Overview:

HYBER has a well-equipped mammalian cell culture laboratory that is mainly used by the Biohybrid Materials group to study cell-material interactions and the potential application of self-assembled biomaterials in the areas of tissue engineering and regenerative medicine. A number of cell-based assays including, but not limited to, cytotoxicity, DNA quantification and cell proliferation assays along with qualitative and quantitative microscopic imaging using live & dead cell-specific fluorescent stains, are available. Furthermore, gene expression assays and tests for biochemical parameters enable studying the effect of biomaterials on the functionality of the cells. Cellular uptake analysis of biomolecules, controlled drug release studies from self-assembled nanocages, targeted cellular and nuclear delivery as well as high throughput liver toxicity assays are some of the approaches used to evaluate the efficacy. Currently, the cell lab has a variety of mammalian cells derived from different tissues in the body such as the liver, kidney, breast, bone marrow stem cells, and cells of the immune system, which can be used to study drug response, immune response, and toxicity- phenomena typical to cell-biomaterial interactions.

Should you have an interest in having your materials tested, please let us know. We are happy to help and collaborate. You might find the attached form helpful when defining your research question. Some methods and the available cell lines are listed below.

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METHODS IN ANALYSIS OF NANOMATERIALS FOR BIOMEDICAL APPLICATIONS

Uptake and intracellular fate	**Cytotoxicity and cell viability**	**Inflammatory response**	**Reactive oxygen species (ROS) assay**	**Immune response and biocompatibility, **disease models (*in vivo* studies)**
• Confocal laser scanning microscopy* • Fluorescent microscopy • Light microscopy • Transmission Electron microscopy • AFM	• MTT/ XTT/ WST assay • Resazurin assay • LDH activity • Live/dead assay using FACS or fluorescence microscopy • DNA quantitation assay	• PCR • ELISA • Western blotting	• DCFDA assay	Interleukin (IL-6, IL-8, IL-10) assay, activation of macrophages. **Immune response on systemic injection/implantation, **Effect on disease models (improvement in conditions, reduction of tumor, etc.)

*facility not available with us (collaboration required); **depending on availability of a well-equipped animal facility

a) Cellular uptake and material-cell interaction:

These studies allow for visualization of the cellular uptake of nanomaterials used as delivery vehicles (either drug delivery or gene delivery). The nanomaterial is tagged with a fluorescent dye (typically FITC), and incubated with the cell line of interest in physiological media (standard medium supplemented with antibiotics and 5-10% fetal bovine serum) for 6-12 hours in a humidified incubator supplied with 5% CO_2. Confocal laser scanning microscopy or simple fluorescent microscopy helps visualize the presence of the tagged nanomaterial within the cell. Electron microscopy techniques are used for visualization in some instances where the nanomaterials are gold-coated.

b) Cytotoxicity and cell viability:

These assays are performed to study the in vitro biocompatibility of the nanomaterials. The nanomaterials are incubated with mammalian cells of interest over a pre-determined period of

time (generally a week). At regular time intervals, the effect of the nanomaterials on the overall growth/proliferation of the cells is studied using any one or all of the following assays:

1. MTT/ XTT/ WST assay:

The MTT, XTT, WST form a group of tetrazolium salts which upon reduction, by enzymes present in the viable cells, form formazan- a purple colored crystal precipitate. The protocol involves incubating the cells treated with nanomaterials (in a 96-well plate) with a (10-20 µL) solution of MTT/XTT/WST (0.2-0.5 mg/mL) for 1-4 hours. In the MTT assay, the insoluble formazan precipitate needs to be solubilized using DMSO for its absorbance to be read at 570 nm using a plate reading spectrophotometer. In XTT and WST assays, the formazan produced is water-soluble and hence absorbance directly read at 490 nm without the need for solubilizing.

2. Resazurin reduction assay (also commercially known as the Alamar Blue assay):

Resazurin is a redox indicator, which is reduced to resorufin by viable cells. Nanomaterial treated cells are incubated with 20 µL of resazurin (a deep blue colored solution; 0.2 mg/mL) for 1-4 hours and fluorescence of resorufin measured at an excitation of 560 nm and emission of 590 nm.

3. Live/dead assays:

These assays involve the simultaneous fluorescence staining of viable and dead cells using calcein-AM and propidium iodide (PI), respectively. Calcein-AM is lipophilic and cell permeable. The esterases present in viable cells convert calcein-AM to calcein, which emits a strong green fluorescence at an excitation of 490 nm and emission of 515 nm. On the other hand, PI is a nuclei staining dye that reaches the nucleus only by passing through the disordered areas of a dead cell's membrane. It intercalates with the double stranded DNA helix giving a red fluorescence (excitation= 535 nm; emission= 617 nm). Cells (1 x 10^6 cells/well) are incubated with nanomaterials in physiological media. The cells are incubated with 100-200 µL of diluted dye solutions in the dark for 15-30 minutes. Since both calcein and PI can be excited at 490 nm, simultaneous monitoring of viable and dead cells can be performed using a fluorescence microscopy. The same can be quantified using flow cytometry where cells treated with nanomaterials can be incubated with approximately 100 µL of diluted stain solution and fluorescence determined using the different channels available in the flow cytometer.

4. LDH activity:

Lactate dehydrogenase is a cell death marker that catalyzes the conversion of pyruvate to lactate. It is typically released upon tissue damage or cell lysis and is known to be elevated in cancer conditions. It reduces NAD to NADH- a conversion that can be colorimetrically detected at 490 nm. 48 µL of the cell media is collected and incubated with 2 µL of the assay substrate (a mix of NAD in appropriate buffer) for 2-3 minutes, ensuring the assay plate is protected from light. Absorbance is measured at 490 nm.

5. DNA quantitation assay for cell proliferation:

This assay is based on the amount of DNA which remains constant for a given cell line or cell type. Hence, DNA content may be used as an accurate estimate of cell number. Approximately 5-100 µL of the sample containing cells treated with nanomaterials is lysed to release the DNA content. The lysate is then incubated with a DNA staining dye (like Hoechst 33258) and the fluorescence measured.

c) <u>Inflammatory response:</u>

Implantation or injection of nanomaterials in vivo may trigger an immune response consequently leading to inflammation because of the release of a number of cytokines from the immune cells of the body. The interleukin IL-6 is secreted by spleenocytes in response to an immune reaction. Increased amounts of IL-6 indicate a severe immune response that may potentially lead to cytotoxicity. The amount of IL-6 secreted is quantified using the enzyme-linked immunosorbent assay (ELISA). The general scheme of ELISA is as follows:

(i) 50-100 µL of the sample (and standard) are coated onto an ELISA plate
(ii) After aspiration and washing, incubate with 100 µL of detection antibody for 1 hour at room temperature
(iii) Aspiration and washing followed by incubation with 100 µL of an HRP conjugate for 30 min at room temperature
(iv) Incubate with 100 µL of stabilized chromogen for 30 minutes in the dark at room temperature, after initial aspiration and washing
(v) Add 100 µL of stop solution and read absorbance at 450 nm.

The cytokines/interleukins involved in inflammatory response can also be detected using Western blotting. Here, the sample (typically 20-50 µL) undergoes protein denaturation, followed by gel electrophoresis. Then, the electrophoresis membrane is washed in a solution

containing the specific antibody for the interleukins. Excess antibody is washed off, and a conjugated secondary antibody that reacts with the first antibody is added. Through various methods such as staining, immunofluorescence, and radioactivity, the secondary antibody can be visualized to show the presence of the interleukin.

d) Reactive oxygen species assay:

In certain conditions, the nanomaterials may result in the release of reactive oxygen species by the cells. These could be assayed using the florigenic dye DCFDA (2',7' –dichlorofluorescin diacetate). After diffusion in to the cell, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2', 7' –dichlorofluorescein (DCF). DCF is a highly fluorescent compound, which can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495 nm and 529 nm respectively. Cells are incubated with DCFDA (100-200 µL) for 30 minutes at 37 ºC followed by washing with buffer and then fluorescence is measured.

CELL LINES AVAILABLE:

HepG2	Human hepatocarcinoma cells(Liver)
NIH3T3	Mouse fibroblast cells
Primary hMSC	Human bone marrow derived mesenchymal stromal cells
HEK293	Human embryonic kidney cells
RAW264.7	Mouse macrophage cells
MCF7	Human breast cancer cells
HeLa	Human cervical cancer cells
Jurkat	Human lymphocyte cells
A549	Human lung carcinoma cells
CHO	Chinese hamster ovary cells
U-937	Human monocyte cells