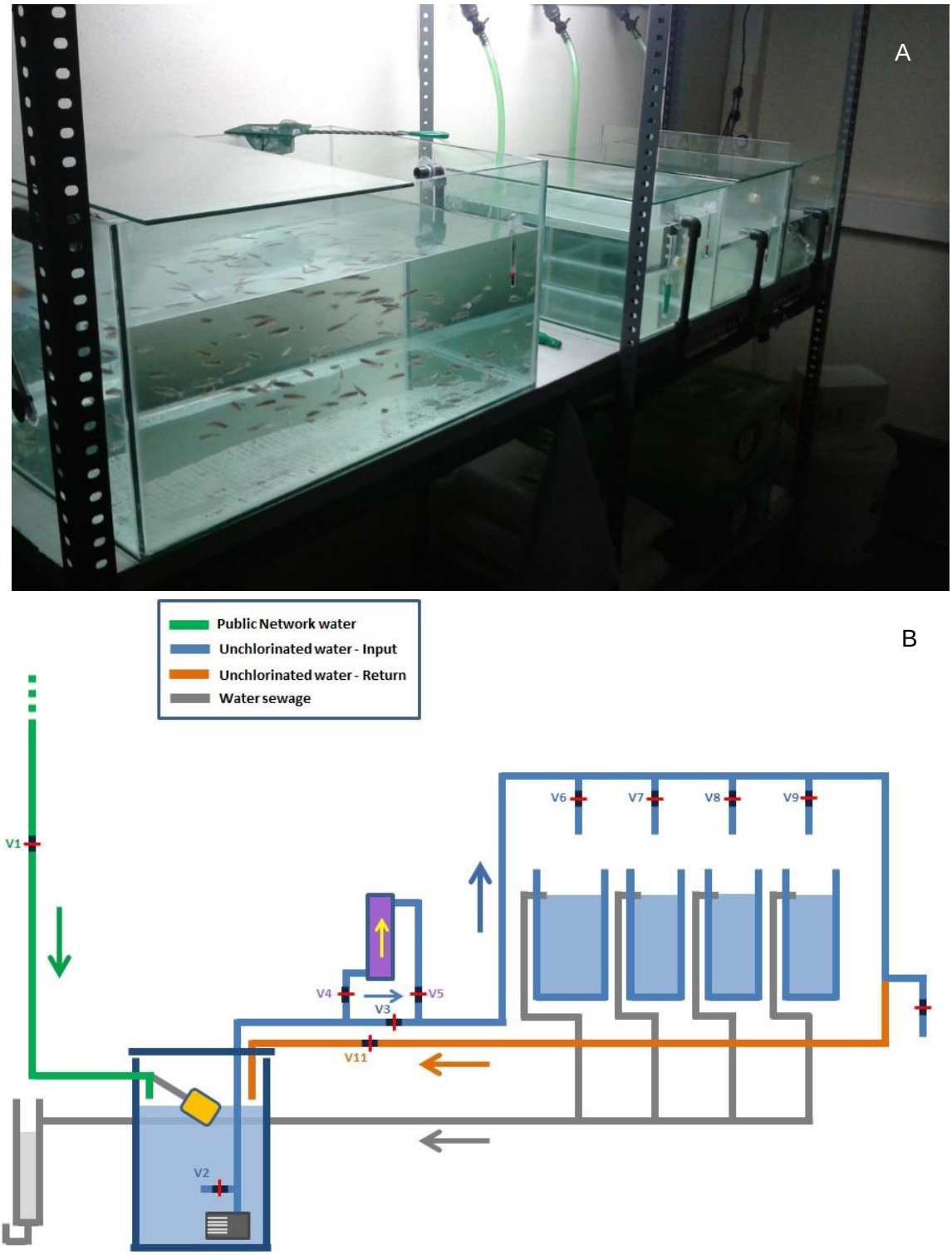

Figure 5: Aquaria system from NanoDanio laboratory. A: From the left to the right, Stock of Reproducers Aquarium, Breeding Aquarium, Hatching Aquarium and Microplates Incubator Aquarium. B: Water system layout.

1.4. Non-metallic (lipid-based) nanoparticles

In biomedical sciences, the arrival of nanosystems and nanomaterial has created a new generation of therapeutic possibilities, having an important role in this area (Immordino, Dosio, & Cattel, 2006; Rizzo et al., 2013).

For safe and effective biomedical application, the development of toxicological and safety tests is required and the application in cell lines and primary human cells isn't enough, this experimentation can provide initial information about toxicity and mechanisms of action of some compounds in contact with biological tissues but, to apply the nanosystem as safe and deduct toxicity about this material, is necessary the application those materials in more complex systems, as a model animal, were the nanosystem go to be in contact with a network of cells and tissues, and his behavior can change or be different (Rizzo et al., 2013). With *in vivo*, the evaluation can be more complete, it's possible to evaluate some factors that can't be possible in *in vitro* assays, as administration, bio-distribution and biodegradability, long-term disposition of the compound on study, evaluate de induction of developmental defects and the activation of some systems as compliment and immune system (Rizzo et al., 2013).

Is known that the tests and maintenance in *in vitro* is more cheap and faster than in *in vivo* testing, so, to cross that line, using more complex models and reducing the costs and time, it's possible with the use of zebrafish, this animal can provide fast toxicological tests with reduced cost in nanosystems experimentation (Rizzo et al., 2013).

The nanosystems presented at this time, have a large base of creation and may be presented in different ways, there is nanosystems based in dendrimers, quantum dots, drug-polymer conjugates, drug-antibody conjugates, nano-emulsions, nano-gold shells, lipid-based nanoparticles, and others, and the with the purpose of delivery of genetic material, drugs, and also imaging agents, switching between them the form of assembly and the outer decoration of the delivery vehicle (Li & Szoka, 2007).

Of this varied type of nanoparticles for material delivery at the cellular level, not all can overcome the clinical tests with positive results, having a large cell barrier, the need for low or inexistent toxicity, that is, the vehicle in question need to be inert both *in vivo* as later, when excreted into the environment (Li & Szoka, 2007).

To pass this barrier, the materials that this nanoparticles are produced need to be studied, and biological compounds are the best way to pass this cellular barrier, biological compounds as lipids, proteins, and carbohydrates (Li & Szoka, 2007).

In this area the nanoparticles produced by basic biological compounds, is emphasized the nanosystems based on lipids, simulating the known liposomes, these non-metallic nanosystems are essentially constituted by layers of lipids, lipids those which form part, also, of biological membranes, what facilitated, in some point, the integration into the cell (Li & Szoka, 2007).

Nanosystems termed as lipoplexes are not more than particles based on non-covalent lipid formation with DNA associated, formed by charge-charge interactions (Li & Szoka, 2007).

Since its development, the liposomes are highly exploited and studied by the scientific community to drug delivery order. In its class, are presented as well studied vehicles, characterized by a lipid bilayer formation of amphipathic phospholipids with his aqueous interior (Li & Szoka, 2007).

The use of lipids for formation of nanoparticles based on the amphiphilic nature of lipids, forming a bilayer, due to having two different sides, a polar head linked to one or two hydrophobic tails (Articles, 2005).

These characteristics of lipids cause, when in contact with an aqueous environment, promotes and the formation of vesicles (closed bilayer) (Fig.6(Articles, 2005).

These liposomes are categorized according to their size, ranging multilamellar (MLVs) and unilamellar (ULVs) which, in turn, can be classified as large unilamellar vesicles (LUVs) and small unilamellar vesicles (SUVs) (Articles, 2005)

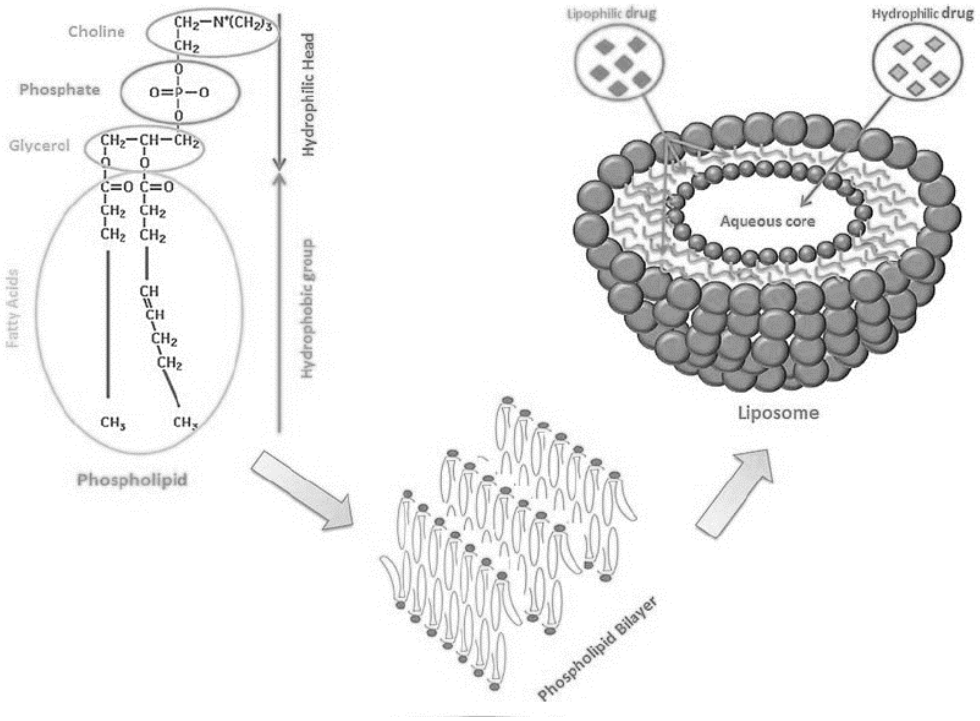

Figure 6: Liposome structure, from (Laouini et al., 2012)

The production of these lipid-based nanosystems can be designed in different ways following techniques well established for this purpose, the order is based on the conversion of a dispersion multilamellar layers into a simple bilayer form, known as unilamellar vesicles. This production can be prepared by sonication, extrusion, and reverse phase evaporation and by injection of a solvent. Important to note that the production of these nanoparticles have to follow safety standards, fulfilling certain parameters such as the particle size, the drug loading efficiency, stability during movement and its capacity in terms of destabilizing membranes for delivery of drug at the target site (Li & Szoka, 2007; Puri et al., 2009).

One class of lipid-based nanoparticles is known as cationic liposomes, and are widely used for efficient DNA delivery due to their physical characteristics (Li & Szoka, 2007; Puri et al., 2009).

1.4.1. Cationic liposomes

1.4.1.1. Cationic liposomes – features and multiple applications

Cationic liposomes have some characteristics that make them an efficient vehicle in the delivery of DNA. The fact of having multivalent cations allows a condensation of the genetic material by charge-charge interaction, with the positively charged particle and the negatively charged nucleic acid, its interaction makes possible the encapsulation of the genetic material. Another key feature for a positive interaction of the nanoparticles with the cell is based, also, on the particle charge, the cell membrane being negatively charged and positive particle, the uptake of the encapsulated material becomes more efficient (Immordino et al., 2006; Laouini et al., 2012; Li & Szoka, 2007).

Despite these cationic lipoplexes being widely studied, they have some adverse effects that need to be worked around in the passage of *in vitro* to *in vivo* assays (Puri et al., 2009).

Aggregation with blood components, and immune reactivity are disadvantages that need to be circumvented for safe administration *in vivo*. As a contour shape of these situations, can be used charged lipids in an acid environment, modification of the cationic group covalently after particle production converting to neutral or anionic, or, even, the formation of a cationic lipid with an attached disulfide as a way DNA encapsulation by changing the surface charge, can be an alternative to the contour of the negative points (Immordino et al., 2006; Puri et al., 2009).

The applications of cationic liposomes are vast, given their characteristics and constituents, these vehicles can be used not only in biomedical applications, as well as in food and cosmetics engineering (Immordino et al., 2006; Laouini et al., 2012).

1.4.1.2. Production and biophysical characterization methods

In the production of these liposomes there are several different methods with specific results, within these methods, some are highlighted. For first the conventional method, that is based on the production of MLVs, this production makes up from phospholipids dissolved in an organic solvent, then inserted in a round bottom balloon, which in turn, through rotary evaporation under low pressure, the solvent is removed leaving the formed particles as a thin film on the balloon wall, and then, through the aqueous buffer, the lipids are hydrated (Articles, 2005; Immordino et al., 2006; Laouini et al., 2012).

Another method often used is called as sonication, in this method, the nanoparticles produced are SUVs. This method is based on sound waves with high frequency pulses, inserting a probe under an inert atmosphere of nitrogen or argon, producing a homogenous dispersion in the nanoparticles, as result the nanoparticles produced are smaller with penetration capacity in tissues. Although a production method used and the nanoparticles be homogenous and with small dimensions, it has some disadvantages, such as the oxidation of links in chains of the compounds, and inactivation or denaturation of thermolabile substances (Articles, 2005; Immordino et al., 2006).

The high pressure extrusion method is a widely used technique, here the nanoparticles are prepared by the conventional method, and then, placed in an extruder, with a controlled pressure on the sample of nanoparticles, causing the pass through a filter with controlled dimensions, producing uniform particles with the desired size. This method is widely used with efficient production of desired particle size, however there is always loss of concentration of particle solution (Articles, 2005; Laouini et al., 2012).

The method for solubilization and subsequent removal of detergent is a widely used technique for biomedical applications of proteins. Here the particles produced are LUVs by detergent solubilization of lipids, these detergents must possess concentrations of easy removal, and this removal is done by column chromatography or dialysis (Articles, 2005; Laouini et al., 2012).

The preparation of nanoparticles through evaporation phase is also widely used, having few limitations in the literature, only the exposure to organic solvents

and mechanical agitation. This method is based on a quickly injection of an aqueous solution in an organic solvent, in combination with sonication, the solution is subsequently dried by a rotary evaporator, finally the compound obtained after dehydration is hydrated and subjected to vigorous mechanical agitation, obtaining large unilamellar nanoparticles (Articles, 2005; Laouini et al., 2012).

Another widely used method is the injection of solvent, ether or ethanol, here the lipids are dissolved in an organic phase, after this, the lipid solution created is injected in an aqueous medium, forming multivesicular liposomes (MVV) (Laouini et al., 2012).

After the production of such liposomes, a characterization of the product is required, the biophysical characterization is prepared through several methods. The parameters analyzed ranging from the lamellarity determination of the nanoparticles, determining the size, zeta potential, encapsulation efficiency and stability of the liposomes. The elaborate analysis will depend on the purpose of the production of the nanoparticles (Laouini et al., 2012).

Determining the lamellarity has the objective of determining the number of lipid bilayers that the particle has, which may influence, the encapsulation capacity of the nanoparticle. This method is based on a fluorescence signal inserting markers into the lipids which constitute the particles, which are activated, before, by reagents, obtaining, thus, the verification of the conformation of the nanoparticles, in terms of the number of lipid bilayers (Laouini et al., 2012).

The analysis of nanoparticle size is important, this parameter influences the insertion capacity of the particle in her target, especially when the aim is therapeutic. This method of average size and size distribution analysis can be analyzed by microscopy techniques, size-exclusion chromatography, field-flow fractionation and static or dynamic light scattering (Laouini et al., 2012).

Zeta potential basically is the total charge that a particle has facing a specific medium, in the case of cationic liposomes, is a key feature for interaction with the cell membrane, such as the measurement of this parameter is very important. This measurement is very common in colloidal systems, that is, if the nanoparticles in suspension are to have a high zeta potential, either negative or positive, will tend to repletion with each other, not allowing the formation of agglomerates, so the particles still stable and homogeneous. If the zeta potential

has low values, the particles will have a tendency to aggregate, experiencing a phenomenon called flocculation (Laouini et al., 2012).

This zeta potential is measured from the illumination of the sample by a light source, the laser beam will focus on the center of the sample, diffusing the light, then applying an electric field, forces the particles to move, then, by calculating frequency proportional to the speed of movement of the particles, this value will be processing by computer, receiving the zeta potential of the sample. The stable value will be considered as> 30 mV or the reverse, <- 30 mV (Laouini et al., 2012).

When the purpose is delivery of drugs, it is necessary to know the particle is encapsulated or not the desired substrate and calculate the encapsulation efficiency rate, this rate is calculated by separation of particles with and without the drug. The method is based on the separation of internalized drug in the particle and the free drug by, for example, mini-column centrifugation, separating by size, is applied drop-by-drop the solution to a column containing gel with a certain spacing, applying, then, a strong agitation, the drug encapsulated will stay in the gel, expelling the particles that do not have internalized drug, subsequently with addition of water and re-centrifugation, the particle with the drug is extruded and remains in the gel the free drug. Another method may be by membrane dialysis or ultracentrifugation (Laouini et al., 2012).

Liposomes stability analysis is a key point in the producing of these particles, it is necessary to maintain the stability of the particle when produced and stored, and can take no risks of adverse effects by an acquired instability. Are necessary physical, chemical and macrobiotic tests to verify the particle stability, not only it as a whole but also in the change of the lifetime of the agent to be introduced as therapeutic, evaluating then aggregate formation over time, the visual appearance of the particles, permeability changes and the size of the particles, since they are composed of lipids and phospholipids, organic constituents which have life time (Laouini et al., 2012).

1.5. DODAX:MO nanosystems

The DODAX based nano- are systems based on the cationic surfactant dioctadecyldimethylammonium (DODA) combined with a counter-ion X. When the counter ion is Cl^- or Br^-, will have the DODAC and DODAB system (Feitosa, Alves, Castanheira, & Oliveira, 2009).

Such surfactants have the capacity to form vesicles with an aqueous interior that is, forming bilayers with a capacity to rescue inside a small amount of the desired product. Its formation simulates a biological membrane, which makes as a very effective vehicle of cell delivery (Feitosa et al., 2009).

The self-assembly in water of these cationic surfactants results in cationic liposomes that will have dependent physical and chemical characteristics depending on the production method used, concentrations of surfactant and the condition of the solvent. When the production is based on a warming of the mixture of the surfactants, the kind of liposomes obtained are large unilamellar vesicles (ULV), in the other hand, if is used large concentrations of surfactant with single inorganic salts, the result are multilamellar (MLV) and multi-vesicular vesicles (MVV) (Feitosa et al., 2009).

In sum, the formation of DODAX nanosystems is based on three considerations, the concentration of the surfactant, the method used for the production and the solvent used during the production (Feitosa et al., 2009; Oliveira et al., 2014; Silva et al., 2011; Silva, Oliveira, Gomes, & Oliveira, 2012).

The insertion of the compound X, bromide or chloride, will make the formation of the vesicle in the same way, however, the stability and toxicity is different between chloride and bromide. In the literature is mentioned differences between DODAB (dioctadecyldimethylammonium bromide) and DODAC (dioctadecyldimethylammonium chloride), making the DODAC more stable than DODAB, however more toxic (Feitosa et al., 2009; Oliveira et al., 2014; Silva et al., 2011, 2012).

The case of dioctadecyldimethylammonium bromide (DODAB) and dioctadecyldimethylammonium chloride (DODAC), although the similarities that show this particles, they differ in the nature of the counterion will go experiments

some differences in the bilayer hydration, what entails some differences in her characteristics in the size and gel-to-liquid crystalline transition temperature (Oliveira et al., 2014).

In the system, DODAX:MO, MO acts as a helper lipid, this addition produces some alterations in the counter-ion that will change the properties of the liposomes, and also changed the efficiency of the lipoplexes (Oliveira et al., 2014).

In another way, the inclusion of monoolein showed that the lipoplex can transfect more effectively some kinds of mammalian cells reducing the cytotoxicity, but the addiction of a helper lipid can't be on excessive concentration, it's proved that the concentration of monoolein must be under 50%, otherwise the lipoplex structure will change leading to the formation of inverted nonlamellar phase decreasing is transfection efficiency (Oliveira et al., 2014).

So, in this system, the helper lipid represents an important role on the efficacy of the lipoplex. MO due to its ability to form nonlamellar inverted cubic or hexagonal phases, described like inductor of fusion of nanoparticles with endosomal membranes, tends to fluidize and stabilize lipoplexes structures and leading to an increase of the silencing efficiency of siRNA-nanoparticles (Oliveira et al., 2014). In addition, the inclusion of MO, increases lipoplex resistance against the biological fluids and some cell components, getting some lifetime, more efficient and biocompatible to the particle (Silva et al., 2012). In other way, the addiction of MO to this system reduce the positive charge to the lipoplexes, reducing, so, the transfection-associated cytotoxicity. With that, to formulate the more efficient lipoplex, we need to now the appropriated characteristics of cell target and the biological barriers that the liposome go to pass, to get an transfection efficiency *in vitro* and *in vivo* (Silva et al., 2012).

1.5.1. DODAC

Dioctadecyldimethylammonium chloride (DODAC) is a cationic surfactant widely used in the production of cationic liposomes (Fig.7) (Feitosa et al., 2009; Oliveira et al., 2014). This compound has a molecular weight of 586.54 M, and his CAS Number is 107-64-2.

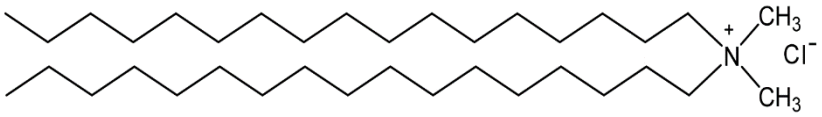

Figure 7: Dioctadecyldimethylammonium chloride (DODAC)

This organic compound has been widely used in the detergent industry however abandoned by their low biodegradability, and can be found also in cosmetics mainly as an ingredient in hair conditioners having an antistatic effect, at biomedical level, that stimulates immune responses, activates macrophages and binds to antigens. The combination of long-chain hydrocarbon groups and the cationic ammonium confers surfactant properties (Feitosa et al., 2009; Oliveira et al., 2014).

It is known that this surfactant has toxicity index to the cells, as such its concentration when be introduced need to be controlled, on the other side its stability, against the toxicity level, shows high, compared to other cationic surfactants (Feitosa et al., 2009; Oliveira et al., 2014).

1.5.2. DODAB

Dioctadecyldimethylammonium bromide (DODAB) it is also a cationic surfactant (Fig.8) (Feitosa et al., 2009; Silva et al., 2011, 2012). This compound has a molecular weight of 630.95 M, and his CAS Number is 3700-67-2.

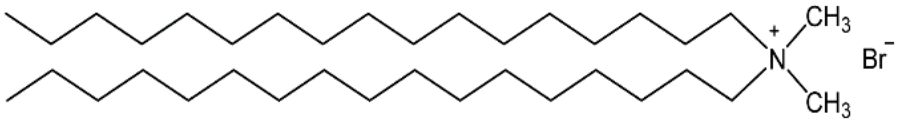

Figure 8: Dioctadecyldimethylammonium bromide (DODAB)

As DODAC, dioctadecyldimethylammonium bromide stimulates the immune responses, activates macrophages and binds to antigens, and is surfactant characteristics is acquired like DODAC, by the combination of long-chain hydrocarbon groups and the cationic ammonium conferring his surfactant properties (Feitosa et al., 2009; Oliveira et al., 2014; Silva et al., 2011, 2012).

Unlike DODAC, this compound has a lower level of toxicity to biological tissues, however its stability is decreased and can be handled with a higher content of the compound, however its stable lifetime is much reduced in comparison with DODAC (Feitosa et al., 2009; Oliveira et al., 2014; Silva et al., 2011, 2012).

1.5.3. Monoolein

The monoolein (MO) is a neutral lipid (Fig.9), used in liposomal formulations, with some purposes, being, the most important, the stability of the conference to the system of nanoparticles at certain physiological conditions and such excretion leakage from the immune system in biomedical applications. Therefore, the insertion of MO into liposome systems allows an escape from endosomes, which are responsible for most of the loss of genetic material in gene therapy (Feitosa et al., 2009; Oliveira et al., 2014; Silva et al., 2011, 2012).

The insertion will allow the formation of non-lamellar structures, performing a fusing role in membrane and membrane destabilizing of the endosomes, maintaining the efficient delivery of genetic material (Feitosa et al., 2009; Oliveira et al., 2014; Silva et al., 2011, 2012).

This compound has a molecular weight of 356.54 M, and his CAS Number is 111-03-5.

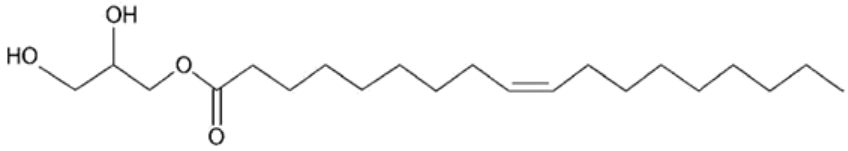

Figure 9: Monoolein (MO)

Work Context and Objectives

2. Msc work-plan and objectives

2.1. Context in the research group

The work-plan of this project is divided into two parts, the production of the compounds to be analyzed and subsequent analysis in zebrafish embryos.

Was prepared several concentrations of cationic surfactants (DODAB and DODAC) and also the lipid helper (monoolein) all alone, then, mixtures was created from the several lipids and, too, lipoplexes formation in various desired proportions, going, after each production, tested on embryos from the animal model in use.

In addition, the buffers where the nanosystems are created and stored, ethanol and Hepes, was tested too, to avoid mistakes in the toxicological analysis.

Since the liposomes are produced in the presence of ethanol, it is necessary to test this compound alone, to obtain a viable concentration curve, that is, several concentrations are tested obtaining, then, the maximum concentration that does not cause harmful effects to the embryos and which will be the maximum concentration of ethanol used in the production of lipid mixtures and nanosystems.

Thereafter, the compounds of liposomes are separately analyzed, that is, both as DODAC, DODAB and MO, are analyzed in isolation mode to obtain results of toxicity of several concentrations, and served, then, these concentrations of template for production of particles and lipid mixtures.

These nanoparticles are no more than mixtures of several compounds tested, placed in ideal conditions for the formation of nanosystems. So, these mixtures have to be tested without initiating liposome formation, that is, the mixture of DODAB and DODAC with MO in the various desired proportions will be prepared but not forced to create nanosystems, being, then, analyzed by ZET assay for a later comparison in terms of toxicity to the liposomes.

Finally, DODAB:MO and DODAC:MO nanosystems, in several proportions (4: 1, 2: 1, 1: 1, 1: 2, 1: 4) are created by lipid film hydration, with the objective to the maximum removal of ethanol, and, then, finally analyzed by ZET assay.

After removal all the ethanol from the lipid film, is afilm hydration is needed in order to form the nanoparticles. This hydration is elaborate through a buffer, Hepes, as such, their separate analysis is necessary, testing the toxicity indices that this compound might have on zebrafish embryos.

2.2. Objectives

This work is in continuation of a combined efforts of several elements, comes from the joint work between elements of the Biology Department and Physics Department of Minho University, as well as the spin-off nanodelivery. This project is under the orientation of Professor Doctor Andreia Gomes of the Biology Department, present in the development of this lipoplexes, responsible for the zebrafish biotherium and a member of the spin-off referenced, co-supervision of Professor DoctorElisabete Oliveira of the Physics Department, responsible for developing formulations of lipoplexes of the family DODAX:MO, and Professor Doctor Marisa Passos, also responsible for the animal facility of the zebrafish, monitoring the development of this project closely.

Having said that this work has as objective the validating zebrafish embryos as a vehicle to test the low toxicity of nanomaterials of the system DODAX:MO previously developed. As this organism has been used with good results in various tests, this work aims to validate their embryos as a way of testing the toxicity of liposomes and protein-based structures.

The liposomes that will be tested in these embryos are inserted into the DODAX:MO family, they are DODAC and DODAB together with monoolein in various proportions (4:1, 2:1, 1:2 e 1:4).

It is known that the DODAC is more toxic than the DODAB and the liposomes with monoolein (neutral), in either of the two formulations, make the general toxicity decreases (less positive charge, so the cells do not suffer as much) than only using cationic lipids.

We have indications that the efficiency of transfection differs in accordance to the quantity of plasmid DNA or siRNA that we want introduce in cells (sometimes it is better DODAC:MO, other DODAB:MO).

This project will be developed in two locations, the development of lipoplexes in the Physics Department and the zebrafish will developed in CBMA, of Biology Department, both of Minho University.

Material and Methods

3. Material and methods

3.1. Zebrafish

3.1.1. Zebrafish maintenance and husbandry

The daily maintenance of the general condition of the animals and aquariums are required. Feeding, measurements of physicochemical parameters, aquariums cleaning, monitoring the development of larvae and juveniles, insulation for breeding and water changes in aquariums without internal water flow, as well as daily activity registrations in laboratory dossier, are daily routine of NanoDanio laboratory.

3.1.1.1. Feeding

Every day the animals need to be fed for the maintenance of health, reducing competitiveness, ensuring success in insulation for breeding and allowing the proper development of larvae and juveniles.

The feeding of the animals of stock of reproducers aquarium can be prepared in three different ways, with packed flakes, staple and standardized for ornamental fish, with live food, *Artemia sp.* produced in the laboratory, it is very nutritional food and, finally, with automatic feeder containing granulated food, designed for ornamental fish as well.

The feeding by packaged flakes is the most used, in its constitution has all the elements necessary for the normal and healthy development of the animals, it is a simple way to feeding, fast and does not require extra treatments to be provided.

In NanoDanio laboratory, is used two types of flake food, *Tropic Mix* and *Tetra Min*, regardless of the brand, both have the same nutritional constitution, as such, the animals are fed alternately by the two kinds of flakes, only once a day.

The volume and frequency of food is beaded in several places as "as needed", however, throughout this year, this volume and frequency has been optimized by the team NanoDanio to one time per day with low volume.

An excessive intake of food leads to the animals an adverse health situation with a harmful nutritional excess, this nutritional excess leads to an excess of adipose tissue development, which prevents the proper insulation for reproduction and the lack of posture and fertilization when necessary, leads, also, to abnormal mortality of reproducers.

Feeding in lower volume is also harmful to animals, low energy charge present in each animal reduces the chance of successful reproduction, there is no good nutritional fitness, the animals will have difficulty maintaining a posture and standard fertilization and can lead, too, to lack of nonviable embryos or postures, and may also in more serious cases, lead to mortality of reproducers.

After optimization by the team, the daily diet for reproducers was stipulated to a low volume once time a day, at midday, with the volume down just enough for each animal able to eat a little food but not leading to satiation, that is, a little hunger in the animals is required to stimulate movement throughout the aquarium and avoid excessive deposits of fatty tissue in the zebrafish.

In terms of live food, food rich in nutrients, feeding such food must be very fractionated, as such, the administration of this food is made very sparingly, usually before isolation for breeding if such insulation is elaborates post end-of-week, where animal feed was prepared by automatic feeder, otherwise is not justified a dose of high nutritional content.

This type of food is used to allow the efficient development in young animals, as larvae or juvenile, making them to have adequate nutritional load when developing and fortification of the animal.

During periods of absence of NanoDanio, where no member of the team is present, an automatic feeder is used, this feeder is fueled by granulated food, and this because it has easy mobility inside the feeder reservoir and allow the calibration for a sufficient discharge to feed all the animals in regularized order.

Usually the feeder is driven Fridays for a period of 24h / 24h, providing just one food dose on Saturday and Sunday, enough to feed all animals.

In terms of younger animals, the food supply is more regular and fortified to allow a faster development and healthy, the animals are fed according the age they have, differentiating the type of food as lavas or juveniles.

Generally, the animals after a week of development require a small food dose, their internal nutrient reserves are already depleted or exhausted phase, thereby, to prevent high mortality, is provided, once a day, a small dose of food. The type of food used by the NanoDanio team for feeding larvae is called Mikrovit, this food is rich in spirulina, a nutritive algae for ornamental fish, or *Artemia sp.* with 24 hours of hatching time, this time of hatching allows small crustaceans to have size enough to be ingested by the larvae, which will allow a positive and rapid larval development to juvenile.

After one month they can be fed twice the times a day and change the type of food, may be provided as food *Artemia sp.* with 48 hours of hatching, the animals are already able to eat the crustaceans with a larger size and high nutritional load, and Mikrovit and single Spirulina, promoting an efficient development to adult.

3.1.1.2. Aquarium maintenance

Despite the flow of water renewal, the aquariums accumulate, daily, detritus on the inner inferior surface, which requires a regular cleaning of the funds of aquariums.

This cleaning process occurs more often in the stock of reproducers aquarium, and aquariums that have animals inserted without internal water flow.

In stock of reproducers aquarium, is only necessary to clean the aquarium floor and his side glass in case of visibly fungi presence. The larger number of animals entered in the aquarium, the greater are the deposit of biological debris in the floor, therefore, after feeding the animals are kept in a clean suction of impurities based on the aquarium floor, taking into account always the volume of water excreted together with the debris. In the case of the stock of reproducers

aquarium, an excretion of a volume of water exceeding 25% may be caused an internal microenvironment change of the aquarium, causing changes in the metabolism of the animals. As such, a standard cleaning of solid waste based on the aquarium floor, the volume of normal water for efficient cleaning, will be around 10% of total volume of water of the aquarium.

In aquariums where younger animals remain, in the case of 5 liters aquariums and juvenile aquarium, the water renewal is not made automatically, the renewal of water in external mode is required, that is, withdraws a volume of between 25 % and 50% and resetting then up with the same volume of heated water. The presence of detritus and possible dead animals, in the case of larval stages, requires a more constantly maintained, as such during the aspiration of these joins the removal of water, then replacing the water in good condition. The volume of water removed should not exceed 50% to not affect the internal environment of the small aquariums, reaching this volume only when the maintenance is not regular, as in the case of weekend, when the maintenance is off until two or three days, otherwise usually removal is about 25% of the total volume of water.

The remaining fixed tanks of 50 liters, except the breeding aquarium, only required maintenance sporadically, as a normal way of general cleaning.

In the case of the breeding aquarium, is always assured the sterilization condition and one optimum temperature for a successful posture. This aquarium is always clean and stay in perfect condition for insulation, the aquarium floor is always free of detritus, however, before each insulation of reproducers to posture and fertilization, the physical and chemical parameters and temperature are always checked.

3.1.1.3. Physical-chemical conditions

An important point of maintenance of zebrafish is the water condition. The animals need to be insert into a good environment to maintain good health and fitness. The several parameters to be evaluated are the alkalinity, pH,

temperature, hardness, un-ionized ammonia, nitrate, nitrite, dissolved oxygen, salinity and conductivity (Avdesh et al., 2012).

This several physic-chemical parameters have an optimum range, above or under that range, animals feel the changes and is detrimental to the internal health of them.

Basically, the pH should remain between 6.8 and 7.5, but this animal can tolerate and large ratio, between the 6.0 and 8.5, the water conductivity should be between 300 and 1500 µS, the alkalinity remaining between 50 and 150 mg/L $CaCO_3$, temperature as was explained before, between 26 and 28.5 degrees, but this animals can tolerate a little higher temperatures, the hardness into 50 and 100 mg/L $CaCO_3$, the ammonia less than 0.002 mg/L, nitrate, NO^{3-}, less than 50 mg/L, nitrite, NO^{2-}, less than 0.1 mg/L, the dissolved oxygen should be more than 6 mg/L and can tolerate, this animals, an index of salinity between 0.5 and 1 g/L (Avdesh et al., 2012).

3.1.1.4. Reproduction

An important process of the NanoDanio is the isolation for reproduction of the animals and the obtainment of embryos, without this process the experimentations are not viable in the laboratory and the assays are not possible.

For a successful isolation, some processes must be taken into account, before and at the time of isolation, new material must be assembled and animals transferred from the stock of reproducers aquarium for the breeding aquarium.

- **Temperature and oxygenation:**

The temperature of the breeding aquarium should be higher than the stock aquarium temperature. This temperature simulates the season of the year that conducts to the breeding of animals, with water been around 28 and 29 degrees,

the animals will have a pleasant atmosphere, favorable to a respective posture and successful fertilization.

In terms of oxygenation, the flow rate should be the maximum, the level of oxygen entering must be set to an intermediate level, causing no oxygen deficit dissolved in water but not causing much unrest in the reproductive environment.

- **Photoperiod:**

An important factor for a successful posture is the photoperiod. The photoperiod should be regulated and never interfered during the breeding season, the night period is important for the accommodation of animals in the new environment to which they were transferred, when the lights are turned on, gives to the animals a trigger for mating and subsequent spawning and fertilization of embryos. The time zone is set to 12 hours with light and 12 hours in the dark, after isolation of the animals, a variation in these light conditions can compromise the success of reproduction.

- **Internal auxiliary aquarium for reproduction:**

Is important that the posture not be missed, as such, another aquarium is inserted into the breeding aquarium, as contact inhibitor between animals and embryos.

This aquarium has a false floor, this fund is a network that allows the passage of eggs from females and sperm from males for the breeding aquarium floor, but prevents be achieved by the reproducers and prevents the ingestion of embryos by the animals. It will be supported by four inert bases that will support the auxiliary aquarium approximately 5 cm above the breeding aquarium floor, thus allowing the safe deposit of the fertilized embryos.

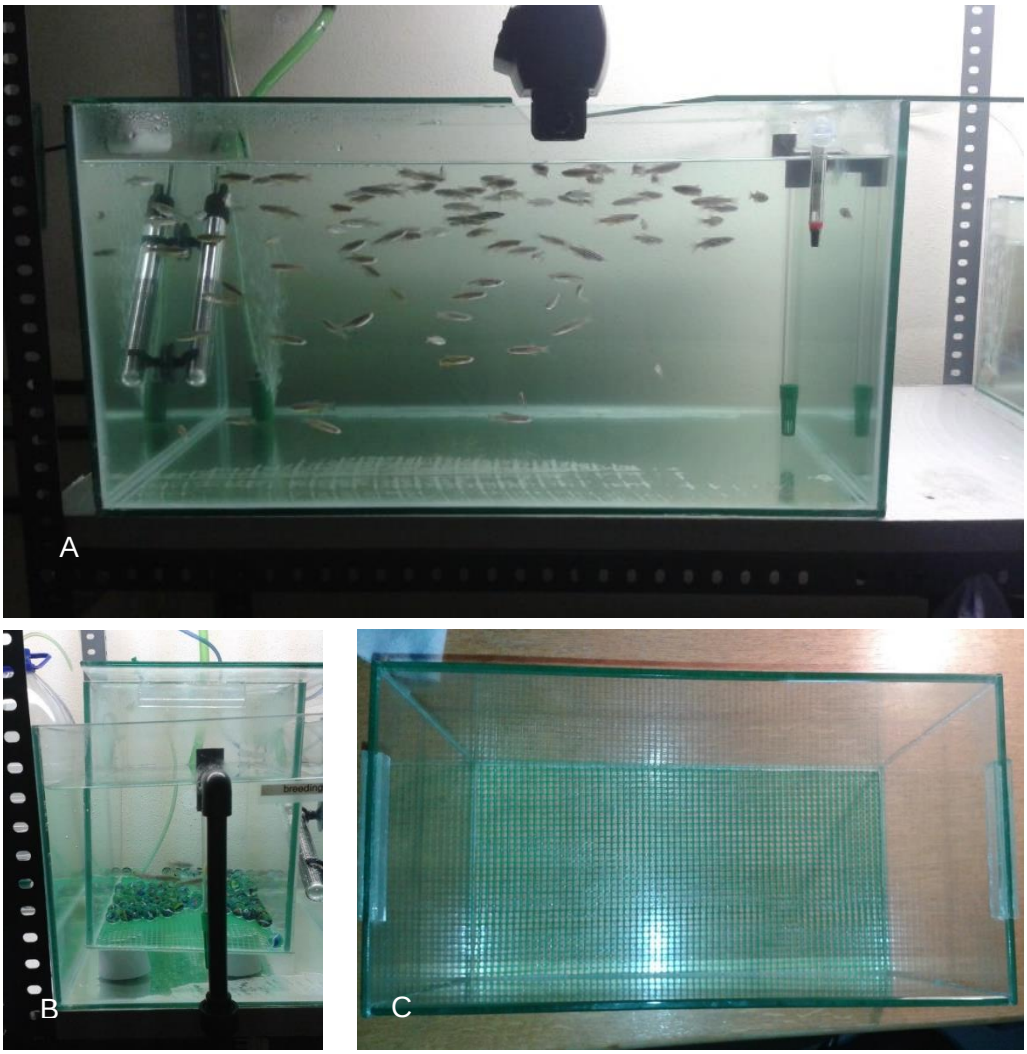

Figure 10: A: Stock of Reproducers Aquarium; B: Breeding internal auxiliary aquarium assembled for use; C: Breeding internal auxiliary aquarium.

- **Marbles:**

This adornment is important for the simulation of an environment favorable to reproduction. These marbles are deposited in the floor of the auxiliary aquarium for reproduction and have as purpose the simulation of minerals present at the time of the year that is favorable to breeding of zebrafish. Spawning occurs as well as the release of sperm, which will pierce between the marbles, through the aquarium's network and depositing it in the breeding aquarium floor.

- **Reproducers:**

The transfer of reproducers to the breeding aquarium must be done carefully to avoid damaging any animal. The animals should go to reproduce place according to a defined ratio, the number of males must always be greater than the number of females, to a number of 5 females, 7 males must be inserted for reproduction.

The animals should be chosen in stock aquarium and later captured and transferred to the breeding aquarium, always trying to choose the animals that appear to have less doubt in terms of gender classification and remaining in a correct reproductive cycle, thus tries to always choose the animals with a more developed stomach, females, and animals thinner and virile, males.

The transference must be about two hours before the cease of light and subsequent spawning occur about an hour after the lights come back on again.

The set of this material properly assembled with the general ambience of the aquarium allows a good likelihood of success in obtaining viable embryos.

The water quality in the breeding aquarium is free from organic load due to the existence of animals only during the time of reproduction, the rest of the time presents no animals, and as such it is always in sterile conditions.

At the end of the reproduction process, the animals are collected back to the stock of reproducers aquarium, the auxiliary material for breeding removed and washed, and embryos collected, washed and ready for use.

3.2. Assessment of effects by compounds and nanosystems

3.2.1. Conditions tested

3.2.1.1. Buffers and solvents

3.2.1.1.1. Ethanol

In the production of these lipoplexes, is used an organic solvent. The use of ethanol is make not only in the production of stock solution, which will be extracted the volume needed to prepare the particles, as well as in the production of the particles, thus there, is need to test ethanol limit supported by the animals.

To realize the limit supported by the embryos of zebrafish, are tested several concentrations of solvent, evaluating what is the maximum concentration at which the embryo will not experiment adversary effect, after this, is determined the maximum ethanol concentration which can be used in the production of the liposomes, thus being able to draw conclusions about the effects of particles in the embryo and do not get false results of the coupling of ethanol in the solution.

It was used, then 100% ethanol, producing multiple dilutions to final concentrations, the percentages by supplementing 0.25%, 0.5%, 1.5%, 3% and 5% of ethanol in a total volume of 50 mL. This final total volume was recorded from the solution of volumes needed for the four days of ZET test.

The percentages in use were deducted from comparisons in the existing literature, obtaining a range of concentrations in which results will have an acceptable window of maneuver for the production of liposome.

The total volume needed to all assay is the 32 mL for each condition, to eliminate pipetting errors, the necessary amount was be rounded to a final 50 mL per condition.

Dilutions of 100% ethanol are prepared in ultrapure water to make up the final volume of 50 mL, respecting the percentages to be tested.

After production of the test solutions, these are stored in cold at 4 degrees, being taken daily, heated to incubation temperature of the embryos, 28 degrees, in aquarium water bath.

After each incubation, the solutions are again sealed and placed in storage at 4 degrees overnight.

All the next solutions prepared are storage and manipulated in the same way as ethanol.

3.2.1.1.2. HEPES

The necessity to test the HEPES, It is owed to its use as a buffer in moisturizing the solution for lipoplexes production in his production process. The mixture of compounds that form the particles after the solvent was removed, ethanol, requires hydration for forming liposomes, therefore, testing the effects of HEPES that can have in the embryos, is elaborated, as in ethanol, one standard curve, the maximum tolerable concentration by zebrafish embryos that it will be used at the time of hydration, during the production process.

The stock solution of HEPES was prepared that will serve not only as more concentrated solution to be tested as well as solution where less concentrated samples shall be prepared.

For this it determined a maximum concentration of 15 mM in a volume of 85 mL to obtain the following pipetting margin, never allowing a smaller volume than 50 mL to the more concentrated condition.

For a concentration of 15 mM are weighed 0.309 g HEPES and diluted with 85 mL of ultrapure water. From this solution the next are calculated, always by pipetting the appropriate volume of the stock solution and diluting with ultrapure water.

3.2.2. Molecules

3.2.2.1. MO

The monoolein is part of the DODAx:MO system tested in this study, with the purpose of helper lipid, so it is necessary to test this lipid on isolated mode, as will later be made with the other constituents of the system in question.

It is necessary to know the critical concentration of this lipid for the embryos, concentration that it will be take into account during the process of formation of lipoplexes.

This study can help to know if the fact of their presence in the lipid mixtures and lipoplexes, can decrease some possible adverse effects or even the opposite.

For the production of monoolein solutions using this assay, the use of ethanol is necessary, having in consideration of the assay of this solvent, the maximum concentration of ethanol used is 0.25%, the limit concentration which shows no toxicological risks to the embryos.

The monoolein concentrations used in this test are based on literature, being used, 5 µM, 15 µM, 25 µM, 50 µM and 75 µM of monoolein.

For a concentration of 75 µM they are pipetted 0.1875 mL from monoolein stock solution that has 20 mM of concentration, completing the volume to 50 mL with 49.813 mL of ultrapure water.

For the next concentrations, the volume that is reduced from the monoolein stock solution is compensated with ethanol, having in consideration the maximum volume of ethanol that will be possible to use.

3.2.2.2. DODAC and DODAB

Both cationic surfactant, as described above, is part of the lipoplexes system tested in this work, therefore, such as monoolein, is necessary to test in isolation. It is well known, as was mentioned earlier, that DODAC has a high toxicity level than the DODAB, as such it is necessary to establish a standard curve for this surfactant and the critical concentration has to be evaluated for modeling the production of these lipoplexes.

The aim of the study will demonstrate that application of those lipoplexes, DODAC / B together with monoolein, have reduced toxicological effects to the embryo, as such must be limited the concentrations that can be administered each surfactant alone.

For the production of DODAC/B solutions using in this assay, the use of ethanol is necessary, having in consideration of the assay of this solvent, the maximum concentration of ethanol used is 0.25%, the limit concentration which shows no toxicological risks to the embryos.

The DODAC/B concentrations used in this test are based on literature, being used, 0.25 µg/mL, 0.5 µg/mL, 1.5 µg/mL, 3 µg/mL and 5 µg/mL of DODAC.

For a concentration of 5 µg/mL they are pipetted 0.0198 mL from DODAC stock solution that has 20 mM of concentration, together with 0.1382 mL of ethanol, completing the volume to 50 mL with 49.842 mL of ultrapure water.

3.2.3. Lipid mixtures (DODAB/C:MO)

To the situation of DODAC/B system in conjunction with monoolein, the purpose is the same as the beaded earlier. In this work are tested lipoplexes consist of cationic DODAC and DODAB surfactants together with helper lipid monoolein, as such lipid mixture of these two surfactants with monoolein is prepared in the same manner, only switched concentrations that were determined in the tests of surfactants in isolation mode, testing these lipid mixtures in the same proportions that will be tested the liposomes, 4:1,.2:1, 1:1, 1:2 and 1:4.

To the mixture of this lipids has been in consideration, too, the concentration of stock solution, 20 mM, to give a concentration of 1 mM in 5 mL, using a final volume of 250 µL of the lipid mixture in several proportions, completing 50 mL with ultrapure water, respecting the critical concentrations of the lipids in isolation.

Table 4: Dilution for DODAC/B:MO lipid mixture ZET assay

Proportion	DODAC/B	MO	Final Volume
4:1	200 µL	50 µL	250 µL
2:1	166.67 µL	83.3 µL	250 µL
1:1	125 µL	125 µL	250 µL
1:2	83.3 µL	166.67 µL	250 µL
1:4	50 µL	200 µL	250 µL

3.2.4. Nanosystems (DODAC/B:MO)

The toxicity studies in zebrafish embryos are terminated with the testing of the nanosystems created by DODAC:MO and DODAB:MO, at several ratios 4:1, 2:1, 1:1, 1:2 and 1:4. After their production, the solutions containing particles in different proportions are administered to embryos.

The results obtained earlier from surfactants and helper lipid in the isolate form, as well as the tests of lipid mixtures, will be compare if the system of liposomes are more stable or less, and the toxicological effects are changed in the case of the mixture without particles and in the case of the presence of particles.

These liposomes are created and tested the level of stability during the test days for not give false results, then with positive results in terms of stability, the liposomes are present to embryos during the assay days. Here, in addition to the comparison with the previous tests, will be evaluated if the presence of monoolein, in various proportions, will change the effect in embryos, stabilizes it, eliminate toxicity or, even, if the toxicity increases and in what level.

First is make a lipid mixture equally to previous assays, respecting the same parameter settings.

The lipids are pipetted from the stock solution, 20 mM, to give a concentration of 1 mM in 5 mL, using a final volume of 250 µL

It uses the lipid film hydration process for the production of particles with nitrogen helps to have any certainty to remove the ethanol present in the solution.

First is pipetted the appropriate volume of each lipid in use for glass tubes.

With nitrogen aid withdraws all the ethanol in the solution leaving the lipid pelleted in the tube wall in the form of lipid film.

Add 5 mL of 25 mM HEPES, as hydration form of film.

It takes then vortexed for 15 seconds each tube is subsequently dipping for 10 minutes in a bath at 60 degrees.

This process is repeated during one hour, vortexing 15 seconds, and bath 10 minutes.

The particles are characterized and evaluated in terms of stability for 72 hours.

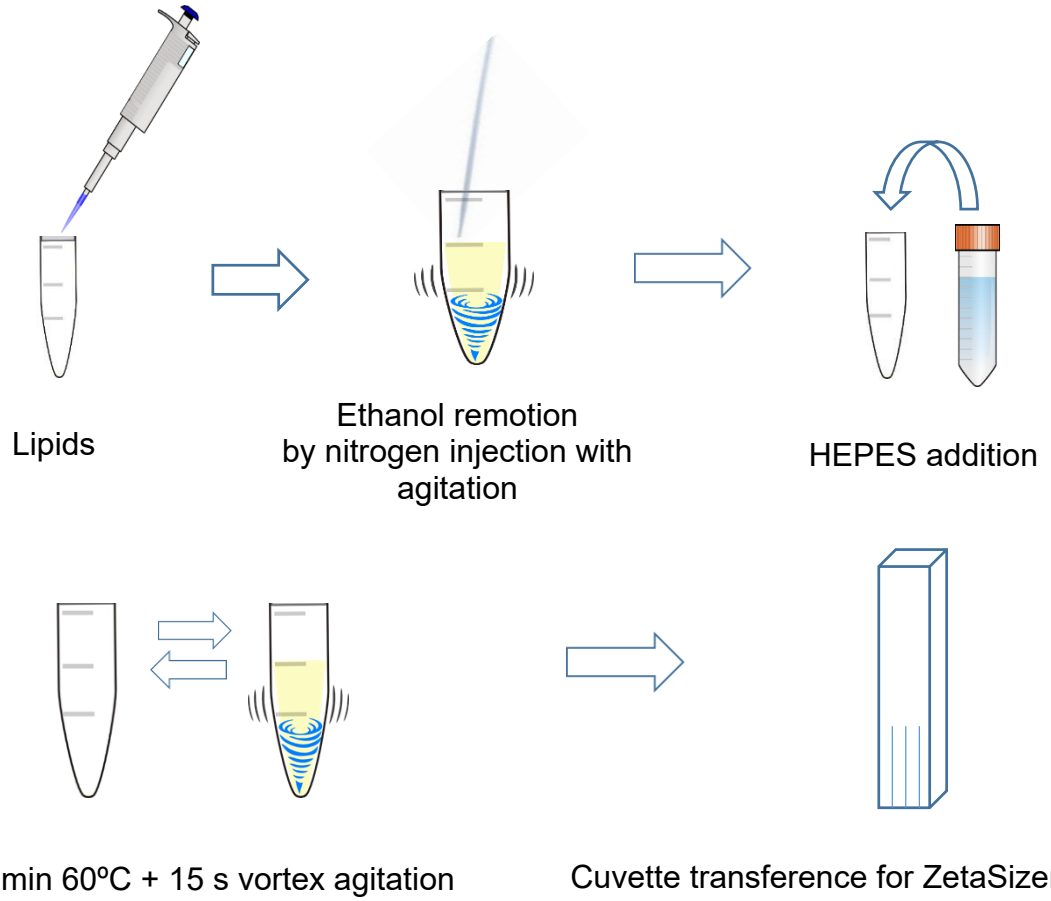

Figure 11: Liposome production process DODAB/C:MO for application in ZET assay.

3.2.5. ZET Procedure

During this work were developed eleven ZET assays to obtain the results described as goal.

The assays have led a logical segment, that is, initially were tested the ethanol, due the need of this compound in the production of nanosystems, and HEPES, because the hydration of the nanosystems with this buffer. Subsequently were tested cationic surfactants, DODAB and DODAC and the helper lipid in an isolated form, and then move to analysis of lipid mixtures of various proportions, DODAB:MO is DODAC:MO, in the proportions 4:1, 2:1, 1:1 1:2 and 1:4, and finally analyzed the nanosystems consisting of particles composed of DODAB:MO and DODAC:MO, also in the previously crimped proportions 4:1, 2:1, 1:1, 1:2 and 1:4.

All the descriptions and materials and methods will be described in each chapter of the mentioned ZET test.

The base, as described above, of the ZET experiment, is designed to test the effect of compounds in the embryonic development of zebrafish embryos. It can, for each compound, being tested various concentrations, following, over time, the effects in embryos subjected to the compounds.

3.2.6. ZET Experiment Protocol

3.2.6.1. Embryo harvest

The animals are transferred from the stock aquarium to the breeding aquarium for isolation to reproduction two hours before the lights turn off. In the next day, about an hour after the illumination be substantially activated, the embryos are collected from the breeding aquarium, transferred to petri plates and washed to remove any contaminants that may exist.

3.2.6.2. Transference

Tem embryos are transferred into each well of the microplate assay. A full 24 well plate requires 240 embryos in its entirety, depending on the number of conditions to be tested, so, for condition, 40 embryos are required, related to 4 repetitions of the same condition, in sum, each condition to be tested requires 4 replications totaling 40 embryos.

Completing the transfer off all embryos, is removed the water from the embryo carrying from each well, and the solutions of the compounds to be tested are placed in the correct well, with a volume of 2 ml per well, in the line for the control assay, the inserted solution is water from the breeding aquarium, simulating the normal development conditions. It is necessary 2 mL of solution per well in four repetitions, totaling 8 mL per condition daily.

At the end of the transferring and insertion of solutions, the plates are sealed and placed in the microplate incubation aquarium, where the incubation is given, between 28 and 30 degrees.

3.2.6.3. Maintenance

The microplates need, apart from renewal the medium daily, maintenance, several times a day, to monitor the development of embryos;The observations are developed from 8 hours post fertilization to 80 hours post fertilization, 4 days, such as, every 4 days, is necessary renewal the medium from each well at the first hour in the morning. That is, at 9 o'clock in the morning, the medium is removed from each well and replaced with the same volume of fresh medium, and possible dead embryos that may exist in each well, will be removed too.

Daily, at about 12 o’clock and 15:30 o’clock, the microplates were observed by eye and retire the dead embryos that may exist, and remaking also the count of existing embryos in each well.

3.2.6.4. Microscope observations

The microscopic observations are produced daily throughout the assay, by 16 o'clock, completing a total of four microscopic observations of embryos per ZET assay. The first observation corresponds to 8 hours post fertilization, the second observation corresponds to 32 hours post fertilization, third observation corresponds to 56 hours post fertilization and the last observation corresponds to 80 hours post fertilization.

Depending in the hours after fertilization, the parameters in analysis will change, always noting the development irregularities in all visualizations.

All parameters to be analyzed are describe on the next table:

Table 5: Several time-pints and parameters observed at each time-point for each ZET assay.

Hours post fertilization	Parameters to be analyzed
8	Mortality Epiboly Hatching
32	Mortality Spontaneous movements Cardiac frequency Hatching
56	Mortality Spontaneous movements Cardiac frequency Hatching
80	Mortality Hatching Free swimming

The daily visualizations are made on two alternate lines, always analyzing two replicates for each condition, describe on the next table.

3.2.6.5. Parameters to be analyzed

3.2.6.5.1. Mortality

The assay is initiated with ten embryos in each well, over time the possible dead embryos are removed from the well, discounting and noting when the embryos are removed, is evaluated, at the end, the mortality rate for each condition.

3.2.6.5.2. Epiboly

At 8 hours after fertilization, it is expected 75% of epiboly of the embryo, is at gastrula stage, at the post blastula embryonic development and prior to the segmentation stage.

With 75% of epiboly, the embryo has not yet completed the full closure around the yolk, assessing the percentage shortfall for the total closure.

3.2.6.5.3. Spontaneous movements

This parameter evaluate the rate of hatched embryos, reaching the larval stage. Hatching is when the animal can break the shell, coming out of this, achieving then free swimming, ranking then as larva.

3.2.6.5.4. Hatching

Are classified as spontaneous movements the movements made by the animal stilling inside the envelope, developing is not enough for their full output, making movements inside the envelope around your own body.

The number of movements and the force of them can be listed as the product of the solutions tested, serving as compared with the spontaneous movements rates obtained in the control group.

3.2.6.5.5. Cardiac frequency

The heart rate is measured at 32 and 56 hours post fertilization, at these times the rate of five animals are recorded for 10 seconds, producing up, then, the average of each well analyzed.

3.2.6.5.6. Free swimming

This parameter is evaluated at 80 hours, at this time of development is expected a full hatching rate, possessing, all the larvae, the ability to swim in all well, reacting to external stimuli.

Free swimming larva regard to the ability to swim in all well, the simple movement of the side fins is not classified as free swimming.

3.3. Statistical Analysis

The embryos are viewed daily at 16 o'clock, being captured images for each embryo, through an optical microscope with built-in camera.

The images are measured by Fiji software.

Post hoc comparisons were conducted using Student-Newman-Keuls. A *P* value of 0.05 was used for significance testing. Analyses were performed in STATISTICA (StatSoft v.7, US).

Results

4. Results

4.1. ETOH (Ethanol)

There are clearly visible differences in the general toxicity, the embryos present at several ethanol concentrations experience several different mortality rates, the higher the percentage of ethanol, the lower the survival of embryos noticed up from 3% a null survival rate immediately post-fertilization for 32 hours, keeping the embryos, partially stable and acceptable survival rate to a maximum concentration of 1.5% ethanol.

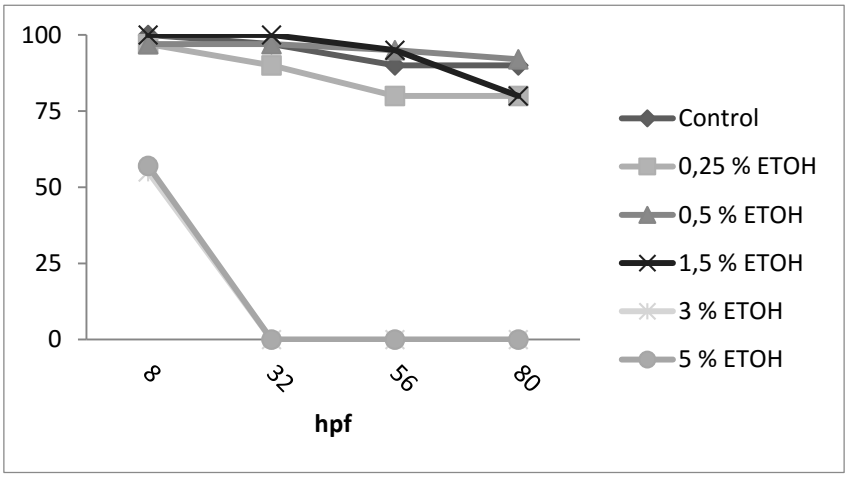

Figure 12: Mean survival rate in percentage for the several conditions of ethanol tested over the several time-points, 8, 32, 56 and 80 hours post fertilization.

In its embryonic development, at 8 hours post fertilization, the presence of ethanol don't show to alter the normal development in a significant way, all embryos appear to be in normal development stage, between the 80 and 100% of epiboly, in accordance with de control group.

In its embryonic development, at 8 hours post fertilization, the presence of ethanol don't show to alter the normal development in a significant way, all embryos appear to be in normal development stage, between the 80 and 100% of epiboly, in accordance with de control group.

Regarding the yolk:egg volume ratio, which indicates a normal embryo development according to their decreasing over all time-points, up to 32 hours post fertilization the volume accompanying the development of all experienced ethanol concentrations still viable, this because in the time-point of 32 hours post fertilization, the mortality, at concentration of 5% of ethanol, proved to be complete.

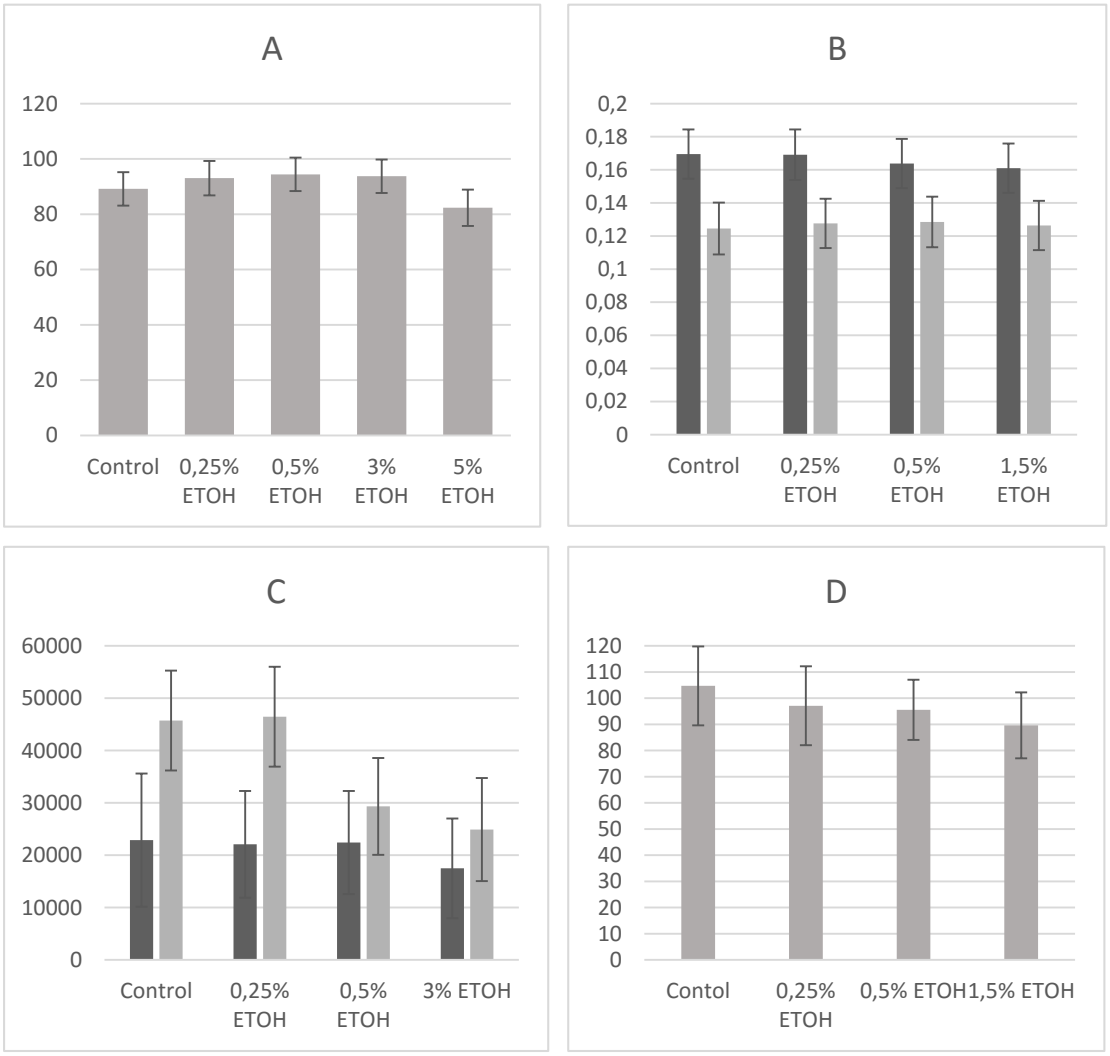

Figure 13: A: Percentage of embryos in epiboly period for each condition of ethanol, assessment at 8 hours post fertilization. B: Yolk:egg volume ratio analysis between the conditions tested of ethanol. From 8 hours post fertilization to 32 hours post fertilization. The conditions will compared goes until 3% ethanol, the conditions 5% of ethanol don't have sufficient data for comparison; C: Eye volume analysis between the conditions tested of ethanol from 32 to 56 hours post fertilization. The conditions will compared goes until 1.5% ethanol, the conditions 3% and 5% of ethanol don't have sufficient data for comparison.; D: Head trunk index analysis between the conditions tested of ethanol on 32 hours post fertilization. The conditions will compared goes until 1.5% ethanol, the conditions 3% and 5% of ethanol don't have sufficient data for comparison.

The development of the eye shows some differences at 56 hours post fertilization, in the presence of 0.5% of ethanol, the volume shows a significate decrease, having a delay in the development of this organ.

The head trunk index, the lower the displayed angle, the greater the delay in development, as shown in ethanol situation, as the ethanol concentration increases, lower is the displayed, however no significate.

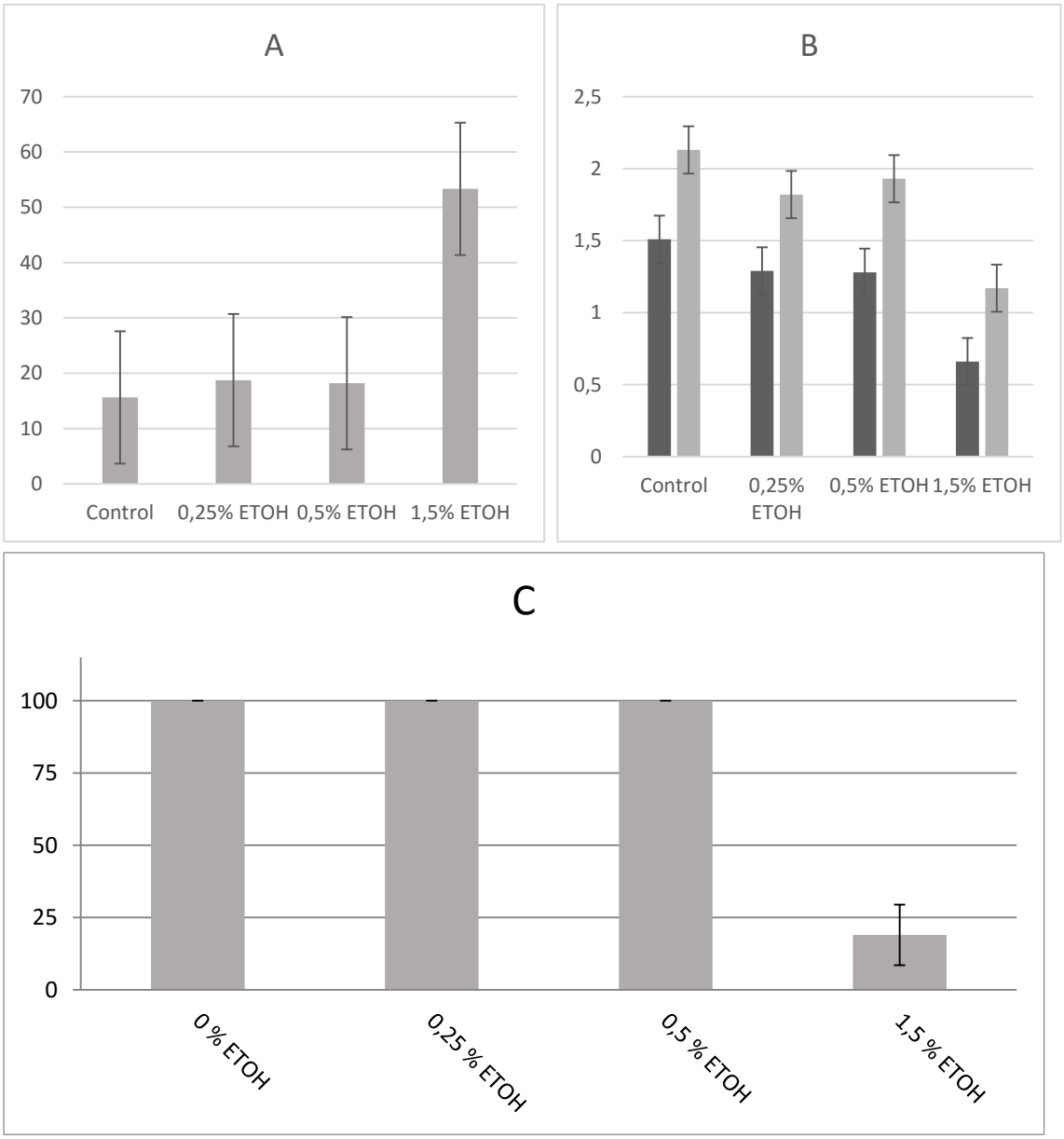

Figure 14: Conditions of ethanol tested until 1.5%, don't exist data for comparison for the concentration of5%. A: Spontaneous movements analysis at 56 hours post fertilization; B: Cardiac frequency analysis from 32 and 56 hours post fertilization respectively; C: Free swimming analysis between at 80 hours post fertilization.

The spontaneous movements viewed during the 32 hours after fertilization, shows up normal until 1.5% ethanol concentration, in this concentration the embryo shows a lot of inner turmoil, leading to a very high accretion rate movement, unusual for this type of animal model.

The cardiac frequency goes according to developmental delays described above, as the ethanol concentration increases, the lower the heart rate.

A serious change is noted in the free-swimming of the animals, starting from 1.5% ethanol, the animals here are shown a difficulty on the moving and responding to stimuli, having a very low free-swimming rate.

The hatching rate also shows significant differences from a concentration also of 1.5% ethanol, begin to have differences in development, getting the total hatching of the animals only at 80 hours post fertilization.

With this analysis it can be concluded that ethanol has a very marked effect of toxicological concern and development of the zebrafish.

The margin of safe concentration for these animals is very low and can remain a safe rate of presence of ethanol between 0:25% and 0.5%, concentrations above 1.5% ethanol show as toxic, giving the embryo a delay in its embryonic development that will lead to death.

4.2. HEPES

The survival rate in all concentrations of HEPES shown to be identical to the control group in all post fertilization hours, not allowing a high mortality level on witch condition at all time-points.

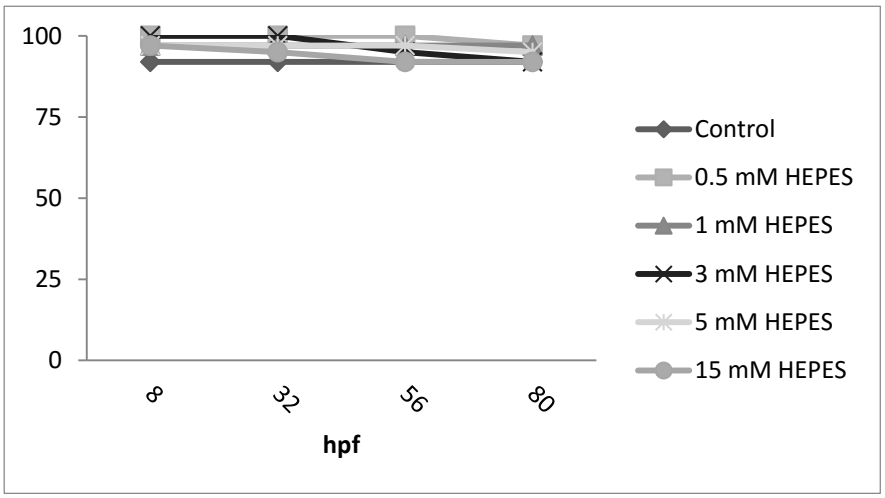

Figure 15: Mean survival rate in percentage for the several conditions of HEPES tested over the several time-points, 8, 32, 56 and 80 hours

All embryos are in epiboly period at 8 hours post fertilization, the yolk:egg volume ratio accompanies the embryonic development correctly at all hours post fertilization regardless the HEPES concentration used, in concentrations of 1 mM and 3 mM of HEPES, the volume appear to be elevated, however not in a significate way. The head trunk index embryo, at 32 hours post fertilization, appears to be in accordance with the control group, don't showing divergences in this evaluation point. The same happens in the eye volume, showing a normal development.

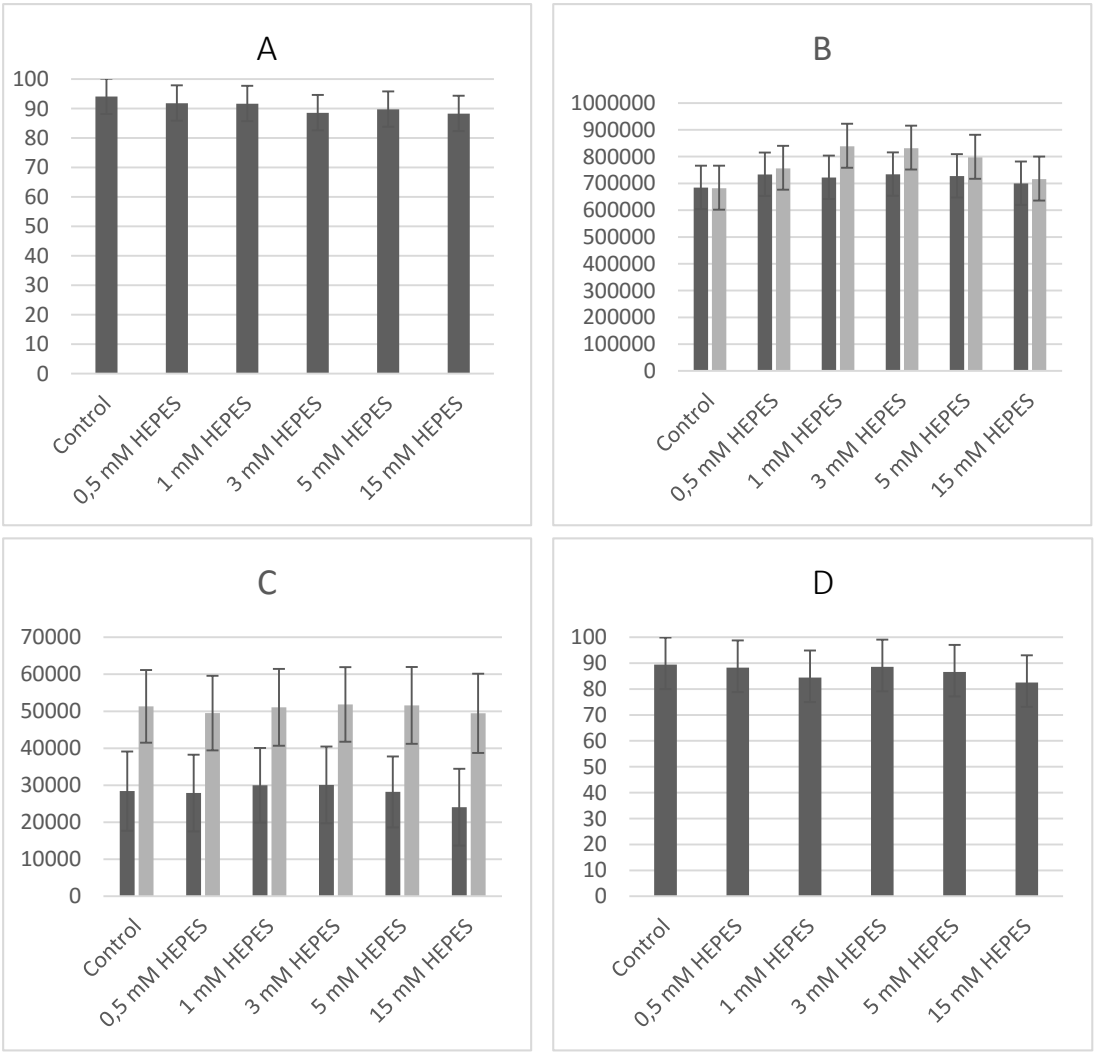

Figure 16: A: Percentage of embryos in epiboly period for each condition of HEPES, assessment at 8 hours post fertilization. B: Yolk:egg volume ratio analysis between the conditions tested of HEPES. From 8 hours post fertilization to 32 hours post fertilization; C: Eye volume analysis between the conditions tested of HEPES. From 32 hours post fertilization to 56 hours post fertilization; D: Head trunk index analysis between the conditions tested of HEPES on 32 hours post fertilization.

On the other hand, the cardiac frequency appears to be within normal parameters for the time-point assessed, 32 hours post fertilization.

In terms of spontaneous movements, the buffer appears to exert increasing this parameter, the animals in the presence of HEPES experience a higher index of internal movements in comparison with the control group.

The total size of the body of the hatched larvae at 56 hours post fertilization is shown within the normal range in the presence of buffer, having, however, variations in the yolk extension, showing a slight delay in development in

concentrations 0.5 mM and 15 mM but it isn't all significant, illustrated on annexes.

The development shows the eye was normal, there are some slight variations along the HEPES concentrations in volume pupil, though the changes are not too far apart to show a toxicological change in development.

The free swimming ability in response to external stimuli shows itself in decreased with increasing concentration HEPES, showing also a slight reduction in hatching at 56 hours post fertilization, however, at 80 hours post fertilization all animals are hatched in larval form, also illustrated in annexes.

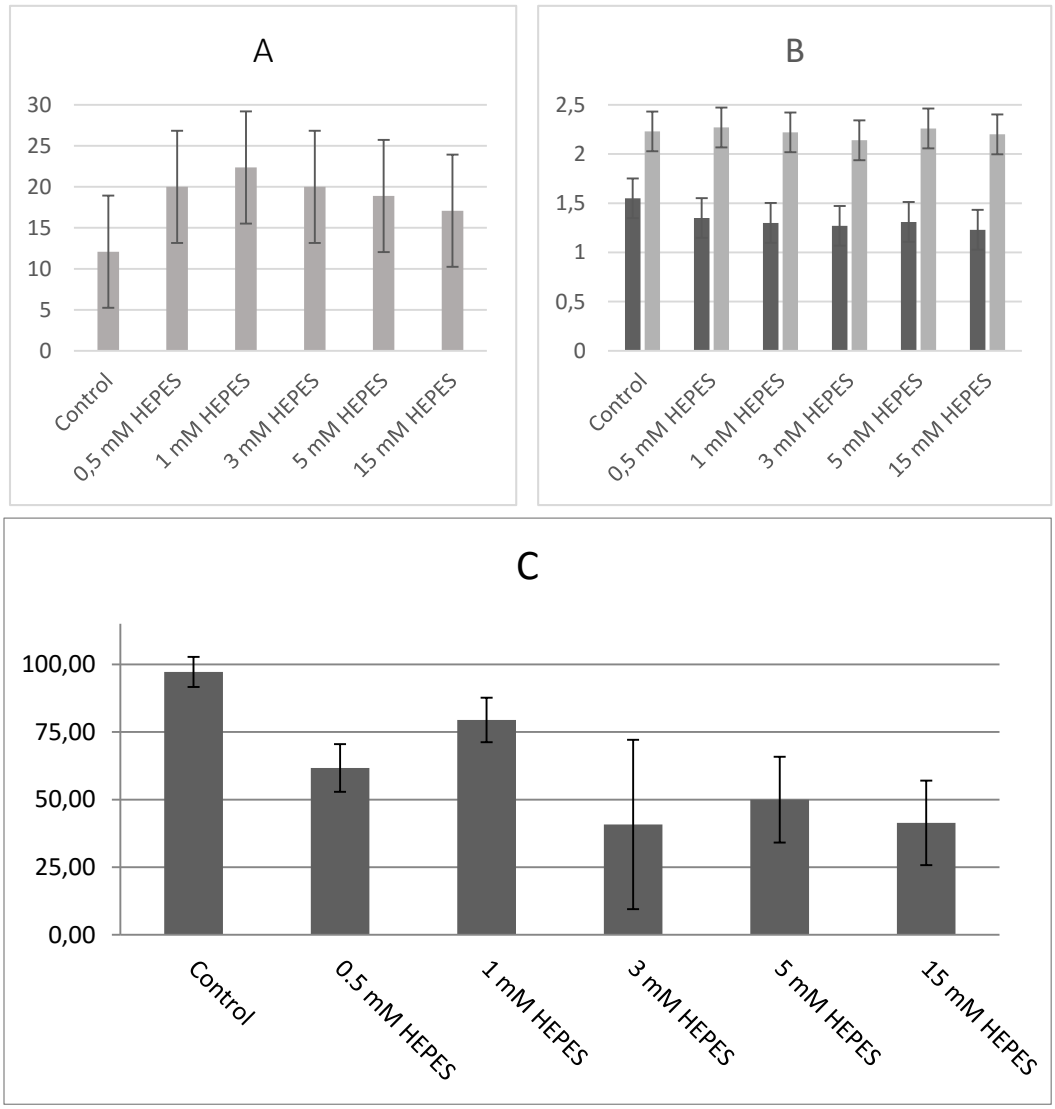

Figure 17: *Conditions of HEPES tested. A: Spontaneous movements analysis at 56 hours post fertilization; B: Cardiac frequency analysis from 32 and 56 hours post fertilization respectively; C: Free swimming analysis between at 80 hours post fertilization.*

In general, this buffer has no visible toxicological effect, not allow his use during the testing of DODAx:MO nanosystems.

Their use will not interfere with the normal development of the animals, the mortality rate is minimal or zero, the embryonic development appears to remain constant, according to the key periods, not affecting the normal development of the animal, not obtaining, by its use, false results in testing other compounds.

The maximum concentration used in this test is shown to be safe for the production of particles, the differences presented are not significant, causing to the animals a normal hatching rate at 80 hours post fertilization, and obtaining an acceptable mortality rate, always in accordance with the control group.

4.3. Monoolein

The several concentrations of monoolein demonstrates, in general, to have a low toxicological index, showing a normal index of survival.

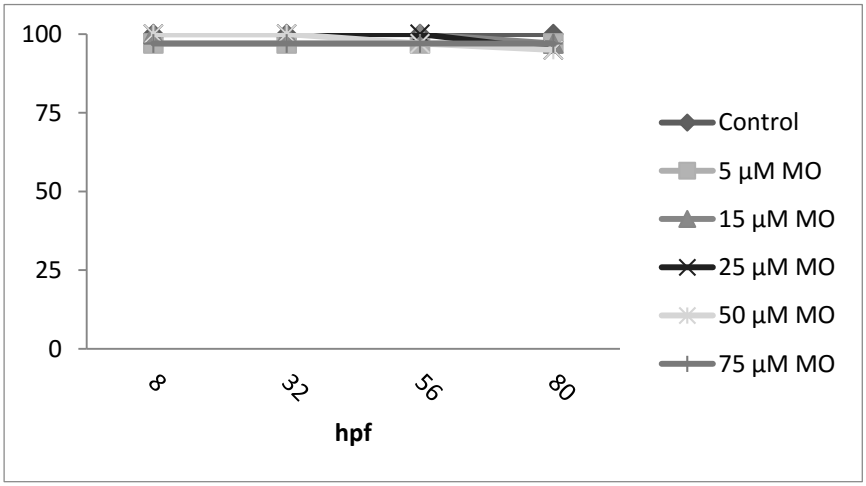

Figure 18: Mean survival rate in percentage for the various conditions tested of monoolein over the several time-points, 8, 32, 56 and 80 hours post fertilization.

The survival rate in all concentrations of monoolein shown to be identical to the control group in all post fertilization hours, keeping all embryos in epiboly period at 8 hours post fertilization.

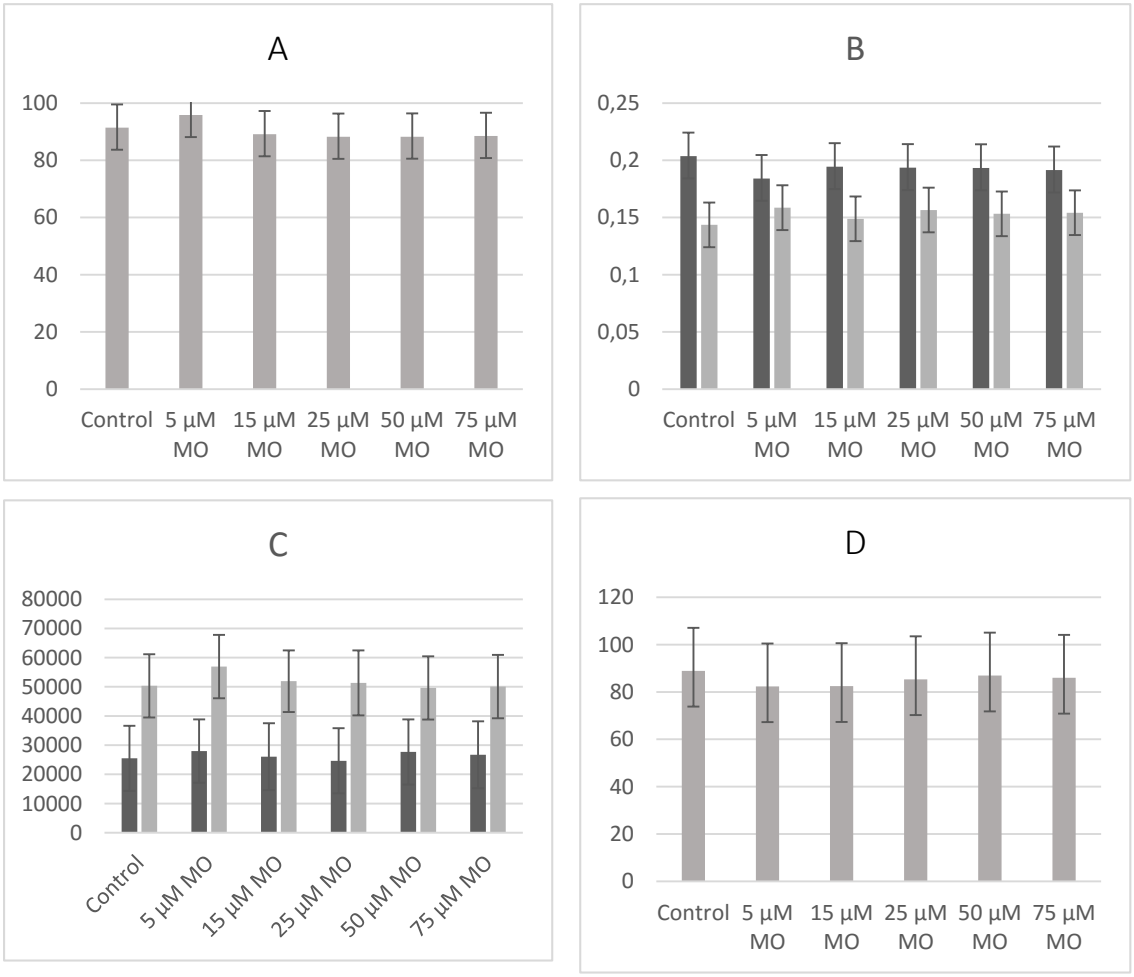

Figure 19: A: Percentage of embryos in epiboly period for each condition of monoolein, assessment at 8 hours post fertilization. B: Yolk:egg volume ratio analysis between the conditions tested of monoolein. From 8 hours post fertilization to 32 hours post fertilization; C: Eye volume analysis between the conditions tested of monoolein. From 32 hours post fertilization to 56 hours post fertilization; D: Head trunk index analysis between the conditions tested of monoolein on 32 hours post fertilization.

The yolk volume accompanies the embryonic development correctly at 8 and 32 hours post fertilization, the same happens in head trunk index and eye volume, showing a normal development.

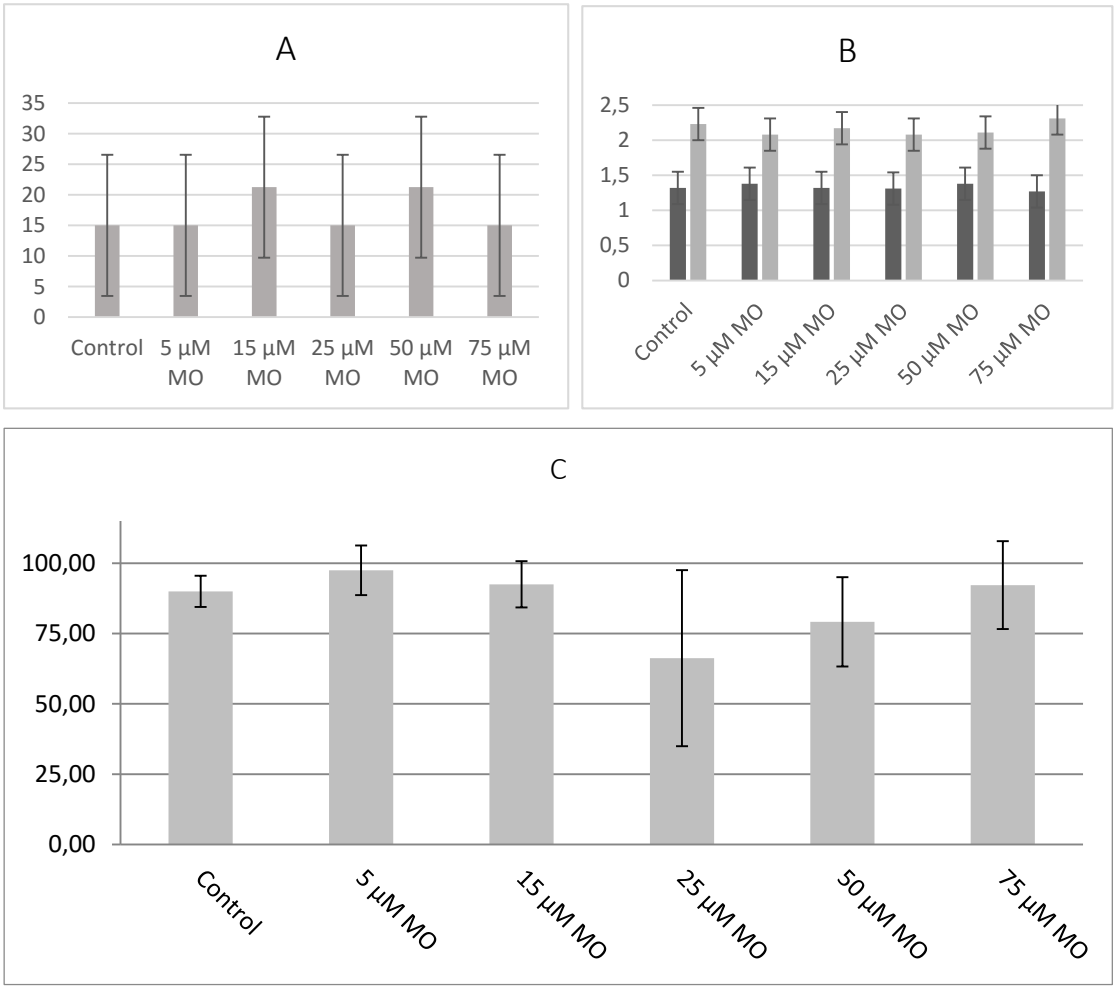

Figure 20: Conditions of monoolein tested. A: Spontaneous movements analysis at 56 hours post fertilization; B: Cardiac frequency analysis from 32 and 56 hours post fertilization respectively; C: Free swimming analysis between at 80 hours post fertilization.

With regard to spontaneous movements, there are some peaks of activity at concentrations 15 µM and 50 µM, reaching 20% more activity compared to the control group.

The cardiac frequency appears to be within normal parameters for the time-point assessed, 32 and 56 post fertilization hours.

The free swimming ability in response to external stimuli itself too regular shows, at 80 hours post fertilization, the animals full respond to the stimuli and experiment free swimming for all microplate well.

The hatching appears to be normal in comparison with the control group, at a concentration of 15 μM, the hatching at 56 hours post fertilization, is somehow low but is shown to be complete the next time-point, in accordance with the control.

The monoolein in a general way, shows no toxicity to embryos, as expected, the helper lipid is shown almost inert to animals, although some developmental delays, all animals showed in accordance with the control group at the end the assay, can be concluded that the differences are acceptable and not some toxic experience from the lipid to the embryos.

So this helper lipid can be used in lipid mixtures and liposome way that liberal, not shown toxic effects on the animals, not stipulates, in this essay, one concentration of monoolein like tolerance limit for zebrafish embryos.

4.4. DODAC

The several concentrations of DODAC demonstrates several indices of toxicity in terms of survival rate, from the concentration of 0.25 µg/mL the mortality begins to noted, in concentrations of 0.5 µg/mL and later, the mortality revels from 32 hours post fertilization, stabilizing at 56 hours post fertilization, wherein the concentration 3 µg/mL has a lower survival rate to 20% and the concentration 5 µg/mL experiment a complete mortality at 56 hours post fertilization.

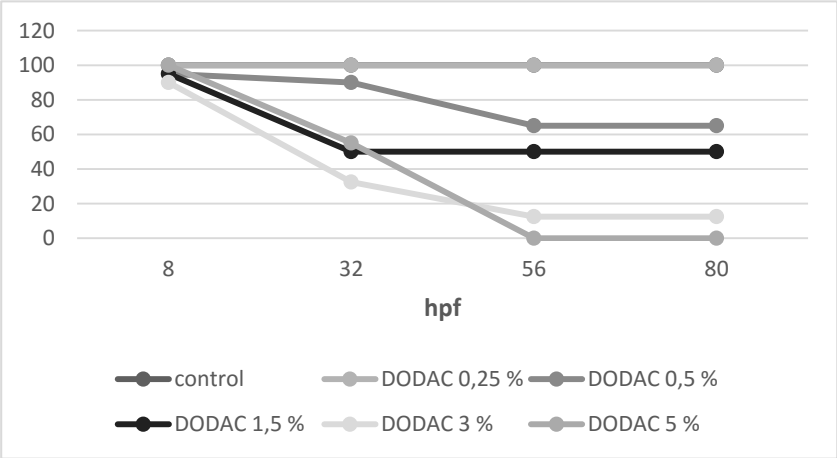

Figure 21: Mean survival rate in percentage for the various conditions tested of DODAC over the several time-points, 8, 32, 56 and 80 hours post fertilization.

As enhancing survival rate, embryo development is shown with a delay at the highest concentrations of DODAC, 5 µg/mL, where the rate of embryos in epiboly have a decrease, showing an evident delay in development.

Regarding the yolk:egg volume ration, at 8 hours post fertilization, variations are noted slightly with growing concentration, more evident in 32 hours, showing an equivalent delay to the large yolk:egg volume ratio.

Contrary to expectations, at 32 hours post-fertilization, embryos have an equivalent opening to the concentration, that is, the higher the concentration of DODAC, the greater the embryo opening angle, the increase shows problems in development, the head trunk index is proportional to the concentration, the higher the concentration, the greater the index. In the eye volume, only exist significante

data for measurement at 32 hours post fertilization, showing a little delay on the higher concetrations of DODAC.

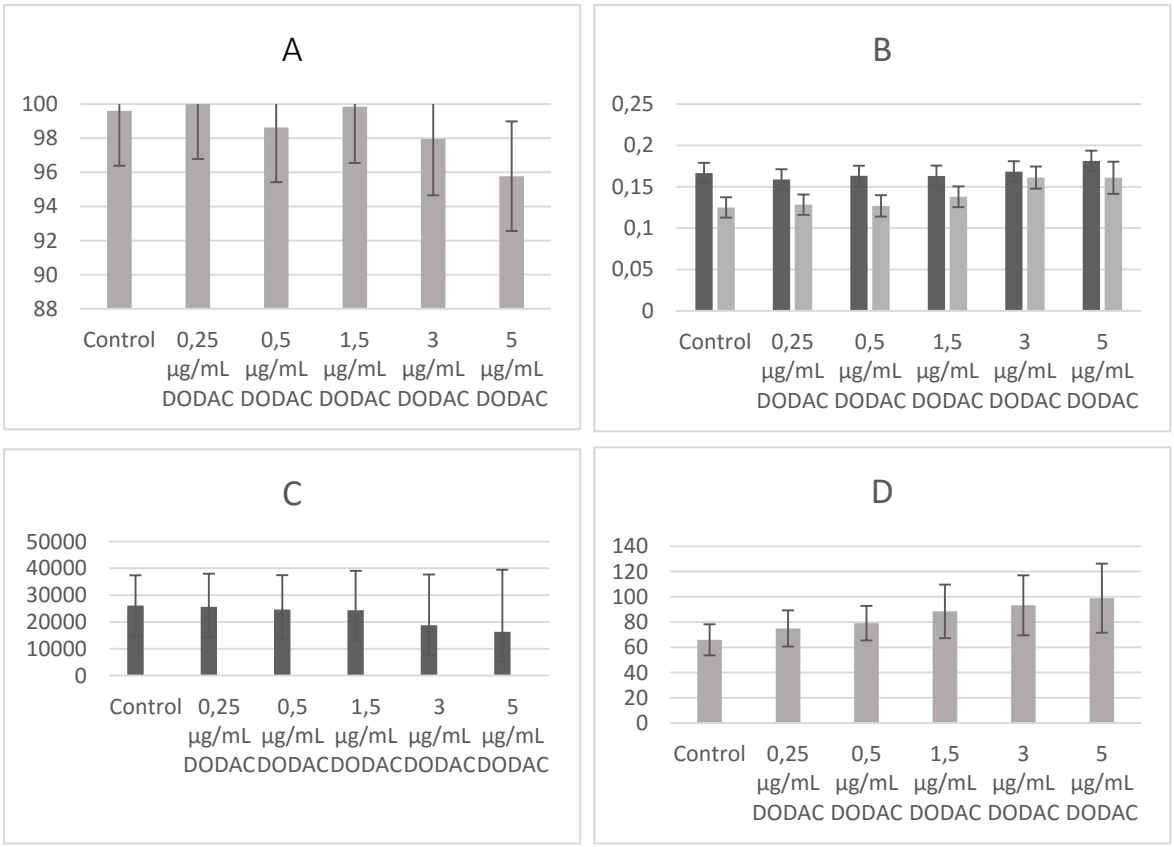

Figure 22: A: Percentage of embryos in epiboly period for each condition of DODAC, assessment at 8 hours post fertilization. B: Yolk:egg volume ratio analysis between the conditions tested of DODAC. From 8 hours post fertilization to 32 hours post fertilization; C: Eye volume analysis between the conditions tested of DODAC at 32 hours post fertilization; D: Head trunk index analysis between the conditions tested of DODAC on 32 hours post fertilization.

The heart rate is shown with differences also during the 32 hours post fertilization, the most obvious difference is noted in concentration of 5 µg/mL. All other feature similarity with the control group at 56 hours post fertilization, there is a fall in heart rate of the animal when the concentration increases, decreasing frequency, don't having values for comparison in concentration of 5 µg/mL.

Spontaneous movements were detected at 32 hours post fertilization, noting an increase in the rate at intermediate concentrations, 0.5 µg/mL and 1.5 µg/mL, with no records to compare in the highest concentration at this time.

The free swimming animals is shown in accordance with the control group, as the hatching rate, animals that managed to reach the 80 hours post fertilization

demonstrate capacity to normalized free swim, in 0.5 µg/mL the swimming experience is above the control value, animals that reached the hatch 80 hours succeeded in the normal way, on the concentration of 3 µg/mL, the hatching rate has decreased by 25%.

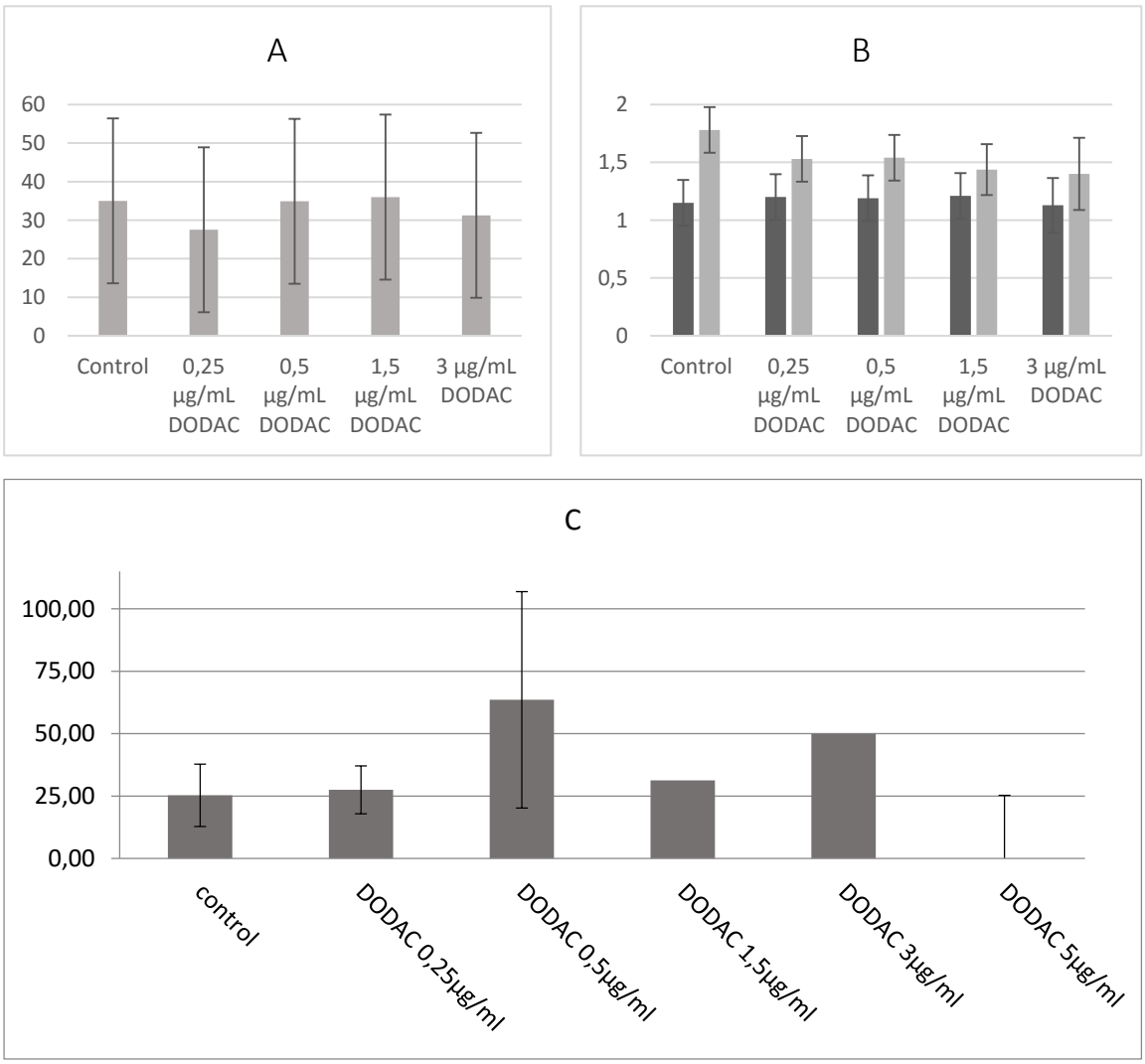

Figure 23: Conditions of DODAC tested until 3µg/mL. A: Spontaneous movements analysis at 56 hours post fertilization; B: Cardiac frequency analysis from 32 and 56 hours post fertilization respectively; C: Free swimming analysis between at 80 hours post fertilization.

As you would expect, this cationic surfactant has toxicity to animals, only the lowest concentration, 0.25 µg/mL, keep up the development noting all parameters required for the evaluation, it appears, therefore, that the limit margin

of this cationic surfactant isolated for the zebrafish is low, with the possibility of severe toxicological effects when the concentration is increased.

There is possibility cardio-toxic and neurotoxic abnormalities and in normal embryonic development of the animal with high concentrations, leaven the embryo to death.

4.5. DODAB

The survival rate of embryos with DODAB shows stable during all hours post fertilization in most part of concentrations. At a concentration of 3 µg/mL, there is a deficit of survival at 80 hours, where the rate of embryos that survive is around 50%.

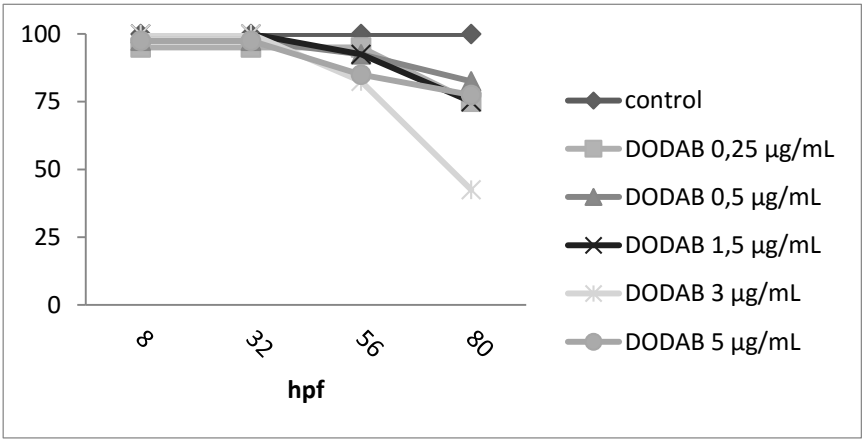

Figure 24: Mean survival rate in percentage for the various conditions tested of DODAB over the several time-points, 8, 32, 56 and 80 hours post fertilization.

In terms of epibolic development, only one concentration force the embryos to deviate of the control, in the concentration 5 µg/mL, embryos shown to have a developmental delay in terms of percentage of embryos on epiboly.

The yolk:egg volume ratio, at 8 and 32 hours post fertilization shown to be normal, at 56 hours, the concentration increases as the yolk volume, suggesting a delay in development at this time.

The angle accompanies the growth of the concentration, that is, from the concentration 1.5 the aperture angle to 32 hours proves to be higher, which suggests an abnormality in the development. On the other hand, the volume of the eye shows a normal development of this organ.

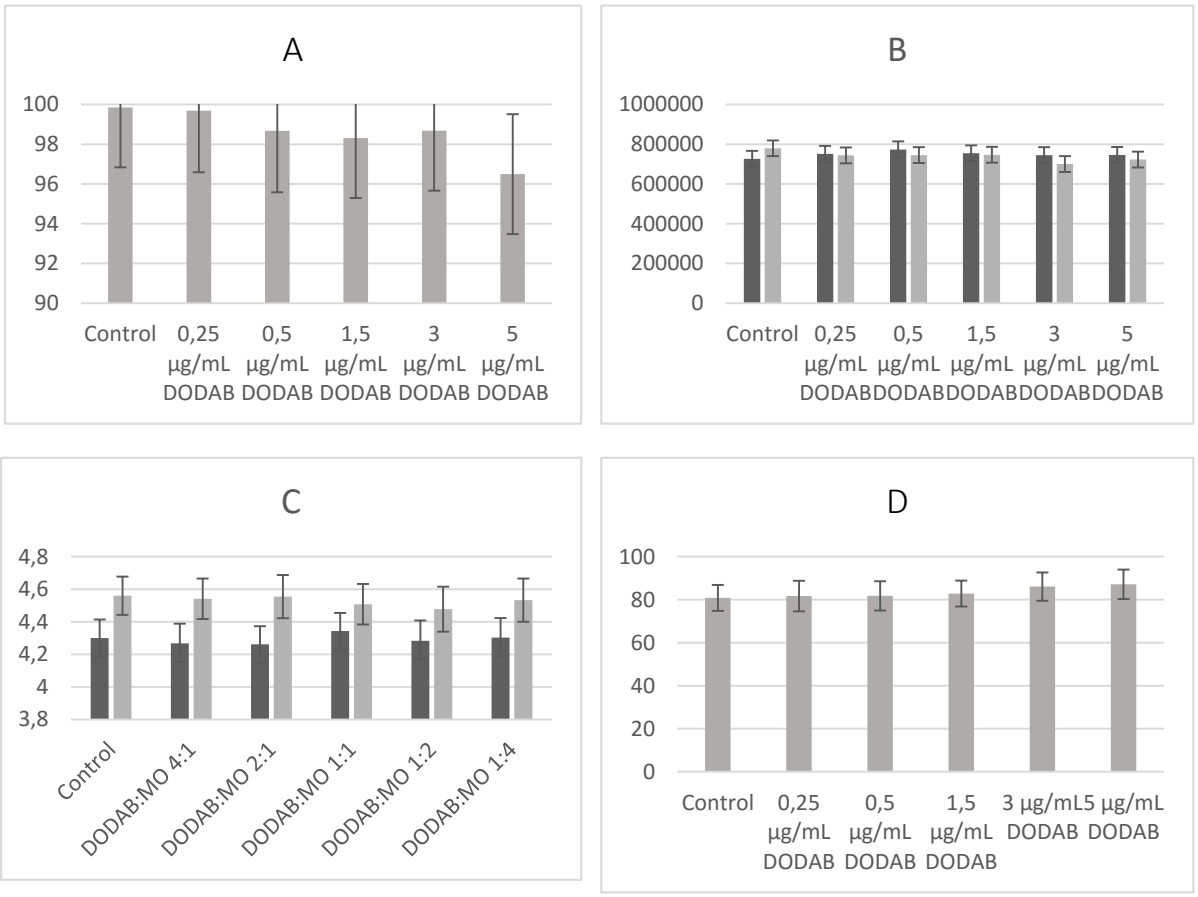

Figure 25: Percentage of embryos in epiboly period for each condition of DODAB, assessment at 8 hours post fertilization. B: Yolk:egg volume ratio analysis between the conditions tested of DODAB. From 8 hours post fertilization to 32 hours post fertilization; C: Eye volume analysis between the conditions tested of DODAB. From 32 hours post fertilization to 56 hours post fertilization; D: Head trunk index analysis between the conditions tested of DODAB on 32 hours post fertilization.

The heart rate is normalized to 32 hours, however at 56 hours shows a peak drop in the presence of DODAB, decreasing dramatically.

The spontaneous movements show up in an alternating form, from the concentration 0.5 µg/mL at 32 hours post fertilization.

DODAB show cause shortage of free-swimming animals in the presence of this surfactant, the animals showed a dramatic decrease in free swimming, with a decrease of around 50%.

The presence of this surfactant will interfere with hatching animals at 56 hours post fertilization, the animals hatched at this time-point is higher compared with the control group.

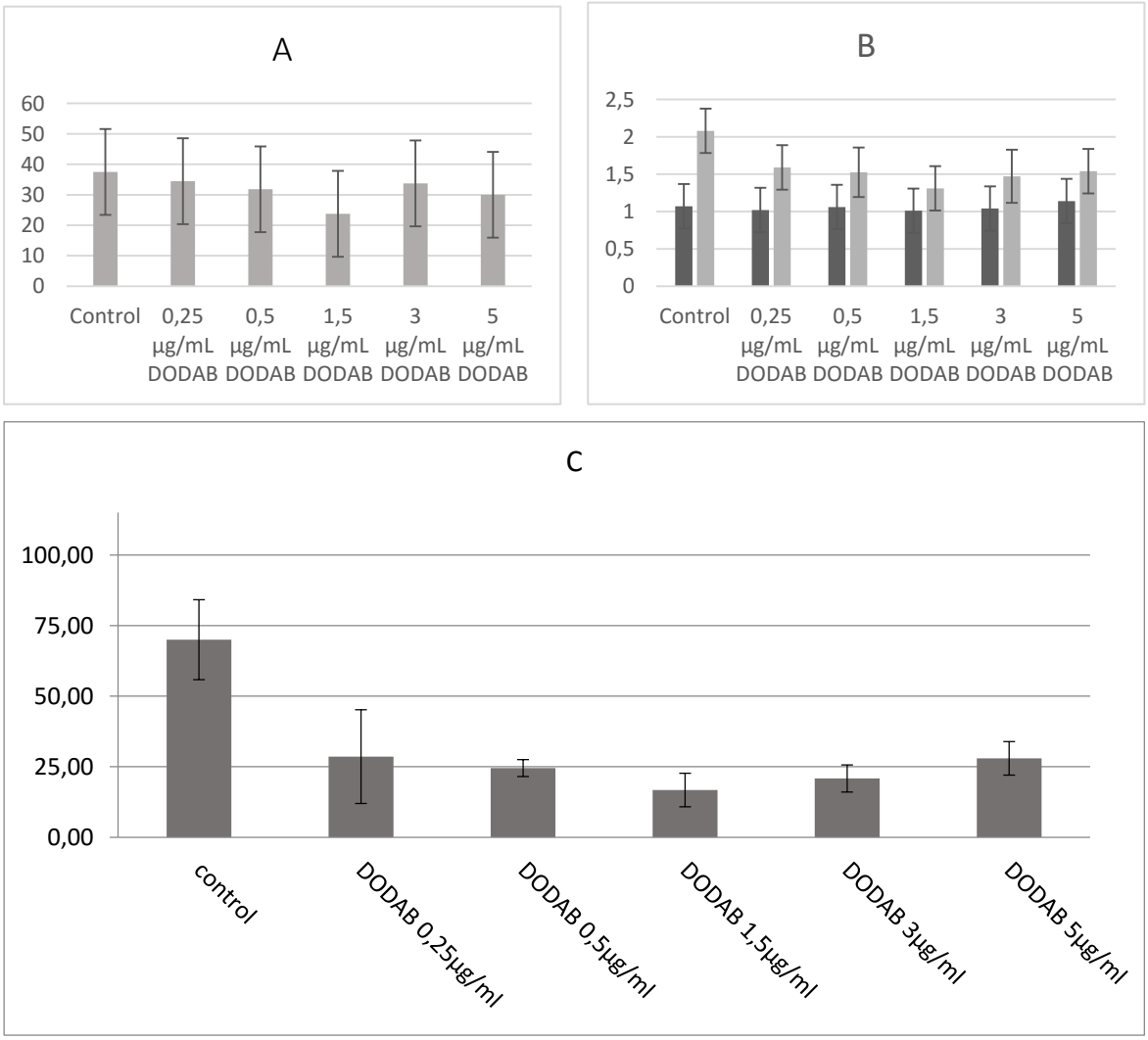

Figure 26: Conditions of DODAB tested. A: Spontaneous movements analysis at 56 hours post fertilization; B: Cardiac frequency analysis from 32 and 56 hours post fertilization respectively; C: Free swimming analysis between at 80 hours post fertilization.

This cationic surfactant, although not dramatically as the DODAC, as expected, the results are lower in a general toxicological concern, the embryos remain viable until the end of the assay, which translates into a toxicological index not harsh at these concentrations, however cardio and neurotoxic level can lead to some anomalies.

Was viewed some abnormal heart rate traces along the concentrations, may suggest an interaction with this organ, on the other hand, the reduced free swimming demonstrate that animals can suggest a delay in neurological development.

4.6. Lipid mixture DODAC:MO

The survival rate is shown along the standard test, the range is between 25% and 100%, with no significant differences.

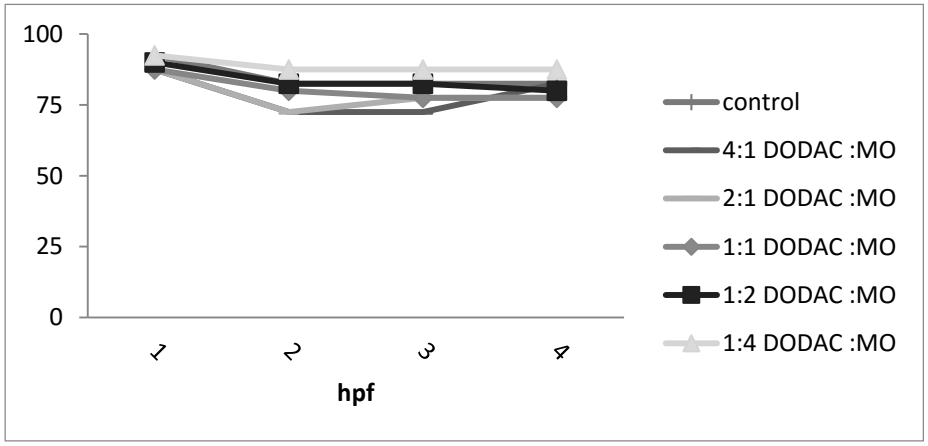

Figure 27: Mean survival rate in percentage for the various conditions tested of DODAC:MO mixture, over the several time-points, 8, 32, 56 and 80 hours post fertilization, respectively.

The embryo development in the first time-point shows a stabilization in the animal development, keeping the normal epiboly.

The same happens in the yolk:egg volume ratio appears to be equivalent to the control group in all mixing ratios, the head trunk index is shown to be around 95 degrees, and the eye volume shows to be normal too.

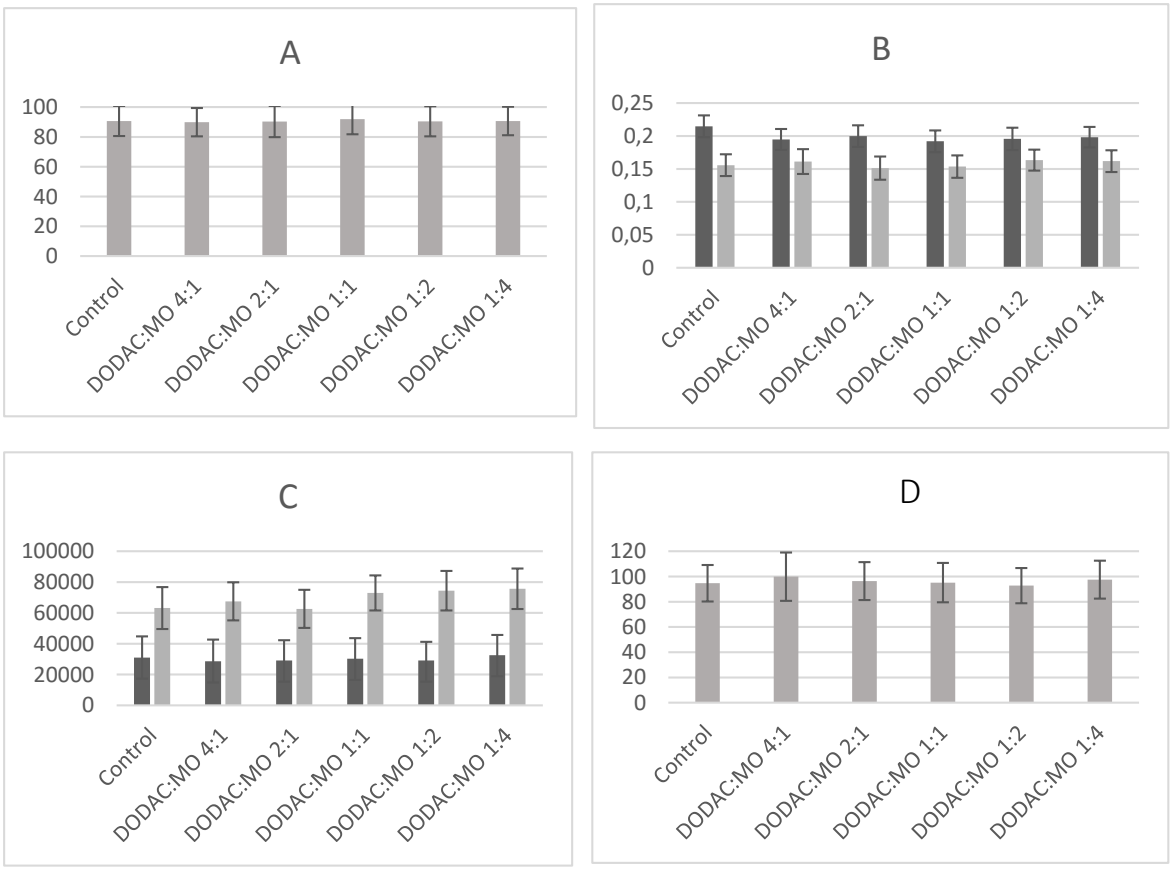

Figure 28: Percentage of embryos in epiboly period for each condition of lipid mixture DODAC:MO, assessment at 8 hours post fertilization. B: Yolk:egg volume ratio analysis between the conditions tested of lipid mixture DODAC:MO. From 8 hours post fertilization to 32 hours post fertilization; C: Eye volume analysis between the conditions tested of lipid mixture DODAC:MO. From 32 hours post fertilization to 56 hours post fertilization; D: Head trunk index analysis between the conditions tested of lipid mixture DODAC:MO on 32 hours post fertilization.

The cardiac frequency shows normal, showing also a distinct change in the percentage of spontaneous movements in the proportions with higher concentrations of DODAC, the movements are lower, thereafter with the decreasing of monoolein, the movements increase gradually.

Both the rate of free-swimming like the hatching rate of the animals remain normal in all conditions, showing no interaction of compounds with proportions of these parameters.

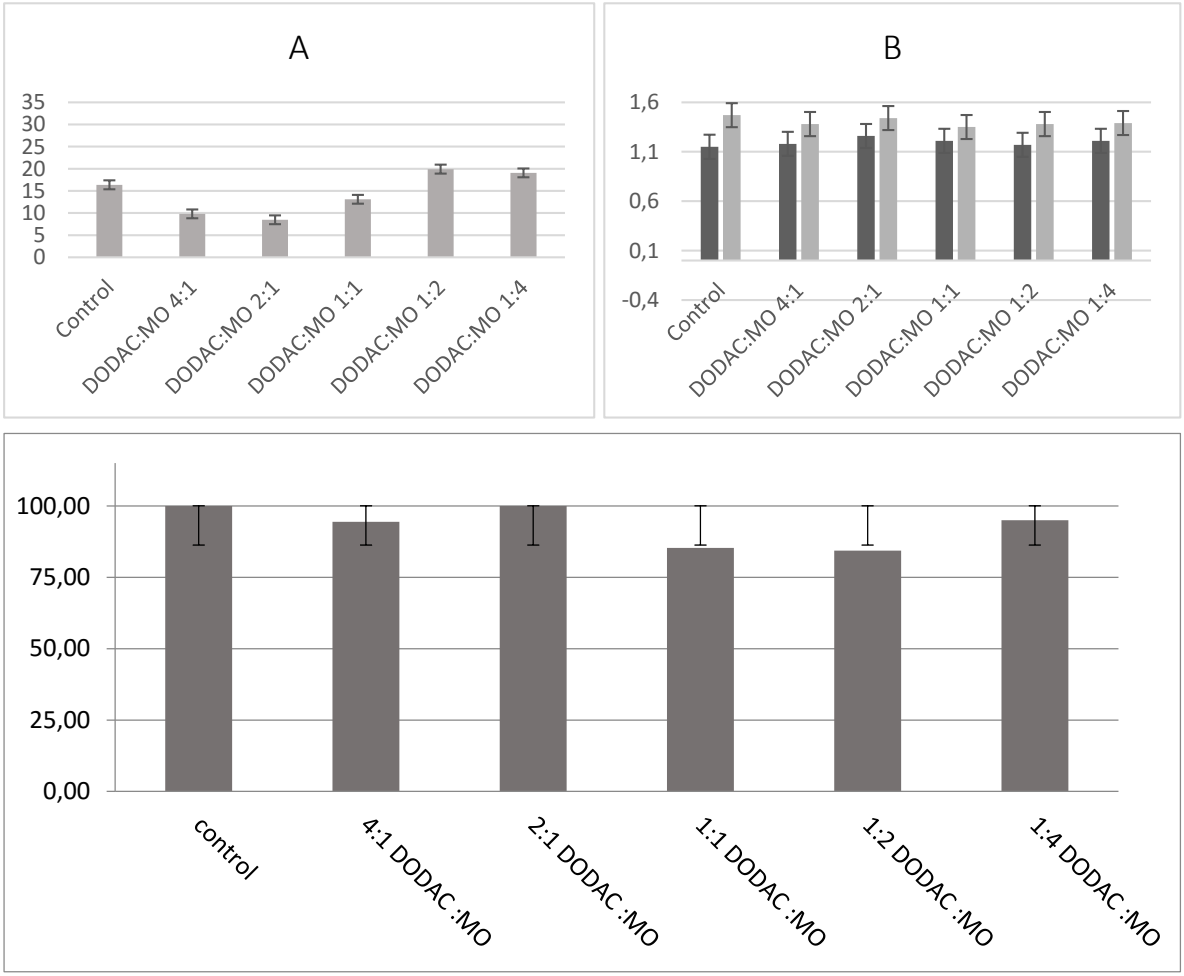

Figure 29: Conditions of DODAB tested. A: Spontaneous movements analysis at 56 hours post fertilization; B: Cardiac frequency analysis from 32 and 56 hours post fertilization respectively; C: Free swimming analysis between at 80 hours post fertilization.

In general, the presence of monoolein break the DODAC toxicity index, their presence keeps stable the mixture to the point of being accepted by the embryos, leading to a survival rate above 75%, contradicting the results of DODAC isolated.

The development of embryos keeps steadily, not significantly altering the normal development, especially when the monoolein is in greater concentration.

It is concluded, therefore, that the presence of the helper lipid reduces the toxic effects of the surfactant, allowing a more standardized embryo development compared to the surfactant alone.

4.7. Lipid mixture DODAB:MO

The lipid mixture with DODAB and monoolein shows more toxic effect from 56 hours post fertilization, leading to an increased mortality, at 80 hour post fertilization, the survival rate shows to be rounding the 70%, however in accordance with the control group.

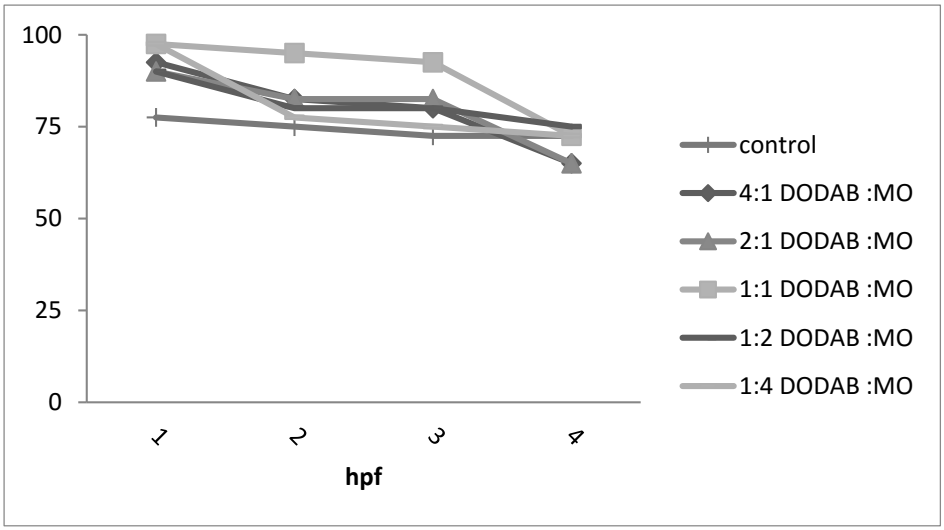

Figure 30: Mean survival rate in percentage for the various conditions tested of DODAB:MO mixture over the several time-points. 1: 8 hours post fertilization; 2: 32 hours post fertilization; 3: 56 hours post fertilization; 4: 80 hours post fertilization.

The embryonic development at 8 hours post fertilization appear to accelerate in the proportions 4:1 and 2:1, the the monoolein try to stabilize this acceleration, reducing the number of embryos in epiboly.

Over the time-points, the yolk volume seems to accompany the normal development, not obtaining significant differences in the conditions.

The head trunk index shows no significate changes in the presence of the lipid mixtures with DODAB, the same happens in the eye volume analysis.

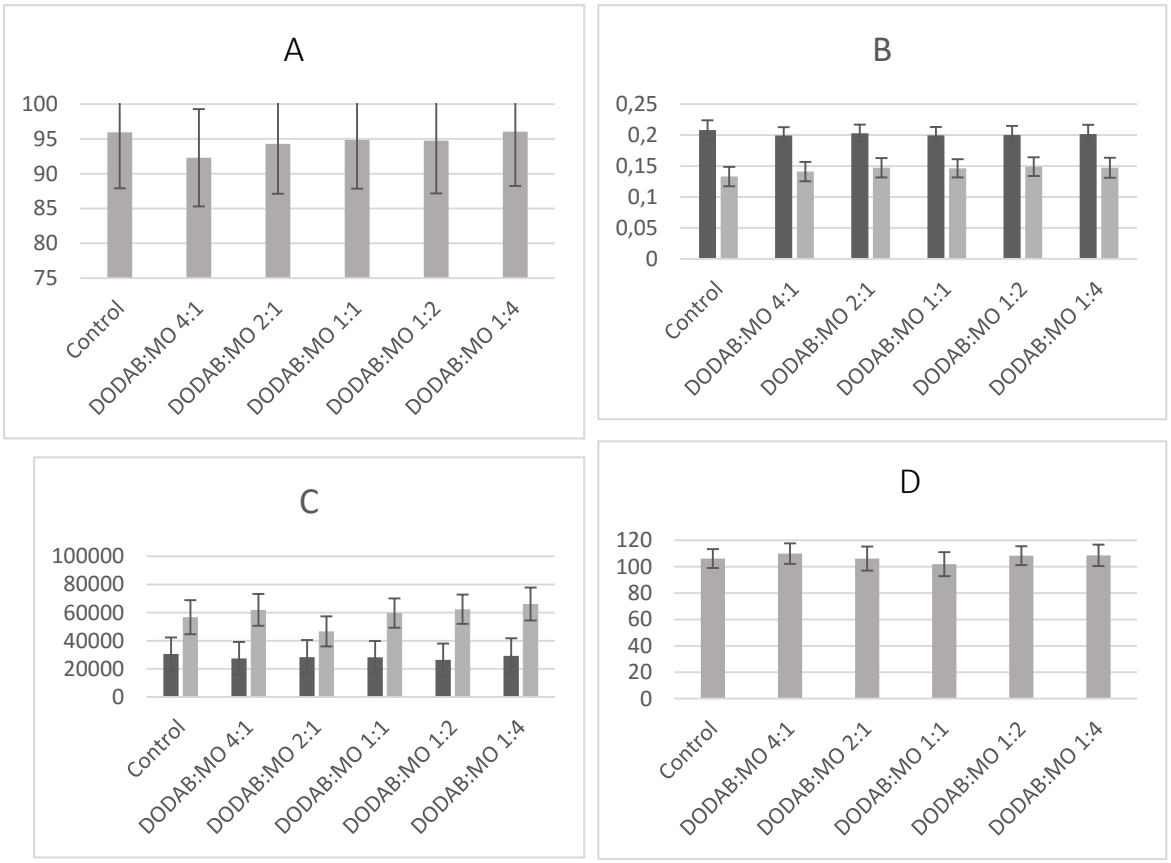

Figure 31: Percentage of embryos in epiboly period for each condition of lipid mixture DODAB:MO, assessment at 8 hours post fertilization. B: Yolk:egg volume ratio analysis between the conditions tested of lipid mixture DODAB:MO. From 8 hours post fertilization to 32 hours post fertilization; C: Eye volume analysis between the conditions tested of lipid mixture DODAB:MO. From 32 hours post fertilization to 56 hours post fertilization ; D: Head trunk index analysis between the conditions tested of lipid mixture DODAB:MO on 32 hours post fertilization.

The presence of this mixture shows not interfere with the heart rate both at 32 and 56 hours post fertilization

There is a variation on spontaneous movements, this movements increase until de proportion 1:1 after that, the next proportions causes a decrease with stabilization.

The free swimming of the animals shows decreased by 50% in the condition 4:1, which worsens when present in a mixture in the proportion 2:1, showing an increase when increasing the concentration of monoolein, stabilizing, however no reaching 100%.

This mixture shows no adverse effect on hatching animals, maintaining a similar rate to the control at both 56 hours and 80 hours post fertilization.

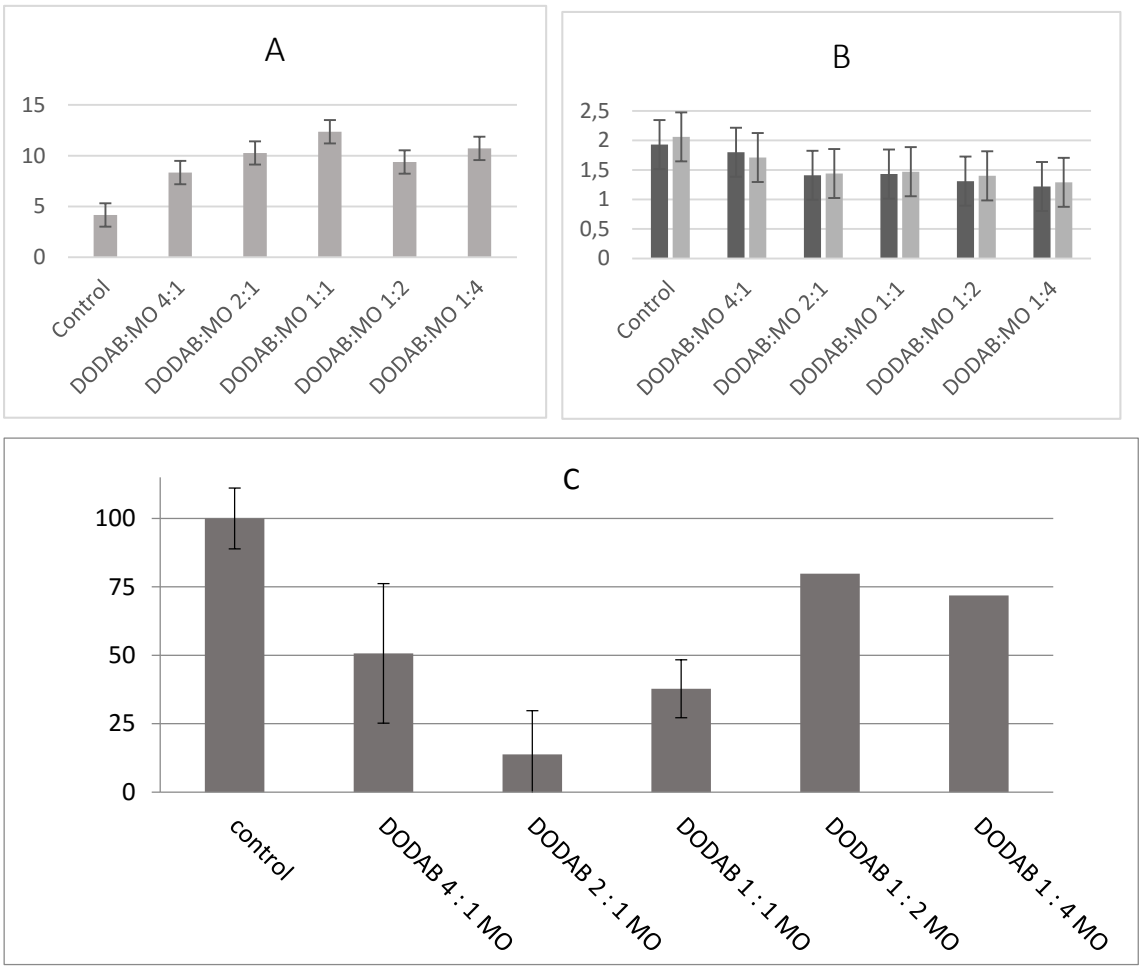

Figure 32: Conditions of DODAB tested. A: Spontaneous movements analysis at 56 hours post fertilization; B: Cardiac frequency analysis from 32 and 56 hours post fertilization respectively; C: Free swimming analysis between at 80 hours post fertilization.

In this mixture of lipids, as above, the monoolein appears to stabilize the system, decreased the surfactant toxicity indices however not as efficient as DODAC, the survival rate immediately decreases to 56 hours after fertilization, while hovering 75 %.

Not affecting the normal development of embryos, appears to have a cardio-toxic effect, namely because the heartbeat will decrease, its frequency have a significant decrease with this mixture, the presence of the monoolein shows to not compensate this decrease, maintaining the low level of cardiac frequency.

Neurotoxicity is also revealed in the free swimming, the animals have difficulty responding to stimuli, leading to a very low index, the monoolein shows to try reduce this toxicity, however cannot do it completely.

The embryonic development, as has been described, it is not affected in a general manner, getting all embryos follow the process and hatch out in the expected time-points.

4.8. Nanosystem DODAC:MO

Performing the analysis of stability of liposomes during 72 hours, they showed to be minimally stable for a period equivalent to the ZET assay, there are slight differences in the distribution of liposomes groups, however in general and the liposomes keep stable for administration in embryos.

All the data about this stability analysis are illustrated in the annexes.

Table 6: Liposome DODAC:MO stability analysis during 72 hours.

Time	Ratio	Size	PDI	Zeta Potential
0 hours	4.1	102	0.19	66.6
	2:1	422	0.72	78.2
	1:1	185	0.46	75.5
	1:2	225	0.49	66.2
	1:4	277	0.2	62.1
24 hours	4.1	106.3	0.175	62.1
	2:1	256	0.736	75.2
	1:1	166.6	0.569	75.2
	1:2	211.6	0.439	66.4
	1:4	293.7	0.182	61.3
48 hours	4.1	107.3	0.189	61.1
	2:1	260.7	0.786	73.8
	1:1	161.2	0.625	73
	1:2	219.7	0.472	67.1
	1:4	290.5	0.165	59.9
72 hours	4.1	105.7	0.185	60.5
	2:1	273.6	0.789	75.1
	1:1	163.2	0.611	74.5
	1:2	224.4	0.48	63.7
	1:4	287.2	0.132	62

With the administration of the DODAC:MO liposomes, the embryos experienced a normal survival rate, no higher than 25% of mortality in most proportions.

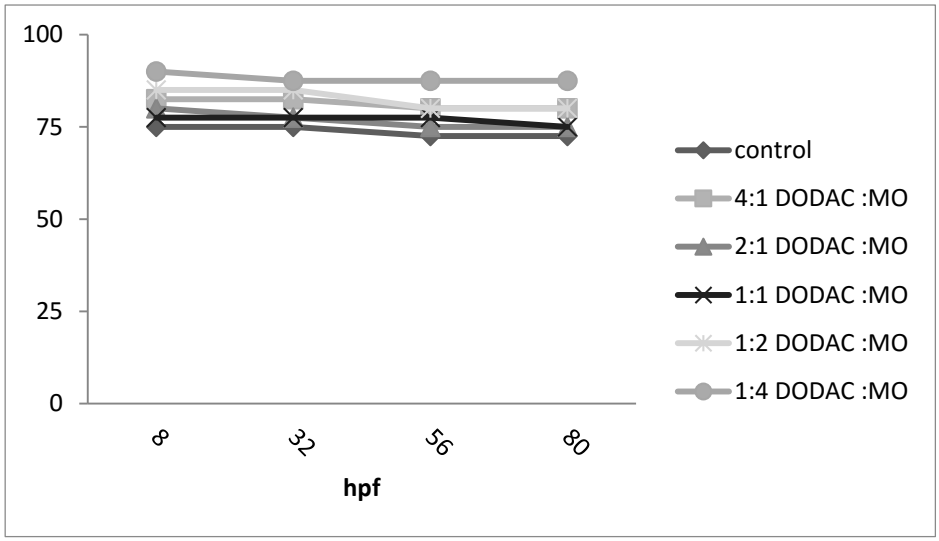

Figure 33: Mean survival rate in percentage for the various conditions tested of DODAC:MO liposomes over the several time-points, 8, 32, 56 and 80 hours post fertilization

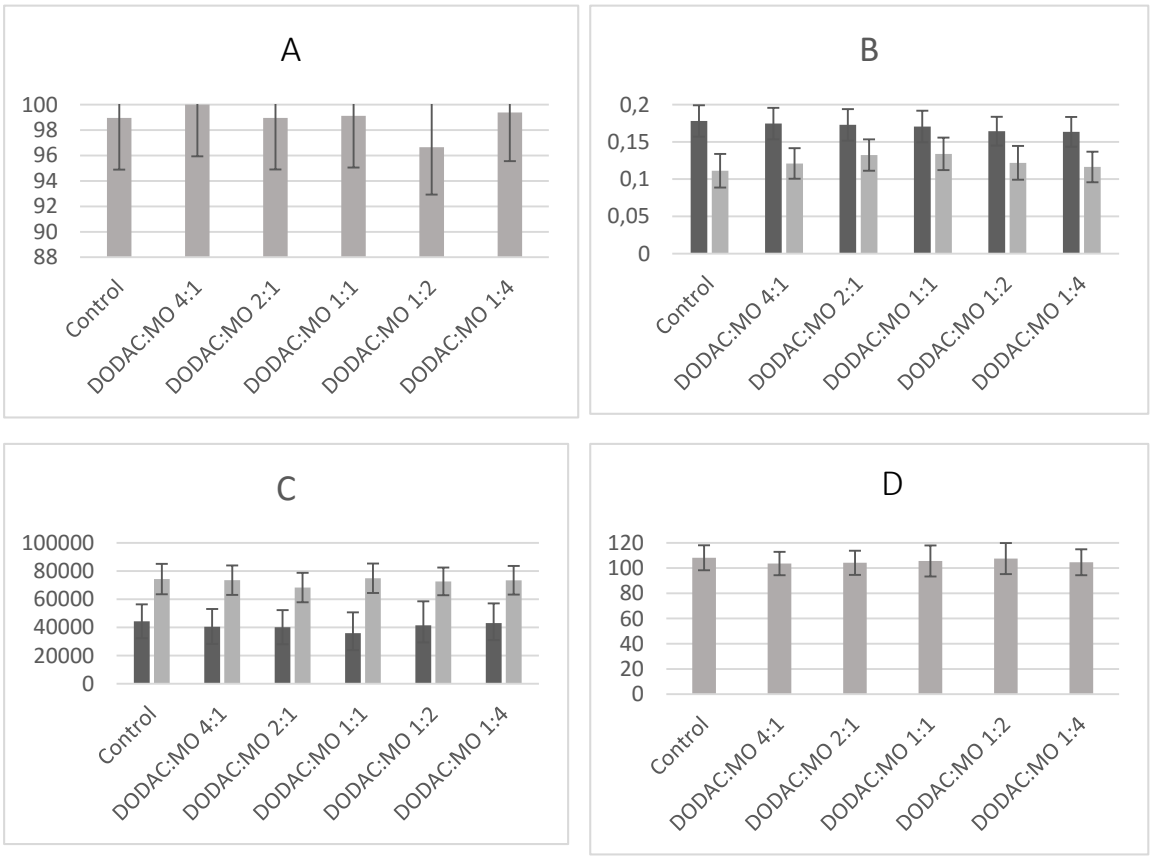

Figure 34: Percentage of embryos in epiboly period for each condition of DODAC:MO liposomes, assessment at 8 hours post fertilization. B: Yolk:egg volume ratio analysis between the conditions tested of DODAC:MO liposomes. From 8 hours post fertilization to 32 hours post fertilization; C: Eye volume analysis between the conditions tested of DODAC:MO liposomes. From 32 hours post fertilization to 56 hours post fertilization; D: Head trunk index analysis between the conditions tested of DODAC:MO liposomes on 32 hours post fertilization.

In comparison with the control group, the presence of monoolein in the proportion 1:2 shows to decrease the epiboly stage in this embryos, showing a decrease in his percentage.

The yolk:egg volume ratio shows to be normal in comparison with the control group, what happens in the head trunk index and eye volume, remaining into the normal angle in all the ratios tested.

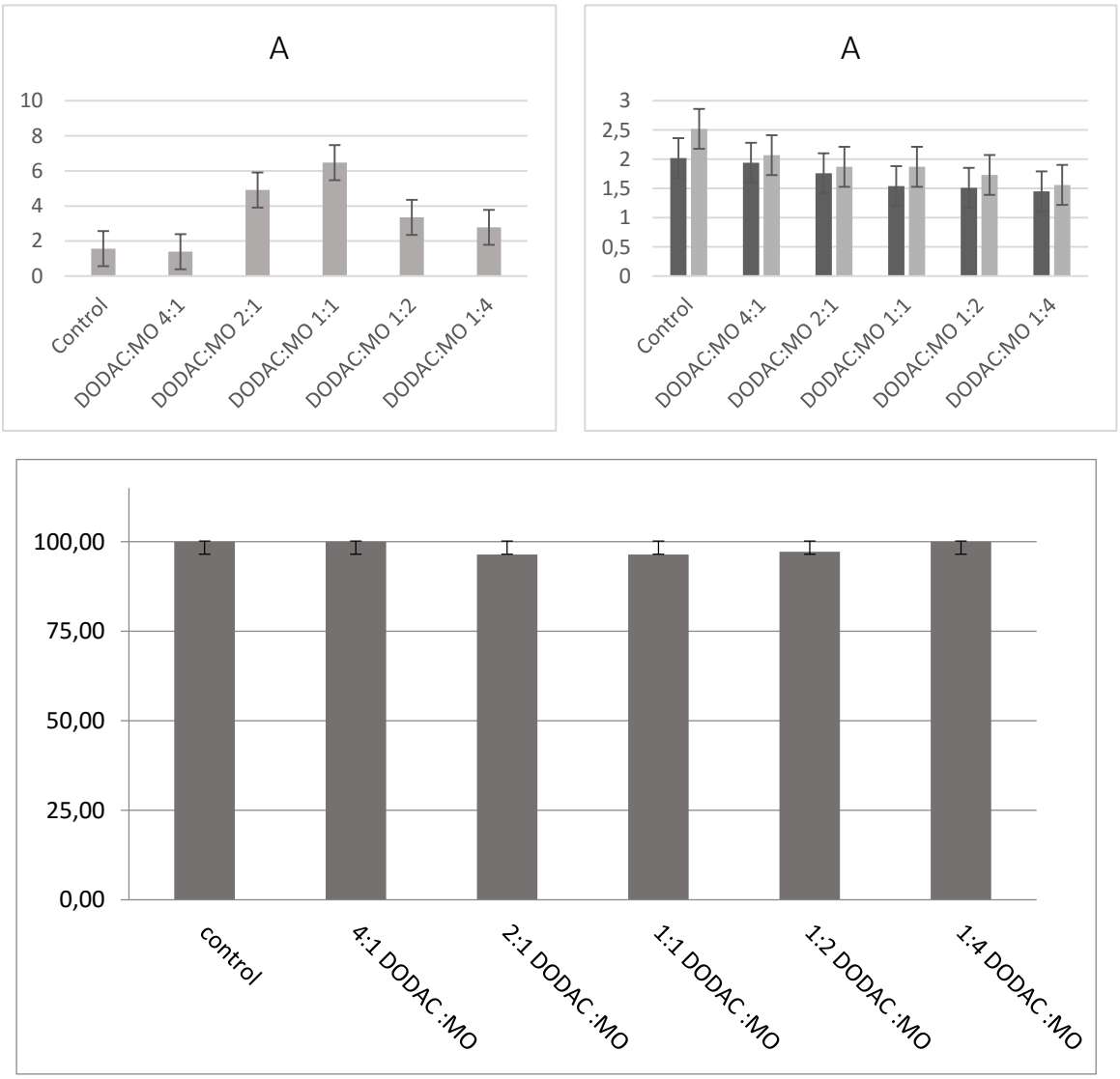

Figure 35: *Conditions of DODAC:MO liposomes tested. A: Spontaneous movements analysis at 56 hours post fertilization; B: Cardiac frequency analysis from 32 and 56 hours post fertilization respectively; C: Free swimming analysis between at 80 hours post fertilization.*

The heartbeat rate is decreased over all proportion, showing a very noticeable reduction both at 32 hours as at 56 hours post fertilization, there is a

slight stabilization when increases the monoolein, however not without decreasing the index of heart rate .

The spontaneous movements tend to grow in advance of proportions, having an abnormal peak in the 1: 1 ratio, decreasing immediately afterwards but never equaled the rate of the control group.

In terms of free swimming and hatching rates, the presence of DODAC:MO nanosystems shows have no influence, every animal have a standard free swimming and a hatching index as expected and equal to the control group.

It can be concluded that the system DODAC:MO generally presents no severe toxicity to embryos, reaching a normalized survival rate and a hatching within normal parameters.

Throughout embryonic development, the system having more monoolein concentration tends to have an acceleration, experienced exactly the opposite with the proportions surfactant, where the yolk tends to be higher, showing a delay in development.

All animals having an index of standard free-swimming, it can be said that the neurotoxic effect are normalized with liposome delivery, unlike a simple mixture of lipids.

Not so with cardiotoxicity, where the nanosystem presence tends to decrease heart rate. The introduction of monoolein in greater concentration tends to normalize, however does not seem sufficient, keeping the low heart rate.

4.9. Nanosystem DODAB:MO

Performing the analysis of stability of liposomes during 72 hours, they showed to be minimally stable for a period equivalent to the ZET assay, there are slight differences in the distribution of liposomes groups, however in general and the liposomes keep stable for administration in embryos.

All the data about this stability analysis are illustrated in the annexes.

Table 7: Liposome DODAB:MO stability analysis during 72 hours.

Time	Ratio	Size	PDI	Zeta Potential
0 hours	4.1	508	0.48	70
	2:1	302	0.56	73
	1:1	293	0.54	69
	1:2	293	0.37	69
	1:4	374	0.23	64
24 hours	4.1	526	0.46	57
	2:1	314	0.54	69
	1:1	290	0.51	71
	1:2	262	0.42	67
	1:4	378	0.19	69
48 hours	4.1	547	0.46	54
	2:1	312	0.53	69
	1:1	292	0.49	68
	1:2	339	0.57	69
	1:4	424	0.26	64
72 hours	4.1	635	0.4	54
	2:1	315	0.53	61
	1:1	298	0.45	67
	1:2	268	0.4	68
	1:4	360	0.15	72

The nanosystem DODAB:MO shows up with a survival rate just below 75%, mainly from the 32 hours after fertilization.

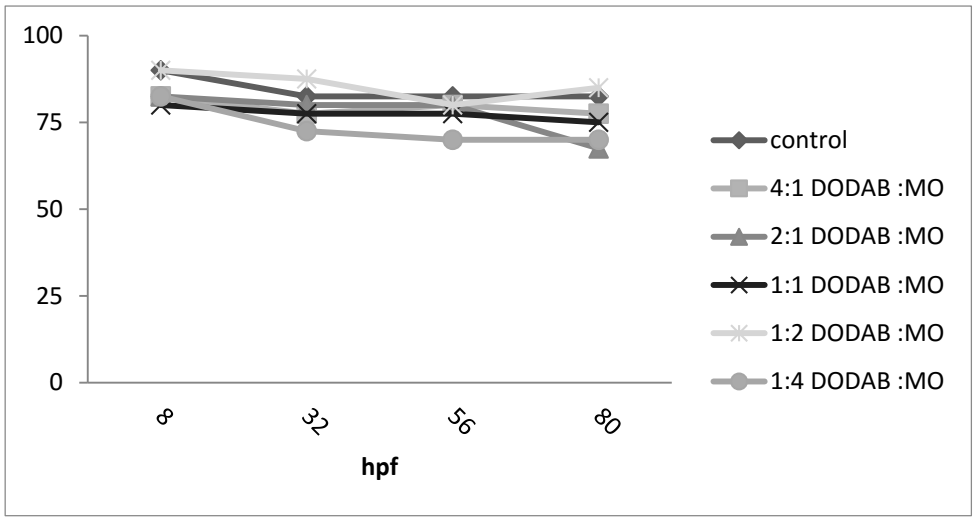

Figure 36: Mean survival rate in percentage for the various conditions tested of DODAB:MO liposomes over the several time-points, 8, 32, 56 and 80 hours post fertilization.

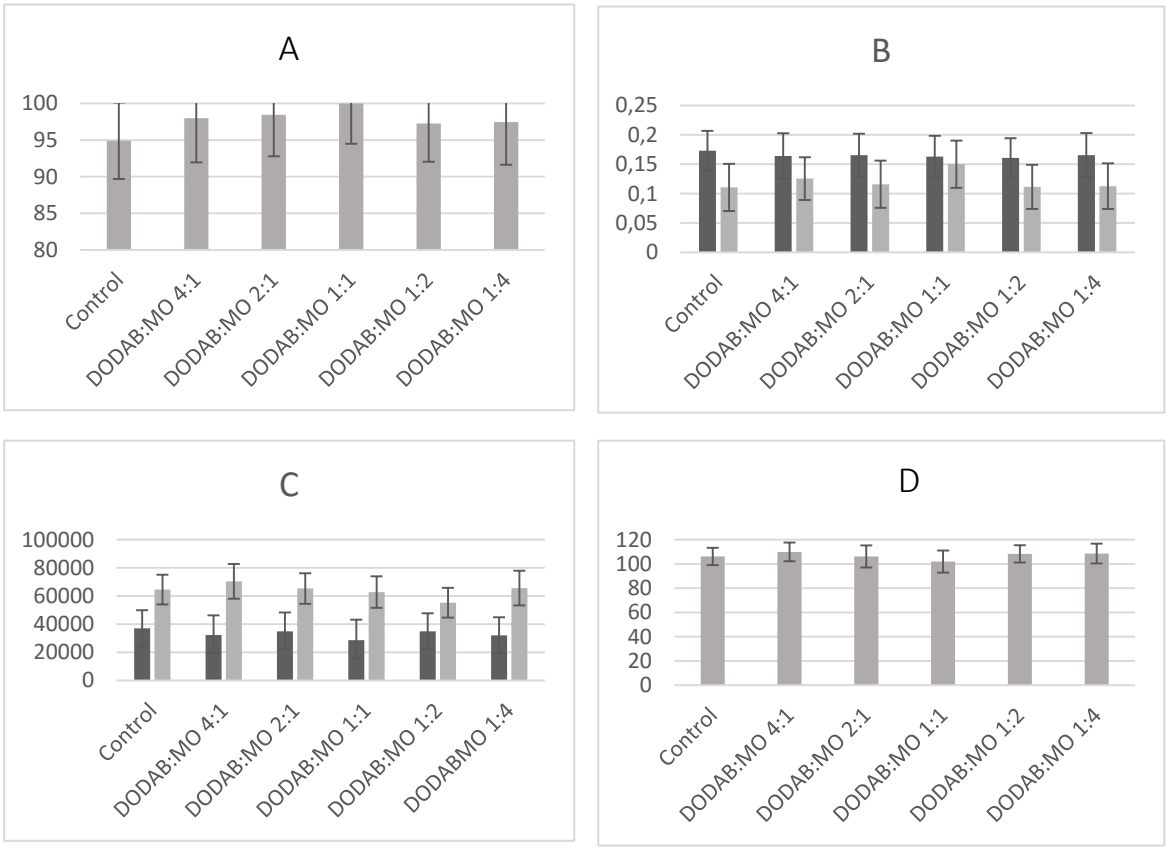

Figure 37: Percentage of embryos in epiboly period for each condition of DODAB:MO liposomes, assessment at 8 hours post fertilization. B: Yolk:egg volume ratio analysis between the conditions tested of DODAB:MO liposomes. From 8 hours post fertilization to 32 hours post fertilization; C: Eye volume analysis between the conditions tested of DODAB:MO liposomes. From 32 hours post fertilization to 56 hours post fertilization; D: Head trunk index analysis between the conditions tested of DODAB:MO liposomes on 32 hours post fertilization.

In terms of embryo development, the presence of more monoolein shows to force the embryos to his normal stage, that is, until the similar proportion of surfactant and monoolein, the percentage of embryos in epiboly increase, but when the surfactant decrease his presence, the percentage of embryos in epiboly stage decrease considerable.

The development in terms of yolk:egg volume ratio, shows stable at all post fertilization hours, only in the ratio 1:1 the volume increase in comparison with the control group at 32 hours post fertilization. In terms of head trunk index, is show a normal development. The eye volume shows to have a delay more evident in the proportion 1:2, having a low volume in comparison with the group control.

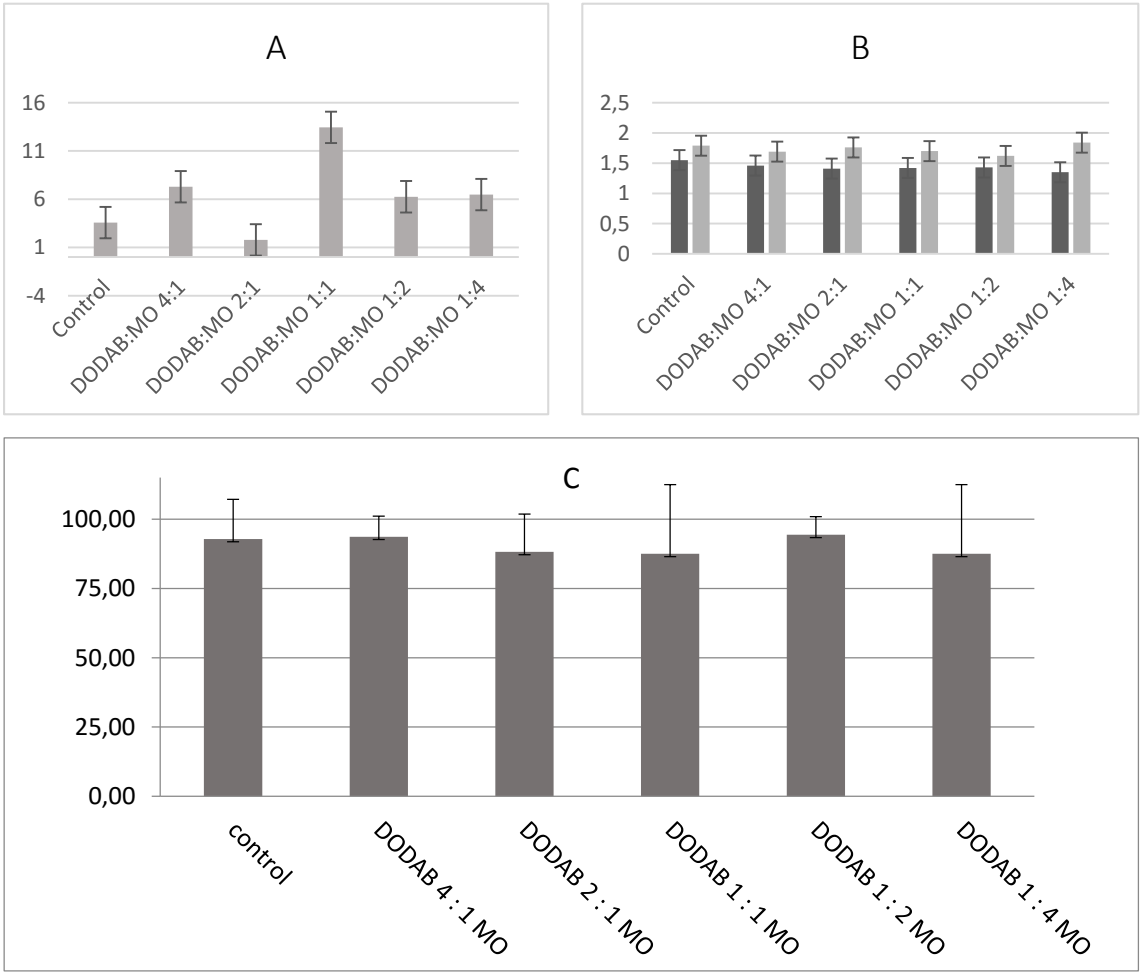

Figure 38: *Conditions of DODAB:MO liposomes tested. A: Spontaneous movements analysis at 56 hours post fertilization; B: Cardiac frequency analysis from 32 and 56 hours post fertilization respectively; C: Free swimming analysis between at 80 hours post fertilization.*

The heart frequency, shows to be stable in both time-points, 32 and 56 hours post fertilization, in the ratio 1:4 the heart beat appears to increase, however no in significant way.

At 32 hours, where the proportion 4:1 has a visible increase in spontaneous movements, stabilized in the ratio 2:1, however the presence of similar proportions of DODAB and monoolein shows an extreme peak in the movements, but decreased after but not efficiently, which leads to a possible neurological interaction of this system in these proportions in animals.

In free swimming for 80 hours the animals appear to have all the normal development index, which shown the visible delays during the remaining hours are compensated up to 80 hours post fertilization, where the percentage of hatching is also standard.

The nanosystem DODAB:MO is shown with a decrease in the survival rate in the ratio 1:4, reaching a little mortality greater than 25% in the very first 32 hours post fertilization.

This proportion of this system, DODAB 1 and 4 monoolein, shown interacting with the animals in one form less favorably, experiencing, the embryos, some cardio-toxic effects on both the evaluated hours, decreased dramatically in the 32 hours post fertilization and increasing significantly at 56 hours post fertilization.

The presence of higher percentage of monoolein is shown always as system stabilizer, however the same ratios of surfactant and lipid helper, 1:1, show a delay in developing embryo, not severe, but not corresponding to the control group in 8 hours post fertilization, compromising, this presence of monoolein in these proportions, the total size of the animal when it reaches the 56 hours post fertilization.

The presence of a higher concentration of monoolein is expected to stabilize the whole nanosystem, however, in a greater proportion, reveals adverse effects to cardiac level, at intermediate concentrations, 1:1, shows interference with normal embryonic development and growth of animal, however none of the tested proportions interfere with the completion of the neurological system, achieving a normal animals free-swimming, as the hatching rate, all animals showed larval stage at the end of the experiment.

Conclusions Remarks

5. Conclusions Remarks

The study of compounds scheduled for this study shows an agreement with the literature.

The cationic surfactants, as well as the lipid helper, when administrator in isolation mode to zebrafish embryos react as expected, following the conclusions drawn *in vitro* assays.

The monoolein, as expected, presents a reaction almost inert to the embryos, as their description indicates, this lipid helper functions as, among other parameters, DODAx system stabilizer, thus their index of toxicity to embryos at various concentrations, showed almost null, with a survival rate of around 90%, in accordance with the control group, although pulse embryonic development in 8 hours post fertilization in lower concentration, causing most of the embryos are in bud stage this progress is not significant, because it comes with somewhat normal embryonic development of animals.

Some concentrations at 56 hours after fertilization show exert a slight delay in the development of animals, having a small decrease in the overall size of the body, which is proven by the size of the yolk extension and a small reduction of the eye volume, however at the last time-point the animals hatched as expected, thus showing the slight delay in the development over the time-points does not compromise the normal grow of the animals, which confirms the *in vitro* test, showing that administration of monoolein does not have much adverse effects.

The cationic surfactant DODAC showed, as expected, has toxicity to embryos, the room for maneuver in terms of concentration is low, as rising concentrations, the survival rate decreases sharply. Although at 8 hours post fertilization all animals follow the standard embryo development, from 32 hours anomalies occur, there is a volume of yolk elevate, suggesting an abnormality in the development, an increasing exponentially of the head trunk index with the increasing of concentrations, a decrease in heart rate, decrease in spontaneous movements as well as the overall size of the animal, despite experiencing an increase in free swimming, the rate of resistant animals during the 80 hours post fertilization is reduced, the hatching of the few animals that reach the last time-point, however in small in number. An increase in concentrations of DODAC leads

to a decrease in survival rate, so, when administrate this surfactant in isolation causes a toxic effect to zebrafish embryos.

DODAB proved to be less toxic than DODAC, as expected.

Faced with the same concentrations than DODAC, the embryo survival rate is higher, starting to prove some mortality only to 56 hours post fertilization, and only the concentration of 3 µg/mL shows more toxic, leading to a mortality of 50% in the others concentrations the survival is generally greater than 75%.

The embryonic development before this surfactant appears to remain normal until 56 hours, where the concentration of DODAB seems to provide a delay, keeping the embryos with an excess of volume of yolk, elevating its head Index high trunk in higher concentrations, and one cardio-toxic index, in the same time-point, drastically reducing the cardiac frequency, which can influence also at the neurotoxic level, demonstrating a low activity at 56 hours in terms of internal embryonic movements, this neurotoxicity is revealed at 80 hours post fertilization wherein the embryos experiencing the surfactant, regardless of concentration, have difficulty in responding to stimuli, having a low free swim rate, however all animals are hatched at 80 hours post fertilization.

In the mixture of lipids, DODAC and monoolein, it is expected that monoolein stabilize the system, which actually happens in terms of survival, keeping always around 75%, as expected by the *in vitro* studies. Even in the analysis of embryonic development, the system monoolein containing shows become more stable, allowing no major changes in general. There are some differences on heart level, the differences mainly in the ratio 2:1 and the presence of high concentration of monoolein show some neurological alterations, leading to greater inner agitation of animals at 32 hours. In terms of deficits in the development, breakage that seem to be caused by systems with more DODAC, tend to be offset by monoolein however shown to have some effect on cardiac level.

DODAB in the presence of monoolein was expected greater stability, however presents a decrease in survival at 80 hours post fertilization lead to obtaining a mortality of 25%.

The presence of DODAB tends to lead to cardio-toxic problems, as when administered alone, trying to this problem compensated by the monoolein but not efficiently when in lipid mixture, expressing also a neurotoxic impact revealed in

a decrease of the free swimming ability, showing, here, also an attempt to stabilize when the monoolein is added in larger concentration.

In general, the surfactants shown to possess cardiac and neuronal toxicity, however the administration of monoolein tends to stabilize the system, leading to significant improvements.

The nanosystems DODAx:MO analysis appears to be possible with this animal model, the results are revealing and with possibility of comparisons, noting improvements with the junction of surfactant and lipid helper. The fact of being formed particles and not just mixing the lipid also shows a system stabilization, that is, particles administered to the embryos show a prevalence of a general survival, however not in large scale, but in a stable manner over all the time-points, the changes to the development level are not very significant in both systems, but the cardio-toxic effects tend to stay as in particles and in simple lipid mixture.

In DODAB:MO system, the monoolein appears to have interference in the development in general, leading to variations in the cardiac level and normal development, leading to some delay peaks, however when prevails the surfactant, the system is shown stable and without much adverse effects to animals.

In conclusion form, this animal model was shown to be a good toxicological testing vehicle for non-metallic particles, showing where is the greater interaction between the particles and in what proportions, achieving stabilize the system to minimize the effects described.

Future Perspectives

6. Future Perspectives

With the results visible through this animal model, showing be agreement with the results from *in vitro* assays we can validate the zebrafish embryos for toxicological analysis of non-metallic particles.

These results open a window for further analysis, with these data, surfactant and helper lipid proportions, may be calibrated these proportions to obtain the concentrations where don’t exist obvious toxicological effects for continuity with others animal models.

After all the tests, the embryos were stored in RNAlater, which impels a future deeper analysis, knowing the visible effects through this elaborate ZET assays, a deeper analysis of the embryo at the molecular level or genetics can deepen their knowledge of interactions that they have been described, obtaining the knowledge how and where the liposomes will interact in the embryo deeper.

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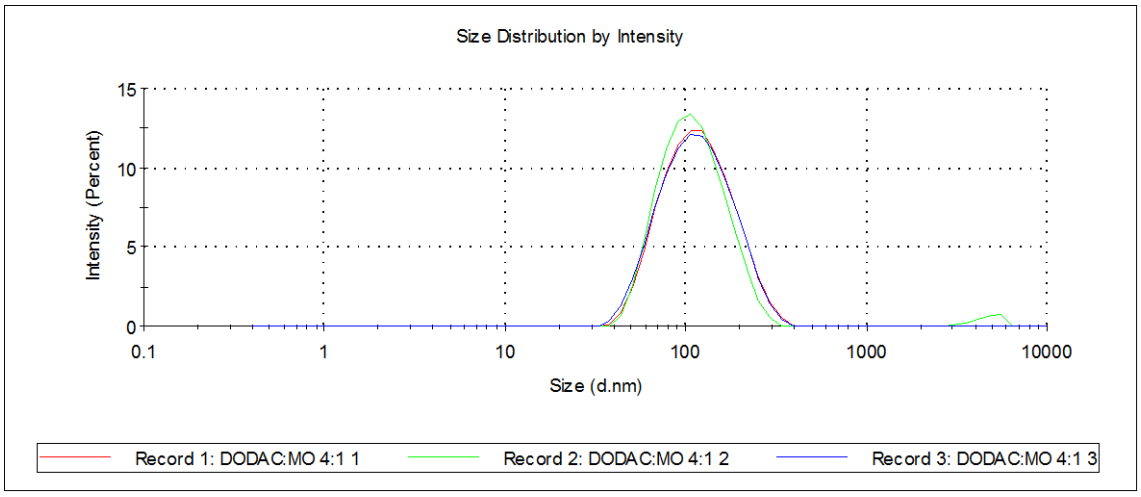
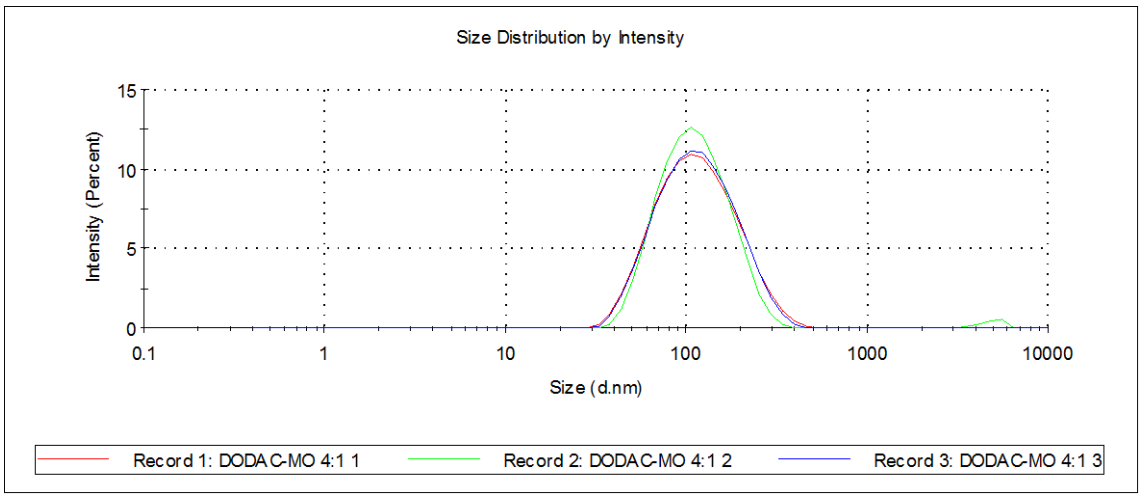
Annexes

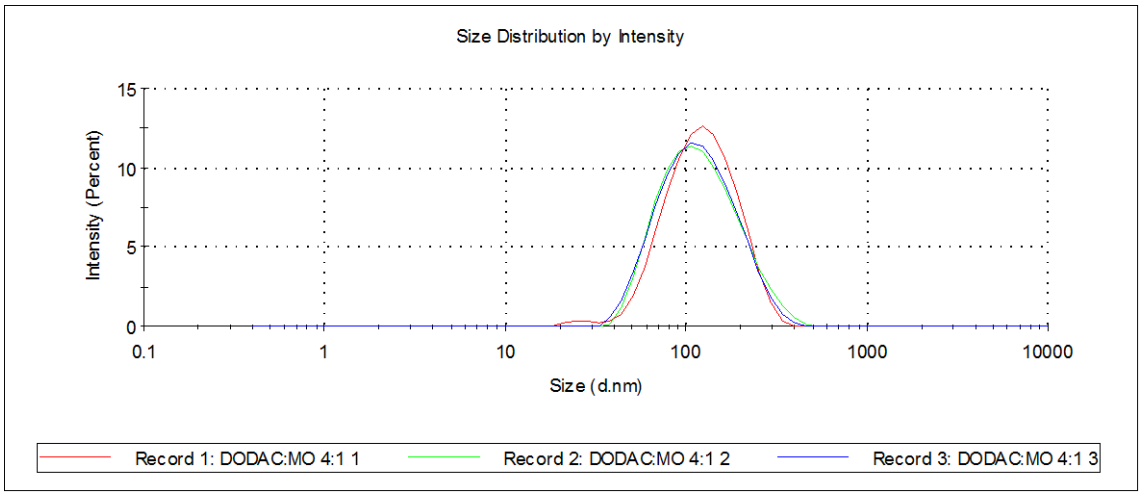
8. Annexes

Annex 1 – DODAC:MO liposomes stability analysis

	0 h						24 h						48 h						72 h					
DODAC:MO	Z-av	SD	PDI	SD	Zeta	SD	Z-av	SD	PDI	SD	Zeta	SD	Z-av	SD	PDI	SD	Zeta	SD	Z-av	SD	PDI	SD	Zeta	SD
4:1	102	0,896	0,19	0,001	66,6	1,69	106,3	1,922	0,175	0,011	62,1	1,32	107,3	3,045	0,189	0,008	61,1	0,153	105,7	1,992	0,185	0,009	60,5	1,34
2:1	422	45,98	0,72	0,02	78,2	0,866	256	11,33	0,736	0,043	75,2	2,48	260,7	3,98	0,786	0,08	73,8	1,85	273,6	10,89	0,789	0,014	75,1	1,33
1:1	185	7,587	0,46	0,02	75,5	1,73	166,6	5,058	0,569	0,023	75,2	2,54	161,2	4,051	0,625	0,025	73	3,35	163,2	3,782	0,611	0,009	74,5	2,37
1:2	225	2,55	0,49	0,01	66,2	1,93	211,6	5,948	0,439	0,058	66,4	2,18	219,7	3,172	0,472	0,012	67,1	1,63	224,4	1,274	0,48	0,007	63,7	0,794
1:4	277	3,402	0,2	0,03	62,1	0,416	293,7	5,003	0,182	0,02	61,3	0,0577	290,5	4,215	0,165	0,004	59,9	0,361	287,2	3,742	0,132	0,009	62	3,68



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Size (d.nm)
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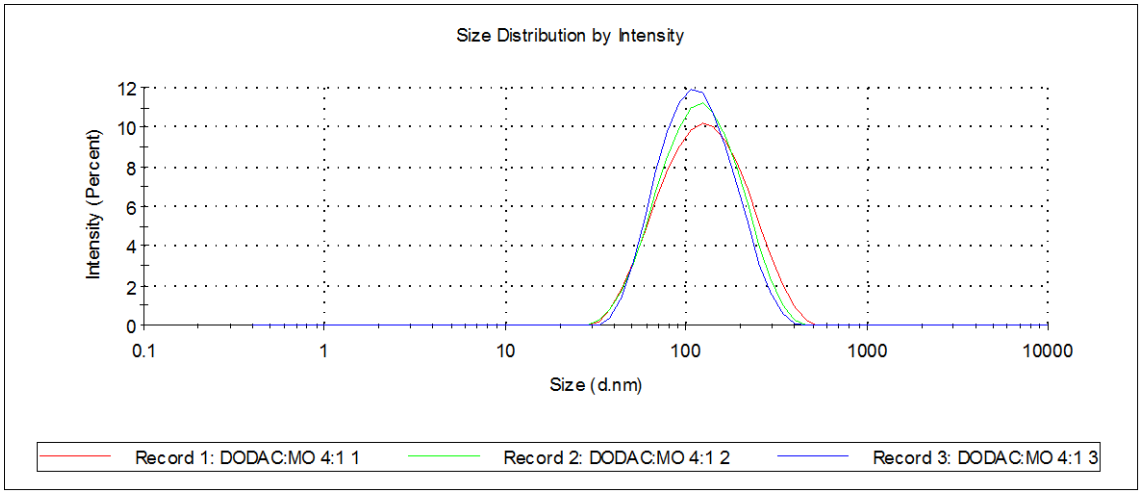
Record 2: DODAC:MO 4:1 2
Record 3: DODAC:MO 4:1 3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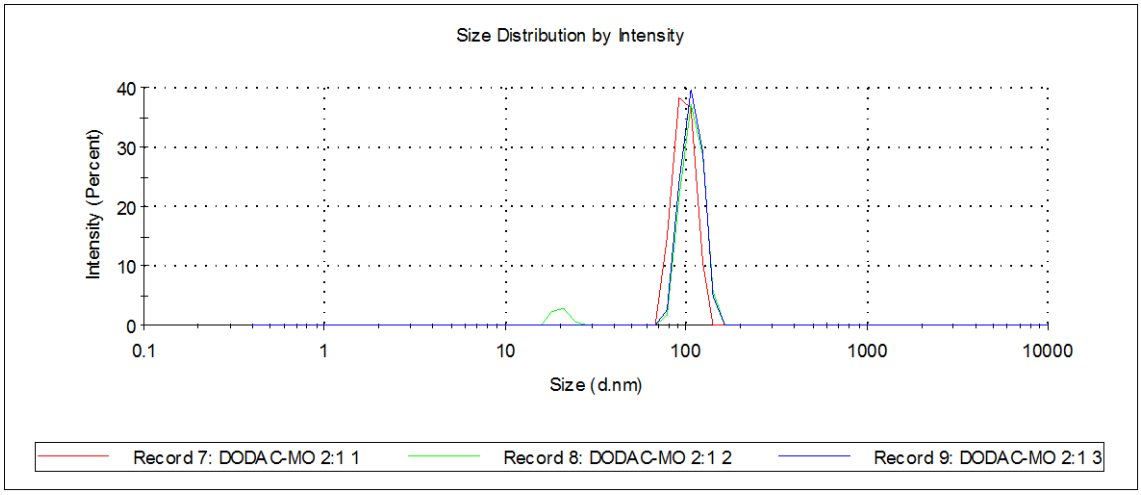
Record 2: DODAC:MO 4:1 2
Record 3: DODAC:MO 4:1 3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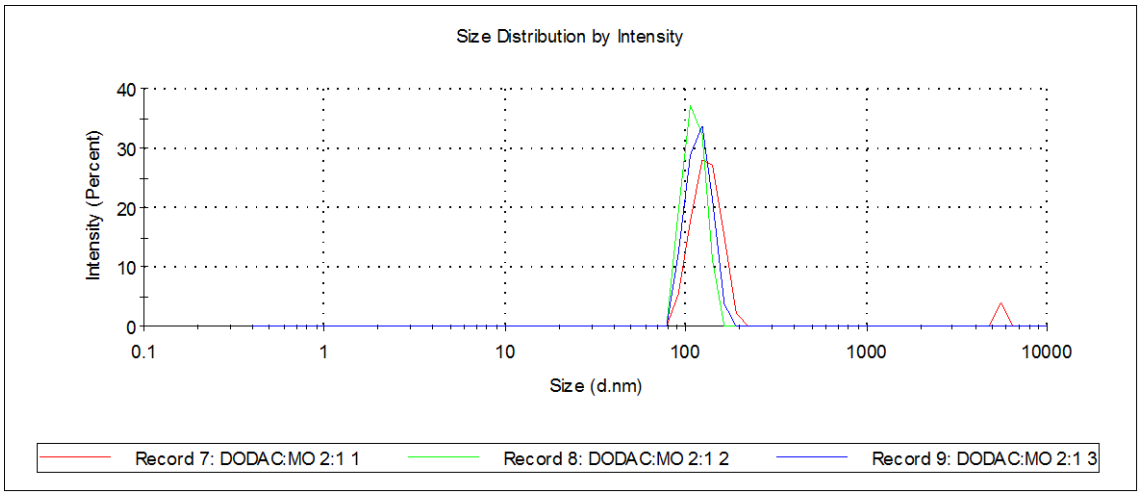
Record 8: DODAC-MO 2:1 2
Record 9: DODAC-MO 2:1 3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Intensity (Percent)
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Record 8: DODAC:MO 2:1 2
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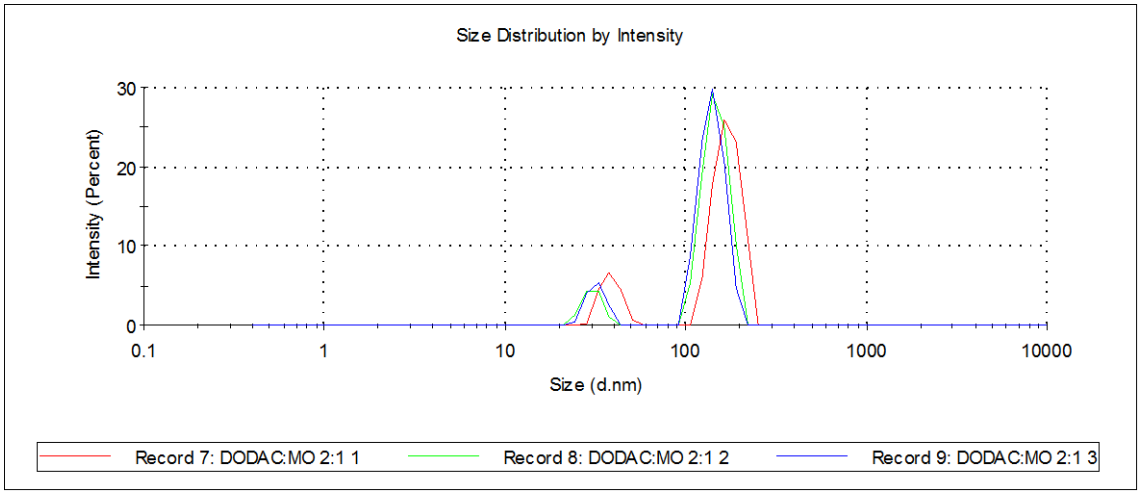
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Intensity (Percent)
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Size (d.nm)
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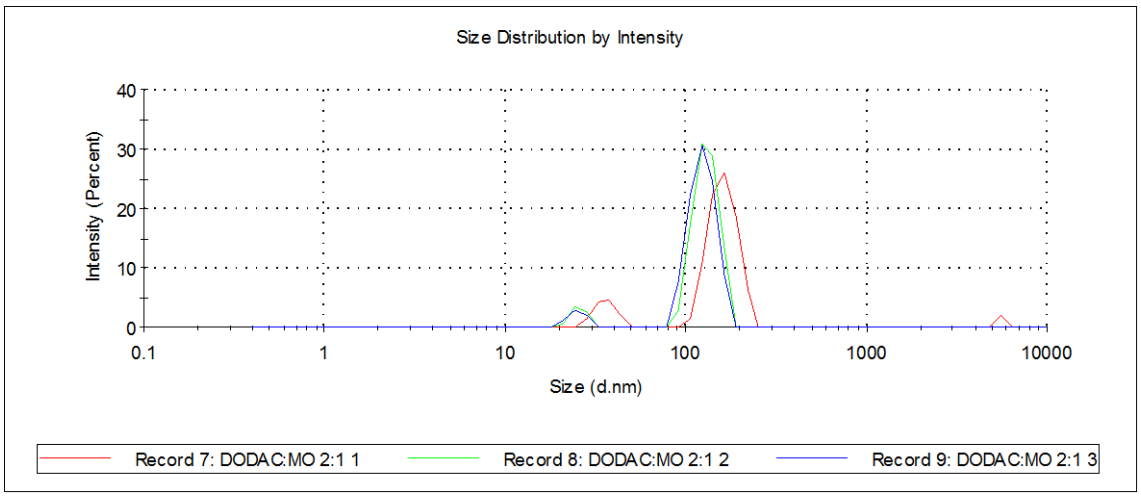
Record 8: DODAC:MO 2:1 2
Record 9: DODAC:MO 2:1 3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Intensity (Percent)
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Size (d.nm)
Record 7: DODAC:MO 2:1 1
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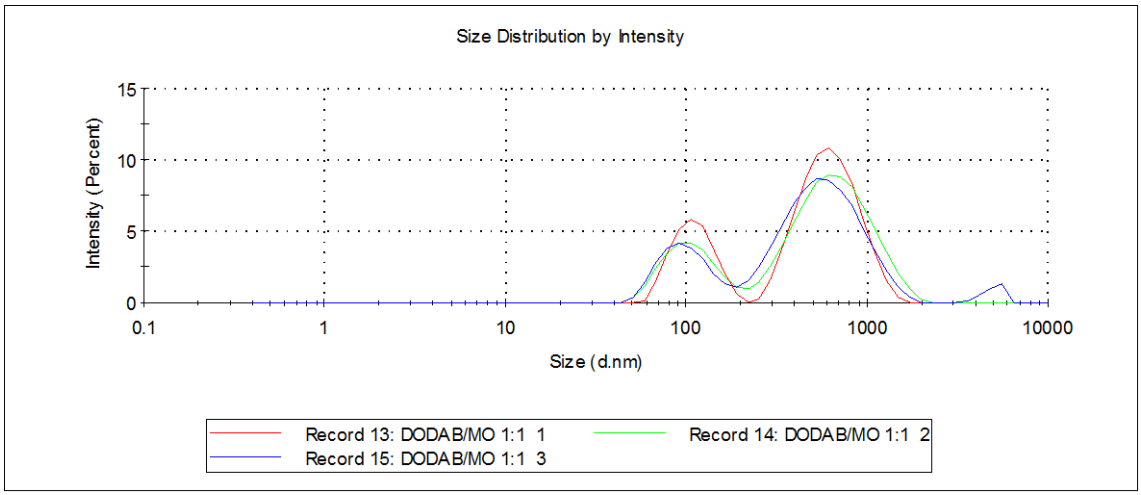
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Record 14: DODAB/MO 1:1 2
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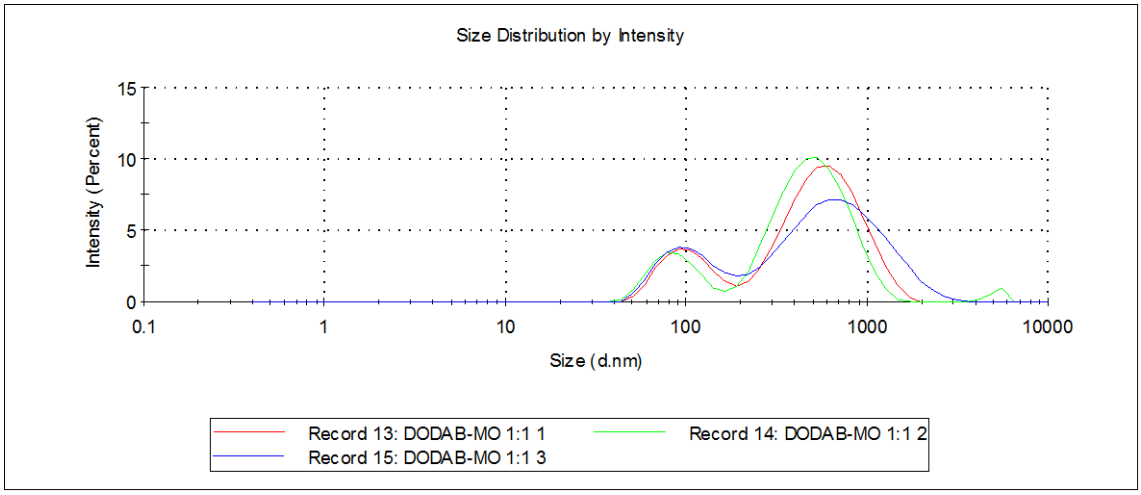
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Size (d.nm)
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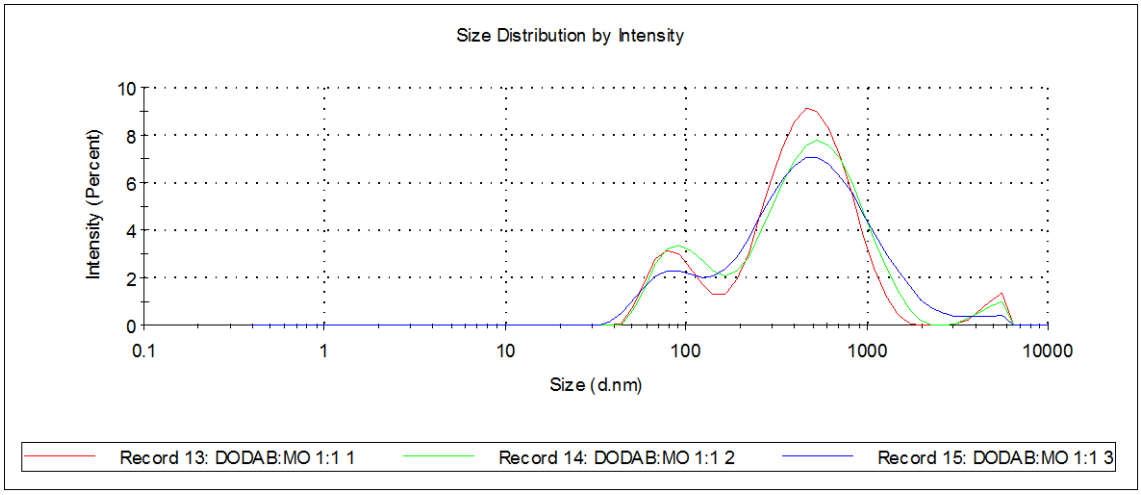
Record 15: DODAB-MO 1:1 3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Intensity (Percent)
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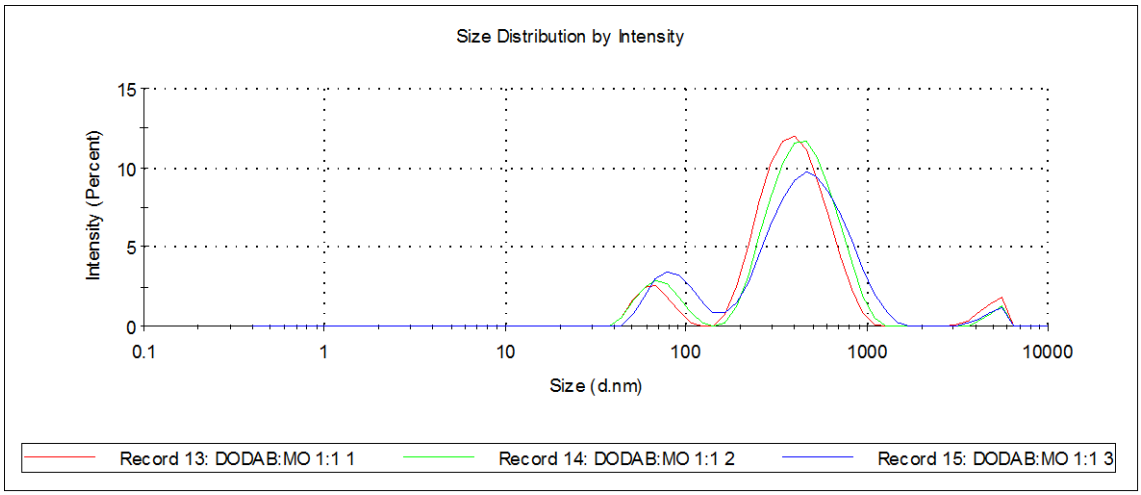
Size (d.nm)
Record 13: DODAB:MO 1:1 1
Record 14: DODAB:MO 1:1 2
Record 15: DODAB:MO 1:1 3


Size Distribution by Intensity
Intensity (Percent)
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Size (d.nm)
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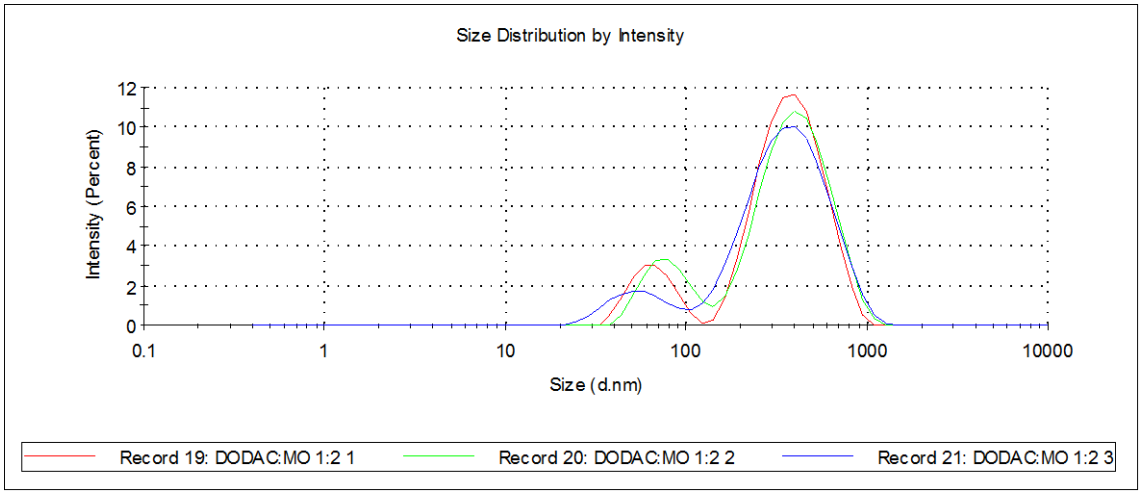
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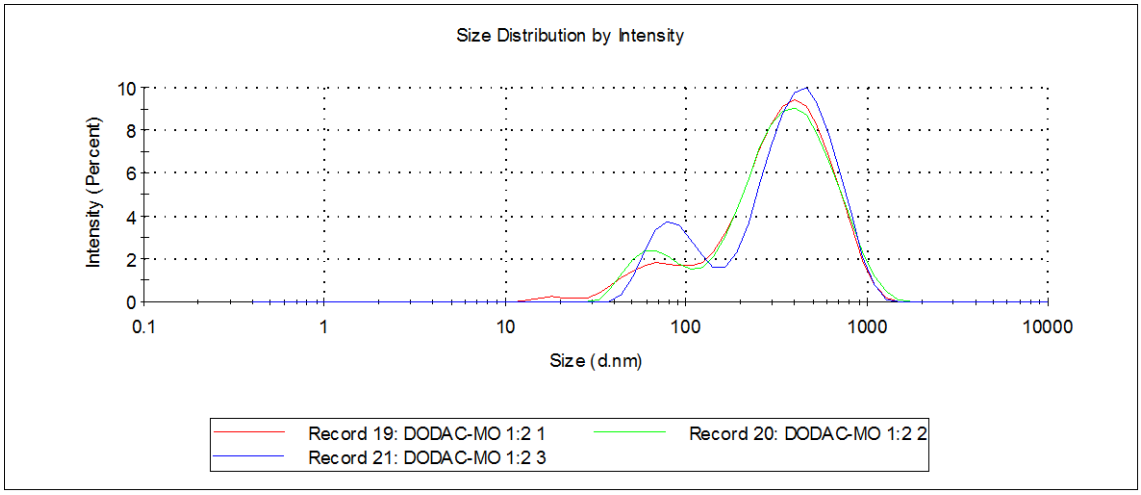
Record 20: DODAC:MO 1:2 2
Record 21: DODAC:MO 1:2 3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Intensity (Percent)
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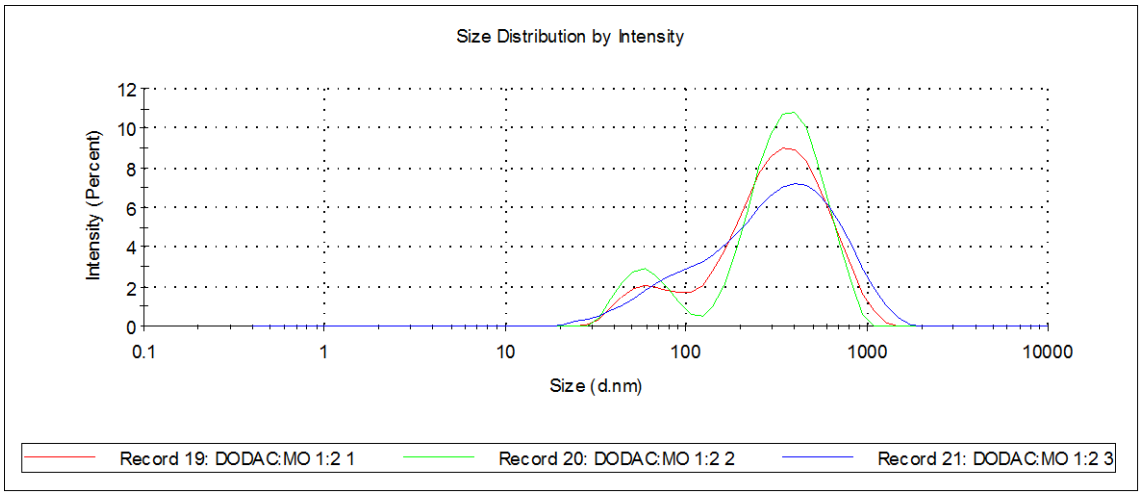
Record 20: DODAC-MO 1:2 2
Record 21: DODAC-MO 1:2 3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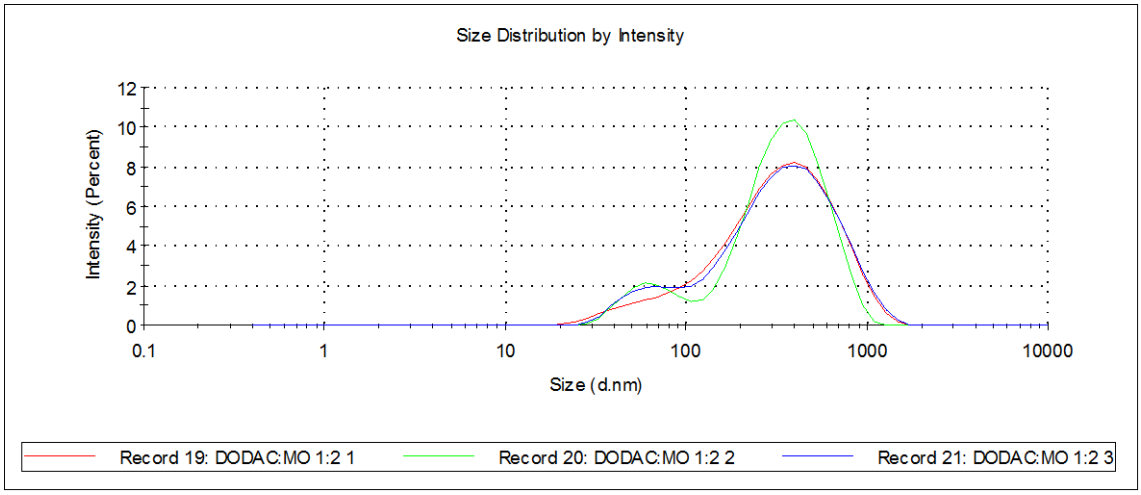
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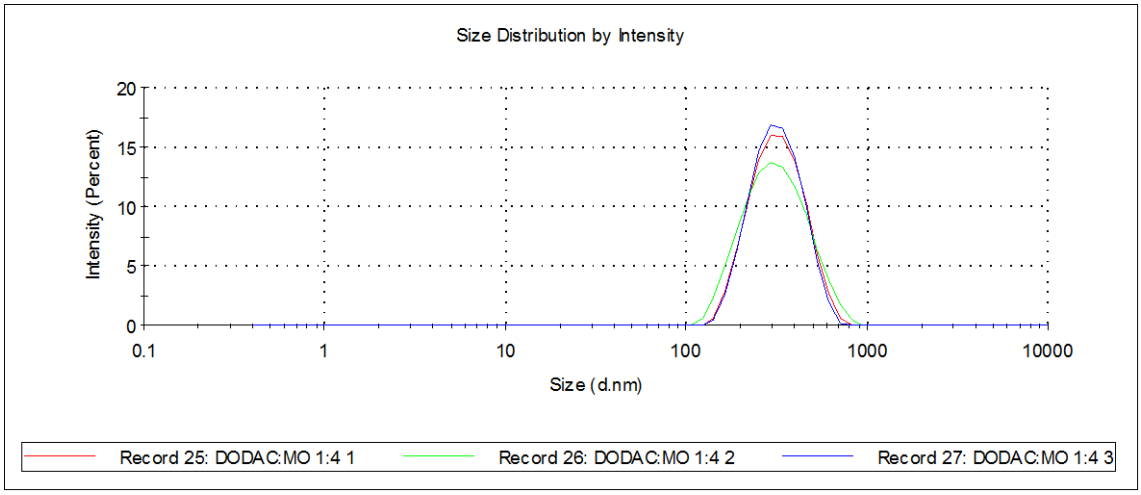
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Intensity (Percent)
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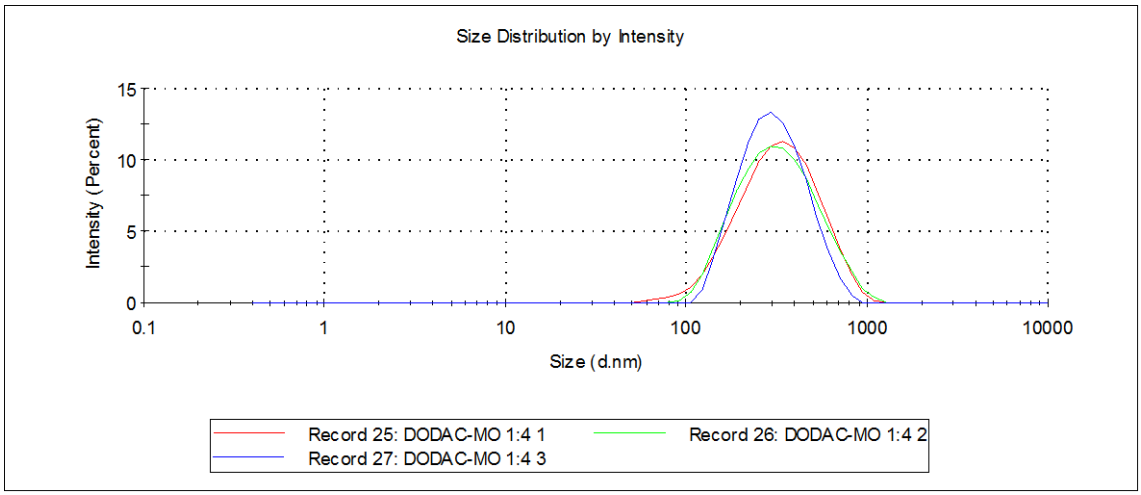
Record 27: DODAC:MO 1:4 3


Size Distribution by Intensity
Intensity (Percent)
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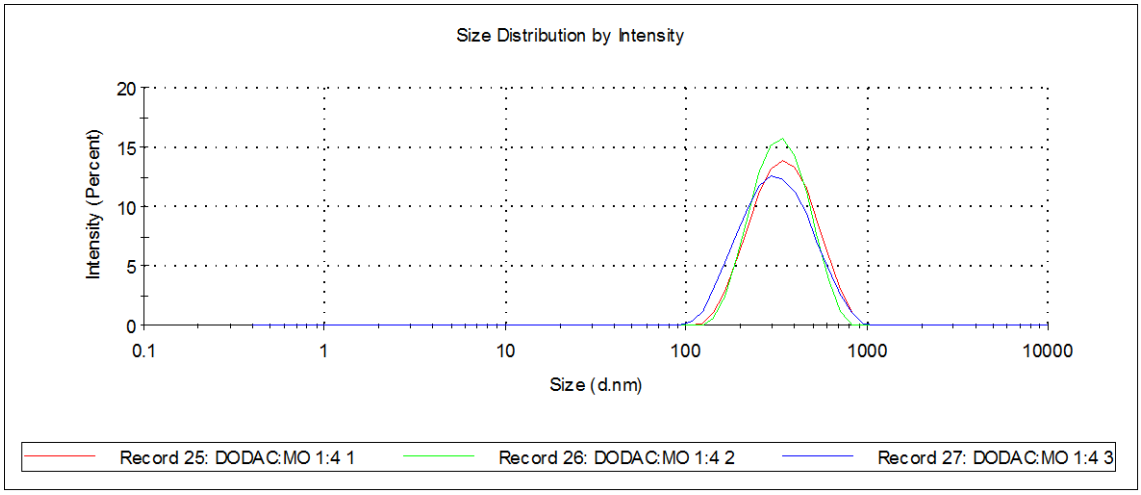
Size Distribution by Intensity
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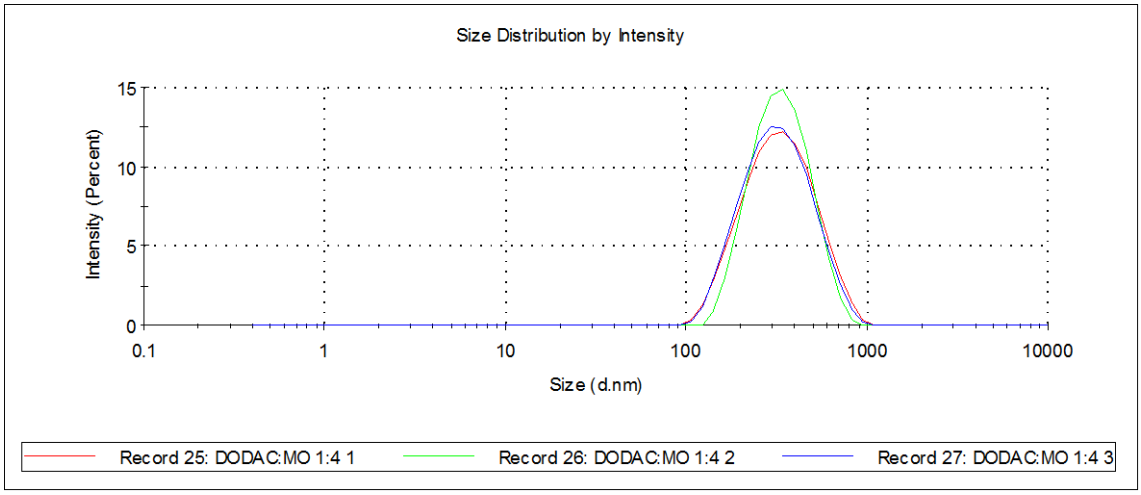
Size Distribution by Intensity
Intensity (Percent)
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Size (d.nm)
Record 25: DODAC:MO 1:4 1
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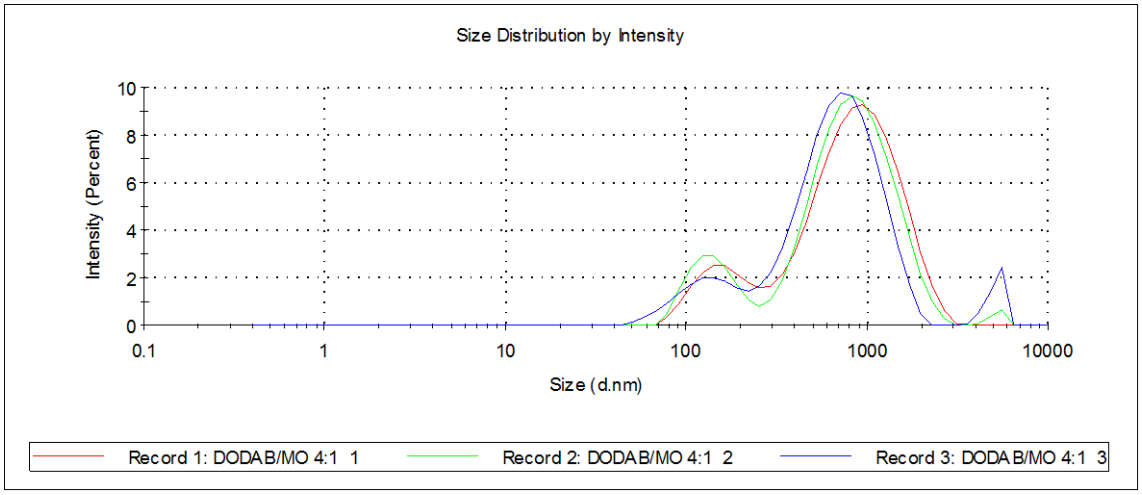
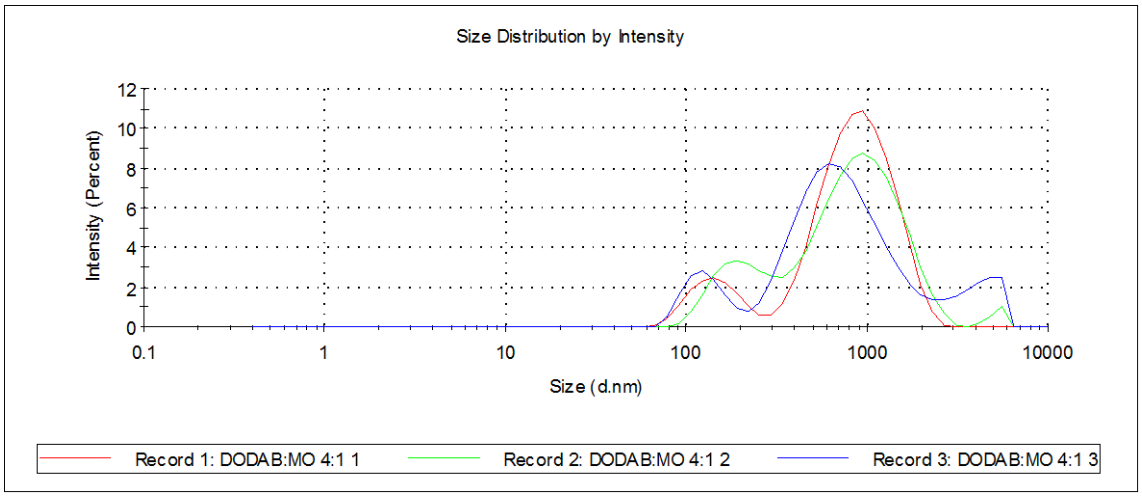
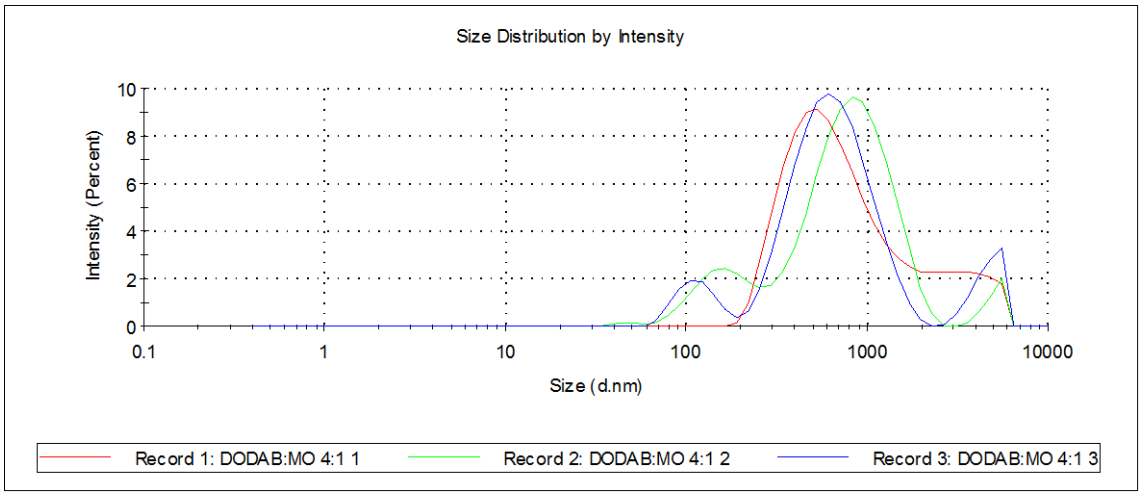
Record 27: DODAC:MO 1:4 3

Annex 2 – DODAB:MO liposomes stability analysis

	0 h						24 h						48 h						72 h					
DODAB:MO	Z-av	SD	PDI	SD	Zeta	SD	Z-av	SD	PDI	SD	Zeta	SD	Z-av	SD	PDI	SD	Zeta	SD	Z-av	SD	PDI	SD	Zeta	SD
4:1	508	13,67	0,48	0,01	70	4,7	526	21,8	0,46	0,09	57	2,9	547	8,64	0,46	0,01	54	1,4	635	53,4	0,4	0,14	54	0,38
2:1	302	0,964	0,56	0,01	73	1,8	314	11,9	0,54	0,03	69	2,12	312	2,51	0,53	0,01	69	2	315	7,17	0,53	0,03	61	2,05
1:1	293	3,669	0,54	0,02	69	1,8	290	4,45	0,51	0,02	71	0,66	292	4,58	0,49	0,01	68	1,3	298	5,51	0,45	0,03	67	2,41
1:2	293	6,466	0,37	0,04	69	1,2	262	6,23	0,42	0,01	67	0,7	339	11,3	0,57	0,11	69	1,5	268	4,42	0,4	0,02	68	1,96
1:4	374	5,112	0,23	0,03	64	1,2	378	5,86	0,19	0,01	69	1,29	424	9,07	0,26	0,01	64	1,2	360	5,09	0,15	0	72	2,26






Size Distribution by Intensity
Intensity (Percent)
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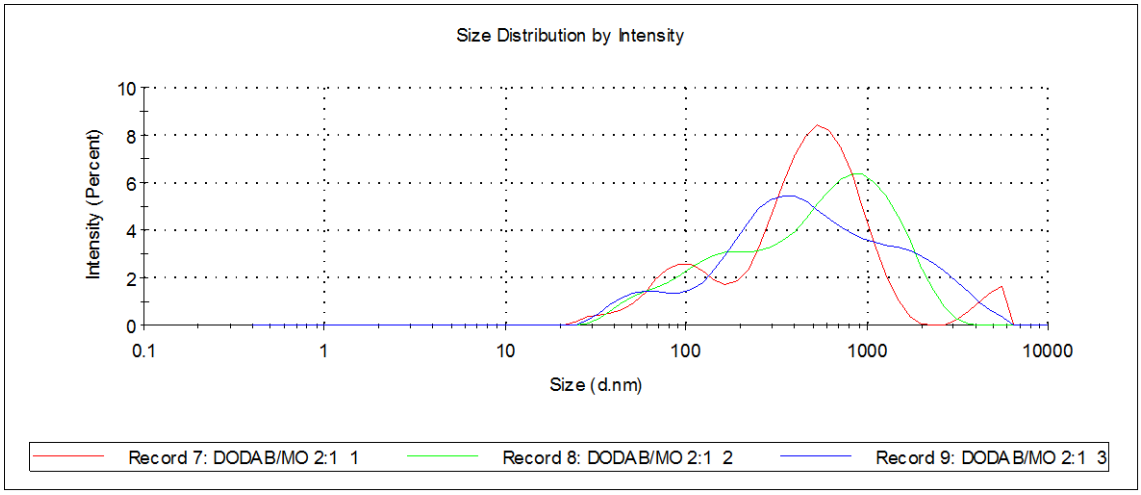
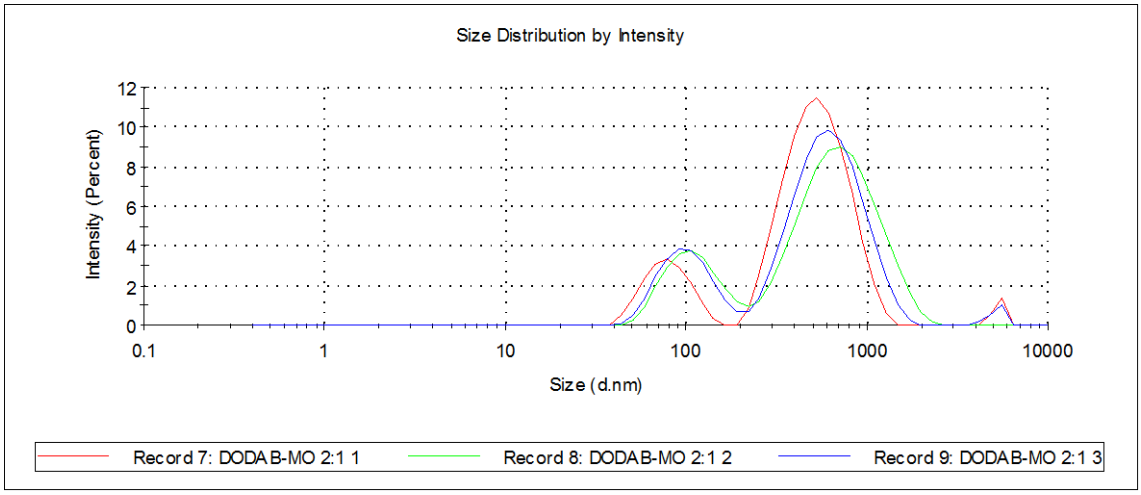
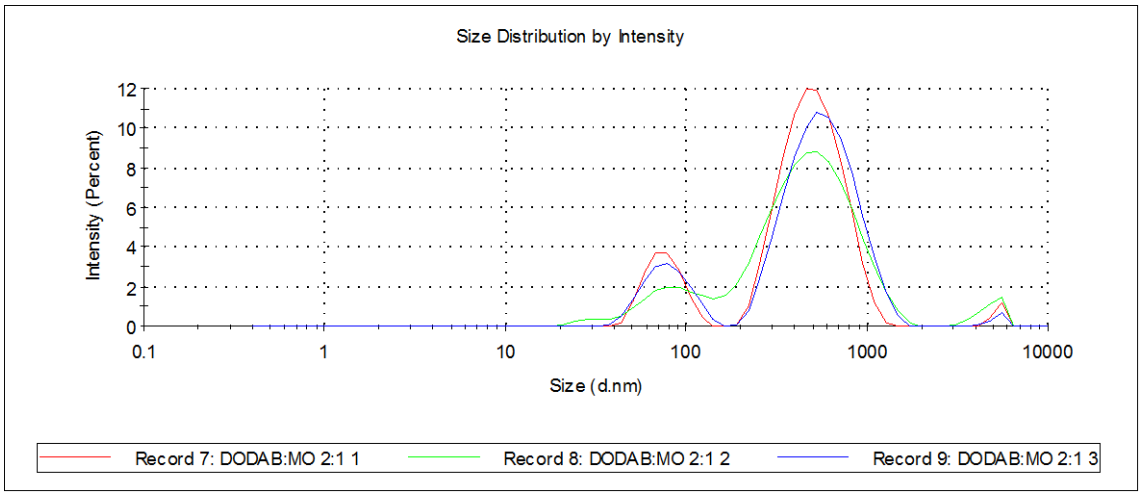
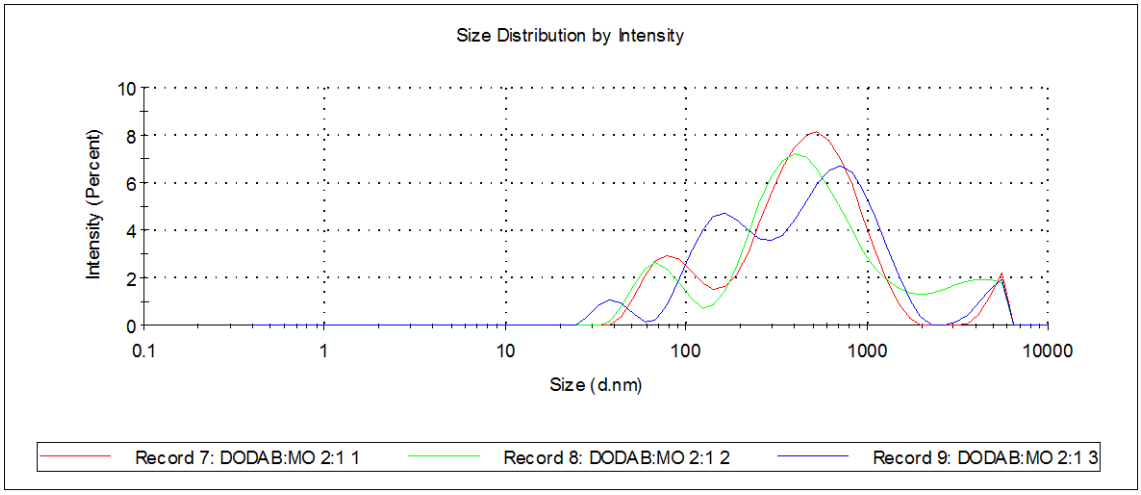
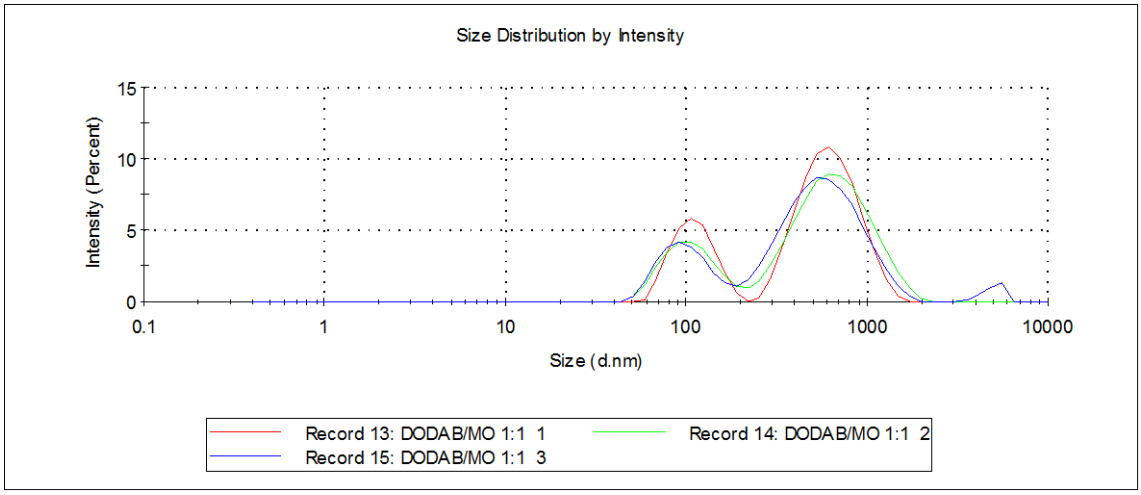
Size (d.nm)
Record 1: DODAB:MO 4:1 1
Record 2: DODAB:MO 4:1 2
Record 3: DODAB:MO 4:1 3

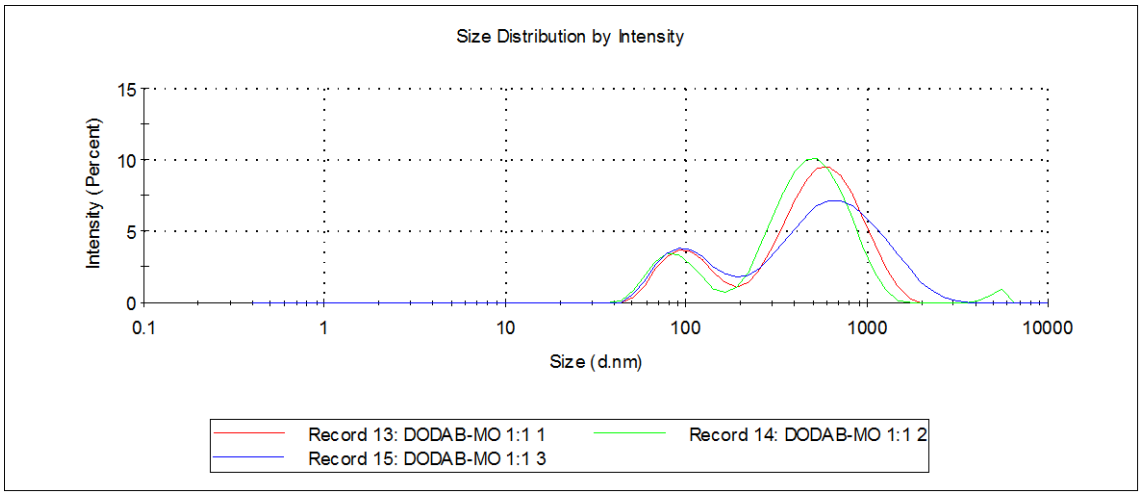

Size Distribution by Intensity
Intensity (Percent)
0
2
4
6
8
10
0.1
1
10
100
1000
10000
Size (d.nm)
Record 7: DODAB/MO 2:1 1
Record 8: DODAB/MO 2:1 2
Record 9: DODAB/MO 2:1 3

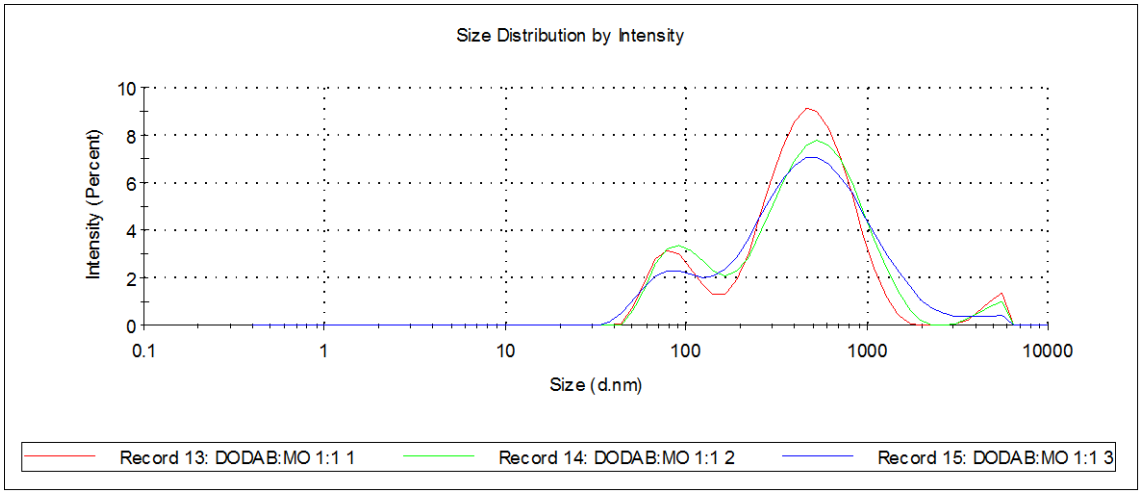

Size Distribution by Intensity
Intensity (Percent)
0
2
4
6
8
10
12
0.1
1
10
100
1000
10000
Size (d.nm)
Record 7: DODAB-MO 2:1 1
Record 8: DODAB-MO 2:1 2
Record 9: DODAB-MO 2:1 3

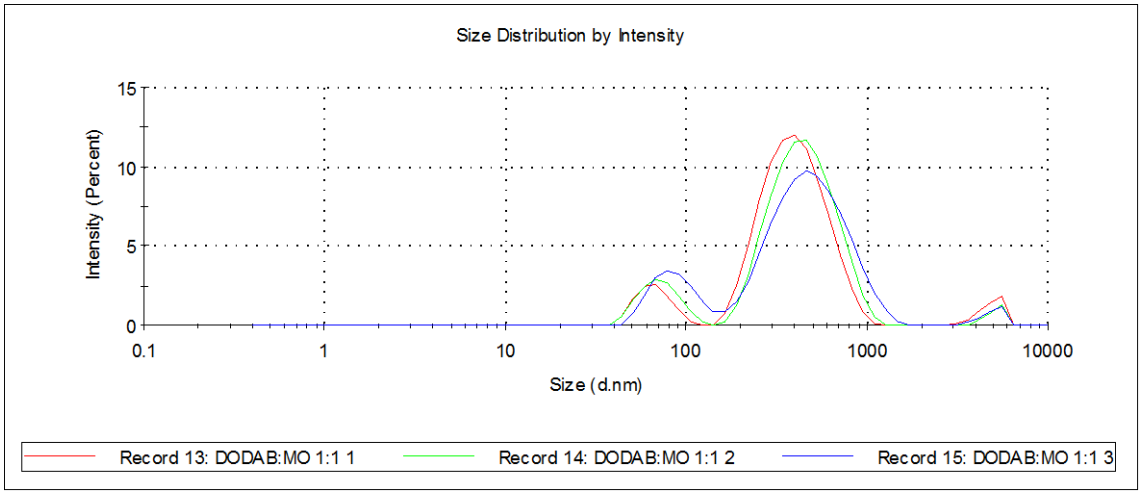

Size Distribution by Intensity
Intensity (Percent)
12
10
8
6
4
2
0
0.1
1
10
100
1000
10000
Size (d.nm)
Record 7: DODAB:MO 2:1 1
Record 8: DODAB:MO 2:1 2
Record 9: DODAB:MO 2:1 3

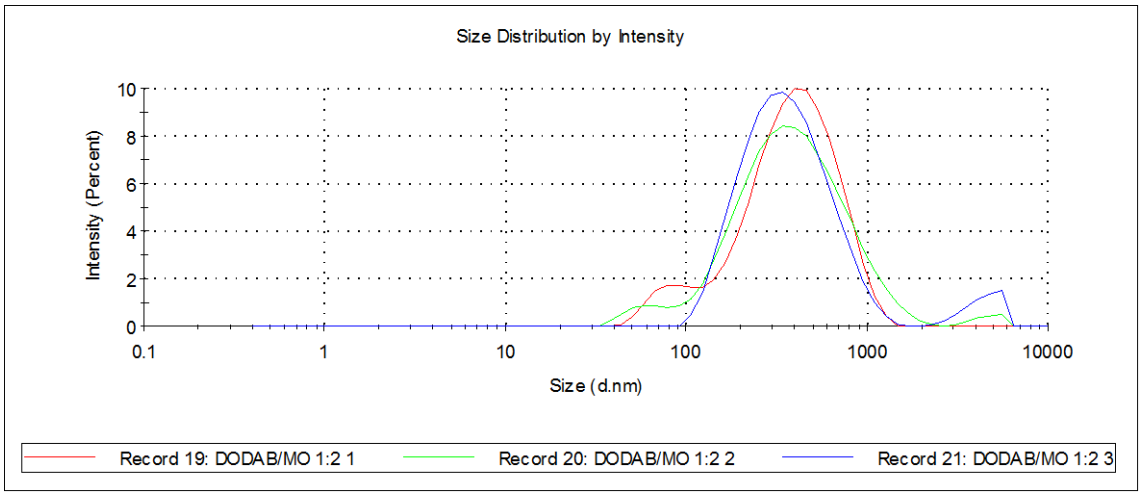

Size Distribution by Intensity
Intensity (Percent)
10
8
6
4
2
0
0.1
1
10
100
1000
10000
Size (d.nm)
Record 7: DODAB:MO 2:1 1
Record 8: DODAB:MO 2:1 2
Record 9: DODAB:MO 2:1 3

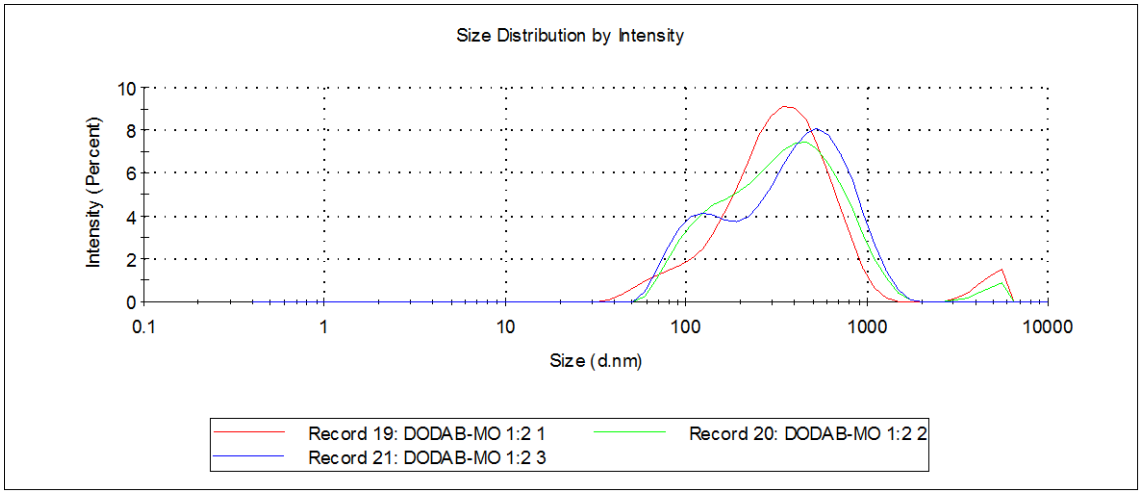

Size Distribution by Intensity
Intensity (Percent)
15
10
5
0
0.1
1
10
100
1000
10000
Size (d.nm)
Record 13: DODAB/MO 1:1 1
Record 14: DODAB/MO 1:1 2
Record 15: DODAB/MO 1:1 3

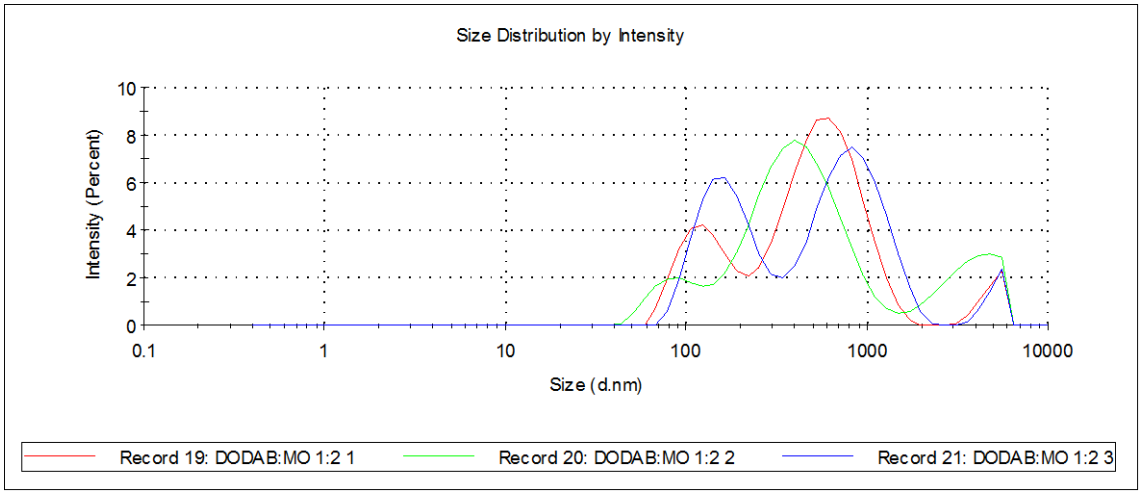

Size Distribution by Intensity
Intensity (Percent)
15
10
5
0
0.1
1
10
100
1000
10000
Size (d.nm)
Record 13: DODAB-MO 1:1 1
Record 14: DODAB-MO 1:1 2
Record 15: DODAB-MO 1:1 3

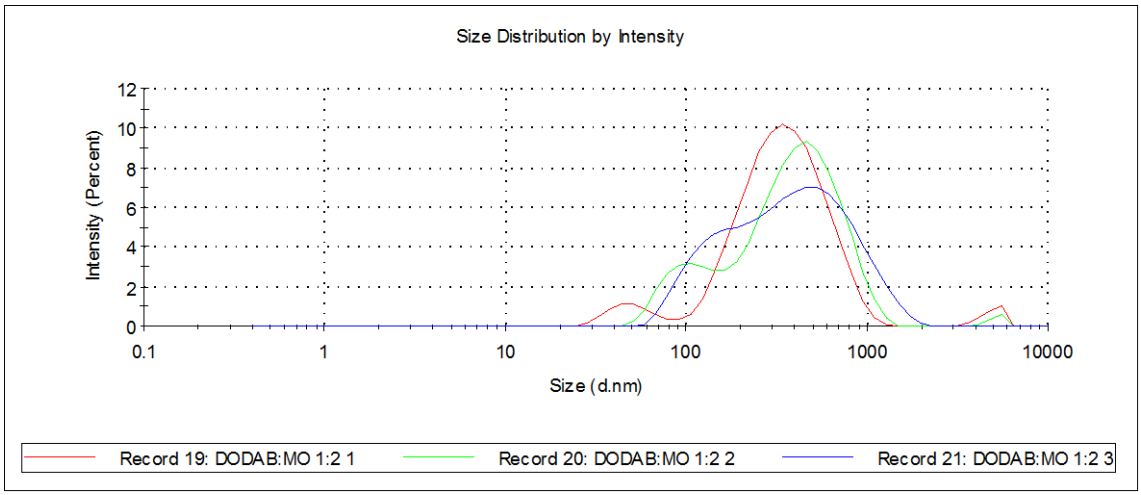

Size Distribution by Intensity
Intensity (Percent)
10
8
6
4
2
0
0.1
1
10
100
1000
10000
Size (d.nm)
Record 13: DODAB:MO 1:1 1
Record 14: DODAB:MO 1:1 2
Record 15: DODAB:MO 1:1 3

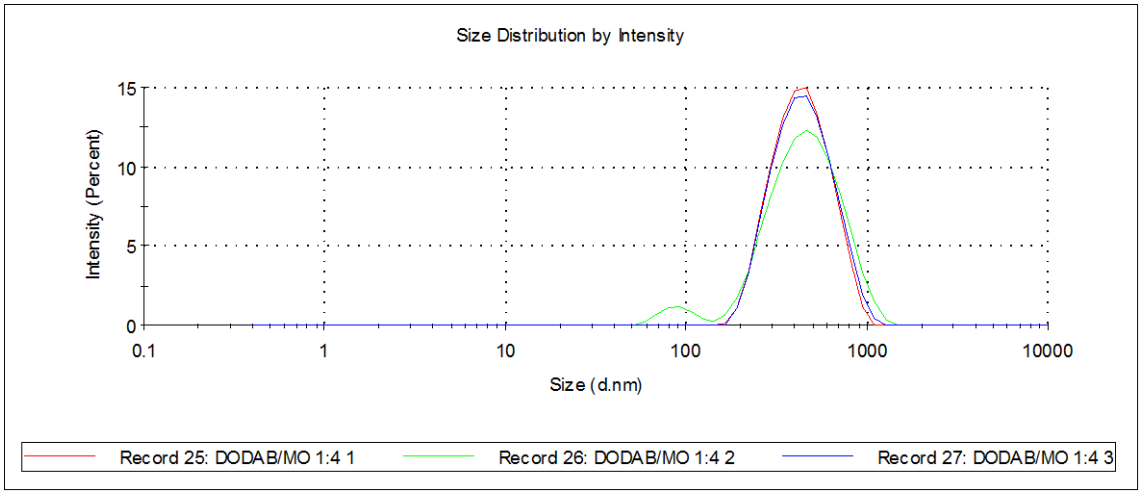

Size Distribution by Intensity
Intensity (Percent)
15
10
5
0
0.1
1
10
100
1000
10000
Size (d.nm)
Record 13: DODAB:MO 1:1 1
Record 14: DODAB:MO 1:1 2
Record 15: DODAB:MO 1:1 3

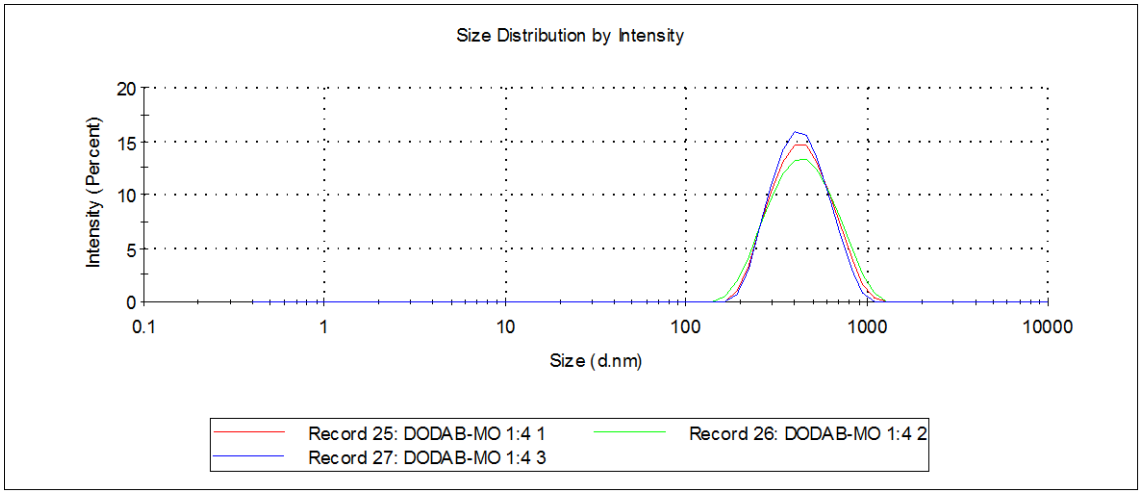

Size Distribution by Intensity
Intensity (Percent)
Size (d.nm)
Record 19: DODAB/MO 1:2 1
Record 20: DODAB/MO 1:2 2
Record 21: DODAB/MO 1:2 3

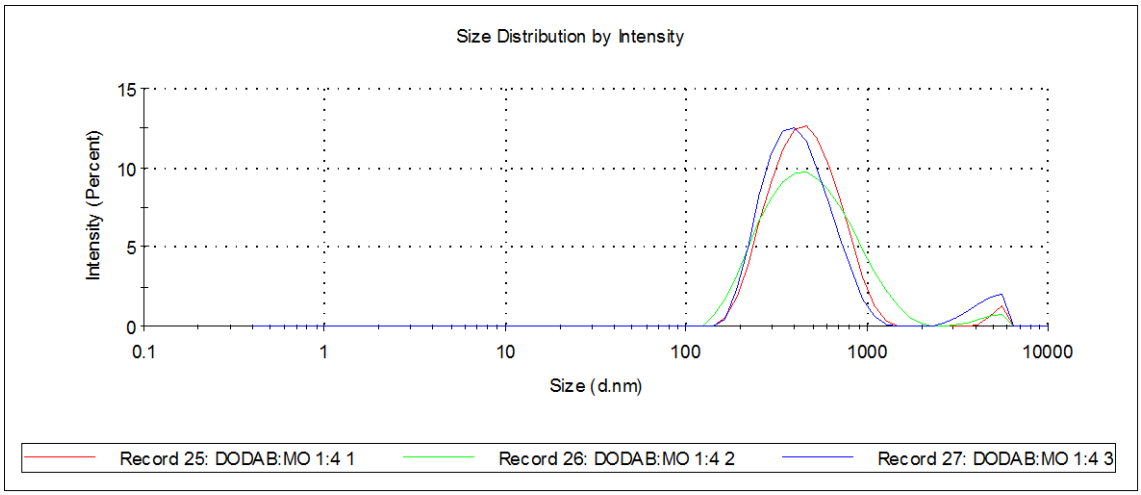

Size Distribution by Intensity
Intensity (Percent)
Size (d.nm)
Record 19: DODAB-MO 1:2 1
Record 20: DODAB-MO 1:2 2
Record 21: DODAB-MO 1:2 3

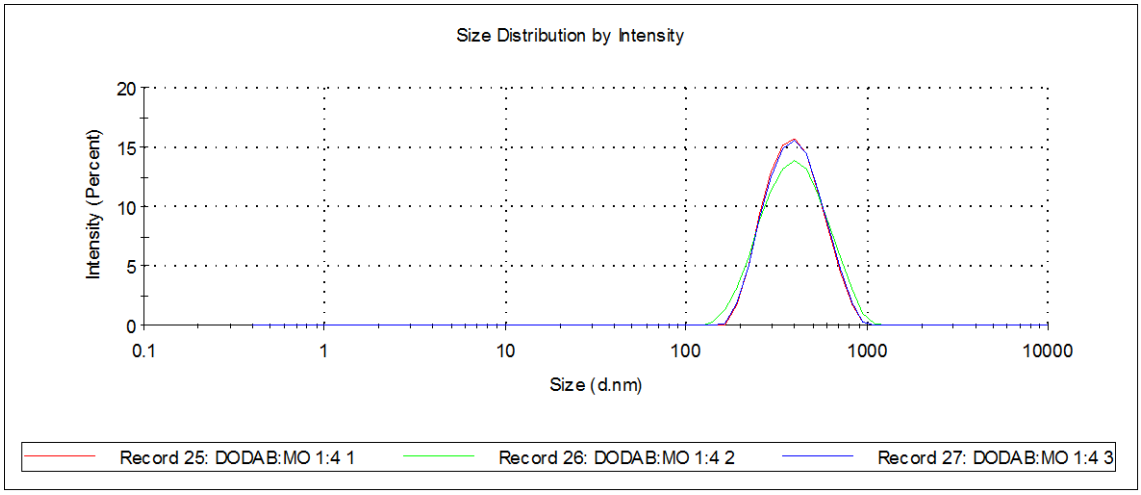

Size Distribution by Intensity
Intensity (Percent)
Size (d.nm)
Record 19: DODAB:MO 1:2 1
Record 20: DODAB:MO 1:2 2
Record 21: DODAB:MO 1:2 3


Size Distribution by Intensity
Intensity (Percent)
12
10
8
6
4
2
0
0.1
1
10
100
1000
10000
Size (d.nm)
Record 19: DODAB:MO 1:2 1
Record 20: DODAB:MO 1:2 2
Record 21: DODAB:MO 1:2 3


Size Distribution by Intensity
Intensity (Percent)
15
10
5
0
0.1
1
10
100
1000
10000
Size (d.nm)
Record 25: DODAB/MO 1:4 1
Record 26: DODAB/MO 1:4 2
Record 27: DODAB/MO 1:4 3


Size Distribution by Intensity
Intensity (Percent)
20
15
10
5
0
0.1
1
10
100
1000
10000
Size (d.nm)
Record 25: DODAB-MO 1:4 1
Record 26: DODAB-MO 1:4 2
Record 27: DODAB-MO 1:4 3


Size Distribution by Intensity
Intensity (Percent)
15
10
5
0
0.1
1
10
100
1000
10000
Size (d.nm)
Record 25: DODAB:MO 1:4 1
Record 26: DODAB:MO 1:4 2
Record 27: DODAB:MO 1:4 3


Size Distribution by Intensity
Intensity (Percent)
20
15
10
5
0
0.1
1
10
100
1000
10000
Size (d.nm)
Record 25: DODAB:MO 1:4 1
Record 26: DODAB:MO 1:4 2
Record 27: DODAB:MO 1:4 3