Development of biocompatible nanogel for sustained drug release by overcoming the blood brain barrier in zebrafish model

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ABSTRACT

A potential delivery system has to be fabricated for crossing the blood–brain barrier (BBB) to reach the brain fluid for effective delivery of drugs for any neurological disorders. The present study is aimed for the delivery of donepezil through functionalized PNIPAM nanogel by overcoming the BBB using zebrafish model. We had synthesized the poly N-isopropyl acrylamide nanogels with 20 nm size for sustained drug release. The entrapment of donepezil in the nanogel was quantified as 87.5% by HPLC and its sustained drug release pattern was achieved at 37°C using Janus green dye release assay. Acetylcholinesterase inhibition assay for the donepezil conjugated nanogel (DCN) has confirmed thermoresponsive drug release by obtaining the donepezil peak at 9.3 min retention time in HPLC. Swim behavior and heart beat rates were found to be biocompatible for the functionalized nanogel DCN in zebrafish. Histological analysis revealed increased pial surface in anterior telenchepalon region of zebrafish brain for the DCN administered fishes. DCN treated embryos exhibited minor developmental deformities above 5 μg/ml and thus confirmed its minimal toxicity and its therapeutic efficiency. This study may shed light on the development of neurospecific nanogel for targeted and sustained drug release to brain by crossing the blood–brain barrier.

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Introduction

Drug delivery to brain is a major challenging task for any neurodegenerative disease condition (Sharma et al., 2012) of the patients because of the blood brain barrier (BBB). BBB is formed by the brain micro blood vessel endothelial cells and prevents uncontrolled passing of amino acids, ions, peptides and small molecules to brain (Brown et al., 2002). The paracellular transports of molecules are restricted by tight
junctons of BBB. It plays a key role in Alzheimer’s and Parkinson’s disease conditions, because the dysfunction of BBB affects the passage of molecules inside brain (Desai et al., 2007). Donepezil is widely used for treating Alzheimer’s disease and its related dementia. Though, it is used for treating Alzheimer’s disease, the side effects and the relatively decreased efficacies are noted on continuous usage of this drug (Tan et al., 2014). To avoid the over dosage, a controlled drug delivery system is needed for sustained and targeted release (Felice et al., 2014) to the brain.

Nanotetchnology based drug delivery vehicles are having the properties of smaller size with effective release of drugs in minimal dosage with target specificity (Ahmad et al., 2014). Poly N-isopropyl acrylamide (PNIPAM) is a thermoresponsive polymer exhibiting hydrophobic – hydrophilic phase transition at its Lower Critical Solution Temperature (LCST). It has significant characteristic of water retaining capacity and permeability properties (Zhang et al., 2014). These smart polymers are successfully used in drug delivery applications for many years (Shao et al., 2011) and they are widely accepted for its biocompatibility (Gandhi et al., 2014) and cytocompatibility in multiple cell lines (Tang et al., 2010; Tekin et al., 2011). Nanogels are reported to be nontoxic materials with greater drug loading capacity and higher BBB permeability (Jain, 2012). Hence, in this study we had synthesized and functionalized the donepezil conjugated nanogel (DCN) with polysorbate 80 by chemical route to overcome the BBB (Kreuter et al., 2005).

Zebrafishes are mainly used due to its genetic similarity with human, as an in vivo animal model for drug development, as well the physiological and toxicological studies to assess the drug efficacy and biocompatibility (Kari et al., 2007). In the present study, we had developed a thermo-responsive nanogel conjugated with donepezil for its sustained drug delivery in brain and studied for its biocompatibility in adult and embryonic zebrafish.

Materials and methods

Fabrication of PNIPAM nanogel

PNIPAM hydrogel was prepared based on the method described by Xia et al. (2013). NIPAM (0.5 g), methyl bis acrylamide (MBA) (0.03 g), potassium per sulphate (0.01 g), sodium dodecyl sulphate (0.1 g) were added and dissolved in Milli-Q water, followed by nitrogen gas bubbling and the polymerization was allowed for 10 min with 300 rpm at 60 °C. The sealed flask was kept on ice to cease the polymerization and dialyzed against Milli-Q water for 10 days. Dialyzed mixture and distilled water in the ratio of 9:1 was bubbled with nitrogen gas. NIPAM (1 M) monomer was added to the above mixture and dissolved well. To accelerate the polymerization, 10 μl of TEMED was added and kept sealed on ice for 2 h, followed by 2 days incubation at room temperature. Further, the samples were freeze dried (Christ Alpha 1-2 LD) under vacuum for 48 h and stored at –80 °C. The freeze dried nanogel was gold sputtered and observed under Field Emission Scanning Electron Microscopy (FESEM) (Supra-55, Carl Zeiss, Germany) for studying their structural morphology. The parameters such as average size and size distribution of the suspension were calculated by dynamic light scattering studies in particle size analyzer (Microtrac Bluewave, Turbotrac).

Functionalization of nanogel

The nanogel was functionalized with polysorbate 80 and donepezil (Sigma Aldrich) for the effective and targeted drug delivery. Functionalization of hydrogel was done by adding 10 mg of nanogel in 100 mM of 1 ml sodium carbonate buffer, treated with 20% of polysorbate 80 and 1 μg/ml of donepezil by continuous stirring for 4 h at room temperature (Vinogradov et al., 2004). The conjugation was confirmed by analyzing 100 μl of functionalized nanogel in liquid Fourier transform infra-red spectroscopy (FTIR) analysis (Perkin Elmer, Spectrum one, USA).

Analysis of thermo responsiveness

Thermoresponsiveness of the PNIPAM nanogel was measured by cloud point measurement method as described by Jafari et al. (2011), using multimode plate reader (Perkin Elmer, EnSpire, USA). PNIPAM nanogel was measured at 500 nm absorbance in series of increasing temperatures (25–45 °C) with 10 min incubation time and 5 s shaking at each temperature. The drug release studies were performed using Janus green dye to ensure the measurement of dye release at 660 nm.

Oral drug delivery in adult zebrafish

Zebrafishes were maintained in a recirculating stand-alone system (Aquaneering, USA) at 28 °C and 10:14 h dark: light cycle. All protocols were reviewed and approved by Institutional Ethical Committee (Approval number for animal usage IBSC/2013/DBT-IDB/RRK-009) of Sathyabama University. Adult zebrafishes with 340 mg average weight and 2.5 cm body length were taken as 5 fishes in each batch for the administration of donepezil, nanogel, DCN and control. Venflon syringe of 24G was used to administer 5 μl volume of the drug containing various concentrations to adult zebrafish by inserting the plastic needle 1 cm below the gills to reach the end of esophagus (Collymore et al., 2013). Control fishes were treated with system water orally instead of drug. As soon as the drug was given orally, these fishes were put in warm water above LCST (~37 °C) for 1 min for the release of donepezil from DCN, until regaining their original physiological function from the anesthetic condition. The drug concentration on each set of experiment was determined after effective dosage calculations. The oral delivery was continued for a week as one time dosage and the changes were observed.

Acetylcholine esterase inhibition assay for the donepezil conjugated nanogel

Acetylcholine esterase inhibition assay was performed using 75 mM acetylthiocholine iodide and 10 mM DTNB chromogen in a 96 well microtitre plate containing homogenized zebrafish brain extract. This change in absorbance was recorded at 412 nm for every 1 min for 10 min using the multimode plate reader (Kannan and Vincent, 2012b).
In vitro drug encapsulation and release studies of nanogel using HPLC

Donepezil standard graph was plotted with known quantity and from the standard graph functionalized nanogel was quantified. The sample was heated at 37 °C for one hour for the drug release from the nanogel and the supernatant was taken and analyzed in RP-HPLC (Waters, USA) at regular intervals. The chromatographic analysis was performed with C-18 column with 100 Å pore size. The mobile phase used was A: B (A – 0.1 M ammonium acetate, B – methanol and acetic acid (80:20)) with pH of 2.5. The flow rate was maintained as 1 ml/min for 30 min and detected at 270 nm in PDA detector.

RP-HPLC analysis of controlled drug release

The orally delivered fishes were anesthetized by ice cold water and the blood was collected by tail vein cut method (Pedroso et al., 2012). The plasma was separated and deproteinized using equal volume of methanol. The mixture was centrifuged at 14,000 rpm for 15 min and the supernatant was analyzed in RP-HPLC (Liew et al., 2014). From the same fish, the brain was dissected out and homogenized in 500 μl of methanol. This sample was centrifuged at 14,000 rpm for 15 min at room temperature and the supernatant was analyzed in HPLC to quantify donepezil concentration.

Zebrafish behavioral analysis

The behavioral patterns of swimming and locomotion were observed for the adult fishes administered with donepezil, nanogel and DCN in three groups. The tracking was recorded by Canon powershot 50X in regular intervals and the graph was plotted using Tracker software (version 4.87) by manually tracking each frame of fish movement by creating a point mark at fish head. These changes were recorded at different time intervals to analyze the effect of drug release responses with respect to the behavior of the zebrafish.

Biocompatibility of blood cells and brain

After 90 min of drug delivery, 10 μl blood was collected as described previously and fixed with 40 μl of 25% glutaraldehyde for 20 h at 4 °C. Finally the samples were centrifuged at 1000 rpm for 10 min and the supernatant glutaraldehyde was discarded. The blood cells were washed with 300 μl of distilled water and again centrifuged. About 20 μl of sample was taken and air dried at room temperature, platinum sputter coated and examined under FESEM (Supra-55 Carl Zeiss) (Suwalsky et al., 2009). Adult fish brain was dissected out after a period of 7 days oral drug delivery and fixed in 4% paraformaldehyde, processed and embedded in paraffin wax. Sections were made at 5 μm thickness and stained with hematoxylin and eosin (Menke et al., 2011).

Cardiac assessment

Cardiac cycle (systole and diastole) and heart beat rate analysis were performed in 3 dpf (days post fertilization) zebrafish embryos to find the cardiotoxicity of the treated drugs with varying concentrations. The 3 dpf embryos were anesthetized by 0.01% Tricane (Sigma) to analyze the heart beat recording for 1 min under Stereo microscope (Leica M165 FC). These embryos were kept at lateral position during imaging, for the standardized view of heart chamber in sequential imaging and analyzed (Kannan and Vincent, 2012a).

Phenotypic and lethal toxicity assessment in zebrafish embryos

Embryos were incubated with drug nanogel, donepezil, DCN separately in series of concentration in 1 ml of E3 medium at 10 hpf and the E3 medium was changed daily with the respective drugs upto 10 dpf. The phenotypical changes were imaged at 48 hpf using Stereo fluorescence microscope (Leica M165 FC).

Statistical analysis

The LC₅₀ for 50% embryos death is calculated according to the standard procedure by OECD (1992) and the results were obtained using the probit regression of statistical software SPSS (IBM SPSS version 20.0). All other data were calculated as means with ± standard deviation (SD).

Results and discussion

The fabricated PNIPAM nanogel showed distinct morphology with cross-linked honey comb and uneven porous structure which is well interconnected to form a mesh like structure under FESEM observation (Fig. 1a and b). Similar honeycomb structure of nanogel was previously reported by Kato et al. (2003). It was observed that the ice crystals in the nanogel had dried and left the porous interconnected structure upon vacuum freeze drying. This network structure of PNIPAM nanogel is preferred for efficient drug loading than the microsphere shaped nanogel (Jafari et al., 2011). The particle size of 20–40 nm was greatly influenced by the polymerization time period and confirmed by dynamic light scattering studies. 70% of mono dispersed nanogel was obtained for 20 nm size range (Fig. 1c and d) with a polymerization period of 10 min. The longer polymerization time of PNIPAM monomers leads to the higher particle size which was also reported by Xia et al. (2013). The smaller pore size and the thermo responsive rate had significantly increased when nanogel itself was used as a cross linker.

The nanogel was functionalized with polysorbate 80 and conjugated with donepezil and confirmed by FTIR analysis (Fig. 2). DCN functionalized with polysorbate 80 had confirmed the vibration stretch of the functional group conjugations of polysorbate 80 at 1104.2 cm⁻¹ and 1358.5 cm⁻¹, secondary amine bends of nanogel at 1632.1 cm⁻¹ and the aromatic bands of donepezil at 2353.3 cm⁻¹ and 2341.6 cm⁻¹ respectively.

The functionalization of DCN had increased the LCST from 32 °C to 37 °C, which was also supported by Haraguchi and Takehisa (2002) as the swelling ratio in response to temperature can be between 20 °C and 50 °C. It was reported that the PNIPAM hydrodynamic volume changes above 32 °C depends on the pH of buffer, molecular weight of the chains and the concentration of the solution (Yim and Kent, 2004). The
transparency of the nanogel turned to white opaque matter above the LCST and stuck to the plate (Fig. 3a, b). The drug release experiments were performed on the same principle and the release of Janus green dye was observed as a change in the absorbance at increasing temperature point (Fig. 3c). As zebrafish is a poikilothermic animal, the in vivo thermo-responsive studies were done at 37°C from the actual water temperature of 28°C in which the fishes were maintained in the controlled laboratory conditions.

DCN showed acetylcholine esterase inhibition in zebrafish brain extract with remarkable inhibition rate above LCST temperature (Fig. 3e). Donepezil loaded inside the nanogel had released above the LCST at 37°C and inhibited the Acetylcholine esterase (AChE) which hydrolyzes the acetylcholine neurotransmitter in zebrafish brain and it is similar to human AChE (Santana et al., 2012).

HPLC quantification revealed the DCN entrapment of donepezil as 875.1 ng/ml (87.51%). The entrapment was quantified by the quantification of non-entrapped donepezil (124.9 ng/ml) found in the aqueous solution of the nanogel at 0 min among the 1000 ng/ml. The sustained release of donepezil was quantified as 89.84 ng/ml at 15 min, 115 ng/ml at 30 min, 131.3 ng/ml at 45 min and 135.4 ng/ml for 60 min respectively at 37°C (Fig. 4). These concentrations were quantified by comparing to the HPLC standard graph (Supplementary Fig. S1). The donepezil entrapped inside the network of nanogel structure was confirmed for its sustained drug release by HPLC detection. Similarly, Mohd et al. (2014) quantified the dissolution rate profile for Gilimepiride in self-nanoemulsifying powder (SNEP) formulation. This is the first report on the quantification of drug release from PNIPAM nanogels by RP-HPLC.

Generally, the drugs are given orally through food (Bruni et al., 2014) or enrichment of the drugs in nauplii Artemia to feed fishes. The amount of food taken by the fishes is not able to be calculated; hence the method of oral gavaging is preferred to deliver the drugs. For zebrafishes, Canadian council on animal care – 2005 had approved the fish drug dosage shall be 1% of the body weight. However, we had administered 5 ml volumes containing various concentrations of drug dose (upto 100 μg/ml) to ensure the entry of at least 3 μl of drug inside the gastrointestinal tract of zebrafish despite spitting out.

The presence of donepezil in adult zebrafish brain and plasma was confirmed by HPLC at 9.3 min of retention time for donepezil and 9.6 min for DCN treated brain and blood plasma (Fig. 5). Although the concentration of donepezil was lower in DCN treated fish brain, the delivery of donepezil was accomplished by the nanogel by overcoming the BBB and confirmed by HPLC (Supplementary Table S1). DCN functionalized with polysorbate 80 had the property of sustained drug release to the brain by crossing the BBB, whereas, the donepezil treated fish had entered directly inside brain, which
may give rise to side effects (Harper and Lawrence, 2010). The slow and sustained release of donepezil from the DCN ensures safer drug delivery with limited dose to the brain which is the necessity to treat neurological disorders (Tan et al., 2014).

In general, the neuroactive drugs are sensitive and resulted in complex behavior and movement patterns (Tierney, 2011). Previous studies revealed that the locomotor responses of zebrafish after treatment with neuroactive drugs are similar to mammals (Lima et al., 2012). In our study, the zebrafish behavior was recorded and the behavior graph was plotted (Fig. 6). Since the half-life period of donepezil is 70 h, the video tracking of swim behavior was recorded up to 72 h. The swim behavioral response after the drug delivery showed unique nocifensive swim behavior (Liao and Fetcho, 2008), whereas in

Fig. 2 – FTIR peaks of fabricated nanogel, donepezil, polysorbate 80 and DCN functionalized with polysorbate 80. The characteristic peak at 1647 cm\(^{-1}\) represents the secondary amine NH bend in the PNIPAM nanogel. Presence of peaks 2383.1 cm\(^{-1}\) and 2128.9 cm\(^{-1}\) show the aromatic combination bands of donepezil. C-O stretch and C-H methylene bends are observed at 1108.2 cm\(^{-1}\) and 1359.6 cm\(^{-1}\) respectively for polysorbate 80. DCN functionalized with polysorbate 80 was confirmed based on the FT-IR spectral stretch at 1104.2 cm\(^{-1}\) and 1358.5 cm\(^{-1}\) and the secondary amine bends of nanogel at 1632.1 cm\(^{-1}\) and the aromatic bands of donepezil at 2353.3 cm\(^{-1}\) and 2341.6 cm\(^{-1}\).
Fig. 3 – Thermoresponsive behavior of nanogel below and above LCST and acetylcholine esterase inhibition assay of DCN.
(a, b) The first row has water and second row has the nanogel. (c) Shows the transformation of nanogel phase below LCST (left) and above LCST (right). (d) The dye Janus green release at different temperature points is measured at 660 nm absorbance. (e) Acetylcholine esterase inhibition assay of donepezil, nanogel and DCN.

Fig. 4 – Drug encapsulation and release studies of DCN in RP-HPLC. (a) The comparative HPLC profile of different concentration of donepezil release from the DCN with different time intervals. (b) Concentration of donepezil analyzed in RP-HPLC after encapsulation at different time intervals at 37 °C which shows the sustained release pattern of donepezil over time.
the current study struggled swim (Blaser and Rosenberg, 2012) and thigmotaxis like swim (Egan et al., 2009) behaviors were also observed immediately after oral administration of drugs. Faster burst swim movement upon donepezil treatment revealed the brain received the drug while comparing the control fish. The nanogel treated fish did not show much movement like freezing (Hurd and Cahill, 2002) for 2 min, but DCN treated fish managed to swim in a clumsy manner. These behavioral changes represented the absence of Donepezil effect in the nanogel and DCN treated fishes. The startle response (Wong et al., 2010) had been decreased for the nanogel treated fishes at 70 and 72 h as they behave like control fishes. Erratic movements (Speedie and Gerlai, 2008) and zigzag movements (like polarization sensitive swimming) were observed up to 70 h for the donepezil treated fishes. Also the distance traveled by the fishes was calculated for 2 min (Supplementary Table S2) and the swim behavior was observed (Supplementary Table S3). All the treated fishes

Fig. 5 – RP-HPLC analysis of donepezil in treated zebrafish brain and plasma. (a–d) Control chromatogram where no donepezil peak is observed in zebrafish brain, Nanogel treated zebrafish brain, plasma and Nanogel treated zebrafish plasma. (a1, b1) Chromatogram of donepezil treated zebrafish brain and DCN treated zebrafish brain shows the donepezil peak at the retention time of 9.383 min and 9.299 min. (c1, d1) Chromatogram of donepezil treated zebrafish plasma and DCN treated zebrafish plasma shows the donepezil peak at the retention time of 9.653 min and 9.697 min.
Fig. 6 – In vivo biocompatibility studies in adult zebrafish. Locomotive and swim behavior of adult zebrafishes before and after drug delivery for 72 h at different time intervals. FE-SEM Morphology of blood cells after oral delivery at 10,000× magnification. Yellow arrows indicate the echinocyte like formation on RBC membrane, red arrows – indicate the fusion of blood cells. Histology of adult zebrafish brain in the medial telenchepalon region. Green arrow denotes the enlarged ventricular space. Red box shows increased pial surface and blue arrow shows missing tela choroidea. (TC – Tela choroidea, SY – sulcus ypsiloniformis, LP – lateral pallial division, PVZ – periventricular zone, VP – Ventral pallial division, DP – dorsal pallial division, V – ventricular area, Dm – medial zone of dorsal telenchepalon area, Dc – central zone of dorsal telenchepalon area, Vv – ventral nucleus of ventricular area, Vd – dorsal nucleus of ventricular area, MP – medial pallium, Ppa – anterior part of parvocellular preoptic nucleus, DIV – diencephalic ventricle, Mot – medial olfactory tract). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
were started to swim normally like control fishes by 70 h of duration, except the nanogel treated fish which suggested that the nanogel itself is not crossing any barrier, since it was not functionalized with any chemical to cross the blood brain barriers.

The blood cell compatibility study was resulted in the observation of fused blood cells together or with neutrophils in donepezil treated zebrafish (Fig. 6). Higher amount of fusion and echinocyte (level 1) (Hasler et al., 1998) like formation was observed in nanogel treated fish. Structural morphology of DCN treated zebrafish blood cells was similar to that of control (untreated) zebrafish blood cells. Donepezil and nanogel treated zebrafish blood cells showed few changes like fusion and echinocyte (level 1) like formation. Previous studies of Joseph et al. (1990) on RBC, and neutrophils fusion showed the Ca²⁺ dependent fusion of blood cells. Similar pattern of fusion was observed in nanogel and donepezil treated fish blood, which might be the biometabolic effects of drugs on Ca²⁺ and other ionic concentrations. However, further biochemical and molecular studies are needed to support the cell aggregation and fusion.

Histological sections of brain were observed to check the organ level toxicity of donepezil, nanogel and DCN in zebrafish brain (Fig. 6). The brain neurotoxicity is mainly observed in the telenchepalon region, which is responsible for emotions and memory (Folgueira et al., 2012) and also act as adult stem cell niche for radial glial cells (Scholpp et al., 2013). In the present study, nanogel and donepezil treated zebrafish brain exhibited broadened T-shaped ventricular space. The pial surface of radial glial cells rich region was increased in anterior telenchepalon for the DCN treated fish brain and in medial telenchepalon for nanogel treated fish brain. In all 3 regions of telenchepalon (anterior, medial, posterior), there was reduced glial cells in the tela choroidea region for the donepezil treated zebrafish brain. In nanogel treated brain, the dorsal pallium (neural tube fold) was found to be smaller size when compared to control. The non-toxicity for any neural stem cell niches is observed clearly in the telencephalon of zebrafish. Thus the DCN is non-toxic in the zebrafish brain. Though the ventricular cavity has been broadened in donepezil and nanogel treated fish brain, which influence the cerebral fluid quantity and flow. However it was not observed in the DCN, which is similar to the control.

Embryonic zebrafish were actively responding to chemicals and other stimuli, and changes their cardiac cycle. The heart beat rate was calculated based on one systole and diastole cycle for the 5 μg/ml treated embryos (Fig. 7) in the 3 dpf embryo. It was reported that the sequential cardiac

![Fig. 7](image_url) - Cardiac assessment studies in embryonic zebrafish. Cardiac cycle analysis (one systole and diastole) at 3 dpf embryos. The red color arrow represents the diastolic start of a systole–diastole cycle. The blue arrow represents the next diastolic start. These results signified that nanogel treated embryos had faster heart beat rate and DCN treated embryos had similar heart beat rate to control embryos. (cs: centi seconds). (a – untreated embryos, b – donepezil treated embryos, c – nanogel treated embryos, d – DCN treated embryos). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
chamber contraction was matured at 36 hpf and starts the cardiac conduction system functions rhythmically (Kirchmaier et al., 2012). In our study, the systole and diastole cycle has been prolonged in the donepezil treated embryos. The time between one diastole and next diastole in control embryos is 68 cs, whereas in nanogel and DCN treated embryos took longer than 72 cs. Thus the heart beat rate (HBR) has been decreased, whereas, the DCN treated embryos showed similar systole diastole cycle to the untreated embryos. HBR was found to be $176.9 \pm 13.3$ beats/min for the experimental

Fig. 8 – Phenotypic assessment in embryonic zebrafish. Phenotypical changes of treated embryos at 2 dpf. Green arrows shows pericardial edema and pericardial bulging. Blue arrow shows the curvature of the body axis at $8 \mu g/ml$ of DCN treatment. D1–D5, G1–G5 and DCN1–DCN5 represents $2 \mu g/ml$ to $10 \mu g/ml$ with $2 \mu g$ increments of the drug donepezil, nanogel and donepezil conjugated nanogel (DCN) treated embryos. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
control and 187.3 ± 17.33 beats/min, 115.5 ± 21.4 beats/min, 173.1 ± 16.18 beats/min for the nanogel, donepezil and DCN treated embryos respectively.

Lethal toxicity assays in zebrafish embryos treated with donepezil, nanogel and DCN showed slow development compared to the control larvae (Fig. 8). The LC50 value for the donepezil, nanogel and DCN are reported in Table 1. Altered axial curvature and cardiac edema (Cheng et al., 2000) were observed for the donepezil treated embryos. The nanogel and DCN treated embryos exhibited hypo pigmentation and this phenotype was previously observed as vsp18 mutant zebrafish (Maldonado et al., 2006). Yolk utilization was found to be less in nanogel and DCN treated embryos (Fig. 9).

In conclusion, nanogels functionalized with polysorbate 80 encapsulated with donepezil crossed BBB for sustainable and controlled release of drug to brain. Further, the present study may help to understand the in vivo biocompatibility of the DCN or any drug conjugated in nanogels using zebrafish model. The continuous and sustained drug release by overcoming the BBB in zebrafish brain will improve the efficacy of the drug with target specificity for any neurological or brain related diseases or disorders.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jab.2016.01.004.

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