CHAPTER 7

Enhanced *In vivo* uptake of drugs in a zebrafish based toxicity screening assay

Keywords: hydrogel • zebrafish • in vivo toxicity assay • high throughput • sodium valproate

Abstract:

Zebrafish (ZF) are an attractive animal model for the high throughput screening of drugs and for toxicity assays. In the current screening assays, drugs enter the ZF embryo via adsorption through the skin or via the oral route, depending on the age of the embryo. A relatively new application of ZF is their use as a high throughput screening assay for toxicity studies. However, when a compound is unable to enter the ZF, its inherent toxicity cannot be determined. To address the issue of ZF uptake, we investigated whether hydrogels can be used as a drug delivery vehicle enhancing the uptake into ZF, either via skin uptake, or via the oral route. In this pilot study, a dextran-based hydrogel was used to deliver the model drug sodium valproate (SV) and study its toxicity by analyzing the phenotype and survival rate of the developing embryos.

Introduction

Toxicology aims to study the adverse effects of chemicals on organisms. These studies can be used to predict the adverse effects of the same chemical entities on human beings and are an important aspect of the commercialization of chemical products. Progress in the pharmaceutical industry, or any chemical industry for that matter, is driven by designing new chemicals with better efficacy for a particular application. However, before new drugs can be tested in a clinical trial, the toxicity has to be established. More general, the chemical industry is required by law to test whether their chemicals are toxic to the environment and humans. For this, biological models have been developed in order to study the toxicity of chemicals and are used to determine the safe concentration range of a particular chemical entity. To date, the determination of the toxicity profile is performed using animal models like mouse, rat, dogs or rabbits.[1] However the process is expensive, labor intensive and time consuming. To overcome these drawbacks, researchers have developed alternative cell based assays which can be high throughput, such as in vitro cell culture,[2] 3D cell culture[3] and stem cells.[4] These cell-based assays are fast and cost effective, but cannot provide all the desired toxicity information, for example, no information on developmental toxicity can be obtained in this manner.

Recently, Zebrafish (ZF) have emerged as a new animal model, which can be used for studying diseases,[5] genetics,[6] developmental biology,[7] as well as in vivo drug discovery[8] and immunology.[9] The transparency of ZF embryos and ease of gene manipulation are an important advantage of this in vivo model system. Female ZF are capable of laying 100-200 eggs in a day, which are fertilized externally and the development from the one cell stage to an adult embryo occurs within one week. This enables the study of the effect of chemicals on the development of an embryo

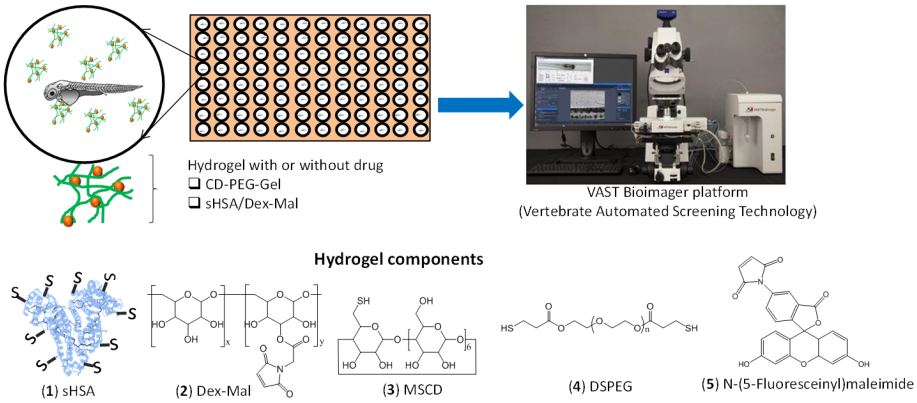
in a short amount of time.[10] Altogether, this makes ZF embryos a suitable model for toxicity research in a medium to high throughput fashion. [11]

For example, ZF have been used as an animal model to screen a wide variety of different materials, such as organic compounds,[12] nanoparticles,[13] various biomaterials[14] and heavy metals.[15] A major problem of using the ZF model for toxicity studies is that many compounds are unable to enter the embryo. To date, the compound of interest is added at different concentration to the fish containing plate and the development and survival rate is studied. However, many compounds of interest are poorly soluble in water. As a result, the effective concentration is significantly lowered and as such the inherent toxicity is underestimated. In addition, when compounds are too polar, uptake via passive skin absorption is limited and as a result, the inherent toxicity is underestimated as well. Therefore new tools are in demand that enhance the uptake of compounds in ZF, and ideally one would like to develop a single tool for all chemical compounds. In this study, hydrogels will be tested for this purpose.

Hydrogels consist dense network of cross-linked hydrophilic polymers containing high amount of water. [16] In biomedical research, hydrogels have proven to be a good candidate in numerous applications such as 3D cell culture, tissue regeneration and drug delivery.[17] From oral medicine point of view, hydrogels are a potential candidate due to their biocompatibility and ability to deliver both hydrophilic and hydrophobic therapeutic chemical entities. In this chapter, a pilot study is described in which a dextran-based hydrogel is used to enhance the uptake of the model drug sodium valproate (SV) in zebrafish. As a readout of the toxicity, changes in the embryo development and the survival rate are studied.

Results and Discussion

To develop a toxicity assay using ZF, it is very important that all compounds (i.e. ranging from hydrophobic to hydrophilic) are taken up efficiently by ZF in order to assess the inherent toxicity. In this study, we investigated whether hydrogels could be used as a delivery tool to ensure the efficient uptake of sodium valproate by zebrafish **(Scheme 1)**. Once the drug-loaded hydrogel are internalized, it is expected to release its cargo and the resulting toxicity can be visualized using quantitative phenotype analysis.[18] Analysis was performed using the Vertebrate Automated Screening Technology (VAST), which is a powerful tool in high throughput imaging.[19] VAST is an automated system where animals are imaged from various angles over time enabling the characterization of the mebryonic development in a high throughput fashion.



Scheme 1: *Experimental set up where every well contains one ZF. The hydrogel containing the model compound is added to each well and after varying incubation times the embryo development is studied using high throughput VAST (Vertebrate Automated Screening Technology) imaging. The sHSA/Dex-Mal hydrogel is composed by mixing thiolated albumin (sHSA) and maleimide functionalized dextran (Dex-Mal) while the CD-PEG-Gel is prepared by mixing mono-6-thio-β-cyclodextrin (MSCD), Dex-Mal and di-thiolated poly(ethyleneglycol) (DSPEG)*

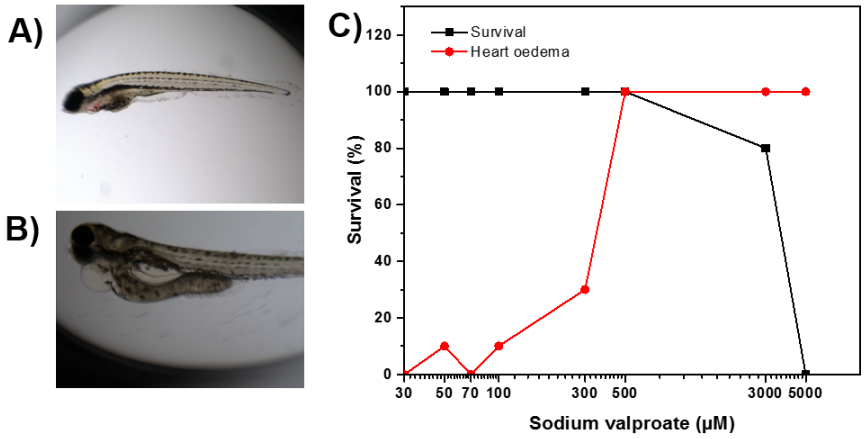


Figure 1: *4 days post fertilization (dpf) ZF embryos (n=10) were exposed to a range of SV concentrations in egg water. After a 24hr exposure time, ZF were imaged using a stereo microscope where;* ***A)*** *Showing a healthy ZF exposed to 100 μM SV while* ***B)*** *shows a ZF with heart oedema due to a SV exposure of 300 μM.* ***C)*** *Survival rate and incidence of oedema as a function of SV concentration. Above 300 μM morphological changes are observed while at higher concentrations lethality is observed.*

Hydrogel-based nanoparticles acting as a drug carrier have previously been developed, composed of cyclodextrin, dextran and poly(ethylene glycol) and their behavior was studied in ZF embryos.[20] In sthis study, maleimide modified dextran (Dex-mal) was functionalized with mono-thio-β-cyclodextrin (MSCD) and subsequently crosslinked using di-thiolated poly(ethylene glycol) (DSPEG) to obtain the resulting cyclodextrin-PEG-gel (CD-PEG-Gel). In these CD-PEG-Gel,

hydrophobic drugs are encapsulated using the cyclodextrin host molecules. More recently, a thiolated albumin/dextran maleimide based hydrogel (sHSA/Dex-Mal) was developed for the delivery of hydrophobic drugs using the albumin both as the crosslinker and as a binding pocket for hydrophobic drugs.[21] Albumin was chosen because it is a most abundant plasma protein in humans with 6 potential binding pockets for drugs and a natural carrier for metal ions, fatty acids or radical scavengers.[22] Upon injection in ZF embryos as nanoparticles, both hydrogel systems did not show any toxicity. Therefore, these two hydrogels were selected for this pilot study to enhance the uptake of a model drug in a zebrafish screening assay.

Sodium valproate (SV) is a well-known therapeutic drug used in the treatment of epilepsy, anorexia nervosa, panic attack, migraine and bipolar diseases was used as a candidate drug. [23] Importantly, SV has shown to be toxic at higher concentrations and therefore it is an ideal model drug to study its *in vivo* delivery in developing ZF embryos.[24] To test the toxicity of the SV, we incubated ZF in egg water with various concentrations of the drug for 24 hours. Next all zebrafish (n=10 in each group) were anesthetized and imaged using a stereomicroscope (**Figure 1**). No toxicity was observed in the group incubated with 100 µM of SV. However, when more than 300 µM of SV was used, severe morphological changes and lethality was observed. Therefore, for further studies, the 100 µM of SV was chosen as it does not interfere with the development of the zebrafish embryos. The reason why no toxicity was observed when ZF were treated with 100 µM of SV might be a result of the inefficient uptake of sodium valproate by ZF due to its polar nature. Therefore, encapsulation of the drug in a hydrogel network could increase the uptake via the oral route resulting in an observed higher toxicity of sodium valproate.

Previously, it was shown that hydrogels composed of Dex-Mal (3 wt%) and DSPEG show a fluid like behavior, which therefore could be used as an injectable gel able to deliver a drug into zebrafish. Importantly, It should be noted that no toxicity was observed for this injectable hydrogel.[20] Therefore, in the case of CD-PEG-Gel, we used a 3 wt% fluid-like hydrogel to study the uptake by the ZF. To study whether the oral uptake is age dependent, two-day post fertilization (2-dpf) ZF embryos were incubated with fluorescein labelled sHSA/Dex-Mal and CD-PEG-Gel. For this, CD-PEG-Gel (3 wt% Dex-Mal) or sHSA/Dex-Mal (3 wt% sHSA) hydrogels were prepared in the presence of fluorescein maleimide (1 mol% with respect to thiol groups). In both hydrogels, the thiol to maleimide ratio of 1:1 (mol %) was maintained. The resulting fluorescent hydrogels were incubated with zebrafish embryos. For this, 20 µl of the fluorescent hydrogel was added to well containing a single ZF in 180 µl of egg water. After 2 hours of incubation, the fluorescent hydrogel was located only in the mouth region of the 2-dpf ZF embryo. However, after 24 hours of incubation (i.e. 3-dpf), the hydrogel was observed in the gut (**Figure 2**). This reveals that 3-dpf ZF can be orally fed enabling the delivery of the drug-loaded hydrogel into the gut.

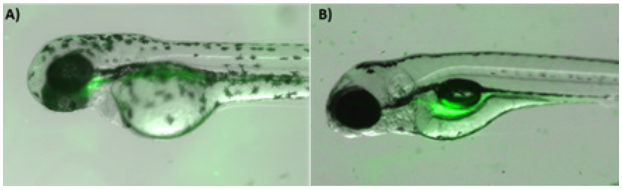


Figure 2: *Representative images of 2-dpf ZF exposed to either of the fluorescently labelled hydrogel gel at 28 °C showing gel is mostly in the mouth region after 2 hrs* ***(A)****. After 24hrs of exposure, the hydrogel was observed inside the gut of the ZF* ***(B)****.*

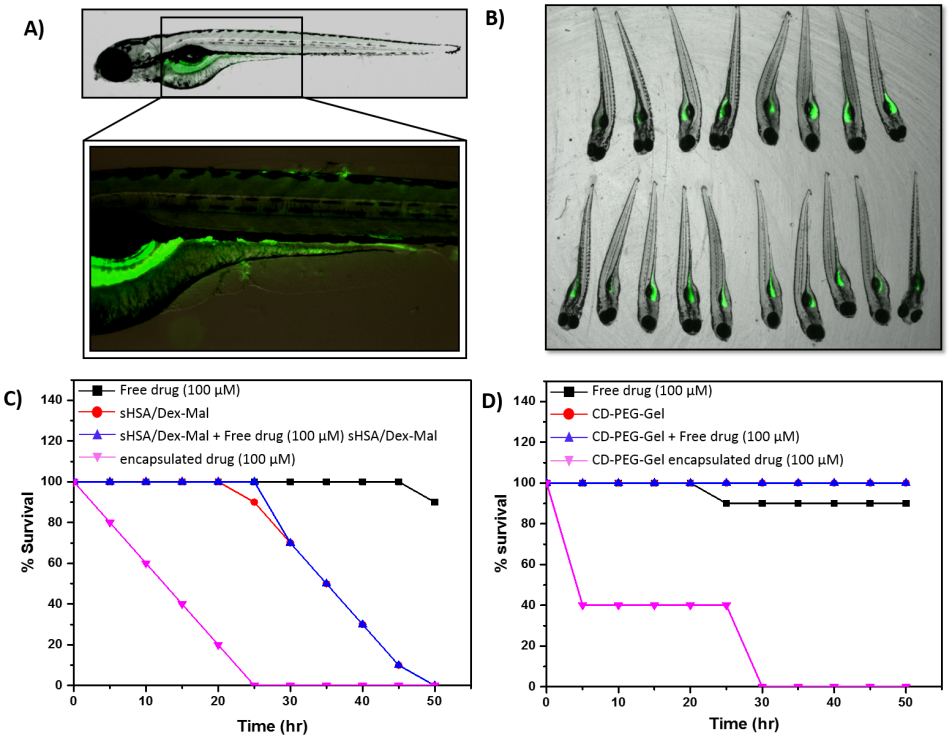


Figure 3: *A) Representative stereomicroscope images showing the uptake of fluorescent labelled (1 mol%) CD-PEG-Gel or sHSA/Dex-Mal hydrogel in the gut of the ZF. B) All the ZF used in the study showed the uptake of the hydrogel in the gut. C) Survival rate of ZF after exposure to 100 µM sodium valproate either administered as a free drug in egg water (control) or administered in sHSA/Dex-Mal hydrogel (C) and in CD-PEG-Gel (D). At a concentration of 100 µM of sodium valproate in the absence of a hydrogel showed no lethality. Upon encapsulation in a hydrogel, severe lethality was observed presuming enhanced uptake of SV. Experiments were performed at 28 °C.*

We also studied the uptake using a stronger sHSA/Dex-Mal (5 wt% sHSA) hydrogel, but here no uptake was observed. These initial studies indicate that the physicochemical properties of a gel have a strong influence on the oral uptake. However, more detailed studies are required to understand this observation.

Therefore, the sHSA/Dex-Mal (3 wt% sHSA) hydrogel and the CD-PEG-Gel (3 wt% Dex-Mal) hydrogel were used in the following studies.

To assess whether the oral uptake is dependent on the chemistry of the hydrogel, two different hydrogels were studied using 4-dpf ZF embryos, as at this stage the organs are more developed making it a better toxicity model. Each ZF embryo (4-dpf) was incubated with CD-PEG-Gel or sHSA/Dex-Mal hydrogels labeled with fluorescein (1 mol% with respect to number of thiol groups). After a 3 hour incubation period at 28 °C, fluorescence was observed in the ZF gut for both hydrogel systems. This confirms that the uptake is hydrogel independent proving their ability to be used as a drug delivery carrier (**Figure 3A** and **B**).

Next, we investigated whether these hydrogels are able to deliver the model drug sodium valproate in zebrafish. For this, 20 µl of the free drug or 20 µl of drug encapsulated hydrogel was diluted with egg water to obtain a final volume of 200 µl containing 100 µM sodium valproate concentration, each well contained a single ZF.

All the fish were monitored for 48 hours. When ZF were exposed to 100 µM SV encapsulated inside the CD-PEG-Gel, within 4 to 5 hours approximately 50% of the fish died. Similar results were also observed in the ZF group exposed to 100 µM SV encapsulated sHSA/Dex-Mal hydrogel (**Figure 3C** and **D**). To study whether encapsulation of the drug inside a hydrogel is required, ZF were incubated with hydrogels and free drug. Interestingly, we observed that free 100 µM SV added together with a CD-PEG-Gel or sHSA/Dex-Mal are not toxic to the ZF. This experiment unequivocally shows that the drug has to be encapsulated in the hydrogel prior to the incubation with ZF. In summary, ZF can be fed orally using dextran-based hydrogels where either DSPEG or thiolated albumin are used as the crosslinker. The drug of interest can be encapsulated in the hydrogel and upon uptake of hydrogel by ZF, the drug is released in the gut of the fish allowing for a better assessment of the inherent toxicity and its effect on the developing embryo.

Conclusion and Summary

In this study, we successfully developed a new toxicity assay in zebrafish based on the oral delivery of a model drug using a dextran-based hydrogel. ZF at the four-day old embryos are able to take up drug loaded hydrogels via the oral route. Using sodium valproate as a model drug, it was demonstrated that this highly soluble drug is not taken up effectively by ZF embryos. However, encapsulation of this drug into a dextran-based hydrogel, the uptake into the gut is enhanced resulting in an increased ZF lethality, showing the inherent toxicity of sodium valproate. This simple approach solves the lack of uptake into ZF of water-soluble drugs. Furthermore, the cyclodextrin conjugated to the dextran-backbone also

enables the solubilization of hydrophobic drugs via the formation of a host-guest complex, thereby raising the effective concentration in solution and increasing the ZF uptake efficiency. The same holds true for albumin-dextran hydrogels as it is known that albumin can bind a wide variety of compounds. This assay holds potential to aid preclinical studies where toxicity screening is a crucial parameter.

Appendix

Abbreviations

VAST, vertebrate automated screening technology; sHSA, thiolated albumin (sHSA); Dex-Mal, maleimide functionalized dextran; MSCD, mono-6-thio-β-cyclodextrin; DSPEG, di-thiolated poly(ethyleneglycol) (DSPEG); ZF, zebrafish; dpf, days post fertilization; SV, sodium valproate; PEG, polyethylene glycol; DMSO, dimethylsulphoxide.

Materials and Methods

β-cyclodextrin was obtained from Acros. Dextran (70 kDa), human serum albumin (HSA), dithiolated poly(ethylene glycol) (PEG, M_w = 2000), SV and N-(5-Fluoresceinyl) maleimide were obtained from sigma. Dextran for CD-PEG-Gel (M_w = 10k, Pharmacia Fine Chemicals, Sweden) and that for sHSA/Dex-Mal (M_w = 70k, Sigma) was dried in the vacuum oven for several days before use. Water used in all experiments was purified through deionization and filtration with a Millipore purification apparatus to the resistivity higher than 18.0 MΩ cm. mono-6-thio-β-cyclodextrin (MSCD)[25] and maleimide modified dextran (Dex-mal)[20, 26] were prepared by previously reported procedures. In this study, we used Dex-mal with 14 maleimide groups per 100 monomer units of dextran. Thiolated human serum albumin (degree of thiolation was 3.4) was prepared and no of thiol groups were quantified using a previously described procedure. [21]

Preparation of fluorescent CD-PEG-Gel

To make a fluorescent CD-PEG-gel (3 wt%), Dex-Mal (7.5mg) was added to a solution of MSCD (0.84 mg) dissolved in 125 µl of Danieau buffer (pH 7.2) and this mixture was vortexed for 5 minutes. To which, DSPEG (6.6 mg) dissolved in 125 µl Danieau buffer (pH 7.2) containing fluorescein maleimide 1.8 µl (5mM in DMSO, 1 mol% of the thiol of MSCD) was added. This resulting mixture was then vortexed for 15 minutes to obtain fluorescent CD-PEG-Gel.

Preparation of fluorescent sHSA/Dex-Mal

To prepare sHSA/Dex-Mal (3 wt%) hydrogel, sHSA (60 mg) was dissolved in 1 ml of Danieau buffer (pH 7.2) and mixed thoroughly for 2 hrs. To which, Dex-Mal (13 mg) in 1 ml of Danieau buffer (pH 7.2) containing 7.5 µl of fluorescein maleimide (5mM in DMSO, 1 mol% of the thiol of sHSA) was added. This resulting solution was vortexed for 15 min and further incubated for 4 hrs at 37 °C to obtain a fluorescent sHSA/Dex-Mal gel. The molar ratio between thiol and maleimide groups was kept at 1.

Preparation of SV encapsulated fluorescent CD-PEG-Gel

To make a fluorescent CD-PEG-gel (3 wt%) loaded with SV; a stock solution of SV (2 mM) was made by dissolving SV in Danieau buffer (pH 7.2). Dex-Mal (7.5mg) was added to a solution of MSCD (0.84 mg) dissolved in 125 µl of SV stock solution and this mixture was vortexed for 5 minutes. To this solution, DSPEG (6.6 mg) dissolved in 125 µl of Danieau buffer (pH 7.2) and fluorescein maleimide 1.8 µl (5mM in DMSO, 1 mol% of the thiol of MSCD) was added. Resulting mixture was then vortexed for 15 minutes to obtain 1 mM SV encapsulated fluorescent CD-PEG-Gel.

Preparation of SV encapsulated fluorescent sHSA/Dex-Mal

To prepare a SV encapsulated sHSA/Dex-Mal (3 wt%) hydrogel, sHSA (60 mg) was dissolved in 1 ml of Danieau buffer (pH 7.2) containing SV (2 mM) and mixed thoroughly for 2 hrs. Next, Dex-Mal (13 mg) was dissolved in 1 ml of Danieau buffer (pH 7.2) to which 7.5 µl of fluorescein maleimide (5mM in DMSO, 1 mol% of the thiol of sHSA) was added. This resulting solution was added to sHSA solution in SV containing Danieau buffer and vortexed for 15 min which was further incubated for 4 hrs at 37 °C to obtain a fluorescent sHSA/Dex-Mal gel containing 1 mM of SV. The molar ratio between thiol and maleimide groups was kept at 1.

Zebrafish embryo experiments

Zebrafish (*Danio rerio*) were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (http://ZFIN.org). After harvesting embryos from single crosses they were grown at 28°C in egg water (60µg/ml Instant Ocean sea salts). Embryos were anesthetized with 200 µg/mL buffered 3-aminobenzoic acid (Tricaine) and imaged with a Leica M205FA stereo fluorescent microscope.

Experimental Set up

96 well plate containing one ZF per well. Initially ZF were in egg water which was further replaced with gel solution with or without drug. Hydrogel was prepared using procedure mentioned above with 10 times concentrated drug (1 mM) and eventually diluted to obtain 100 µM effective SV concentration (20 µl of gel containing 1000 µM drug in 180 µl of egg water). To investigate if the hydrogel is taken up by ZF, gel was fluorescently labelled with fluorescein dye. In each experimental group 10 ZF were used. The whole plate was kept in an incubator at 28 °C and monitored for the number of survivors over two days.

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