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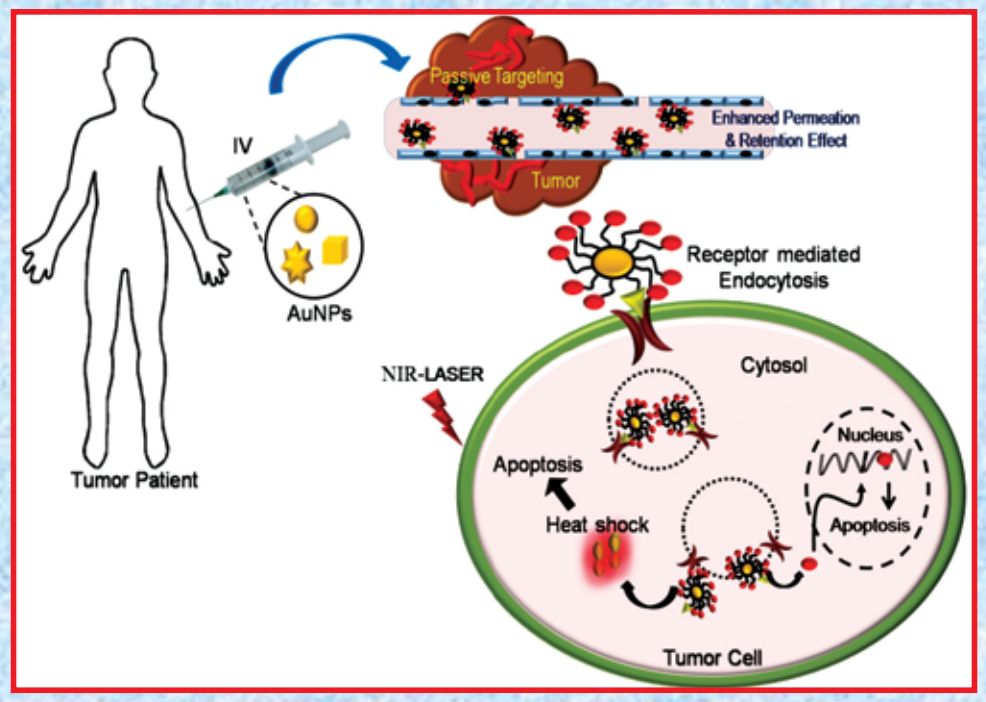

Special Issue on

Nano-Biomaterials in Theranostics

SOCIETY FOR MATERIALS CHEMISTRY

Society for Materials Chemistry

Society for Materials Chemistry was mooted in 2007 with following aims and objectives:

(a) to help the advancement, dissemination and application of the knowledge in the field of materials chemistry,

(b) to promote active interaction among all material scientists, bodies, institutions and industries interested in achieving the advancement, dissemination and application of the knowledge of materials chemistry,

(c) to disseminate information in the field of materials chemistry by publication of bulletins, reports, newsletters, journals.

(d) to provide a common platform to young researchers and active scientists by arranging seminars, lectures, workshops, conferences on current research topics in the area of materials chemistry,

(e) to provide financial and other assistance to needy deserving researchers for participation to present their work in symposia, conference, etc.

(f) to provide an incentive by way of cash awards to researchers for best thesis, best paper published in journal/national/international conferences for the advancement of materials chemistry,

(g) to undertake and execute all other acts as mentioned in the constitution of SMC.

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Guest Editor

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Front cover shows passive targeting of metal nanoparticle uptake in tumor tissue administered intravenously.

Guest Editorial

Dr. Anuj Tripathi

Biomaterials encompass a broad spectrum of research and development areas of science and technology, and deliver imperative humanitarian requirements. Designing of a biomaterial requires a specific expertise of design and evaluation by the scientists for confirming the nature of biocompatibility and application potential. The field of biomedical research is rapidly increasing as seen by the number of publications and technology patents in this field with potential outlook.

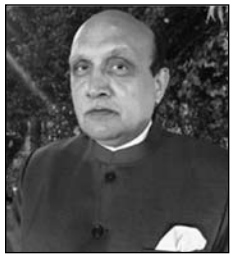
In this special issue of SMC Bulletin, we have sought to bring together the expertise of established woman scientists involved in the biomaterial's chemistry and theranostics, with the aim of providing overview on contemporary preparation and diversified applications of biomaterials.

The article of Dr.Aurelien Forget and Dr.NehaArya highlights application of biomaterials for recapitulating tumor's extracellular matrix towards screening of therapeutics on patient-derived tissue-engineered 3-D tumor models presents a significant advancement towards personalized medicine.Theranostic approach of metal nanoparticles is vital in the current scenario. In this series, Dr.Sreelekha's group is exploring the multifaceted function of polysaccharide coated metal nanoparticles on cancer management since past few decades and discussed the viability of approach in her contributed article. Since nano-theranostics promises imaging, diagnosis and therapy in a single nano-unit, Dr. Gandhi's group has outlined the progression and reworking of nano-theranostics, along with a perspective of its effect on precision medicine. Dr.MuniaGanguli has extensively worked on the potential nano-delivery systems. In this quest, her group has explored possible mechanism for long term expression of therapeutic gene using non-viral vectors that have emerged as potential delivery systems with increased mucus permeation ability towards treatment of lung diseases. An article on peripheral nerve tissue engineering by Dr.Vishnoi is giving insight on factors involved in peripheral nerve tissue injury and ideal properties for regeneration of functional nerve guidance channels and neural tissue. In the last article of this issue, Dr.Verma and her colleagues have addressed a comprehensive overview on colloidal stability of hydroxyapatite nanostructures and surface functionalization methodologies for developing it as a suitable nano-carrier for drug delivery.

I am extremely privileged to be a guest-editor for this special issue on 'Nano-Biomaterials in Theranostics'. I thank all authors, who are acclaimed experts of their field, for the quality of their contributions.

(Guest Editor)

From the desks of the President and Secretary



Dr. V. K. Jain
President

Dr. R. K. Vatsa
Hon. Secretary

Dear Esteemed SMC Members and colleagues,

Warm greetings from newly elected Executive Council of ***Society for Materials Chemistry*** (SMC),

The SMC and the Editorial Board of SMC Bulletin have been making consistent efforts to bring out thematic issues on contemporary subjects of materials chemistry. Biomaterials are one such broad area which has gained momentum in the recent past, although such materials have been in use for more than a half century. This special issue of SMC Bulletin is in succession of the last issue on 'Materials for Healthcare' highlighting the emerging role of materials science for bio-medical applications.

Biomaterials are used for diagnostic, therapeutic or prosthetic applications and can be of natural or synthetic origin. A biomaterial when comes in contact with tissue, blood or biological fluids should not affect the living organism, its components and functioning. The challenge in designing these materials is the ability of the material to be acceptable by the biological system and optimum performance under specific conditions for prolonged use. A variety of materials, like materials for hip bone joints, knee, shoulder joints, dental implants, etc., have been designed keeping in mind the properties of material and the intended application. Continuous R & D is required so as to improve the quality of materials with increased lifetime and cut down cost to make them affordable to common man

This special issue of SMC Bulletin contains six invited articles on biomaterials, nano-theranostics, gene therapy, nerve tissue engineering and hydroxyl apatite nanostructures. We place on record our sincere appreciation to Dr. Anuj Tripathi, Guest editor, who has taken keen interest to bring out this special issue in a timely manner. We also thank all the members of SMC for their continued support and cooperation in the growth of the Society.

CONTENTS

Biomaterials for recapitulating tumor's extracellular matrix: towards personalized therapy

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Abstract

Due to inter-and intra-tumorheterogeneity, there is a need toshift the treatment paradigm towards patient-specific therapies. These therapies aim at the identification of optimal drug and its dosage for a specific patient. In this context, tissue engineering-based 3-D models utilize biomaterials that can reproduce patient-specific cancer environments *in vitro* and demonstratethe potential to be used as drug screening platforms. These platforms can also be adapted to high throughput screening assays and would help the surgeons identify the right drug regimen on patient-to-patient basis. This review focuses on defining the role of biomaterials towards the'new treatment' approach and how versatile biomaterials could be used to reproduce the cancer environment of individual patients.

Keywords: In-vitro tumor models, personalised chemotherapy, tissue-engineering, drug screening, synthetic extracellular matrix.

1. Introduction

Tumor progression is a multi-step process during which the tumors acquire the 'hallmarks of cancer' listed by Hanhanan and Weinburg. These hallmarks include"sustenance of proliferative signals, evasion from growth suppressors, resistance from cell death, activation of invasion and metastasis, induction of angiogenesis, enabling uncontrolled multiplication, modulation of cellular energetics and evasion of immune destruction" [1]. These characteristics emerge as a result of interaction between various components of physiologically abnormal tumor microenvironments (TME). The TMEincludescomponents of the solid tumors other than the tumor cells such as cancer-associated fibroblasts, immune cells, vascular network (including endothelial cells and pericytes), progenitor cells, inflammatory cells and the extracellular matrix (ECM) (**Figure 1**) [2]. The TME is not only crucial for tumor growth, invasion, and malignancy but also contributes to chemoresistance [2]. Additionally, tumors are heterogeneous and dynamic and exhibit inter-tumor heterogeneity [3]; therefore, traditional chemotherapeutics targeting tumor cells may not demonstrate the same response in tumors belonging to different cancer patients. Apart from demonstrating inter-tumor heterogeneity, a solid tumor possesses a complex architecture and may exhibit intra-tumor heterogeneity owing to varying molecular signature of cancer cells, even within the same solid tumor. More specifically, intra-tumor heterogeneity may be contributed by genetic and non-genetic (epigenetics, plastic gene expression, and signal transduction) factors as well as unequal microenvironments [4].Therefore, therapeutics that simultaneously targets the tumor cells and its microenvironment may offer a more efficient way to treat cancer. In order to make sure that the patient is responsive to a particular chemotherapeutic regimen, it is imperative to design personalized *ex vivo*screening protocols that screen the patient's tumor and its microenvironment against therapeutics.

2. Modeling the tumor microenvironment *in vitro* using tissue engineering

Understanding cancer development, predicting its evolution *in vitro* and identifying molecules as potential therapeutics require establishing reliable laboratory models that can accurately reproduce the *in vivo* natural behavior of cancer cells. Traditionally available methodologies for screening small molecules include two-dimensional (2-D) culture of cancer cells and small *in vivo* animals [5]. 2-D culture involvesculturing the cells on 2-D tissue culture plastic, which are then tested against various small molecules. While this is convenient and relatively inexpensive, the cells do not recapitulate the *in vivo* tumor microenvironment [6] and hence the drug-sensitivity data cannot be correlated to the next screening steps, i.e., *in vivo* testing. Patient-derived xenografts based on the generation of tumors in immune compromised mice [7] are the next most common model for understanding tumor biology and drug screening. Although *in vivo* models recapitulate

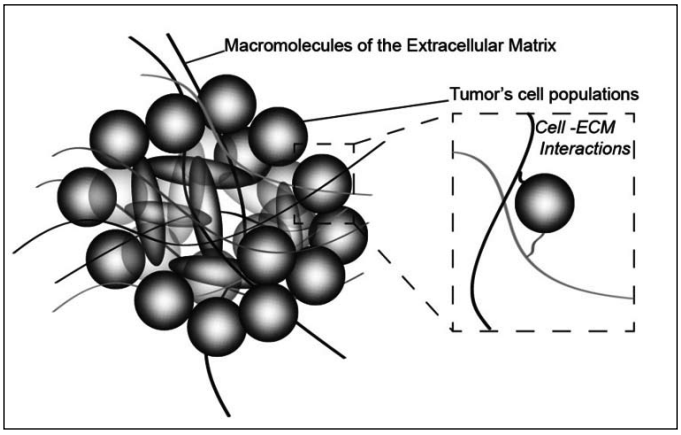

Fig. 1: *Tumor microenvironment composed of the different types of cells attached to a dense network of macromolecules forming the extracellular matrix.*

the tumor microenvironment to some extent, they do not mimic the natural response coherent to human tumors and are also associated with enhanced incubation time as well as ethical constraints. As a result, three-dimensional (3-D) tumor models have emerged, which involve simple culturing practices and have also shown to recapitulate properties of *in vivo* tumors. In addition to this, they also allow direct monitoring of tumors following drug treatment.

Cancer cell-based spheroids reproducing the 3-D architecture of tumors are extensively used for drug development [8]. In spheroids, cells organize with each other and produce their own ECM [9].Various studies have compared the drug responsiveness of 3-D spheroids to 2-D planar cell culture, and response to anti-cancer drugs was found to be different [10]. It has been demonstrated that the 3-D architecture has a tremendous impact on the behavior of the cells, but cancer spheroids often rely on the cells obtained from a cell culture library, expanded on tissue culture plastic and reseeded in the 3-D context. Recent advances in the field of tissue engineering have demonstrated the generation of physiologically relevant *in vitro* tumor models using 3-D polymeric matrices [11]. These matrices are transient structures based out of ECM-mimicking biomaterials and are of various types ranging from 3-D freeze-dried scaffolds to hydrogels to nanofibrous substrates to 3-D printed scaffolds [11]. Tissue-engineering based 3-D models can be used to investigate the role of ECM in cancer pathophysiologyas well as to screen various anti-cancer drugs or small molecules. To be a reliable assay for drug discovery, 3-D in vitro models need to be compatible with drug screening platforms and reproduce the characteristics of the tumor ECM. It is now clear that many factors of the ECM such as composition, degradation and stiffness play a role in modulating cell behaviour [12], and mimicking these functions *in vitro* can be used to predict *in vivo* cancer treatment [13]. Theupcoming sections will focus on strategies to establishbiomaterialsrelevant for tumor models and their role in the development of personalized 3-D tumor models.

3. Biomaterials as ECM mimics

Tissue engineering aims to reproduce the complex interaction between cancer cells and their microenvironment with the use of transient 3-D structures called scaffolds. These scaffolds can be fabricatedusing natural polymers, synthetic polymers or their blends [14]. Both natural and synthetic polymers have been widely used in tissue engineering, and are associated with certain limitations. Nevertheless, these materials or their blends have been utilized to fabricate 3-D scaffolds that serve assubstrates for 3-D culture of cancer cells.

Collagen, the most commonly occurring macromolecule in the ECM,can be classified into two types of polymer architecture, fibrillar collagen for type I and type II, and two-dimensional network collagen for type IV.While collagen type I has a long history of application in 3-D cell microenvironment [15], collagen IV can also be used to form a hydrogel [16]. Both these macromolecules have been linked to influence the remodeling of the tumor environment and cancer cell migration [17]. **Laminin**, on the other hand, it is based on the aggregation of the trimmers, α (5 different types), β (3 different types) and γ (3 different types), and can form 45 possible combinations. It is a cell binding macromolecule which can be cleaved by bone morphogenetic protein-1 (BMP-1), matrix metalloproteinase -2 (MMP-2), Membrane Type 1 Matrix Metalloprotease (MT1-MMP) and plasmin [18]. It can form hydrogels, and it was demonstrated in a study that the culture of breast cancer cells in this environment promoted their metastatic behaviour [19]. **Fibrin**, a hydrogel formed by the secretion of fibrinogen monomer that is polymerized by thrombin, is primarily found in blood clots [20]. Because fibrin can be degraded by plasmin, MMP-2, MMP-3, and MMP-9, it allows recapitulation of the natural remodeling processes of the ECM in the tumor microenvironment [21]. As an example, it was demonstrated that stiffness of fibrin hydrogel plays a role in isolation and enrichment of tumor colonies of colorectal cancer cells.[22]Interestingly, colony formation and expression of a stemness marker, Nanog,was inversely proportional to the stiffness of salmon-fibrin [22]. **Hyaluronic acid**, composed of repeating units of D-glucuronic acid and D-N-acetylglucosamine, is yet another macromolecule of the ECM that has been utilized for the 3-D culture of cancer cells. In a study, Gurski et al. utilized hyaluronic acid-based hydrogels to understand

aspects of invasion in encapsulated prostate cancer cells [23]. In another study, hyaluronic acid promoted stem cell-like behavior of U87 astrocytoma cells [24].

While each of these natural ECM macromolecules play a specific role in cancer progression and modulate the response of tumor cells to anti-cancer drugs, these molecules could also have a concomitant and competitive effect. Therefore, more sophisticated formulations can be used to reproduce the full microenvironment. In this regard, **Matrigel®**,an ECM hydrogel material isolated from decellularized mouse carcinoma, allows cell growth in a complex cell microenvironment. This hydrogel also provides the required growth factors and supports cancer cells to form tumorsboth *in vitro* and *in vivo* [25].

Although natural macromolecules of the ECM have been well established for the generation of 3-D tumor models, they demonstrate batch-to-batch variability, inconsistent ligand presentation, and low modulus especially in case of scaffolds derived from Matrigel® and collagen. In contrast, non-mammalian naturally occurring macromolecules, have the advantage to provide a biologically neutral environment that can then be engineered to reproduce natural cancer environment. In this regard, polysaccharides obtained from seaweeds are quite attractive. **Alginate**, a polymer comprising of repeating units α-L-guluronic acid and β-D-mannuronic acid, is obtained from seaweeds and has demonstrated popularity for encapsulation of many cancer cells types [26-28]. In one study, alginate-based hydrogels were used to explore the tumor-initiating properties of head and neck squamous cell carcinoma cells as a function of polymer stiffness [27]. Another polymer extracted from seaweed, **agarose** (made of repeating units of D-galactose and 3,6-anhydro-L-galactopyranose), has been employed for encapsulation of cancer cells [29]. **Chitosan**, a glycosaminoglycan mimic, obtained from crustaceans and comprising of D-glucosamine and N-acetyl-D-glucosamine units, has been utilized along with other polymers such as alginate, gelatin and hyaluronic acid for the growth of tumor cells [30-32]. One such study utilized chitosan-gelatin 3-D scaffolds to generate lung cancer cell line-based tumoroids and these tumoroids were shown to demonstrate more *in vivo* like properties as compared to the cells grown on 2-D substrates.

Alternatively, synthetic polymers offer the possibility of designing the right chemical environment due to the ease of functionalizing the macromolecule backbone and modifying their mechanical properties. **Polyethylene glycol (PEG)** is one of the most explored synthetic polymers for the culture of cancer cells [33, 34]. As an example, Jabbari *et al*. utilized 3-D PEG-diacrylate of varying stiffness to investigate its effect in the maintenance of stem cell properties of cancer cells of different origin [35]. Other synthetic polymers such as **poly(lactide-co-glycolide) (PLGA)** have been utilized as 3-D matrices for the growth of oral, liver and ovarian cancer cells [36-38]. Nanofibrous scaffolds based on PLGA and a block copolymer of poly(lactic acid) (PLA) and mono-methoxy poly(ethylene glycol) (mPEG) induced epithelial-to-mesenchymal transition in cancer cells of different origin [39]. In another study, electrospun nanofibers based on **poly(ε-caprolactone) (PCL)** was used to generate an *ex-vivo* model of Ewing sarcoma. In this example, the 3-D model demonstrated more resistance to traditional chemotherapeutics as compared to the cells grown on a 2-D substrate, similar to *in vivo* conditions [40]. While these materials can be processed in many different forms such as hydrogels or fibers, they do not offer adhesion sites for the cells. To reproduce the cell-ECM interactions, these polymers need to be functionalized with molecules that can bind to the cells.

4. Towards personalizedtissue engineered screening platforms

Specific platforms for personalized therapeutics have been reported. In one system, patient-specific tumor stromal matrix proteinscocktail were engineered by matching the composition of the patients' ECM to support tumor explants; such models can be then used to identify the response of patients' tumor to anti-cancer drugs [41]. Such patient-specific tumor stromal matrix proteins cocktail incorporate only the most abundant protein-based macromolecules of the patients' ECM. However, the tumor ECM comprises of other macromolecules and their interaction with tumor cells is a key to tumor progression. Therefore, in order to reproduce a patient's ECM, a fully synthetic cell microenvironment that recapitulates the ECM could beused. Cell attachment can be reproduced by decorating polymers such as agarose [42, 43], alginate [44], cellulose [45], PEG [46] or PLA [47] with a variety of peptides termed cell adhesion peptides [48]. Although RGD is the most commonly used sequence as cell adhesion peptides, other peptide sequences have been used for the study of cancer cells. For instance, when presented to cancer cells spheroids,the peptides IKVAV,GFOER, and RGD immobilized on a substratehave been shown to differentially modulate the invasion of cancer cells [49]. With the constant growth of cell adhesion peptide library [48], the precise role of many sequencesmostly remains unknown. Screening the impact of these peptides on the behavior of cancer cells would allow designing precise tumor microenvironment that reproduces the

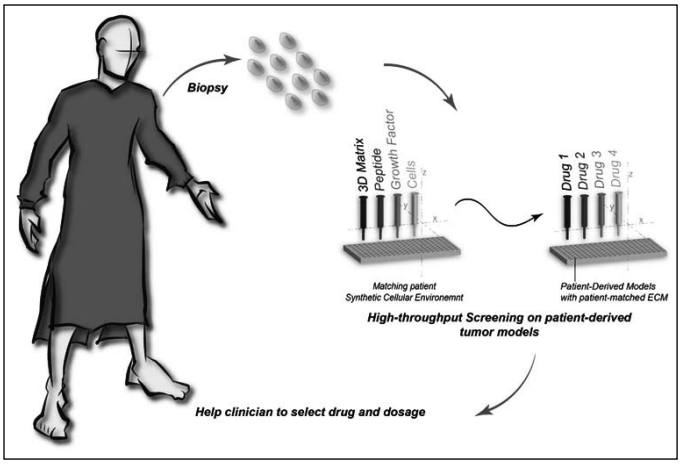

Fig. 2:*The paradigm for personalized medicine in cancer. Scheme demonstrating the seeding of patient-derived tumor cells on a synthetic cellular environment comprising of a synthetic hydrogel conjugated with adhesion peptides to simulate the natural tumor environment. The system would also be incubated with additional growth factors to complete the tumor niche followed by the screening of potential therapeutics.*

natural cancer niche without the need to use human-derivedECM macromolecules.Using hydrogels that are amenable to automated screening platforms, an entire peptide library could be screened on different cancer cells and selected to design patient-specific synthetic microenvironment (**Figure 2**) [50]. To achieve this, peptides terminating with a fast reactive moiety could be attached to a hydrogel functionalized with a compatible reactive functional group. This approach would permit rapid immobilization of peptide sequences onto the hydrogel and automatize the creation of a variety of synthetic cellular microenvironmentin which patient-derived cells could be grown. Alternatively, analytical methods such as mass spectroscopy could provide insights into the abundance of these peptides in the patients' ECM, so that the synthetic environment could be matched to the patients' tissue. Once mature, these cancer models could be used to test anti-cancer drugs and identify the dose-response of patient cancer cells to singleor combinatorial therapeutics (**Figure 2**). The *ex vivo* models could also be extended to radiation testing to help the surgeon personalize the treatment regime for each patient.

Screening of therapeutics on patient-derived tissue engineering-based 3-D tumor models in the field of translational cancer research presents a significant advancement towards personalized medicine. The recent development in the field of biomaterials and engineering would aid in automation of screening the cell-ECM interactionsconducive to tumor growth and investigate a broad range of therapies based on patient-derived tumor cells. Further, development of robotics-based assay would help translate such technologies to bedside therapeutics and help oncologist design patient-specific treatment regimen.

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Advances in polysaccharide metal nanoconstructs and its impact in cancer theranostics

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Abstract

Polysaccharides are high molecular weight biopolymers present in various flora and fauna in the ecological systems. Due to its high biocompatibility and versatile functions, these polymers are employed in various biomedical applications. Biocompatible polysaccharide coated metal nanoparticles are widely used for the theranostic management of cancer. Numerous literatures have reported the use of gold and iron oxide nanoparticles to be a potential agent for drug delivery and imaging. There are several commercially available biopolymer based metal nanoparticles, but none of these exhibits excellent biocompatibility and multifaceted functions. Based on these rationales, the current review attempted to focus on polysaccharide coated gold and iron oxides nanoparticles and its impact on cancer management in past few decades.

Keywords: Polysaccharides, cancer, AuNPs, nanoparticles, therapy.

1. Introduction

1.1 Polysaccharides

Polysaccharides belong to a class of bio-macromolecules enriched with hydroxyl groups with the occurrence of versatile monosaccharide units covalently jointed with O-glycosidic bonds[1]. Special enzymes bind to these monomers and catalyse polymerization reaction to form the polysaccharides or glycans. A polysaccharide may be a homo-polysaccharide or a hetero-polysaccharide depending on the monomer units present in it. In homo-polysaccharides all the monomer units are same were as in the hetero-polysaccharides different monomer units are present. Structural variations and complexities are the main hallmark of polysaccharides which describes the biological activities of these compounds[2]. Polysaccharides generally do not have definite molecular weights and there is no template for the polysaccharide synthesis. Polysaccharide biosynthesis is intrinsic to the enzymes and that catalyse the polymerization of monomer units. Among the macromolecules, polysaccharides show the highest capacity for carrying biological information because they have the greatest potential for structural variability. They are ubiquitous and possess low processing costs. These polymers serve as an agent of energy source (eg: starch & glycogen) and structural component (eg: cellulose & chitin)[3-5]. Their structure range from linear to branched and can be derived from microbes, plants, fungi and animals. In current scenario, polysaccharides have been well appreciated in the field of biomedical sectors including osteology, cardiology and oncology[6-11]. They play a vital role in storing energy, structural functions, cell-cell recognition, cellular communication and immunology [12-16].

Polysaccharides based on the presence of charged side chain molecules, classified into ionic and non-ionic electrolytic polymers[17, 18]. Figure 1 illustrates structure of different polysaccharides. Chitosan, de-acetylated form of chitin, is soluble in weak acids reported to possess various application in drug delivery systems (DDSs) that contributes greatly for the management of dreadful diseases including cancer. These polymers are negatively charged biomolecules used in photography, cosmetics, skin substitution, wound dressing, ophthalmology and textile industry[19]. Anionic polysaccharides including alginate, hyaluronic acid, gellan gum and pectin were widely used in DDSs in cancer management as well as for tissue engineering applications. Non-ionic polysaccharides are highly hydrophilic, biocompatible, soluble at neutral pH and hence used for the synthesis of biodegradable DDS. Microbial derived polysaccharide (eg., dextran) as well as plant polysaccharide (eg., xyloglucan) belongs to neutral polysaccharides serve as major role in the production of multivalent nanoparticles for efficient targeted DDSs [20, 21].

1.2 Polysaccharide - Metal nanoparticles

Metal nanoparticles comprise nano sized unit cells made of pure metals (e.g., gold, platinum, silver, iron, cobalt etc,) or their compounds (e.g., oxides, hydroxides, phosphates, and chlorides) within the size range of

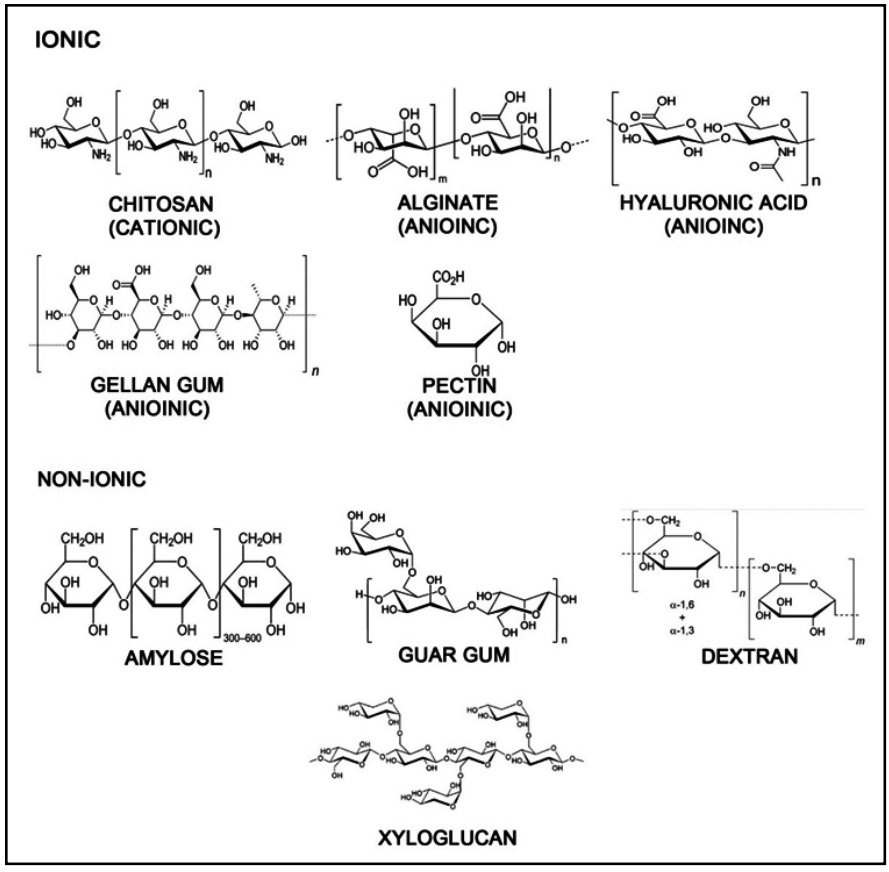

Fig. 1: Classification of polysaccharides: polysaccharides are classified into ionic and non-ionic electrolytes.

1-100 nm in dimension. Studies have shown that metal nanoparticles were thermodynamically unstable in its colloidal state and that the metal nanoparticles had to be stabilized kinetically to prevent aggregation. Thus, different biopolymers like protein, polysaccharides and oligonucleotides were introduced to stabilize the metal nanoparticles in aqueous solutions. The current review focuses on the recent developments in the polysaccharide coated metal nanoconjugates for biomedical applications especially in the field of oncology.

Gold nanoparticles (AuNPs) employed in cancer treatment were highly biocompatible and occurs in various sizes ranging from 2 to 100 nm in diameter. Versatility in its shape and size extends its wide applications in many biomedical fields especially in bio sensing and drug delivery systems[22, 23]. The properties of AuNPs behind the success of its use in the landscape of nanoscience and nanotechnology are (i) high stability and cytocompatibility; (ii) greater possibility of surface functionalization and (iii) possess optical properties[24-26]. Ionic electrolytes as well as non-ionic electrolytic polysaccharides have been widely used for capping and stabilizing these gold nanoparticles. For past few decades, polysaccharide coated gold nanoparticles were surface functionalized with drug, antibody, ligands and reporter dyes for theranostic application in tumor management.

Superparamagnetic Iron oxide magnetic nanoparticles (IONPs) are widely under research on Magnetic Resonance (MR) contrast enhancement focusing on the alterations of proton relaxation in the tissue microenvironment[27]. The storage as well as preparation of these nanoparticles favours the stability of the colloid that in turn benficial for MRI. IONPs are prone to aggregate in fluid which limits its applicationand the coating of IONPs surface using polymers is vital[28]. Liu et al. synthesized IONPs decorated with the mono carboxyl-terminated-poly (ethylene glycol) via a one pot reaction approach. The results have shown that the so-prepared IONPs possessed excellent biocompatibility and exhibited a long blood circulation time[29]. Reports have shown that super paramagnetic maghemite nanoparticles could be prepared via a two-step layer-by-layer technique using poly-(ethylene imine) as the and poly(ethylene oxide)-block-poly(glutamic acid). Initial investigations have shown that the biocompatible particles provided strong contrast [30]. The recently developed SPIO SHU 555 A (Resovist, Schering, Germany) is made-up of a colloidal sol of IONPs coated with carboxydextran, which shows promising results in terms of safety, characterization and detection of focal liver lesions[31]. The particle diameter ranges between 45 and 60 nm inferring thatthe larger particles are taken up by Kupffer cells; the smaller ones remain longer in the vessels, displaying blood pool characteristics.

2. Applications of polysaccharide coated metal nanoparticles for cancer therapy

2.1 Mechanism of nanoparticle transport and uptake

With snowballing requisite to develop drugs with greater specificity to target cancer tissue and improve chemosensitivity, there is a prerequisite to develop or improve drug delivery strategies. Therefore, researchers focused to develop novel metal nanoparticles that facilitates drug loading and preferentially makes cancer cells more responsive. Usually cancer tissues tend to synthesize their own blood vessels via neovascularization leading to the formation of leaky tumor blood capillary system. The

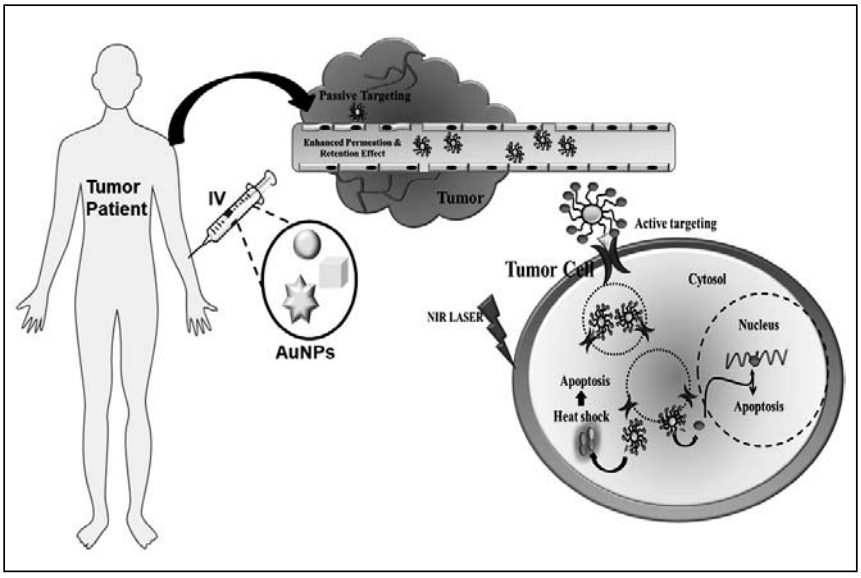

Fig. 2: *Mechanism of nanoparticle uptake in tumor tissue: Metal nanoparticle administered intravenously enters the tumor site via leaky vasculature by passive targeting strategy and ligand conjugated metal nanoparticles tend to internalize the tumor cell via active targeting strategy*

nanoparticle exudates into the tumor site via the dynamic pits formed in the tumor vasculature leading to its passive targeting. The poor lymphatic system around the tumor microenvironment favours the retention of these particles in the tissue site after its penetration and is technically known as Enhanced Permeation and Retention (EPR) effect as depicted in figure 2 [32].

2.2 Drug delivery systems

Polysaccharide coated gold nanoparticles were widely used for targeted drug delivery system for tumor reduction with abridged systemic toxicity. For example, anionic chitosan was chemically modified by grafting carboxymethyl functional group followed by reducing and capping gold nanoparticles from gold chloride solution. The synthesized particles exhibited an ionic interaction with amino group of chemotherapeutic drug doxorubicin favouring its high percentage of encapsulation with pH-triggered drug releasing property. The particles exhibited excellent cytotoxicity against cervical cancer cells with higher accumulation of intracellular drug offer a promising tumor site specific DDS[33]. Hexanoyl-chitosan-PEG copolymer coated, paclitaxel (PTX)-loaded, and chlorotoxin (specifically binds to matrix metalloproteinase-2 (MMP-2) that is overexpressed on primary brain tumors) conjugated IONPs was developed for targeted delivery of PTX to human glioblastoma (GBM) cells. This study focuses on the development of copolymer system for loading and delivery of many hydrophobic drugs and can be modified with prominent cell surface markers for targeting and imaging purposes[34].

Gemcitabine, efficient chemotherapeutic drug for the treatment of pancreatic cancer, was loaded on chitosan coated IONPs with highest drug release (65%) at pH4.2, while it was 8% at pH7.2. The inhibitory concentration of the particles was lower than free-drug on SKBR-3 and MCF-7 cell lines, indicating the increased efficacy of drug when loaded onto nanoparticles[35]. Phytic Acid-chitosan-iron oxide nanocomposite induced apoptosis and cell cycle arrest via an intrinsic mitochondrial pathway through modulation of pro-apoptotic proteins and the release of cytochrome-c from the mitochondria into the cytosol. At concentration of 90μg/mL of nanocomposite could transcriptionally activate JNK1 and iNOS leading to G0/G1 cell cycle arrest in HT-29 cells[36].

Doxorubicin loaded dextran coated super paramagnetic iron oxide nanoparticles (DOX-DSPIONs) exhibited cytotoxicity against HepG2 cells without obvious toxicity on LO2 cells. The conjugate acts as a new drug magnetic delivery platform, with much lower systemic toxicity both in vitro and in vivo with pH dependent drug release. A pH sensitive chitosan coated iron oxide -Dox nanoparticles have been developed with higher anticancer activity with IC_{50} at 1.4 μM than free Dox with IC_{50} at 4.8 μM in ovarian cancer cell (SKOV3) and breast cancer cell line (MCF7)[37].

Citrate reduction method leads to the production of uniformly mono-dispersed metallic gold nanoparticles and further surface modification with carboxylate bearing ligands facilitate loading of anti-metabolites like 5-Fluorouracil (5-FU). These particles usually exhibited pH sensitive drug release with better anti-cancer effect compared with free drug, which reduce the dose and subsequent deleterious side effects. Green synthesis route of nanoparticles are platform for eco-friendly, non-toxic, clean formulations for drug delivery system and other biomedical applications which gained great impulse in the last few years[38]. The use of polysaccharides for the synthesis of NPs is getting remarkable influence because of the fact that these do not generate environmental hazard and needs negligible input energy usage. Salem et al synthesized and characterized 5-FU loaded gold nanoparticles using chitosan as a reducing and stabilizing agent. The amount of drug needed to attain IC_{50} was lowered compared to the free 5-FU. The 5-FU-Au nanocomposites showed the highly efficient photothermal conversion which led to seven-fold decrease of the IC_{50} value after

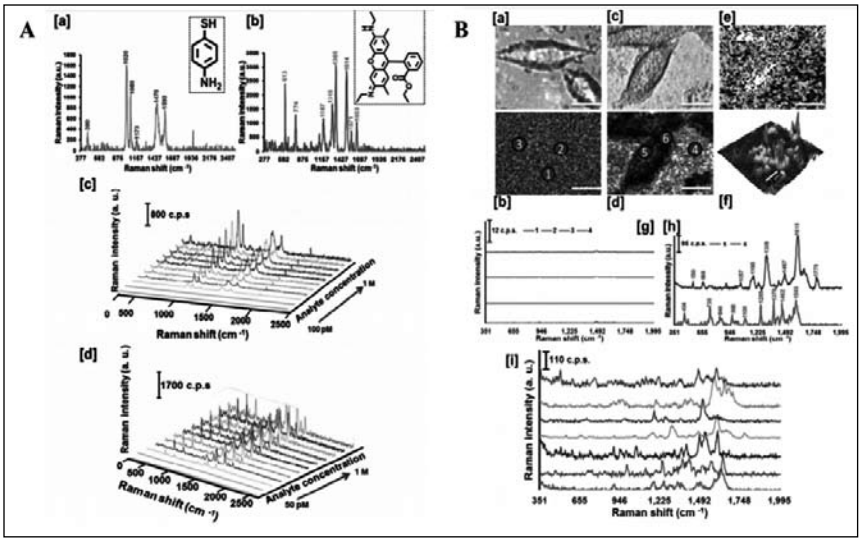

Fig. 3: SERS analyis of PST-Gold Nnaoparticles (A):(a) Raman spectra of the analytes (1mM)4-Aminothiophenol and (b) Rhodamine 6G. SERS enhancement with decreasing concentrationsfrom 1mM of (c) 4-Aminothiophenol and (d) Rhodamine 6G6 up to the limit of detection; (B)Bright field and Raman images ofuntreated (a, b) and nanoparticles treated (c, d) HeLa cells.Cluster mapping of the Ramanimage (e, f) of cells treated with the NPs for 30 minutes. The Raman spectra (g) represent theareas 1-3 from control and 4 from treated cells; (h) represents the labelled area 5, 6 and (i)represent spectra abstracted from various regions of PST-GNPs treated cells.Reprinted with permission from (Joseph MM, Nair JB, Maiti KK, Therakathinal T S.Biomacromolecules.2 017;18(12):4041-53). Copyright (2017) American Chemical Society)

20 min of laser exposure[39]. Dey et al have reported the synthesis of dual drug carrying alginate-curcumin conjugate stabilized AuNPs via green synthesis. The so-called hemocompatible particles were conjugated with methotrexate conjugate of bis(aminopropyl)terminated polyethylene glycol (PEG) and exhibited synergistic effect of two anti-neoplastic agents as well as pleiotropic effect of curcumin[40]. Polysaccharide mediated cancer immunotherapy has been widely accepted that could overcome the limitations of conventional chemotherapy and radiation therapy. *Ganoderma lucidum* polysaccharide coated AuNPs internalized via Toll-like receptor-4 (TLR4) and induces dendritic cell maturation along with T cell activation compared to free polysaccharides. These particles were demonstrated to have better therapeutic effects on tumor suppression when combined with doxorubicin. Also these AuNPs significantly induced memory T cells, which contribute for effective inhibition of tumor metastasis[41]. Ferumoxytol (carboxymethyl dextran-coated iron oxide) polarizes M1 macrophage leading to cancer cell apoptosis through the Fenton reaction. These IONPs recruit monocytes to malignant tumours leading to local expression of chemotactic cytokines and are typically polarized to anti-inflammatory M2 phenotypes[42].

Our laboratory has previously reported the synthesis of anti-tumor polysaccharide PST001 coated gold nanoparticles and its potential applications in therapeutics and SERS imaging with reduced side effects. The study focused on the green synthesis of PST001 coated AuNPs to eradicate the tumor by modulating immune system in tumor bearing Balb/c mice. PST-AuNPs were examined for the non-invasive label free SERS live-cell spectral imaging to assess the fingerprint molecular particulars of cellular processes. The characteristic SERS feature of PST-AuNPs enabled to examine the dynamic and multifaceted nature with metal nanoparticle bio-distrubution in tumor-bearing mice on a SERS platform. Henceforth, these results emphasized a innovative clinically relevant scenario for sketching the in vivo NP distribution in a label-free fashion[43-45].

2.3 Targeted drug delivery systems

Most of the cancer cells express cell surface receptors like folate receptor, epidermal growth factor receptor and so on that enhance the cancer cell proliferation and integration[46, 47]. Nowadays, ligand conjugated gold nanoparticles were used for active targeting of cancer tissues that increase more specificity and sensitivity. Akinyelu et al assessed the effect of folic acid and chitosan functionalization of Poly (lactide-co-glycolide) (PLGA) and colloidal gold loaded PLGA NPs with respect to their cytotoxicity and transfection efficiency. The novel NPs displayed the highest cellular uptake in the folate receptor positive MCF-7 cells and confirmed the receptor mediated uptake of folate via the cognate receptors especially in the breast cancer cells (MCF-7), and to a lesser extent in the hepatic cells (HepG2). In addition, NPs were of favorable size, stable and could effectively bind plasmid DNA. The higher efficient transgene capacity, ability to protect plasmid DNA from nuclease degradation in vitro, and the possibility of gold to be tracked via imaging in vivo, provides support for the use of these NPs in nanomedicine[48]. NDong et al. evaluated Trastuzumab conjugated aminodextran coated IONPs for its potential in BT-474 tumor model. Non-targeted 30 nm IONP were efficiently delivered to the tumor by blood flow, but inside the tumor these particles exhibit only low level while ligand conjugated IONPs is removed from circulation followed by its interaction with tumor tissues[49]. Similarly, a quaternary chitosan derivative, N-(2-hydroxy)propyl-3-trimethyl ammonium chitosan chloride (HTCC), which

is highly soluble, possess anti-oxidant and anti-microbial properties has been used for synthesizing AuNPs tethered with folic acid molecule. Highly stable and cytocompatible folate-HTCC capped AuNPs have shown higher rate of endocytosis in Caco-2, HepG2, and HeLa cancer cells confirmed by *ex vivo* cellular uptake flow cytometry study. Thus, folate HTCC-AuNPs has been promised as targeting vector for cancer imaging, diagnostics, or therapeutic purposes[50]. Hyaluronic acid is an anionic, non-sulfated glycosaminoglycan that possesses affinity towards CD44 marker that participates in cell adhesion interactions required by tumor cells. Hyaluronic acid capped AuNPs loaded with Metformin binds easily on the surface of the hepatic cancer cells achieving IC_{50} value around 4μg/ml compared to free drug ranged from10 μg/ml. These particles were found to be non-toxic against zebra fish embryos indicating its high compatibility for drug delivery applications [51]. Nandagopal et al prepared quercetin loaded IONPs in order to increase the bioavailability at the target site of cancer tissue and there was a significant increase in the induction of apoptosis in prostate and breast cancer cells along with MR imaging[52]. PEGylated methotrexate prodrug (MTX-PEG) and Cy5.5 dye were functionalized on the surface of the chitosan coated IONPs were employed as multifunctional and therapeutic NPs for multimodal (fluorescence and magnetic resonance) imaging cooperated with self-targeted cancer therapy. In vivo anticancer effect of MTX-PEG-CS-IONPs-Cy5.5 NPs was evaluated in HeLa tumor-bearing xenograft BALB/C mice exhibited tumor inhibition compared to free drug with the longer circulation time and greater tumor accumulation[53]. Monodispersed ferrimagnetic iron oxide nanoclusters of 22 nm coated with glycol chitosan conjugated with hydrophobic 5β-cholanic acid allowed to react with peptide sequence CSNRDARRC and Cy5.5 was conjugated to free amine groups on the chitosan molecule. The improved targeted NPs had excellent accumulation in small tumors, minimal non-specific binding to other organs, and longer retention making them better MRI contrast agents with improved tumor reduction[54]. Gum kondagogu is an anionic polysaccharide collected from the bark of the *Cochlospermum gossypium* tree used for reducing, stabilizing and capping AuNPs along with coupling to folic acid and fluorescein isothiocyanate (FITC) to produce a targeted and fluorescently labelled AuNPs. The particles were highly cytocompatible against MCF-7 cells and synthesized AuNPs is as an effective nanocarrier for different applications in drug delivery, such as site-specific or targeted delivery, cellular imaging and diagnostic purposes [55].

2.4 Radiosenitization

Conventional chemotherapy for cancer treatment is coupled with radiation therapy for the complete eradication of tumor. However, radiation therapy fails to control tumor progression in case of hypoxic condition because they are resistant to ionizing radiation therapy[56]. Literature states that AuNPs conjugated with chemotherapeutic drugs can improve the efficiency of chemoradiation in cancer patients. In Iran University of Medical Sciences, cisplatin and AuNPs co-loaded alginate hydrogel network (ACA nanocomplex) was assessed for the in vivo antitumor efficiency against CT26 colon adenocarcinoma tumor in the presence of 6 MV X-ray. Besides the high chemotherapeutic potency, nanocomplex possesses radiosensitizing properties due to the presence of AuNPs and cisplatin. The nanocomplex along with radiation stimulates immune cells and cause stress in the cells leading to autophagy and apoptosis of tissues [57].

2.5 Photoacoustics and photodynamics

Photo acoustics is a currently emerging biomedical modality that allows the generation of an acoustic wave resulting from the absorption of optical energy for imaging applications[58]. Manivasagan et al reported the use of multifunctional biocompatible chitosan-polypyrrole nanocomposites (CS-PPy NCs) as novel agents for photoacoustic image-guided photothermal ablation of cancer. The particles exhibited both in vitro and in vivo photothermal anticancer activity using NIR 808-nm laser irradiation along with enhancement in photoacoustic imaging conducted to accurate localizing of cancerous tissue, as well as precise guidance for photothermal therapy[59]. Photodynamic therapy (PDT) is a treatment process that uses photosensitizers (PS), which are light-activatable chemicals, to generate cytotoxic reactive oxygen species (ROS) under light activation, thus causing cell apoptosis and tissue destruction[60]. A novel type of core/shell mesoporous silica nanocomposite of M-MSN(Dox/Ce6)/PEM/P-gp shRNA containing the Fe_3O_4–Au nanoparticles, photosensitizer chlorin e6 (Ce6), antitumor drug doxorubicin (Dox) and P-gp shRNA modified with biocompatible alginate/chitosan polyelectrolyte multilayers (PEM) were designed for monitoring a real-time imaging-guided PDT therapy, chemotherapy and gene therapy. The particles were biocompatible with the hemolysis rate less than 5% and capable of pH-responsive release of drugs with lesser side effects in treated animals[61].

2.6 Photothermal effect

In case of gold nanoparticles, oscillations of free electrons or plasmons in nanoparticle surface with

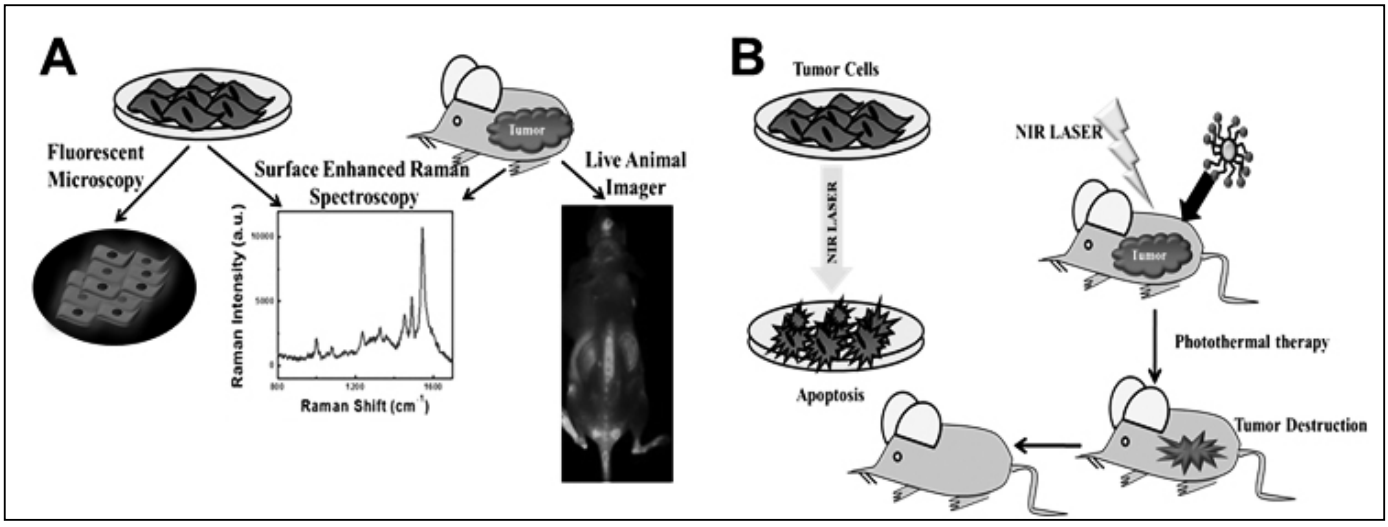

Fig. 4: *Applications of polysaccharide coated gold nanoparticles: SPR effect of AuNPs were utilized for SERS, fluorescence imaging and PTT*

size much smaller than photon wavelength are non-propagating excitations called localized surface plasmon resonance (LSPR). The dipole oscillation over the metal surface is dominant and the extinction cross section is simplified by solving Maxwell equations to give rise to following equation:

$$C_{ext} = \frac{24\pi^2 R^2 \varepsilon_m{}^{3/2}}{\lambda} \frac{\varepsilon_i}{(\varepsilon_r + 2\varepsilon_m)^2 + \varepsilon_i{}^2}$$

C_{ext} = extinction cross section; R = Radius of the particle; λ = Wavelength of the light; ε_m = dielectric constant of the medium; ε = complex dielectric constant of the metal[62].

With the continuous modelling and advancement in the study of LSPR, gold nanoparticles were currently employed in photothermal therapy (PTT), Surface-enhanced Raman spectroscopy (SERS) and optical sensing[63-65]. Gold Nanorods (AuNRs) were widely applied for PTT because of their strong scattering and absorption in the near infrared (NIR) region, including a better heat generation rate than other shapes. Multidentate chitosan oligosaccharide modified gold nanorods exhibit a strong NIR absorption peak at 838 nm rapidly reached 52.6°C for 5 min of NIR laser irradiation at 2 W/cm^2. The study demonstrated the use of these nanoparticles to thermally ablate the breast cancer cells in both in vitro and in vivo models[66]. Mauran (MR) is a novel sulfated polysaccharide extracted from a halophilic bacterium, *Halomonas maura* and has been used for reducing as well as stabilizing AuNPs. These nanoparticles found to produce heat when irradiated with laser lines for 1–10 min of exposure and acts as good photothermal agent for breast cancer cells[67]. The efficiency of previously stated ACA nanocomplex as an efficient strategy for concurrent delivery of heat and drug to the tumor was reported earlier synergistic therapeutic construct. The tumors treated with nanocomplex followed by laser exposure showed an increased temperature rise compared to laser irradiation alone, and reached to the thermal ablation range (>45°C), leading to localized hyperthermia in tumor tissues[68]. On the other hand, imaging technologies are currently used for monitoring the progression of treatment in cancer patients. Zhang et al develop multifunctional chitosan coated hybrid nanoparticles (h-NPs), denoted as GNR/Gd–DTPA–CS@PEG, via a simple method for MRI-guided photothermal therapy of cancer. The NPs maintained the optical properties of GNR and evaluated photothermal efficiency by determining the temperature variations of the h-NPs solutions at different concentrations which were irradiated by a NIR laser (808 nm, 2W/cm^2) for 10 min. An effective tumor ablation was achieved by applying the NIR light irradiation at proper time after the IV injection of h-NPs under the guidance of MR imaging[69]. Many targeted drug delivery systems were under development for past few decades based on gold nanoparticles. However, certain cancer like triple-negative breast cancer (TNBC), characterized by tumors that do not express the estrogen receptor (ER), the progesterone receptor (PR), or human epidermal growth factor receptor 2 (HER-2), represent clinical challenge as these cancers do not respond to targeted therapies. Successful application of siRNA against these cancers is highly dependent on the progress of delivery vehicles that are non-toxic and enable the selective and efficient transport of siRNA to a specific site. RNA interference delivery system via Chit-Au NR/ siRNA complexes was reported to be able to escape or avoid the lysosome, which contains nucleases. This system

could successfully silence (pyruvate kinase isozymeM2 (PKM2) gene in TNBC along with PTT effect, resulting in reduced cell migration and viability[70]. Injectable hydrogels are emerging recently with the advancement with multimodality incorporating system to achieve controlled and selective tumor damage with reduced risk of local recurrence and systemic cytotoxicity.

PTX/GNR/gel) co-encapsulates PEGylated (PEG = polyethylene glycol) GNRs and paclitaxel (PTX)-loaded chitosan polymeric micelles (PTX-M) in a thermal-reversible poly (F127) hydrogel matrix which had the drug-loading ability of 34.8% and the encapsulation efficiency of 89%. The gel performed as sustained and localized drug cargo to eliminate the residual tumors cells that survived the GNR-mediated photothermal treatment, thereby improving the extensiveness of the tumor regression and attributing to elimination of tumor reappearance[71].

2.7 Non-invasive imaging

Computed tomography (CT) is based on the principle that the density of the tissue on passing the x-ray beam can be measured from the calculation of the attenuation coefficient. Mannan-capped AuNPs were developed for the targeted CT imaging of lymph nodes using green chemistry-based synthesis. The particles were spherical with average hydrodynamic size of 9.18 ± 0.71 nm and demonstrated selective targeting of lymph nodes, internalized into antigen-presenting cells via mannan receptor-mediated endocytosis and could effectively enhance the contrast of popliteal lymph nodes during CT imaging[72]. ACA nanocomplex enhance CT contrast than uncoated AuNPs reflects that alginate coating can facilitate the cell membrane crossing of the nanoparticles, resulted in enhanced drug delivery to tumor cells. Thus, imaged guided DDSs were developing rapidly with the aid of polysaccharide coated metal nanoparticles[73].

Aminated, cross-linked starch and aminosilane (A) coated iron oxide nanoparticles modified with polyethylene glycol (PEG) chains can potentially enhance magnetic tumor targeting. Modified starch MNPs, showed 7 to 10 fold less uptake in RAW264.7 macrophages, much longer half-lives, improved plasma stability and enhanced tumor IONP exposure. Sustained tumor exposure in a 9L-glioma rat model (12 mg Fe/kg) using MRI proved that a modified polyethylene glycol, cross-linked starch-coated IONPs is a promising platform for enhanced magnetic tumor targeting[74]. A long-circulating polyethylene glycol modified, cross-linked starch IONPs (PEG-IONP) suitable for magnetic targeting was developed by Adam et al., in 2011. This work explores the bio-distribution patterns of PEG-IONPs in liver, spleen, lung and kidney in rat models. It possesses enhanced magnetic brain tumor targeting due to the relatively long circulation lifetime of the nanoparticles. Selective magnetic brain tumor targeting of PEG-MNPs (12 mg Fe/kg) was confirmed in 9L-glioma tumors, with up to 1.0% injected dose/g. Carboxylic mannan (CM)-coated super paramagnetic iron oxide nanoparticles (CM-SPIONs) were able to target immune cells including antigen-presenting cells (APCs), macrophages. The high negative charge of uniform-sized CM-SPION nanoparticles helps it for longer blood circulation, and it could more effectively target macrophages bearing mannose receptors than polyvinyl alcohol (PVA) or dextran-coated SPION (Dex-SPION). iIn vitro uptake study visualized by MR phantom imaging, the intracellular uptake of CM-SPION was much faster than those of Dex-SPION and PVA-SPION at the initial hours of incubation and increased drastically up to 24 h post-incubation. The in vivo uptake of CM-SPION in lymph nodes was tracked by MRI after subcutaneous injection in a rat model. It was found that the CM-SPION predominantly accumulated in the popliteal nodes, and the in vivo accumulation rate with CM-SPION in the lymph nodes was comparable to that of Dex-SPIONsas measured by a signal drop in MR intensity. The accumulation of SPION was confirmed by histological analysis with Prussian blue staining [75]. Yuhaan et al. developed surface immobilization of Hyaluronic Acid on monodisperse magnetite nanocrystals in aqueous solution. This possesses cancer targeting capability via CD44-HA receptor-ligand interactions[76]. Omid et al. developed a nanoprobe composed of an iron oxide nanoparticle coated with polyethylene glycol–grafted chitosan copolymer, to which a tumor-targeting agent, chlorotoxin, and a near-IR fluorophore were conjugated. This nanoprobe exhibited the ability to cross the blood-brain barrier and specifically target brain tumors in a genetically engineered mouse model. The nanoprobe showed an inoffensive toxicity profile and constant retention in tumours. With the multipurpose affinity of surface marker and the supple conjugation chemistry for alternative diagnostic and therapeutic agents, this nanoparticle can be possibly used for the diagnosis and treatment of a diversity of tumor types[77].

Bimetallic systems are favoured recently rather than single metal system as it gives a chance to synergize the properties of two metals in a single unit making it a smartly engineered nanostructure. The gold nanoclusters with size range less than 5 nm tends to fluorescence in the presence of UV light which marked its potential as an imaging agent. Dutta et al reported that the possibility of bimetallic nanoconjugate formed using silver nanoparticles and gold nanoclusters for anticancer activity and bioimaging better

than monoatomic system against HeLa cell lines. The study provides a stable idea of cancer nanotheranostics enables cellular imaging due to the presence of luminescent nanoclusters without tha use of any dye and offered anti-tumor properties due to the presence of silver nanoparticles, resulting in successful killing of cancer cells through apoptosis[78].

2.8 Immunosensors and imaging probe

Immunosensors is based on the principle of antigen-antibody interaction that has attracted greatly for very sensitive detection of biomarkers due to its simplicity, low cost, high sensitivity and miniaturization. A nanocomposite film consisting of chitosan and AuNPs for designing sandwich disposable immunosensor for the detection of prostate specific antigen (PSA) was developed by Lakkavarapu Suresh et.al. The surface of chitosan-AuNPs casted screen printed electrodes was immobilized with Ab1 monoclonal antibody interacts with PSA and displayed a linear response with wide range (1-18 ng/ml), low detection limit (0.001ng/ml), acceptable reproducibility, selectivity and stability[79]. In another report, an efficient bimodal imaging probe for biological systems was developed using chitosan encapsulated multifunctional magneto-fluorescent nanocomposites for fluorescence as well as for MR imaging. The particle containing chitosan encapsulated iron oxide (as MRI contrasting agent), CdS NPs (as fluorescent probe) and podophyllotoxin (PD) as anticancer drug can find its application in future healthcare diagnostic system[80].

2.9 Other biomedical applications

Most of the data available regarding the use of polysaccharide coated or uncoated metal nanoparticles (NP) are on diagnosis and treatment of cancer. The nanoparticles can be further used in several other biomedical conditions including wound healing, arthritis etc. and have several other properties such as anti-oxidant, anti-microbial, hepato-protective effects which could be beneficial in treating various ailments. A very few studies are available recently on benefits of nanoparticles other than using as imaging agents and drug carrier systems.

Antimicrobial activity is a desired feature for nanoparticles which are to be used in wound healing. Silver (Ag), copper (Cu), zinc (Zn), gold (Au) nanoparticles have reported to have antimicrobial properties. Muhammad et al published a work on polysaccharide coated silver nanoparticles for wound healing. The polysaccharide used as stabilizing and capping agent was glucuronoxylan, which was isolated from *Mimosa pudica*. The antimicrobial activity was proved against several bacterial and fungal species and they prepared a dressing using the nanoparticles and proved its wound healing potential in rabbit wound models[81]. Sanyasi et al prepared polysaccharide capped AgNPs which will inhibit biofilm formation and inhibit multidrug resistant bacteria. An implausible investigation arose of a dual modality nanoparticle wherein chemotherapy in fusion with antibacterial efficacy is obtained. The particles are green synthesized using galactoxyloglucan and silver nitrate to produce polysaccharide coated silver nanoparticles, which displayed biocompatibility with an upgraded selective cytotoxicity toward cancer cells. The non-toxic particles found to be anti- bacterial and exhibited a unique SERS platform[82]. Carboxyl-methyl derivative of tamarind polysaccharide coated particles showed antibacterial activity by altering the expression of ftsZ-ftsA and affecting bacterial cell elongation and cell division[83]. Chitosan is a polysaccharide having antimicrobial property. This has been used for the synthesis of AgNPs and activity studies have been done by Kalaivani et al[84]. They have proposed the use of low-cost waste products for the preparation of cost effective products in biomedical field. Cu is an extensively used in several fields of human life including electricity, catalysis and biomedical field. El-Batal and group tried to prepare CuNPs using different polysaccharides such as chitosan, citrus pectin and sodium alginate and compared its activity. The particles showed improved antimicrobial and antioxidant activities[85]. According to the authors the antioxidant activity was due the hydrogen providing capability of CuNPs. Similarly the same group has prepared ZnNP using the same polysaccharide and analyzed its bioactivities and found that ZnNP were cytotoxic, antimicrobial and antioxidant in nature[86].

Selenium nanoparticles can balance the redox balance in human body and major drawback in synthesis is the aggregation of the particles therefore, capping is very crucial in particle synthesis. Zhang et al and Xiao et al have synthesized polysaccharide coated selenium nanoparticles. Polysaccharides from *Lycium barbarum* and *Cordyseps sinensis* have been used for the fabrication of nanoparticles. Antioxidant activity was proved and the authors proposed to use this as anti-oxidant food supplement and neuro-protective agent [87, 88]. Green synthesized palladium nanoparticles have been synthesized using gum ghatti polysaccharide by Aruna et al for its application as an anti-oxidant agent[89].

3. Conclusions

Theranostic approach of metal nanoparticles with regard to both therapeutic and imaging of tissues in better

management of cancer is vital in the current scenario. Metal nanoconjugates are not stable for long extent due to its incompatibility in body fluids and higher cytotoxicity in normal tissues surrounding the tumor. In order to minimize the toxicity and increase the stability, these nanoconjugates needed to be coated with a biopolymer. Low-cost and abundant availabilityof polysaccharides could be employed for the synthesis of metal nanoparticles. Gold and iron oxide nanoparticles were widely used for drug delivery, imaging, hyperthermia and adjuvant therapy for tumor management. The polysaccharide coated metal nanomaterials conjugated with cell surface receptors and drugs are yet to be prepared in order to use in the field of oncology. Synthesis of polysaccharide coated metal nanoconjugates to be used for in vivo imaging of tissues like MRI appears promising.

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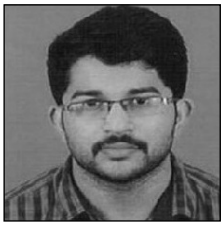	**Mr Unnikrishnan B. S.** *(CSIR-UGC SRF) is pursuing his PhD in Biotechnology from Univesity of Kerala under the guidance of Dr TT Sreelekha, Assistant Professor, Laboratory of Biopharmaceuticals and Nanomedicine, Division of Cancer Research, Regional Cancer Centre, Thiruvannathapuram, Kerala, India. He is working on polysaccharide metal nanoconjugate for cancer diagnosis and therapy. He has co-authored many publications and book chapters.*
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Emerging trends and advances in targeted nanotheranostics

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Abstract

The current human population is rapidly falling prey to numerous illnesses owing predominantly to compromised immune systems and severe multi-drug resistance (MDR). These growing incidences of diseases entails for an elaborate diagnostic system which can aid in early stage diagnosis, especially in the case of life threatening maladies like cancer. Nanotheranostics is one such field which exhibits enormous potential for early stage diagnosis of a disease as well as in curing it at the point of care itself. Nanotheranostics signifies nanoscale level prognosis by combining biomedical payloads with techniques like MRI and PET-CT scan along with providing therapy at the nanoscale level *via.*chemotherapy, radiotherapy, photodynamic therapy etc. This is achieved by conjugating the diagnostic and the therapeutic entities in the form of a biomedical payload on nano carriers which can be polymers, lipids or inorganic materials, depending upon the ailment in question. This new age nanomedical system has further expanded avenues for personalized/precision medicine (e.g., individual tumor examination) as nanotheranostics offers to combine imaging, diagnosis and therapy in single nano unit, thereby rendering precision medicine a unique, multidirectional approach for achieving path-breaking headways in the field of medical research and sciences. Through this comprehensive review, we aim to delineate the progression and reworking of nanotheranostics, along with a perspective of its effect on precision medicine.

Keywords: biomedical payloads, carriers/platforms, surface-modifiers, nanotheranostics and precision medicine.

1. Introduction

Nanomedicine is the combination of nanotechnology with medicine, wherein nanomaterials are key to facilitating disease diagnosis, treatment and post-treatment monitoring. Some of the most commonly employed nanomaterials are inclusive of carbon nanomaterials, polymers, liposomes, dendrimers and antibodies [1]. In contrast to several other standard, low molecular weight carriers, nanomaterials proffer numerous benefits, such as, (i) preventing premature clearance and enzymatic degradation; (ii) improving bioavailability (iii) enhancing target site accumulation of drugs and imaging agents; (iv) increasing the *in vivo* efficacy of diagnostic and therapeutic mediations; (v) ensuring targeted delivery; and (vi) reducing the incidence and intensity of side effects [2-5].

However, to better understand and optimize the efficacy of these nanomaterials in the field of nanomedicine, it is critical to develop a singular platform for simultaneous diagnosis, treatment and disease monitoring, and this can be achieved by a latest technology introduced into the domain of biomedical engineering, namely, theranostics. Theranostics is an amalgamation of therapeutics and diagnostics. This combination of diagnosis with therapeutics accelerates the process of disease identification and treatment along with making it a cost-effective process with minimal side effects.

2. Nanotheranostics

A well-developed theranostic model would aid in facilitating high-resolution, real-time analyses, treatment and disease monitoring, thereby enabling us to examine the ailment, basis its molecular profiling and progression rate, at individual patient levels. A theranostic tool's most imperative part is an ideal diagnostic probe, which must be endowed with the following features, namely; (i) ability to identify the target location, (ii) non-toxic nature, (iii) high signal-to noise ratio, (iv) high target specificity, and (v) early and rapid detection rate. As has been discussed in numerous reports earlier, the Ferrari's classification delineates the theranostic agent/nanoparticle to be a fusion of three major components; namely, (i) carrier/platform, (ii) biomedical payloads, and (iii) surface modifier (fig.1a and 1b) [6-18].

Biomedical payloads are composed of therapeutic drugs and/or genes, targeting moieties, performance enhancers, and imaging agents. Some of the most popularly

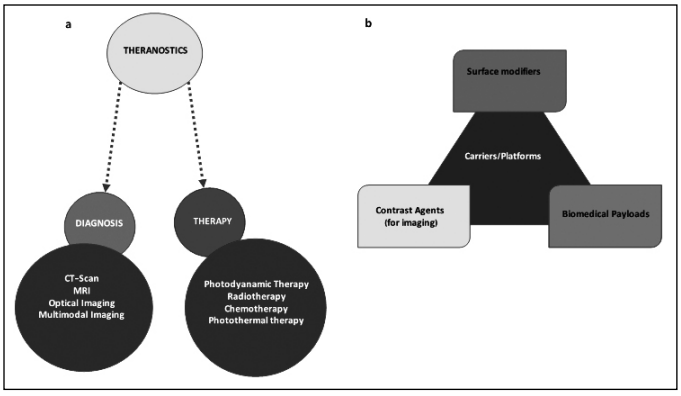

Fig. 1: *(a) Theranostics as a combination of diagnostics and therapeutics, and (b) Theranostic agents in specific.*

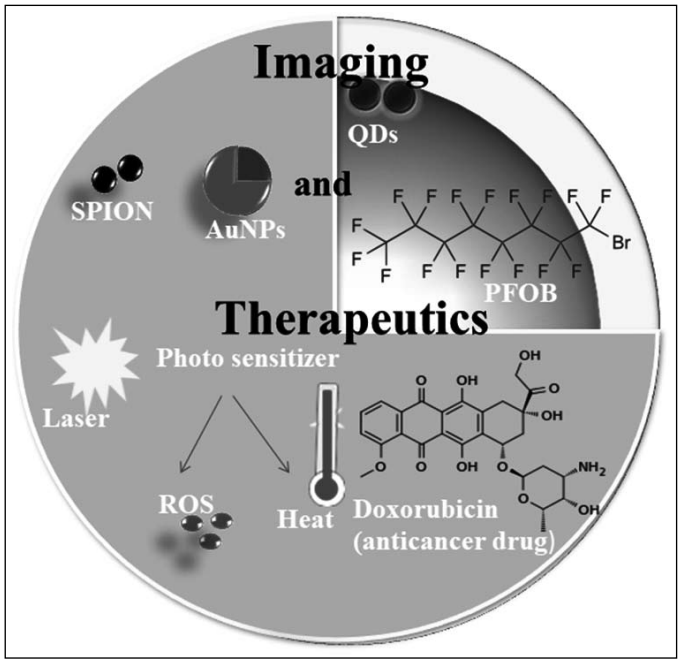

Fig. 2: *Nanoplatforms with theranostic functionalities: Gold nanoparticles (AuNPs), Superparamagnetic iron oxide nanoparticles (SPION) as MRI active agents, Quantum dots (QD), Laser treatment, drugs as targeting agents, photo sensitizer, PFOB nanocontrast agent.*

adopted biomedical payloads are quantum dots (QDs), Magnetic Resonance Imaging (MRI) contrast agents, Computerized Tomography (CT) contrast agents, and therapeutic agents (DNA, small interfering RNA, anti-cancer drugs, ROS-generating nanoparticles, etc) (fig. 2).

Further, carriers are exploited to shield the biological payloads under several unsuitable physiological conditions, thereby promoting target-specific delivery of the biomedical payload. Subsequently, surface modifiers are bound to the theranostic nanoparticle to endow it with supplementary attributes such as target-specific binding ability and improved bioavailability.

2.1 Carriers

A wide array of carriers/platforms are being developed and explored for the establishment of a bankable, theranostic probe. The platforms are developed keeping in mind the disease, its therapeutic requirement and the desired imaging modalities. Carriers are defined as substrates that have the potential to host and deliver the therapeutic agent along with the imaging modality and contrast agents to the desired site of action. The physiological conditions of the target area, chemical nature of the drug to be delivered and the imaging modes being applied play a decisive role in choosing the right platforms. Majorly, carriers/platforms can be broadly classified into (i) Inorganic platforms (e.g., Gold nanoparticles, Magnetic nanoparticles, Quantum dots, etc.), (ii) Organic/carbon based platforms (e.g., Carbon nanotubes, Carbon nanodots etc.), (iii) Polymeric platforms (e.g., Dendrimers, hydrogels etc.) and (iv) Lipid based platforms. Their pliability to form different shapes and sizes also attributes to their acceptance in nanotheranostics as different shapes confer different properties. For example, a 10 nm spheroid would ideally absorb in the UV range of 520 nm, however; the nano-rods absorb in the near infrared radiation (NIR) in the range of 690-900 nm. Important features that make these nano-platforms a viable nano-theranostic option are [19]:

(i) Biocompatibility and low toxicity.

(ii) Facile and targeted binding to drugs and biological molecules.

(iii) Tunable core size.

(iv) Monodispersivity.

(v) Light scattering properties.

2.2 Biomedical Payloads:

2.2.1 Imaging

Latest imaging modalities occupy a key spot in nanomedicine, and this is ascribed to the biomedical payloads, with a host of advantages such as non-invasive imaging, specific targeting and the ability to track biomarkers involved in the disease progression by allowing a real-time scan. The primary characteristics of major imaging modalities are discussed herewith:

a. Optical Imaging (OI)

OI is extremely sensitive and flexible in its approach. *In-vivo* OI uses contrast agents that interact with the visible and near-infrared region of light and can be classified into diffusive and ballistic imaging systems. Diffusive OI uses near-infrared spectroscopy (NIRS) or fluorescence-based methods, whereas; on the other hand,

ballistic imaging system employs ballistic photons, which are light photons, traversing in a straight line, through scattering media.

In comparison with other imaging modalities, OI offers numerous advantages such as probing both functional and structural changes with a high spatial resolution, non-invasive and non-ionizing nature, cost-effectiveness, and provides macroscopic as well as microscopic scale imaging options [20-22].

The most extensively employed OI techniques for monitoring drug delivery include Fluorescence reflectance imaging (FRI) and fluorescence molecular tomography (FMT) [23-25]. An example of concurrent drug release and imaging using OI was stated by Ferber *et. al.* [26]. They synthesized a dual polymeric system specific to breast cancer. Herein, its diagnostic moiety was made up of high loading, self-quenching, turn-on system with NIR fluorescent dye Cy5 (SQ-Cy5), while the chemotherapeutic agent paclitaxel (PTX) represented the therapeutic segment, and consequently, both were conjugated to N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer *via* GFLG linkage.

Additionally, J. Chen *et. al.* developed quantum dots (QD) based OI for examining the membrane protein structures with a diffraction-unlimited spatial resolution [27]. The QDs are known to possess excellent photostability and due to their high photo-luminescence, it remarkably enhances the signal-to-noise ratio and the reproducibility of the optical data.

Some of the most widely practiced applications of theranostics are inclusive of cancer detection, real-time drug-release, monitoring and therapy and post-therapeutic screening. These recent advances in the field of theranostics also brought about a paradigm shift for personalized/ precision medicine.

b. Positron Emission Tomography and Computed Tomography

Positron Emission Tomography (PET) is a form of nuclear medicine imaging. In PET, the images are obtained by imaging the decay of radioisotopes bound to molecules with established biological characteristics. PET utilizes positron-emitting radionuclides like ^{11}C, ^{13}N, ^{15}O, ^{44}Sc, ^{18}F, ^{62}Cu, ^{64}Cu, ^{18}Ga, ^{72}As, ^{76}Br, ^{86}Y, ^{82}Rb, ^{89}Zr and ^{124}I [28-34]. Due to its exceptionally high sensitivity, PET requires trace amounts of radioisotopes and aids in examining the ailment at metabolic level. Ascribing to its remarkable sensitivity and high tissue-penetration, PET is routinely employed in nanotheranostics for monitoring bioavailability, pharmacokinetics and pharmacodyanamics of the nanomedicines.

Computed tomography (CT) is an X-ray based imaging technique, which enables a 2D as well as 3D visualization of organs, bones and tissues of interest. CT generates anatomical and cross-sectional images by utilizing highly electron dense contrast agents such as iodine. CT scans are a commonly favored imaging option for the diagnosis of tumors, in addition to diagnosis of circulatory disorders, kidney and bladder stones, abscesses, inflammatory diseases, bone injuries and internal organs.

Recently, a lot of emphasis has been laid on the development of nanoparticles such as lipoproteins, micelles, and polymeric nanoparticles [35-41]. Nanoparticles hold many advantages, such as prolonged blood circulation half-life [41], biocompatibility, better *in-vivo* cell tracking [42] and targeted imaging application, to be used as contrast agents when compared to other small molecules.

c. Magnetic Resonance Imaging (MRI)

A medical application of Nuclear Magnetic Resonance (NMR), MRI is an imaging technique where the spins of specific atomic nuclei are visualized within the body. Numerous magnetic nanoparticles (MNPs) have been utilized for several biomedical applications, especially as contrast agents for MRI [43]. Recently, Mikhaylov and colleagues have developed universal lapidated magnetic nanocarrier (ferri-liposome) that not only enhanced MRI's contrast abilities but were also effectively taken up by tumor cells and the surrounding stromal cells. These ferri-liposomes, comprising of MNPs contained inside a liposome, were used as a delivery vehicle for cathepsin protease inhibitor to mammary tumor and the surrounding stromal cells in a mouse model. These as-synthesized MNPs resulted in an appreciable decrease in the size of the tumor when compared to systemic route of delivery for the same drug [44].

d. Multimodal Imaging

Multimodal Imaging is a fusion of different imaging modalities, focused at providing panacea to counter the existing limitations of the independent imaging techniques. Multimodal imaging brings together the functional and/ or structural advances from several imaging techniques, resulting in a more accurate diagnosis. Moreover, the combined therapeutic advantages of the assorted techniques render an improved, synergistic, therapeutic efficacy [45-49]. The most widely exploited multimodal imaging techniques are SPECT-CT, PET-CT and PET-MR.

Single photon emission computed tomography (SPECT), an analog of PET, is based on utilizing the non-coincident gamma-rays which are generated by radionuclides like ^{99m}Tc, ^{111}In, ^{123}I and ^{201}Tl. These radionucleotides are known

Table 1: Various Carriers/platforms and their properties.(Charlene M. Dawidczyk, 2014)

	Particle type	Composition/Structure	Properties	Applications
	Polymer	e.g., PLGA, glycerol, chitosan, DNA; monomers, copolymers, hydrogels	Some biodegradable	Drug delivery; passive release (diffusion), controlled release (triggered)
	Dendrimer	PAMAM, etc.	Low polydispersity, cargo, biocompatible	Drug delivery
	Lipid	Liposomes, micelles	Can carry hydrophobic cargo, biocompatible, typically 50–500 nm	Drug delivery
	Quantum dots	CdSe, CuInSe, CdTe, etc.	Broad excitation, no photobleaching, tunable emission, typically 5–100 nm	Optical imaging
	Gold	Spheres, rods, or shells	Biocompatibility, typically 5–100 nm	Hyperthermia therapy, drug delivery
	Silica	Spheres, shells, mesoporous	Biocompatibility	Contrast agents, drug delivery (encapsulation)
	Magnetic	Iron oxide or cobalt-based; spheres, aggregates in dextran or silica	Superparamagnetic, ferromagnetic (small remanence to minimize aggregation), superferromagnetic (~10 nm), paramagnetic	Contrast agents (MRI), hyperthermia therapy
	Carbon-based	Carbon nanotubes, buckyballs, graphene	Biocompatible	Drug delivery

to possess higher sensitivity, high penetration depth and exceptionally quantitative results. Usage of radioactive probes, low spatial resolution and lack of anatomical information are some of the disadvantages coupled with SPECT, which are now countered by integrating SPECT with CT. Chrastina et. al reported the use of SPECT-CT for screening nanomedicine based drug targeting to lungs [50]. Till date, PET is reported to suffer from relatively poor spatial resolution. This drawback can be overcome by combining PET with MRI, which is known to proffer not only excellent spatial resolution but also has better soft tissue contrast. Thus, PET-MRI has found its way into clinical applications such as cancer diagnosis, neurological studies, and stem cell therapy [51-53]. Further, Yang et. al. prepared cRGD functionalized ^{64}Cu labeled SPIO nanocarriers loaded with DOX for both tumor targeted drug delivery and PET-MRI imaging [54].

2.3 Surface modifiers:

Usually, tumor blood vessels are tainted with anamolies like comparitively higher ratio of proliferating endothelial cells due to rapid vascularisation [55-56]. This combination of perforated vasculature and compromised lymphatic drainage results in the accumulation of nanoparticles. This accumulation is further aggravated in tumors due to decreased glomerular excretion, owing to enhanced permeability and retention (EPR) effects [57-60]. EPR conciliated nanoparticle accumulation requires their extended circulation in the blood. Therefore, these nanoparticles (i) need to be highly soluble in blood, (ii) shouldn't be eliminated by the reticuloendothelial system (RES), (iii) their size range should be appropriate for EPR processes, and (iv) they should be biocompatible with minimal toxicity [61-63]. Moreover, these nanoparticles should get seized by the cells upon reaching the target site, prior to the release of their therapeutic component. This requires a perfect mix of surface hydrophobicity and charges. These aforementioned aspects entail advance-stage nanoparticle surface engineering and several recent researchers are making significant efforts for the same.

As previously mentioned, nanoparticle carriers/ platforms like polymeric micelles using biological (e.g., starch, dextran, cellulose, and hyaluronic acid) or synthetic polymers and hydrophilic surface-re-modelled inorganic

nanomaterials have attracted huge research interests [64-68].

Although, the therapeutic potency of theranostic nanomaterials are heavily dependent on the above-mentioned parameters like biomedical payloads, carriers and surface modifiers; nonetheless, their ultimate purpose of serving as a successful theranostic tool emanates from the final action of these nanomaterials at the therapeutic level. Therefore, it is imperative to discuss "therapeutics" post "diagnoses" which are mostly non-invasive, imaging techniques, as has been stated earlier.

Since the past decade, nanostructures are being largely employed, primarily in cancer therapy to mitigate these loopholes. This shift in focus has led to plethora of therapeutic options, wherein, nanoparticles with appropriate biomedical payloads have been incorporated into therapeutic strategies like Nano-chemotherapy (NCT) [69], photothermal therapy, photodyanamic therapy and radiotherapy with nanoparticles that can be divided into, but not restricted to, liposomes, polymeric micelles and polymer-drug conjugates, dendrimers, oil nano-emulsions, inorganic nanocarriers, etc.

3. Perspective

Nanotheranostics for precision medicine:

Recently, a new domain, namely, precision medicine, also known as personalized medicine has been attracting huge attention. Eponymous to its name, it relies on the fact that a single drug cannot be suited for all mankind. As has been previously discussed, theranostics can aid us in developing therapeutic strategies that are patient specific [70], thereby rendering the treatment individual centric.

Since nanotheranostics promises imaging, diagnosis and therapy in a single nano-unit, it confers precision medicine with a unique multidirectional approach. Therefore, by bridging the gap between precision medicine and nanotheranostics, early diagnosis, staging of a disease, selection of a personalized treatment, treatment follow ups, recognizing the side effects and changing the course of treatment can be performed at nanoscale level.

Achieving the goal of an ideal, disease specific, personalized, nanotheranostic tool would further strengthen biomedical technology and benefit the health sector by leaps and bounds as it would impart several following advantages, to enlist a few:

(i) A more specific diagnosis

(ii) Lesser side effects

(iii) Better chances at cure

(iv) Cost-effectiveness.

Currently, the investigations and breakthroughs in the fields of nanotheranostics as well as precision medicine are still very rudimentary. Leading researcher's world over are working towards a quick, large-scale and economic implementation of the systems that would be capable of performing an individualistic diagnosis.

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Mucus penetrating Non-Viral Vectors in Lung Gene Therapy

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Abstract

Thick layer of mucus underlying the airways epithelium is a major hurdle for delivery of therapeutic molecules to the lung. The presence of endogenous and exogenous bacterial DNA and actin filaments from degraded neutrophils potentiate the barrier property of the mucus in various diseased conditions like Cystic fibrosis (CF) and Chronic Obstructive Pulmonary Disease (COPD). There are several gene therapeutic approaches reported in literature with respect to various lung disorders where the focus is on the design of gene delivery vectors that take into account the composition and dynamic properties of the mucus and are fine-tuned to overcome the mucus barrier for effective penetration and delivery of the therapeutic gene. Selection of appropriate vector for gene therapy is decided by the type and complexity of various lung disorders. There is a need for long term expression of therapeutic gene in certain diseases which could be achieved by viral vectors but their repeated dose of administration lead to immune responses and their relative inability to cross the thick mucus barrier decreases the therapeutic gene delivery efficiency. To tackle these problems non-viral vectors with mucus penetrating strategies have emerged as potential delivery systems with increased mucus permeation ability towards treatment of lung diseases.

Keywords: Gene therapy, mucus barrier, mucoadhesive, mucus penetrating.

1. Introduction

Gene therapy is currently being evaluated for a wide range of acute and chronic lung diseases including Asthma, COPD, cystic fibrosis. For example, over expression of TLR heterodimers through plasmid DNA delivery could be thought as one of the therapeutic strategy in asthma pathogenesis. Polymorphisms in TLR1, 6 and 10, which can form heterodimers with TLR2, have shown protective effects on atopic asthma in humans and is further associated with elevated peripheral blood mononuclear cell secretion of Th1 cytokines [1]. Yamada et al first demonstrated that the large size of 5′-flanking polymorphism ((GT)n repeat number) in the HO-1(one of the isoforms of heme oxygenase enzyme) gene was associated with chronic pulmonary emphysema [2]. So delivery of the HO-1 gene would result in reduced inflammation and mucus secretion in COPD. Gene therapy has gained more attention for monogenic disorders than for complex diseases where both external (environmental and behavioural risk factors) and internal (biological) factor play role [3]. For example, mutations in the CFTR (cystic fibrosis transmembrane conductance regulator) gene leads to imbalanced ion and water movement across the airway epithelium, resulting in accumulation of sticky mucus, chronic bacterial infection and inflammation. CFTR gene is the reported therapeutic target in cystic fibrosis.

Although lung has been chosen as a target for systemic drug delivery due to enormous surface area of the alveoli and a relatively low enzymatic, controlled environment for systemic absorption of medications [4], targeted delivery to lung itself for treatment towards lung disorders like COPD and cystic fibrosis is challenging due to airway mucus obstruction. In healthy lungs, a thin layer of mucus traps the foreign particulates and pathogenic strains, which are then phagocytosed by mucociliary clearance mechanism and consequently are also barriers to the therapeutic effectiveness of inhaled medications. The barrier property of the mucus is enhanced in patients suffering from cystic fibrosis and COPD due to the presence of bacterial DNA and actin filaments from degraded neutrophils [5, 6]. In these cases, where gene therapeutic approaches are adopted as strategy towards cure, the non-viral gene delivery system has to efficiently penetrate the hard to- breach human airway mucus barrier of lung tissue. Usually, it needs to be muco-inert or coated with some hydrophillic molecules or could also be conjugated to some mucolytic agents. Coating gene delivery system with low molecular weight (MW) PEG is widely studied as a strategy for mucus penetration [7].

The aim of present study is to summarize the mucus barrier property, approaches for designing non-viral vectors to conquer the mucus barrier and stress upon the therapeutic genes which could be delivered by non-viral

vectors in various lung diseases like COPD, Asthma and Cystic Fibrosis.

2. Therapeutic Genes to be targeted in various Lung Disorders

Gene therapy is currently being evaluated for a wide range of acute and chronic lung diseases including acute respiratory distress syndrome (ARDS), cancer, asthma, emphysema and cystic fibrosis.

2.1 Asthma

Asthma is the most common chronic disease. Several studies indicate that children having been exposed to an environment rich in microbes in their early childhood show less frequent atopy and allergic asthma [8, 9]. Toll-like receptors (TLRs) are the primary sensor of immune system that is responsible for recognizing and responding to microbes and microbial components (pathogen-associated molecular patterns (PAMPs)). TLR stimulates the secretion of certain "instructive" cytokines and influence T-cell development, mainly towards a T helper cell type 1 (Th1) dominant phenotype [10]. Multiple studies demonstrated that an imbalance of T helper cell responses plays an important role in asthma development [11, 12]. In this disease predominance of a Th2 pattern leads to an increased production of chemokines, as well as allergen-specific immunoglobulins, thus causing airway inflammation, eosinophilia and mucus hypersecretion in the lung [13, 14]. The clinical presentation of atopic asthma eventually consists of wheezing, airway obstruction, breathlessness and cough, often accompanied by recurrent bronchitis or pneumonia [15, 16].

Polymorphisms in TLR1(Toll Like Receptor), 6 and 10, capable of forming heterodimers with TLR2, have shown protective effects on atopic asthma in humans [17]. Franziska Zeyer *et al* found that treatment with either Tlr1/2 mRNA or Tlr2/6 mRNA resulted in better lung function as well as reduced airway inflammation *in vivo* but treatment with Tlr2 alone did not reduce inflammation. *In vivo* studies revealed that percentages of neutrophils and eosinophils in bronchoalveolar lavage fluid (BALF) were reduced after Tlr1/2 administration when compared to the HDM (House Dust Mite) control group, and absolute numbers of eosinophils in lung tissue were diminished [18]. Furthermore, those mice showed notably improved lung function and significantly reduced pulmonary mucus production. It is possible that over expression of TLR1/2, TLR6 through gene delivery can help to alleviate the disease.

2.2 COPD (Chronic Obstructive Pulmonary Disease)

Repeated exposure to noxious particles, usually tobacco smoke, can trigger a distinct inflammatory cascade in the small airways and lung parenchyma involving several different cell types and inflammatory mediators resulting in COPD [19]. Therefore, reducing oxidative stress and airway inflammation is supposed to be effective in the treatment of COPD. A number of clinical trials and system reviews have shown that NAC (N-acetyl cysteine) leads to dose dependent reduction in the risk of re-hospitalization for COPD [20-23].

HO-1, one of the isoforms of heme oxygenase enzyme, is traditionally thought to be a fundamental "sensor" of cellular stress and directly contributes toward limiting or preventing tissue damage [24]. HO-1 is a pleiotropic protein and has multiple roles, including a heme degradation function as well as a nonenzymatic signaling function, modulation of protein translation, and binding to DNA repair proteins [25, 26]. So HO-1 can be taken as one of the therapeutic genes to be explored in COPD.

2.3 Cystic Fibrosis

CF is the most common lethal autosomal recessive disease in the Caucasian population and affects approximately 70 000 individuals worldwide. Although several organs are affected, severe lung disease is the cause of most of the mortality in CF individuals [27, 28].

Cystic Fibrosis is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that encodes a cAMP(cyclic AMP) dependent anion channel that conducts chloride and bicarbonate ions and plays an important role in secretion of these anions and fluid by epithelial lining of the conducting airways and submucosal glands [29, 30].[0] In the healthy condition, airway surface liquid(ASL) is isotonic in nature and is maintained in isotonic equilibrium by the secretion of fluids from the epithelium (especially serous cells in the sub-mucosal glands where *CFTR* is highly expressed) [31]. CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) controls the secretion of Cl- and the absorption of Na+ by the regulation of the epithelial sodium channel, ENaC [32]. In CF, the ASL remains isotonic, but loss of CFTR activity leads to an increase in Na+ absorption by ENaC and leads to excessive fluid uptake, which dehydrates the ASL [33]. The concentration of the ASL increases the viscosity and impairs mucociliary clearance and ultimately leads to recurrent bacterial infection.

The low-level, but widespread pattern of *CFTR* gene expression in the airways suggests that most cells require CFTR gene transfer to correct the physiological defect. However, the expression of *CFTR* in only 6 – 10% of cells in a polarized CF epithelium was sufficient to correct the Cl- ion transport defect [34]. It has been also suggested

that down-regulation of ENaC may help to restore airway hydration and mucus clearance.

3. Mucus Structure and Composition

Body's first line of defence, mucus, is a viscoelastic secretion from epithelial cells and forms the interface between an organism and its external environment. Mucus consists of 90-95% water which serves as a medium for mucin protein, lipid and electrolyte [35]. Mucins can be classified into two groups, membrane bound mucins (MUCs 1, 3, 4) and secreted gel forming mucins (MUCs 2, 5AC, 5B). MUC 2 forms the intestinal mucus and MUC5AC and MUC5B is found in airways mucus lining [36]. These mucin proteins are heavily gycosylated (both N and O-glycosylation), which contribute 50-80% of the mucin weight, and primarily glycosylated through Gal NAc, GlcNAc, Fuc, Gal and sialic acids [37]. The size of Mucin fiber is 10-40MDa [38].

Thickness of mucus layer differs in various organ systems - in trachea, the mucus layer thickness is 10µm [39] and in GI tract it varies from 200µm to 800µm [40]. Moreover the mucus thickness declines with age [41]. MUC5AC and MUC5B are involved in mucus production in lungs and their expression varies among individuals. MUC5B variants regulate airways homeostasis, mucosal immune function and disease pathogenesis in humans [42]. MUC5B levels affect macrophage mediated expression in lungs and inflammation. MUC5B deficient mice showed intensely affected mucociliary clearance indicating critical role of MUC5B in mucociliary clearance (MCC).

4. Pathophysiology of mucus in diseased condition

The pathogenesis of mucus hypersecretion varies in different lung diseases. In COPD, chronic inflammatory stimuli result in hypertrophy and hyperplasia of submucosal bronchial glands and metaplasia of bronchial epithelial globlets cells, consequently leading to obstruction in airflow and blockage of respiratory tract [43]. Asthma is characterized by lymphocyte Th2 ($CD4^+$) mediated eosinophilia and it affects the airway rather than lung parenchyma [44]. In contrast to COPD, exocytosed mucins from globlet cells in Asthma are not fully released and are tethered to the airway epithelium which contributes to the mucus plug formation [45]. Cystic Fibrosis (CF) on the other hand is a monogenetic disorder, which occurs due to the defect in Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) channel as described above. Defects in CFTR channel in CF leads to imbalance between absorption and secretion of salt and water resulting in dehydrated, viscous mucus and impaired mucociliary clearance [46-48]. Additionally, neutrophillic inflammation were shown to induce abnormal mucus secretion.

The upregulation of mucin gene in diseased condition is triggered by various inflammatory cytokines like IL-4 and IL-13 [49, 50]. STAT6 has a key role in IL-4 and IL-13 mediated overexpression of mucin [51]. Xiaoyun Wang and group found the role of Lyn kinase, a non-receptor cytoplasmic tyrosine kinase in mucin hypersecretion, which was earlier reported to be associated with Asthma and their studies revealed that Lyn overexpression down regulates STAT6, which binds to MUC5AC promoter and hence ameliorates the airway mucus hypersecretion [52].

Thickened mucus not only affects the epithelial cilia movement and mucociliary clearance but also motility of neutrophils get hampered in airway mucus due to the increased viscosity and thickness of mucus layer in CF, which further prevents bacterial capture and killing, leading to early and chronic stages of bacterial infection [53]. Defect in CFTR channel in CF is reported to affect all organs which produce mucus. Some adults with CF also showed distal intestinal obstruction syndrome (DIOS) [54]. Also severe intestinal phenotype has been observed in experimental animal models (mice or pigs) of Cystic Fibrosis [55-57].

The solid weight of mucus varies in different lung disorder. David B. Hill and group postulated and showed that weight% solids of lung mucus can serve as a candidate for easily applied clinical biomarker for airways disease [58].

5. Hindrance to delivery system by mucus barrier

5.1 Micro and Macrorheology

It is pertinent to consider the microrheology and macrorheology of mucus, while developing a delivery system to overcome the mucus barrier. Mucus is a viscoelastic gel at the macro scale and is characterized by a non-Newtonian viscosity non-linear with shear stress and gives more resistance to deformation at low shear rates and less resistance at high shear rates. Macrorheology is critical for macroscale functions like mucociliary clearance and lubrication while microrheology deals with the viscoelasticity encountered by various micro and nanoscale entities [59]. It has been shown that mucoinert particles as large as 500nm were able to cross undiluted human cervicovaginal mucus which disproved the existing notion that particles more than 100nm in size were hindered by viscous and elastic forces of mucus during penetration. This implies that development of the drug and gene delivery system for transmucosal delivery needs to be focused upon

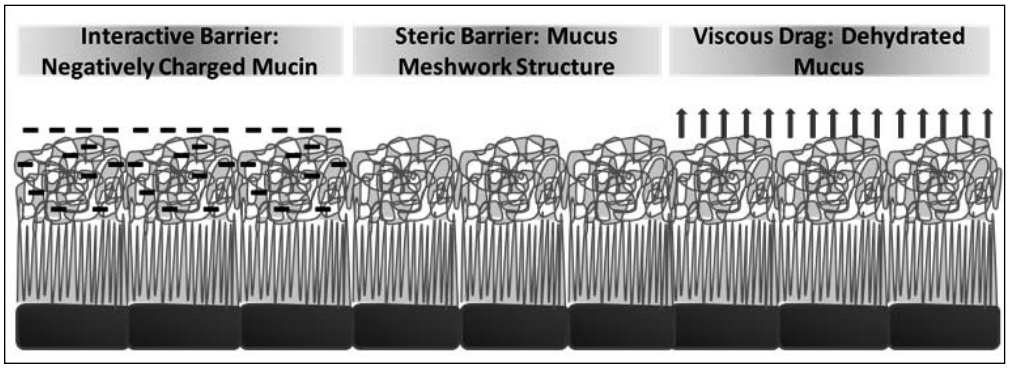

Fig 1: *Schematic diagram representing obstacles that mucus layer poses to delivery systems*

the property which would reduce nanoparticle adhesion to mucus [60].

It was also observed that dehydration is sufficient to cause mucus obstruction inabsence of mucus hypersecretion which further paves the way for hydrating agents as promising therapeutic strategy to unplug the mucus in COPD and CF.

5.2. Interactive and Steric Barrier Property

In diseased condition like CF and COPD the barrier property of the mucus layer is reinforced due to the bacterial and endogenous DNA and actin filaments from the degraded neutrophils [61, 62], which results in elevated adhesivity and tighter mesh size of mucus.

Mucins are heavily glycosylated glycoprotein and the sialic acid residue imparts the negative charge on mucus. Therfore, regardless of the size, positively charged delivery system interacts with negatively charged mucin, which affects its mobility in mucus.The different barriers of the mucus are shown in Fig.1.

6. Delivery Systems to overcome mucus barrier

A delivery system which needs to be sent across the mucus layer needs to have one of the following two properties- it can either prolong drug residence time at the target membrane, or increase the permeation across the mucus layer to reach the underlying epithelium. Therefore the choice of delivery system depends upon the property of mucus layer (thickness, turnover rate) and the therapeutic target [63].

6.1 Mucoadhesive delivery system

Mucoadhesive delivery system promotes intimate contact between mucus layer and drug and it also prolongs the local residence time of the drug. Both natural and synthetic polymers serve as the mucoadhesive transmucosal delivery platform. Chitosan, alginate and cellulose derivatives are amongst the synthetic mucoadhesive systems and silk and silk like derivatives are gaining attention as natural mucoadhesive delivery systems [64].

Another class of mucoadhesive systems are thiomers; the immobilization of the thiol groups on the mucoadhesive polymer improve the mucoadhesive property by mimicking the disulfide bond formation [65]. Cysteine is the widely used thiol donor molecule and has been conjugated to different cationic and anionic polymers like chitosan, poly (acrylic acid) and carboxy-methylcellulose. Immobilization of thiol group on these polymers improved their mucoadhesive property. Thioglycolic acid has also been utilised for thiol group functionalization on the polymer [66].

6.2 Mucopenetrating delivery system

Mucopenetrating particles can easily penetrate the mucus layer and are able to reach the underlying epithelium [67, 68]. These systems can either change the property of mucus on their own to impart the mucus penetration ability. While designing and developing the mucopenetrating delivery system, surface property of the particle is engineered such that it interacts minimally with the mucus component. Additionally mucus rheology can be altered by introducing hydrating agent and mucus structure disrupting moieties onto the delivery system. The various classes of mucopenetrating agents are described below.

6.2.1 Mucolytic enzyme conjugated delivery system

Due to the presence of mucolytic enzymes on the surface of the nanoparticle, these particles can partially and transiently disrupt the 3-D structure of the mucus. These particles can decrease the elastic property and dynamic viscosity of the mucus by breaking down its internal structure [69, 70]. These enzymes cleave the amide bonds in mucus glycoprotein. According to literature papain (PAP) and proteinase strongly decrease the mucus viscosity in pH independent manner whereas another proteolytic enzyme Bromelain (BRO) tends to show mucolytic activity below pH 6.5. Trypsin and Chymotrypsin needs basic pH for their activity which make them less appropriate for the application [71, 72]. PAP and trypsin also aid in paracellular transport across the epithelium due to their additional property to open tight junction [73].

Poly(acrylic acid) (PAA) nanoparticle has been conjugated with mucolytic enzymes BRO and PAP via

carbodiimide chemistry and it was demonstrated that BRO decorated nanoparticles showed better mucopenetration as compared to PAP conjugated nanoparticles [74].

6.2.2 Thiomers

Thiomers represent another class of mucolytic agents-in addition to being mucoadhesive. In this category, cysteine and N-acetyl cysteine are utilised which transiently disrupts the mucus structure by substituting with disulfide bond in mucus. S. K□lner and group explained the synergistic effect of cysteine and PAP, when both are present on PAA nanoparticle, the mucus permeability increases and 2 fold increase in penetration across mucus layer was observed as compared to only cysteine modified PAA nanoparticles [75].

6.2.3 Hydrating Agents

There are studies that suggest that improvement of mucus hydration provides a rational therapeutic strategy to tackle mucus plugging in CF and COPD. Studies have been done for inhaled hypertonic saline (HS) and dry powder mannitol, both are osmotically active agents that improve airway surface hydration by creating an osmotic gradient that draws water into the airway lumen and decreased viscosity in turn facilitates the nanoparticles mobility in mucus layer. In controlled trials in patients with CF Long-term inhalation with these prototypical rehydration therapies improved lung function and reduced pulmonary exacerbations [76].

7. PEGylation and POZylation

Conjugating biodegradable polymers like (polyethylenimine (PEI), Poly β Amino Ester(PBAE)[HC], poly-L-lysine (PLL) and coating nanoparticles with low molecular weight (MW) Poly(ethyleneglycol) (PEG) is the most widely studied mucus penetrating strategy.

PEG is an uncharged hydrophilic polymer which has been used to reduce the interaction between the particle and the mucus. 2 kDa and 5 kDa polymer are widely used to mask the positive surface charge on the therapeutic system. As the mucus is composed of negatively charged glycans, PLL and PEI polymers having positive charge density get immobilised on to the mucus. PEGylating these polymers decrease the charge density and increase their mucus penetrating ability. Mastorakos and group have shown that the high density PEGylation of the PBAE polymer decreases the polymer charge and also size of the polymer was reduced so that it was able to pass through the nanoporous structure of the mucus barrier [77].

To determine the effect of PEG molecular weight (MW) on the interactions of coated particles with mucus, Hanes et al. studied the diffusion rate of NPs (nanoparticles) modified by different MW (2, 5 and 10 kDa) and densities (42 ± 3%, 65 ± 1% and 69 ± 1%) of PEG in CVM (human Cervicovaginal Mucus). The experimental results showed that low MW (e.g. 2 kDa) and high-density (e.g. 65-70%) PEG coating can facilitate the NPs to pass through mucus [77, 78].

Mansfield and group demonstrated that 5 Kda poly (2-ethyl-2-oxazoline) functionalization on silica nanoparticles have comparable transmucosal drug delivery efficacy as to PEGylation when studied using nanoparticle tracking analysis (NTA) and fluorescence microscopy. POZylation similarly makes the system muco-inert, minimizing the interaction of system with mucin polymer [79].

8. Hydrophilic Coating

Hydrophillic surface of nanoparticles are reported to serve as "muco-inert" and facilitate the diffusion of nanoparticles through mucus layer. HPMA(N-(2 hydroxypropyl)methacrylamide) copolymer has been explored as hydropllic macromolecule and Wei Shan and group validated it as dissociable "muco-inert" coating material and demonstrated that upon coating of HPMA on cell penetrating peptide(CPP)-insulin nanocomplexes, mucus permeation was enhanced [80].

Another group studied the correlation between the molecular weight of HPMA copolymer and the mucus permeation of trimethyl chitosan nanoparticle and the optimal weight of the copolymer was found to be 26kDa, which showed maximum mucus permeation [81]. In another study chitosan nanoparticles when formulated with hydrophilic biopolymer chondroitin sulphate A (CS-A) showed decline in zeta potential and increased diffusion ability in mucus [82].

The details of the different types of mucopenetrating systems and their mechanisms of action are shown in Fig. 2 and Table 1.

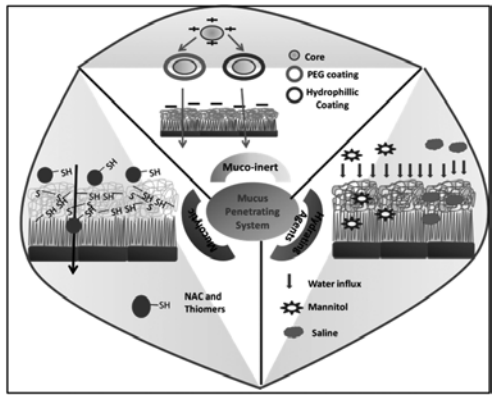

Fig. 2: *Schematic diagram representing different category of mucopenetrating systems*

Table 1:Mucus penetrating Agents with mechanism of action as reported in literature

S. No.	Mucus penetrating agent	Mechanism of Action
1	Low MW (molecular weight) PEG(Poly Ethylene Glycol)	Neutralises the charge and reduce the interaction between positively charged vector and negatively charge glycans present in mucus
2	Hydrophillic polymer	Neutralises the positive surface charge on the nanocomplexes, thus making it mucoinert
3	N-Acetyl Cysteine (NAC) and Thiomers	Disrupts the structure of the mucus polymer by substituting free thiol (sulfhydryl) groups for the disulfide bonds connecting with mucin proteins
4	Mucolytic Enzymes	Hydrolyses the mucin peptide (like trypsin, papain) and transiently disintegrates the mucus structure
5	DNase (rhDNase)	Hydrolyse the DNA that forms dense entanglements with mucin glycoproteins and other mucus constituents
6	Mannitol	Osmotically active agents that increase the hydration of mucus and thus decreasing its viscosity

9. Conclusions

COPD, Asthma and CF are the chronic lung disorders which can be targeted for effective gene therapy by delivering therapeutic gene complexed with appropriate mucus penetrating vectors. Mucus layer covering the exposed epithelial surfaces of the body has vital protective and lubrication effect. However, the adhesive and viscous property of mucus is one of the main barriers for mucosal drug delivery. A promising strategy to tackle this problem is use of non-viral vectors conjugated with mucolytic agents which can readily infiltrate into the mucus layer before turnover occurs. Moreover hydrophillic coating, PEGylation and presence of mucolytic enzyme on the surface of nanoparticles can help to overcome the mucus barrier.

However simultaneous use of mucus penetrating delivery system and delivery of therapeutic gene in a particular lung disorder requires careful system design. Delivery of required gene/nucleic acid in particular diseased condition using mucus penetrating delivery system would help in breaching the mucus barrier as well as improve local concentration and efficacy of drug at the target site.

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An overview of Peripheral Nerve Tissue Engineering

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Abstract

Peripheral nerve injuries have been reported to be one of the major causes of upper/lower extremity disorders leading to restricted physical activity/ bed ridden life. It not only causes physical and mental stress to an individual but also overall affects the growth and economy of a country. It has been well established that injuries less than the critical size defect undergoes physiological changes *in vivo* and is capable of self-regeneration. However, when the gap generated, due to accident, cut etc., is more than critical size there is a need to provide a physical guidance or support which acts as a bridge for the proximal and distal ends generated. These structures have been formulated in various shapes and sizes by various methods available to provide optimum physical and mechanical strength for the regenerating axon. Apart from mechanical cues, these scaffolds have also been provided with various chemical cues in the form of growth factors, supporting cells and neurotrophins which further assist the growth of the axon towards its distal end. Electrical cues have also been thoroughly studied as one of the important factors in directing this growth as neural cells are excitable in nature and communicate to each other via polarization and depolarization of the membrane. The success of the peripheral nerve regeneration mainly depends on how much duration the two cut ends of the nerve are able to start communicating else the distal nerve eventually degenerates leading to atrophy of the muscle which it innervated. The regeneration of the nerve and degradation of the implanted scaffold has to be in tune with each other to prevent regenerated nerve compression or loss of the scaffold before providing the required cues to the proximal end.

Keywords: Peripheral nervous system, neural-disorders, tissue-engineering, scaffolds, regeneration

1. Introduction

Neural cells, known as excitable cells, have the ability to communicate with each other through nerve impulses. These nerve impulses are a collective result of the change in membrane potential of these cells due to the alteration in the sodium/potassium ion gated channels thus resulting in action potential and thus a nerve impulse. However, the term "bioelectricity" dates back to late1970's and its role has well been documented in various physiological processes like cell division, cell polarity, muscle contraction, etc [1]. Nervous system in vertebrates has evolved into a complex network from diffuse nerve net in hydra, since evolution [2]. It is mainly divided into central nervous system(brain and spinal cord) and peripheral nervous system (cranial, spinal nerves and autonomic nervous system) [3]. The major role players in this complex network are neurons (structural and functional unit of nervous system) and glial cells or the supporting cells [4]. Oligodendrocytes, astrocytesand microglia are the supporting cells of the CNS whereas Schwann cells represent the supporting cell population in PNS [5]. The neurons consist of a cell body and various threadlike extensions called dendrites which are responsible for receiving impulses from the surrounding environment and transmitting them unidirectionally along the axon (among these dendrites one extend to form the axon) [6]. These axons can either be myelinated or non-myelinated depending on whether

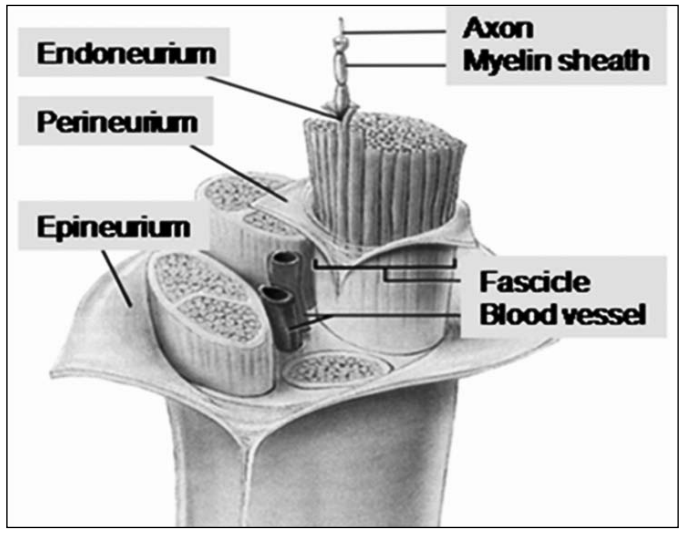

Fig. 1: *anatomical structure of a nerve bundle showing the layers of connective tissue. (Source : Campoy, L. and Read, M. (2013). Small animal regional anesthesia and analgesia. Hoboken, New Jersy, U.S.A: John Wiley and sons, Inc. Publication.)*

their axon is surrounded by a layer of myelin sheath. This structure however, varies in PNS wherein the myelin sheath is further surrounded by layers of connective tissue. Each axon with myelin sheath is surrounded by a dense layer of connective tissue called endoneurium and is referred to as a fascicle. Group of fascicle is enveloped by perineurium, the second layer, and finally these structures are enveloped by the final layer of connective tissue called epineurium [7]. These layers together with blood vessels and fibroblast cells form the nerve trunk. In this review article our main focus would be PNS and its injuries [8].

2. Peripheral nerve injury

Depending on the site of injury and its severity Seddon classified peripheral nerve injury into three main categories. These injuries are majorly result of trauma, accidents etc. In additionstudies with diabetic neuropathy have shown to play an important role in nerve degeneration [9-11]. The mildest form of injury with no damage to the nerve continuity is neuropraxia (Type I). It is the result of nerve compression or ischemia leading to alteration in nerve conduction velocity. The damage is restored within few weeks or months. In axonostemesis (Type II), there is loss in nerve continuity leading to damage in axon and myelin sheath. It is mainly result of birth defects, lacerations etc. The complete transaction of nerve due to loss of its connective tissue layer is referred to as neurostmeis (Type III). However, neurostmesis was further classified by Sunderland into Type III, IV or V depending on the damage to axon and endoneurium, perineurium or epineurium, respectively [12].

3. Physiology of peripheral nerve regeneration

The repair mechanism of human body prevents the degeneration and loss of the target organs provided the damage is within the critical size defect beyond which external support mechanism is required. Similarly the proximal and distal ends generated, after a nerve transection, has shown to undergo inherent regeneration if the defect size is less than 15mm. The rate of axonal regeneration is 1-3 mm per day as a consequence of which nerve gaps generated above this defect size might take months for regeneration resulting in the complete loss of the nerve ends due to lack of proper tropic and trophic support thus leading to muscle atrophy. The lack of any communication between the cell body and its target organs inhibits the transportation of any cargo across the cut end leading to swelling or neuroma formation which is a source of constant pain. In injuries less than critical size, the distal end axonal and myelin sheath breaks down leading to formation of ovoids and the process is referred to as Wallerian degeneration [13]. This two weeks degeneration process is then replaced by the regeneration process in which the generated debris is removed from the site by macrophages and other Schwann cells and monocytes to pave the way for the formation of aligned channels called "Bands of bunger" by Schwann cells to provide trophic and cellular support [14]. These channels guide the transacted proximal and distal end toward each other to establish their primary contact and thus the functional recovery. The nucleus (proximal end) undergoes chromatolysis leading to release of signalling molecules from nissl granules and endoplasmic reticulum following their degranulation. These factors further assist the regeneration process by promoting the Schwann cell proliferation in the distal segment of the nerve [15].

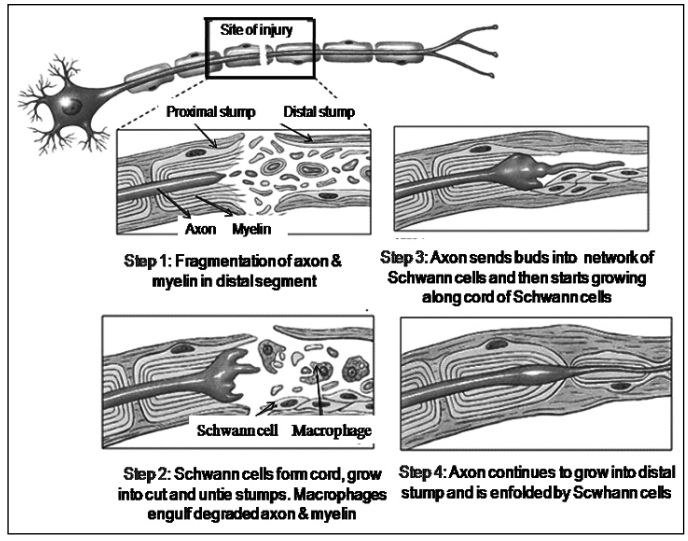

Fig. 2: *Physiology of peripheral nerve regeneration showing Wallerian degeneration (Source: Blain, A.M. (2008). Invetsigating molecular mechanism of neuronal regeneration: A microarray approach. Ph.D thesis. Faculty of Biomedical and Life Sciences, University of Glassgow; pp 19.)*

4. Peripheral nerve repair strategies

4.1 Direct repair

In this type of repair process, the proximal and distal ends of the transacted nerve are sutured against each other. In this end to end repair either the epineural layer or the perineural layer of the connective tissue is involved. Epinueral repair is the most common method employed for small nerve gaps generated [16, 17]. However, in cases where long nerve gaps are generated the suturing results in tension of the proximal and distal nerve ends leading to nerve compression and poor vascularity [9]. In addition studies have also shown that suturing can lead to inflammation, fibrosis and scarring thus delaying the process of regeneration [18]. Alternatives like fibrin glue are being used to limit these disadvantages.

4.2 Cell therapy

In this method the cells are injected at the injury site where they assist the regeneration mechanism by secreting growth factors and other signalling molecules to initiate growth and proliferation of the native cells. Neural stem cells, glial cells, stem cells, olfactory ensheathig cells are the few examples of the injected cells. Although cell transplantation is a less invasive approach it is limited by cell retention at the site of injury, cell death and tumor formation mainly with the injection of stem cells [12, 19].

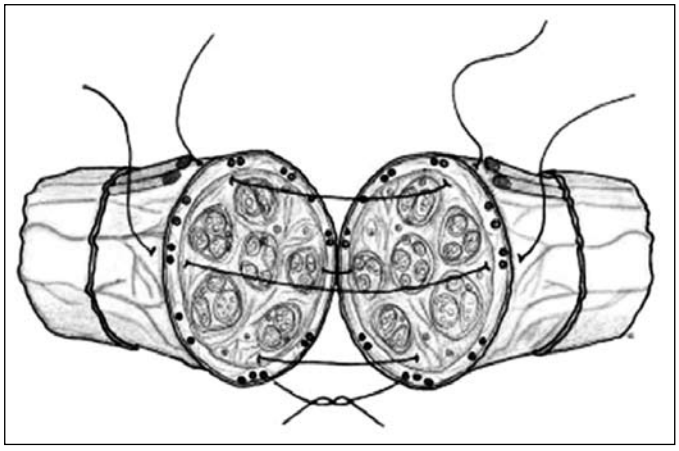

Fig. 3: *Direct end-to-end repair via placement of epineural sutures using the epineural blood vessels as a guide for alignment. (Source: Calvo, I., Espadas, I., Hammond, G., and Pratschke, K. (2014). Epineurial repair of an iatrogenic facial nerve neurotmesis after total ear canal ablation and lateral bulla osteotomy in a dog with concurrent cranio-mandibular osteopathy. J S Afr Vet Assoc 85, 1-4.)*

4.3 Conventional strategies

Autograft is still considered the gold standard method for the repair of the transacted nerve as it lacks the disadvantage of graft rejection and immune response [20]. Sural nerve is the most commonly used graft nonetheless the technique has its ownlimitations like donor site morbidity, difference in the dimensions of the nerve graft used [21]. Non nerve grafts like muscles, veins have also shown potential in nerve repair after their decellularization such that it provides 3D support to the native cellsused [22-24]. (Chiu et al., 1982)The abovementioned limitations made it obligatory to search for better alternatives which brought into focus the tissue engineering approach. Tissue engineering which laid its foundation in 1980′s turned to be an impending force in the field of nerve tissue engineering. Although the use of conventional electrodes for neural cells regeneration and growth has an established background and their lack of biological interface do pose a constrain stimulation [25]. Tissue engineering combines the principles of engineering and biology to synthesize biomaterials capable of cell proliferation and regeneration. The materials architecturally mimic the 3D structure of the native extracellular matrix (ECM) to enhance the growth and proliferation of cells [26, 27]. Various approaches like solvent extraction, cryogelation, electrospinning, 3D printing are being employed to synthesize such biomaterials [28-30]. Polymers, natural and synthetic, have been usedfor the synthesis as these can be processed in various shapes, sizes and strength as per the application. Natural polymers like gelatin, collagen, hyaluronic acid closely resemble the native ECM and thus provide a supportive microenvironment for their growth [31]. Synthetic polymers like have properties like mechanical strength, degradation etc. which can be fine-tuned to make them suitable candidates for tissue engineering [32]. Enormous work have shown that combining the natural polymers with synthetic polymers can provide optimum mechanical properties as well as support better growth of the seeded cells.

5. Scaffold

Scaffolds are 3D structural frame works which provide suitable environment for the growth of the seeded cells unlike the 2D tissue culture plates which lack the third dimension or the "Z" axis. It has been shown that cells physiologically respond better in terms of the release of growth factors, signalling molecules, growth markers etc. when grown in 3D cultures compared to their 2D counterparts. Moreover these synthesized scaffolds can be modified with various growth factors, signalling molecules or native ECM motifs like RGD to mimic the native ECM. In addition they do not suffer from the major limitation of contact inhibition as imposed on 2D culture plates.

6. Hydrogels

Hydrogels are crosslinked 3D network structures with increased water holding capacity due to the presence of hydrophilic groups in the polymers used for its synthesis. These hydrogels are either physically crosslinked due to electrostatic interactions, molecular and physical entanglement, hydrogen bonding or covalently linked to form permanent hydrogels [33, 34]. Cryogels are a type of hydrogels synthesized at subzero temperature leading to porous network formation which allows better transportation of gases and nutrients [35]. The permeable nature of these hydrogels also supports vascularization which is one of the important parameters in the field of tissue engineering.

7. Electrospinning

Electrospinning is another approach for the synthesis of nano range fibres for the growth and regeneration of cells. These nano fibres have a high surface area to volume

ratiofor improved cell seeding and growth. The properties of the synthesized fibres can be varied by altering the voltage, viscosity of the polymer solution, distance between electrodes, etc. The technique is usedto synthesize either aligned fibres or non-aligned fibres depending on its application [36]. However, for neural tissue engineering aligned fibres have shown improved results [37].

8. 3D Printing

3D printing is an emerging technology in the field of tissue engineering which is rapidly capturing market due to its precise synthesis of 3D structures in xyz axis thus closely resembling the native physiological structures. The 2D slices assimilated from the CT/MRI image of the damaged tissue are converted to '.stl' format which is then used by the printer head to closely mimic and synthesize the 3D biomaterial using "bottom up" approach. The major advantage of this technology is precision, reproducibility and simultaneous seeding of single type or multiple types of cells on the scaffold which provides uniform distribution of cell and thus better cell-cell contact [38-40].

9. Nerve guidance channel Nerve guidance channel

Nerve guidance channels (NGC's) are widely available in market and used for the regeneration of transacted nerve axonal sprouts to its target. These nerve guidance channels are hollow channels used for providing an enclosed spatial area for the growing nerve endings to prevent their turning or deviation from their unidirectional growth [41]. The growth factor, plasma exudate and signalling molecules secreted from the native cells are encased in these guidance channels this preventing their diffusion form the injury site. These NGC allows the formation of Fibrin Bridge which provides guidance to the growing sprouts and also directs the Schwann cells to their distal target end. These channels are now supplemented with various growth factors, signalling molecules as well as supporting cells to enhance the regeneration process [42]. In addition these hollow channels have been improved in their micro as well as macroarchitecture by microgrooving, injection molding etc. these channels are commercially available as nerve wraps or connectors as well [43]. The pore size ranging from 100 μm to 220 μm is considered optimum for neural tissue engineering [44, 45].

To enumerate a few commercially available NGC's [42] which are FDA approved;

Neurolac which is made up of poly ε-caprolactone is commercially available but lacks optimum mechanical properties and thus collapse inhibiting regeneration process.

Neurotube is capable of regeneration of nerve gaps of 20 mm in size but its degraded acidic products lead to hostilemicroenvironment by altering the pH.

Neuragen channels are collagen I composites which have a degradation period of 4 years. It is most efficient among all the available NGC's.

Axoguard nerve protector and connector are used to bridge nerve gaps of 40mm and 5mm, respectively. The nerve connector apart from providing protection to the damaged nerve site from inhibitory microenvironment also has multi laminar ECM which provides additional guidance cues to the growing axonal sprouts.

These NGC's compared to the natural nerve grafts have controlled mechanical properties, porosity, degradation as well as promote angiogenesis.

9.1 Nerve guidance channels with optimum biodegradability and porosity

The conventionally used NGC's were non degradable in nature and thus posed a limitation in the proper nerve regeneration as a second surgery was required to remove these guidance channels to prevent build up of strong immune rejection as well as prevent the compression of the neo nerve. The non-porous and non-degradable nature prevents proper angiogenesis and thus proper diffusion of nutrient and gases for optimum growth.

10. Conducting polymer in neural regeneration

The presence of endogenous electric fields to determine nerve polarization apart from division, morphogenesis and axonal migration laid the foundation for the use of electrical conductivity in the field of neural regeneration [46-50]. *In vitro* studies with different neural cell lines have been conducted to study the role of electrical stimulation for nerve regeneration and various theories have been formulated. Firstly, the opening and closing of Na^+/K^+ channels in response to electrical stimulation is altered as a consequence of which there is change in the release of neurotransmitters and growth factors affecting their regeneration. Secondly electrical stimulation affects the release of calcium ions, secondary messengers, which play an important role in the cascade of signalling mechanism and thus influence the gene expression and signalling molecules [51, 52]. Thirdly studies have reported alteration in protein adsorption leading to change in neurite length as well as cell adhesion and morphology [53]. Piezoelectric materials are few of the conventionally used conducting materials for nerve regeneration but they lack controlled stimulation which makes them less liable for the use of tissue engineeringstimulation [25]. Use of conducting polymers was initially limited to the field of chemistry

and physics have paved its way in the field of biomedical due to its biocompatible nature along with their ease of synthesis. Conducting polymers can be synthesized either by electrochemical method leading to the formation of thin films or electrodes or by chemical method leading to their bulk synthesis [54, 55].

Conducting polymers are organic polymers capable of conducting electricity because of the presence of their alternate single and double bonds which upon doping with sodium dodecyl sulphate, tosylate etc. result in charge carriers and thus increased conductivity [56]. Nevertheless doping with biological moieties like NGF, neurotrophins have reduced their conductivity although an increment in cell adhesion and growth was observed [54, 57-60]. Conducting polymers use in biomedical field is restricted due to its brittle nature as well as non degrading nature [61, 62]. However, incorporating these materials with natural polymers like chitosan, gletain etc. have shown to improve their mechanical properties. Moreover it was observed in various studies that over the period of time during regeneration the polymer degraded to size less than which was capable of being removed from the body through circulatory system without causing any toxicity [63]. The conductivity of these polymers follows percolation behaviour when incorporated with natural polymers for better biological properties [64]. According to percolation theory, which is applicable to the composites in which discontinuous or the dispersed phase has entirely different conductivity compared to the continuous phase. The concentration of the conductingpolymer to be incorporated in this composite should be above the percolation threshold

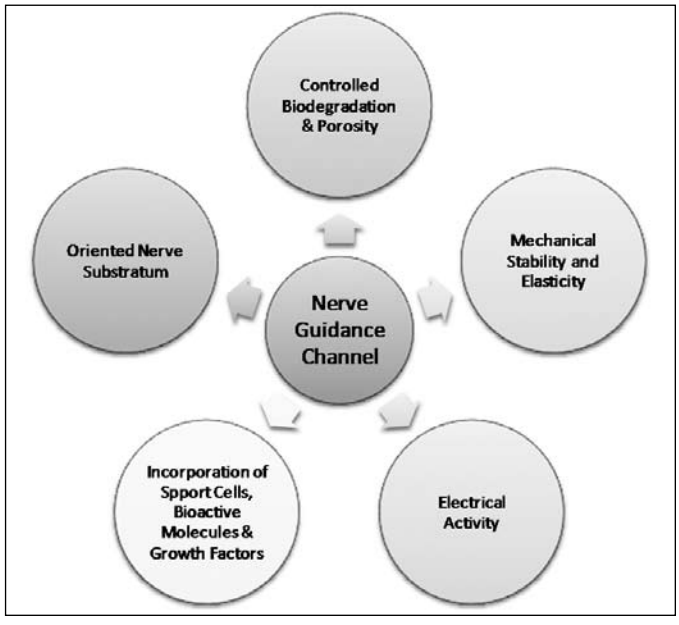

Fig. 4: *Factors affecting neural regeneration in neural tissue engineering.*

such that it would result in continuous conduction paths and thus induce conductivity to the composite.

11. Various parameters to study electrical stimulation of neural cells

In order to measure the consequence of electrical stimulation on seeded cells neurtie outgrowth measurement is an important factor. It is responsible for cell-cell communication and is an important parameter of differentiation for sympathetic neurons. Neurite outgrowth is manifested in three steps mainly neuronal sprouting, elongation and maturation. In a transacted nerve there is scarring at the sprouting end which therefore inhibits its regeneration. Nonetheless studies with neural cells like PC12, N_2A cells have shown increased neurite extension at an optimum electrical stimulation compared to its control [65, 66]. Apart from this, studies have shown that electrical stimulation also increases the secretion of growth factors like NGF, BDNF etc [67, 68]. It has been reported to be in response to either neurite extension or axonal regeneration, respectively. Additionally there was an increase in release of neurotransmitters like dopamine, acetylcholine when analysed after electrical stimulation [69, 70].

12. Factors affecting neural regeneration

Regeneration of neural tissue is limited due to non dividing nature of the neural cells as they exit from the cell cycle. However, tissue engineering and other approaches utilize the fact that axonal regeneration as well as the supporting cell has the capacity to divide and proliferate. Various studies have led to the formulation of theories wherein injury proximal to the cell body is difficult to repair compared to the one near its distal end [71]. Lacerations with sharp knives are better regenerated compared to crush injury as the layers of connective tissue are better maintained in the sharp cuts. Treatments within six weeks of injury leads to improved rate of regeneration compared to the delayed injury models [72, 73]. Apart from the site as well as the extent of injury few studies have shown a correlation between the age and neural regeneration capacity wherein younger patients showed better recovery compared to their older counterparts however, there are contradictory results pertaining to this observation.

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Hydroxyapatite Nanostructures: Implications of Surface Passivation on Colloidal Stability and Drug Delivery

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Abstract

Hydroxyapatite (HAp) is one of the most important materials for biomedical applications owing to its excellent biocompatibility, biodegradability, bioactivity and osteoconductivity.HAp has non-inflammatory properties and compositional similarity to natural bones and teeth, henceextensively being used in various orthopaedic applications. In recent years, there is a growing interest in developing HAp-based nanostructures as a carrier for the delivery of drugs, proteins, genes and other biomolecules. However, in spite of having various favourable features, the uncontrolled growth and poor colloidal stability of HAp nanoparticles limit their potential to be fully exploited for drug delivery applications. Through this brief review, we provide a comprehensive background on various surface functionalization methodologies employed for increasing the colloidal stability of HAp-based nanocarriers in aqueous/solvent media. Further, the role of surface functionalization in enhancing the drug delivery efficacy of HAp-based nanocarriers, in particular for targeted cancer therapy and theranosticshas been discussed.

Keywords: Hydroxyapatite, surface functionalization, colloidal stability, drug delivery, targeted therapy, stimuli-responsive drug delivery.

1. Introduction

Hydroxyapatite (HAp) is an inorganic material belonging to apatite group of minerals.It is composed of calcium, phosphate, and hydroxyl group with calcium to phosphorous ratio as 1.67. The chemical formula of HAp is $Ca_5(PO_4)_3(OH)_2$, however it is commonly written as $Ca_{10}(PO_4)_6(OH)_2$,representing the presence of two entities in the hexagonal crystal unit cell. Carbonatedand calcium-deficient HApnanocrystals arethe major inorganic constituents of the bone, while the organic part is composed of collagen matrix.Synthetic HAp possess excellent properties including non-toxicity, biological degradation, integration with bonesand composition similarity with bone and tooth minerals, hence extensively being used for repair, regeneration and reconstruction of damaged and degenerative bones. HAp is also used for tissue engineering, coating of metallic implants, for bone cavity filling, replacement of joints etc. The bioactive and porous nature of HAp allows in-growth of bone tissues and its integration with the living bones, which results into compact fixation of the implant material without considering it a foreign material.

In recent years, a great deal of attention has been paid for developingHAp based nanostructures for drug delivery applications,in particular,for treatment of various carcinoma, osteosarcoma, glioblastoma multiforme, breast cancer, lung cancer etc. For drug delivery applications, it is very important to synthesize HAp nanostructures with desirable physicochemical features such as size, morphology and surface properties. In addition to this, the homogenous dispersion and good colloidal stability of nanocarriers in the suspending media are also crucial parameters to be considered for drug delivery applications. Hence, along with morphological features, it is important to take account of dispersion ability of nanoparticles during preparation for their effective utilization. A variety of experimental methods, including chemical precipitation, hydrothermal, electrodeposition, solid state synthesis, sol-gel method, mechano-chemical and emulsion techniquehave been employed by several research groupsto tailor the structural parameters of HAp nanostructures to the desired extent. However, the tendency of HAp nanoparticles to agglomerate in the aqueous or solvent environment poses a major concern and in spite of having all the benefits, the uncontrolled growth and poor colloidal stability of HAp nanoparticles limits their potential to be fully exploited for drug delivery. Consequently, the preparation of HAp nanoparticles, having homogenous dispersion with good colloidal stability is a big challenge. The efforts have been made to

address this problem by reducing the agglomeration either by mechanical stirring or sonication during the processing of HAp [1, 2]. However, these approaches do not provide anenduring solution because as soon as the external energy is removed, nanoparticles lead to agglomeration. Besides, the modification of surface via adsorption of ions or molecules, encapsulation, or surface reactions through chemical techniques provides a more reasonable solution to improve the colloidal stability of HAp in aqueous/ solvent media [3].

2. Surface functionalization: colloidal stability

The unique surface characteristics of HAp allow easy surface functionalization due to the presence of hydroxyl groups and calcium cations, which can effectively adsorb organic molecules having carboxylic and phosphoric groups. The surface modifiers interact with the medium by chemical or physical interactions and greatly improve the colloidal stability of nanoparticles. In order to improve the long-term colloidal stability of HAp nanoparticles through surface functionalization, commonly two approaches have been adopted, namely the increase in the electrostatic repulsion between nanoparticles due to the presence of surface charges, and the steric stabilization effect attained by the adsorption of long-chain organic molecules. In addition, the effect of electrosteric repulsive forces, i.e. a combination of electrostatic repulsion and steric repulsion has also been studied on colloidal stability of HAp. Fig.1 shows the schematic illustration of different approachesused for enhancing colloidal stability of hydroxyapatite nanostructures.

2.1 Colloidal stability: Electrostatic stabilization

The modification of HAp surface by calcium, citrate, and phosphate ionscan significantly improve the stability of HAp colloids under physiological conditions due to emergence of electrostatic repulsion [4].Several researchers have shown the effect of electrostatic repulsion on colloidal stability of HAp nanoparticles. The surface functionalization of HAp nanoparticles with citrate moieties rendered them colloidal stabilityfor over a period of 7 months with no visible creaming or sedimentation with its zeta potential value at -45 mV. Moreover, the stability of these nanoparticles could further be increased by exchanging the citrate ions with hexametaphosphate ions. Thecolloidal stability of these nanoparticles has been assigned to the electrostatic repulsive interactions between the citrate/phosphate ions adsorbed on the surface of HAp [5]. The effect of fluoride ions on enhancing the stability of HAp aqueous colloid has also been reported [6, 7]. Tanaka and co-workers investigated the effect of alkyl phosphates on colloidal stability of HAp[8, 9]. According to them, the modification of surface with alkyl phosphates leads toan increase in the number of surface P–OH groups and helps in increasing the colloidal stability of HAp nanostructures. In another study, HAp hydrocolloids were prepared by employing a series of aminoalkyl phosphates as surface modifiers. The long-term colloidal stability of resulting hydrocolloids could be attributed to the electrostatic repulsion among the suspending particles due to formation of an ionized layer of calcium complex around each HAp core [10].

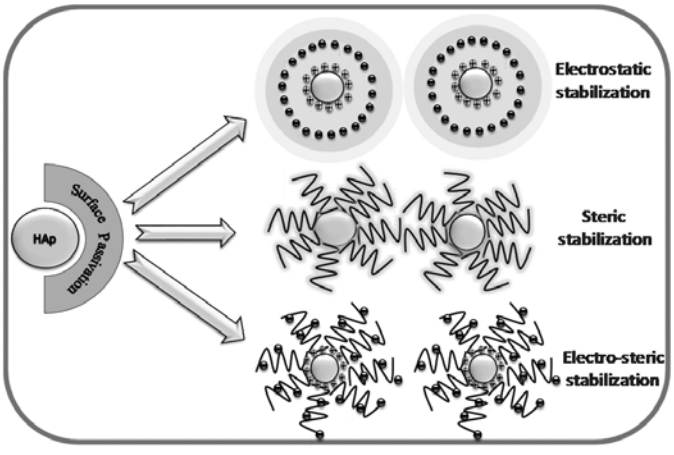

Fig.1:*Schematic illustration of different approachesused for enhancing colloidal stability of hydroxyapatite nanostructures.*

2.2 Colloidal stability: Steric stabilization

Though surface functionalization with ionic moieties provides colloidal stability to HAp nanostructures, however, charge stabilization has limitation in providing desired level of dispersion stability to nanostructures in different solvents. Hence, in order to attain good dispersion stability, different types of surface modifications such as grafting polymerization, esterification reaction as well as the use of capping reagents of biological polyelectrolytes on the surface of HAp has been explored. There are several reports in which HAp surface was modified with small molecules such assilane coupling agents, dodecyl alcohol, organophosphonic acidsand polymer chains such as polyethylene glycol, poly(methyl methacrylate), poly(acrylic acid), polylactic acid, polycaprolactoneetc. in order to attain good colloidal stability[11-18].In this method, the surface hydroxyl groups of HAp react with the coupling agents and polymers following the relevant chemical reaction.For example, Lee *et al*increased colloidal stability and interfacial adhesion of HAp nanoparticles by surface modification through grafting ofε-caprolactone. The presence of poly(ε-caprolactone)imparted enhanced colloidal stability and excellent dispersion propertiesto nanoparticles in methylene chloride without inter-crystal

aggregation as compared to bare HAp nanocrystals [18]. The reaction ofalcohols with acidic surface functional groups modifies surface of HAp through esterification reaction and enhances the colloid stability. The influence of esterification reactions on the surface chemistry and colloid stability of HAp was studied by treating HApwith dodecyl alcohol at elevated temperatures. Sedimentation studies revealed that esterified HAp at elevated temperature and suspended in ethyl alcohol had better dispersibility (over 65 days) as compared to HAp dispersed in ethanol (1–7 days) and esterified HAp dispersed in water (a few hours). The differences in colloid stability were attributed to Lewis acid/base interactions for the untreated HAp and steric stabilization effects for the nanophase HAp[3].Block copolymers can also significantly enhance the colloidal stability of HAp nanoparticles by forming organic–inorganic hybrid nanostructures. The Pluronic F127, an ABA-type triblock copolymer consisting of hydrophilic polyethylene oxide (PEO) units and hydrophobic polypropylene oxide (PPO) was covalently attached to HAp surface via esterification reaction between carboxylated F127 and the hydroxyl groups of HAp. The F127 chains grafted on the surface provided a shell structure around HAp core leading to decreased agglomeration and improved dispersion ability [19].

2.3 Colloidal stability: Electrosteric stabilization

Another method of obtaining satisfactory dispersion stability of HAp nanoparticles is to modify the surface with biocompatible organic molecules viz., chitosan, gelatin, collagen, alginate, block copolymers, polyelectrolytes, and lipids. They provide a protective organic layer around HAp nanoparticles, which offers a steric barrier that counterbalances the attractive van der Waals forces responsible for particle agglomeration and keep them dispersed in the liquid phase.Also the presence of functional groups such as carboxyl, amine etc. in these organic molecules provides stability to the nanostructures due to electrostatic repulsion. Zhang *et al*. prepared water-dispersible HAp nanoparticles in the presence of grape seed polyphenol (GSP) solution by chemical precipitation method. The modified nanoparticles were found to be colloidally very stable as compared to non-modified nanoparticles. The increased colloidal stability of GSP modified HAp nanoparticles is assigned to the existence both electrostaticrepulsion between the particles (zeta potential: −26.1 mV) and steric hindrance of GSP molecules on the surface of HAp nanoparticles [20].In another study, gelatin, a typical protein havingample hydroxyl, carboxyl and amine groups was coated on the HApnanorods during *in-situ* preparation.The resulted HAp-gelatin nanoparticles were colloidally stable and were easily re-dispersed in water after centrifugationwithout any agglomeration for more than 24 h. The reaction temperature, pH, Ca/P ratio and amount of gelatin were major stakeholders in maintaining the colloidal stability of HAp in aqueous solution. The proposed reason for the stability of HAp is the excess of calcium ions, which gets adsorbed on the surface of HAp nanoparticles and produces an electrical double layer, thus providing electrostatic repulsive interaction. In addition, the chemisorption of thick layer of gelatin on HAp surface also contributed towards increased stability through spatial stabilization [21]. Li *et al* investigated the effect of electrosteric repulsive forceson colloidal stability of HApby incorporating polymer/ surfactant pair. The HApnanosphere were functionalized with polymer/ surfactant pair of poly(ethylene oxide) and sodium oleate. The time dependant sedimentation studies showed that the functionalized HApparticles have good dispersion stability and less conglomeration due to the presence of both electrostatic repulsion and steric hindrance effects [22].

3. Surface functionalization: Drug delivery

As discussed in previous section, surface functionalization provides good colloidal stability and dispersibility to HAp nanoparticles in different mediaand makesthem a promising carrier for deliveryof drugs and biomolecules. The surface functionalization not only renders the colloidal stability to nanoparticles but also provides large number of uncoordinated functional groups on the surface of nanostructures favouring the binding of biomolecules and drugs. The organic molecules having functional groups such as hydroxyl, carboxyl, amine, phosphate, etc. are very promising as surface modifiers becausethey provide large number of active sites for the conjugation of drug and other biomolecules and enhance the drug loading efficiency of the nanocarrier. A number of functionalities have been incorporated on HAp surface and their drug delivery behaviour has been investigated. For example, Zhang *et al* prepared PEG coated HAp nanoparticles and conjugated them with insulin (for its hypoglycaemic effect) and gallic acid (for its antioxidant property) with an aim to be used for oral delivery of insulin. The oral delivery of insulin poses a major challenge due to its poor gastrointestinal stability and poor absorption. In this study,the introduction of PEG protected the nanosystem from the destructive effect of digestive enzymes thereby prolonged the duration of action in the digestive tract. Also, the nanocarrier didn't show toxicity in either *in-vitro* or *in-vivo* studies [23].

In recent years, a great deal of attention has been paid to the targeted delivery of drugs, in particular for

cancer therapy. During cancer treatment, the patient experiences serious side effects of the drug due to its potent nature. Though, the side effect of drugs can't be completelyeliminated, however, their magnitudecan be reduced with the help of targeted drug delivery. Therefore, to overcome this challenge, targeted drug delivery systems are widely being studied. Stimuli responsive systems also called as 'smart drug delivery systems' are one of the targeted drug delivery systems as they release the drugin response to external or internal stimuli such as light, heat, pH, radiofrequency etc.In particular, the pH-responsive drug delivery systems are very advantageous for cancer therapy as the tumour micro-environment is considered to be acidic in nature due to the increased metabolic by-products like lactic acid etc.Hence, the characteristic microenvironment of cancer cells could be exploited to selectively release the drug attached to the carrier in cancer cells only,in response to change in pH. Therefore, the selective and targeted release of drugs can be achieved using pH-sensitive carriersand thereby reducing the potential side effects of drug by a significant margin. Fig. 2 shows the schematic diagram of different methodologies used for fabrication of HAp-based nanostructures for targeted drug delivery and theranostics.

3.1 pH-responsive drug delivery

HAp has a unique property to dissolve under acidic pH conditions, hence are promising carriers to be used for pH-triggered sustained release of drug. Several researchers have exploited this property and designed HAp-based, pH-responsive drug delivery systems. Yang *et al*. reported pH-responsive release of an anticancer drug, doxorubicin hydrochloride (DOX) from HAp nanostructures with a hollow core and a mesoporous shell. The drug loaded system showed a higher release rate under acidic environment, which has been assigned to the dissolution of HAp under acidic conditions [24]. pH-responsive release of drug was also reported from hollow magnetic HAp microspheres loaded with an antibiotic vancomycin due to faster dissolution of HAp in acidic medium [25].

Though HAp shows pH-responsive release of drug due to its tendency to dissolve under acidic conditions, however the drug delivery efficacy of HAp nanoparticles could be enhanced by functionalizing the surface with different pH sensitive functional groups.For example, Verma *et al* reported a higher loading efficiency for DOX in citrate functionalized HAp nanoparticlesas compared to pure HAp. The modified HAp nanoparticles showed pH-responsive release of DOX, which has been assigned to the weakening of electrostatic bond between negatively charged citrate molecules and positively charged DOX as well as faster dissolution of HAp under acidic pH conditions.The DOX-loaded nanoparticles showed dose and time dependent toxicity and significant cellular uptake in WEH-164mouse fibrosarcoma cancer cells [26]. Mesoporous HAp nanoparticles functionalized withpolyacrylic acid (PAA) enhanced the drug loading efficiency of DOX due to the presence of drug binding sites on PAA. PAAalso provided a pH-responsive switch to modulate the release of theloaded DOX with a higher release rate at pH 5.0 as compared to pH 7.4 and 6.5, which has been ascribed to the dissociation of electrostatic interactions [27]. Venkatesan *et al* fabricated a chitosan coated nanocomposite of HAp and loaded with celecoxib. Celecoxib is an anticancer agent used for colon cancer, breast cancer and lung cancer, however, due to its serious side effects it has limited use. The tumour inhibitory efficacy studiesin HCT 15 and HT 29 colon cancer cell lines showed significant anti-proliferation, apoptosis and time-dependent cytoplasmic uptake of celecoxib-loaded nanocarriers. Further, *in-vivo* studies in nude mouse human xenograft modeldemonstrated significantly greater inhibition of tumor growth with modified nanoparticle system as compared to free celecoxib without showing any serious sideeffects [28].

The drug molecules can also be covalently conjugated to nanoparticles via acid-cleavable bonds such as hydrazones, Schiff bases (imines), acetals/ketals, oximes, and boronate esters, etc. Under acidic conditions, the drug releases in a sustained manner after acid hydrolysis of the bond.Following this approach, Verma *et al* prepared glycine functionalized HApnanocarriers and DOX was chemically conjugated to nanoparticles viapH-sensitive imine linkage using gluteraldehyde as a cross-linker. The nanoparticles showed five times higher release of DOX under acidic conditions as compared to physiological pH due to cleavage of imine bond. The *in-vitro* cytotoxicity studies showed insignificant cytotoxicity of nanoparticles towards WEHI-164 cancer cells, however the DOX loaded nanoparticles exhibited significant dose and time dependent cytotoxicity along with time dependant cellular uptake [29]. Mesoporous HAp nanocarriers having lactobionic acid-conjugated bovine serum albumin molecules as end-caps, and 4-carboxyphenylboronic as intermediate linkers also exhibited good loading capacity for DOX and a pH-triggered release. At physiological medium (pH 7.4), only ~ 8.0% of DOX was released as compared to ~ 34% and 53% of DOX at pH 6.5 and 5.0, respectively due to cleavage of cyclic ester linkages[30].

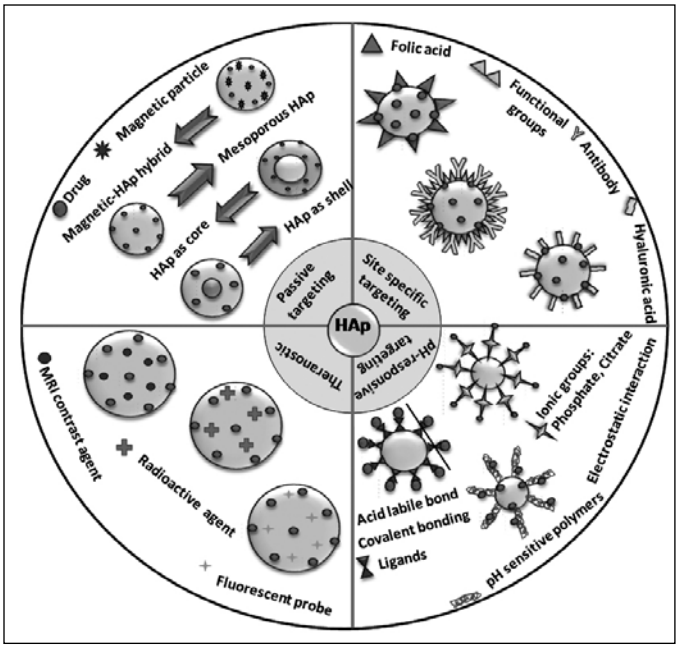

Fig. 2:*Schematic diagram showing different methodologies for fabrication of HAp-based nanostructures for targeted drug delivery and theranostics.*

Another approach for targeted delivery of drug is by conjugating special ligands,also called as site specific targeting ligands to the nanocarriers.

3.2 Site-specific drug delivery

Cancer cells have increased expression of certain types of receptors to meet their increased demands for nutrients and supplements. Upon attaching site-specific ligands such as folic acid (FA), hyaluronic acid (HA), transferrin, biotin to drug nanaocarriers, the probability of their uptake by cancer cells overexpressed with receptors increases. This leads to more localization of drug in cancerous tissue thereby decreasing the side effects on healthy cells.Kong *et al* fabricated HAp based nanocarrier coated with polyethyleneimineand decorated with hyaluronic acid (HA). HA is an efficient targeting ligand, which has the ability to selectively bind the CD44 receptors overexpressed on the surface of certain cancer cells. The nanoparticles showed pH-responsive release of DOX along with enhanced cellular uptake in CD44 receptors overexpressed A549 cells as compared to U87 cells having low CD44 receptors expression [31].Xiong*et al*employed DOX loaded HAp decorated with HA in order to deliver drug to both nuclei as well as mitochondria of tumour cells. The mitochondrial and nuclei targetability of nanosystemwas evidenced from the cellular uptake studies performed using confocal laser scanning microscopy. In addition, *in-vivo*studies in mice bearing Heps xenografts showed tumor-targeting capacity and enhanced antitumor efficacy of nanocarrier with less harmful effects [32].Folic acid (FA) is another site specific targeting ligand, which is overexpressed on some of the cancer cells. Hence, by decorating the drug nanocarriers with FA, they can be used for site-specific delivery of drug. Venkatasubbu*et al* fabricated polyethylene glycol (PEG) coated HAp and conjugated them with FA for the delivery of paclitaxel, an anticancer drug. PEG protects the nanocomposite from the clearance by macrophages and plasma proteins thereby increasing its circulation time. The drug released gradually with 100% release over a period of 50 h [33]. To increase the transfection efficiency of HAp, Wang and co-workers functionalized it with arginine during hydrothermal synthesis. *In-vitro* transfection assay showed that arginine coated HAp has high transfection efficiency in Hela cells and has the capability to protect DNA against degradation in DNase I [34].

4. Surface functionalization: Theranostics

Theranostics i.e. therapy conjugated with diagnostics is a topic of current interest. The simultaneous delivery of drug as well as bio-imaging leads to increased accuracy and efficiency of cancer therapy.Since HAp has proven to be a good drug delivery vehicle, various attempts have been made to convert it into a theranostic tool by conjugating it with an agent capable of being detected by magnetic resonance imaging, fluorescence imaging, positron emission tomography (PET), single photon emission computed tomography (SPECT) etc. Some of the theranostic approaches using HAp has been discussed here. Victor *et al* designed an oral drug delivery system containing HApnanoplatform doped with neodymium for treatment and monitoring of colon cancer. The system was loaded with a model drug, 4 acetyl salicylic acid (4ASA) by interaction of carboxylic group on ASA and calcium ions of HAp. The system was coated with alginic acid to impart pH responsive drug release in colonic alkaline medium. This system has the ability to specifically deliver the anticancer drugs to the colon site as well as makes simultaneous imaging possible. Thus this could be a promising theranostic system for early tumour detection, targeted tumour therapy and monitoring of colon cancer [35].In another study, a pH-sensitive nanoplatform made up of mesoporous HAp and chitosan, a pH-sensitive polymer was decorated with FA for both tumor targeted delivery of adenosine 5′-triphosphate (ATP) and bio-imaging. *In-vitro*biological studies suggested that as compared to free ATP, thenanosystem loaded with ATP has significant cytotoxicity towards tumor cells (Saos-2, T47D, and MCF7) in a dose-dependent manner. Moreover, no significant cytotoxic effect was observed in the normal

cells (HEK-293).Further, the nanocarriers internalized into the tumor cells in a time-dependent manner and exhibited strong fluorescence within the cells [36].FA functionalized, gadolinium-doped HAp nanorods were synthesized by Ciprest*et al*with an aim to use as a theranostic system for osteosarcomas. Phosphorous and gadolinium in the sample were activated by neutron capture.The successful production of ^{159}Gd-^{32}P-HAp and functionalization with FAmakes it a promising agent to act as a theranostic system. The presence of activated gadolinium ions offers them capability of radiotherapy as well as an MRI contrast agent.In addition, the presence of FA on the surface of porous HAp carriers makes them promising for targeted chemotherapy towardsosteosarcoma[37].

5. Conclusion and Future Aspects

As summarized in this review, surface functionalization plays a very prominent role in increasing the colloidal stability and drug delivery efficacy of HAp-based nanocarriers. The colloidal stability of nanocarriers could be increased by incorporating different ions/molecules on the surface of nanoparticles, which stabilizes them due to electrostatic interactions, steric repulsion or by electrosteric interaction.The surfacemodificationof HAp not only renders them colloidal stability but also furnishes active sites, which makes themapromising candidate for targeted drug delivery. The incorporation of pH-responsive moieties, such as citrate ions, amino acids, phosphate, and pH-sensitive biopolymers enhances the drug delivery efficacy of nanocarriers and facilitates pH-triggered release of drug. Further, the conjugation of active targeting ligands such as FA, HA with nanocarriers enable themfor site-specific delivery of drug. Though, passive delivery of drugs using HAp-based nanocarriers is well studied, however, the use of HAp for site-specific delivery of drugs is less explored. In addition, there are fewer reports on the theranostic behaviour of HAp-based systems. Hence, it opens up new avenues for fabricating novel HAp-based drug carriers having site-specific ligands as well as have the ability to integrate with bio-imaging contrast agents for variousdiagnostic techniques such asfluorescence imaging, magnetic resonance imaging, and ultrasound imaging.

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Dr. K. Indira Priyadarsini *joined Chemistry Division, BARC after completing 26th batch of BARC training school from Chemistry. Currently she is the Head of Chemistry Division, BARC. She is also a senior professor at Homi Bhabha National Institute, Mumbai. She received her Ph.D from Mumbai University in 1990 and did her post-doctoral fellowship as European Communities Marie Curie Fellow at Gray Laboratory Cancer Research Centre, UK.She has worked in the multidisciplinary research areas of Chemistry and biology on development of antioxidants and radioprotectorsfrom natural products and organoselenium compounds. She holdstwo patents and transferred two technologies to Indian industries to prepare turmeric based nutraceutical products. She is a recipient of young investigator award in International congress on radiation research in 1991, Homi Bhabha Science & Technology Award, 2003 and is an elected Fellow of National Academy of Sciences, India and Royal Society of Chemistry, UK. In 2015, she received Indian Nuclear Society's Outstanding Service award in the field of Radiation and Radioisotopes Technologies.In 2018, she received DAE-Group achievement award.*

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