**-Biocompatibility study of ultrashort peptide**

CYTOTOXCICITY ASSAY:

Cat # G1781 and G1782

Biocompatibility studies were carried out in 96-well plates (Corning, USA). HDFn

cells (10,000 cells/well) were seeded into a 96-well plate and incubated overnight, in 200 μL complete growth medium. After incubation, media in the wells were ultrashort peptide at different concentrations viz 5 mg/mL, 4mg/mL, 2 mg/mL, 1 mg/mL and 0.5 mg/mL were added to the wells were added to the wells. Untreatedwells were used as positive controls. 20 μL of lysis buffer was added in the control well then incubated for 45 min at 37°c.50 μL of media was Transferred from each well, including (lyses well) into new plate. Then, 50 μL of cytotoxicity reagent was added into each well and covered with aluminum foil then incubated for 30 min. After that, 50 μL of stop solution was added.Finally, Read the plate at 492 NM.

The plates were incubated for 24 hours. Cell viability was determined by Non-Radioactive Cytotoxicity Assay is a colorimetric alternative to radioactive cytotoxicity assays. The CytoTox 96® Assay quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis.Released LDH in culture supernatants is measured with a 30-minute coupled enzymatic assay that results in the conversion of a tetrazolium salt (INT) into a red formazan product. Finally, the absorption of individual wells was read at 490 nm using a plate reader (PHERAstar FS, Germany).

Cell Culture:

Human dermal fibroblasts, neonatal (HDFn, C0045C) was procured from Thermo

Fisher Scientific, USA. Cells were cultured in medium 106 (Thermo Fisher Scientific,

USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

The cells were maintained either in a T175 or T75 cell culture flask (Corning, USA)

at 37°C in a humidified incubator with 95% air and 5% CO 2 . The cells were

subcultured by trypsin at approximately 80% confluence. The culture media was

replenished every 48 hours.

MTT Assay:

Biocompatibility studies were carried out in 96-well plates (Corning, USA). HDFn

cells (10,000 cells/well) were seeded into a 96-well plate and incubated overnight

in 200 μL complete growth medium. After incubation, media in the wells were

changed. Ac-CH-01- NH 2 and Ac-IVZK- NH 2 were weighed out and dissolved in milliQ

water. To test the compatibility, peptides at different concentrations viz 5 mg/mL, 4

mg/mL, 2 mg/mL, 1 mg/mL and 0.5 mg/mL were added to the wells. Untreated

wells were used as positive controls. The plates were incubated for 24 hours. Cell

viability was determined by means of a colorimetric microculture assay (Vybrant ®

MTT Cell Proliferation Assay Kit, Thermo Fisher Scientific, USA) according to the

manufacturer’s protocol. Briefly, the plates were taken, the medium was carefully

removed and fresh serum free medium containing 10% MTT reagent was added.

After 2 h of incubation at 37°C, the supernatant media was removed and 200 μL of

DMSO was added to each wells to dissolve the formazan crystals. Finally, the

absorption of individual wells was read at 540 nm using a plate reader (PHERAstar

FS, Germany).

Live/Dead Staining:

HDFn cells were seeded and treated with peptides according to the protocol

described above. After 24 h of incubation, the spent media were removed and

replaced with DPBS solution containing approximately 2 mM calcein AM and 4 mM

ethidium homodimer-1 (LIVE/DEAD ® Viability/Cytotoxicity Kit, Life Technologies TM )

and incubated for 40 min in dark. Before imaging, the staining solution was

removed and fresh DPBS was added. Stained cells were imaged under an inverted

confocal microscope (Zeiss LSM 710 Inverted Confocal Microscope, Germany).

Cytoskeletal staining:

Immunostaining was performed after 24 h of culture. In brief, the cells were fixed

with 3.7% paraformaldehyde solution for 30 minutes and incubated in a cold

cytoskeleton buffer (3 mM MgCl 2 , 300 mM sucrose and 0.5% Triton X-100 in PBS

solution) for 10 minutes to permeabilise the cell membranes. The permeabilised

cells were incubated in blocking buffer solution (5% FBS, 0.1% Tween-20, and

0.02% sodium azide in PBS) for 30 minutes at 37°C, followed by incubation in

antivinculin (1:100) for 1 hour at 37°C and subsequently with anti-mouse IgG

(whole molecule)-FITC and rhodamine-phalloidin (1:200) for 1 hour at 37°C.

Further, the cells were incubated in DAPI for 1 hour at 37°C to counterstain the

nucleus. These fluorescent dye treated cells were observed and imaged using laser

scanning confocal microscope (Zeiss LSM 710 Inverted Confocal Microscope,

Germany).

3D Culture of Cells in Peptide Hydrogels:

HDFn cells were encapsulated in peptide hydrogels in 24 well tissue culture plates.

Peptide solutions of Ac-CH-01- NH 2 and Ac-IVZK- NH 2 were added to the plate at 200

μL per well. HDFn cells resuspended in 3X PBS were added to each well at 100,000

cells/well and gently mixed. The final concentration of the peptide hydrogel was 1X

after the addition of 3X PBS containing cells. Gelation occurred within 3-5 minutes

and subsequently, culture medium was added to the wells. At pre-determined time

points, the 3D cell viability assay, live/dead assay and cytoskeletal staining were

performed.

3D Cell Proliferation Assay

The CellTiter-Glo ® luminescent 3D cell viability assay is a method to determine the

number of viable cells in 3D hydrogels based on quantification of the ATP present,

which signals the presence of metabolically active cells. After each time point, the

hydrogels cultured with cells were washed twice with DPBS. Fresh medium was

added to each well and equal amount of CellTiter-Glo ® luminescent reagent was also

added to the gels. The contents were mixed for 2 minutes to digest the hydrogels

and then incubated for 10 minutes. After incubation, the luminescence was recorded

using a plate reader (PHERAstar FS, Germany).

Morphological observation of bacteria treated with Np:

Bacteria incubated with in situ silver encapsulated peptide nanoparticles solution (0.4 μg/500 μL water) for 30 min . Bacterial cell were condensed by centrifugation at 3600 rpm for 4.0 min at 40, and then fixed with 4%paraformaldehyde for 1h. bacteria were dehydrated by treated with 30%,50%,60%,70%,80%,90%,100% ethanol for 2 min, then a drop of the dehydrated bacterial cell was placed on a silica chip and air dried.the mounted sample were sputter –coated with gold (60 s,50 mA) and viewed under a SEM(FEI Quanta 200 FEG SEM with an accelerating voltage of 2-3 kV) and fot TEM samples of dehydrated E.coli was immediately deposited on bar copper grids.

Finally, dehydrated immediately deposited on bare 200-mesh copper grids. And then examine using TEM.

Characterization of AgNPs

**Transmission Electron Microscopy (TEM) Analysis**

The TEM studies were carried out on two different instruments; Tecnai G2 Spirit Twin with accelerating voltage of 120 kV and FEI Titan G2 80-300 CT with 300kV emission gun. The TEM samples for peptide hydrogel were prepared by transferring a small portion of hydrogel on carbon coated copper grid using clean cotton swab. Non-hydrogel samples were directly dropped on the grid as long as they can be dissolved in either water or ethanol. Staining was not performed for most of the samples unless otherwise stated. The grids were dried in normal air for at least 1 hour before imaging.

**Scanning Electron Microscopy (SEM) Analysis**

The morphology of the samples was visualized using FEI Quanta 200 FEG SEM with an accelerating voltage of 2-3 kV. The freeze-dried samples were adhered to carbon conductive tape on SEM stub and sputter coated with 3 nm of Platinum.