Nanoparticles as Anti-inflammatory and Pro-regenerative Therapeutic Molecules

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The saying 'size matters' is the basis of the revolutionary technology that has taken this decade by storm. Nano-sized particles, on account of their small size, show many interesting, and hitherto unseen and unknown, properties, like self-recognition at zero energy expenditure. As a result, they can be called 'intelligent' molecules.

2.1 Background and Novelty of This Translational Project

2.1.1 Chemistry and Synthesis

Nanomaterials are widely used in cosmetics, foods and medicinal products for improved performance.[1] In addition, various nanomaterials are under development for different biomedical applications. However, it is gradually becoming evident that many of the nanomaterials can induce substantial inflammation. For example, it is shown that metal and metal oxide nanoparticles generate oxidative stress that can induce acute pulmonary inflammation and other adverse health effects.[2] Moreover, it has been shown that the inflammatory effects of nanoparticles can be modulated by changing surface chemistry[3] and the chemical composition.[3] Some nanomaterials even show anti-inflammatory effects.[4] Thus evaluation of the inflammatory effect of potential nanomaterials, ways to suppress inflammation and to investigate their anti-inflammatory properties are important for translational research. Furthermore, our laboratory has synthesized a library of nanomaterials useful as bioimaging probe and as drug delivery carriers.[5] However, their inflammatory effects have not yet been investigated.

Tissue engineering represents an attractive option for regeneration of several organ systems.[6] Nanoporous 3D scaffolds are attractive materials for tissue engineering as the structures can be modulated as per requirements.[7] However, their application as a scaffold for lung regeneration is not well explored. There are only few reports that use poly-lactic acid-based foam[8] and Gelfoam sponge[9] as a scaffold for lung regeneration and have shown a partial success. In addition, graphene and porous carbon/silica based new generation scaffolds are not yet explored for lung regeneration.

2.1.2 Translational Outcomes Study in Biology

Increasing solubility, enhancing bioavailability by protecting a therapeutic molecule (be it a peptide, a small molecule, an antibody protein or a cell) by protecting it from degradation and metabolism, graft rejection, and metabolism, and engendering unique epitope recognition and self-assembly properties of nanoparticles (NPs) may be exploited. Nanoparticle (NPs)-like polymeric NPs, polymeric micelles, nano-/microemulsions, liposome/phospholipids, solid lipid NPs, nanogels, self-assemblies, polymeric conjugates and so on, can be suitable for these purposes [1–3].

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Nano biotechnological applications are envisaged in translational studies for the following reasons [4]: they self-assemble, have self-healing properties, are able to recognize their destined tissue as well as their own kind, and are able to seamlessly separate by virtue of their selective reactivity. A three-pronged biological application envisioned for the nanomaterials synthesized by my chemist co-PI are by tapping these precise properties as elaborated below.

Specific Aim # 1. Translational Outcomes Research in drug discovery or drug delivery systems in Inflammation and Degeneration I have in vitro models and in vivo preclinical models of tissue-specific inflammation (allergic asthma in lung and Inflammatory Bowel Disease in intestine) and systemic inflammation (septic and aseptic peritonitis), as well as degeneration (bleomycin-induced lung fibrosis and Chronic Kidney Disease) in my lab where drug discovery studies are ongoing, using-small molecules as novel drugs, herbal extracts as functional foods, probiotics as nutraceutical's, novel antibody-mediated therapy using camelid antibodies, and cell-based therapy using embryonic stem cells, adult stem cells and umbilical cord-derived stem cells [5, 8–14].

Specific Aim # 2. Translational Outcomes Research in tissue engineering and transplantation studies in Regenerative Medicine My lab also focuses on three important areas of Regenerative Medicine and Biology, viz. repair, replace, regenerate, and for that employs:

(a) Tissue engineering of tissue-specific (lung and kidney) cells from
   - Mouse and human embryonic stem cells
   - Mouse and human umbilical cord-derived mesenchymal stem cells
   - Mouse amniotic fluid stem cells
   - Mouse bone marrow-derived stem cells

(b) Characterization and validation of the induced differentiated cells in transplantation models of tissue degeneration [6, 7].

Nanomaterials customized and synthesized by my chemist co-PI shall be validated in our (i) tissue engineering platform (2D and 3D culture) and (ii) transplantation models where the processes of mobilization, homing, engraftment, repairment/replacement/regeneration shall be assessed.

2.2 Objective

2.2.1 Chemistry Part

Development of nanodrugs or nanodrug delivery system in Inflammation and Degeneration In this project, we will prepare 1–100 nm size nanomaterials that can be used for detection of inflammation and for delivery of anti-inflammatory drugs. In addition, we will study the inflammatory effects of some useful nanoparticles, deriving appropriate design to lower their inflammatory effect and to investigate the anti-inflammatory roles of any nanoparticles.

Nanoparticle will be prepared and functionalized with appropriate molecules for targeting and imaging of inflammation sites. In addition, anti-inflammatory drug-loaded porous colloidal nanoparticle will be prepared for specific delivery of drugs to inflammation sites. Therapeutic response of drug will be investigated via standard methods.

Development of nanomaterial scaffold for tissue engineering from stem cells and lung and kidney regeneration In this project, we will develop a 3D nanoporous scaffold materials

for cell-based tissue engineering and lung regeneration. In the first step, 3D nanoporous scaffold will be prepared using an assembly of different nanomaterials such as graphene, porous carbon, and porous silica. The pores and surface of the scaffold will be functionalized with affinity molecules for efficient attachment and proliferation of cells. In the second stage, lung cells will be grown on this scaffold. The successful attachment and growth of cells will be optimized by changing the nature and functionality of scaffold. Next, the cells, along with scaffold, will be injected or implanted in the injured lung. The therapeutic responses of injured lung and lung disease will be monitored via conventional approaches.

2.2.2 Biology Part

A. The nanomaterials synthesized shall be used for improving anti-inflammatory properties and anti-degenerative or pro-regenerative properties in the following areas:
(i) drug delivery
(ii) drug availability
(iii) as nanodrugs themselves.
B. The nanoscaffolds will define culture conditions in tissue engineering and as engraftment scaffold for regeneration.

2.3 Outcome

Thorough screening of a large library of materials synthesized in the Chemistry lab of Dr. Jana, through achievement of Specific Aims # 1 and 2 conducted in Biology lab of Dr. Ray Banerjee and rigorous Structure–Activity-Relationships to further fine tune that library, shall yield a large body of nanomaterials with thorough characterization in the above two important fields of biomedical research, namely, inflammation and degeneration, making up more than 80% of all known diseases and exacting a heavy price in economic burden to the nation. These materials shall form a part of a library of nanobiotechnical tools and may be developed accordingly.

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Details of the project **'Synthesis and Characterization of Nanomaterials for Application in Translational Studies of Drug Discovery in Inflammation and Regeneration'.**

1. **Scope of application indicating anticipated product and processes**

Inflammation and degeneration are the twin banes of a progressively complicated network of biological and biochemical circuitry that must be completed for the cycle of onset, development, maintenance and exacerbations, the various ramifications of a disease pathophysiology to set in. The first task of the drug hunter is, therefore, to look for potential 'druggable' materials and rationalize the chemistry of the same in the context of biological translation pertinent to the particular disease.

Nanomaterials are widely used in cosmetics, foods and medicinal products for improved performance. In addition, various nanomaterials are under development for different biomedical applications. They are preferred because of certain special characteristics. They are considered to be 'special' because of certain attributes:

(1) their property of self-assembly through intelligent recognition,
(2) their power to integrate through seamless fusion and merger with pre-existing materials, including biomaterials,
(3) their ability to 'photoactivate',
(4) their versatility to be modulated into flexibly adept forms that fit into the dynamic flux of the various nuances of the disease and effectively modulate them in a controlled fashion.

In addition to the pharmacological properties, ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) properties of a compound are very important to the ultimate success of the compound/molecule as a drug candidate. It has been seen that almost 50% of drugs fail unsatisfactory efficiency, especially due to poor absorption in the intestine or unsuitable metabolic stability. Also, almost 40% of drug candidates fail due to safety issues. These have led to the introduction of ADMET screening during the process of drug discovery, so that drugs with questionable ADMET properties can be screened out.

This project is therefore aimed at addressing:

(a) Determination of efficacy of a library of nanomaterials synthesized in my co-PI's Chemistry lab in three well-characterized preclinical disease models of tissue-specific and systemic inflammation and degeneration;
(b) Determination of suitability of the nanomaterials screened in (a) as drug delivery vehicles for tissue-specific targeting;
(c) Identification and characterization of nanomaterials further screened through (b) for their potential use as scaffolds in these diseases where inflammation invariably precedes degeneration and regeneration of lost tissue in the correct atanomical-physiological configuration is a critical step to modify the disease.

In summary:

- Anticipated product(s):
  - (I) Anti-inflammatory and/or regenerative nanodrugs
  - (II) Tissue-specific delivery nanovehicle (lung, intestine and peritoneum)

(III) Nano-scaffold for tissue regeneration (directly in vivo or in directly developed ex vivo followed by re-transplantation)

- Anticipated process(s):

(I) The synthetic chemistry of such nanomaterials
(II) The correct delivery system or mode of administration as a drug

2. **Project Summary**

Chemistry and synthesis

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2.4 Introduction

2.4.1 Nanoparticles as a Novel Anti-inflammatory Molecule

Nanotechnology is a broad term that is used to describe the products, procedures and features at a nano- or micro-scale, which have culminated from the union of chemical, physical and life sciences (1, 2). It involves manipulation of atoms to make lighter, stronger and more efficient materials of size ranging from 1 to 100 nm, called nanomaterials, with customized properties (1, 2). Recently, it has become a rapidly growing field with varied applications in different fields of science and technology, especially as drug delivery systems (3, 4), in cleanup of environmental pollution (5, 6), medical imaging (7, 8), as well as in the military (9, 10). The present study investigates the role of nanoparticles as anti-inflammatory molecules.

2.4.2 Inflammation

Inflammation is a complex process that is triggered by factors like bacterial infection, chemical injury and environmental pollution. These factors lead to cell injury or cell death (11, 12) and release of inflammatory factors like cytokines, tumour necrosis factor (TNF-α) or interleukin- 1 (IL-1) from leukocytes, macrophages and monocytes (13). The release of these factors further stimulate the upregulation of pro-inflammatory cytokines, chemokines, immunoglobulins and some cell adhesion molecules (CAMs) (14). When inflammation is caused by bacteria or other foreign particles, these are phagocytosed by the immune cells. During the process of phagocytosis, the uptake of oxygen by neutrophils increases, leading to the release of reactive oxygen species (ROS) like superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (HO•) (15), and also to the increase in ROS-generating enzymes like NADPH oxidase, xanthine oxidase, and myeloperoxidase. Expression of Phospholipase A2, 5-lipoxygenase (5-LOX), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) also increases (16). Transcription factors and nuclear factor kappa B (NFκB) are also activated. It has been found that NFκB has an important role in the regulation of inflammatory cytokines, inducible enzymes, cell adhesion molecules and other mediators of inflammation.

An anti-inflammatory molecule is any material that can effectively reduce or inhibit the above-mentioned characteristics. Over the years, various organic and inorganic products have been investigated for their potential anti-inflammatory properties. Among these, a number of plant-derived products (phytochemicals) are most important. Using the advances in the field of nanotechnology, several phytochemicals have been formulated with nanoparticles (like gold or silver nanobodies), to target the phytochemical-nanoparticle complex to the target site.

2.4.3 Nanoparticles as Anti-inflammatory Molecule

Among the different types of nanoparticles, the metallic nanoparticles (gold, silver, zinc, iron and metal oxide nanoparticles) have the greatest potential in biomedical applications. This is because of their large surface area-to-volume ration (17, 18), and also because of their varied biomedical activities (19). These properties have been established in experiments showing the anti-tumour properties of gold nanoparticles, and anti-inflammatory properties of cerium nanoparticles. Silver was earlier used as an anti-microbial agent and as a disinfectant with minimal adverse effects (20). With the development in antibiotics, however, the use of silver agents has now been limited to topical silver-sulfadiazine cream to treat burns (21).

(A) **Silver nanoparticles**

From the 1990s, colloidal silver has been promoted as an alternative treatment for various

diseases and as a mineral supplement (22). Despite the availability of colloidal silver products as health supplements, in the USA, it is illegal, as claims about the products are often scientifically unsupported (23).

(B) **Cerium oxide nanoparticles**

Hirst et al., in 2009, showed that the valence and oxygen-deficient properties of cerium oxide nanoparticles, or nanoceria, may play a role in their activity as auto-regenerative-free radical scavengers. Free radical nitric oxide (NO) is overproduced during inflammation, by the action of iNOS, and is responsible for tissue damage. The fact that nanoceria can scavenge free radicals and inhibit the production of inflammatory mediators has been tested in J774A.1 murine macrophage cell lines. These in vitro studies have shown that the nanoceria can be internalized by the cells, where it can reduce oxidative stress, as well as pro-inflammatory expression of iNOS, without being toxic to the cell. In vivo studies have shown that nanoceria show no pathogenic effect in mouse tissues. These studies show that nanoceria can be used as a therapy for chronic inflammation (24).

(C) **Gold nanoparticles**

Gold nanoparticles (AuNPs) have low cytotoxicity, can target cells, have functionalized surfaces and have tunable optical properties. These properties of AuNPs make it useful in biomedical applications. Gold compounds in an active oxidized form, like in gold sodium malate, has been used to treat forms of arthritis (25, 26). However, in nanoparticles, gold is present in a more inert metallic form. Though AuNPs can be synthesized into different morphologies, the most common are spheres and rods.

Macrophages have the ability to phagocytose particulate matter, and they remove dead cellular material and other foreign particles in chronic inflammation. When AuNPs are introduced to a body, there is a chance that macrophages will recognize them as foreign material and elicit an immune response. However, studies have shown that this is not the case (27). Whether AuNPs are toxic to the body is a topic of controversy (28). There appear to be no adverse effects either in vitro or in vivo (29–35). Whatever effects have been observed, have been due to substances attached or adsorbed to the AuNPs, rather than the AuNPs itself (32–36). Since it is possible that naked AuNPs introduced into a body may get coated with host proteins (37), it seems better to coat the AuNP with poly-ethylene glycol (38). Size and shape of the AuNPs appear to determine the in vivo activity of the nanoparticles.

(D) **Carbon nanoparticles**

Carbon nanotubes (CNTs) are tube-shaped nanomaterials made of carbon. They can be classified as either single-walled (SWCNTs) or multi-walled (MWCNTs). Therapeutic agents can be conjugated to CNTs via covalent or non-covalent bonds. It has been shown by Liu et al. that functionalized CNTs can be administered intravenously, and can be subsequently eliminated by the biliary pathway without any adverse effects (39).

2.4.4 Formulation of Nanoparticle-Mediated Drug Delivery

Research has shown that administration of anti-inflammatory drugs like dexamethasone and cortisone acetate before the administration of anti-cancer drugs, increases the efficiency and reduces the toxicity of the latter (40, 41).

For decades, polymeric nanoparticles, made of synthetic or natural polymers, have been studied extensively as systems for drug delivery (42). Polyesters are the main constituent of synthetic polymers. Polyesters like poly-lactic-co-glycolic acid (PLGA), poly-lactic acid (PLA), poly-glycolic acid (PGA) and PLA-PEG, have been used for their favourable properties biodegradability and low antigenicity (48). They have also been approved by the FDA for clinical use (48). However, these are difficult to encapsulate to allow the slow release of the drug, as

they show burst release phenomenon (the drug is released rapidly), leading to lower therapeutic efficacy (43, 44). Also, when degraded, the products lead to the formation of an acidic environment in the tissues, which may lead to inflammation.

PGA is made of glycerol and adipic acid, both of which can be naturally metabolized by the cell. The polyester is synthesized by a process of enzymatic polycondensation, which is better and cleaner than chemical synthesis (45, 46). This polymer is favoured because it can attach various groups to its hydroxyl groups that are present on the polymer backbone. Drug encapsulation is better in PGA nanoparticles than in PLGA (47, 48). Thus, formulations like microspheres (49), nanoemulsions (50), nanoparticles (51) and liposomes (52) have been used to enhance the effects of, and to decrease the side effects of, drugs like ibuprofen and ketoprofen. Due to the higher stability of polymeric nanoparticles, however, they have been preferred as carriers. Thus, the use of non-acylated (e.g. 0% C18-PGA) and stearoyl-acylated (e.g. 40% C18-PGA and 100% C18-PGA) have been studied as potential carriers for ibuprofen and ketoprofen. PGA polymers are suitable for studying the parameters for the formation of nanoparticle, the interactions between the polymer and the drug, and the ability of the carrier to load and release the drug optimally.

2.4.5 Nanomedicine

Recent advances in science have led to a better understanding of the biology of inflammation. It is now known that certain genetic changes lead to abnormal cell division, abnormal differentiation, and loss of control over the production of reactive oxygen and nitrogen species. This ultimately leads to the complex process of inflammation, either acute or chronic, that causes changes in the tissue. Traditional therapy often fails to treat inflammatory diseases due to lack in the bioavailability of the drug, harmful side effects and development of drug resistance.

To overcome the drawbacks of conventional medicine, nanomedicine has come into importance in the recent years. First-generation nanomedicines include drug-loaded nanoparticles, liposomes, micelles, carbon nanoparticles, and other inorganic nanoparticles. With better understanding of the mechanisms of inflammation, second-generation nanomedicines can be developed which can allow for targeted therapeutic strategies. Nanotechnology provides opportunities to develop strategies for the diagnosis, prevention, treatment and eradication of potent diseases and conditions. It also attempts to resolve pain and to enhance existing medical techniques.

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2.5 Some Inflammatory Diseases

Asthma and idiopathic pulmonary fibrosis (IPF) are very common chronic disorders that affect people all over the world. Improper management of the diseases lead to their aggravation, and ultimately to increase in mortality and morbidity. Though these diseases are similar in that they are both inflammatory diseases of the respiratory tract and can be treated with similar drugs, they vary in the underlying mechanisms. Both being lung diseases, asthmatic patients

suffer from reversible obstruction of the airways, whereas patients with IPF suffer a progressive deterioration in lung function. Diseases like asthma and chronic obstructive pulmonary disorder (COPD) form the third leading cause of death, and both are gaining prevalence. It has been predicted that by 2020, India alone will contribute to 18% of all tobacco-related deaths globally (1). IPF is a leading cause of death in China (2).

Asthma is characterized by the phenomenon of airway narrowing, which is caused by the interactions between several immunological, biochemical and biomechanical processes. In allergic asthma, many inflammatory and structural cells are activated and recruited to the site of inflammation. These cells release mediators and cytokines which lead to the pathological and structural changes that are usually observed in asthma. The chronic form of the disease is characterized by an infiltration of the airway walls by Th2 cells, eosinophils, macrophages, monocytes and mast cells. Other characteristics of the disease include accumulation of inflammatory cells in the lungs, desquamation of the epithelial cells, goblet cell hyperplasia, hypersecretion of mucus, and bronchoconstriction and airway hyper-responsiveness caused due to the thickening of the submucosa. Circulating leukocytes like Th2 cells, IgEs, eosinophils, neutrophils and mature plasma cells (3), as well as resident local cells and structural cells that make up the 'respiratory membrane' (e.g. fibroblasts, airway epithelial cells, bronchial smooth muscle cells, resident macrophages and mast cells) contribute to the symptoms of asthma (4). The common symptoms of asthma, like episodes of wheezing, breathlessness, coughing and chest tightness, are due to the phenomenon of airway hyper-responsiveness, which causes airflow obstruction. This obstruction may or may not be reversed with treatment. While acute asthma involves airway hyper-responsiveness, the chronic form involves certain structural changes called airway remodelling. This contributes to the irreversible element of airway obstruction that is seen in some patients.

IPF is a disease of the lung's lower airways. It is featured by a progressive limitation of airflow, and this obstruction is increased when the disease aggravates. The pathological indications of IPF are pulmonary emphysema (destruction of the lung parenchyma and the air sacs), respiratory bronchiolitis (inflammation of the airways) and inflammation of the parenchyma. In IPF, eosinophils, neutrophils, macrophages and $CD8^+$ T cells can be found in the lung compartments (5). There is an increase in the production of mediators and cytokines, like TNF-α, IL-8, Leucotriene B4 (LTB4), Endothelin-1 (ET-1), and adhesion molecules like ICAM-1 (6). Upregulation of the above-mentioned mediators during exacerbation of the disease may be via activation of transcription factors like NFκB and activator protein-1 (7). Acute aggravation of the disease is linked with an increase in inflammation of the airways and increase in oxidative stress (5, 6). These factors are the main reasons for the mortality and morbidity associated with this disease (5, 6). Existing methods of treatment for IPF are inadequate (8).

Inflammatory Bowel Diseases (IBD) are a group of complex and multi-faceted diseases, caused by a variety of reasons (9). Two major IBDs are ulcerative colitis (UC) and Crohn's disease (CD). 'Intermediate colitis' includes chronic infectious, non-infectious and undiagnosed IBD, is gaining prevalence in developing countries (10).

Peritonitis is a systemic inflammation of the peritoneum (membrane lining the peritoneal cavity), usually caused by some trauma suffered during surgery or by some unknown immunological causes. Usually, this disease cannot be treated with antibiotics and steroids only. It can be fatal as it progresses rapidly by uncontrolled inflammation. The mucus membrane is usually the first point of defense of the body. In peritonitis, the mucus membrane is affected, leading to the disease.

2.6 Origin of the Project

2.6.1 Unmet Needs in These Inflammatory and Degenerative Diseases

For all the inflammatory diseases mentioned above, especially asthma and IPF, there are a lot of unmet requirements. The most important of these needs include the need for efficient anti-inflammatory therapy, since existing therapies can neither arrest nor cure the diseases completely. Other needs include the need to find methods to prevent and control the aggravation of IPF and asthma, and to develop therapies for refractory asthma, where patients suffer from persistent symptoms despite administration of medication. Thus, it is imperative to develop drugs that can address the underlying mechanisms of the diseases. Advances in understanding the mechanisms of a disease, both cellular and molecular, can be helpful in drug development.

There is no absolute cure for asthma and IPF, and current therapy options have a number of limitations, and are further impeded by aggravation of the diseases. A number of oral treatments are available, but all of them are associated with adverse side effects. In many of these cases, the adverse effects exceed the beneficial effects of the oral drugs, thus limiting the success of IPF management (11).

Current medical therapy for IBD includes drugs like sulfasalazine, 5-amino-salicylates, antibiotics and corticosteroids. Although these can restrict the disease, they have serious side effects and can make the host more susceptible to other infections, auto-immune disorders or even multi-drug resistance. This therapy system leads to an increase in drug use, the consequent escalation of drug-related toxicity and development of resistance, and ultimately to unbridled exploitation of the ecosystem (12).

The main impediment to research and drug discovery in the field of IBD is the lack of knowledge about the etiology of the disease, and the necessity to use novel methods to control or prevent the disease. This lacuna in the field of drug discovery for inflammatory diseases is the basis for this project.

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2.7 Rationale of the Study Supported by Cited Literature

2.7.1 Nano-formulation for Targeted Drug Delivery of Anti-inflammatory Molecules

For efficient drug delivery, we require a system which is highly biocompatible and is able to penetrate the organs of interest to deliver the payload, and for which a proof of concept exists demonstrating its efficiency using some model system. On literature survey, we find that a polymer-based nano-material exists which has these properties. Polyesters such as poly-lactic-co-glycolic acid (PLGA) have been used for nano-formulation since they are biodegradable, biocompatible, have versatile degradation kinetics (Park 1995) and have been approved by the U.S. Food and Drug

Administration for pharmaceutical applications. Such materials have been demonstrated to be favourable for hydrophobic molecules and for the bioavailability of poorly water-soluble agents (Anand et al. 2007). Since several known natural products (like Curcumin, etc.), as well as bioactive agents, used in inflammation fall in this category, such delivery systems are likely to be highly useful. The method of preparation of such nano-delivery materials is relatively easy and the introduction of variations is possible to change its characteristics, which is a requirement to optimize the properties. The material for preparations is commercially available.

Tsai et al. (2011) have shown how curcumin, which is an anti-inflammatory and anti-cancer molecule but sparingly soluble in water, can be effectively delivered using nanoparticulation to increase its circulation time in the body, which is one of the main hurdles to its efficacy. They have shown that in distribution studies, curcumin could be detected in the evaluated organs, including liver, heart, spleen, lung, kidney and brain. Curcumin-nanoparticles (C-NPs) were found mainly in the spleen, followed by the lung. Formulation led to a significant increase in the curcumin concentration in these organs with increases in the AUC (area under the curve in a plot of concentration of the drug in the blood plasma against time), $t_{1/2}$ (the half-life) and MRT (mean residence time of the drug molecule inside the body) of curcumin, though this was not apparent in the heart. Curcumin and C-NPs have the ability to cross the blood–brain barrier (BBB) to enter brain tissue, where it was concentrated chiefly in the hippocampus. Nanoparticulation caused a significant elongation of the retention time of curcumin in the cerebral cortex (increased by 96%) and hippocampus (increased by 83%).

2.7.2 Drug-Nanoparticles Encapsulation Procedures

Tsai et al., in 2011, prepared curcumin encapsulated in PLGA nanoparticles, using a technique of high-pressure emulsification-solvent evaporation. They dissolved 50 mg of PLGA and 5 mg of curcumin in 1.25 ml of dichloromethane. This constituted the oil phase, which was then added to 10 ml of the aqueous phase. The aqueous phase was made of 2% poly-vinyl alcohol (PVA) and 20% sucrose. This was then homogenized at 28,000 rpm for 10 min, forming an emulsion. The emulsion was filtered twice using a 0.1 μm filter at a pressure of 5 kg/cm^2. The resulting filtrate is the curcumin nanoparticle (C-NP). These C-NPs were then incubated overnight, while stirring at 500 rpm, to air-dry the organic solvent to evaporation (Jaiswal et al. 2004). This yielded the final C-NP solution.

According to Tsai et al. (2011), the size of the resultant particle was approximately 165 nm (Table 2.1; Tsai et al. 2011), which is suitable for the purpose of encapsulation.

Tsai et al. also demonstrated in vitro how the curcumin is released from the PLGA nanoparticles (Fig. 2.1 of Tsai et al. 2011). The release profile shows a biphasic pattern, with an initial burst release in 12 h, when 59.0 ± 6.7% of the curcumin is released. After this, it is gradually released, till up to 6 days, when 89.7 ± 1.4% is released. As anti-inflammatory treatments are often long-term, systems like this are useful.

The system of drug delivery using nanoparticles increases the $t_{1/2}$, as well as the AUC, as shown in Table 2.2 (Tsai et al. 2011).

Such nano-delivery systems are worth exploring as they have the potential to give good results in studies to understand the anti-inflammatory effects of chosen drug molecules.

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Table 2.1 Properties of C-NPs before and after condensation (mean ± SD; $n = 3$)

Groups	Particle size (nm)	Polydispersity index	Zeta potential (mV)	Encapsulation efficiency (%)
Original	163 ± 8.1	0.053 ± 0.021	−12.5 ± 2.8	46.9 ± 8.2
Condensation	168 ± 9.0	0.085 ± 0.011	−14.1 ± 1.9	44.8 ± 3.6

Ref: Tsai et al. (2011)

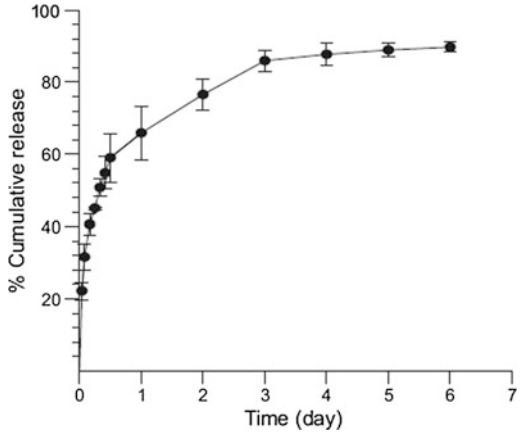

Fig. 2.1 In vitro release profile of C-NPs (Tsai et al. 2011)

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2.8 Hypothesis

Pharmacological intervention has to be judiciously designed to enhance efficacy and tissue specificity while minimizing toxicity and side effects. To this end, a strategic drug designing approach and validation/modulation/customization of the same in tandem in a biological platform is necessary. Clean green approach to drug discovery to ameliorate inflammation and degeneration in diseases such as the ones mentioned above is necessary to optimally leverage available resources in chemistry and biology.

2.9 Key Questions

1. Screening of certain nanomaterials for 'druggability' while eliminating pro-inflammatory properties;
2. Screening such nano-drugs for efficient tissue-specific delivery (lung, intestine and peritoneum);
3. Screening nanoscaffolds to minimize inflammation and degeneration and optimize homeostasis and regeneration in situ.

2.10 Current Status of Research and Development in the Subject (Both International and National Status)

Nanomaterials are widely used in cosmetics, foods and medicinal products for improved performance (1). In addition, various nanomaterials are under development for different biomedical applications. However, it is gradually becoming evident that many of the nanomaterials can induce substantial inflammation. For example, it is shown that metal and metal oxide nanoparticles generate oxidative stress that can induce acute pulmonary inflammation and other adverse health effects (2). Moreover, it has been shown that the inflammatory effect of nanoparticles can be modulated by changing surface chemistry (3) and the chemical composition (3), and some

Table 2.2 Pharmacokinetic parameters of curcumin and C-NPs in rat organs following i.v. administration

Organs	Particle	$t_{1/2}$ (min)	AUC (min μg/ml)	MRT (min)
Liver	C	17.6 ± 2.20	9.06 ± 1.55	33.0 ± 1.94
	C-NP	19.8 ± 1.50	71.3 ± 11.7*	35.4 ± 2.40
Heart	C	37.5 ± 9.31	3.03 ± 0.85	57.0 ± 11.1
	C-NP	13.4 ± 1.81*	2.82 ± 1.14	27.1 ± 2.80*
Spleen	C	12.6 ± 2.46	5.71 ± 1.14	25.6 ± 2.65
	C-NP	14.2 ± 0.96	1213 ± 102*	28.1 ± 1.18
Lung	C	13.2 ± 1.16	8.98 ± 1.82	26.1 ± 1.11
	C-NP	15.1 ± 0.48	196 ± 23.1*	30.6 ± 0.36*
Kidney	C	19.7 ± 1.13	12.0 ± 0.88	35.4 ± 1.38
	C-NP	48.8 ± 0.81*	16.0 ± 1.78	75.7 ± 1.22*
Brain	C	9.20 ± 1.84	4.04 ± 0.22	20.4 ± 0.95
	C-NP	14.8 ± 1.31*	5.68 ± 1.44	27.1 ± 2.04*

$T_{1/2}$ half-life; *AUC* area under the concentration-time curve; *MRT* mean residence time; *C* curcumin; *C-NP* curcumin-nanoparticles; (mean ± SEM; $n = 4$)
*$p < 0.05$, vs curcumin
Ref: Tsai et al. (2011)

nanomaterials even show anti-inflammatory effect (4). Thus, evaluation of inflammatory effect of potential nanomaterials, ways to suppress inflammation and to investigate their anti-inflammatory property are important for translational research. Furthermore, our laboratory has synthesized a library of nanomaterials useful as bioimaging probe and as drug delivery carriers (5). However, their inflammatory effects are under investigation.

Tissue engineering represents an attractive option for regeneration of several organ systems (6). Nanoporous 3D scaffolds are attractive materials for tissue engineering as the structures can be modulated as per requirements (7). However, their application as a scaffold for lung regeneration is not well explored. There are only few reports that use poly-lactic acid-based foam (8) and Gelfoam sponge (9) as a scaffold for lung regeneration, and these have shown a partial success. In addition, graphene and porous carbon/silica based new generation scaffolds can be explored for lung regeneration.

At the national level, several research groups focus on nanoparticle synthesis and characterization (10), and a relatively less number of groups are working on biomedical application of nanoparticles (11, 12). The synthesis and characterization involve variety of materials such as noble metal, metal oxide, semiconductors, carbon and Au cluster. Applications involving nanomaterials include gas sensing (11a), protein detection (11c), cell labelling (11g), electrochemical detection (11j) and drug delivery (12). These nanoparticles include quantum dot based fluorescence imaging, magnetic nanoparticle-based MRI, Au cluster based fluorescence imaging and carbon nanoparticle based fluorescence imaging (10g, 10h, 11).

In the past 10 years, work has been done on biomedical applications of nanoparticles, specifically on the development of cellular imaging nanoprobes (13). Various nanoprobes of 5–100 nm size having different surface charge and surface affinity molecules have been synthesized, which have been used for detection and imaging of molecules in cells. It has been observed that cellular interaction and entry of nanoprobe depends on particle hydrodynamic diameter, particle surface charge, particle shape anisotropy, and particle hydrophobicity. It has also been found that there exists an optimum size range of

10–50 nm for which cellular interaction and uptake can be modulated by varying the particle size, surface charge and surface ligand density.

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2.11 The Relevance and Expected Outcome of the Proposed Study

2.11.1 Relevance for Conceiving the Project Proposal

The three disease sectors in total constitute a health hazard condition that leads to progressive scarring and destruction of the lungs, gut and other tissues. The most common of the idiopathic interstitial pneumonias, idiopathic pulmonary fibrosis (IPF) is of increasing prevalence, affecting 5,000,000 people worldwide, with a median survival time of less than 3 years. In regards to onset, incidence and financial impact of IBD, about one-third of all persons with IBD have the onset of their illness before adulthood. The peak age of onset is between 10–30 years and the disease persists for a large part of a person's life. Males and females are affected almost equally. IBD tends to run in families. When one family member has IBD, there is a 15–30% chance that there is another affected family member. It is estimated that almost one million Americans are affected. The incidence of IBD varies from country to country; however, IBD has been increasing worldwide. IBD, in its different forms, affects over 4 million people over the world. In the United States alone, IBD accounts for approximately 1,52,000 hospitalizations each year. Chronic UC is being reported with increasing frequency from various developing countries such as India and China. The incidence and prevalence of chronic UC are well defined in

the industrialized countries, amounting 40–100 cases per 100,000 people of the total population.

2.11.2 Chemistry Part

Development of nanodrugs or nanodrug delivery system in Inflammation and Degeneration

This project aimed at preparing 1–100 nm sized nanomaterials that can be used for detection of inflammation and for delivery of anti-inflammatory drugs. In addition, the inflammatory effects of some useful nanoparticles would also be studied, deriving appropriate designs to lower their inflammatory effects and to investigate the anti-inflammatory roles of any nanoparticles.

The nanoparticles will be prepared and functionalized with appropriate molecules for targeting and imaging of inflammation sites. In addition, anti-inflammatory drug-loaded porous colloidal nanoparticles will be prepared for specific delivery of drugs to inflammation sites. Therapeutic response of drug will be investigated via standard methods.

Development of nanomaterial scaffold for tissue engineering from stem cells and lung regeneration

This project also aimed to develop 3D nanoporous scaffold materials for cell-based tissue engineering and lung regeneration. In the first step, 3D nanoporous scaffold will be prepared using an assembly of different nanomaterials such as graphene, porous carbon and porous silica. The pores and surfaces of scaffolds will be functionalized with affinity molecules for efficient attachment and proliferation of cells. In the second stage, lung cells will be grown on this scaffold. The successful attachment and growth of cells will be optimized by changing the nature and functionality of scaffold. Next, the cells, along with the scaffold, will be injected or implanted in the injured lung. The therapeutic responses of the injured lung and lung disease will be monitored via conventional approaches.

2.11.3 Biology Part

The nanomaterials synthesized shall be used for improving anti-inflammatory properties and anti-degenerative or pro-regenerative properties in the following areas:

(i) As a drug delivery system
(ii) As a drug availability system
(iii) As nanodrugs themselves.

The nanoscaffolds will define culture conditions in tissue engineering and as engraftment scaffold for regeneration.

Expected outcome of the project

Thorough screening of a large library of materials, synthesized in a Chemistry lab, through achievement of Specific Aims # 1 and 2 conducted in a Biology lab and rigorous Stucture–Activity Relationships to further fine tune that library, shall yield a large body of nanomaterials with thorough characterization in the above two important fields of biomedical research namely, inflammation and degeneration, making up more than 80% of all known diseases and exacting a heavy price in the economic burden to the nation. These materials shall form a part of a library of nanobiotechnical tools and may be developed according.

2.12 Preliminary Work Done for Capacity Building for Project Execution

A variety of nanomaterials have been synthesized for this project, including graphene, porous carbon, porous silica and other nanoparticles. Several anti-inflammatory drugs have been loaded into porous carbon or graphene, and their efficacies tested on lung cells. Graphene-based porous nanoscaffold has also been synthesized (Figs. 2.2 and 2.3).

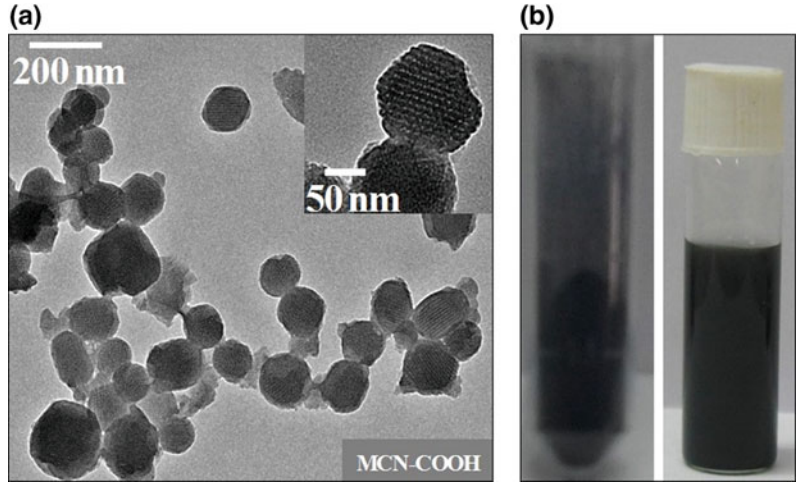


Fig. 2.2 **a** TEM image of porous carbon nanoparticle that we have synthesized. **b** Digital image of solid sample (*left*) and their colloidal solution (*right*) in water

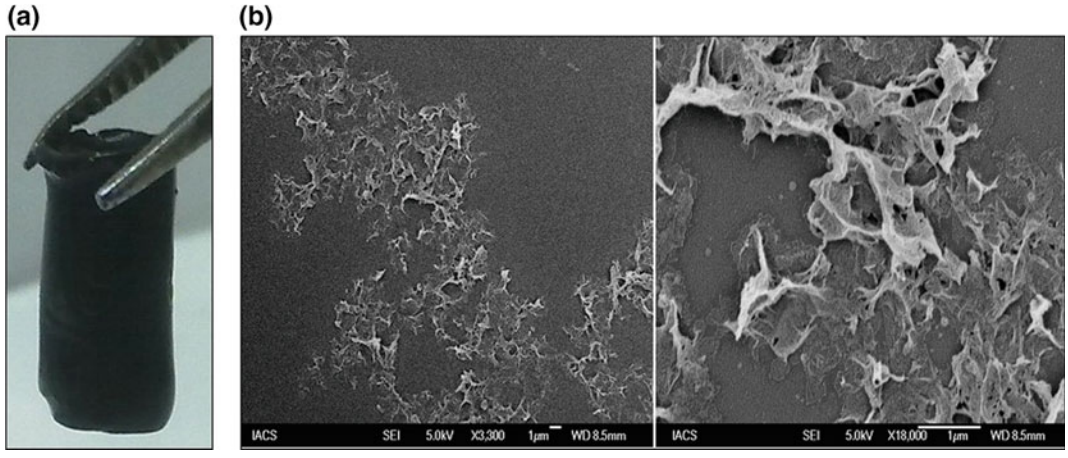


Fig. 2.3 **a** Digital image of graphene-based scaffold we have synthesized. **b** Scanning electron microscopic (SEM) image of scaffold under different magnification

2.12.1 Anti-inflammatory Activities of Nano Particles

To evaluate the anti-inflammatory properties of fisetin-loaded carbon nanoparticles, the following experiments have been done:

1. RAW cells (murine macrophage cell line) and HEK cells (Human embryonic kidney cell line) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS and 1% penicillin-streptomycin. On reaching confluency, they were seeded into the wells of a 96-well plate, and treated with different concentrations (1 μM, 500 nM, 250 nM, 100 nM, 50 nM, 25 nM) of both the drug (fisetin) loaded onto the nanoparticle, and the nanoparticle alone as vehicle. The cells were incubated for 3 hours, and the cytotoxicity of the particles and the drug was assessed using the MTT reagent.

2. On confirming that the nanoparticle itself is not cytotoxic, its anti-inflammatory effects were then assessed. The above-mentioned cell lines were seeded in the wells of a 96-well plate, and treated with 1 µg/ml *Escherichia coli* lipopolysaccharide (LPS) to induce septic inflammation, and by 3% thioglycollate (TG) (aseptic model of inflammation), for the same duration. This was followed by treatment with different concentrations of the drug (1 µM, 500 nM, 250 nM, 100 nM, 50 nM, 25 nM), and assessment of cell viability using MTT assay.

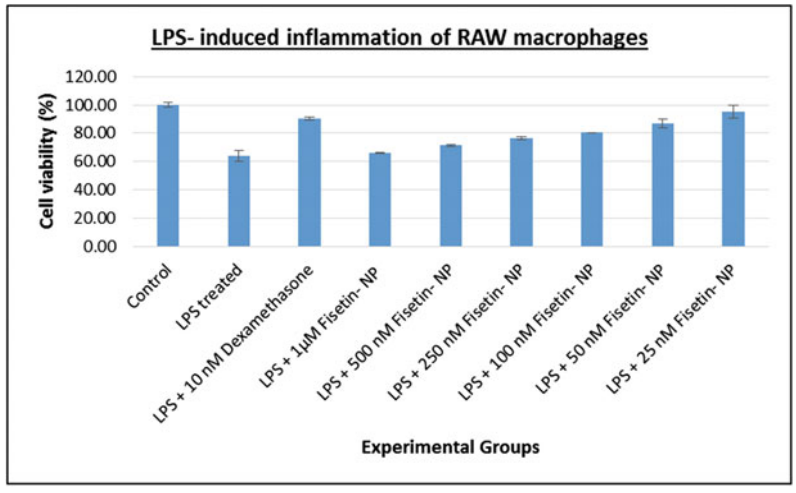
2.12.2 Cell Viability Determination

Cell viability was determined by using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. 5×10^4 cells were seeded per well of a 96-well plate. After 24 h, cells were treated with LPS or TG, followed by various concentrations of drug-loaded nano bodies for 3 hours. Then, the cells were incubated with 5 mg/ml MTT working solution for 3 h at 37 °C, followed by treatment with 100 µl DMSO to dissolve the formazan crystals. Absorbance was read at 570 nm using a microplate reader (Shimazdu), and the cell viability was determined from the absorbance.

2.12.3 Results

LPS-induced inflammation on RAW macrophages

Experimental groups	Cell viability (%)
Control	100.00 ± 1.96
LPS treated	63.94 ± 3.69
LPS + 10 nM Dexamethasone	90.55 ± 1.01
LPS + 1 µM Fisetin-NP	66.22 ± 0.46
LPS + 500 nM Fisetin-NP	71.30 ± 0.61
LPS + 250 nM Fisetin-NP	76.30 ± 0.90
LPS + 100 nM Fisetin-NP	80.39 ± 0.14
LPS + 50 nM Fisetin-NP	86.57 ± 3.09
LPS + 25 nM Fisetin-NP	94.92 ± 4.45

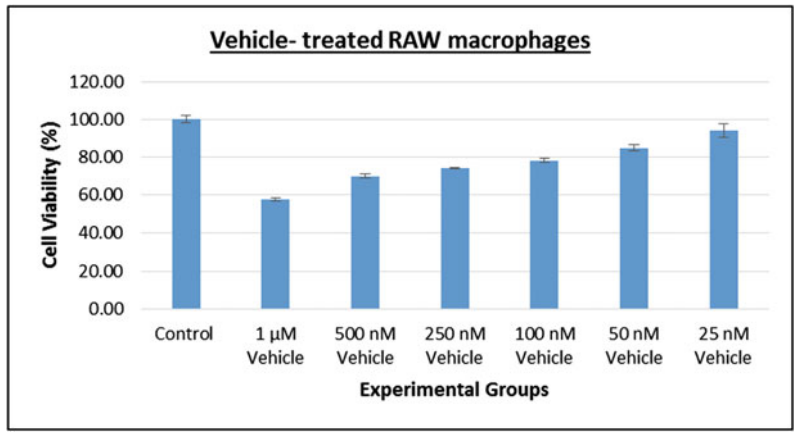


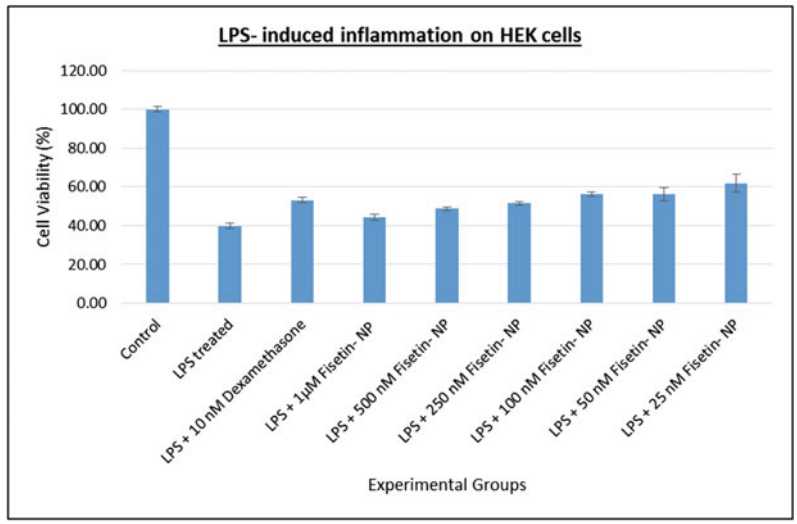
Vehicle-treated RAW macrophages

Experimental groups	Cell viability (%)
Control	100.00 ± 1.96
1 μM vehicle	57.68 ± 0.79
500 nM vehicle	70.03 ± 1.12
250 nM vehicle	74.21 ± 0.34
100 nM vehicle	78.30 ± 1.15
50 nM vehicle	84.84 ± 1.68
25 nM vehicle	94.11 ± 3.65

LPS-induced inflammation on HEK cells

Experimental groups	Cell viability (%)
Control	100.00 ± 1.32
LPS treated	39.83 ± 1.35
LPS + 10 nM Dexamethasone	53.23 ± 1.51
LPS + 1 μM Fisetin-NP	44.35 ± 1.60
LPS + 500 nM Fisetin-NP	48.88 ± 0.93
LPS + 250 nM Fisetin-NP	51.58 ± 0.85
LPS + 100 nM Fisetin-NP	56.28 ± 1.16
LPS + 50 nM Fisetin-NP	56.22 ± 3.30
LPS + 25 nM Fisetin-NP	61.98 ± 4.50



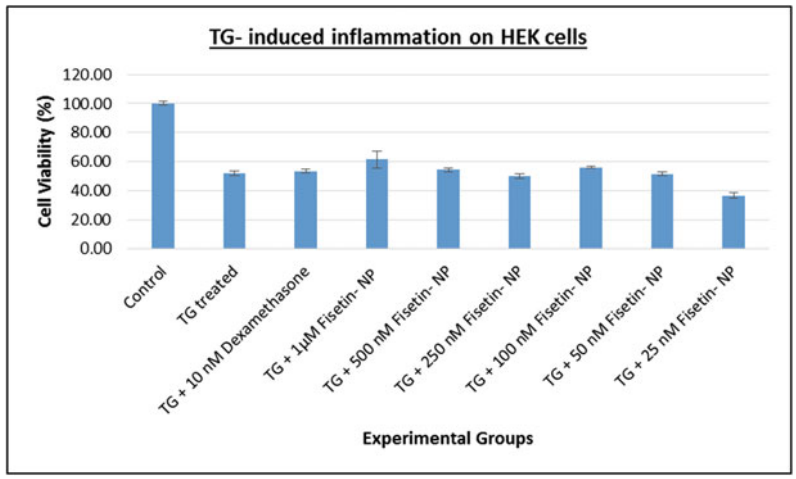


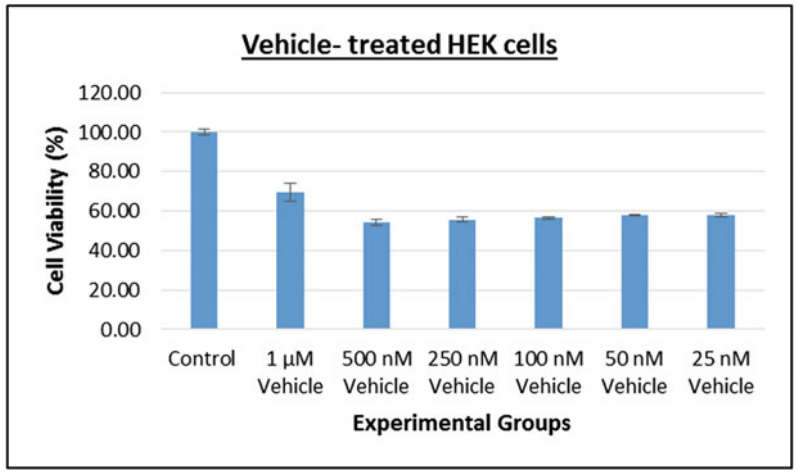
TG-induced inflammation on HEK cells

Experimental groups	Cell viability (%)
Control	100.00 ± 1.32
TG treated	51.70 ± 1.50
TG + 10 nM Dexamethasone	53.46 ± 1.22
TG + 1 μM Fisetin-NP	61.33 ± 5.92
TG + 500 nM Fisetin-NP	54.16 ± 1.47
TG + 250 nM Fisetin-NP	49.93 ± 1.47
TG + 100 nM Fisetin-NP	55.87 ± 0.61
TG + 50 nM Fisetin-NP	51.28 ± 1.34
TG + 25 nM Fisetin-NP	36.42 ± 1.88

Vehicle-treated HEK cells

Experimental groups	Cell viability (%)
Control	100.00 ± 1.32
1 μM vehicle	69.44 ± 4.81
500 nM vehicle	54.34 ± 1.60
250 nM vehicle	55.75 ± 1.43
100 nM vehicle	56.57 ± 0.50
50 nM vehicle	57.81 ± 0.33
25 nM vehicle	57.92 ± 0.71





2.12.4 Conclusion

The basic studies have revealed that this carbon nanoparticle is not itself cytotoxic to cells, as shown by the studies on the murine RAW macrophage cell lines as well as on human embryonic kidney cell line. Its efficacy as an anti-inflammatory molecule after drug loading has shown a significant dose-dependent pattern. For example, it can carry the loaded fisetin to the murine macrophage cells effectively at a very low dose, i.e. at a concentration of 25 nM, this molecule can restore the cell viability of LPS-treated cells by about 92%, but it is not as effective at higher concentrations. This is also seen in the HEK cells, where 25 nM of the drug is successful in restoring the viability of LPS-treated HEK cells by about 63%. In the aseptic model of inflammation, induced by 3% TG, the observation was different. Fisetin loaded onto nanoparticles have succeeded in restoring the viability of TG-treated cells by about 61.50% at a concentration of 1 μM, but only by 36.50% at a concentration of 25 nM. Thus, this type of molecule can deliver the anti-inflammatory drugs inside the cell in a dose-dependent manner, and its efficacy also depends on the type of antigen.

2.13 Specific Objectives

I. **Development of nanodrugs or nanodrug delivery system in Inflammation and Degeneration**

In this project, we will prepare 1–100 nm size nanomaterials that can be used for detection of inflammation and for delivery of anti-inflammatory drugs. In addition, we will study the inflammatory effect of some useful nanoparticle, deriving appropriate designs to lower their inflammatory effects and to investigate the anti-inflammatory roles of any nanoparticles.

The nanoparticle will be prepared and functionalized with appropriate molecules for targeting and imaging of inflammation sites. In addition, anti-inflammatory drug-loaded porous colloidal nanoparticle will be prepared for specific delivery of drugs to tissue-specific inflammation sites. Therapeutic response of drug will be investigated via standard methods.

II. **Development of nanomaterial scaffold for tissue engineering from stem cells and lung regeneration**

In this project, we will develop 3D nanoporous scaffold materials for cell-based tissue engineering and lung regeneration. In the first step, the 3D nanoporous scaffold will be prepared using an assembly of different nanomaterials such as graphene, porous carbon and porous silica. The pores and surfaces of the scaffold will be functionalized with affinity molecules for efficient attachment and proliferation of cells. In the second stage, lung cells will be grown on this scaffold. The successful attachment and growth of cells will be optimized by changing the nature and functionality of the scaffold. Next, the cells, along with the scaffold, will be injected or implanted at injured lung. The therapeutic responses of injured lung and lung disease will be monitored via conventional approaches.

III. **Biological validation of nanomaterials derived in I and II**

A. Standardization of dosage and formulation of nanodrug/nanovehicle using in vitro models of inflammation and regeneration
B. Characterization of tissue-specific delivery, SAR for nanovehicles for lung, intestine and peritoneum specific delivery
C. Optimization of nanoscaffold for expeditious regeneration of the lung.

2.14 Work Plan

Synthesis of nanodrug and nanodrug delivery system: In this part of the project, we will prepare water-soluble nanomaterials which will act like anti-inflammatory drug or act as nanocarrier for anti-inflammatory drugs. Nanomaterials will be appropriately functionalized for targeted and responsive drug delivery. Nanoparticles such as magnetic iron oxide, fluorescent quantum dot, and fluorescent carbon/silicon will be synthesized using established organometallic approaches. Next, these nanoparticles will be converted into water soluble and amine/carboxylate-terminated nanoparticle via established polymer coating approaches.

We will synthesize mesoporous carbon nanoparticle (MCN) via hydrothermal treatment of a mixture of phenol and formaldehyde based precursor and pluronic surfactant. Next, particles will be carbonized at 700 °C under inert atmosphere. At this stage, surfactants/polymer will be removed, leaving pure and mesoporous carbon particles. The mesoporous carbon will be further oxidized by HNO_3 to generate carboxyl acid functional groups.

Porous silica particles will be synthesized following conventional hydrolysis of silane (e.g. tetraethoxysilane, aminopropyltrimethoxysilane, etc.) in acidic or basic media using surfactant templates. Then surfactants will be removed using appropriate solvent. The porous silica will be appropriately functionalized for targeted and responsive drug delivery.

Synthesis of nanomaterial scaffold: In this part of the project, we will focus on the synthesis of nanomaterial based porous scaffold. Specifically, carbon- and silicon-based nanomaterials, such as graphene, porous nanocarbon, and colloidal silica, will be used for the synthesis of scaffold materials. For example, graphene-based porous scaffold will be synthesized from colloidal graphene oxide. The colloidal graphene oxide will be synthesized from graphite powder via established Hummer's method and then ascorbic acid-based reduction will be used to transform them into porous graphene scaffold. Similarly, porous silica-based scaffold will be synthesized and appropriately functionalized for cell attachment and proliferation.

Testing of inflammatory property: In this part of the project, we will focus on cell-based performance study of nanodrug and nanodrug delivery systems towards anti-inflammation.

A. **In vitro screening for anti-inflammatory properties**

Murine macrophage-like RAW 264.7 cells were cultured in DMEM medium with 10% (v/v) FBS and 1% Pen-Strep in 5% CO_2 at 37 °C. At a density of 5×10^4 cells/well, various concentrations (1 μg/ml, 500 ng/ml and 200 ng/ml) of LPS and 3% thioglycollate were added with or without nanodrugs/nanovehicles. After suitable incubation, the following inflammatory parameters were assessed: proliferation, cytotoxicity, oxidative and nitrosative stress by the following assays-cell viability determination using MTT assay, measurement of super oxide radicals by NBT reduction assay, and NO estimation by Griess reagent.

B. **In vitro screening for cell/tissue regeneration**

HEK cells were cultured in DMEM with 10% FBS and 1% Pen-Strep, and incubated with LPS or PMA in suitable doses and times of incubation, and pre- or post-incubated with the nanodrugs/nanovehicles. Regenerative potential was assessed by CFU-c assay in matrigel (with IMDM and supplemented with stem cell factor) (Fig. 2.4).

C. **In vivo validation for disease amelioration using preclinical disease models of inflammation**

Murine models will be used for investigating the efficacy of the nanoparticles screened and fine-tuned through rigorous SAR in sections A and B. Detailed patterns of cellular traffic and identification and characterization of molecular switches operative for the

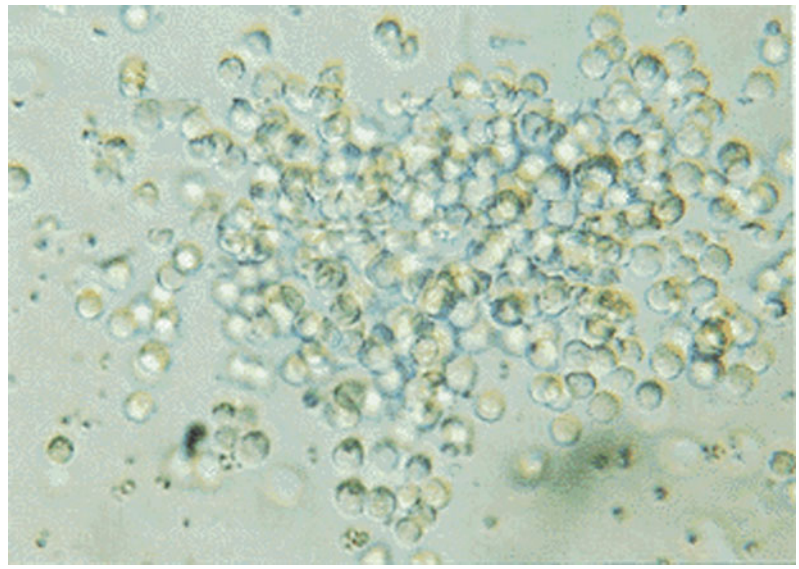


Fig. 2.4 CFU-c assay of bone marrow

- Onset
- Development
- Maintenance
- Exacerbation of the diseases will be chartered (both in context with inflammation as well as tissue-specific degeneration).

In vivo murine models

Treatment protocols and downstream assays for the above preclinical models

From the data of various experiments we know that mice do not spontaneously develop asthma; for this reason, to investigate the processes underlying the asthma, an artificial asthmatic-like reaction has to be induced in the airways of the experimental animals. The BALB/c mouse strain is the most commonly used strain to study asthma, as they show a good Th2 response. For the chronic model of the disease, the C57BL/6 strain is more preferred. So these two strains are used, and when required, knockout strains will also be generated from these.

(I) **Acute asthma model**: Asthma will be induced in mice using ovalbumin (OVA). 100 μg OVA, complexed with 200 μl aluminium sulphate, will be administered by intraperitoneal injection on day 0. On days 8, 15, 18 and 21, mice will be anesthetized and challenged with 250 μg of OVA (day 8) and 125 μg OVA (days 15, 18 and 21) intratracheally. The control group will be treated with normal saline with aluminium sulphate (1).

(II) **Chronic asthma model**: This model also makes use of OVA. Mice will be sensitized with 50 μg OVA in 0.5 mg aluminium hydroxide by s.c. injections on days 0, 7, 14, and 21, and then challenged with OVA (20 μg per mouse) intranasally on days 23, 25, and 28, followed by additional intranasal challenges with OVA twice a week for 8 week. Control mice will be administered PBS instead of OVA for sensitization and challenges (1).

(III) **IPF model**: Mouse model of idiopathic pulmonary fibrosis (IPF) will be created with a single i.t. dose of 0.075 U/ml of bleomycin in 40 μl saline on day 0. The mice will be sacrificed 14 and 21 days later. One week after bleomycin administration, mice have been found to develop marked interstitial and alveolar fibrosis, detected in lung sections by Masson's trichrome stain. Cell populations and

subsets were identified and quantified by Flow Cytometry (FCM), and total and differential count of hematoxylin and eosin (HE)-stained cytospin smears of single cell suspensions show loss of type I and type II alveolar epithelial cells and influx of macrophages (2).

Ancillary assays for I-III

Bronchoalveolar lavage fluid (BALF) and lung tissue collection

After the mice are sacrificed, various tissues are collected. For asthma and IPF, BALF and lung tissue are the most important. Bronchoalveolar lavage fluid (BALF) will be pooled after three washes with saline (0.5 ml each). Total and differential cell counts will be done, and BALF supernatants will be stored at −70 °C for further evaluation. Right lungs will be snap-frozen, and left lungs will be perfused with 4% paraformaldehyde to preserve the pulmonary structure, fixed in 4% paraformadehyde, and paraffin-embedded sections prepared for histological analysis.

Assessments of cell viability, cell number and cell shape and size

Specific cell counter-cum image analyzer will be used to count and assess the number of cells collected from the BALF, bone marrow and spleen and their viability and shapes are observed.

Determination of ROS and RNS levels

To detect the ROS and RNS following methods will be applied:

1. Assessment of myeloperoxidase (MPO).
2. Thiol detection method.
3. Glucose Assay.
4. Nitrate and nitrite estimation: using Greiss reagent.
5. Flow cytometric study to detect ROS production: using dihydrorhodamine 123 and 2, 7-dichlorofluorescein diacetate.
6. Electron spin resonance spectroscopy: to assess hydroxyl radical formation.
7. Immune cytochemical methods to detect nitrotyrosine

Cytochrome c reduction or nitro blue tetrazolium (NBT) reduction method (used for leucocyte NADPH oxidase activity)

Cytochrome c reduction and NBT reduction both can accurately predict whether ROS have been produced by leukocytes. The two most commonly used reagents to detect superoxide anion radicals are NBT and ferricytochrome-c (Cyt). Superoxide formed by electron transfer from a donor to molecular oxygen can be quenched by NBT and Cyt. These reagents are reduced to diformazan and ferricytochrome-c, respectively. Superoxide radical can be detected when the addition of superoxide dismutase (SOD) leads to the reduction in production of diformazan from NBT, or to the complete absence of ferricytochrome-c from cytochrome c. Thus, cytochrome c reduction helps measure superoxide released extracellularly only, whereas NBT has no such stringency.

Biochemical assessments of ROS and RNS regulating enzymes

Different enzymes, like superoxide dismutase (SOD) and catalase regulate the reactive oxygen and nitrogen species. These will be biochemically evaluated, along with the quantitative measurements of ascorbic acid, which is an antioxidant. SOD, an essential antioxidant that catalyzes superoxide radicals to hydrogen peroxide, is present in three forms in the mammalian system: as (i) the copper-zinc superoxide dismutase (Cu-ZnSOD) located in the cytosol, (ii) the manganese superoxide dismutase (MnSOD) primarily a mitochondrial enzyme and (iii) extracellular superoxide dismutase (EC-SOD) found outside the plasma membrane. In asthmatic lungs, activities of both the extracellular and intracellular SOD is reduced, which may be due to inactivation of the SOD or nitration of SOD isoforms, leading to loss of its activity. Catalase converts hydrogen peroxide to water and oxygen.

NO estimation

Nitric oxide (NO), a relatively stable free radical, is increased in exhaled air of asthmatic individuals. There is almost 3 times more NO in the lower airway and exhaled breath of asthmatic

people, as compared to healthy individuals. This increase in the NO production plays a role in the pathogenesis of asthma. Exhaled NO levels are inversely correlated with airflow parameters in asthmatic patients.

Immunoblots

After extraction and quantification of proteins from the lung, spleen and lymph nodes, 10% (w/v) homogenate will be prepared for western blot. Equal amounts of proteins (50 μg), determined by Folin's method, will be loaded on SDS PAGE (10%) for electrophoresis. Thereafter, proteins will be transferred electrophoretically to nitrocellulose membrane (NC) overnight at 4 °C. NC will then blocked for 60 min with 3% BSA in Tris buffered saline (TBS), and then incubated with primary antiserum, diluted in the blocking buffer, for one hour. Then membranes will be washed for 10 min (3 washes) in TBS-Tween 20. Then NC membrane will be incubated with secondary antibody conjugated with serum immunoglobulin (anti-rabbit IgG HRP) (1:500) for 30 min and then again washed in TBS for 10 min (3 times). Signals will be detected using an ECL kit. Blot for each protein will be repeated for three times. The densitometric analysis of the blots will be performed by scanning and quantifying the bands using an image analysis software. The densitometric data will be plotted as mean ± SEM. A pre-stained multicolor broad range marker will be also run along with sample proteins to clarify the position of bands obtained.

Assessment of GLUTs

To correlate the inflammatory pathways with the metabolic alternations, targeting the glucose transporters (GLUTs) that help in the transportation of main energy fuel (glucose) inside a cell, is very essential. As reported, GLUT 1 plays a crucial role in mouse lung. Thus, after allergen treatment, there may be alterations in the expression of GLUT1. These alterations may be targeted for therapy. Research has also shown that GLUT4 and GLUT8 can also be targeted.

Expressional profiling of MUC5AC

One of the major features of airway inflammatory diseases, like asthma, are hyperproduction of Goblet cells and mucin. The expressional profiling of MUC5AC is an important biomarker for both the acute and chronic forms of asthma.

Expressional profiling of NOS2 protein

Nitric oxide synthases (NOS) are enzymes responsible for the synthesis of endogenous NO. These enzymes, present in three isoforms NOS1 (neuronal/nNOS), NOS2 (inducible/iNOS) and NOS3 (endothelial/eNOS) convert L-arginine to nitric oxide and L-citrulline with the help of oxygen and NADPH. Airway epithelial cells of the healthy lung are a major source of NOS2. At the transcriptional level, murine NOS2 protein is regulated by a combination of the interferon γ (IFNγ) activation of Janus kinase (JAK)/signal transducer and activator of transcription1 (STAT1) pathway with interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α) and/or endotoxin-mediated activation of nuclear factor κB (NF-κB).

Expressional profiling of cell adhesion molecules

Previous studies have shown the involvement of various families of adhesion molecules, viz. a4b1, b2, VCAM-1 and selectin, facilitate leukocyte transmigration, adherence to parenchymal cells, and Th2 response, in inflammatory diseases like allergic asthma. Assessment of these key cell adhesion molecules in response to high ROS and RNS level in allergen treated animal model in relation to metabolic alterations is a key goal to investigate in this project.

Expressional profiling of cyclooxygenase 2 (COX-2) as well as lipoxygenase (LOX)

As the experimental data suggests, Cyclooxygenase 2 (COX-2), as well as lipoxygenase (LOX), are two enzymes involved in inflammation, so the expressional profiling of these two molecules is a key feature in this pathway.

Expressional profiling of pro-inflammatory molecules

TNF-α is a pro-inflammatory cytokine that activates various signal transduction cascades, improving the insulin sensitivity and glucose homeostasis, advocating the fact that metabolic,

inflammatory and innate immune processes are coordinately regulated. So our aim is to correlate the expression of TNF-α along with the metabolic alterations in case of inflammatory responses in asthma.

TGF-β is another important mediator involved in tissue remodelling in the asthmatic lung. TGF-β is believed to play an important role in most of the cellular biological processes leading to airway remodelling, involving itself in epithelial changes, sub-epithelial fibrosis, airway smooth muscle remodelling, and microvascular changes. As in the lungs, almost all structural immune and inflammatory cells are recruited to the airways during an exacerbation of asthma. These cells are able to express and secrete TGF-β1. In healthy individuals, the airway epithelium seems to be the major site of TGF-β1 expression. However, other structural cells in the airways, such as fibroblasts, vascular smooth muscle cells, endothelial cells, and ASM cells, are also potential sources of this cytokine. Here, we have aimed to find out the molecular switch which correlates the expression of these cytokines with the metabolic alternations at the genomic levels.

Use of two-dimensional electrophoresis system to assess the novel proteins

Two-dimensional electrophoresis is a useful tool to analyze the protein pattern of various and complex biological materials to connect the genome to the proteome and to provide valuable information on various protein expressions by which we can get a picture of some novel protein molecule involved in this pathway, except the traditional ones.

Fluorescein-activated cell sorter (FACS) analysis

Hemolzed peripheral blood (PB) cells, bone marrow (BM) cells, bronchoalveolar lavage fluid (BALF) cells, cells from the lung parenchyma, spleen cells, and cells from the lymph nodes (cervical/CLN, axillary/ALN, and inguinal/ILN) will be analyzed on a FACS Calibur. The cells will be stained with antibodies conjugated to various fluorochromes. Markers for different cells, like CD45, CD3, CD4, CD8, B220, CD19, CD21, GR-1, and F4/80, and also for different molecules, like α4- integrin, IgM, selectin and VCAM, are used.

Measurement of lung cytokines by cytometric bead array

Levels of cytokines in lung tissues will be assessed using Th1/Th2 cytokine and IL13 cytometric bead assay (CBA). Lung tissues will be homogenized in lysis buffer (PBS, with 1% Triton X-100, 1 mM PMSF and protease inhibitor cocktail). The total protein of the sample will be estimated using suitable methods. The levels of the different cytokines, as well as that of IL13, will be assessed with the CBA kit, where the levels will be expressed as picograms of cytokine per milligram of total protein.

Quantitative assessments of specific protein molecules

To estimate certain proteins, ELISA may be done. TNFα, MIP2 and IFNγ in the BALF and serum will be estimated using ELISA.

Histological assessments

1. Hematoxylin and eosin staining for lung tissues: Paraffin-embedded sections of lung tissue will be stained with hematoxylin, and counter-stained with eosin, to study the differences in the architecture of the lung.
2. Masson's trichrome staining for collagen fibres: This method is used to detect the amount of collagen deposited in the airways. Collagen is an important marker for airway remodelling.
3. Alcian blue/PAS for acid and neutral mucopolysaccarides.
4. Toluidine blue stain for mast cells.
5. Wright's stain for differential blood cell counting.

Immuno-histochemical techniques

Immuno-histochemical methods will be applied for the localization of GLUT1, GLUT4, GLUT8, MMP9, MMP12, TNF-α, TGF-β1, NOS2, nitrotyrosine, MUC5AC, VCAM1, selectin and MIP-2.

Collection of airway smooth muscle cells and its culture

Collection and treatment of airway smooth muscle (ASM) cells will be done by the method proposed by Willems et al. (2011). After collecting the ASM, these will be treated by TNF-α, TGF-β1 and allergen to detect the different ROS and RNS biomarkers, along with the measurement of GLUT1, GLUT4, GLUT8, NOS2, as well as the screening of different cytokines to correlate them in an in vitro system.

Collection of alveolar macrophages and their treatment

Collection and treatment of macrophage cells from the lung will be done by the method proposed by Willems et al. (2011). Such cells will be similarly treated and assessed mentioned above.

Use of specific inhibitors

Specific inhibitor molecules will be used for this purpose to investigate our goal of which few inhibitors are enlisted below:

1. NEM: NADPH oxidase inhibitor
2. S1: MMP12 inhibitor
3. TIMP1: endogenous inhibitor of MMP9
4. Di deoxy glucose: specific inhibitor of glucose metabolism
5. Mercaptoethylguanidine (MEG): inhibitor of iNOS

Use of RNAi techniques

Specific siRNAs will be used for this purpose to correlate the metabolic alternations with the inflammatory responses in asthma. Here some siRNAs are enlisted which will be extensively used in this purpose: GLUT1, GLUT4, GLUT8, MMP9, MMP12, NOS2.

(IV) **Septic and aseptic peritonitis model** Mice are injected intraperitoneally with 1 ml of 3% thioglycollate (TG), and sacrificed by cervical dislocation at intervals. The peritoneal cavity is lavaged with 5 ml of ice-cold PBS, containing 5 mM EDTA, to obtain the peritoneal fluid (PF). The number of cells is counted, and various other assays, like chemokine estimation and FACS, are performed (3).

Testing of nanomaterial scaffold for tissue engineering: Here we will focus on the growth of lung cells in nanomaterial scaffold, and then use them for lung regeneration.

Cell-based lung repair or regeneration is unquestionably the most promising agenda of regenerative medicine (Bishop AE, 2006, Expert Opin Biol Ther, 6(8): 751–8; Gomparts BN, Annu Rev med, 2007: 58: 11–24). Regulation of tissue injury and repair is a carefully orchestrated host response to eliminate the causal agent and subsequently, restore the tissue's integrity. A range of coordinated host responses work together to restrict the structural damages and initiate repair of the injured tissue. Studies are underway to understand the mechanisms that regulate host responses to tissue injuries. Chronic disease states are associated with anomalies in the host's repair response. These anomalies may lead to excess deposition of extracellular matrix (ECM) that causes fibrosis in the organs.

Embryonic stem cells (ESCs) can differentiate, under in vitro conditions, into cells of all the three germ layers (Evans MJ, 1981, Nature, 292: 154). Different methods have been used to guide these cells to differentiate into specific lineages, like endothelial (Lisheng, W, 2004, Immunity, 21: 31–41) and hematopoietic (Chang K-H, Blood, 108(5): 1515–23) lineages. Mostly mouse embryonic cells have been used for *in vitro* differentiation studies (Van Vranken, Tissue engineering, 2005, 11(7/8): 1177; Samadikuchaksaraei A, 2006, 330: 233, Meth Mol Biol; Ali NN, Tissue Eng, 2002, 8(4): 541; Rippon, NJ, 2006, Stem cells, 24: 1389; Nishimura Y, Stem Cells, 2006: 24: 1381; Qin, M, Stem cells, 2005, 23: 712). In few cases, human ESCs have been used (Samadikuchaksaraei A, 2006, Tissue Eng. 12(4): 867).

Tissue engineering of stem cells of embryonic, foetal and adult origin into lung lineage specific cells will be done in the following ways:

(a) Guided endodermal differentiation with or without nanoscaffolds to see if they improve the time taken for such differentiation;
(b) Whether differentiation of one or more types of cells is preferentially orchestrated by varying composition of such nanoscaffolds;
(c) Whether in xenograft transplantation experiments, nanoscaffold yield better homing and engraftment of cells differentiated by nano-scaffold-aided ex vivo tissue directed engineering.

Detailed methodology for tissue engineering and engraftment in in vivo model of lung regeneration

Materials

Cells: Undifferentiated H7 from WiCell (Enver T, 2005, Human Mol Genet, 14(21): 3129–40) Wisconsin, MA were cultured following established protocol (LaFlamme M, 2005, Am J Pathol, 167(3): 663) and differentiated in SAGM from Clonetics without triiodothyronine (T3) and retinoic acid (RA) based on observations from Ali, N, 2002, Tissue Eng, 8(4): 2002 and Rippon HJ, 2004, Cloning and Stem cells, 6 (2): 49) with our own modifications. GFP + mouse ES cells on a C57Bl/6 background were also similarly cultured. For H7 cell culture, cells were either grown on MEF feeder prepared from CF-1 timed pregnant mice or conditioned medium prepared by following standard techniques from WiCell or Geron Corp were followed. For cell culture, 6 well tissue culture plates, 10 cm^2 plates or T75 and T225 flasks were used according to confluence needed and for collecting conditioned media from MEF. 0.1% gelatin from Sigma was used for coating culture surfaces and all cultures were done in humidified 5% CO_2 incubator at 37 °C under absolutely sterile conditions.

Antibodies used were from BD Pharmingen, San Diego, CA or Santa Cruz Biotechnology, Santa Cruz, CA or Chemicon, Temecula, CA. For intracellular or surface detection, established methods were followed: for FACS, single cell suspensions were labelled by directly conjugated antibodies and readouts analyzed on a FACSCalibur (BD Immunocytometry Systems, San Jose, CA) by using the CELLQuest program. For immunofluorescence (IF), standard protocols were followed with slight modifications on fixed cells in chamber slides from Nunc and stained and viewed and photographed with a Leica DMIL and a Zeiss Apotome.

Animals: Rag2γC double knockout mice from Taconic (Cao X, 1995, Immunity, 2(3): 223-228) were housed under specific pathogen free conditions in the University of Washington facilities and treated according to a protocol approved by the UW IACUC.

Methods:

MEFs for huES culture: Day 13 pregnant CF-1 female were sacrificed and their embryos removed from the sac. Their heads and liver masses were surgically removed and then placed one by one in a 10 ml syringe containing 5 ml MEF media and then a 18G needle was attached to the syringe and the plunger pushed. This was repeated twice and then the needle was replaced by a 25G and the same procedure repeated. MEFs were grown (3 embryos/100 mm^2 plates) for 2 days (P0). On reaching 95% confluency, the cells were passaged into 5 plates, and grown for another 2 days (P1). These were again split in a ratio of 1:8 (P2). On day 2 of P2, the cells were irradiated for 5 min at 3000 rads, and plated at a density of 0.75×10^6/ml (Ware CB, Biotechniques, 2005 38(6): 879-80, 882–3).

H7 cell culture: Human ESC line H7 was cultured, either in feeder-free form or with MEF-feeders, according to the protocol from WiCell.

Simultaneous surface and intracellular staining of trans-differentiated lung cells from human ES H7: To stain cell-surface antigens, the cell suspension was treated with 1 μl conjugated antibody/million cells for 30 min on ice. After thorough washing, cells were fixed in Fixation Solution (4% Paraformaldehyde/PFA in PBS) by vortexing and incubated in the dark at room temperature for 20 min. Intracellular markers were stained by the same procedure, with an extra stem of permeabilization (with 0.1% Treew-20, or with 0.25 Triton X-100). Readouts were taken on a FACScalibur. Different conjugates with greatly separated excitation

wavelengths were used (Chen DS, PLoS Med. 2005 Oct; 2 (10): e265).

ABC immunoperoxidase IHC: The following protocol was followed for IHC with non-conjugated antibodies. The paraffin-embedded sections were deparaffinized in xylene (twice for 5 min each) and rehydrated in 100% ethanol, followed by 95% ethanol. Endogenous peroxidase was quenched by treating the sections with 0.3–3% hydrogen peroxide in methanol, for 30 min at room temperature. Blocking was done for 1 h at room temperature, in PBS containing calcium and magnesium, supplemented with 1.5% serum from the species from which the secondary antibody has been obtained. The sections were then incubated with primary antibody, suitably diluted in blocking buffer, for 1 h at room temperature, or overnight at 4 °C. This was followed by three washes in 1X PBS for 5 min each. The sections were then incubated in secondary antibody, diluted in blocking buffer, for 1 h at room temperature. The last step of ABC staining is done according to the manufacturers' protocol (Laflamme M, 2005, Am J Pathol, 2005, 167(3): 663).

Immunofluorescence staining: This was done following standard protocol with slight modifications as required and readouts taken under a Leica fluorescent microscope (LaFlamme M, Am J Pathol, 2005).

Research Design

To induce differentiation of mouse embryonic stem cells to establish lung-specific cell lineages, viz. alveolar epithelial cells type I and II (AEI, AEII) and Clara cells.

Differentiation into lung lineage specific cells will be first induced by the following strategies:

(a) **Guided endodermal differentiation to pulmonary epithelial cells**, using known factors for lung morphogenesis. Embryoid bodies (EBs) will be grown to crowded confluency following established protocols with slight modifications and then the following growth factors added in combination or separately. Small airways growth medium (SAGM) or bronchial epithelial growth medium (BEGM) are routinely used as lung cell differentiation medium, and so these growth factors will be used for culture of cells. All of them have been shown in previous studies to either have regulatory roles in lung morphogenesis or to affect remodelling in injury-induced lung regeneration. Thus, these growth factors, selected as they seem most likely to trigger signaling pathways, can imitate the inherent environment present during the formation or the regeneration of a lung. To understand which factor plays the most important role in the guided differentiation, variables will be introduced one at a time and in combination. Functional status can be measured by engraftment studies, or intracellular cAMP and calcium-ion mobilization assays. Cell lines developed this way will then be thoroughly characterized.

The rationale for using the following growth factors:

(i) **Endothelin (ET1-3)**: This has been found to promote growth in smooth muscle cells (SMC) of lung (Panettieri, RA Jr. 1996, Br J Pharmacol 118(1): 191), stimulate collagen synthesis by fibroblasts in IPF and induce growth of myofibroblasts (Shi-Wen, X. 2004, Mol Biol Cell 15: 2707–2719; Teder P, 2000, Am J Respir Cell Mol Biol 23: 7–10), induce matrix-associated gene expression in myofibroblasts by MEK/ERK pathway (Shi-Wen, X. 2004, J Biol Chem 279: 23098–23103), and regulate lung morphogenesis (Sharma, A. 2006, Cancer Res 66(16): 8200–9). So, it can be considered to influence the differentiation of the epithelial cells.

(ii) The **TGF-β** family is known to show inhibitory effects on the development of lung (Warburton D, 2005, Pediatr Res, 57 (5 Pt 2): 26R-37R). Thus, the use of low

concentrations of isomers of TGFβ or an antibody of TGFβ may induce the differentiation of lung cells.

(iii) **Mitogen Activated Protein (MAP) kinases** play a role in the regeneration of lung tissue after any injury. Among the known MAP kinases, p38, ERK1 and ERK2 may influence the differentiation of lung alveolar cells.

(iv) Epidermal Growth factor (**EGF**) is already a component of commercially available SAGM and BEGM. Earlier research shows that EGF, vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) play a role in the progression of lung diseases. Our preliminary studies have shown that EGF guides the cells to differentiate to an AEII phenotype.

(v) Hepatocyte growth factor (**HGF**) is a humoral mediator of epithelial-mesenchymal interactions, acting on a variety of epithelial cells (Akiyama K, Chest, 2006, epub). Since hepatocytes and pulmonary epithelia arise from the endoderm, it may be hypothesized that HGF may guide differentiation of lung epithelia.

(vi) Epithelial specific keratinocyte growth factor (**KGF**) (Ware LB, 2002, 282: L924) is expressed by mesenchymal cells, and receptors for KGF are expressed only by epithelial cells. KGF is known to regulate morphogenesis of lungs (Simonet WS, 1995, PNAS, 92: 12461), and also has a role in repair of injured lung (Yi ES, Am J Pathol, 149: 1963).

Of the 6 molecules to be used, the factors that are found to be most satisfactory for differentiation, will be further used to scale-up the differentiated cells. This will be done by inserting the gene for that factor into the ES cells, with a suitable promoter.

(b) **Conditioning the culture media** with primary lung cells. H7 will be co-cultured with primary human cells, and murine ES with human stem cell line. Isolation and enrichment will be done according to established protocols. ESCs will be either transferred to conditioned medium for induction, or to differentiation medium and co-cultured with primary cells.

(i) **Pulmonary neuroendocrine cells (PNEC)** are among the first of the lung epithelial cells to differentiate during the gestation period. They originate from the endoderm, and have secretory granules. They can be isolated and cultured in αMEM with 10% FBS and 5–50 ng/ml neuronal growth factor (NGF) for a week, and co-cultured with undifferentiated H7.

(ii) **Primary fibroblasts** from lungs will be isolated and co-cultured with undifferentiated human/mouse ES cells for the same reasons as explained above (White AC, 2006, Dev, 133(8): 1507–17).

(iii) **Primary endothelial cells** and (iv) **smooth muscle cells** will be isolated (Kobayashi M. 2005, 12(3): 138–42 J Atheroscler Thromb.) will also be co-cultured with undifferentiated ES in commercially available endothelial growth medium (EGM) or smooth muscle cells growth medium (SMCGM).

(iv) **Human umbilical cord (huUC)-derived mesenchymal stem cells** (Lu L, Hematopoietic stem cells, 2006, 91(8): 1017) will be co-cultured with EBS and study their differentiation pattern. Of the UC-derived cells enzymatically detached from UC by collagenase II, the adherent ones are MSC which will be further purified by magnetic activated cell sorting (MACS). MSC phenotype (positive for CD166, CD105, CD90, CD73, CD49e, CD44, CD29, CD13, MHC I; negative for CD14, CD34, CD45, MHC II). Mesenchymal marker expression on cells will be plotted periodically and their signaling pathways traced by introducing inhibitors of MAP kinase (commercially available p38 inhibitors) and GPCR (pertussis and cholera toxin).

(v) **Primary AEII** (Isolation and Primary Culture of Murine, Alveolar Type II Cells, Corti M, Brody AR, Harrison JH. m J Respir Cell Mol Biol. 1996 Apr. 14(4): 309–15; Elbert KJ, Pharmaceutical res, 1999, 16(5): 601).

Stem cells of adult tissues depend on their surrounding stromal and accessory cells. These cells help stem cells maintain their 'stemness', and also to begin differentiating in response to some signals. Knowledge about the accessory cells is limited. So, the proposed study will help explain the role of these cells in the process of differentiation of ESCs. The knowledge acquired will help in the ex vivo manipulations of the stem cells.

Original work generated from the above project: Original work done based on the above proposal has been published by the author in the following papers:

1. Mitra S, Paul P, Mukherjee K, Biswas S, Jain M, Sinha A, Jana NR, Banerjee ER (2015) Mesoporous nanocarbon particle loaded fisetin has a positive therapeutic effect in a murine preclinical model of ovalbumin induced acute allergic asthma. J Nanomedine Biotherapeutic Discov 5:132
2. Kar S, Konsam S, Hore G, Mitra S, Biswas S, Sinha A, Jana NR, Banerjee ER (2015) Therapeutic use of fisetin, curcumin, and mesoporous carbon nanoparticle loaded fisetin in bleomycin-induced idiopathic pulmonary fibrosis. Biomed Res Ther 2(4):250–262
3. Mitra S, Biswas S, Sinha A, Jana NR, Banerjee ER (2015) Therapeutic use of fisetin and fisetin loaded on mesoporous carbon nanoparticle (MCN) in Thioglycollate-induced Peritonitis. J Nanomed Nanotechnol 6:332

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