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**THE ART OF FORMULATION OF
PERFLUOROCARBON ENCAPSULATED
POLYMERIC PLGA NANOPARTICLES**

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Chapter 1

Introduction to Perfluorocarbon

1. Perfluorocarbons: A Beginner's Guide

Perfluorocarbons (PFCs) are a class of molecules characterized by a hydrocarbon backbone where hydrogen atoms are replaced with fluorine atoms, resulting in linear, cyclic, or polycyclic structures. Initially produced during World War II for the Manhattan Project [1], PFCs were utilized to handle reactive uranium isotopes due to their exceptional inertness, a property derived from the strong carbon-fluorine bonds that resist chemical or enzymatic degradation [2]. This stability led to their use in various applications, including as non-stick coatings in cookware (e.g., Teflon or polytetrafluoroethylene) or as lubricants in spacecraft [2]. PFCs' biomedical potential was recognized in the 1960s, notably in Professor Leland Clark's experiment (figure 1) where a live mouse submerged in oxygenated fluorocarbon liquid survived, illustrating PFCs' high oxygen solubility [3]. This property enables PFCs to carry respiratory gases, making them valuable in medical scenarios such as oxygen delivery during severe blood loss, organ preservation, and liquid ventilation for premature infants with respiratory issues. In the human body, PFCs remain inert, lacking enzymatic degradation pathways. They are taken up by the reticuloendothelial system's (RES) phagocytic cells and eventually released into the bloodstream, transported by plasma lipids to the lungs, and exhaled as vapor [4], thanks to their high vapor pressure. This inert nature and the absence of reactivity in biological systems underscore the versatility of PFCs in both industrial and clinical settings. PFCs are distinguished by their colorless, odorless nature and a specific gravity approximately double that of water [5].

These chemically inert and stable compounds exhibit a high density, hydrophobicity, and lipophilicity. They are non-toxic even at elevated doses, characterized by high vapor pressure, and low surface tension. PFCs remain unaltered at physiological pH, are not metabolized by enzymes, and are expelled from the body through pulmonary exhalation, with a circulatory half-life varying from minutes to several days [6]. PFCs' remarkable stability, biocompatibility, and safety profile, devoid of carcinogenic, mutagenic, or teratogenic risks, make them highly valuable [7]. Their inertness is attributed to the optimal orbital overlap between carbon and fluorine, forming the strongest single bond in molecular chemistry. The electron-withdrawing nature of fluorine strengthens the carbon-carbon bonds by contracting the carbon orbitals. This results in a lack of accessible low-energy molecular orbitals for binding gases like O_2 , CO , or NO , while the fluorine atoms provide steric protection to the carbon skeleton [8]. The molecular size of the PFC chains determining their physical state—shorter chains generally exist as gases, whereas longer chains form liquids that are heavier than water.

The introduction of PFC into magnetic resonance imaging (MRI) in 1977 by Holland and colleagues, using sodium fluoride (NaF) and perfluorotributylamine as probes, represented a pivotal development [9]. This breakthrough led to comprehensive research efforts focused on the development, refinement, stabilization, and detailed analysis of PFCs, particularly for their potential in biomedical imaging applications. Since this period, PFCs have been systematically investigated as contrast agents (CA) for Fluorine-19 Magnetic Resonance Imaging MRI (^{19}F MRI).

2. Magnetic Resonance Imaging (MRI)

Magnetic Resonance Imaging (MRI) is a non-invasive medical imaging modality that offers intrinsic anatomical contrast and high resolution without the use of harmful ionizing radiation, such as X-rays or γ -rays [10]. It is particularly adept at imaging soft tissues like blood vessels, muscles, the brain, and joints, aiding in the diagnosis of diseases and tumors. MRI operates with a powerful magnet generating an intense magnetic field (1.5 – 7T) and radio waves [11]. The most MR-sensitive and abundant atom in the body, the hydrogen isotope



Figure 1 : A living mouse submerged in a liquid containing dissolved oxygen and fluorocarbons. Figure adapted from ref (1)

^1H , primarily in water, aligns with the magnetic field. The imaging process leverages radiofrequency pulses to perturb these aligned nuclei, which then relax through longitudinal (T_1) and transverse (T_2) relaxation processes, varying by isotope and tissue type. These relaxation rates, unique to each tissue, provide excellent anatomical detail and delineation of diseased tissue. The contrast in ^1H -MRI images is derived from the differences in T_1 and T_2 relaxation times across tissues, reflecting their proton/water content. The ubiquity of the ^1H isotope on earth further underscores its significance in MRI technology. In MRI, achieving optimal image contrast is crucial for distinguishing between normal and pathological tissues. However, certain scenarios present challenges due to insufficient contrast. To address this, contrast agents (CAs) are introduced into the patient's body to enhance the differentiation between tissues by altering the relaxation rates of water protons, particularly in areas where the CA accumulates [12]. These agents can effectively decrease the T_1 and T_2 relaxation times, leading to a more rapid realignment of protons with the magnetic field and producing images with greater clarity.

2.1 Gadolinium-Based Contrast Agents (GBCAs)

Among the various CAs utilized, gadolinium-based contrast agents (GBCAs) are prevalent [13]. Gadolinium (Gd^{3+}), the active element in GBCAs, is known for its potential toxicity when free in the body, posing risks especially to individuals with renal impairments. To mitigate this, Gd^{3+} is chelated with an organic ligand, which encases the ion, preventing it from interacting with biological processes while preserving its imaging capabilities. GBCAs are categorized into linear and macrocyclic types, with the latter being favoured due to their enhanced stability [14]. Once administered, Gd^{3+} shortens the T_1 relaxation time of nearby water protons, intensifying the MRI signal and improving soft tissue visualization. Despite chelation, trace amounts of gadolinium may persist in the brain post-scan. Gadolinium is primarily excreted through the kidneys, with a half-life that can be prolonged in patients with renal insufficiency, raising concerns about conditions like nephrogenic systemic fibrosis [15]. Recent observations of gadolinium retention in various human tissues have prompted regulatory bodies to reassess the safety of GBCAs. In 2015, the Food and Drug Administration (FDA) and the European Medicine Agency (EMA) issued new guidelines on gadolinium-containing contrast media, and has since recommended greater caution in GBCA administration, discontinuing the use of certain linear agents [16, 17]. Current evidence suggests that macrocyclic GBCAs release fewer Gd^{3+} ions in the brain compared to their linear counterparts, leading to continued usage with specific restrictions [18].

The quest for new CA in MRI that enhances image quality with minimal physiological impact has been a driving force in research since then. Beyond the commonly used ^1H isotope, other NMR-active nuclei like ^{19}F , ^{23}Na , ^{31}P , ^{13}C , and ^3He can also produce MRI signals. Among these, the ^{19}F nucleus stands out as a viable alternative to hydrogen due to its clear detection capabilities and feasible *in vivo* acquisition times. A notable advantage of ^{19}F is the absence of background signals from normal tissue, although its use is limited by sensitivity constraints that necessitate high local concentrations. This limitation can be addressed by employing compounds rich in fluorine, such as PFCs, which allow for enhanced sensitivity in ^{19}F MRI applications.

3. Fluorine-19 (^{19}F) MRI

Fluorine-19 (^{19}F) MRI offers several benefits for medical imaging. ^{19}F is naturally abundant and shares the same nuclear spin as protons ($\frac{1}{2}$), facilitating high-contrast imaging due to the minimal presence of fluorine in biological tissues, except in trace amounts within teeth and bone [19]. This allows for the precise visualization of small quantities of exogenous fluorinated compounds, producing a signal that is less complex than that of protons, which can vary widely due to different relaxation times. Additionally, ^{19}F has a distinct chemical shift of over 350 ppm, aiding in its clear identification. In MRI, liquid PFCs such as perfluorooctylbromide (PFOB), perfluoro-15-crown-5-ether (PFCE), and the superfluorinated contrast agent PERFECTA (figure 2) are widely used due to their non-toxicity, inertness, and highly abundant fluorine. PFCE is particularly noteworthy for its chemical and biological inertness and the presence of 20 equivalent fluorine-19 nuclei due to the symmetry of

the molecule as shown in *figure 2*, yielding a singular nuclear magnetic resonance (NMR) peak crucial for quantification and imaging [20].

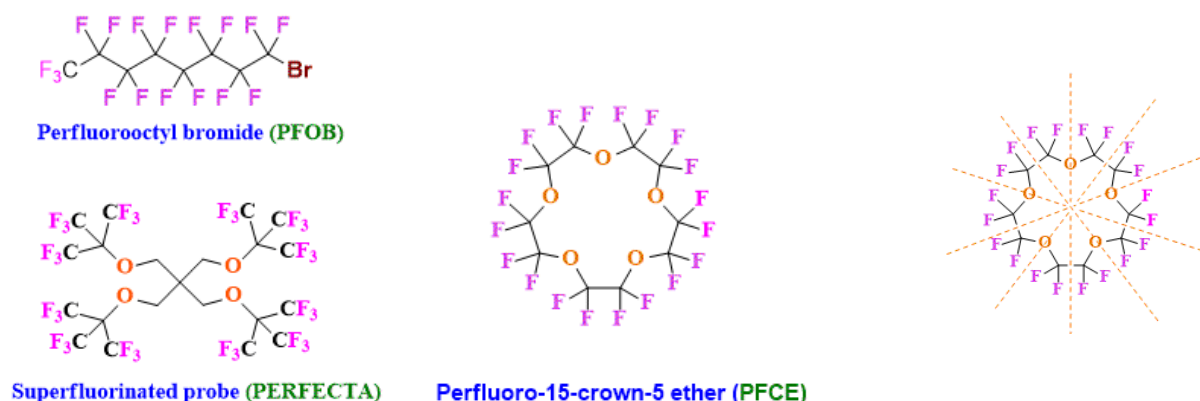


Figure 2. Commonly Used Perfluorocarbons in ^{19}F MRI - PFOB, PERFECTA, and PFCE, Including the Molecular Symmetry of PFCE

The efficacy of ^{19}F MRI is limited by the sparse presence of ^{19}F nuclei within fluorine-based probes, which necessitates the use of high-fluorine-content agents to produce a discernible signal. PFCs are particularly beneficial in this context due to their abundant fluorine content. However, the intrinsic hydrophobic and lipophobic characteristics of PFCs challenge their stabilization in aqueous mediums as they do not blend with water or organic solvents. To address this, PFCs are commonly encapsulated in emulsions or micro/nano formulations, akin to drug delivery systems, which provide a protective layer and enhance their biopharmaceutical properties. The foundational research by Geyer *et al.* in 1968 [21] highlighted the potential of PFC emulsions to act as blood substitutes in experimental animal models, thus emphasizing the extensive potential of PFCs in medical applications.

4. Drug Delivery Systems (DDS) for PFC Delivery Using Polymeric Nanoparticles

Drug delivery systems are pivotal in enhancing the pharmacological profiles of therapeutics, including their solubility, *in vivo* stability, pharmacokinetics, and biodistribution. Recent advancements in nanomedicine have catalysed the development of novel drug formulations. Utilizing nanomaterials, it is feasible to augment drug transport and efficacy, minimize dosage requirements and toxicity, and facilitate targeted drug release. Among various nano-formulations, polymeric nanoparticles (NPs) are extensively employed for the encapsulation of bioactive molecules, contributing significantly to nanomedicine's evolution. These NPs, crafted from biodegradable polymers, are favoured due to their biocompatibility, controlled release capabilities, non-toxic nature, and harmonious interaction with biological tissues and cells.

Polymeric NPs are defined as colloidal systems made of solid polymers and are a potential platform for *in vivo* nanomedicine. In the realm of nanotechnology, polymeric NPs stand out as one of the most thoroughly investigated carriers in nanomedicine. Characterized as hydrophobic, these NPs are versatile in drug delivery and diagnostic applications. They are categorized into two primary types: nanospheres, which are matrix systems where the drug is either interspersed within or adsorbed onto the polymer matrix, and nanocapsules, which are reservoir systems with the drug typically solubilized in an oil core encased by a polymeric shell [22].

The advantages of NPs are manifold [23, 24]:

- ✓ They enhance the solubility and stability of hydrophobic/hydrophilic molecules through protective encapsulation.

- ✓ Surface functionalization of NPs facilitates active targeting, directing the drug to specific tissues, thereby improving therapeutic efficacy, reducing side effects, and increasing patient adherence.
- ✓ Diverse preparation techniques, such as nanoprecipitation, emulsion using microfluidics, allow for the creation of NPs with unique properties.
- ✓ NPs can be synthesized using both natural and synthetic polymers.

Predominantly, natural polysaccharides like alginate and chitosan are utilized in fabricating natural polymeric NPs, while synthetic polymers such as poly(lactic acid) (PLA), polycaprolactone (PCL), polyhydroxybutyrate (PHB), and poly(lactic-co-glycolic) acid (PLGA) serve as the foundation for synthetic NPs formulations. Notably, PLGA is the most widely adopted polymeric base for the synthesis of synthetic NPs [22].

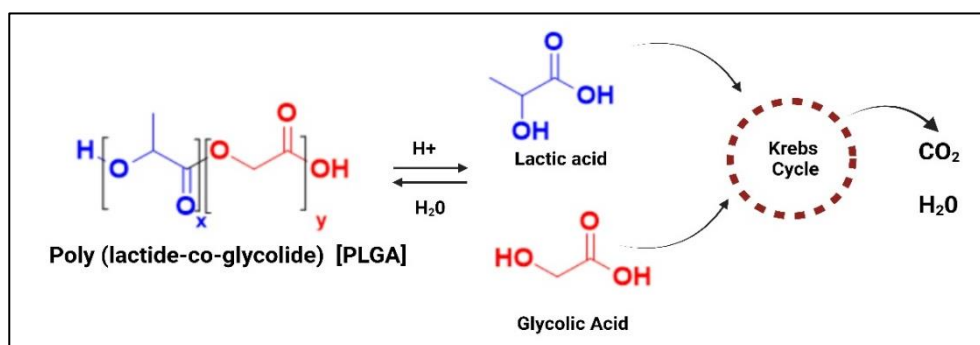


Figure 3: Hydrolysis of PLGA to form lactic acid and Glycolic acid

Poly(lactic-co-glycolic acid) (PLGA), represents a class of highly biocompatible polymers extensively researched for their efficacy as carriers of drugs, proteins, and a variety of macromolecules, including DNA, RNA, and peptides. PLGA's widespread adoption among biodegradable polymers is attributed to its extensive clinical track record, advantageous degradation properties, and capability for sustained drug release [25]. As a copolymer, PLGA has garnered FDA and EMA approval for numerous drug formulations, owing to its excellent biocompatibility, biodegradability, safety, modifiable surface properties, storage stability, and rapid *in vivo* degradation that ensures drug protection [23]. PLGA's attributes render it an ideal matrix or shell for NP, facilitating controlled drug or CA release via gradual degradation. Its applications extend to biomedical products and devices, including biodegradable sutures, and it plays a significant role in tissue engineering.

This synthetic linear copolymer, composed of lactic and glycolic acid at varying ratios, is renowned for its biodegradability. The degradation process (as shown in *figure 3*), which hinges on the polymerization degree, unfolds through hydrolysis, yielding non-toxic metabolites. The degradation mechanism, illustrated in *figure 3*, involves hydrolytic cleavage of ester bonds, resulting in lower molecular weight chains with increased hydrophilicity and ultimately, water-soluble fragments that are endogenously produced and metabolized by the body. These fragments that degraded into glycolic acid and lactic acid, are metabolized to produce energy, water, and carbon dioxide. The molecular weight of PLGA not only influences the size of the resultant NPs but also dictates the degradation pace. Higher molecular weights correlate with extended degradation periods, a process modulated by the relative concentration of lactic acid to glycolic acid, which affects the copolymer's hydrophobicity [26]. The presence of a methyl group on LA's α -carbon adjacent to the carbonyl group is a contributing factor. Commonly utilized LA:GA ratios in research include 50:50, 65:35, 75:25, and 85:15 [27].

4.1 Preparation of nanoparticles (NPs)

Choosing the right method for NPs formulation is pivotal to meet specific requirements. Prevalent strategies include nanoprecipitation, emulsion solvent evaporation (single or double), salting out, dialysis, or supercritical fluid technology [28]. Nanoprecipitation, also termed solvent diffusion or displacement, relies on interfacial

deposition following solvent-non-solvent displacement [29]. The emulsion solvent evaporation technique, specifically the single emulsion (oil-in-water, o/w) method, necessitates a volatile, water-insoluble organic solvent like dichloromethane (DCM). Here, the polymer-containing organic phase is mixed with an aqueous surfactant solution to form an o/w emulsion. Effective blending can be accomplished through the use of homogenizers, such as high-speed or high-pressure homogenizers, or an ultrasonicator [30]. The double emulsion solvent evaporation method (water-oil-water, w1/o/w2), often employed for hydrophilic drug encapsulation, mirrors the single emulsion process but includes an additional emulsion step to create a w1/o/w2 emulsion. The salting out technique employs salts to induce NPs precipitation and separation [31, 32]. Dialysis leverages a dialysis tube or semi-permeable membrane to hold the polymer and drug, which is then dialyzed against a non-solvent, resulting in a colloidal suspension within the membrane [32]. Supercritical fluid technology, an environmentally benign approach, utilizes supercritical fluids and dense gases for particle production [32]. Every method can impart distinct physicochemical characteristics to the resulting formulation, making the selection of an appropriate technique essential.

4.2 Protein corona formation

When NPs are exposed inside the human body, by virtue of their large surface area to volume ratio and a high surface free energy, they inevitably interact with bodily fluids, thereby adsorb hundreds of biomolecules and this has strong impact on drug pharmacokinetics [33, 34]. NPs on entering the blood stream undergo an identity change, they become coated with proteins. They not only give NPs a new biological identity but also, they are triggering an immune (physiological) response in the host system. The immune recognition of NPs can seriously affect the drug delivery efficacy and toxicity. These newly formed biological identities are very different from their original synthetic identity derived from the laboratorial or engineered chemical reactions and alter NP size, shape, hydrophobicity, surface properties as well as other physicochemical properties providing the NP with a “biological identity” [35]. The dynamic interactions of NPs with living organisms are driven by an interface called the “protein corona”, also known as “bio-corona” or “nano-bio interface” [33, 36]. This interface is formed when NP is introduced in biological milieu and proteins are adsorbed at NPs’ surfaces which form a dynamic layer around them and decide on the fate of NPs in the body [37]. This newly recognized bio-nano interaction between the protein and NPs is the first step of interaction when NPs are introduced to living systems and likely ‘what the cell sees.’ Hydrophobic polymers are more prone to opsonization (NPs are more readily identified and eliminated by the immune system, enhancing the clearance of NPs from the body) and integrate readily with macrophage membranes, enhancing NP clearance. Figure 4 shows a TEM image of the protein-NP complex for 100 nm polystyrene NPs [38].

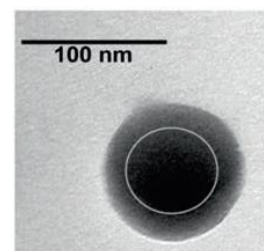


Figure 4: Protein NP complex for 100 nm polystyrene NPs. The white circle is drawn as a guide to the eye (figure taken from ref. 38).

The term corona is an appropriate description for proteins adsorbed to a particle surface as it comes from the Latin word for circle or wreath [39]. This biological interface involves physicochemical, thermodynamic and kinetic interactions between the NP surface and the surfaces of biological components such as proteins, phospholipids, or DNA contained in biological fluids. It is considered that different forces are responsible for the interaction between proteins and NPs, e.g., Van der Waals, electrostatic, hydrophobic, π - π stacking or hydrogen bonding [40]. When NPs are exposed to biological fluids, high-abundant proteins will initially be adsorbed onto the NP surfaces and then be replaced by proteins with high affinities, which are known as the Vroman effect (Vroman *et al.* 1980) [41]. According to its affinity, the protein corona can be classified as ‘hard corona’ and ‘soft corona’. Hard corona is a firmly bound, equilibrium state layer, having a high affinity for the particle surface with high-affinity proteins that bind irreversibly due to a low dissociation constant, forming rapidly post-administration. It is more stable, tightly associated with the

NPs and is considered to play an important role in NP interaction with its surrounding cells [38]. The long residence times of biomolecules in the hard corona make it easier to isolate and identify them. Conversely, the 'soft corona' consists of lower-affinity proteins and is more dynamic and is in rapid exchange with those in the medium and displays low association rates [33, 38].

The protein-corona formation influences the conformation and surface properties of NPs and thus can significantly influence their circulating lifetime, cytotoxicity, body distribution, and endocytosis into specific cells, which can possibly reduce the targeting efficiency [42]. The protein corona is known to trigger physiological and pathological changes including protein aggregation, blood coagulation, protein's secondary (2°) structure change and complement activation [39]. It can also ensue in undesirable localization of NP resulting in unintended toxicities like accumulation in tissues and causes inflammatory response [43]. Research on protein adsorption and its role in biological interactions with surfaces and materials began in the 1950s by Bangham and Vroman. Dawson and co-workers first investigated the impact of the protein corona on targeting capabilities and raised concerns about it [43]. They demonstrated that transferrin-functionalized silica NPs lose their targeting capabilities when a protein corona is adsorbed onto the NPs.

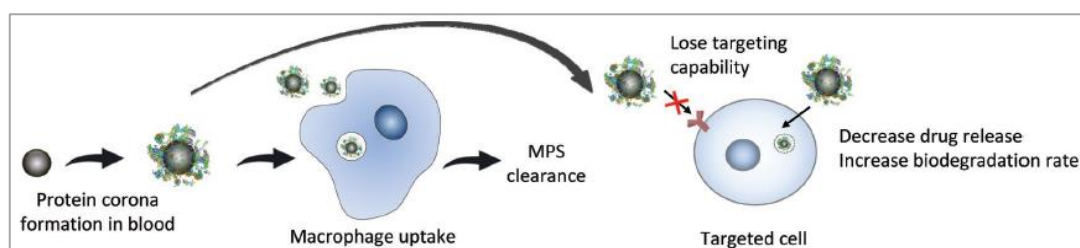


Figure 5. Effects of the protein corona on the pharmacokinetics of nanomedicine (Figure adapted from ref 44)

The first line of defence that any NP encounters within the body is the immune system. Opsonization is one of the main factors responsible for fast blood clearance of NPs. Opsonin are any protein that assists in the immune recognition of a certain body entity [44]. These mainly include immunoglobulins, complement proteins, and fibrinogen [45]. Typically, when a drug is injected into the bloodstream, it is rapidly recognized by the mononuclear phagocyte system (MPS), also known as the reticuloendothelial system (RES) and removed following opsonization by plasma proteins [44]. After opsonization, phagocytosis can occur, which is the engulfing and eventual destruction or removal of foreign materials from the bloodstream. Together these two processes form the main clearance mechanism for the removal of undesirable components from the blood. This leads to their removal from circulation and accumulation in RES organs like the liver, spleen, and kidneys. Macrophages within these organs facilitate the clearance of these opsonized particles, which are internalized into phagosomes that merge with lysosomes to form phagolysosomes, where enzymatic degradation occurs. For example, the biochemical cascade that removes pathogens is known as the complement system. NPs are recognized as foreign substances that can be sequestered, degraded, and eliminated by the MPS, leading to rapid clearance, low efficiency, and high liver accumulation [45].

The surface adsorption of serum proteins by particles (opsonization) was first postulated by Wright and Douglas in 1903. After administration of a nanomedicine, when protein such as albumin and some apolipoproteins enrich the protein corona on NPs, they prolong the blood circulation time, while complement and immunoglobulins reduce the circulation time. Eventually when NPs reach the targeted site, the protein corona hinders the accessibility of the NPs to reach the targeted organ as pictorially represented in Figure 5 [44], which affects both drug release and biodegradation of NPs [45]. Therefore, macrophage recognition can dramatically reduce the effectiveness of the nanoparticulate treatments. Hence the therapeutic effect of drug-

loaded NP is relatively poor after administration inside the body. To mitigate macrophage uptake, it is suggested to cloak NPs with suitable polymers, such as poloxamers or polyethylene glycols, that are covalently bonded or adsorbed to the matrix [28, 46]. Upon entering a biological fluid, NPs promptly interact with blood proteins to diminish their surface energy, resulting in the formation of a protein corona.

4.3 PEGylation of NPs

As noted in the works of Gref *et al.*, the macrophages of the MPS have the ability to remove unprotected NPs from the bloodstream within seconds of intravenous administration, making them ineffective as site-specific drug delivery system [47, 48]. Although protein interactions are not fully understood, surface chemistry of NPs has been shown to play a fundamental role in protein adsorption. Polyethylene glycol (PEG) is the current gold standard for reducing nonspecific cellular uptake of drug delivery vehicles making particles to remain camouflaged or invisible to phagocytic cells and has multifaceted applications [49]. The preferred method of imparting 'stealth' or sterically stabilized properties to NPs is through the PEGylation of these particles to prevent protein absorption. Since PEG is electrically neutral in a wide range of pH, it can block the electrostatic interactions between the substrate and protein and can resist nonspecific adsorption. Extensively used PEGylation has been shown to create a hydrophilic brush border around NPs, confers the ability to repel the absorption of opsonins via steric repulsion forces that prevents access of proteins consequently hinders the phagocytosis of the carriers [50]. In the presence of enzyme-catalysed alcohol dehydrogenase, PEG is metabolized by oxidation of its alcohol group to harmless by-products and higher molecular weight PEG readily undergoes renal clearance thereby possessing a safe toxicity profile and bio-tolerability [51]. PEG chains can be incorporated as diblock copolymers with PLGA or PLA and form the stealth border layer when NPs are made using this copolymer. Therefore, surface modification of PLGA NPs with PEG is expected to enhance NPs' long circulating properties.

PEGylation, the process of attaching PEG chains to NP surfaces, enhances the hydrophilicity of NPs. PEG is synthesized by polymerizing ethylene oxide monomers and its attachment to NPs can occur through covalent bonds or adsorption. This modification reduces opsonin adsorption, which is typically mediated by hydrophobic interactions. As Owens *et al.* have indicated [44], the interaction between opsonins and PEG chains can induce a repulsive force that counteracts the opsonin attraction, although this is not the sole factor in preventing opsonization. The molecular weight (Mw) of PEG and its surface density are crucial in forming an effective barrier against the protein corona. A Mw of 2 kDa is generally adequate to diminish immune cell recognition [34], with a more pronounced effect observed at 5 kDa [48]. However, low surface density of PEG may result in incomplete coverage, allowing opsonins to bind to the exposed areas of the NPs. Conversely, high density can restrict the mobility of PEG chains, reducing their protective function [44]. Thus, a balance of these parameters is vital. Studies have demonstrated that NPs with adsorbed PEG have shorter blood circulation times compared to those with covalently bound PEG. Hence the promising approach to enhance circulation time is the use of PEG copolymers with biodegradable polymers like PLGA.

5. PLGA modification using state of the art cerium catalyst.

To counteract the protein corona's alteration of NP biopharmaceutical properties, increasing system hydrophilicity through polymer PEGylation is a viable strategy. Controlling the physicochemical properties of the NPs becomes paramount for their bioavailability and effective delivery across biological barriers as protein corona can compromise the targeting capacity of NPs and subsequently negate any therapeutic potential. Poly(ethylene glycol)-poly(lactic-co-glycolic acid) (PEG-PLGA) is a widely used amphiphilic block copolymer for this purpose, effectively preventing protein corona formation. PLGA is a versatile polymer available commercially and can also be synthesized through ring-opening polymerization (ROP) [52]. ROP is a type of chain-growth polymerization where the end of a polymer chain acts as a reactive site, enabling the incorporation of cyclic monomers like lactones and lactams to extend the polymer chain. During PLGA

synthesis, lactide and glycolide, cyclic diester derivatives of lactic and glycolic acids, are opened by a metal catalyst in the presence of an alcohol, which serves as a co-initiator. This co-initiator not only activates the catalyst but also influences the polymerization degree, depending on the monomer-to-alcohol ratio. Industrially, tin catalysts such as Sn(II)2-ethylhexanoate are favoured for their cost-effectiveness and high reactivity at increased temperatures [53]. However, the toxicity of tin compounds necessitates careful consideration, particularly in pharmaceutical applications [54]. Strategies to mitigate tin residue include using lower catalyst concentrations or implementing solvent-based purification to comply with FDA limits. Exploring alternative catalysts, we have investigated cerium trichloride heptahydrate as an eco-friendly, water-tolerant, non-toxic option for ROP [55].

This research work is focused on the encapsulation of perfluoro-15-crown-ether (PFCE) within polymeric NPs using the PLGA polymer as a contrast agent (CA) for MRI and examines multiple facets of the formulation process. The thesis is structured into 5 chapters :

Chapter 2 “*Nanotechnology as a Versatile Tool for ^{19}F -MRI Agent’s Formulation: A Glimpse into the Use of Perfluorinated and Fluorinated Compounds in Nanoparticles*” : This chapter offers an in-depth literature review on cutting-edge carrier systems for encapsulating PFCs. It provides insights into the utilization of perfluorinated and fluorinated compounds within NP frameworks.

Chapter 3 “*The Art of Preparations of PFCE Encapsulated PLGA Nanoparticles : Formulation and optimisation using High Speed Homogenization and Ultrasonication*” The focus of this chapter is the formulation and optimization of PLGA NPs containing perfluoro-15-crown-ether (PFCE). It examines the impact of varying molecular weights of PLGA and the role of surfactants in the formulation process, utilizing techniques such as homogeniser and ultrasonic sonication for emulsion formation.

Chapter 4 “*Central Composite Design of Experiment Based Optimization of Key Formulation Parameters for PFCE-Encapsulated PLGA Nanoparticles*”. A statistical approach is adopted to explore the parameters critical to the nanoformulation of PLGA-PFCE. This chapter delves into the methodical aspects that underpin the successful preparation of these NPs.

Chapter 5 “*Strategic Enhancement of PLGA Nanoparticles: PEG and Fluorine Additions for PFCE Encapsulation*” – These chapters document the exploration of PLGA modification with polyethylene glycol (PEG) to increase the circulation time of the NPs and fluorine to increase the PFCE loading, employing advanced cerium catalysts. The modifications aim to enhance the preparation process of PFCE-encapsulated PLGA NPs.

Finally, **Chapter 6** “*Conclusion and future perspectives*” This section provides a comprehensive overview and analytical examination of the results derived from this thesis. It also contemplates potential enhancements and the broader clinical implementation of the formulations.

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Chapter 2

Review

Nanotechnology as a Versatile Tool for ^{19}F -MRI Agent's Formulation: A Glimpse into the Use of Perfluorinated and Fluorinated Compounds in Nanoparticles

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Abstract: Simultaneously being a non-radiative and non-invasive technique makes magnetic resonance imaging (MRI) one of the highly sought imaging techniques for the early diagnosis and treatment of diseases. Despite more than four decades of research on finding a suitable imaging agent from fluorine for clinical applications, it still lingers as a challenge to get the regulatory approval compared to its hydrogen counterpart. The pertinent hurdle is the simultaneous intrinsic hydrophobicity and lipophobicity of fluorine and its derivatives that make them insoluble in any liquids, strongly limiting their application in areas such as targeted delivery. A blossoming technique to circumvent the unfavorable physicochemical characteristics of perfluorocarbon compounds (PFCs) and guarantee a high local concentration of fluorine in the desired body part is to encapsulate them in nanosystems. In this review, we will be emphasizing different types of nanocarrier systems studied to encapsulate various PFCs and fluorinated compounds, headway to be applied as a contrast agent (CA) in fluorine-19 MRI (^{19}F MRI). We would also scrutinize, especially from studies over the last decade, the different types of PFCs and their specific applications and limitations concerning the nanoparticle (NP) system used to encapsulate them. A critical evaluation for future opportunities would be speculated.

Keywords: magnetic resonance imaging; perfluorocarbons; imaging agent; nanosystems; nanoparticles; fluorine

1.

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1. Introduction

For an early stage detection of a disease or a routine medical checkup, it is preferable to have a non-invasive, cost-effective, and patient-friendly technique that makes it approachable and reassuring [1]. In vivo molecular imaging is one such technique that can visualize, quantify, and characterize biological processes at the cellular and molecular levels in a living entity for pretreatment planning, prognostics, and post-treatment surveillance. Screening (detecting diseases in early stages) and identifying the extent of the disease, monitoring for disease recurrence, personalized medicine (selecting patient and disease-specific therapeutic treatment), measuring molecular-specific effects of treatment, predicting and monitoring response to therapy are within the realm of its possibilities [2]. The parameters taken into consideration before choosing an imaging modality are its depth of penetration, temporal resolution (how quickly the image can be acquired/acquisition time), anatomical resolution, spatial resolution, sensitivity (ability to depict molecular features of imaging areas), multiplexing capabilities (ability to simultaneously image/visualize multiple molecular targets), etc. [3]. In most cases, the imaging modality requires the presence of an entity called a contrast agent (CA) to enhance the distinction between the target tissue and the background that overcome the issue of low sensitivity and hence obtain a good quality image. Some of the modalities for molecular imaging are optical, photoacoustic (PAI), ultrasound (US), computed tomography (CT), positron emission tomography (PET)/single-photon emission computed tomography (SPECT), X-ray, and MRI.

2. Types of Molecular Imaging

In optical imaging, the near-infrared (NIR) and visible part of the optical spectrum is feasible for detection with the help of fluorochromes [4]. Fluorochromes injected into the bloodstream emit wavelengths up to about 700 nm. Microscopic lenses are utilized throughout the near infrared spectroscopy and imaging (NIRS) ranging from 600 to 900 nm, with near-transparency of living tissue. Two modes of optical imaging are fluorescence and bioluminescence imaging [5]. Higher intensities (above approximately 1200 W/cm²) overheat the

tissues, preventing deeper penetration by upheaving the light intensity used [4]. Optical imaging is an inexpensive technique with temporal resolution in minutes and spatial resolution in millimeters (mm) and is well-suited for diagnostic and microscopic studies of cells and tissue sections in real-time scans. However, the penetration depth, which is limited to a few millimeters, autofluorescence, and poor spatial resolution at greater depths limits the present applicability of optical molecular imaging in clinical practice [6,7].

Photoacoustic imaging (PAI), also known as optoacoustic or thermoacoustic imaging, is a modality for non-invasive visualization based on converting laser into heat [8]. Known for good penetration depth (mm–cm), it can image semi-transparent objects, soft biological tissue, and biological samples. Imaging agents are frequently used, including methylene blue, gold NPs, etc. that have a superior ability to absorb light to produce vivid photoacoustic images [9]. The technique still suffers from certain technical hurdles like the indispensable coupling of the instrument to the subject and the possibility to image only soft tissues and not bones or air structure, and due to the moderate laser energy, a small part of the body can only be imaged at a time.

Ultrasound (US) is a rapid, real-time, soft-tissue imaging technique that is rather inexpensive [10]. However, the spatial resolution (mm–cm) is inconsistent depending upon the required penetration of depth, and it is unsuitable for adult brain imaging as it does not penetrate air gaps or bone. Currently, US is used in the clinic and has an excellent sensitivity [11]. Unsuitable for multiplexing, and its imaging is limited to soft tissues with the unavoidable physical coupling of the device to the subject.

Computed tomography (CT), positron emission tomography (PET)/single-photon emission computed tomography (SPECT) imaging, often used in sequence, uses ionizing radiation. Notwithstanding the use of radiations, CT is the most commonly used clinical imaging modality with its advantage of limitless depth penetration. CT provides mm–cm resolution and a good contrast between hard and soft tissues with a typical scan taking up to 3–4 min to acquire [12]. CT provides mainly anatomical information but has poor sensitivity, specificity, and temporal resolution [13]. PET/SPECT is a radionuclide molecular imaging technique that allows for whole-body imaging of molecular targets or processes, has 1–2 mm resolution, and typically has scan time in minutes. Yet, the need for freshly prepared radioactive chemicals makes it a costly and complex technique. Even though this technique has excellent sensitivity, it has a poor spatial resolution [7,14,15]. PET/SPECT has the great advantage of identifying diseases at early stages since it visualizes molecular targets affected by changes at an earlier stage than that occurring in structural tissue.

The state-of-the-art X-ray imaging uses an X-ray source to get the images and have an inherent high spatial resolution. The instrumentation and use of it are relatively inexpensive, though the imaging process should be precisely monitored. The absorption of X-rays is directly proportional to the atomic number of the absorptive element [16]. A contrast medium is used otherwise soft tissues will not be visible in the image. Except for using an ionizing radiation source like X-rays which can cause radioactive damages in the human body if exceeded a safe dosage, it is essentially a very economic diagnostic technique with a straightforward image acquisition [4]. Refer to the review by Gambhir et al. [3] and Debbage et al. [4], for further explanations on each of the previous techniques.

MRI is an extremely versatile anatomical, functional, and diagnostic imaging technique which excels at deep soft tissue imaging and provides disease information [17]. It can stipulate finer distinctions between soft tissues at higher resolution (mm range) than the previously mentioned imaging techniques without the need for ionizing radiation [18]. In 2010, the Food and Drug

Administration (FDA) center for devices and radiological health started an initiative to reduce unnecessary radiation exposure from medical imaging [19], favoring the use of techniques that do not require the use of radioactive sources. Compared to other imaging techniques, limitless sample penetration, the possibility to manipulate contrast between tissues of interest by altering the scan acquisition parameters, and a better differentiation among fat, water, muscle, and other soft tissues make MRI one of the most sought imaging techniques despite low sensitivity (including coil sensitivity), lower temporal resolution (scan time depends on the required resolution and the field of view size) and a time-consuming data acquisition process [3]. Its safety profile allows repetitive imaging sessions, an exigent aspect for prolonged, chronic disorders [20].

Since every imaging technique has its unique benefits and drawbacks, combining imaging modalities (multimodal imaging) can offer synergistic advantages over a single modality to compensate for each imaging method's inherent limitations, subsequently to obtain more accurate and informative images. In fact, in most studies, multimodal imaging has become a trend both in research and clinic applications for meticulous examinations [21–23]. Table 1 compares the different parameters of all the imaging modalities discussed including optical imaging, PAI, US, CT, PET, SPECT, X-ray, MRI.

Table 1. Features of in vivo imaging modalities including their emission source, technique's requirement of a contrast agent, their penetration depth, acquisition time and the targeted region for imaging.

Technique	Emission Source	Need of Contrast Agents	Spatial Resolution	Acquisition Time	Target
Optical Imaging	Visible and Near-Infrared Light	✓	millimeters (mm)	Seconds (Sec) to Minutes (Min)	Soft tissues
Photoacoustic (PAI)	Laser	✓	centimeters (cm)		Soft tissues
Ultrasound (US)	Sound Waves	✓	cm	Sec	Soft tissues
Computed Tomography (CT), Positron Emission Tomography (PET) Single-Photon Emission Computed Tomography (SPECT)	Gamma Rays	✓	mm	Min	Hard tissues and soft tissues
X-ray	X-rays	✓	micrometer (μm)	Sec	Hard tissues and soft tissues
Magnetic Resonance Imaging (MRI)	Radiofrequency Waves	✓	μm –mm	Seconds (Sec) to Hours	Deep soft tissue

3. Principles of NMR and MRI

Depending on the appropriately tuned amplifiers and transceiver coils, in theory, any nuclear magnetic resonance (NMR) active nucleus can be used for imaging by MRI [24]. A nucleus with a spin quantum number of $\frac{1}{2}$ (e.g., ^1H , ^3He , ^{13}C , ^{14}N , ^{15}N , ^{19}F , ^{19}O , ^{31}P , etc.) is designated to be in two spin states and the direction of spin alignment depends on the sign (+/–) of the gyromagnetic ratio, one of the two spin states will align along the magnetic field (ground state, lower energy), whereas the other one will align against it (excited state, higher energy). When an external magnetic field is applied, the spins in the ground state can be promoted to the excited state after absorbing the energy [25]. Upon the termination of the external magnetic field, the spin returns to its equilibrium state

(ground state) by a process called relaxation. There are two processes involved, each with an exponential time constant (T_i , $i = 1, 2$): ' T_1 ' (longitudinal or spin-lattice) or ' T_2 ' (transverse or spin-spin) relaxation times [26]. These parameters help in determining the signal/contrast-to-noise ratio (SNR) and the image resolution.

The distinctiveness in the color density of the images of the biological tissue obtained in the MRI (which is the contrast) is fundamentally due to the difference in the rate of relaxations of the nucleus under study. Standard proton MRI (^1H MRI) imaging relies upon the detection of differences in relaxation of water protons to their ground state (relaxation rates) among tissue types, whose signal strengths are reconstructed to give a well-defined distinctive final image [27]. While conventional MRI does not necessarily require the addition of an external CA, there are circumstances when there is not sufficient difference in the relaxation rates of protons among the tissue types (bones, bodily fluids (soft tissues), fat, etc.) to produce a decent contrast. In such cases, an external CA is administered to alter the endogenous proton relaxation times (T_1/T_2) to obtain highly enhanced tissue contrast signals. Gadolinium(III)-based CAs (GBCA) are among the widely used examples of inorganic substances used for ^1H MRI. Currently, a few others are also being explored as potential MRI CAs, including perfluorocarbon (PFC) compounds and fluorinated molecules, which will be extensively considered in this review.

3.1. Gadolinium Based Contrast Agents (GBCAs)

GBCAs are paramagnetic coordination complexes comprising of a Gadolinium-III (Gd(III)/Gd^{3+}) ion and a chelator that independently do not emit MR signals but can bring about a significant reduction of the T_1 of nearby water protons [28]. Annually, millions of patients globally undergo MRI scans who receive GBCAs. The lanthanides like Gd are highly coveted CAs due to their intrinsic paramagnetic properties, favorable relaxation time, [29], and stable shelf life. GBCAs permit the imaging of tissues that are less sensitive to motion (hence better quality images) and higher throughput by shortening T_1 of the proton [28]. The contrast enhancement function comes from Gd^{3+} that has seven unpaired electrons. After administering the CA, the diagnostic image is procured while the patient is in the scanner. Generally, the diagnostic and prognostic information attained from MRI predominates the information given from other techniques. Several GBCAs have gained regulatory approvals, including Eovist® (gadoxetate disodium), Omniscan® (a gadodiamide), Gadavist® (gadobutrol), Optimark® (gadoversetamide), etc. [30,31]. The free Gd^{3+} ion is toxic since its ionic radius is relatively close to zinc, calcium, or iron [32]. Likely interference with calcium ion channels in the living entity is plausible. Gd^{3+} , therefore, needs to be cocooned within chelator (most often used is organic ligands) to avoid those toxicity issues [33,34]. Two classes of chelates developed to complex Gd: linear or macrocyclic organic ligands evade the release of free Gd^{3+} and make the resulting complexes kinetically and thermodynamically stable [35].

However, in 2006, GBCAs were associated with a devastating and potentially fatal condition called nephrogenic systemic fibrosis [36], recurrently reported in patients suffering from renal deficiency, and its onset can occur months after the last GBCA administration [28]. Furthermore, it is prevailing that some fraction of the residual Gd^{3+} can remain in the body for long periods, although the chemical form or its whole-body distribution is still obscure [28]. Because of the low sensitivity of MRI, formulation stipulates a high concentration of Gd^{3+} , typically 0.1 mmol kg^{-1} body weight (approximately 0.5 M aqueous solution) that is hypertonic relative to body fluids [37]. Notwithstanding this, some macrocyclic GBCAs are still sanctioned and can be administered to the patients but in the

lowest possible doses. Together, these conclusions have led to renewed interest in finding alternatives to using Gd^{3+} for MR contrast [38,39]. Further, in 2017, the European medicines agency (EMA) and FDA confirmed the necessity of restricting the use of some linear GBCAs because they tend to release Gd ions in the biological environment [40,41]. For a deeper understanding of GBCAs, the reader is referred to the following reviews [27,28,34,39].

3.2. Fluorine as a Contrast Agent

There is variation among different elements of an NMR active nucleus for their relative natural abundance and response to a magnetic field, meaning that the NMR signal per mole of the compound varies from element to element [24]. Choosing an imaging nucleus from the different NMR active elements depends on its properties entailing to its inherent physical, chemical, and biological properties. In 1977, shortly after the invention of ^1H MRI, Holland et al. [42], have demonstrated the feasibility of fluorine-19 suited for fluorine-MRI (^{19}F MRI), which paved the way for new research avenues in molecular and cellular imaging. ^{19}F MRI is anticipated to be a promising imaging tool in the future due to unambiguous detection, acceptable in vivo acquisition times, and relatively high spatial resolution [43]. The external addition of a suitable fluorinated compound (also called a probe/tracer/label) is a prerequisite for ^{19}F MRI/magnetic resonance spectroscopy (MRS).

Only insignificant amounts of endogenous fluorine are embedded in the teeth and bone matrix of the human body. This immobilized fluorine ($<10^{-6}$ M) has only a very short T_2 relaxation as they are in the solid-state and cannot be detected by ^{19}F MRI (that is below the detection limit), which extinguishes the possibility of intrinsic background signals, implying potentially high SNR [44]. Using the same scanner and the receiver electronics of ^1H MRI with retuned radiofrequency coils/dual-tuned coils, ^{19}F -images can be superimposed on anatomical, high-resolution ^1H images, generating hotspot ^{19}F -images (hybrid $^1H/^{19}F$ MRI) [45–47]. The MR effect of the additional element (^{19}F here) does not disturb the local magnetic field either and adds a second colored layer of complementary information to the corresponding grayscale ^1H image, hence called “hot spot” [48,49]. Aside from that, ^{19}F is a natural halogen, non-radioactive stable isotope of fluorine [50], unlike the radioactive isotope ^{18}F used in PET imaging [51], and thus it is not necessary to have advanced synthetic skills to introduce fluorine into a probe.

3.3. Similarity between Fluorine and Hydrogen

^{19}F exhibits the NMR phenomenon like ^1H , which has one unpaired proton and no unpaired neutrons, and thus with a net spin of $\frac{1}{2}$. Many fluorinated compounds that are non-toxic and chemically inert provide a non-invasive means to study biological systems. When an NMR-active nucleus is placed in an external magnetic field of strength B , it can absorb a photon of frequency ν that depends on the gyromagnetic ratio (γ) of the particle.

$$\nu = \gamma B \quad (1)$$

In Equation (1), B is the strength of the applied magnetic field (in Tesla [T]), and γ is the gyromagnetic ratio of the nucleus (in $MHzT^{-1}$). The similarity of ^{19}F 's gyromagnetic ratio to ^1H is another strong suit that makes ^{19}F the second most sensitive stable nuclei for MRI followed by ^1H (Table 1) [52,53]. At 3T, the typical field strength for clinical MRI scanners, ν is 128 MHz for ^1H and 120 MHz for ^{19}F [37]. These frequencies (commonly known as ‘resonance frequencies’) lie in the radiofrequency (RF) range, and hence, MRI signals are RF signals. ^{19}F resonates at a resonant frequency of 94% that of ^1H [54]. A huge advantage of MRI over other

imaging methods is that RF pulse is non-ionizing radiation and per se can penetrate deep into soft tissues [18]. Once the wave packet of frequency (in this case RF pulse) is applied, as already disclosed, the ground state spins obtain the energy to transition to the excited state, whose energy can be posited by Equation (2)

$$E = h \nu \quad (2)$$

where h is Planck's constant (6.626×10^{-34} joules (J)-second (S)). Denoting the population of the ground state as N_G and that of the excited state as N_E , the MR signal intensity is proportional to the population excess between the two states that can be secured by Equation (3) [37]

$$\text{Population excess (Spin polarization)} = \frac{(N_G - N_E)}{(N_G + N_E)} \quad (3)$$

At thermal equilibrium, the distribution of spins between the two states obeys Boltzmann's law. The population ratio, which is the ratio between the spins in the excited state to the ground state, (N_E/N_G), is obtained by Equation (4) which is 0.9999802 for ^1H and 0.9999814 for ^{19}F [37].

$$\frac{N_E}{N_G} = e^{\frac{-\Delta\epsilon}{kT}} \quad (4)$$

where $\Delta\epsilon$ is the energy difference between the excited and ground state, k the Boltzmann constant (1.381×10^{-23} JK $^{-1}$), and T , the absolute temperature in kelvin (K). Hence, the MR signal is the output of a tiny population difference between the two states as only 9–10 spins out of almost 10 lakhs contribute to the sequel. It sums to the fact that in the absence of CAs, MRI is an intrinsically low-sensitive technique. NMR receptivity is the absolute NMR sensitivity of a nucleus at its natural abundance [24]. ^1H has the most distinguished receptivity of any nucleus. To identify an absolute value of receptivity for other nuclei, it is represented relative to ^1H , with ^1H having a receptivity of 1. ^{19}F atom with a natural abundance of 100%, has a receptivity of 0.834 relative to ^1H , and the fact that it is not a particularly rare (or expensive) element [52] makes it exemplary suitor for replacing ^1H . It has a relative sensitivity of 83% compared to ^1H and is essentially devoid in biological tissues [52]. Table 2 compares the properties of hydrogen and fluorine that present a large extent of similarity between them except for the chemical shift, for which fluorine is electron-rich, so possesses a high chemical shift.

Table 2. Comparative properties between hydrogen and fluorine.

Parameter	^1H	^{19}F
Natural abundance (%)	99.98	100
Spin	1/2	1/2
Gyromagnetic ratio (γ) in MHz/T	42.576	40.076
Relative sensitivity	1.0	0.834
Van de Waals' radius (in Å)	1.2 (H–C)	1.35 (F–C)
The population ratio (N_E/N_G)	0.9999802	0.9999814
$\Delta\epsilon/kT$ at 3T	1.98×10^{-5}	1.86×10^{-5}
Lattice spacing	4.97 Å	5.9 Å
	(Hydrocarbon)	(fluorocarbon)
Chemical shifts in ppm (NMR)	0 to 15	>350

4. Perfluorocarbons (PFCs) as Contrast Agents for ^{19}F MRI

4.1. Physicochemical and Biological Properties of Perfluorocarbon (PFC) Molecules

The signal from ^1H MRI originates from nearly two-thirds of all protons present in the body, and for ^{19}F MRI to produce an equivalent image, a very high density of ^{19}F nuclei needs to be comprised in the CA to reach an optimal concentration. One way around the prerequisite of high F-concentration is by using PFCs and their derivatives, where all the protons (H 's) of the hydrocarbon chain are switched to ^{19}F nuclei [54–57]. Other options would be fluorine-rich macromolecules, nanosystems, and paramagnetic metal-containing agents. PFCs are one of the most biologically inert organic molecules ever known and have been under scrutiny for the last few decades [55]. Usually, fluorination enhances the bioavailability of the new drug by increasing lipophilicity [58]. Since fluorine is the highest electronegative element in the periodic table, the covalent C-F bonds are among the strongest known bonds that attribute to the high thermal, chemical, and oxidative stability [44,59].

In addition, they have higher compressibility, higher gas-dissolving capacities, extreme corrosive resistance, high density, high vapor pressure, high fluidity, low cohesive forces, lower dielectric constants, low refractive indices, low polarity, weak intermolecular interactions, and lower surface tension [44,57,60]. The high density and compressibility enable PFCs to reduce the contact force. Even at very high in vivo doses, this class of molecules is biologically compatible with no toxicity partly because of their high hydrophobicity and significant lipophobicity that gives them the tendency to segregate from the surrounding environments [55,60,61].

Between the degree of hydrophobicity and lipophobicity, the former outstands the latter [62,63]. Furthermore, they are xenobiotic, there are no known enzymes that metabolize PFCs in vivo [62], and are degradation resistant [50] at typical lysosomal pH values or in the volatile form such as Freon®. Furthermore, most PFCs, within the molecular weight range of 460–520 Da, exhibit no significant toxicological risks, carcinogenicity, teratogenicity, or mutagenicity [53,54]. The notable properties of PFCs are represented graphically as a tree and its leaves in figure 1.

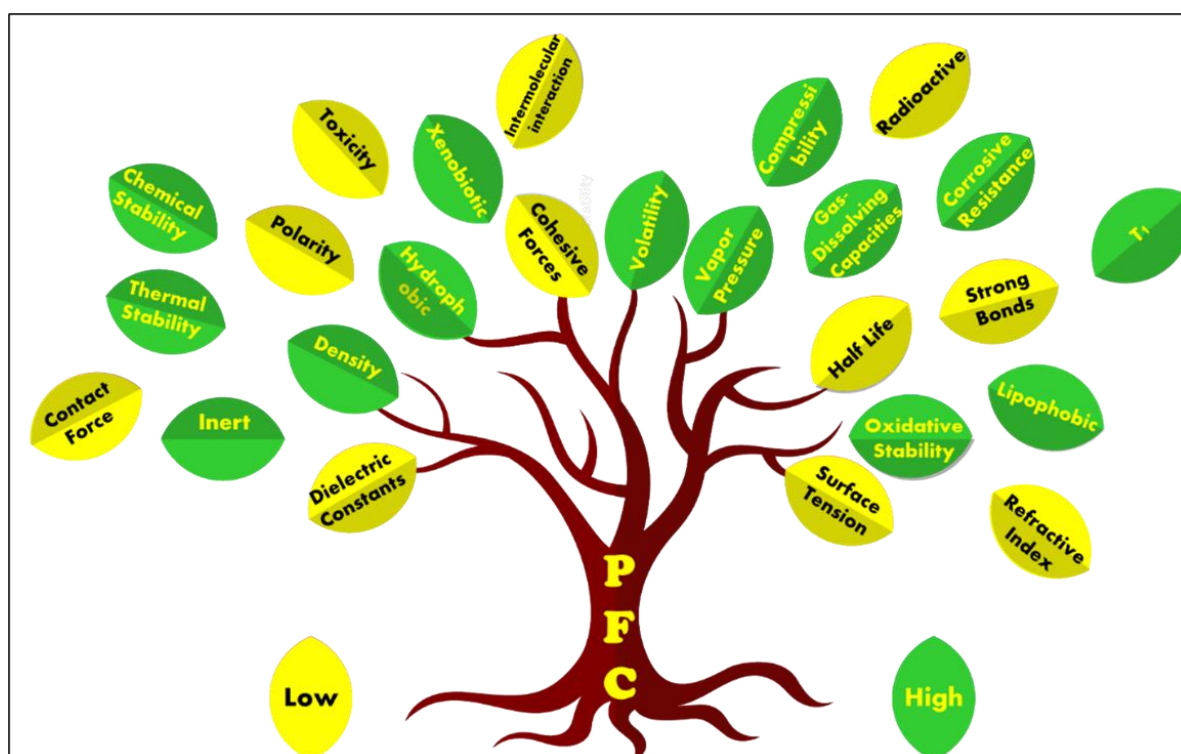


Figure 1. The unique properties of perfluorocarbon compounds represented by a tree and its leaves. The green leaves represent the properties of PFC in which the values are higher, and the yellow leaves represent lower values.

4.2. PFC Molecules in a Nanoparticle Formulation

The bottleneck factor for manipulation of most PFCs, bearing in mind its high fluorinated nature, is their simultaneously hydrophobic and lipophobic character, which makes them, in most cases, insoluble in any medium [61,64]. This peculiar feature has an ultimate implication for the design and formulation of MR imaging agents. One way around is to encapsulate/hide PFCs inside a biocompatible coating or capsule to optimize their biopharmaceutical properties. These formulations are accomplished by making nanoemulsions or microemulsions stabilized by surfactants whose employment might also influence cellular uptake [65]. Some of the frequently used surfactants are pluronics and phospholipids, the surface-active agents that can form a film around the dispersed globules of PFC by adsorbing at PFC–water interfaces and reducing the interfacial surface tension (water–PFC interfacial tension is around 60 mN/m) [54]. In many instances, one or more surfactants are blended to get the desired characteristics. By way of alternative, nanoscale micelle had been reported that can self-assemble without the need of surfactants in an aqueous solution like amphiphilic poly-fluorinated polymers [66,67]. It is a pragmatic choice since the emulsification process with a surfactant is adding yet another complexity to the system in addition to equipment and different reagents, and practically the outcome is the formation of a heterogeneous system with disparate NP size.

There are various preparation procedures reported for formulating a stable emulsion with longer shelf life. The techniques are identified as a top-down and bottom-up approach [68]. The commonly used methods are from the bottom-up approach, including emulsion-solvent evaporation method, double emulsion, and evaporation method, the emulsions-diffusion method using a homogenizer or a sonicator, nanoprecipitation (solvent displacement), salting out method, microfluidization, etc. [69]. A perfect nanoemulsion would depend on the definitive desired application, and it is always a balance of emulsion stability, the desired outcome, and body clearance. The therapeutic effect of the nanosystem can be further reduced after administration into the body by proteins adsorbed to the nanosystems surface by so-called ‘protein corona’ formation [70]. There are camouflaging ways to prolong the nanosystems circulation in the blood, including modification of its surface with polyethylene glycol (PEG) [71] shell, dextrose, polysaccharide (chitosan, hyaluronic acid, fucoidan), albumin, or zwitterion, etc. [72]. Research has found that PFC emulsions have no adverse renal toxicity [73], thereupon might be the best alternative for people with kidney complications.

The nanosystems can be chemically/physically modified with a targeting ligand (antibodies and peptides) to amplify their accumulation to the target site [74,75]. Compared to non-targeted nanosystems, targeted nanosystems seem to stay longer in the blood circulation. The former immediately accumulates in the liver and spleen post-injection. Active targeting involves conjugating the NPs with ligands that can specifically bind to cellular antigens in the pathological site of interest. On the other hand, a passive targeting strategy exploits the abnormalities of tumour vasculature that cause leakage of macromolecular agents and NPs into the tumour interstitium, the phenomenon known as the enhanced permeability and retention (EPR) effect [76]. Concomitantly it is possible to equip the nanosystems with other payloads (drugs, genes, protein, etc.) to craft them as a therapeutic agent. Likewise, a theragnostic agent could potentially combine an imaging and a therapeutic agent into a single formulation [77]. There are also

“smart” systems that can respond to the biochemical or physiological abnormalities (pH, temperature, the concentration of ions or metabolites, hypoxia, enzyme, etc.) to modulate their SNR by their physical-chemical properties [78–80].

The primary clearance system of the majority of the nanosystems in humans is the immune system. The first line of defense that they encounter within the body is the reticuloendothelial system/mononuclear phagocyte system (RES), which can, later on, undergo opsonization (surface adsorption of serum proteins to the nanosystem), and phagocytosis (engulfing and destruction/removal of foreign materials from the bloodstream into organs like liver and spleen) [81]. In principle, the duration of PFCs remaining in the body/exact clearance depends on their chemical structure, individual intrinsic properties, the mode of administration (intravenously, orally, or inhalation), molecular weight, and vapor pressure/volatility [73]. The half-life of the PFCs has an inverse relation to their volatility, which can range from minutes to years. Due to the hydrophobicity of PFCs, they have slow diffusion in their natural form that can prolong their stay in the target site.

After being drawn up by RES, PFC emulsions are diffused back into the blood, where they dissolve in plasma lipids and are carried to the lungs to be expelled out mainly through exhalation by the lungs [56,73,82]. Even though PFC is intrinsically inert, there are reports of severe retention of PFCs in the liver, spleen, and lungs [83–85], and the effect of the PFC when stayed too long in vivo or their intracellular fate is currently undetermined [53,86]. In general, nanosystems of size less than 10 nm, exceedingly are devoured by the renal clearance system, 20 to 100 nm by far, is the optimal size range to avoid physiological barriers, 100 to 200 nm particles have extended potential for prolonged circulation, and size greater than 200 nm is retreated almost certainly to spleen and liver and has the possibility for capillary clogging [87]. Frequently, a formulation contrived by design considerations including droplet sizes ranging from 10 to 200 nm (to take advantage of passive targeting by EPR effect), a low polydispersity index (narrow size range) of less than 0.2, and a high fluorine concentration [88]. So far, reported, PFCs have a half-life for blood clearance ranging from 3 h (h) to 42 h and tissue half-life ranging from 4 to 8 days for PFOB, up to 65 days for PFTA, and over 100 days for PFCE [53,89,90].

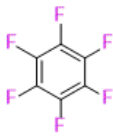
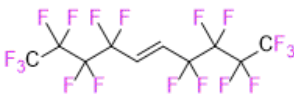
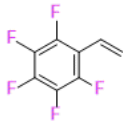
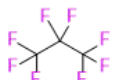
4.3. *Types of PFC Molecules*

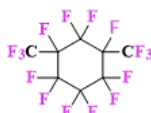
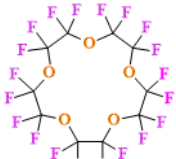
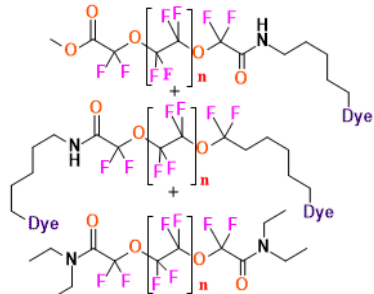
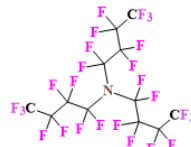
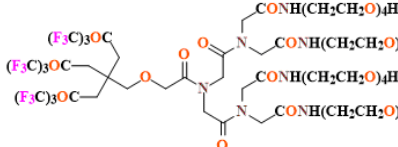
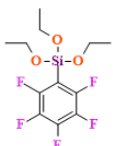
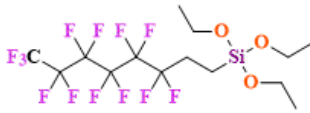
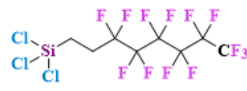
One of the critical aspects of fabricating an optimized ^{19}F MRI CA-using PFC is the chemical structure of the CA itself. The sensitivity of PFCs as MRI CA is highly dependent on the number of fluorine atoms present in the CA, and to increase the signal intensity, the number of fluorine atoms per imaging agent molecule is a vital parameter. In addition, the dosage, magnetic field strength, detector design, etc. affect the sensitivity. PFCs can be detected and quantified directly by ^{19}F NMR, an excellent technique for preliminary studies in ^{19}F MRI. One of the colossal benefits of using PFCs is that their ^{19}F NMR has an extensive chemical shift range (~400 ppm), which asserts the marginal possibility of signal overlap when multiple agents are simultaneously studied [91].

PFCs are clear colorless dense liquids, and their molecular structures generally fall into several classes, including, aromatic-hexafluorobenzene (HFB) [92], trans-1,2-bis(perfluorobutyl)-ethylene (TBPE), 2,3,4,5,6-pentafluorostyrene (PFS)), saturated linear-perfluoro-tert-butanol (PFTB), perfluoropropane (PFP), perfluorohexane (PFH), perfluorononane (PFN), perfluorooctyl bromide (PFOB), perfluorooctanoic acid (PFOA)), saturated ring system-perfluorodecalin (PFD), perfluoro-1,3-dimethylcyclohexane (PFDCH), perfluoroperhydrophenanthrene (PFPHP), perfluoroethers and polyether-perfluoro-15-crown-5 ether (PFCE),

perfluoro-2,2,2',2'-tetramethyl-4,4'-bis(1,3-dioxolane) (PTBD) [93], fluorescent 'blended' PFPE amides (FBPA) [94], superfluorinated probe (PERFECTA), perfluoropolyether (PFPE), perfluoroamines–perfluorotriethylamine (PFTA), perfluorotriethylamine (FC-43), ^{19}F imaging tracer (^{19}F IT) [95] and perfluorosilanes –(pentafluorophenyl)triethoxysilane (PFPTS), $^1\text{H},^1\text{H},^2\text{H},^2\text{H}$ -Perfluorooctyltriethoxysilane (PFOTS), Trichloro($^1\text{H},^1\text{H},^2\text{H},^2\text{H}$ -perfluorooctyl)silane (TCPFOS) [65] (refer to Table 3 for detailed information on each of the PFCs mentioned). Depending on its structure, the same could be classified as cyclic, branched, linear, and non-linear. Currently, even though not all of the described PFCs are applied in ^{19}F MRI, most of them hold the potential to be trialed for biomedical applications and then sieve them for clinical trials. There is a variety of PFC's presently available, in which some of them are commercially used for applications such as an ultrasound probe or cell tracking agent.

Table 3. Survey of PFC molecules for potential MRI applications. The MF stands for the molecular formula/chemical formula, Mw is the molecular weight in g/mol, B.P is the boiling point, the density (D) is expressed in g/mL at 25 °C (lit.), the FNMR signals are estimated based on the molecular structure and based on the fluorine environment: S—Singlet, M—multiple peaks.

Aromatic PFCs	 Hexafluorobenzene (HFB) MF = C_6F_6 Mw = 186.05 B.P = 80.2 °C FNMR signals = S	 Trans-1,2-bis(perfluoro-N-butyl)ethylene (TBPE) MF = $\text{C}_{10}\text{H}_2\text{F}_{18}$ Mw = 464.09 B.P = 64.3 °C D = 1.675 FNMR signals = M	 2,3,4,5,6-Pentafluorostyrene (PFS) MF = $\text{C}_8\text{H}_3\text{F}_5$ Mw = 194.10 B.P = 139–140 °C D = 1.406 FNMR signals = 3 major peaks with spitting
	 Perfluoro-tert-butanol (PFTB) MF = $\text{C}_4\text{HF}_9\text{O}$ Mw = 236.04 B.P = 45.0 °C FNMR signals = S	 Perfluoropropane (PFP) MF = C_3F_8 Mw = 188.02 B.P = -36.6 °C FNMR signals = 2 major peaks with spitting	 Perfluorohexane (PFH) MF = C_6F_{14} Mw = 338.04 B.P = 56.6–57.2 °C FNMR signals = 3 major peaks with spitting
	 Perfluorononane (PFN) MF = C_9F_{20} Mw = 488.06 B.P = 125–126 °C D = 1.799 FNMR signals = 3 major peaks with spitting	 Perfluorooctyl bromide (PFOB) MF = $\text{C}_8\text{BrF}_{17}$ Mw = 498.96 B.P = 142 °C D = 1.93 FNMR signals = M	 Perfluorooctanoic acid (PFOA) MF = $\text{C}_8\text{HF}_{15}\text{O}_2$ Mw = 414.07 B.P = 189.0–192 °C D = 1.792 FNMR signals = M

Saturated Ring System PFCs	 Perfluorodecalin (PFD) MF = C ₁₀ F ₁₈ Mw = 462.08 B.P = 142 °C D = 1.908 FNMR signals = M	 Perfluoro-1,3-dimethylcyclohexane (PFDCH) MF = C ₈ F ₁₆ Mw = 400.06 B.P = 101–102 °C D = 1.828 FNMR signals = M	 Perfluoroperhydrophenanthrene (PFPHP) MF = C ₁₄ F ₂₄ Mw = 624.11 B.P = 212–218 °C D = 2.03 FNMR signals = M	
	 Perfluoro-15-crown-5 ether (PFCE) MF = C ₁₀ F ₂₀ O ₅ Mw = 580.07 B.P = 145 °C D = 1.780 FNMR signals = S	 Perfluoro-2,2,2',2'-tetramethyl-1,4,4'-bis(1,3-dioxolane) (PTBD) MF = C ₁₀ F ₁₈ O ₄ Mw = 526.08 B.P = ~ 160 °C D = ~1.9 FNMR signals = M	 Fluorescent 'blended' PFPE Amides (FBPA) MF, Mw, B.P = Depends on repeat unit and the dye attached FNMR signals = 1 major peak, 4 minor peaks	
	Perfluoroethers and Polyethers	 Superfluorinated probe (PERFECTA) MF = C ₂₁ H ₈ F ₃₆ O ₄ Mw = 1008.23 FNMR signals = S	 Perfluoropolyether (PFPE) MF, Mw, B.P = Depends on repeat unit and the R-group attached FNMR signals = 1 major peak and 1 minor peak	
Perfluoroamines	 Perfluorotriethylamine (PFTA) MF = C ₆ F ₁₅ N Mw = 371.05 B.P = 68–69 °C D = 1.736 FNMR signals = 2 major peaks with spitting	 Perfluorotributylamine (FC-43) MF = C ₁₂ F ₂₇ N Mw = 671.09 B.P = 178.0 °C D = 1.884 FNMR signals = M	 ¹⁹F Imaging Tracer (¹⁹FIT) MF = C ₆₃ H ₉₄ F ₂₇ N ₇ O ₂₇ Mw = 1894.41 FNMR signals = S	
	Perfluorosilanes (Pentafluorophenyl)triethoxysilane (PFPTS)	Perfluorooctyltriethoxysilane (PFOTS)	Trichloro(¹ H, ¹ H, ² H, ² H-perfluorooctyl)silane (TCPFOS)	
	 MF = C ₁₂ H ₁₅ F ₅ O ₃ Si Mw = 330.32 B.P = 69 °C D = 1.242	 ¹ H, ¹ H, ² H, ² H- MF = C ₁₄ H ₁₉ F ₁₃ O ₃ Si Mw = 510.36 B.P = 220 °C D = 1.329 FNMR signals = M	 MF = C ₈ H ₄ Cl ₃ F ₁₃ Si Mw = 481.54 B.P = 192 °C D = 1.30 FNMR signals = M	

**FNMR signals = 3 major peaks
with spitting**

1. Perfluorooctyl bromide (PFOB/perflubron) is one of the most used PFC materials in biomedicine [96,97]. It is a tasteless and odourless liquid and is extensively adapted for artificial oxygen carriers [73]. It is a dense liquid with a low diffusion coefficient inside the blood, has a longer blood circulation time, and excreted out faster than most other PFCs. It has a linear structure, low surface tension, high specific gravity, finite lipophilicity due to a covalent bond to bromine which enhances its clearance rates from the body [98]. PFCs with additional chemical elements, such as a bromine atom in PFOB, tend to have a short biologic half-life value [99]. They are scarcely absorbed in the gastrointestinal tract, wherefore could be ingested in large doses for bowel imaging [62]. Albeit PFOB displays multiple ^{19}F peaks (eight peaks, one for each CF_n moiety) that compromises its sensitivity, it is possible to minimize undesired resonance peaks by including pre-saturation RF pulses with MRI pulse sequences before readout [98,100].
2. Perfluoropolyether (PFPE) is the simplest linear polymer that is an excellent ^{19}F MRI probe as they provide a single sharp resonance for hassle-free identification, maximizing the SNR and eradicating any chemical shift artifact of the PFC [101]. This class of molecule is known for its remarkable thermal stability and high molecular mobility that improves ^{19}F MRI sensitivity [102]. Linear PFPE possesses end groups susceptible to chemical modification by synthetic strategies [103,104] to bolster additional scope in multimodal imaging. This polymer has short T_1 (600 ms) and adequately long T_2 (160 ms), the desired trait for an imaging agent. They own a linear structure and high content of MR equivalent ^{19}F nuclei per molecule, with >40 chemically equivalent fluorine [88] that should theoretically give them single resonance. The carbon-oxygen bonds stipulate an increased bond rotation that aids them to be better biodegradable [59].
3. Using macrocyclic perfluoropolyethers (PFPEs), e.g., the 12, 15, or 18 crown ethers with their high number of equivalent fluorine atoms (16, 20, and 24, respectively) assure an outstanding NMR performance, notably of chemical shift artifacts, SNR, single sharp resonance peak that enable for unambiguous identification, etc. Macrocyclic PFCs such as the perfluoro-15-crown-5 ether (PFCE) assure a substantial improvement in MRI sensitivity with 20 chemically equivalent fluorines (NMR resonance at around ~ 92.5 ppm) [98] and is one of the most explored PFC [105–112].
4. PERFECTA (suPERFluorinatEdContrasT Agent) has 36 chemically equivalent fluorine atoms per molecule, which gives them a single major resonance in FNMR [113]. Unlike other PFCs, they have a polar hydrocarbon core. They are found to have reliable cellular compatibility from the preliminary in vivo F-MRI experiments [113,114].

4.4. The Sine Qua Non of Fluorinating Agents in ^{19}F MRI for Clinical Translation – Chemical, Physical and Biological Traits

Even after four decades into the research of ^{19}F MRI, none of the PFC formulations have gained clinical approval [83]. Umpteen requisites should be actualized to extend a formulation into the clinic. Until August 2021, as many as six PFC ^{19}F MRI agents (phase 1) are in clinical trials, mainly employing PFP for cell labelling and lung imaging [115]. There are several parameters and requirement norms for any CA formulation to be spanned to the clinic in ^{19}F MRI. In a nutshell, some of the particulars taken into consideration for the optimal formulation of a probe with PFCs or fluorinated molecules are depicted in Figure 2. In short,

1. Significant biological stability and possessing desired chemical traits [91]. The probe must be chemically inert to such an extent that it can endure all of the omnifarious chemicals in the biological milieu until it performs its mission and will be degraded. Most organofluoride compounds easily match this precondition given the strong C–F bond.
2. An ideal tracer should possess a restrained T_1 relaxation time (reduce acquisition times and increase the number of scans per unit time) and an adequately long T_2 relaxation time (to avoid signal intensity loss) [116]. A constant relaxation is anticipated in the complex biological environment. T_2/T_1 ratio close to unity is desirable for a better SNR. One of the drawbacks of PFC is their long T_1 . When a PFC has intrinsically long T_1 relaxation (PFOB and PFCE have T_1 relaxations >1 s), it will severely limit the rate of data acquisition and its sensitivity [98]. Invariably, in the literature, T_2 is an easily manipulable parameter, and this is inspected by modulating the length of fluorinated chains in the probe.
3. High number of magnetically equivalent ^{19}F -content: ^{19}F NMR spectrum, a characterization technique used in the initial analysis, for an ideal CA should be simple, preferably with a single, sharp, narrow resonance and intense peak to maximize sensitivity and prevent chemical shift imaging artifacts. The integral of an NMR signal is quantitative [117,118], directly proportional to the imaging agent concentration. The probe should also have a high fluorine content to give a single dominant signal and a good sensitivity, customarily accomplished employing PFCs. One of the undesired attributes of PFCs is that some of them lack proper symmetry, so they have a split signal in the NMR due to the disparate chemical environment of the fluorine in the molecule. This issue is surmounted by methodically applying ^{19}F MRI probes with high symmetry like PFCE/PFPE or polymeric species like dendrimers.
4. Prominent SNR enhancement: ^{19}F MRI often suffers from low SNR [119]. The commonly performed strategies to enhance the SNR are to use a CA, modulate the magnetic field strength [120], to improve pulse sequences [121] or hyperpolarization techniques such as dynamic nuclear polarization, chemically induced dynamic nuclear polarization, spin-exchange optical pumping, and parahydrogen-induced polarization that can achieve the same goal [122]. In the case of ^{19}F MRI, using the fluorinated component CA with a considerable amount of fluorine in the probe is also one of the commonly used approaches. However, high concentrations of CAs might potentially result in toxicity issues.
5. Nominal/no in vitro and in vivo toxicity: neither should it modify any biological functions nor degrade to give by-products detrimental for other tissues/organs and hence should possess low immunogenicity.
6. Easy and scalable synthesis and formulation of CA: a reproducible synthesis that can sustain the purity of the formulation with as simple as a single-step reaction and adeptness of scaling up.
7. Water solubility would be an advantageous feature that would help in the easy application of fluorine. The approach to effectuate water-soluble fluorinated moiety is by chemically modifying the system with hydrophilic compounds or employing hydrophilic hyperfluorinated organofluorine molecules [123]. One requirement for such a probe is possessing a suitable conjugation site. One of the most explored PFC in this regard is PFPE.
8. A long shelf life is favoured for a probe (at least six months).
9. Finally, it is always preferred to have an easy clearance from the living system to be approved for clinical application.

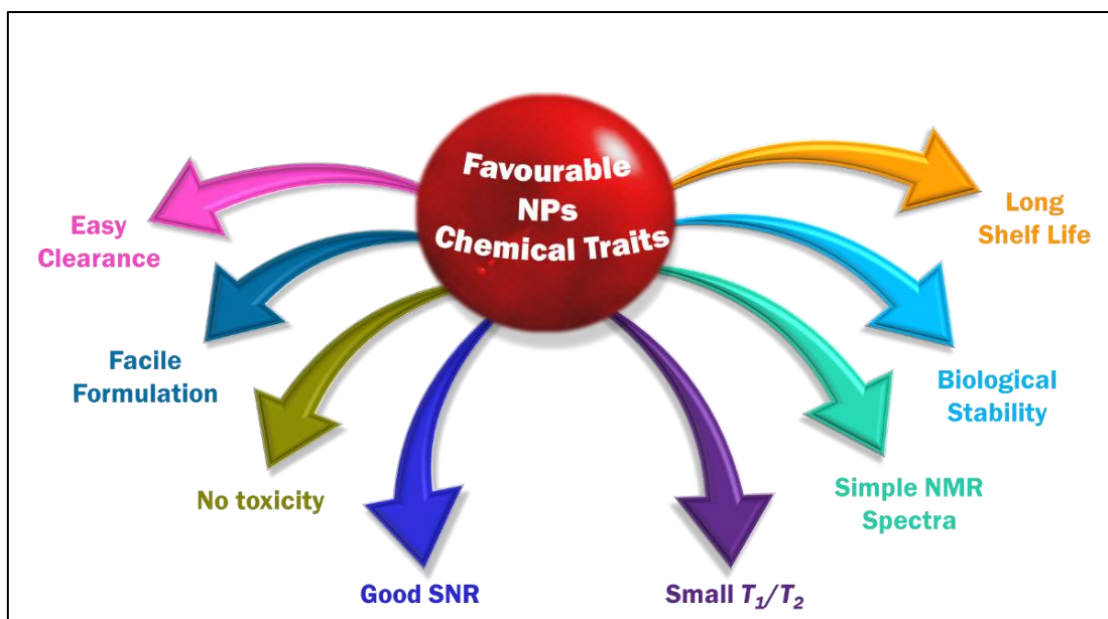


Figure 2. The favorable characteristics of a CA for ^{19}F MRI.

4.5. Biomedical Applications of PFC Molecules

PFC emulsions are flourishing in an array of biomedical applications, including molecules with high oxygen solubility for respiration and blood substitution, anaesthetics, chemotherapeutic agents, etc. Time after time it is being used in inflammation studies. Some of the early biomedical applications of PFCs include approved use as artificial oxygen transport vehicles and blood substitutes for human use, as PFC can readily dissolve oxygen and, at a constant temperature, the concentration of O_2 in the liquid PFC linearly correlates with the partial pressure of O_2 [124]. Hence, the safety profile of PFCs inside the human body is vastly explored. It is also capable of dissolving carbon dioxide and nitrogen. Over and above, incorporating other imaging agents like fluorescein isothiocyanate (FITC), Alexa647, and boron-dipyrromethene (BODIPy) can further extend their application in the field of multimodal imaging. The unique characteristics of PFCs in unison with their hydrophobic nature favored them to be suitable for US imaging as injectable emulsions of PFCs [125]. Some of the PFC-containing formulations are approved by the FDA for CAs in the ultrasound. They are Definity® and Optison® [126,127], both of which avail PFP/perflutren in the gaseous state [30]. Alike, there are two commercially available PFPE emulsions used for in vivo ^{19}F imaging in cell tracking studies—cell sense and V-Sense [128–131]. Perftoran®, rebranded under the name Vidaphor™, is a drug approved for clinical application in Russia, Mexico, Kazakhstan, Kyrgyzstan, and Ukraine, and is in the progression to be introduced in the US and European markets [132,133], to be used as a blood substitute [134]. It consists of PFD and perfluoromethyl cyclohexyl piperidine as PFCs and is stabilized by proxanol-268-polymeric surfactant and electrolyte mixture [85,133,135].

A plethora of studies are underway to evaluate the various properties of PFCs. They are found feasible for applications such as hypothermic total or partial liquid ventilation of the lungs [135–139], in vivo visualization of the effects of antibiotic therapy [140], oligonucleotide therapeutics [141], cell tracking [142], including stem cells [143–145], specific detection of organ rejection [146], identifying penumbra in stroke patient [147], quantifying immune cells (tumour-associated macrophages) in the tumour microenvironment [148–150], organ preservation [151], quantifying renal vascular damage [152], ^{19}F -oximetry [153],

inflammation imaging of various diseases [154–159], gasification-enhanced photoacoustic cavitation [160] etc. There are some excellently written reviews for further reference on PFCs used for oxygen delivery [55,63,73,124,161–164], imaging inflammation [48,91,165,166], and cell tracking [65,98,129,167–169]. Refer to the reviews for profound understanding on ^{19}F MRI used in biomedicine [44,47,56,170–172], PFCs used for various applications [53,74,84,89,173–176] and fluorinated compounds including PFCs used for imaging and/or drug delivery [17,24,54,177–183]. Being in the tight grip of SARS-CoV-2, PFCs have been proposed as a source of gas exchange in patients in critical conditions and be employed to protect blood cells [184].

5. Examples of Nanosystems Used for ^{19}F MRI Studies

A miscellaneous collection of NP probes has evolved and is employed to overcome the present limitations and drawbacks of the ^{19}F MRI CA. These nanosystems will be reviewed scrupulously, in particular, nanosystems loaded with PFCs besides fluorinated molecules like dendrimers and polymers will be considered in dept. With the intention to make the study more comprehensible and coherent, the nanosystems have been broadly classified as organic, inorganic, and hybrid systems. The organic nanosystem comprises polymeric, hyperbranched, dendrimer, hydrogel, lipid, and micelle systems. The inorganic system consists of metal and carbon-based nanoparticles. The hybrid or the mixed system consists of a fusion of organic and inorganic systems.

5.1. Organic NPs

5.1.1. Polymeric NPs

Due to the sparsity of ^{19}F nuclei per molecule, ^{19}F MRI possesses a low sensitivity which subsists as the major stumbling block. These, in turn, can help in increasing the sensitivity, thus reducing chemical shift artifacts. Figure 3 represents a general polymeric nanosystem where the NPs are pieced together with either one polymer or a combination of two or more polymers that can encapsulate the payload with a manipulable outer surface. Such a system increases the practicability to make it chemically fine-tuned, smart NPs that hold the possibility to add drugs moieties or target ligands compliant with its physicochemical properties.

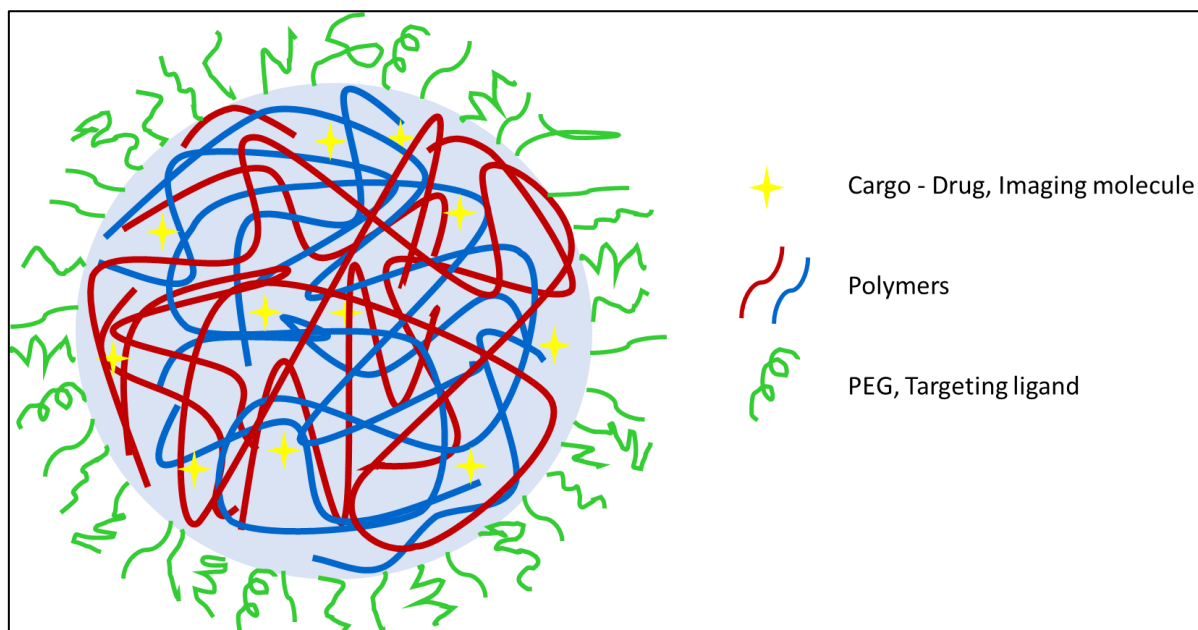


Figure 3. Polymeric nanoparticle complex with random polymer (blue and maroon) coils and a modifiable surface PEGylated surface here (green)). They can encapsulate/hold the payload (yellow star) in the polymer matrix.

The additional advantages of using polymeric species include high stability, manipulative chemical synthesis based on the desired properties, and depending on the polymers, they can be easily eliminated from the body [185,186]. In polymeric NPs, generally, two types of system are encountered—nanosystems made of CA-modified polymers that are fluorinated polymers and physical encapsulation of CA (mainly using PFCs) into nanosystems.

Geared towards overcoming the quandary faced by typical PFC encapsulated NPs—emulsion localized in diseased tissue and long-term accumulation in off-target tissue, Wallet et al. prepared low-molecular-weight fluorinated polymeric colloidal NPs [187]. The NPs from the copolymer synthesized by atom transfer radical polymerization (ATRP) using an azide-terminated initiator consisted of trifluoroethyl methacrylate and oligo (ethylene glycol) methyl ether methacrylate, and they proved effective for breast and ovarian cancer models with little off-target accumulation. NPs have been prepared from ^1H , ^2H -perfluoro-*n*-octyl acrylate, *N*-vinylformamide, (1,5-*N*-vinylformamido) ethyl ether, and (*E*)-2,20-(diazene-1,2-diyl)bis(2,4-dimethylpentanenitrile) as an initiator by one step, free radical polymerization technique [188]. The NPs appear to be promising carriers for ^{19}F MRI CAs, and their in vivo and ex vivo studies are yet to be carried out to confirm the results. A salient amalgamation trio of the fluorophilic–lipophilic–hydrophilic system was developed by Kaberov et al. using poly(2-oxazoline) block copolymers [189] to be an imminent ^{19}F MRI CA. Di- and tri-block-low molecular weight copolymers were synthesized based on 2-(^1H , ^1H , ^2H , ^2H -perfluorooctyl)-2-oxazoline, 2-methyl-2-oxazoline, and 2-*n*-octyl-2-oxazoline, which self-assembled in aqueous solution and DMSO to micelles like structure and polymersomes possessing a core–shell structure.

A new class of fluorinated MRI agents, synthesized by one-pot reversible addition-fragmentation chain transfer (RAFT) polymerization of ^{19}F -containing functional copolymer poly(oligo(ethylene glycol) methyl ether methacrylate-*co*-2,2,2-trifluoroethyl acrylate-*b*-poly(styrene-*co*-3-vinylbenzaldehyde) (poly(OEGA-*co*-TFEA)-*b*-poly(St-*co*-VBA)) was introduced to study the effect of morphology on different properties of NPs [190]. The core–shell structured particles formed from the copolymers proved that polymeric nano-objects of varied morphologies could be potential ^{19}F MRI agents. The prepared NPs were of spherical, worm-like, or vesicle particles morphologies. Comparative studies showed that worm-like NPs had the highest uptake, and vesicle NPs were less likely to be taken up by the cells as the former has a higher aspect ratio. Levels of cytotoxicity (Chinese hamster ovarian cells) were in the order of spherical NPs > vesicle NPs > worm-like NPs, although none of them were toxic, while ^{19}F MRI sensitivity was in the order of spheres > worm-like > vesicles (which depends on the fluorinated segment's motion in the corona). Interestingly, there was not any variation in the T_2 . This study showed the influential role of morphologies in NPs.

Fu et al. developed novel ^{19}F polymeric imaging agents activated by reactive oxygen species (ROS) such as H_2O_2 or low pH [191]. The monomers (thioether- and fluorine-containing methacrylate) were gathered from a PEG-based initiator by ATRP. The NPs formation was ensued in an aqueous solution by self-assembly with compact hydrophobic cores to give core–shell structured nanoaggregates. The imaging agent acted as a molecular switch by variation of T_2 relaxation in the presence of ROS, depending on the oxidation of the hydrophobic thioether group of the agent into hydrophilic sulfoxide group. These were pertinently useful for

imaging cancer cells as the environment is hypoxic. For specific imaging of bio-thiols using ^{19}F MRI, Huang et al. developed intracellular reducing microenvironment-induced amino-activatable nanoprobe [192]. The copolymers for the nanoprobe acquired by RAFT polymerization from 2-((2,4-dinitro-*N*-(3,3,3-trifluoropropyl)-phenyl)sulfonamido)-ethyl methacrylate monomers. This nanoprobe could specifically detect bio-thiols, including cysteine, homocysteine, and glutathione. Initially, the fluorinated segments immobilized in the hydrophobic core quenched the MRI signal (OFF state). When encountered a sulfhydryl moiety, a change induced in the molecular substitution of the nanoprobe ultimately dissembled the nanoprobe, and MRI signals were regained (ON state).

To evaluate the influence of NP's charge on the stability of ^{19}F MRI CAs, a fluorinated multifunctional monomer was used to prepare cationic NPs. The study started up with the development of six forms of NPs with poly(methyl methacrylate) as the hydrophobic block and differing in hydrophilic block [PEG, mannose, fructose, two different 2,2,2-trifluoroethylamide L-arginine methacrylamide ratios (10 and 20 mol%)]. This study concluded that the choice of the hydrophilic copolymer had an immense implication on stabilizing the NP corona, thereupon the performance of the CA. The conclusion stemmed from the finding that PEG with bulky side chains prevented the aggregation of the fluorinated moieties in the NP corona, hence exhibiting extended T_2 . Other studies in the polymeric system include Nafion (sulfonated tetrafluoroethylene based fluoropolymer-copolymer) based nanocarriers that were experimented with for ^{19}F MRI [193] together with poly-*L*-lysine and pegylated poly-*L*-glutamic. Cascaded, multi-responsive, self-assembled nanoprobe was identified for sensing and imaging by the sequential redox-triggered and NIR irradiation-induced ^{19}F MR signal activation/amplification [66]. The nanoprobe consisted of amphiphilic polymers containing monodisperse PEG (mPEG_{2k}) and ^{19}F bearing moiety with NIR-absorbing indocyanine green (ICG). The ICG NPs dissociated in a reductive environment leading to the formation of ultrasmall NPs that could further dissociate to small and water-soluble molecules under the response to photothermal therapy.

Srinivas and co-workers have harnessed the benefits of poly(D, L-lactide-*co*-glycolide) (PLGA) (Resomer RG 502H, lactide: glycolide molar ratio 48:52–52:48) particles of sizes ranging from 200 nm to 2000 nm formulated by single and double emulsion techniques under sonication [194]. The effects of the moiety confined inside the NPs—different PFCs, and the surface coating (targeting agent, antibody)—have been studied and shown that the NPs were exceedingly flexible in terms of encapsulated contents (imaging agent, fluorescent dye, drug), particle size, charge (–40 to 30 mV), and the bound moiety. These resulted in a versatile system with the capability to optimize parameters depending on the application. PLGA NPs were already documented for the detection of the labeled cells and direct quantification of cell migration in a diabetes model, using the PFPE by the cellular MRI method with the anticipated expansion of the PFPE-imaging platform to a wide range of cell and disease models. It was also proposed that the PFPE imaging platform could be outstretched to a wide range of cell and disease models [195].

PFCE/perfluron with different fluorescent dyes were encapsulated inside the PLGA NPs for simultaneous imaging of distinct cell populations [196]. The PLGA-PFCE-NPs with ICG or fluorescein fluorescent dye was applied simultaneously to obtain the images in less than ten minutes, making it expeditious. The slow processing and poor resolution images from unsatisfactory penetration of MRI were prevailed over by fluorescence imaging. PFCE encapsulated PLGA NPs were recognized to have many applications such as

imaging using ^{19}F MRI in conjunction with the US discerning the NPs were stable on exposure to high-pressure ultrasound [197]. With ICG dye incorporated in the formulation, its application was extended to PAI and fluorescent imaging [198] and to obtain cardiac ^{19}F MRI using PFCE labeled cells [199]. These PLGA-PFCE-NPs were used alongside gold NPs to assess the bone fillers and images using MRI and CT [200].

Srinivas et al. did an exhaustive study with PLGA (resomer RG 502H, lactide: glycolide molar ratio 50:50) for the formulation of triphasic NPs containing PFCs applicable to clinical imaging [201]. Exploring different parameters such as surfactant type and concentration, polymer concentration, and solvent type affecting the miniemulsion formation of PLGA NPs loaded with PFCE, such as their size, stability, release properties, and cell viability. The insight on the ultrastructure of the NPs is crucial for determining their exclusion from the body. The PLGA-PFCE NPs established to have a multicore structure in contrast to the anticipated simple core one, which helped in the easy clearance of NPs from the body, as proven by in vivo studies conducted on mice [202]. The simultaneous loading of two PFC agents (PFCE and PERFECTA) yielded a two-color MRI probe [203]. When modified with ^{111}In -DTPA to the PFCE-PLGA NP, they had the aptness for combined SPECT/PET and ^{19}F MRI in vivo cell tracking [204]. Since these particles are powerful theranostic agents evidenced from the previous discussions, their production was aspired to be scaled up. It was achieved by a modular microfluidic system, with sufficient yields for clinical use [205].

Chitosan-coated PLGA-PFOB NPs (RG Resomer 504H) attained by homogenization under emulsion evaporation method followed by sonication was applicable for tracking in vivo cell migration [206]. The encapsulation efficiency of PFOB was $67.1\% \pm 10$ (*w/w*). Cyanine dyes like IR Dye 800CW used are advantageous for biomolecule labeling and in vivo clinical diagnostic. NPs derived using single emulsion and solvent extraction methods with NIR fluorophores and PFCE give an entrapment efficiency of PFCE of around 240 $\mu\text{g}/\text{mg}$. The same group prepared PLGA-PEG-folate polymer, encapsulated with PFOB and either ICG (for NIRS) or the chemotherapeutic agent doxorubicin, showed enhanced uptake on human nasopharyngeal epidermal carcinoma (KB) cells, and in vitro cytotoxic studies showed that folate-targeted NPs were able to kill cancer cells more efficiently than non-folate conjugated counterpart [207]. With an encapsulation efficiency of 80% PFOB, the same NPs had been taken advantage of rheumatoid arthritis diagnosis [208]. Poly(styrene sulfonate), an ionic polyelectrolyte polymer, was used to modify the PLGA-PFOB NPs, to be used for cell labeling [209]. It was demonstrated from the in vivo and in vitro studies that the prepared NPs could be effective for cell tracking studies with MRI, least affecting any cellular functions.

PEGylation of PLGA polymer is a widely used approach for increasing the half-life of the NPs in the bloodstream, and it was reestablished with PLGA-PEG nanocapsule encapsulated with PFOB [210]. For probing ultrasound-triggered drug release, PFOB loaded PLGA-PEG NPs encapsulated with Nile red had been investigated, which proved that the mentioned NPs are least suitable for the function due to the requirement of robust inertial cavitation [211]. Cruz et al. manipulated PEGylated PLGA NPs for the detection and monitoring of ischemic diseases and traumatic brain injury, using optical microscopy and ^{19}F MRI [212]. Tumour-associated macrophages (TAM) are tumour-promoting inflammations that could be potential biomarkers for diagnosis, prognosis, and therapeutic targets for cancer [213]. Zambito et al. used PLGA-PEG-mannose NPs encapsulated with PFCE to visualize TAMs by optical imaging and ^{19}F MRI [214] with higher specificity and robust signal strength. NIR dye encapsulated PLGA-PEG NPs were adapted for monitoring and imaging in osteoarthritis by

modifying the NPs with trifluoroacetamide [215]. In vitro, in vivo and, ex vivo ^{19}F MRI and optical imaging studies proved their prospect to be multi-modal nanoprobe.

The combined effect of PFCs capability to diffuse oxygen into the tumour tissue and the possibility to modify the surface of PLGA NPs have been exploited for the enhanced antitumour efficacy in colon cancer using PLGA NPs functionalized with epidermal growth factor and co-loaded with 5-fluorouracil (chemotherapeutic drug) and PFC [216]. The aforementioned system proved to be more fitting in accumulating in tumours via ligand-targeting interactions and amended the hurdle of hypoxia-induced chemotherapy resistance. A very distinct approach was adopted by Neri et al. for the PLGA polymers—they had been fluorinated with two different fluorinated amine ligands (coupling reaction) to form F_3 -PLGA and F_9 -PLGA that contained three and nine equivalent fluorine atoms, respectively [217]. They displayed a higher efficacy to load hydrophobic drugs. Preliminary in vitro studies of F_9 -PLGA NPs were done using the drug (dexamethasone) loaded NPs to assess their cellular availability and drug release showed a greater efficacy.

The linear PFPEs possess a functional group, unlike most PFCs, to allow for facile chemical modification, and this trait was maneuvered to achieve the desired effect. A nanoemulsion with tyramide modified PFCE with NIR dye, surfactants, and hydrocarbon oil was designed for hydrophobic drugs delivery and dual imaging [88]. By inhibiting the function of the cyclooxygenase-2 enzyme by selective inhibitor-Celecoxib is an anticancer strategy to reduce cancer risk and suppress tumour growth. Janjic et al. have reported the PFPE nanoemulsions loaded with Celecoxib and NIR dye for theranostic application including, three complementary imaging modalities—fluorescence, NIRS, and ^{19}F MRI [218,219]. The application of the developed nanoemulsions has been extended for in vivo monitoring and modulating tumour-infiltrating immune cells [218]. The authors were the first to show the two-color PFC nanoemulsion [220]. PFPE was modified with oligo(ethylene glycol) methyl ether acrylate by RAFT polymerization to form a CA that had high imaging sensitivity and was hydrophilic [221]. To investigate the aggregation behavior of nanosystems that can have a role in the interaction between the NPs and living entities, doxorubicin-loaded polymeric PFPE-based NPs were reported [222]. Evaluation of fluorinated NPs on 3D spheroids concluded that for greater efficacy of drug delivery, it was efficacious for the NPs to have a smaller fluorinated core and the fluorinated segments to have greater exposure to the external environment.

RAFT polymerization intended to combine 2,2,2-trifluoroethyl acrylate with 2-(methylsulfinyl)ethylacrylate resulted in an MRI CA that was exceedingly hydrophilic and displayed intense in vitro/in vivo MRI signals [223]. With the solid-phase peptide synthesis of disordered fluorinated peptides by sequential addition of amino acid–trifluoroacetylated lysine, a platform conceivable for in vivo targeting applications was made [224]. Copolymers developed from perfluoropolyether methacrylate and oligo(ethylene glycol)methacrylate and modified with a green fluorescence dye–*N*-(5-fluoresceinyl)maleimide, had a hydrodynamic size around 12 nm and molecular weight $\sim 75000 \text{ g mol}^{-1}$ [225]. From the in vivo studies, it was acknowledged to have favorable non-phagocytic cells uptake profiles and outstanding MRI performance. With the widely used PEG, a novel, low cost, hydro-soluble, highly flexible, easily tunable with a facile synthetic route, PEG-based fluorinated esters were built up using 2-(trifluoromethyl)-3,3,3-trifluoro-propanoic acid [226] and PFTB [227]. Polydispersity in PEG is an inherent trait of the polymer, and recent years have seen the development of mPEG with improved biodegradability [228]. A thermoresponsive imaging probe with fine-tunable lower critical solution

temperature pioneered from peptidic mPEG combs [229] was explored for their smart drug-carrying ability using doxorubicin.

5.1.2. Hyperbranched

Coupled with linear, cross-linked, and branched-chain polymers, dendritic polymers are the fourth subclass of polymers that are invariably branched irregularly [230]. Hyperbranched polymers (as shown in Figure 4) are a subclass of dendritic polymers whose polymeric structures are bestowed with abundant functional groups, intramolecular cavities, low viscosity, and high solubility [231]. This class of molecules has been ventured mainly to overcome the intrinsic drawback of PFC formulations like low stability, limited aqueous dispersibility, and a limited possibility to functionalize.

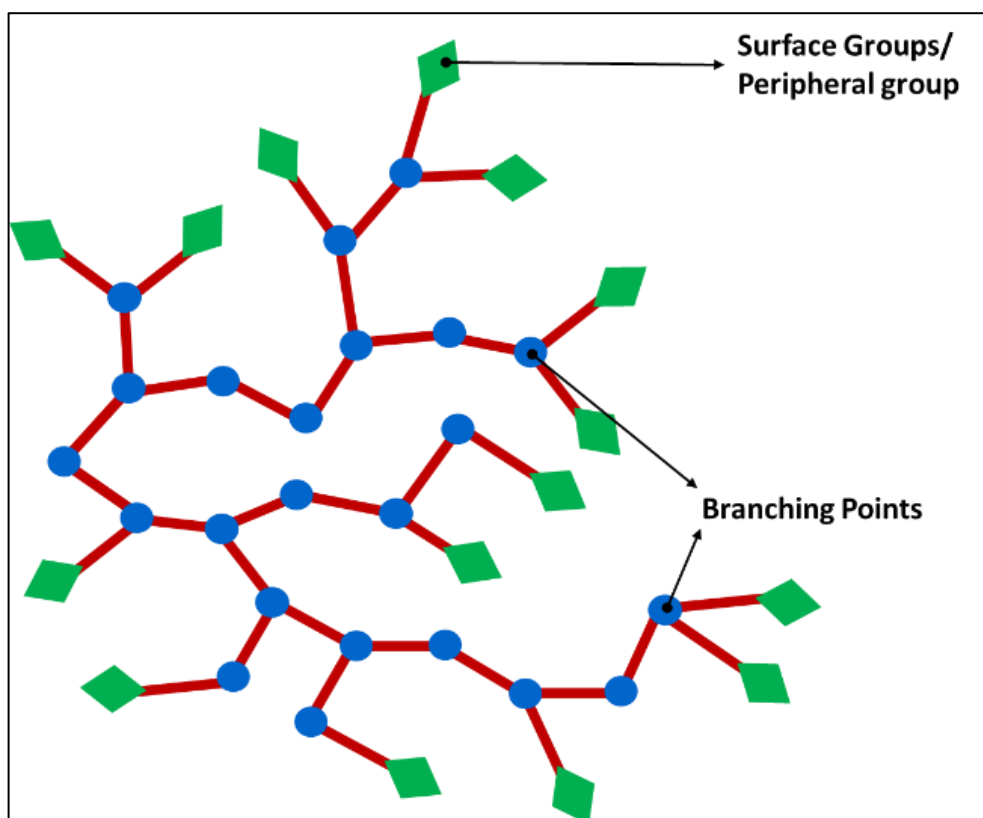


Figure 4. Hyperbranched polymers structure - the polymer structure is a randomly branched polymer with circles (blue) representing the branching points and rhombus shapes (green) for surface groups.

As already highlighted regarding the benefits of multimodal imaging—combining the high resolution, 3D anatomic images for soft tissues with MRI, and high spatial resolution for hard tissue by CT, a more accurate diagnosis is guaranteed, facilitating treatments. Multifunctional hyperbranched polymers containing iodine and fluorine were synthesized by initially using a hyperbranched iodopolymer via RAFT polymerization. The 2-(2',3',5'-triiodobenzoyl)ethyl methacrylate was incorporated to provide X-ray opacity along with poly-(ethylene glycol) methyl ether methacrylate (PEGMA) to provide hydrophilicity, and bis2-(methacryloyl)oxyethyl disulfide was chosen as a crosslinker to achieve biodegradability [232]. HBIP was chain extended with 2,2,2-trifluoroethyl acrylate (TFEA) and PEGMA to obtain hyperbranched iodopolymer containing ^{19}F (HBIPF). From the in vivo degradation studies, the polymers were proven to be biodegradable. Thereby, this study demonstrated

that multifunctional hyperbranched polymers were promising molecular imaging agents for CT/ ^{19}F MRI bimodal imaging.

To boost the local fluorine concentration, segmental mobility of the fluorine-containing moieties, and for active and specific targeting of diseased tissues, a multifunctional PFPE-based NPs conjugated with a peptide aptamer, Hsp70, as targeting ligand was pioneered (Hsp70—specifically to target the heat shock protein 70 overexpressed in breast cancer cells) [233]. They were attained from RAFT polymerization with hydrophobic PFPE segments and oligo(ethylene glycol) methyl ether acrylate (OEGA) as the hydrophilic monomer. The poly(OEGA)₃-PFPE polymer, further chain extended with OEGA and ethylene glycol dimethylacrylate (EGDMA) monomers gave rise to a hyperbranched PFPE-based polymer. After polymerization, fluorescence dye molecules, Cy5.5 were conjugated by reduction at the termini and aptamer peptide by click chemistry. The series of outcomes from the *in vivo* detection of breast cancer on a murine tumour model indicated that PFPE based NPs are efficacious theranostic agents for the specific detection of *in vivo* breast cancer. The explored properties included scrutinizing the ^{19}F NMR and MRI properties, *in vivo* and *ex vivo* molecular imaging, *in vitro* cell uptake, intracellular distribution, and trafficking, elimination of polymers from the body alongside tumour-penetration analysis.

Self-assembled colloids prepared using fluorinated hyperbranched polyglycerols were macromolecules germane to therapeutic functions [234]. By ring-opening multibranching polymerization (ROMBP) of glycidol followed by copolymerization with a fluorinated glycidyl ether (2-[(2,2,2-trifluoroethoxy)methyl]oxirane), hyperbranched polyglycerols were formed and explored for their ability to perform both as ^{19}F -MRI nanoprobe and drug-loaded nanocarrier. A synthetic steroidal anti-inflammatory drug—dexamethasone, was used as the model drug. The formation of the micelles gave a narrow size distribution after the drugs were incorporated inside.

5.1.3. Dendrimers

Even though multiple fluorines could be incorporated into a single molecule in fluorinated polymer, they suffer from pitfalls that often split signals are obtained in the FNMR. A group of macromolecules belonging to the family of dendritic polymer is of great use in enhancing the ^{19}F signal intensity per imaging agent molecule since they possess a spherical symmetry that can provide an identical chemical environment to the multiple fluorine atoms [235]. Frequently obtained from convergent synthesis methods, they self-organize to form well-defined 3D structures called dendrimers, with radically distributed branches, growing out from a focal point as illustrated in Figure 5. As they flare out to wide branches, the ‘generation’, as well as the number of peripheral groups of a dendrimer can be recognized from each subsequent branching unit. Even though both hyperbranched polymer and dendritic polymer have a 3-dimensional (3-D) macromolecular structure, the difference between them is that the latter has a regular topology as pictorially represented in Figure 5 with a multistep synthesis, while the former has an irregular topology as shown in Figure 4 with relatively facile one-step preparation [230].

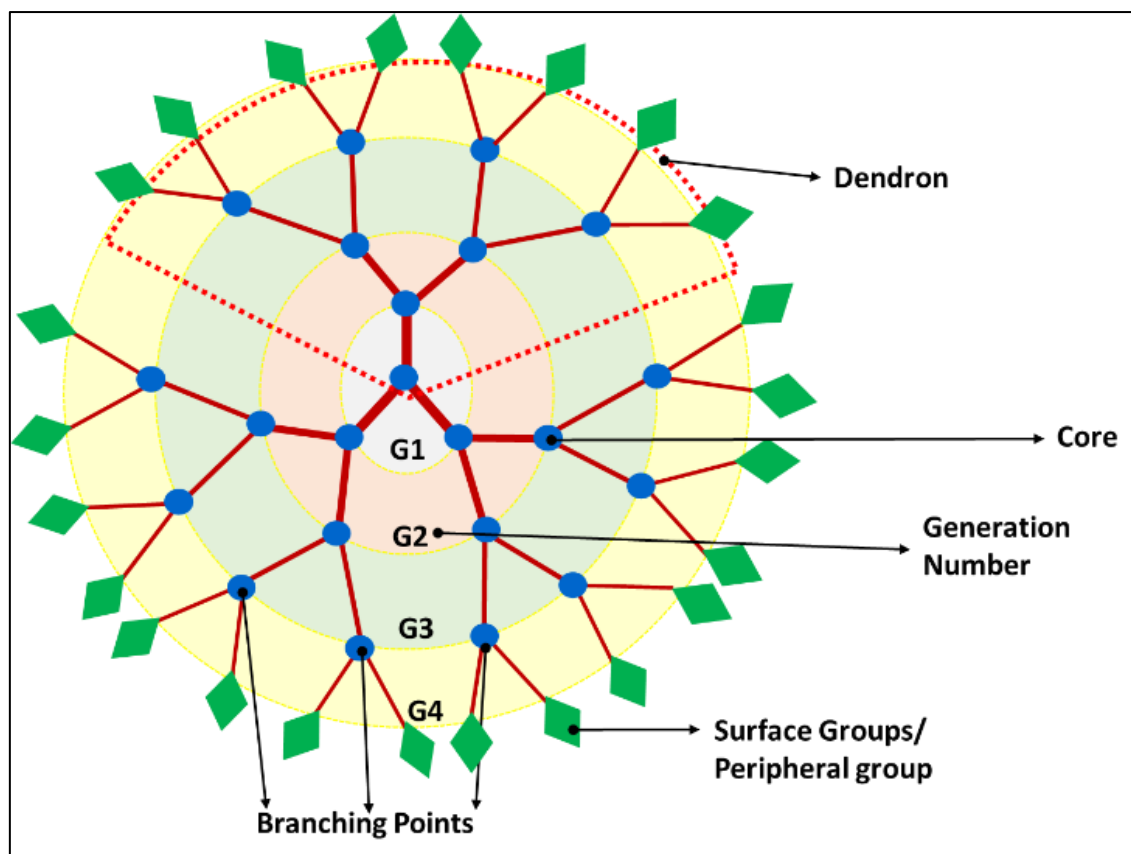


Figure 5. Dendrimers structural components include the core, branching points (blue circles), surface/peripheral groups (green rhombus), and the a dendron segment in a dotted red triangle.

Dendrimers have unique properties including monodispersity, multivalence, uniform and well-controlled size and shape, modifiable peripheral surface groups, and available internal cavities that make them a strong candidate for both imaging and drug delivery [236]. Their internal cavity can incorporate other imaging agents or drugs and have high intrinsic payload capability. When these polymer chains are fluorinated, they are adequate for ^{19}F MRI. The first fluorinated dendrimer studied for MRI was a small Janus dendrimer, a polymer assembly with a core attached to two different side chains [237]. Multiple studies had been carried out to study the dendrimers as nanocarriers.

The intricacy of ^{19}F MRI is their high T_1 , nuclear anisotropy, and frequently, NPs made by emulsions result in a size greater than 200 nm that can hardly pass through the capillaries of the blood vessel [238]. To evade these demerits a bifunctional Gd^{3+} chelate (DOTA—1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) was prepared and characterized to be employed as dendrimers. The dendrons, synthesized using fluorinated amino acids (BOC-L-4-trifluoromethylphenylalanine and 3,5-bis(trifluoromethyl)-DL-phenylalanine) along with carboxylic acids of the repeat branch unit. Different dendrimers with a size around 3 nm and T_1 decreasing with increasing dendrimer generation, were tested with animal studies (Sprague Dawley female rats), and they exhibited less toxicity (KB cells) and had better SNR [239]. Kolmél et al. described the synthesis of polyfluorinated second-generation dendrons consisting of 72 magnetically equivalent fluorine atoms and displaying a single sharp resonance in its ^{19}F NMR spectrum. The polymer was prepared by repeating iteratively Sonogashira coupling, alkyne deprotection, and copper-catalyzed azide-alkyne cycloaddition (CuAAC) for the generation build-up [240]. For a plenitude of pseudo

symmetrical fluorines and excellent ^{19}F MRI properties, the target dendrimer was convergently synthesized on a gram scale over 11 steps with an overall yield of 8%. Through assembling of the building block, the acidic bis(trifluoromethyl)carbinols, 540 fluorines were symmetrically distributed on each spherical layer, in unison emitted a single ^{19}F peak with high signal intensity and therefore had high ^{19}F MRI sensitivity [241].

Fluorinated self-assembled dendrimers were observed as promising ^{19}F NMR/MRI-traceable drug-delivery vehicles for in vivo tracing and quantifying drugs, detecting drug microenvironments, and weak interactions [242]. It was established that co-self-assembly of fluorinated amphiphile dendrimers could determine weak interactions between the drug and the drug-delivery vehicle because of the changes in the self-assembling profile (π - π stacking, hydrophobic interactions, etc.) that sensitively effectuated corresponding ^{19}F FMR responses. To study drug-amphiphile interactions in micelle- and liposome-based drug-delivery systems, a total of 15 model molecules with structural diversity such as (R)-carvone, cholesterol, the anesthetic propofol, and the anticancer drug doxorubicin, were chosen. In comparison to the per-hydrogenated dendrimers, fluorinated counterparts had different traits due to the fluorophobic effect relative to solubility and micro-segregation effect. Like PFCs, their degradation pathway and toxicities were still ambiguous after being retained for a longer time in the body [242].

Although dendrimers seem like a scintillating prospect, the enigma faced by this class of molecules is their arduous synthetic procedure and use of organic solvents that limit them from being an easily approachable technique. Often, for the formation of dendrimers, different chemical groups can be fine-tuned depending on the outcome. Its cytocompatibility, biodegradability, cellular toxicity, and cellular uptake are complex and abstruse, and on top, it requires further investigations and inferences. There is also a condition called "hydrophobic aggregation-induced signal attenuation" that happens when the ^{19}F -content in the molecular structure is greater than 10 *wt.%* [243]. The nanoprobe cannot exceed a threshold concentration of fluorine, for stimulation in biological systems, as exceeding a base concentration result in hydrophobic aggregation of fluorinated segments.

5.1.4. Nanohydrogel

Hydrogels are 3D hydrophilic cross-linked or self-assembled polymer networks (Figure 6) that have high loading capacities of payloads (30% *wt.*), self-healing ability, viscoelastic behavior, ample stability, and can be triggered to release the payload through swelling in response to environmental changes in pH, ionic strength, or temperature [244,245]. The payloads can be encapsulated in nanohydrogels through various means, such as (i) passive/diffusion-based, (ii) covalent conjugation to either the interior or exterior, (iii) physical entrapment within the polymer network [246].

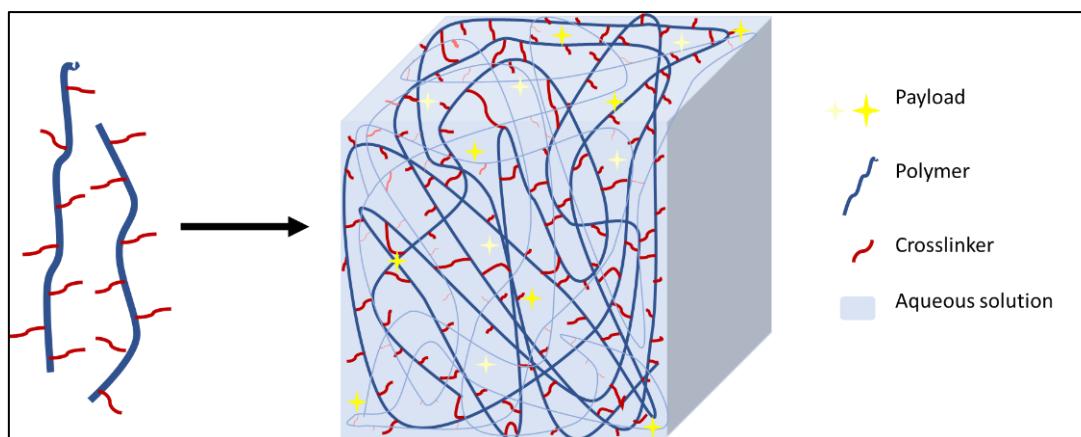


Figure 6. Nanohydrogel matrices are formed by polymers that can form the 3D network with the help of crosslinkers (red) that act as linking ligands. The payloads (yellow star) can be trapped inside the 3D matrix.

Designing NPs targeting the lymphatic system (a vital part of the immune and circulatory system), both PEGylated and fluorinated chitosan were synthesized to fathom their application for encapsulation of probes and MRI lymphography (relating to the body's lymphatic system) experiments [247]. The nanohydrogels were prepared by ionic gelation, the spontaneous supramolecular assembly of cationic chitosan with anionic compounds. After resolving the dilemma of determining the degree of substitution of PEGylated and fluorinated derivatives with chitosan, *in vivo* experiments affirmed good biocompatibility and prospective use of nanohydrogel for the relevant applications. Similarly, a thermoresponsive hydrogel was reported by Kolouchova et al. where the structure of the nanohydrogel was based on amphiphilic copolymers containing two blocks: one hydrophilic biocompatible block-poly[N-(2-hydroxypropyl)methacrylamide] (PHPMA) or poly(2-methyl-2-oxazoline) (PMeOx) and one fluorinated thermoresponsive block-poly[N(2,2difluoroethyl)acrylamide] with excellent sensitivity and non-cytotoxic for cell lines like human cervical carcinoma, murine monocyte/macrophage, HF-primary fibroblasts, and human B lymphoblast cell lines [248].

To prevail over the crucial challenge of aggregation in fluorocarbon substitutions that induced the segments of polymers hydrophobic, Munkhbat et al. had used an intelligent chemical play using nanohydrogels [249]. It facilitated in fully realizing the potential of polymeric tracers. Firstly, polymeric assembly was constructed with degradable hydrocarbon moieties and a high fluorocarbon core, and by chemical cross-linking, preserved the morphology of assembly. Eventually, segmental mobilities were amplified within the nanohydrogel interior by triggered degradation of cleavable hydrocarbon parts that decreased the density of the assembly's interior. That prompted escalated T_2 relaxation time and propelled signal intensities enhancement in ^{19}F NMR and ^{19}F MRI phantom imaging.

To delve into the controlled release of bioactive agents, ^{19}F MRI was used to quantify the degradation rate of implantable or injectable hydrogels and provide the precise location in a real-time and non-invasive manner, without interruption of endogenous background signals and limitation of penetration depth. Traditionally, gravimetric methods are being used to provide this information *in vitro* but offer limited insight on the *in vivo* fate and sequential tracking [250]. Ergo, a zwitterionic, fluorinated and alkynyl ^{19}F MRI molecular CA was designed, namely *N*-(carboxymethyl)-*N*-methyl-*N*-(3,3,3-trifluoropropyl) prop-2-yn-1-aminium (termed PA-CBF₃), with zwitterionic carboxybetaine structure, which

was superhydrophilic and had superior resistance to protein adsorption and was capable to tether with different hydrogels [243]. The probed nanohydrogels included polyacrylamide hydrogel, injectable alginate hydrogel, thermosensitive poloxamer hydrogel, and poly(ethylene glycol)-*b*-poly(L-valine) polypeptide hydrogel.

Besides manoeuvring of PFPE modified polymeric nanoemulsion for various applications [65,88,94,163,218–220,251], Janjic et al. had extended their use in hydrogels too. Anti-tumour necrosis factor- α (anti-TNF α) therapy had been a proven strategy for treating inflammatory bowel disease, where TNF α -binding lactococci bacterium can also act as infrared fluorescent protein. For localized delivery of anti-TNF α therapy, the PFPE nanoemulsion loaded with theranostic TNF α -binding lactococci (*Lactococcus lactis*) was incorporated into a thermoresponsive polymer (Pluronic®F127) hydrogel [77]. The resulting nanoemulsion-based hydrogel (nanoemulgel) was ^{19}F MRI and NIRS visible. The same group had used a slightly modified hydrogel for increasing its ability to load different payloads (fluorescent dyes, pH sensors, chelators, drugs, and antibodies) and therefore adapt the hydrogel for a broad range of biomedical imaging and delivery applications. PFPE nanoemulsions were crosslinked with polyethylenimine to form hydrogels hence ridding the necessity of any energy utilizing the emulsification step [252].

5.1.5. Lipids

The primary component for the vaccine technologies (Pfizer/BioNTech and Moderna) used during the outbreak of the novel coronavirus causing severe acute respiratory syndrome is lipid NPs [253]. This system helped in the translocation of the mRNA/self-replicating RNA (responsible for producing the immune response) across the plasma membrane. Typically, liposomes are derived by the self-assembly of the phospholipids like phosphatidylethanolamine, phosphatidylcholine, phosphatidylglycerol, or cholesterol. As portrayed in Figure 7, they possess a hydrophilic core and a lipid bilayer, thereupon have the stupendous advantage over other NPs in encapsulating hydrophilic (in the core), hydrophobic (between the bilayer), and even amphiphilic drugs in addition to the possibility of surface modification [175]. They are one of the widely used systems with less known toxicity compared to conventional drugs. For instance, they are the primary component of the first FDA-approved nanodrug Doxil® for the treatment of Kaposi's sarcoma, ovarian, and breast cancer [254].

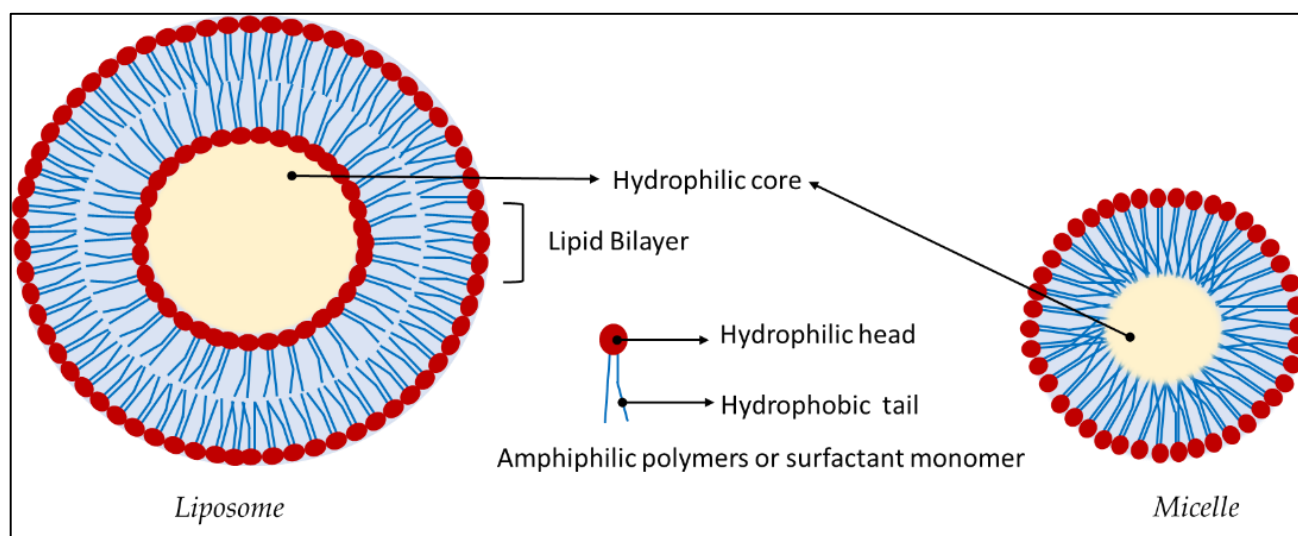


Figure 7. Liposome and micelle consist of an assembly of amphiphilic polymers or surfactant monomers that possess a hydrophobic tail (blue) and a hydrophilic head (maroon).

Cellular therapeutic dendritic cells (DC)-based vaccination is an ex vivo modified DC with tumour-associated antigens and had been used to initiate anti-tumour immune responses. Hence, it is vitally substantial to track the fate and location of the injected DC. Intending to collectively load antigenic proteins into DC and enable high-resolution tracking of the antigen-loaded cells ^{19}F -MRI CAs, antigen-coated PFC particles for DCs were prepped. The cationic particle consisted of 1,2-dioleoyl-3-trimethylammonium-propane, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)-2000], and cholesterol. The PFC component was either PFH or PFCE, and it was emulsified in the presence of excess lipid solution through high frequency shaking. Particles were loaded electrostatically by negatively charged ovalbumin (commonly used model antigen) [255]. The same particles were used for in vivo imaging the transplantation of pancreatic islets and tracking the autoreactive T-cell migration in the pancreatic region [256]. Liposomes were formulated with hydrophilic organofluorine molecules with a fluorine encapsulation up to 22.7 mg/mL [257] that could concomitantly image multiple targets without any chemical shift artifacts.

In an attempt to mimic the temperature range in tumours (37–39 °C) that has a different microenvironment than normal cells, Lima et al. demonstrated the change of the ^{19}F NMR signal of F—containing compound in thermally responsive lipid nano-emulsion particles, mainly the T_1 and T_2 values, depending on the temperature change (37–42 °C). The carriers were tripalmitin, tristearin, and triarachidin, favored based on high melting point neutral saturated fatty acid, and the fluorine compound was a modified α -tocopherol. The study concluded that T_2 changed more than T_1 , and the change in T_2 was mainly given by the increased molecular motion of the modified α -tocopherol, highlighting that the local temperature might impact the ^{19}F NMR signal intensity [258]. With MRI multimodal imaging, it was demonstrated that PFC NPs were prospective to be delivered for lung cancer [259]. The PFCE emulsions with rhodamine-phospholipid surfactants consisted of 20% (*v/v*) PFCE, 2% (*w/v*) of a surfactant commixture, 1.7% (*w/v*) glycerin and water. Surfactant commixture consisted of dipalmitoylphosphatidylcholine, cholesterol, Gd-diethylenetriaminepentaacetic acid-phosphatidylethanolamine, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-*N*-(lissaminerhodamine B sulfonyl), and 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol). The PFCE emulsions were exposed to human bronchial epithelial (BEAS-2B) and human lung squamous carcinoma (H520) cell lines following intratracheal or intravenous administration. This study established evidence that with minimal extratumour systemic exposure, PFC NPs can be locally delivered into lung cancers intratracheally (reported for the first time) in high concentrations.

5.1.6. Micelle

Typically, micelles are obtained via the self-assembly of the amphiphilic molecules similar to liposomes. They engineer into the core-shell architectures that possess outer hydrophilic surfaces that can impart steric stability, and prolong their circulation lifetimes while the entire interior of the NP is hydrophobic, which portends that only hydrophobic drugs could be encapsulated as depicted in Figure 7 [175]. This scenario leaves hydrophilic cargo to be attached to the surface. They are smaller (10–100 nm) than liposomes, making them

suitable for leaky vasculature of tumours. A biosynthesized fluorinated protein was presented by Hill et al. [260] as a “fluorinated thermoresponsive assembled protein” (F-TRAP) that could encapsulate small-molecule chemotherapeutic doxorubicin in its self-assembled micelle structure and release them in response to temperature and concentration, owing to its inherent stimuli-responsive properties. They bore a coiled-coil pentamer corona and a hydrophobic, thermoresponsive elastin-like polypeptide core. When exposed to increased concentration and temperature, they assembled into nanoscale micelles characterized by nearly a constant ^{19}F T_1 relaxation times and a remarkable decrease in ^{19}F T_2 relaxation. Furthermore, through thermally induced in vitro coacervation of the proteins at 45 °C, free doxorubicin was collected in the supernatant. The therapeutic efficacy of the precedent had been assessed in mammalian tumour cells, MCF-7 human breast adenocarcinoma cells, and discerned that it was significantly effective at reducing cell viability.

5.2. Inorganic NPs

Inorganic NPs such as metallic, magnetic, quantum dots consist of a central core made of inorganic material, as illustrated in Figure 8, which defines their unique characteristics. We would be focusing on metal, silica, and carbon-based NPs in particular, due to the vast literature published for exploring them. Hequet and coworkers [35] had prepared a CA containing a paramagnetic center and chemically equivalent fluorine atoms using a cycloaddition reaction for dual $^1\text{H}/^{19}\text{F}$ MRI. T_1 agents (mostly lanthanide complexes) are called positive agents because of the hyperintense signal they engender in the accumulation areas, whereas T_2 agents (usually iron oxide NPs) are “negative” CAs since they induce darker contrast in the accumulation area. A series of cyclen derivative lanthanide (Gd(III), dysprosium (III), terbium (III), and europium (III)) complexes associated with nine chemically and magnetically equivalent fluorine atoms were synthesized, and the study showed that gadolinium, dysprosium, and terbium complexes were promising transition metals for future use in ^{19}F MRI in terms of their relaxation time.

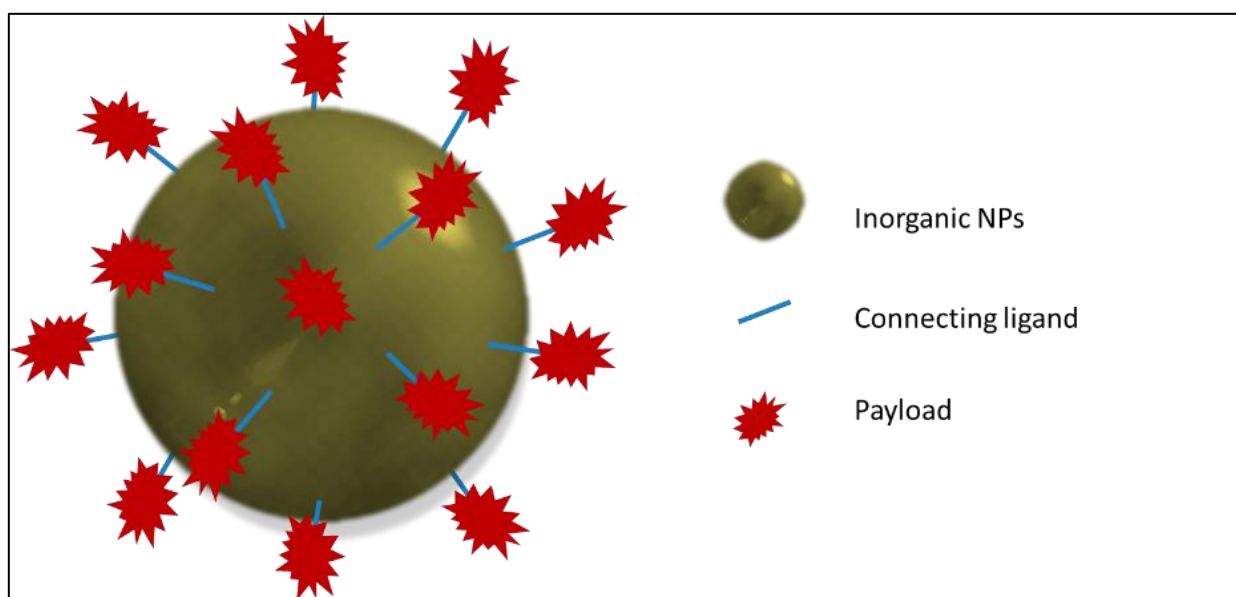


Figure 8. General representation of inorganic NPs where payloads (red star) are attached to the surface of the nanosystem. They include gold, silica, iron oxide, quantum dots, nanotubes etc.

Lanthanide-based upconversion (UC) NPs have potential applications in MRI or drug targeting or carriers due to their luminescent properties, such as large anti-stokes shifts, photostability, narrow emission peaks, and low toxicity. NaYF_4 is an efficient well-known UC host material where Y^{3+} can be replaced in any ratio by rare-earth ions, of which Gd^{3+} is the most attractive one for their intrinsic magnetic properties. To track down the possibility of their UC application in the biological field, water-soluble $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Tm}^{3+}$ nanorods were prepared using the hydrothermal method [261]. The samples were conductive to visible light, and luminescence images were obtained in laser diode excitation. PLGA was reported to encapsulate doxorubicin and inorganic nanocrystals– $\text{NaYF}_4\text{:Yb,Er@NaGdF}_4$ used for cancer cell imaging and exhibited pH-responsive drug-releasing behavior [262].

Another inorganic material of interest is nanofluoride (calcium fluoride (CaF_2))-based inorganic nanocrystals. The unique characteristics like controllable content, sizes, and shapes, are often outperformed with the disadvantage of the restricted mobility of the elements within the crystal that leads to NMR line broadening and impedes their use as MRI tracers. Ashur et al. established a synthetic, water-soluble, small (<10 nm) fluoride-based nanocrystals that average out homonuclear dipolar interactions and thus allow high-resolution ^{19}F NMR spectroscopy of the nanocrystals in aqueous solutions. The formulated PEG-coated CaF_2 nanocrystals were used as an imaging tracer, combining the advantages of nanocrystals (small, high ^{19}F equivalency, surface modifiability, maximal ^{19}F density) with the merits of ^{19}F MRI tracer after being used for in vivo ^{19}F MRI in mouse models [263]. To increase the relaxivity of NPs that could help improve the SNR, fluoride doped iron oxide ($\gamma\text{-Fe}_2\text{O}_3$) NPs were considered [264]. Doping citric acid- on the mentioned NPs chemically induced intensification of magnetic anisotropy, unaffected either its crystal structure or electronic configuration.

5.2.1. Metal NPs

Metal NPs are picked apart for their unique electrical, optical, and mechanical properties [265]. Widely explored for their size, shape, surface chemistry, and optical properties, this class of NPs had opened a broad array of applications when stumbled upon the possibility of obtaining advanced materials with the required properties by various modifications.

The combination of metal NPs with fluorinated ligands anchored on its surface is suitable for ^{19}F MRI as this combination of NPs has superior properties in terms of their function. Boccalon et al. [266] prepared gold NPs (F-MPC) for dual-mode imaging, whose surfaces were grafted with fluorinated organic monolayer and ligands ending with a fluorescent dye. The exclusive properties included a central fluorinated chain to generate the ^{19}F signal, the ability to solubilize/disperse in water (hence in biological media) without any additives and impart solubility in many solvents due to the presence of terminal hydrophilic triethylene oxide or PEG_{550} chain. Further, the outer monolayer capable to solubilize small hydrophobic molecules laid the groundwork for the development of drug nanovectors. Thanks to these features of the gold NPs that made them a novel imaging platform. The gold core had less than 2 nm size, and the overall size with attached ligands less than 10 nm, permitted them to penetrate smaller blood vessels. F-MPC-cell interactions were evaluated with human cervical carcinoma cells (HeLa), showed that more than 95% of the cells were viable after the uptake.

The aforesaid group had also functionalized the gold NPs with Gd(III) chelates [267] in an endeavor to develop a probe for dual imaging. Some of the impediments considered to get around were the NPs' detectability and the usual

size range of emulsions (200–300 nm), which limit NPs in vivo applications. Increasing the number of fluorine reinforces the odds of making it hydrophobic, hence the development of probes containing enough fluorine to reach detectability in ^{19}F MRI is one of the captious challenges in the field. The Gd chelating unit of the ligand was based on the DOTA scaffold, deeply embedded in the monolayer of water-soluble gold NPs that gave good quality MRI images at a 20 mg/mL concentration with Gd(III) units. In pursuance of the ligands with fluorine atoms in the same chemical environment, PFTB ligands were set up for the preparation of gold NPs. Out of the prepared NPs (fluorinated/non-fluorinated, attaching PEG/thiolate to its side chain, etc.), long-chain PEGylated compounds were proven to be the best option to obtain colloiddally stable NPs in vitro and hence obtain a single chemical shift, narrow ^{19}F -NMR signal, and high fluorine loading [268].

5.2.2. Silica NPs

Another widely used metal for NPs preparation is mesoporous silica. Massive surface areas (1000 m^2/g), tunable pore sizes (2–20 nm), facile surface modification capability via various synthetic approaches, and controlled release of numerous drugs from its pore make mesoporous silica NPs (MSN) unique for diversified applications [269]. Hollow mesoporous silica particles filled with PFCE were put to use to demonstrate the type of cargo in the mesoporous silica drug vectors may have substantial influences on the biodistribution [270]. Since the outer surface of these particles is mesoporous as illustrated in Figure 9, the PFCE will be exposed at every stage to the biological environment. Subsequently, the fate of the NPs can be accurately deciphered from the protein adsorption behavior and how cargo is affecting the protein adsorption. Protein adsorption studies and in vivo ^{19}F quantification results proved that adsorbed amount of protein (apolipoprotein A-1 and A-2) was much higher for the PFCE-filled NPs as compared to the native particles and that PFCE-loaded particles were eliminated by liver 72 h post-injection.

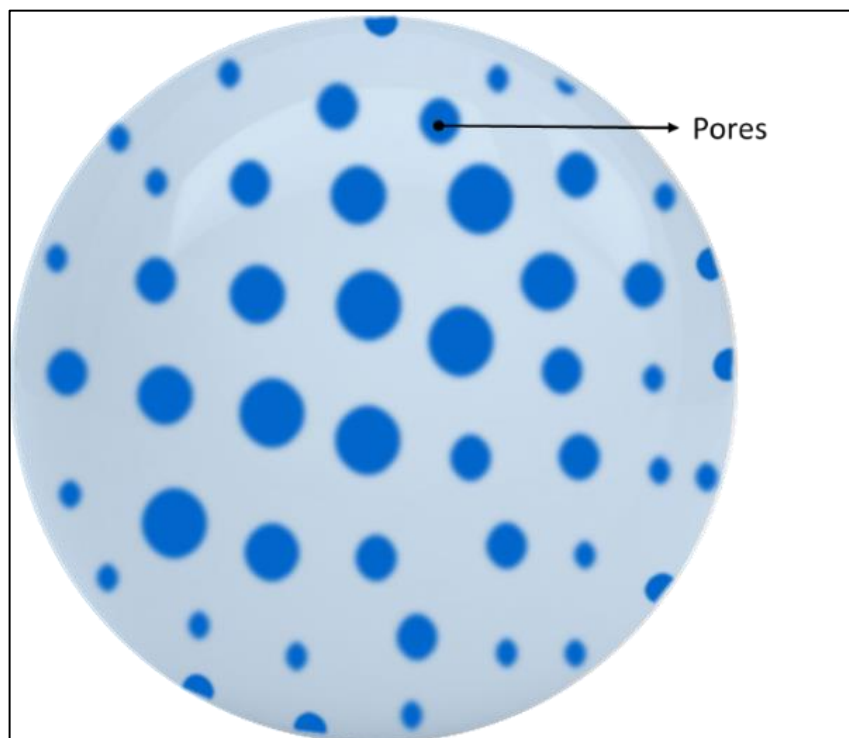


Figure 9. Mesoporous silica nanoparticles structure represented by a blue sphere and the darker small circles shows the pores present on its surface.

Lee et al. have communicated the PFC-loaded ultraporous mesostructured silica NPs (PERFUMNs) for ^{19}F MRI detectable oxygen-sensing probes [271]. They had used a post-synthetic loading method for experimenting with three different PFCs (PFCE, PFD, and perfluoro(*tert*-butylcyclohexane)) which made it possible to encapsulate more PFCs, around five times more than usually encapsulated by MSN. Post-synthetic loading methods were explored to find their influence on the loading yield and efficiency, and it was pinpointed that sonication time is a crucial factor [272]. It was revealed that for silica NPs, as the leaching of PFCs was typical due to their porous structure, it was better to graft fluorine probes onto its surface by covalent bonds. To put this into practice, Bouchoucha et al. [273] reported the synthesis of mesoporous silica NPs with covalent modification with either fluorosilane or polyfluorosiloxane together with a paramagnetic Gd chelate grafted at the surface. NPs (MCM-48-type) were synthesized and functionalized with fluorine-containing molecules and Gd chelates (Gd-DTPA). It helped in the production of imaging probes which induced a strong “positive” contrast enhancement effect, and NPs with the potential for dual ^1H and ^{19}F MRI. Even though the metal Gd on NPs surface aided improvement of the ^{19}F relaxation time, a strong effect on T_2 relaxation had the possibility to prevent the detection of probes. This dual detection represented a potential alternative to MRI-PET or in MRI-SPECT and hence the use of radioactive molecules in them.

Kikuchi et al. [274] developed novel multifunctional core-shell NPs utilizing the PRE effect to detect enzymatic (Caspase-3) activity and to use ^{19}F MRI for detecting gene expression. Paramagnetic relaxation enhancement (PRE) is the liaison between two magnetic moments of paramagnetic nuclei and observed nuclei, resulting in efficient curtailment of T_1 and T_2 of the nuclei under observation [275,276]. A probe was designed by connecting a ^{19}F containing moiety with a Gd^{3+} complex via an enzyme cleavable linker. Anticipating the T_2 of ^{19}F would be shrunk by the existing PRE effect in the enzyme reaction (hence low/no signal) as ^{19}F containing moiety was close to the Gd^{3+} complex, and when the enzyme cleaved the substrate, ^{19}F MRI signal would emanate as T_2 increased in the guise of no longer effective PRE (distance between ^{19}F and Gd^{3+} became infinite). However, in mouse experiments with small molecule-based ^{19}F MRI probes, there were sensitivity issues, and to prevail over, PFCE inclusive silica-NPs were synthesized with surface modified with PEG.

Being mindful of the ingrained downside of predominant ^{19}F MRI probes—arduous modifiability of the surface of nanoemulsions and low sensitivity of small molecule-based probes—multifunctional core-shell silica NPs were introduced for successful detection of gene expression in living cells and tumour tissue in living mice by ^{19}F MRI. The biological inertness, favorable colloidal properties, and ease of surface modification of silica with the acquiescence of PFCE resulted in the formation of fluorine accumulated silica NP for MRI contrast enhancement (FLAME) [277]. The surfactant was *n*-cetyltrimethylammonium bromide (CTAB), and the surface was tailored with a folate receptor. The identic system had been modified for ^{19}F MRI traceable silica NPs as drug carriers (mFLAME) by improving the silica coverage of the PFC core, reforming the mesoporous silica shell with a NIR dye (Cy5), and functionalizing it with a folate receptor. By this, it was possible to extend its application to dual-modal imaging (NIRS/ ^{19}F MRI) and drug delivery [278]. Flow cytometric analysis confirmed mFLAME internalized by KB cells (HeLa or cervical adenocarcinoma). Further loading it with doxorubicin had a top-tier cytotoxic effect on KB cells. The

combination of fluorine atoms with a paramagnetic ion reduced the ^{19}F relaxation times, which was attributed to the PRE effect.

In like manner, to create an OFF/ON switching ability of Ln^{3+} complexes by PRE effect, FLAME NPs were attached to Gd^{3+} diethylenetriamine pentaacetate (DTPA) complexes on its surface by disulfide linkers (FLAME-SS-Gd^{3+}). The study had been engaged on the FLAME-DTPA complex after treating with a reducing agent–tris(2-carboxyethyl)-phosphine. This inspection facilitated to show for the first time that the PRE effect of surface Gd^{3+} complexes was effective for fluorine compounds in NPs over 50 Å [279] that were contradictory to the study of De Vries et al., who previously observed that the distance between Gd^{3+} complexes and the fluorine core was less than 22 Å for the PRE of PFCE in Gd^{3+} modified nanoemulsions [280]. FLAME NPs were also reported for their use in multicolor MRI probes (PFC@SiO_2 , FLAME). Five different types of PFCs were employed–PFCE, PFOB, FC-43, perfluorodichlorooctane (PFDCO), and 1,1,1-tris(perfluorotert-butoxymethyl)ethane (TPFBME) and to render multicolor fluorescence imaging capabilities, rhodamine B isothiocyanate, sulfo-cyanine 5, and fluorescein-4-isothiocyanate were covalently modified to silica shells [281]. Nanoprobes (PFCE@SiO_2 , TPFBME@SiO_2 , and FC-43@SiO_2) enabled the triple-color ^{19}F MR imaging in vivo for the first time.

5.2.3. Carbon Based

Carbon nanotubes are nanoscale hollow cylindrical tubes consisting of rolled-up sheets of layer (single-walled or multi-walled) of graphene as sketched in Figure 10 [282]. The unique 1D structure, high mechanical tensile strength, high surface area, high thermal conductivity, chemical stability, effective resistance to any chemical impact, rich electronic polyaromatic structure, lightweight, possible surface functionalization, and the possibility to stuff the hollow interior with various imaging agents/drugs/molecules of interest renders them with manifold potential for applications [283,284].

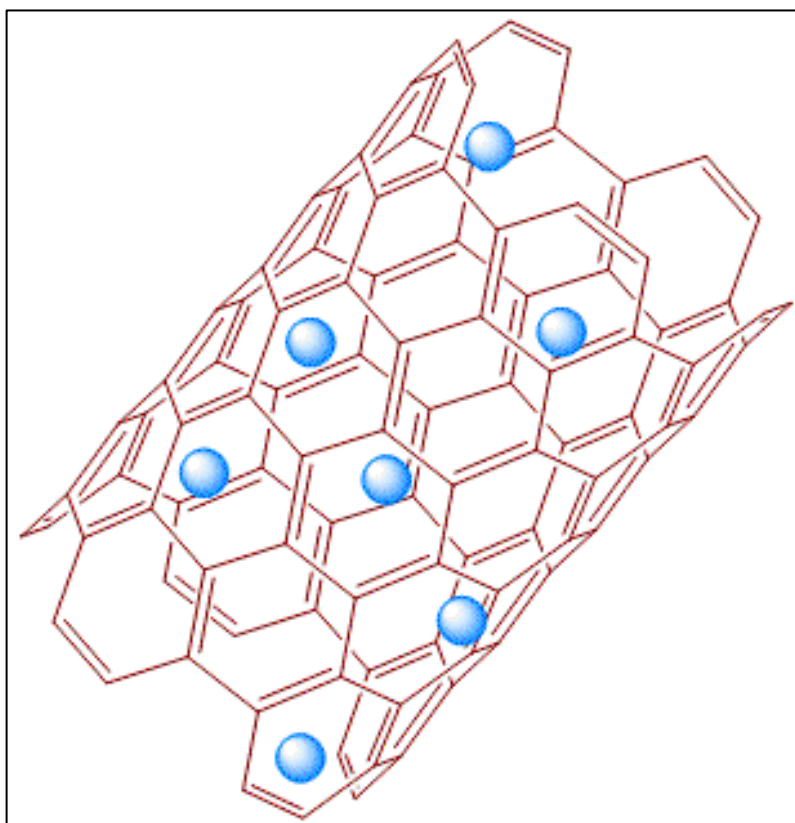


Figure 10. Carbon nanotubes with payload (blue circles).

Among the ROS, H_2O_2 plays an indispensable physiological role. Monitoring its pathological level gives valuable information on the multiple abnormalities occurring in the living entity. Fluorinated halloysite nanotube (HNT) was used to detect the low concentration of H_2O_2 by ^{19}F NMR probe prepared using halloysite nanotubes $((\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4 \cdot n\text{H}_2\text{O}))$ and 3,5-bis(trifluoromethyl) benzeneboronic acid [285]. The halloysite nanotube with minor modifications uncovered its implementation as a fluorescent probe for selective and sensitive response to hyperoxide using 1-pyrenylboronic acid [286] in conjunction with a smart halloysite-based hydrogel prepared for H_2O_2 -responsive drug delivery system [287].

5.3. Mixed/Hybrid NPs

To impart multifunctionality to an NP-based system, the convergence of organic and inorganic components is often pivotal, resulting in hybrid theranostic NPs. Ingenious and intelligent combinations of discrete functional nanostructured materials will enable the development of versatile nanomedical platforms for multimodal imaging or simultaneous diagnosis and therapy.

'FETRIS'/Iron(III) tris- β -diketonate with PFPE were prepared with the idea that high-spin paramagnetic metal ions can profoundly alter the relaxation times T_1 and T_2 for the cell detection via ^{19}F MRI [288]. The PFPE-based ligand-fluorinated β -diketones (FDK) was made using Claisen condensation between PFPE and p-methoxyacetophenone. Formed FDK was blended with an assortment of PFC derivatives (PFPE, PFPE diethylamide, PFOB, short PFPE oligomer perfluorotetraglyme), and the obtained blended oils were formulated into lipid-based paramagnetic nanoemulsions using microfluidization. Cytocompatible FETRIS agents were formed as FDK efficiently and irreversibly extracted Fe^{3+} ions from an aqueous solution into the fluorous phase in the PFPE-in-water nanoemulsions. The system had stretched out its application for intravenous injection and in vivo inflammation imaging [289]. To forge a multifunctional nanoprobe, Chen et al. used the strategy of the one-pot encapsulation method, where the PFCE was anchored through hydrophobic-hydrophobic interactions to $\text{Cu}_{1.75}\text{S}$ NPs and then trapped within the silica shell ($\text{Cu}_{1.75}\text{S}-^{19}\text{F}@\text{OFP}-\text{SiO}_2$) [290]. Co-encapsulation agents were oleylamine-functionalized polysuccinimide and trimethoxy(octadecyl)silane. The resulting nanoprobe contained up to $\sim 2.0 \times 10^8$ fluorine atoms per particle with an ultrahigh ^{19}F MRI signal.

Star polymers were contrived with a polyhedral oligomeric silsesquioxanes (POSS) core and partly fluorinated arms expecting the star's arm to be visible by ^{19}F MRI, while the POSS core to load drug molecules [291]. The arm of the star polymer consisted of TFEA and PEGMA monomers. The formation of eight partly fluorinated copolymer arms and a POSS core, with different sizes of NPs, molded depending on the length of the chain. The fabricated NPs were dissolved in the water, and the characterization showed a singlet for ^{19}F NMR and augmented T_2 . Hybrid functionalized 2D carbon nanomaterials like graphene oxide and iron oxide (Fe_3O_4) NPs blended the magnetic characteristics of Fe_3O_4 and the photoluminescence of graphene oxide in MRI and fluorescence imaging [292]. Fluorinated graphite polymer was oxidized by strong oxidizing agents, to give highly fluorinated graphene oxide. Hybrid of fluorinated graphene oxide and iron oxide were prepared by co-precipitation technique using iron sulfate and iron chloride.

Another nanocomposite based on multifunctional $\text{Cu}_7\text{S}_4\text{-Au}@ \text{PSI}-^{19}\text{F}/\text{PEG}$ was reported by Cui et al. [293]. Plasmonic nanostructures, including gold (Au)

with a localized surface plasmon resonance (LSPR) peak in the transparent window (800–900 nm), had been recognized as a promising agent for photothermal therapy. LSPR in metals is due to free electrons whereas, for doped semiconductors, it is the cation vacancies (holes) that can be tampered with doping. Heterodimers (combining plasmonic nanostructures with chalcogenide) can aid in tuning the LSPR peak position. To attune the LSPR (~808 nm), the nanocomposite was prepared by growing a small Au domain on a heavily doped Cu_7S_4 to form a Cu_7S_4 -Au heterodimer. Furthermore, by click chemistry, adding the fluorine component that had an inconsequential background, good sensitivity combined with high spatial resolution enhanced photothermal efficacy and decreased the optical damage to normal tissues.

A multifunctional hybrid vesicle was synthesized from PEGylated magnetite/PFOB-loaded organic/inorganic hybrid that could be used in dual-modality US/MR imaging and intensified image-guided high intensity focused ultrasound ablation [294]. The organic component was amphiphilic block copolymer-polystyrene-block-poly(acrylic acid). The hybrid shell layer was formed by shell cross-linking of the micelle of Fe_3O_4 NPs and PFOB using thiol-silane (3-mercaptopropyltrimethoxysilane). Hu et al. used copper sulfide (Cu_7S_4) coated with oleylamine functionalized 3,5-bis(trifluoromethyl)benzaldehyde fluorinated ligands for ^{19}F MRI and photothermal ablation [295]. Ionic liquids (IL) based on 1-butyl-2,3-dimethyl-imidazolium (BMMI, BMMIBF₄), 1-ethyl-3-methyl-imidazolium (EMI, EMIOF), and 1-ethylpyridinium (EPy, EPyBF₄) were explored with fluorinated anions, such as tetrafluoroborate (BF₄) and trifluoromethanesulfonate (OTf) engendering a fluorinated IL-based active ^{19}F MRI platform (FILAMP) [80]. At 50 to 60 °C, these ILs confined inside the hollow mesoporous silica, and the pores sealed by stimuli-responsive copolymers could respond and release free IL upon biological stimulation.

A promising dual $^1\text{H}/^{19}\text{F}$ MRI probe was derived from lipid NPs, whose surface was modified with paramagnetic calcium-responsive Gd-chelates and encapsulated with PFCE [79]. The surfactants were dipalmitoylphosphatidylcholine with 5% of PEGylated phospholipid. Calcium helped in the contrast enhancement of ^1H MRI. Water-soluble fluorinated NPs with metal-core and fluorinated ligands were reported by Arango et al. [116] with interesting MR features. A simple phase transfer method was used to bind gold NPs with fluorinating building blocks, and in vivo mice studies showed that they are suitable for ^{19}F MRI/MRS. Magnetite (Fe_3O_4) NPs were modified with oleic acid, and this system formed self-assembled magneto-micelles with amphiphilic copolymers [296]. The fluorine-containing copolymers were synthesized using 2,2,3,4,4,4-hexafluorobutyl methacrylate (for holding hydrophobic drugs) and PEGMA (increased the hydrophilicity of the hybrid system). 5-fluorouracil was used to study the capability of the magneto-micelles to carry and load drugs and for in vitro release studies.

Some of the other hybrid systems include fluorinated polymer and manganese-layered double hydroxide NPs (Mn-LDH NPs) that benefitted the specific and sensitive detection of breast cancer [297]. Layered double hydroxides (LDHs) are 2D nanomaterials consisting mainly of divalent and trivalent cations in layers with anionic species intercalated between the layers. The cations could be metals, and the anions could be drug/polymers/siRNA, etc. pH-activated ^{19}F MRI agents based on the PRE were specifically activated within the acidic tumour environment and were assembled with PFPE-based polymer and Mn-LDH NPs. Furthermore, Wang et al. reported the function of fluorine and nitrogen co-doped carbon dot complexed with Fe(III) put together as T_1 CA in ^{19}F MRI [298]. Using HeLa as a model cell line, the CA exhibited the lowest T_1 relaxation, and in BALB/c

mice, it displayed an accurate tumour image effect in addition to efficient renal clearance, low toxicity, high relativity, and bright luminescence.

A recent review by Mali et al. discusses recent advances in ^{19}F -nanoparticles for ^{19}F MRI [299], with a detailed analysis on the nanotechnologies employed for the design and applications of ^{19}F -based nanoprobe. Table 4 gives information on most of the nanosystems discussed so far, including their preparation technique, fluorine component, characterization technique, their interesting pros and cons, and their applications.

Table 4. The selected examples of studied fluorinated and PFC nanosystems explaining their preparation technique, fluorine component, characterisation techniques used for studying various aspects of the nanosystems, pros and cons based on the synthesis/preparation and the practicality, and applications. * The abbreviations are expanded at the 'Abbreviation' session.

Type	Name	Preparation Technique	Fluorine Component	Characterisation *	Pros and Cons	Application	Ref
POLYMERIC	Fluorous colloidal NPs	Copolymer by ATRP. NPs formation by self-assembly to micelle	Trifluoroethyl methacrylate	DLS (260 nm), TEM, FMRI, FC, CM, UV-Vis, CyA-on macrophage cells, animal studies–female athymic NCR nude mice for breast cancer	Simple preparation of copolymer No surfactant Little off target accumulation Tumour-homing	Immune cell tracking and systemic disease monitoring	[187]
	Poly(OEGA- <i>co</i> -TFEA)- <i>b</i> -poly(<i>St-co</i> -VBA)	Polymerisation by RAFT and NPs by PISA	2,2,2-trifluoroethyl acrylate	FMRI and NMR, DLS, TEM, CM	Little or no cytotoxicity–Chinese Hamster Ovarian cells Multiple NPs morphologies by controlling reaction time and polymer chain length in one preparation (spherical, worm, vesicle)	In vivo cell tracking	[190]
	ROS-responsive fluorinated polymers	Polymer by ATRP and NPs by self-assembly	2,2,2-trifluoroethyl methacrylate	H and F NMR, FMRI, DLS (62, 32 and 18 nm), UV-Vis	Enhanced sensitivity for acidic microenvironment and the presence of ROS The concentration of H ₂ O ₂ studied (~1 M) were higher than biological levels (50–100 μM) 6-step synthesis that requires purification “OFF–ON” regulation of NPs to acidic environment	ROS/pH dual-responsive ¹⁹ F MRI agent	[191]
	Amino activable nanoprobe-	Copolymers by RAFT polymerisation and		H and F NMR, DLS (33 nm), FMRI, TEM, FTIR,	2 step preparations for monomers	In vivo bio-thiols imaging	[192]

p(mPEGMA)- <i>co</i> -poly(AMA-DNBS-F) (PEDF nanoprobe)	nanoprobe by nanoprecipitation	Trifluoromethyl-containing segments	CLSM, in vivo imaging in tumours–xenograft tumour models in mice	Highly sensitive to bio-thiols Water soluble		
Fluorinated block copolymers NPs	RAFT for the block polymers and NPs by self-assembly in aqueous solution	2,2,2-trifluoroethylamide L-arginine methacrylamide	H, F- NMR, DLS (25 to 60 nm), TEM	Fluorinated functionalities in the hydrophilic shell Increased T_2	MRI Imaging	[300]
^{19}F MRI-detectable drug delivery system	Layer-by-layer technique deposition of polyelectrolyte shells on nanoemulsion drops	Polyelectrolyte Nafion–fluorinated anionic polymer	DLS (170 nm), LDV, NTA, C-SEM, QCM, FMRI	Sufficient SNR ratio Highly cationic particle (+ targeting and drug 68 ± 5 mV)	Passive tumour delivery	[193]
Self-assembled ^{19}F nanoprobe	Self-assembly of amphiphilic redox-responsive ^{19}F -containing polymers and NIR-absorbing ICG molecules	Bis(trifluoromethyl) benzoic acid part in the polymer	TEM, DLS (40 nm), UV–Vis, FNMR and MRI, TEM	Water-soluble	Accurate sensing and imaging of tumours	[66]
				In vivo and in vitro studies–HepG2 tumour-bearing cells and mice		
				High SNR ratio		
				Good biocompatibility		
				5 steps for preparation with purification requirement and moderate yield		
Multi-functional fluorocarbon NPs	Single and double emulsion	PFD, PFH, perfluorooctane, PFOB, PFCE	DLS (200 nm–200 μm), SEM, CM, FC, FI, FMRI, Cell viability–primary human dendritic cells, histology	Novel system which has potential to be extended for imaging other tumour targets	In vivo imaging and targeting applications	[194]
				Customizable NPs, minimal toxicity Size smaller than 200 nm is not formed by this NP formation		
PLGA PFPE	Emulsification (Sonicator)–1:1 molar	PFPE	DLS (103 nm), FNMR and MRI, FM, cellular	Specificity for the labelled cells	Non-invasive monitoring the	[195]

ratio of autoclaved PFPE and sterile filtered Pluronic		viability–diabetogenic mice T cells		Reliable estimates of the apparent number of cells from image data	trafficking of cellular therapeutics
PFCE encapsulated PLGA	Single emulsion	PFCE	DLS, FNMR and MRI, SANS, animal studies– male Wistar rats, mouse, mice, cell studie–primary murine/human dendritic cells	Biocompatible NPs	US and ¹⁹ F MRI [197]
				Better acquisition time	Murine cardiac ¹⁹ F MRI/MRS [199]
				Obtains complimentary information when in combination with other imaging agents	In vivo PAI, ¹⁹ F MRI and fluorescent imaging (FI) [198]
				NPs loaded with chemotherapeutic drugs could give it a theranostic effect, Resomer RG 502 H, lactide: glycolide molar ratio 48:52 to 52:48 is the mostly used PLGA. The other ratios of lactide: glycolide and also their end group might give interesting results. The encapsulation efficiency of PFC could be studied each time to better understand the sensitivity	FMRI and CT (with gold NPs) [200]
					SPECT/PET and ¹⁹ F MRI [204]
Chitosan coated PLGA -PFOB NPs	Single emulsion by homogenisation followed by sonication using 1.5% sodium cholate	PFOB	DLS (170 nm), CLSM, FC, FNMR and FMRI, TEM	Background-free signal compared to Gd (III) and super paramagnetic iron oxides NPs As chitosan coating is just a physical adsorption, the stability of it has to be verified in biological environment	Labelling and tracking therapeutic cells in vivo [206]

HYPERBRANCHED					Size of NPs is increased (200–400 nm) after the chitosan coating	In vivo imaging	[212]
	PEGylated PLGA NPs (PLGA NP (NIR700 + PFC)-PEG-800 CW)	O/W emulsion and solvent evaporation-extraction method	PFCE	DLS (240–250 nm), TEM, FMRI, TEM, FM, histology, cell culture–murine breast carcinoma cell line	Quantitative 3D information from deeper tissues Rapid qualitative optical monitoring		
	PLGA–PEG folate-receptor-targeted NPs	Single emulsion-evaporation (1.5% sodium cholate surfactant)	PFOB	DLS (150 nm), FC, CLSM, F MRI, NIRS, CyA-KB cells	Encapsulate imaging agent and drug Insufficient SNR in vivo for FMRI The loading capacity of the NPs is low for doxorubicin and ICG (0.04% and 0.127%)	Theranostic NP	[207]
	Doxorubicin-conjugated PFPE NPs	Polymers by RAFT polymerization	PFPE	DLS (8.1, 9.3 and 8.3), FNMR, MD	Improved cellular uptake Deep tumour penetration Studies done using 3D tumour spheroids	Improved therapeutic efficacy	[222]
	F ₃ -PLGA and F ₉ -PLGA	Nanoprecipitation–surfactant free	Fluorinated PLGA (2,2,2-trifluoroethanolamine, nonafluorobutoxyethylamine)	DLS (~54 nm and 58 nm), TEM, F NMR, FM, CyA–immortalized human glomerular endothelial cells and podocytes	No surfactant used Encapsulate hydrophobic drugs The reaction yield of the fluorinated polymer is not understood	Theranostic NPs	[217]
	Multifunctional hyperbranched polymers containing ¹⁹ F	RAFT polymerization for polymer, NPs by self-assembly in water	2,2,2-trifluoroethylacrylate	DLS (~13 nm), GPC, TEM, FNMR and MRI, CT	Direct dissolution in water Biodegradable 3 step preparation and the final product is not pure (3 mixture products) FNMR with multiple peaks	Quantitative ¹⁹ F MRI CA	[232]

DENDRIMERS					T_2 shortened		
	PFPE based hyperbranched NPs conjugated with targeting aptamers	RAFT polymerization–for NPs, click chemistry for aptamers attaching	PFPE	F-DOSY (<10 nm), FM, FC, CrM, MD, FNMR and MRI	Superior MR imaging sensitivity and fluorine content -breast cancer cells Low-cost fluorescence imaging	Quantitative ^{19}F MRI CA	[233]
					Unsuitable for long term studies due to faster clearance from the body Accumulation of polymer in the liver was observed after 48 h and the ^{19}F signal could be still detected in the liver		
	Fluorinated hyperbranched polyether copolymers	ROMBP and copolymerization for polymers and self-assembly of the colloids	2-[(2,2,2-trifluoroethoxy)methyl]oxirane/epifluorohydrin	DLS (160–200 nm), H NMR and F MRI, FM, HPLC, cytotoxicity studies - immortalized human glomerular endothelial cells and immortalized human podocytes	Repair damaged kidney glomerular cells in vitro Negligible cytotoxicity Narrow size distribution Relatively long T_1 Higher amount of F gives less SNR	New generation ^{19}F MRI nanotheranostics	[234]
	Fluorinated Gd(III)-DOTA complexes	Convergent synthesis for polymer and self-assembly for NPs	Fluorinated amino acid group	F NMR, DOSY, H and F MRI, KB cells for in vitro cytotoxicity study, animal imaging—Sprague Dawley female rats	Substantial improvement in relaxation rate and SNR ratio Easily cleared through the kidneys The fluorine in the surface layer of dendrimers is toxic which can be diminished by burying the fluorine further into the dendrimer interior	CA for high field imaging	[239]
				FNMR	Higher number of equivalent fluorine than	Probes for ^{19}F MRI	[240]

NANOHYDROGELS	Second-generation dendron	Sonogashira coupling, alkyne deprotection and CuAAC	PFTB group attached to the dendron		commercially available ^{19}F MRI probes Too unpolar to be water-soluble Just one characterisation technique used		
	Pseudo-symmetrical fluorines dendrimers	Polymer prep-bromination and Williamson ether synthesis, NPs by self-assembly	Bis(4-fluorophenyl) trifluoromethyl carbinol group	FNMR and MRI	Large amount of fluorine with a single NMR peak Optimize ^{19}F relaxation time High sensitivity Reliable quantification Comparatively low yield (8%) for 11 synthesis steps	^{19}F MRI-guided drug therapy	[241]
	Self-assembled fluorinated amphiphiles	Convergent way–Sonogashira coupling and Williamson ether synthesis for polymer, NPs by self-assembly	Fluorinated benzyl group	FNMR, DLS (6.3 nm), TEM	Quantifying drugs, detecting drug microenvironments and weak interactions Several synthetic step for the preparation with most of them requiring separation	^{19}F NMR/MRI guided drug therapy.	[242]
	Chitosan	Ionic gelation using hyaluronic acid and tripolyphosphate	4,4,4-trifluorobutyric acid	DLS (274 nm), ELS (+30 mV), FNMR (–66 ppm), HNMR, TGA, DOSY, IR	Good biocompatibility toward murine macrophages cell line Degree of substitution is comparatively low (0.3% and 20%) and varies between different substitutes, and determination is laborious	Chitosan drug delivery systems for MRI lymphography	[247]
	Diblock polymers	Self-assembly by heating in aqueous solution	Poly[N(2,2-difluoroethyl)acrylamide]	SLS (100 and 67 nm), TEM, C-TEM, FNMR	Good sensitivity Non-cytotoxic for several cell lines	^{19}F MR imaging–angiogenesis imaging or the	[248]

LIPIDS					Long synthesis steps for preparation of polymers	labelling of pancreatic islets	
	Fluorinated amphiphilic polymers	Self-assembly of polymers–direct dissolution of amphiphilic polymers in PBS buffer	-CF ₃ groups attached to the chains of polymer	DLS (6- 14 nm), FNMR, FMRI–phantom and animal imaging, CM, CyA-HeLa cells	Enhancement in T_2 relaxation times by increasing the segment mobility	Multimodal imaging and therapeutic applications.	[249]
	Superhydrophilic ¹⁹ F MRI CA	Hydrogel matrix attached to zwitterionic, fluorinated and alkynyl molecule by click chemistry	The fluorine atoms on trifluoromethyl groups	HMRS, FTIR, GPC, CD, Rheometer, SEM, FMRI, degradation study–female BALB/c mice, CyA-Dendritic cells, NIH 3T3 cells	Gelation properties of hydrogels unaffected by labelling CA	Real-time FMRI to precisely locate and quantify the degradation rate of hydrogel scaffolds in vivo	[250]
	Antigen-loaded PFC particles	High-frequency mixing of the liquid PFC with a cationic lipid mixture-particles coat with PEG	PFH or PFCE	CM, TEM, F NMR, Cytotoxicity in transplanted pancreatic islets and beta cell-like cells and T-cell proliferation assay	Improving pancreatic islets transplantation technique Good cell viability and no change in cells' phenotypical properties High resolution localization of transplanted cells	Theranostic PFC NPs	[255,256]
	Thermally responsive lipid nano-emulsion	Nano-emulsion	Modified α -tocopherol	FNMR, DLS (50 nm), ZP	The use of PFCE is better than PFH because the latter have 3 peaks in FNMR which reduces its sensitivity Proved that T_2 changes more than T_1 due to variation in temperature for FNMR The temperature studied is extreme (37 and 42 °C) compared to real tumour	Potential tumour diagnosis	[258]

MICELLE	Multifunctional paramagnetic PFC NP	Microfluidization	PFCE	DLS (132 nm), AFM, UV-vis, FM, cellular toxicity on bronchial epithelium, FC, clinical pathology, FMRI	Enhanced intratumoural penetration NPs stored under very special condition The lipid surfactant used have a laborious preparation The studied NPs contain Gd ³⁺ as Gd-lipid chelates	PFC NP delivery from intravenous applications to intratracheal use (for lung cancer)	[259]
	Fluorinated thermoresponsive assembled protein (F-TRAP)	Self-assembly micelle	Fluorinated amino acids within a protein (5,5,5-DL-trifluoroleucines)	DLS (30 nm), FA, SLS, CD, MALDI-TOF-MS, TEM, turbidometry, FNMR, FMRI, Animal studies-mouse xenograft model of human breast cancer	No change in T_1 Doxorubicin encapsulation and thermoresponsive release Zero echo time ¹⁹ F MRI was used to get the direct imaging of protein as after micelle formation, there is a reduction in T_2 The release of drug is at 45 °C (usually tumour temperature range is 37 °C to 39 °C)	Thermoresponsive ¹⁹ F MRI/MRS-traceable theranostic agents	[260]
	Gold NPs protected by fluorinated ligands (F-MPC)	Homogeneous phase synthesis	Fluorinated tetraethylene glycol part of the ligand	DLS (10 nm), TEM, HAADF-STEM, FNMR, UV-Vis, ESR, CLSM, cell interaction with HeLa cells	Elimination of the use of surfactants Size may help to reach small vasculature vessels Soluble in many organic solvents The preparation of fluorinated ligands contains 6 steps, most of them requiring purification Water-soluble	Nanovector	[266]
INORGANIC						Dual ¹ H/ ¹⁹ F MRI	[267]

Functionalized gold NPs	Homogeneous phase synthesis	Fluorinated tetraethylene glycol part of the ligand	DLS, HNMR, TEM (1.5–2 nm), UV-Vis, FNMR and MRI	<u>Good quality MRI images</u> Same as ref [266] + Gd(III) is embedded deep in the layer of Au NPs that causes reduction in T_1 relaxation times of bulk water proton		
Gold NP functionalised with fluorine atoms	Reduction of HAuCl_4 in the presence of NaBH_4	PFTB	ICP-MS, F-NMR/MRI, UV-Vis, TEM, Cell viability and apoptosis assays - MDA-MB-231, C33-A and MDA-MB-435S cell lines, MTS CyA	<u>Colloidal stability in water and other solvents</u> <u>Single chemical shift</u> <u>Long storage</u> <u>High fluorine loading</u> Long preparation and purification procedure for the fluorine ligands The position of fluorine in the NPs is not established	^{19}F MR imaging	[268]
Hollow mesoporous silica NPs (HMSN-PFCE)	Modified protocol from [301]	PFCE	DLS, SEM (290 nm), TEM, MRI, NMR, PAGE	<u>Prolonged circulation time</u> Helps in understanding the effect of loading agent on the biodistribution of NPs <u>Better biodistribution of NPs</u> The study is majorly applicable to systems whose cargo is on the outer surface	Dual MRI (^1H and ^{19}F)	[270]
Fluorinated mesoporous silica NPs (FMSNs and polyFMSNs)	Repeated impregnation-calcination process	Fluorosilane or polyfluorosiloxane	TGA, TEM (140 nm), DLS, FNMR and MRI, XPS, relaxometric properties	<u>Colloidal stability</u> <u>Increase in ^{19}F relaxivities</u> <u>Meticulous NPs preparation</u> Contains Gd^{3+}	Dual MRI (^1H and ^{19}F)	[273]

				The detection of probe might be impeded by the strong reduction of T_2 after NPs formation		
				High sensitivity		
				Water stability		
PEG modified silica NP	Dehydration polymerizing reaction–PFCE including micelle as a platform	PFCE	TEM, DLS (50 nm), $^1\text{H}/^{19}\text{F}$ MRI	Information on long term stability, encapsulation efficiency of PFCE is deficient	Tumour imaging	[274]
				High sensitivity		
				Modifiability of the surface, biocompatibility		
				In vivo stability		
Silica multifunctional core-shell NPs (FLAMEs)	PFCE-phospholipid nanoemulsion by sol-gel process using a novel surfactant, PAP	PFCE	DMS (76 nm), F NMR and MRI, TEM, biocompatibility by MTT assay-colon-26 cells, Passive targeting, and accumulation- mice bearing a tumour	The FLAME NPs needs to be PEGylated as naked NPs is trapped immediately by the RES The information on long term stability of NPs is lacking	Detection of gene expression and in vivo tumour imaging	[277]
				Ample cellular uptake and drug release in folate receptor-overexpressing tumour cells		
Mesoporous FLAME (mFLAME)	PFCE emulsion by Sol-gel process	PFCE	DLS (165 nm), TEM, FNMR and MRI, CLSM, FC, MTT CyA-KB cells, FM	Drug release abilities at lower pH. (pH 5)	Theranostic cancer treatment	[278]
				Efficient tumour cell internalization		
Gd ³⁺ complexes on FLAME NPs surface (FLAME-SS-Gd ³⁺)	Gd ³⁺ complexes were attached to the FLAME surface by disulfide linkers	PFCE	DLS (53.4 nm), FNMR and MRI, ICP-AES	Smart nanoprobe-based on PRE effect In vivo imaging High SNR ratio	Novel ^{19}F MRI probes that visualize reducing environments	[279]

PFC based ^{19}F MRI nanoprobes (PFC@SiO ₂ , FLAME)	PFC emulsion by sol-gel process	PFCE, PFOB, FC-43, PFN, PFDCO, TPFBME	DLS, TEM (40–120 nm), FI-RAW264.7 cells, H MRI and FMRI, hepatic uptake in mouse	T_2 values -relatively longer than polymer-based or inorganic ^{19}F MRI nanoprobes In vivo triple-colour ^{19}F MRI The shelf-life information is lacking for the NPs	Multicolour MRI probes	[281]
Fluorinated paramagnetic CAs	Multistep synthesis–cycloaddition reaction	Nonafluorinated carboxylic acid	FNMR, relaxivity measurements, MD	Relaxation times depending on the lanthanide ion Low solubility in aqueous media	^{19}F MRI	[35]
Hexagonal-phase NaGdF ₄ :Yb ³⁺ /Tm ³⁺ NPs	Hydrothermal method	NH ₄ F/NaF	XRD, SEM, EDX, UV, photoluminescence spectra, EPR	Conductive to the UV light Good water solubility Lanthanide-based upconversion NPs	IR tomography and MRI	[261]
Inorganic nanocrystals-PEG-coated CaF ₂ nanocrystals	Solvothermal approach	CaF ₂	H and C and F-NMR, DLS (<10 nm), TEM, XRD, EDX, FTIR, TGA, mouse model of inflammation	Maximal ^{19}F density Average out homonuclear dipolar interactions Direct and real-time in vivo ^{19}F MRI Chemically surface modifiable Long T_2	Imaging tracers for in vivo ^{19}F MRI	[263]
Halloysite nanotubes-benzenboronic acids (HNTs-6FBB)	One-pot synthesis	3,5-bis(trifluoromethyl) benzenboronic acid	FNMR (–60 ppm), XRD, FTIR, XPS, TEM, EA (0.31% F)	Relatively long T_2 Water dispersibility Detection of H ₂ O ₂ is based on a very minute shift in FNMR (0.2 ppm) Low cell cytotoxicity	Selective response toward H ₂ O ₂	[285]
MIXED/HYBRID Fe(III) tris- β -diketonate with PFPE ('FETRIS')	Microfluidization –metal-binding β -diketones	PFPE and PFPE derivatives, PFOB	DLS (140 nm to 200 nm), FNMR and FMRI, cell	Ability to tune T_1 by Fe concentration Low cytotoxicity	In vivo detection of cell therapies	[288]

	conjugated to PFPE using pluronic surfactant		labelling-rodent glioma cell line	Small rates of metal leakage in the presence of EDTA in vitro and after cell labelling	and inflammatory cells	
				<u>Ultrahigh F signal</u> <u>Biocompatible</u> Capable of both in vivo imaging (F-MRI) and photothermal ablation <u>Presence of excess of metals in a single probe!</u> The degradation of this complex should be evaluated since without the SiO ₂ coating it is cytotoxic	Ablation and sensitive multimodal imaging	[290]
Cu _{1.75} S- ¹⁹ F@OFP-SiO ₂	One-pot encapsulation method-PFCE anchored to Cu _{1.75} S NPs and trapped within the silica shell	PFCE	DLS (20.8 nm), TEM, FMRI, PTT			
				<u>High imaging intensity</u> <u>No surfactants</u> The yield for the formation of star polymers is low and extreme conditions for preparation	Theranostic agents for cancer diagnosis and treatment	[291]
Fluorinated POSS-star polymers	Synthesis of star polymers by RAFT polymerization and polymer formation in water	2,2,2-Trifluoroethyl acrylate in the ligands attached to POSS	DLS (8–10 nm), FNMR and FMRI			
Hybrid of fluorinated graphene oxide and iron oxide (IFGO)	Graphene oxide-Hummer's method. Hybrid-co-precipitation	Fluorinated graphene	DLS (8–10 nm), FMRI, XRD, XPS, SEM and HRTEM, FTIR, MTT CyA-benign breast epithelial cell line, Raman, UV-Vis, hysteresis	<u>Additional imaging modality-magnetic targeted drug delivery</u> Increased magnetic saturation-better contrast	Superior CAs for MRI and fluorescent imaging	[292]
Cu ₇ S ₄ -Au heterodimer Cu ₇ S ₄ -Au@PSI- ¹⁹ F/PEG nanocomposites	Wet-chemical method for Cu ₇ S ₄ -Au nano seeds followed by click chemistry	2,2,2-trifluoro-N-2-propyn-1-yl-acetamide	DLS, HRTEM (27 nm), XRD, EDX, HAADF-STEM, XPS, STEM, F NMR and MRI, CT, cell viability-4T1 cell lines, PTT-liver of female mice	<u>Deep penetration</u> <u>High spatial resolution</u> <u>Enhances the photothermal efficacy</u> Long preparation for the nanocomposite	Multimodal imaging guided photothermal therapy	[293]

Mn-LDH@PFPE NPs	Composite system by conjugating a PFPE onto the surface of manganese-incorporated layered double hydroxide	PFPE	NMR and MRI, DLS (10 nm), TEM, GPC, CM, MTT assay–MDA-MB- 468 breast cancer cells, histopathologic examination	High specificity to breast cancer cells	Potential “smart” ¹⁹ F MRI agent for detection of cancer diseases	[297]
Fe ³⁺ @F,N-CD (fluorine and nitrogen co-doped carbon dot)	Simple microwave-assisted thermal decomposition method–from glucose and levofloxacin	Levofloxacin	DLS (16 nm), TEM, GPC, FTIR, XPS, FM, ESR, cytotoxic studies–HeLa cells, In vivo experiments - 4T1 tumour bearing BALB/c mice, FMRI, CLSM	High T_1 relaxivity Strong photoluminescence Low synthetic cost Low toxicity Cannot be used for long term imaging in the body as they are excreted in a very short time from the body	T_1 -weighted MRI CA	[298]

6. Conclusions and Future Perspectives

Even though we see massive progress in the field of ^{19}F MRI with fluorinated compounds, and in particular with PFCs, its clinical translation requires an in-depth understanding of their exact behavior from its intake until its complete degradation. PFCs are atypical molecules and overcoming specific barriers can shorten the distance to its clinical reality. Even though emulsion is one of the most used techniques to encapsulate PFC compounds, there are still various shortcomings that must be subjugated, including instability, heterogeneity, complex formulation procedure, split ^{19}F signals, excessive retention of the agent within organs for months or longer are some of them [226]. The bottleneck attributes for PFC compounds are that they are immiscible with water or lipids, besides their organ retention and inefficacious chemical modification. It is simultaneously intriguing as well as exciting. These will open new approaches to drug delivery besides making scientists think differently. It is quintessential to comprehend the degradation of PFCs at extreme (pH/temperature/anoxic/hypoxic) conditions and the effects on high doses of PFCs as the knowledge of this is scarce considering PFCs are not naturally occurring compounds. Storage conditions need to be ascertained as long-term storage of PFC NPs remains a persisting setback.

Reviewing the recent works on nanosystems using fluorinated ligands and PFCs for ^{19}F MRI, we see a prodigious stride in our understanding of formulation of the nanosystems and bringing novel ways of producing ^{19}F signals. Not restricted to the fact that PFC is a unique liquid, the expedient means explored to encapsulate PFCs, above and beyond uncovering ways to produce molecules with a strong and single ^{19}F signal, have been remarkable. The frequently confronted issues when attempting to make molecules equal to or better than PFCs is the complexity of the molecules' preparation itself, in addition to the required number of synthetic steps, utilization of organic solvents, purification contingency, and reproducibility of the same. We can always see that the products that ultimately manage to pass to clinical stages are straightforward and, typically, would not require complex synthetic skills. One of the concerning issues is the usage of Gd^{3+} yet in these nanosystems, especially for making hybrid $^1\text{H}/^{19}\text{F}$ MRI. The unreserved fact that Gd has a plethora of advantageous properties cannot be denied. An effectual CA from another element can pave the way for unexplored possibilities and conceivably higher quality images.

The aerial perspectives for the future use of PFCs in an emulsion would be to experiment with the use of fluoro-surfactants instead of commonly used surfactants [302], to find a carrier system that has an affinity to PFCs and could hold PFCs inside with some interactions, to explore the therapeutic effect of PFCs, to make PFCs more hydrophilic by modifying them or to make them less hydrophobic so that they could be soluble in an organic solvent and to study the effect of using more than two PFCs concurrently. Similarly, one of the emerging and compelling nanosystems would be to combine different classes of NPs to produce a complementary system with a synergic effect. It is like a jigsaw puzzle where pieces are fit together to create a serendipitous combination.

The anticipated challenges faced when the chemical modification is performed, as already pointed out, is the usage of organic solvents, the increase in the number of reaction steps that decreases the overall yield

of the final compound, which can hinder its progress to the clinic. Formulating a synthesis/preparation towards a 'green' approach can make its way to the clinic swifter. PFCs have some inimitable exclusive characteristics, and not only could this be utilized in imaging but also in other research areas like cell tracking, ^{19}F -oximetry, inflammation probing, etc. Some of the PFC-based compounds are already FDA-approved for ultrasound-based CAs and it is only a matter of time before discovering the most befitting CA for ^{19}F MRI, which would open a floodgate of applications.

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Abbreviations

^{19}F MRI/fMRI	Fluorine-19 Magnetic Resonance Imaging
^{19}F NMR	Fluorine-19 Nuclear Magnetic Resonance
^1H MRI/HMRI	Proton Magnetic Resonance Imaging
AFM	Atomic Force Microscopy
ATRP	Atom Transfer Radical Polymerization
BALB/c	Albino, laboratory-bred strain of the house mouse
BODIPy	Boron-dipyrromethene
C NMR	Carbon-13 Nuclear Magnetic Resonance
CA	Contrast Agent
CD	Circular Dichroism Spectroscopy
CLSM	Confocal Laser Scanning Microscopy
CM	Confocal Microscopy
CrM	Correlation Microscopy
C-SEM	Scanning Cryo-Electron microscopy
CT	Computed Tomography
C-TEM	Cryo-Transmission Electron Microscopy
CuAAC	Copper-Catalyzed Azide-Alkyne Cycloaddition
CyA	Cytotoxicity Assays
MTT Assay	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay
DC	Dendritic cells
DLS	Dynamic Light Scattering
DOSY	Diffusion Ordered 2D-NMR Spectroscopy
EA	Elemental Analyzer
EDX	Energy-Dispersive X-Ray Spectroscopy
EGDMA	Ethylene Glycol Dimethylacrylate
ELS	Electrophoretic Light Scattering
EMA	European Medicines Agency

EPR	Electron Paramagnetic Resonance Spectrum
FA	Fluorescence Anisotropy
FC	Flow Cytometry
FDA	The Food and Drug Administration
FDK	Fluorinated β -diketones
Fe ₃ O ₄	Iron Oxide/Magnetite
FI	Fluorescence Imaging
FITC	Fluorescein Isothiocyanate
FLAME	Fluorine Accumulated Silica NP for MRI Contrast Enhancement
FM	Fluorescence Microscopy
FTIR	Fourier Transform Infrared Spectroscopy
GBCAs	Gadolinium Based Contrast Agents
Gd(III)/Gd ³⁺	Gadolinium-III
GPC	Gel Permeation Chromatography
HAADF-STEM	High-Angle Annular Dark-Field Scanning Transmission Electron Microscopy
HBIPF	Hyperbranched Iodopolymer Containing ¹⁹ F
HepG2 Cells	Hepatocellular carcinoma cells
HNMR	Proton Nuclear Magnetic Resonance
HNT	Halloysite Nanotube
HPLC	High-Performance Liquid Chromatography
HRTEM	High Resolution Transmission Electron Microscopy
ICG	Indocyanine Green
ICP-AES	Inductively Coupled Plasma Atomic Emission Spectrometry
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
KB cells	Human Nasopharyngeal Epidermal Carcinoma
LDV	Laser Doppler Velocimetry
LSPR	Localized Surface Plasmon Resonance
MALDI-TOF-MS	Matrix-Assisted Laser Desorption Ionization-Time-Of-Flight Mass Spectrometry
MD	Atomistic Molecular Dynamic Simulations
mPEG	Monodisperse Poly (ethylene-glycol)
MR	Magnetic Resonance
MRI	Magnetic Resonance Imaging
MRS	Magnetic Resonance Spectroscopy
NIR	Near Infrared
NIRS	Near Infrared Spectroscopy and Imaging
NMR	Nuclear Magnetic Resonance
NP	Nanoparticle
NTA	Nanoparticle Tracking Analysis
OEGA	Oligo(Ethylene Glycol) Methyl Ether Acrylate
PAGE	Polyacrylamide Gel Electrophoresis
PAI	Photoacoustic Imaging
PEG	Poly (ethylene-glycol)
PEGMA	Poly-(ethylene glycol) methyl ether methacrylate
PET	Positron Emission Tomography
PFC	Perfluorocarbon
PFCE	Perfluoro-15-crown-5 ether
PFDCO	Perfluorodichlorooctane
PFOB	Perfluorooctyl Bromide
PFP	Perfluoropropane
PFPE	Perfluoropolyether

PFTB	Perfluoro-tert-butanol
PLGA	Poly (lactic-co-glycolic acid)
PRE	Paramagnetic Relaxation Enhancement
PTT	Photothermal Therapy
QCM	Quartz Crystal Microbalance
RAFT	Reversible Addition–Fragmentation Chain-Transfer Polymerization
RF	Radiofrequency
ROMBP	Ring-Opening Multibranching Polymerization
ROS	Reactive Oxygen Species
SANS	Small Angle Neutron Scattering
SEC	Size Exclusion Chromatography
SEM	Scanning Electron Microscope
SLS	Static Light Scattering
SNR	Signal/Contrast-to-Noise Ratio
SPECT	Single-Photon Emission Computed Tomography
STEM	Scanning Transmission Electron Microscope
TAM	Tumour-associated macrophages
TEM	Transmission Electron Microscope
TFEA	2,2,2-trifluoroethyl Acrylate
TGA	Thermogravimetric Analysis
TM	Turbidometry
TPFBME	1,1,1-tris(perfluorotert- butoxymethyl)ethane
UC	Upconversion
US	Ultrasound
UV–Vis	UV/Vis Absorption Spectra
XPS	X-Ray Photoelectron Spectroscopy
XRD	X-Ray Diffraction
ZP	Zeta Potential

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Chapter 3

Article

The Art of Preparations of PFCE Encapsulated PLGA Nanoparticles : Formulation and optimisation using High Speed Homogenization and Ultrasonication

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Abstract

Perfluorocarbons (PFCs), known for their dual hydrophobic and lipophobic properties, present challenges in intravenous administration, necessitating stable dispersions of PFC by emulsions or encapsulation into nanosystems. This study explores the encapsulation of PFCs, specifically perfluoro-15-crown-5-ether (PFCE), into biocompatible nanocarriers, aiming to develop them as contrast agents for fluorine magnetic resonance imaging (^{19}F -MRI). Polylactic-co-glycolic acid (PLGA) polymers, renowned for their biodegradability and numerous Food and Drug Administration (FDA)-approved formulations for clinical use, are employed in the encapsulation of PFCE due to their proven safety and efficacy, facilitated the development of a PFC-nano delivery system via the emulsion solvent evaporation technique. We investigated the physicochemical properties of PFCE-PLGA nanoparticles (NPs) formulated via homogenization, sonication, and a hybrid of both. The study examined the influence of molecular weight (MW) variations in PLGA (MW 7,000-17,000 Da & 24,000-38,000 Da) and polyvinyl alcohol (PVA) surfactants (9,000 -10,000 Da & 13,000 – 23,000 Da) on the NPs' characteristics when different technique of preparation is applied on them. The resulting NPs exhibited sizes ranging from 150 nm to 900 nm, polydispersity indices (PDI) between 0.05 to 0.5, zeta potentials (ζ) from -15 to -33 mV and loading capacities (LC) from 5 wt/wt%.

The findings reveal that the formulation technique and polymer-surfactant MW combination significantly influence NP size, PDI, ζ , and LC. Homogenization produced the largest, most variable NPs, while sonication yielded intermediate sizes with the highest LC, indicating efficient PFCE encapsulation. The hybrid technique generated the smallest, most uniform NPs but with variable LC and ζ . Structural analyses via Scanning Electron Microscopy (SEM) and confocal microscopy confirmed spherical morphology and distribution of PFCE respectively. Cytotoxicity assays demonstrated the non-toxicity of the NPs, and investigations into the protein corona were conducted using bovine serum albumin (BSA) and human plasma to understand their interactions and implications. The study underscores the importance of technique selection and material MW in designing NPs, offering insights for optimizing NP-based drug delivery systems.

1.Introduction

Perfluorocarbons (PFCs) are organic compounds with carbon-fluorine bonds instead of carbon-hydrogen bonds. They have remarkable properties, such as high oxygen and carbon dioxide solubility, low toxicity, and high thermal stability, *etc.* [1]. These properties enable their use in various biomedical applications, which include but are not limited to blood substitutes, artificial oxygen carriers, and contrast agents for Magnetic Resonance Imaging (MRI). One such application is fluorine-19 Magnetic Resonance Imaging (^{19}F MRI), which employs the Fluorine-19 (^{19}F) nucleus as a contrast agent for imaging. ^{19}F MRI is a non-invasive and quantitative molecular imaging technique that has several advantages over conventional proton (^1H) MRI, such as high sensitivity, low background signal, and multiplexing capability. It can monitor various biological processes, such as cell tracking, inflammation, tumour detection, oxygenation measurement, *etc* [2, 3]. Some of the most used PFCs for clinical studies are perfluorooctyl bromide, perfluorodecalin, perfluoro-15-crown-5-ether (PFCE), perfluorinated probe PERFECTA, perfluoropolyethers to name a few [3]. PFCE has been utilized in these studies due to its structure, which contains 20 fluorine atoms within a cyclic perfluoroether framework, ensuring clear and unambiguous detection [4].

However, ^{19}F MRI has some challenges. Fluorine is less abundant than proton, so it needs a higher concentration of fluorine-containing compounds to produce a detectable signal in MRI. Additionally, PFCs exhibit both hydrophobic and lipophobic properties, which prevent their direct injection into the human body for medical imaging purposes due to the formation of a distinct fluorine phase that is insoluble in both aqueous and organic solvents. One way to overcome these challenges is by using nanocarriers, specifically polymeric nanoparticles (NPs) like poly(lactic-co-glycolic acid) (PLGA), to

encapsulate PFCs to form PFC-nano-delivery system. PLGA is a biodegradable and biocompatible polymer widely used in drug delivery systems. PLGA is composed of lactic and glycolic acids, which are natural metabolites produced in the human body, ensuring its safe biodegradation.

Extensive research has demonstrated the successful encapsulation of PFCs within PLGA matrixes. An important use of PLGA is in the micro- and nano-encapsulation of PFCs, which act as contrast agents in ultrasound imaging techniques [5-8]. The encapsulation process prefers low-boiling-point PFCs for their superior contrast enhancement during the phase change from liquid to gas, and utilizes a thin PLGA shell to optimize acoustic properties [9]. Modified PLGA variants have been explored for PFC encapsulation, including erythrocyte membrane-coated PLGA for hypoxia [10], PEGylated PLGA for cell reoxygenation enhancement [11], trifluoroacetamide-modified PLGA for osteoarthritis imaging [11], and epidermal growth factor-modified PLGA for investigating anti-tumor efficacy in colon cancer [12]. A substantial body of research has been devoted to the utilization of PLGA as a carrier for PFCE encapsulation, serving a spectrum of purposes from contrast enhancement in various imaging modalities to cellular tracking and multimodal imaging techniques [13-18]. Despite this, the exploration into how the specific parameters of these carrier systems affect their physicochemical attributes—such as particle size/PDI, charge, and stability of contrast agent incorporation is sparse. Notably, Staal *et al.* [19] have shown that altering the PFCE distribution within the carrier from a core-shell to a multicore structure can significantly decrease the half-life from over 250 days to merely 16 days. While investigations like those conducted by Swider *et al.* [20] have shed light on the impact of different surfactants, their concentrations, and the choice of solvents and PLGA concentrations on the preparation of PLGA-PFCE systems, there is a clear opportunity for further research. The research presented in this paper illuminates the intricacies of nanoencapsulation within triphasic systems, which encompass an organic PLGA phase, a surfactant aqueous phase, and a PFC fluorine phase. Specifically, it provides an understanding of the influence of parameters such as the formulation technique and the molecular weight (MW) of the polymer & surfactant might furnish crucial insights that could aid in the advancement of this domain.

Our study explores three critical elements of PFCE-encapsulated PLGA NP formulation: (1) the formulation technique, (2) MW of the polymer, and (3) the surfactant. We evaluated their influence on size, PDI, ζ , and LC. Scanning electron microscopy (SEM) was employed to analyse the external morphology, and we attempted to visualize the distribution of PFC within the NPs using confocal microscopy. Furthermore, we conducted *in vitro* tests, including protein corona assessments with bovine serum albumin (BSA) and human plasma, on the NPs, with a particular focus on those formulated via various methods and cytotoxicity studies using fibroblasts cell lines. Finally, we measured the signal-to-noise ratio (SNR) using a phantom study in ^{19}F MRI.

2. Materials and Methods

2.1. Materials

PLGA (Resomer® RG 503 H, acid terminated, lactide: glycolide 50:50, Mw 24,000-38,000 and Resomer® RG 502 H, acid terminated, lactide: glycolide 50:50, Mw 7,000-17,000, Polyvinyl alcohol (PVA) (average Mw 9,000-10,000, 80% hydrolysed and average Mw 13,000-23,000, 87-89% hydrolysed), Nile Red, deuterium oxide, bovine serum albumin were purchased from Sigma-Aldrich. Dichloromethane (DCM, CH_2Cl_2 , Mw 84.93 g/mol) used to prepare the NPs, was obtained from CarloErba (Milan, Italy). The contrast agent-loaded into the NPs is perfluoro-15-crown-5-ether; 98% (PFCE) ($\text{C}_{10}\text{F}_{20}\text{O}_5$, Mw 580.07) and was supplied by abcr GmbH (Karlsruhe, Germany).

2.2. Preparation of NPs

2.2.1. Homogenisation

PFCE-encapsulated PLGA NPs were prepared using the PFCE/O/W double emulsion solvent evaporation method, with PLGA employed to achieve NP formation. The PFCE-loaded PLGA was formulated through a homogenization process, as depicted in Figure 1. Initially, a first emulsion was created by adding PFCE (900 μ l) dropwise into 3 ml of DCM containing 90 mg of polymer (30 mg/mL). This mixture was then homogenized using the Ultra-Turrax® T10 basic, S10N-5G (manufactured by IKA in Staufen, Germany), which features a two-blade rotor and a stator with six vertical, narrow rectangular slots. The homogenization was performed at 5,000 rpm for 20 minutes to form the PFCE/O in an ice bath to mitigate heat generation from the high-speed process. Following this, the PFCE/O was carefully pipetted into 25 ml of a 2% *w/v* PVA water solution and homogenized using the Ultra-Turrax® T25 digital, S25N-18G (also from IKA), which has a two-blade rotor and a stator with ten vertical, narrow rectangular slots. This second emulsion was conducted at a speed of 13,500 rpm for 20 minutes in an ice bath.

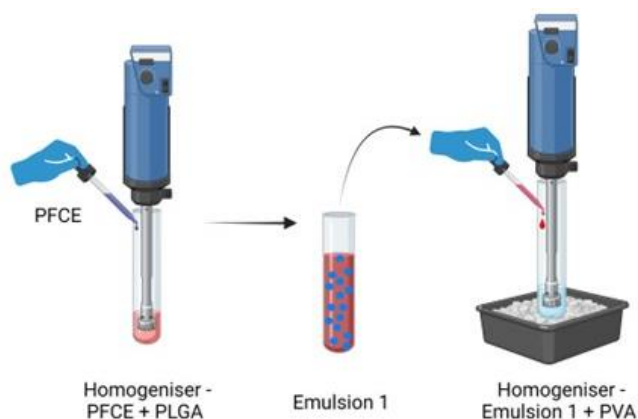


Figure 1: Double emulsion solvent evaporation method for PFCE-encapsulated PLGA NP formulation using high speed homogenisation

2.2.2. Sonication

PFCE encapsulated PLGA NPs for sonication was formulated by PFCE/O/W single emulsion solvent evaporation method as described in [20] with slight modifications. The process of formulating PFCE-loaded PLGA via sonication is depicted in Figure 2. In summary, a mixture of 900 μ l PFCE and 3 ml DCM, containing 90 mg of PLGA polymer (30 mg/mL), was manually stirred using a pipette to form the PFCE/O phase. This mixture was then incorporated into 25 ml of a 2% *w/v* PVA water solution and sonicated using a Sonicator (Qsonica, Newtown, CT, US) with a 6 mm sonicator tip for 3 minutes at 40% amplitude in an ice bath to create the PFCE/O/W phase. For the preparation of empty PLGA NPs (control), the initial PFCE/O emulsion step was omitted. In this case, only the PLGA was used, and the second O/W emulsion was created under the same conditions as previously outlined.

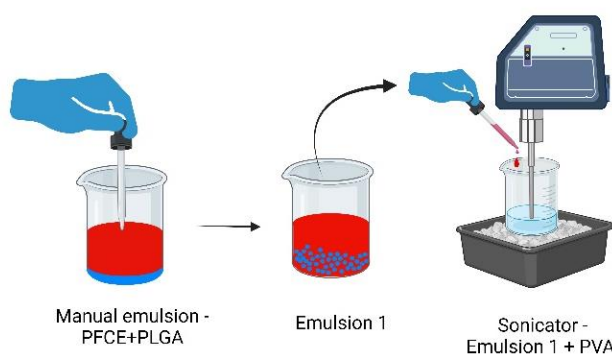


Figure 2: Formulation of PFCE-Encapsulated PLGA NPs via emulsion solvent evaporation method utilizing ultrasonic sonicator

2.2.3. Hybrid

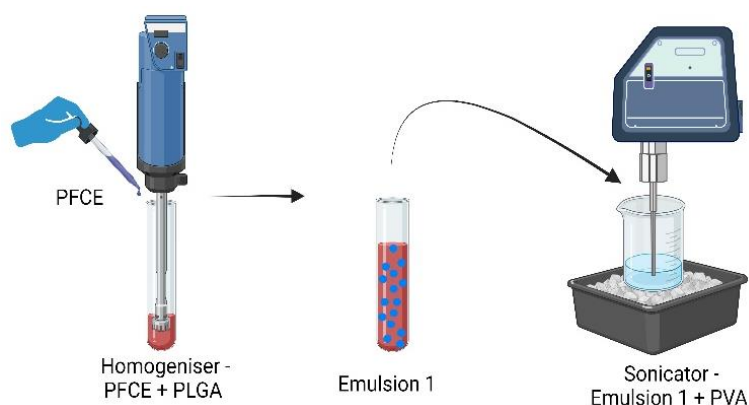


Figure 3: Hybrid Methodology Employing Ultraturrax for Primary Emulsion Formation and Sonicator for Secondary Emulsion

PFCE-encapsulated PLGA NPs were formulated using a hybrid technique as follows: The formulation process, which combined both homogenization and sonication, is illustrated in Figure 3. Initially, a primary emulsion was formed by adding PFCE (900 μ l) dropwise into 3 ml of DCM containing 90 mg of polymer (30 mg/mL). This mixture was then homogenized using the Ultra-Turrax® T10 basic at 5,000 rpm for 20 minutes in an ice bath.

Subsequently, the primary emulsion was immediately transferred into 25 ml of a 2% *w/v* PVA water solution and sonicated for 3 minutes at 40% amplitude in an ice bath, culminating in the formation of the PFCE/O/W.

2.2.4. Washing and characterisation

Irrespective of the preparation method used, the organic DCM solvent was eliminated either by magnetic stirring (using IKA® RCT basic ikamag™, Staufen, Germany at 400 rpm) for overnight evaporation or by rotary evaporation (IKA® RV 8, Staufen, Germany) for about 30 – 40 minutes under reduced pressure, facilitating the solidification of NPs. Following this, the NPs were separated by centrifugation (high speed micro-centrifuge, D3024R, Scilogex, Rocky Hill, CT, USA) at 8,000 g for 30 min at 4°C. To remove PVA and any unformed polymers from the NP formulation, the NPs were then rinsed with deionised (DI) water and each rinse was followed by a centrifugation step. The NPs were then frozen with liquid nitrogen, lyophilized (using a Freeze dryer - FreeZone, Labconco, Kansas City, MO, USA) for 15-20h, and preserved at -20°C.

2.3 Characterisation

2.3.1. Size, polydispersity index (PDI) and Zeta Potential (ζ)

The particle size (size) and PDI of NPs were measured using dynamic light scattering (DLS) technique on the Zetasizer Nano (DLS Zetasizer Nano S90, Malvern Instruments (Worcestershire, UK)). PDI was measured to determine the distribution/ uniformity of particle size. Small PDI values approaching zero indicate monodisperse particles while large values (>0.4) indicate heterogenous particles. Size and PDI of NPs were measured by dispersing the formulation in DI water at 25°C and an angle of 90°. ζ was determined using the same instrument in a capillary zeta cell and the measurements were performed at 25°C. All samples were measured in triplicate and the average values and standard deviation of the measurements were calculated.

2.3.2. Loading Capacity (LC %)

PFCE load content of PLGA NPs was determined by Fluorine-19 nuclear magnetic resonance spectroscopy (¹⁹F-NMR) using Varian Mercury plus 500, Crawley, UK. 5 mg of lyophilized NPs were

resuspended in deuterium oxide (D₂O) to achieve a concentration of 10 mg/mL. Trifluoroacetic acid (TFA) at a concentration of 0.1 M was added from the stock solution to serve as an internal standard. The quantity of perfluorocarbon (PFCE) was determined by calculating the integration ratio of the PFCE peak to the TFA peak. Fluorine contents were calculated according to the *equation 1*:

$$\text{Loading capacity (wt/wt \%)} = \frac{\text{Mass of PFCE in gram}}{\text{Mass of NPs in gram}} * 100 \quad (1)$$

2.3.3 ¹⁹F MRI Signal to noise ratio (SNR) acquisition and data processing

All MRI scans were performed on a 9.4T ultra shielded MR scanner (Bruker Avance Neo) with a 25mm resonator tunable to both ¹H (linear) and ¹⁹F (quadrature) located in the Institute for Molecular Cardiology at Heinrich Heine University (HHU), Düsseldorf. 25 mg of the lyophilised sample was resuspended in DI water for the measurements. ¹⁹F scans of phantoms were acquired with RARE sequence (Rapid acquisition with relaxation enchantment), size = 128x128, FOV = 30x30 mm², slice thickness = 3mm, TE = 9.78ms, TR = 2500, Avg = 60, RARE factor = 32, Bandwidth = 15151,5 Hz

2.3.4. Morphological Analysis

2.3.4.1 Field Emission-Scanning Electron Microscope (FE SEM)

The morphology and particle size of the NPs were evaluated by a field emission-scanning electron microscope (FE-SEM Zeiss Sigma 300, Zeiss, Germany). The SEM sample stage was prepared by placing double-sided adhesive carbon tape on an aluminium stub. One small drop of 1 mg/mL NP sample suspended in deionised water was placed on the sample stab and then dried at room temperature overnight. Subsequently, the dried sample was sputtered under vacuum with a chromium layer of approximately 100 Å thickness (Quorum Q150T ES, Quorum Technologies, Lewes, UK) prior to analysis.

2.3.4.2 Confocal microscopy

Lyophilized samples, stained with Nile Red, were resuspended in DI water and left to dry on a glass slide overnight. The slides were then examined using a Nikon C2 plus confocal scanning laser microscope. A 60X objective (NA 1.40, oil immersion) with optical zoom 3X was used for the examination. Red fluorescence was observed using a long pass 561nm emission filter under 570-1000 nm laser illumination. The pixel size used for the analysis was 0.05 µm/px (3x 0.14µm/px). The pinhole diameter was set at 30 µm. Image stacks were collected every 0.5 µm along the z-axis. The images were subsequently analysed using NIS-Elements Software. Measurements were carried out in the equatorial plane of each capsule to minimize positional error. For NP preparation for confocal microscopy experiments, Nile Red was added to the organic solution prior to emulsification. Typically, about 100 µL of a concentrated Nile Red solution (0.057 mg/mL in DCM) was added to the organic PLGA solution prior to the PFCE/O emulsification.

2.4 Invitro studies

2.4.1 Protein corona studies

Investigations into the protein corona were performed utilizing bovine serum albumin (BSA) sourced from Sigma Aldrich and plasma procured from healthy human volunteers, with the latter isolated via blood centrifugation. NP concentrations were established at 1.5 mg/ml, while BSA concentrations were sustained at 20 mg/ml, yielding an NP to protein ratio of 1:13. Temporal analysis was conducted at three junctures: immediately (0 hours), 2 hours, and 24 hours post-incubation. BSA in isolation manifested peaks at 10 nm and 100 nm within the dynamic light scattering (DLS) spectrum, culminating in a z-average of approximately 13 nm. This denoted a reduced z-average size when considering the combined presence of BSA and NPs. To discern the influence of BSA on the nanosystem, emphasis was

placed on the peak mean intensity (PMI) of the first peak, indicative of the NP size exclusively. Comparative analyses of NP size were conducted using baseline measurements obtained from NPs resuspended in phosphate buffer solution (PBS) prior to exposure to BSA and plasma proteins.

2.4.2 Cytotoxicity studies : MTS assay

The metabolic activity of viable cells was determined by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay. NCTC clone 929 [L cell] (connective mouse tissue) (ATCC) fibroblasts were plated at the density of 5000 cells/well in 96 culture plates and were grown until 80% of confluence. Cultures were incubated for 24 h with empty (control) or PFCE loaded PLGA NPs samples at concentrations at 5 and 10 µg/mL in complete medium. Then, cells were incubated with 20 µL/well of CellTiter 96 Aqueous One Solution Reagent (Promega Italia, Milano, Italy) for 2 h in a humidified, 5% CO₂ atmosphere. The quantity of formazan product is directly proportional to the number of living cells in the culture. The colored formazan was measured by reading the absorbance at 490 nm using a 96-well plate reader.

In our study, we have selected a combination of PLGA with a MW of 7,000-17,000 and PVA with a MW of 9,000 – 10,000 to characterize the system using various techniques such as SEM, confocal studies, protein corona behaviour analysis, cytotoxicity studies, and SNR measurements. The PLGA empty system, which we used as a control, consisted of NPs without PFCE. These NPs were synthesized using PLGA 502H and PVA with a MW of 9,000 - 10,000 Da, employing a sonicator for the process.

2.5 Statistical analysis

To assess the variance among groups derived from different formulation techniques, a one-way analysis of variance (ANOVA) was employed, complemented by the student's *t*-test for pairwise comparisons. A *p*-value less than 0.05 (*p* < 0.05) was deemed indicative of statistical significance. The results are reported as the mean ± standard deviation (SD).

3.Results

In the present investigation, we compared three NP formulation methods—homogenization, sonication, and a hybrid technique (the combination of homogenisation and sonication)—across four different combinations of PLGA polymer and PVA surfactant molecular weight, as outlined in Table 1. Two PLGA types, 502H and 503H, with MW ranges of 7,000-17,000 and 24,000-38,000, respectively, were used in conjunction with PVA of MW ranges 9,000 – 10,000 Da and 13,000 – 23,000 Da. The nomenclature for the polymer mixtures was defined as follows: H_{PLGA}H_{PVA} (HH) for high MW PLGA with high MW PVA, H_{PLGA}L_{PVA} (HL) for high MW PLGA with low MW PVA, L_{PLGA}H_{PVA} (LH) for low MW PLGA with high MW PVA, and L_{PLGA}L_{PVA} (LL) for low MW PLGA with low MW PVA. The aim was to elucidate the effects of these polymer combinations, applied through distinct formulation techniques, on the physicochemical attributes of the NPs, such as size, PDI, ζ, and LC. The particle size, PDI, ζ and LC % distributions were measured for each formulation technique and for four groups of different MW of PLGA and PVA (HH, HL, LH, LL). The students *t*-test and ANOVA revealed significant differences among various groups and techniques. The concentration of the PLGA (30 mg/mL), the percentage of surfactant (2 *wt/wt* %), and the volume of contrast agent (PFCE – 900 µL) was kept constant throughout the study.

Table 1. Overview of formulation Parameters: PLGA and PVA MW Combinations

	MW of PLGA (Da)	MW of PVA (Da)	Notation
1	24,000-38,000 (503H)	13,000 – 23,000	H _{PLGA} H _{PVA} (HH)
2	24,000-38,000 (503H)	9,000 – 10,000	H _{PLGA} L _{PVA} (HL)
3	7,000-17,000 (502H)	13,000 – 23,000	L _{PLGA} H _{PVA} (LH)
4	7,000-17,000 (502H)	9,000 – 10,000	L _{PLGA} L _{PVA} (LL)

3.1 Sizes

The findings offer insights into the impact of various techniques (homogenisation, sonication, and hybrid) on particle size as well as their behaviour when using different MW combination of polymer and surfactant (HH, HL, LH, LL). The particle size distributions for each formulation method were as follows: homogenization yielded particles ranging from 300 to 450 nm (*figure 4A*), sonication produced particles between 200 and 270 nm (*figure 4C*), and the hybrid technique resulted in particles of 170 to 230 nm (*figure 4E*) in size. Statistical analysis using the *t*-test revealed significant differences in particle sizes among various groups. For the homogenization method, the most pronounced differences were observed between groups HH & HL, HH & LL, HL & LH, and LH & LL. In the case of sonication, significant differences were noted between groups HH & HL and HL & LH. For the hybrid technique, the groups showing significant differences were HH & HL and HH & LH. The pattern observed for homogenization demonstrates larger particle sizes, approximately 400 nm, for combinations HH & LH, and comparatively smaller particle sizes, around 300 nm, for combinations HL & LL (*figure 4A*). The *t*-test suggests that the sizes are nearly identical for both groups (HH & LH and HL & LL), indicating that using the same MW of PVA resulted in the same particle size, despite the use of different MW of PLGA. In contrast, for sonication, the most significant difference was observed between group HH with HL, and group HL with LH. The groups HH & LH, HH & LL, HL & LL and LH & LL were found to be of similar size, suggesting a lesser influence of either the polymer or PVA for the sonication process (*figure 4C*). The hybrid technique demonstrated the least variation, with group HH exhibiting the smallest size that significantly differed from groups HL and LH (*figure 4E*). Group LL displayed a larger SD in the hybrid technique, suggesting an anticipated lack of significant size change with this combination and other 3 groups (HL, LH, LL under the same SD) with almost similar sizes.

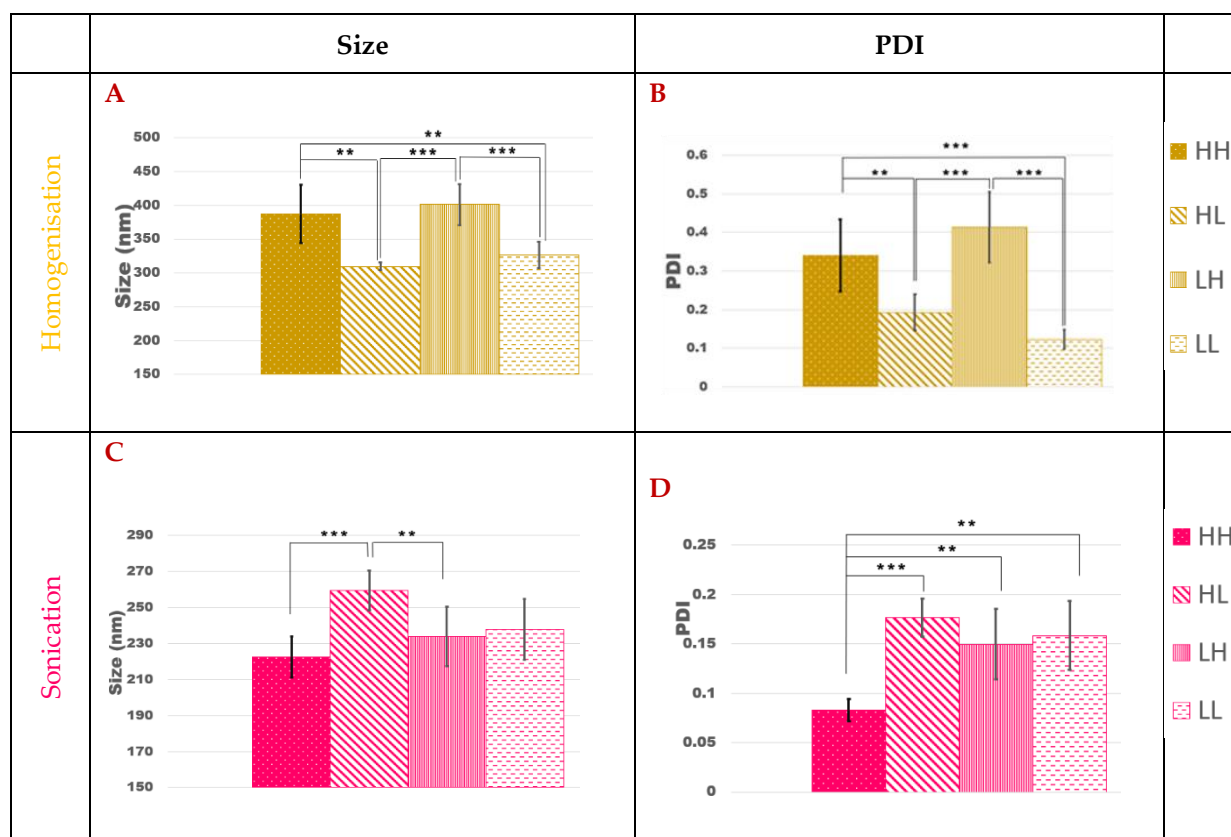
A thorough observations emerge when all the techniques are evaluated collectively as illustrated in the *figure 4G* . The results showed that the hybrid technique produced the smallest particles, followed by sonication and homogenization. The *t*-test was utilized to determine the significance of particle size differences between the techniques, and an ANOVA test (supplementary information *table 1S*) was employed to assess the significance of each group (HH, HL, LH, LL) when compared across the three techniques. The line graph (*figure 4G*) shows that homogenization increased the particle sizes for groups HH and LH, while it decreased them for groups HL and LL. This indicates that the effect of homogenization on particle sizes depends on the polymer-surfactant combination. For sonication the largest particle size was observed for the HL combination, which differed from the homogenization result, and it was significantly different from the HH and LH combinations. This demonstrates that the same polymer-surfactant combination can have different size outcomes depending on the preparation technique. The line graphs for the hybrid technique closely resembled the sonication trend line, without any regular patterns. As indicated by the data in *table S1* (supplementary information), each combination resulted in a notably significant difference, which was corroborated by the ANOVA results, demonstrating significant variations across all four groups when compared across the three techniques. The comparative analysis indicated that the homogenization method yielded larger particle sizes, whereas the hybrid technique resulted in the formation of the smallest nanoparticles. Notably, each polymer-surfactant combination exhibited unique size discrepancies when subjected to the three distinct formulation methods.

3.2 Polydispersity Index (PDI)

The PDI, indicative of the system's homogeneity, varied across the three techniques: the homogenization technique produced the largest and most heterogeneous particles (0.1 to 0.5) (*figure 4B*), while the sonication and hybrid techniques produced smaller and more homogeneous particles (0.05 to 0.2) (*figure 4D & 4F*). The *t*-test revealed significant differences between various groups for each

technique. For homogenization, significant differences were found between groups HH & HL, HH & LL, HL & LH, and LH & LL. For sonication, the groups with significant differences were HH & HL, HH & LH, and HH & LL. For the hybrid technique, the groups were HH & HL and HH & LH. The observed variations in PDI were consistent with the particle size distributions, except in the case of sonication. Specifically, homogenization showed significant PDI differences among the same groups that exhibited notable disparities in particle size. In the hybrid method, the LL group's PDI paralleled its high SD in particle size, with marked PDI differences occurring between the HH & HL and HH & LH groups, mirroring the distinctions noted in particle size measurements. However, with sonication, the HH group yielded a significant PDI variation when compared to the HL, LH, and LL groups, which remained relatively unchanged.

The comparative graph (*figure 4H*), which aligns all the techniques and combinations side by side, reflects certain aspects of the particle size trend. The PDI was highest for the homogenization technique, followed by sonication and the hybrid method, albeit the difference between sonication and the hybrid method was minimal. For both sonication and hybrid techniques, the PDI followed a straight-line graph without any discernible patterns. The ANOVA test (supplementary *table S2*) corroborated significant group differences in HH, HL, and LH, with no noticeable difference in group LL between the techniques.



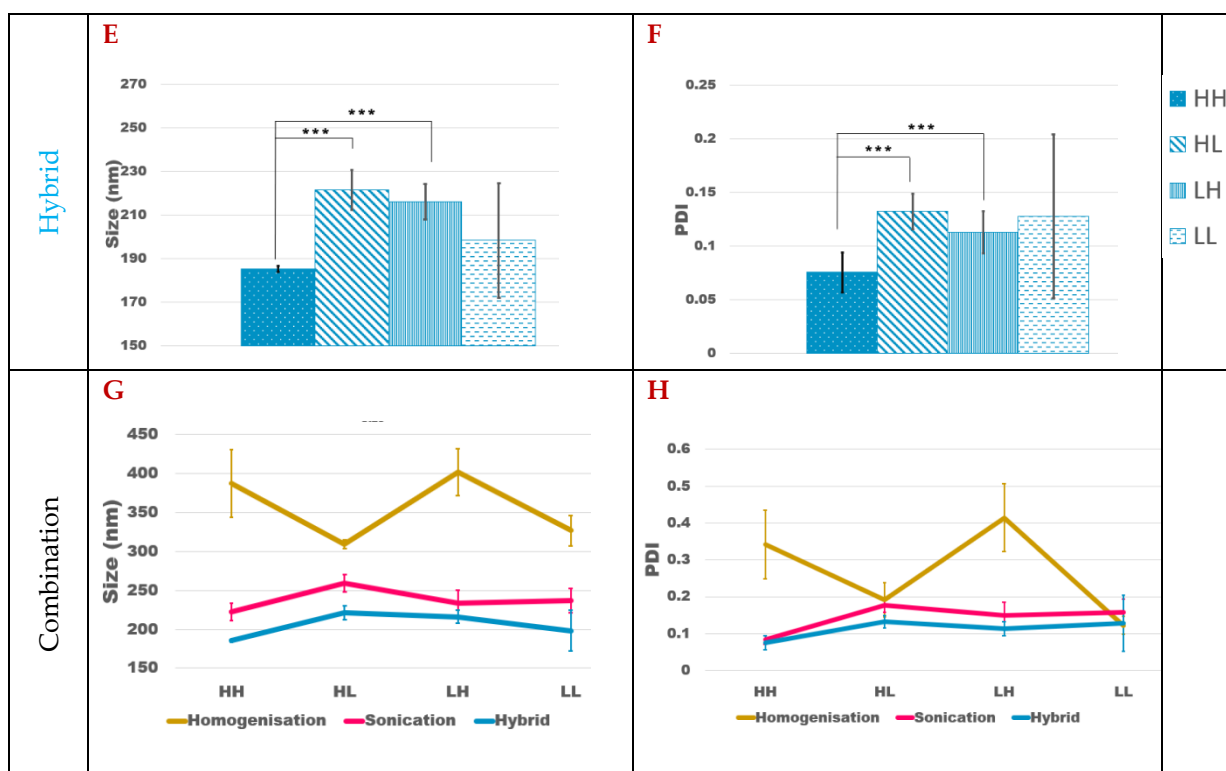


Figure 4. Size and PDI analyses for the three different techniques, along with their comparative graph. The homogenization technique resulted in size (A) and PDI (B), sonication in size (C) and PDI (D), and the hybrid technique in size (E) and PDI (F). Comparative analysis of the three techniques for size (G) and PDI (H). Significant values are marked with an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

3.3 Zeta Potential (ζ)

The ζ which represents the overall charge of the system, varied across the three techniques: homogenization (-25 to -32 mV) (figure 5A), sonication (-18 to -28 mV) (figure 5C), and the hybrid method (-20 to -30 mV) (figure 5E). The homogenization technique achieved the lowest ζ (-32 mV), while sonication resulted in the highest ζ (-18 mV) among the techniques. The t -test revealed significant difference between groups HH & HL, HH & LH, and HH & LL for homogenization, between groups HH & HL and HL & LH for sonication, and for all six combinations (HH & HL, HH & LH, HH & LL, HL & LH, and LH & LL) for the hybrid technique. The ζ did not show any consistent pattern with respect to other parameters such as size or PDI. The smallest group difference in ζ was observed for sonication, while the most substantial difference was noted for the hybrid technique, indicating a significance level ranging from considerable ($p < 0.01$) to very significant ($p < 0.001$) for all six possible combinations among the four groups. Homogenization exhibited only a minor significance change ($p = 0.4$) between groups HH & LH, which differ in the polymers used. The variation in ζ for sonication closely echoed the changes in the PDI for the same technique, particularly between groups HH & HL and HL & LH. Upon examining the comparative graph of ζ (figure 5G), the line graph demonstrates a lack of discernible trends respect to other parameters such as size, or PDI. This is particularly noteworthy when juxtaposed with the size and PDI measurements, where the sonication and hybrid methods show similar patterns, in contrast to the divergent trends observed with homogenization.

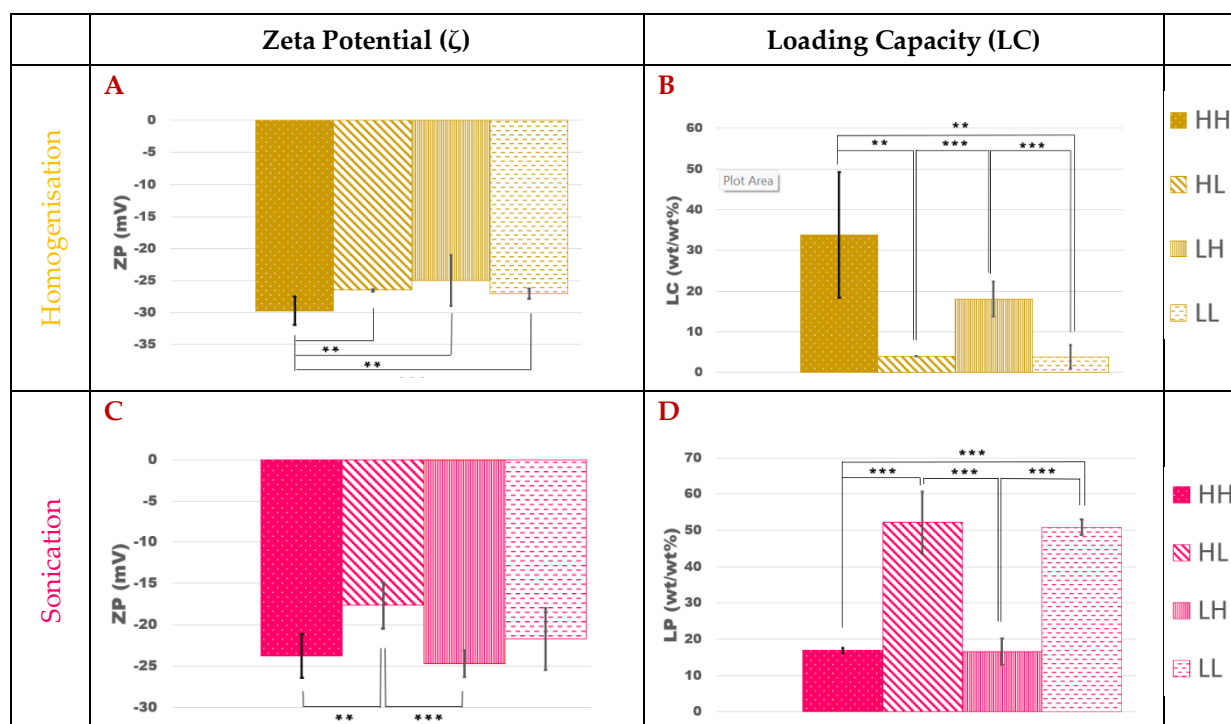
3.4 Loading Capacity

The term “loading capacity” is used to denote the quantity of contrast agent PFCE present in the system. It is calculated as a weight percentage using the following equation (2):

$$\text{Loading capacity (LC \%)} = \frac{\text{Mass of PFCE (gram)}}{\text{Mass of nanoparticles (gram)}} * 100 \quad (2)$$

The LC varied across the three techniques: homogenization (4 to 45 wt/wt%) (figure 5B), sonication (15 to 60 wt/wt%) (figure 5D), and the hybrid method (10 to 35 wt/wt%) (figure 5F). Significant differences were observed in the groups for each technique. For homogenization and sonication, these differences were between groups HH & HL, HH & LL, HL & LH, and LH & LL. For the hybrid technique, the differences were between groups HH & HL, HL & LH, and HL & LL. The substantial disparities observed among different groups in the homogenization process closely reflect the variations seen in the size and PDI for homogenization. These variations within the group were also consistent in the context of the sonication process. In both cases, the only non-differing groups were HH & LH and HL & LL. In the hybrid technique, the LC for groups HH, LH, and LL were very similar, with the only varied value presented by group HL, which showed a significant difference compared to all the other three groups.

Figure 5H presents a detailed comparison of the NPs formulation techniques in terms of their PFCE LC. Homogenization yielded the highest particle sizes and PDIs when compared to sonication and the hybrid method. Despite this, homogenization produced the lowest LC, particularly notable within the HL and LL combinations, which registered LC percentages of 3-4%, the lowest across all groups and techniques. Conversely, within the homogenization technique, the HH and LH combinations, which exhibited larger particle sizes and PDIs, corresponded to higher LC percentages, ranging from 18-34%. In the sonication technique, no specific trends were observed for size and PDI. However, the HH and LH combinations demonstrated lower LC percentages (around 17%), while the HL and LL combinations achieved higher LC percentages (around 52%). The hybrid method mirrored the sonication technique in the absence of a specific trend for size and PDI, with LC percentages spanning 10 to 29%, and the HL combination achieving the highest LC. The ANOVA test (supplementary table S4) corroborated the significance of these differences across all groups. The findings suggest that the choice of technique and the MW of the polymers can have a significant impact on the physicochemical properties of the nano-formulation of PFCE encapsulated PLGA NPs.



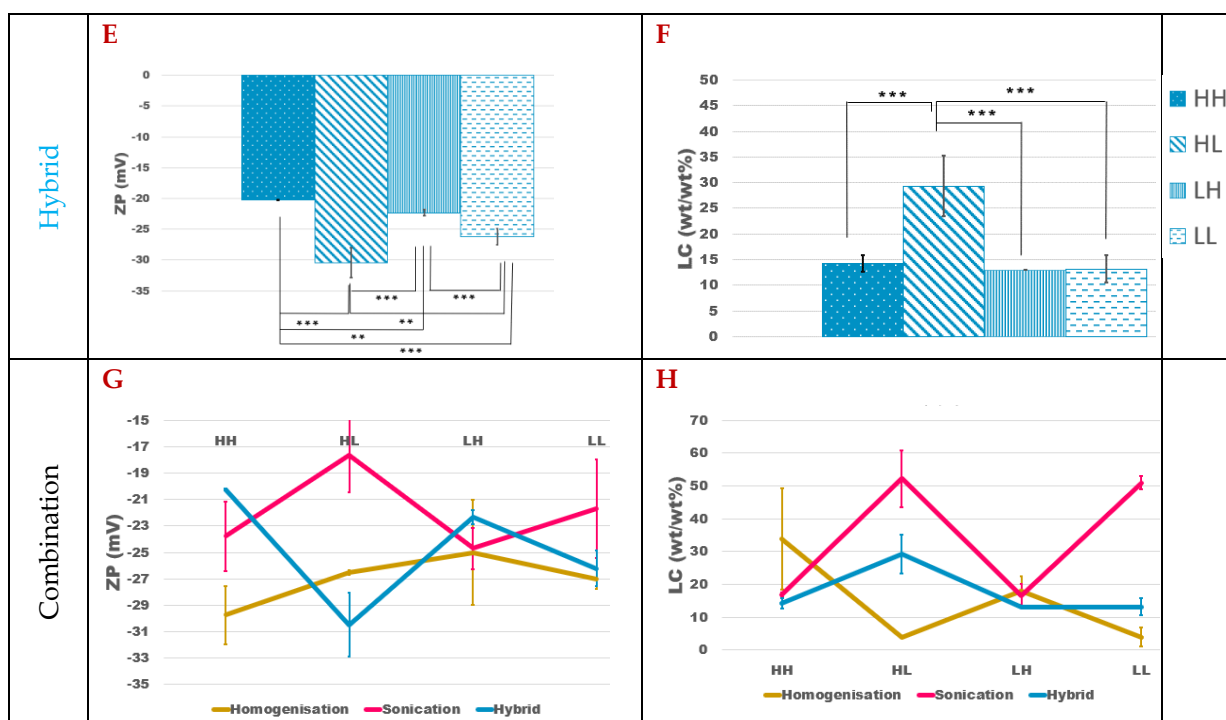


Figure 5. The zeta potential (ζ) and loading capacity (LC) analyses were conducted for three different techniques: homogenization (ζ (A), LC(B)), sonication (ζ (C), LC(D)), and the hybrid technique (ζ (E), LC(F)). Comparative analysis across these techniques revealed distinct patterns for ζ (G) and LC(H). Significant values are marked with an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

3.5 Comparative Analysis of Homogenization, Sonication, and Hybrid Techniques

PFCE-PLGA NPs had been formulated using homogenization, sonication, and a hybrid approach, with variations in the MW of PVA and PLGA, which led to the measurement of their size, PDI, ζ , and loading capacity percentage. The homogenization technique yielded particles with the largest size range, from 300 to 450 nm, and exhibited the most variability in terms of PDI, ranging from 0.1 to 0.5. This was in comparison to the sonication and hybrid techniques. The ζ measurements for the homogenization technique did not display a consistent pattern, fluctuating between -25 to -32 mV, and the PFCE loading was observed to be lower or comparable to the other techniques, ranging from 4 to 45 wt/wt% (table 2). Using the homogenization technique, the employment of PVA with a higher MW range of 13,000 – 23,000 Da alongside polymers of high (HH) and low (LH) MW resulted in increased particle size, PDI, and PFCE loading; specifically, the HH and LH variants exhibited particle sizes of 410 ± 50 nm and 400 ± 30 nm, PDIs of 0.4 ± 0.1 , and LC of 34 ± 15 wt/wt% and 18 ± 4 wt/wt%, respectively. In contrast, using a lower MW range of PVA (7,000 – 17,000 Da) in combination with polymers of high (HL) and low (LL) MW resulted in smaller particle sizes (310 ± 5 nm and 330 ± 20 nm, respectively), lower PDI values (0.2 ± 0.01 and 0.16 ± 0.03 , respectively), and reduced LC % (3 ± 1 wt/wt% and 4 ± 3 wt/wt%, respectively) (table 2). No significant differences were observed in particle size, PDI, and LC between the HH & LH and HL & LL groups when the MW of the polymer, PLGA, was varied while maintaining a constant PVA MW. The surfactant's MW emerged as the primary factor influencing the size, PDI, and LC of NPs produced via homogenization. With respect to ζ , only the HH combination exhibited a distinctively lower value of -31 mV, diverging from the other combinations (HL, LH, and LL). The specific influences of polymer and surfactant MW on this parameter appears to exert minimal influence.

Application of the sonication technique resulted in particle sizes ranging from 200 to 270 nm and PDI values between 0.05 to 0.2, positioning it between the homogenization and hybrid techniques in terms of these absolute values. The ζ varied from -18 to -28 mV, and the LC was recorded between 15 to 60

wt/wt%, the highest observed among the techniques (table 2). Notably, the sonication method did not exhibit discernible trends in particle size, PDI, and ζ when comparing the different MW combinations of polymer and surfactant (HH, HL, LH, and LL). Contrary to the homogenization technique, the LC trends were inverted; a higher MW of PVA (13,000 – 23,000 Da) in conjunction with both high (HH) and low (LH) MW polymers yielded a lower PFCE loading, approximately 17 ± 5 wt/wt% for both. Conversely, a lower MW of PVA (7,000 – 17,000 Da) paired with high (HL) and low (LL) MW polymers led to a higher PFCE loading, 52 ± 10 wt/wt% and 51 ± 2 wt/wt%, respectively. The LC did not significantly differ between the HH & LH and HL & LL combinations, akin to the homogenization technique, where the polymer MW was altered, and the surfactant MW remained constant. Employing the hybrid technique revealed a consistent pattern for particle size (170 to 230 nm) and PDI (0.05 to 0.2), yet no clear trend was discernible for ζ (-20 to -28 mV) or LC (10 to 35 wt/wt%) between the groups HH, HL, LH, and LL (table 2). In terms of size and PDI, this technique exhibited the smallest values in comparison to homogenization and sonication, closely mirroring the trends observed with sonication. In this study, the group demonstrated a range of ζ across the four groups, which were significantly distinct. The reported LC was intermediate between homogenization and sonication methods.

Table 2. The size, PDI, ζ , and loading capacity percentages characterized for NPs formulated by homogenization, sonication, and hybrid techniques.

	Homogenisation	Sonication	Hybrid
Size (nm)	300 - 450	200 - 270	170 - 230
PDI	0.1 - 0.5	0.05 - 0.2	0.05 - 0.2
ζ (mV)	-25 to -32	-18 to -28	-20 to -28
LC wt/wt%	4 - 45	15 - 60	10 - 35

3.6 Signal to noise ratio (SNR)

The Signal-to-Noise Ratio (SNR) was quantified utilizing ^{19}F MRI across three distinct techniques, detailed in Table 3 using phantom studies, with NPs at a concentration of 25 mg/mL. The samples analysis was for the group LL (combination of PLGA with a MW of 7,000-17,000 and PVA with a MW of 9,000 – 10,000). Analysis of the data demonstrated that samples subjected to sonication possessed superior SNR values. This was succeeded by those processed through homogenization and hybrid methods, respectively. Notably, these trends were found to be in close alignment with the corresponding LC metrics obtained using ^{19}F NMR.

Table 3. The Signal-to-Noise Ratio (SNR) obtained from three different techniques (PLGA with a MW of 7,000-17,000 and PVA with a MW of 9,000 – 10,000 (LL))

	Technique	LC	SNR
1.	Homogenisation	7	17
2.	Sonication	59	69
3.	Hybrid	10	6.4

3.7 Optimizing solvent removal and centrifugation conditions for NP formulation

Optimal conditions for the removal of DCM from samples were investigated using both rotary and overnight evaporation methods, as detailed in *supplementary table S5*. The outcomes of both methods did not exhibit significant differences; hence, overnight evaporation was selected for its convenience. Additionally, the impact of centrifugation parameters on the size, PDI, and LC of the NPs was examined, varying the centrifugal force (4,000 to 20,000 g), duration (20 to 60 minutes), and temperature (4 to 18°C), with the results presented in the *supplementary table S6*. Based on these findings, the centrifugation conditions of 8,000 g, 4°C, and 30 minutes were determined to be the most suitable for this study.

3.8 PFCE solubility

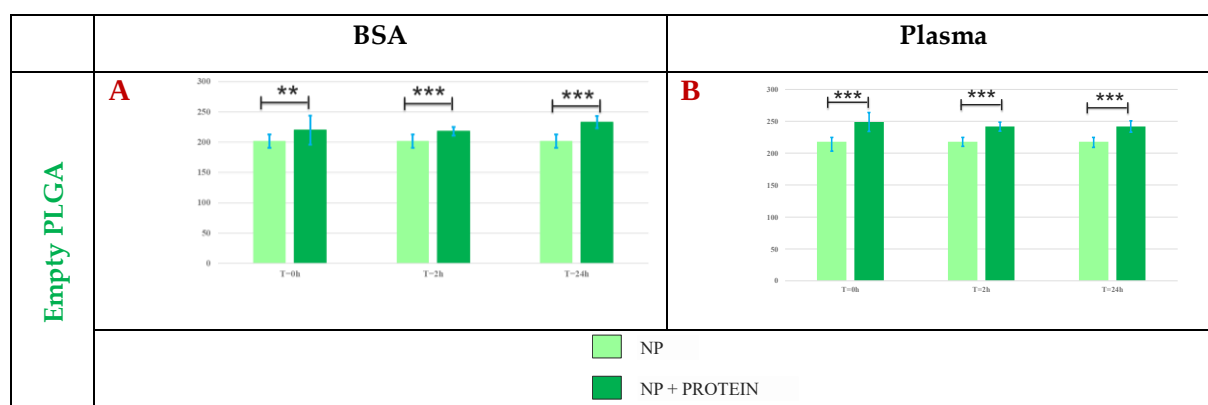
The solubility of PFCE was examined in various solvents, including selected eutectic mixtures, detailed in the supplementary information (*supplementary table S7*). PFCE, characterized by its hydrophobic and lipophilic properties, exhibited solubility in certain solvents, notably at lower concentrations (10 μ L in 1 mL of solvent). Solubility was observed in solvents like pentane, diethyl ether, and hexane, corroborating literature findings that suggest a greater hydrophobicity over lipophilicity for PFCs [21, 22]. Diethyl ether had the highest solubility, up to 30 μ L in 1 mL of solvent. Significantly enhanced outcomes were noted upon the application of homogenization and sonication energies to the solution. With solvents such as ethyl acetate and DCM, the PFCE was temporarily soluble when sonicated at 40% amplitude for up to 1 minute, and then immediately precipitated out. This behaviour was not seen with ultraturrax T10.

3.9 Morphological study using SEM and confocal.

The SEM studies (*figure 6*) revealed the spherical morphology of the NPs formulated by all three techniques including control NP formulated without PFCE (*figures 6A & 6B*), homogenization (*figures 6E & 6F*), sonication (*figures 6I & 6J*) and hybrid (*figures 6M & 6N*). The confocal studies were performed after adding the fluorescent agent Nile Red to the solution during NP formulation. For the confocal imaging, only particles of micrometre size were seen in the instrument. These particles corresponded to the very small population of microparticles around 5 μ m that was seen in the DLS number average graph. The aim was to observe the distribution of PLGA and PFCE in the NPs. Nile red is a hydrophobic drug that dissolves only in the organic phase of the PLGA polymer. PFCE do not dissolve Nile red and appeared darker (since it did not emit any fluorescence in the analysis). In the *figures 6C & 6D*, the PFCE-free empty PLGA NPs showed a homogeneous spherical shape in the confocal studies. When PFCE was added to the solution (homogenization (*figures 6G and 6H*), sonication (*figures 6K & 6L*), and hybrid (*figures 6O & 6P*)), a hollow space was observed, which was identified as PFCE.

3.10 Protein corona studies

Dynamic Light Scattering (DLS) was utilized to ascertain the size variation of the NPs to study the protein corona behaviour. The size measurements were conducted prior to and subsequent to protein exposure, with assessments occurring immediately, as well as 2 hours and 24 hours after exposure to BSA and plasma proteins, as documented in *Figure 7*. The PLGA empty system (created as a control without PFCE inside) exhibited a significant interaction at all three time points (*figures 7A & 7B*), similar to the NPs made by homogenization (*figures 7C & 7D*), sonication (*figures 7E & 7F*), and hybrid techniques (*figures 7G & 7H*).



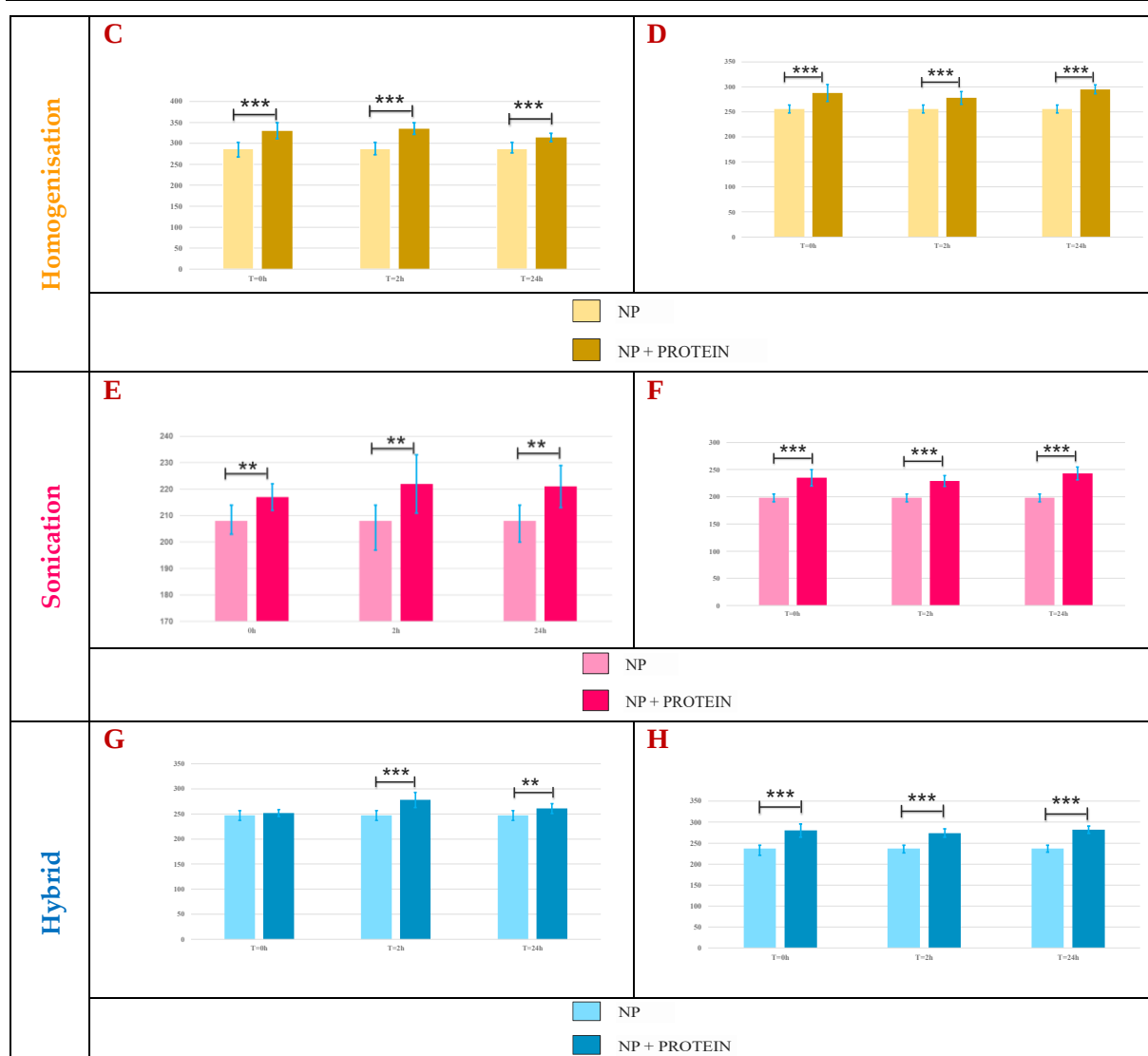


Figure 7. Analysis of Protein Corona Formation on PLGA NPs for PLGA control (A & B), formulated by homogenization (C & D), sonication (E & F), and a hybrid technique (G & H) (PLGA with a MW of 7,000-17,000 and PVA with a MW of 9,000 – 10,000 (LL)). Significant values are marked with an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

3.11 Cytotoxicity studies

To quantify the potential impact on the cells viability, the MTS assay was used. To test the biocompatibility of PFCE loaded PLGA NP, MTS assay was performed. *Figure 8* illustrates that the control and the empty NPs did not impact the viability of fibroblasts, which was consistent with the results observed for NPs loaded with PFCE using homogenization, sonication, and hybrid techniques.

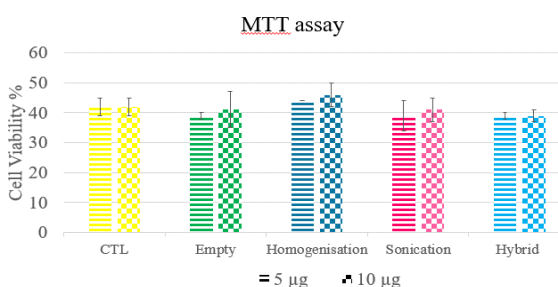


Figure 8. Biocompatibility studies determined by MTS assay in fibroblasts cells. Values are expressed as mean $\pm$ S.D. ($n = 3$) (PLGA with a MW of 7,000-17,000 and PVA with a MW of 9,000 – 10,000 (LL))

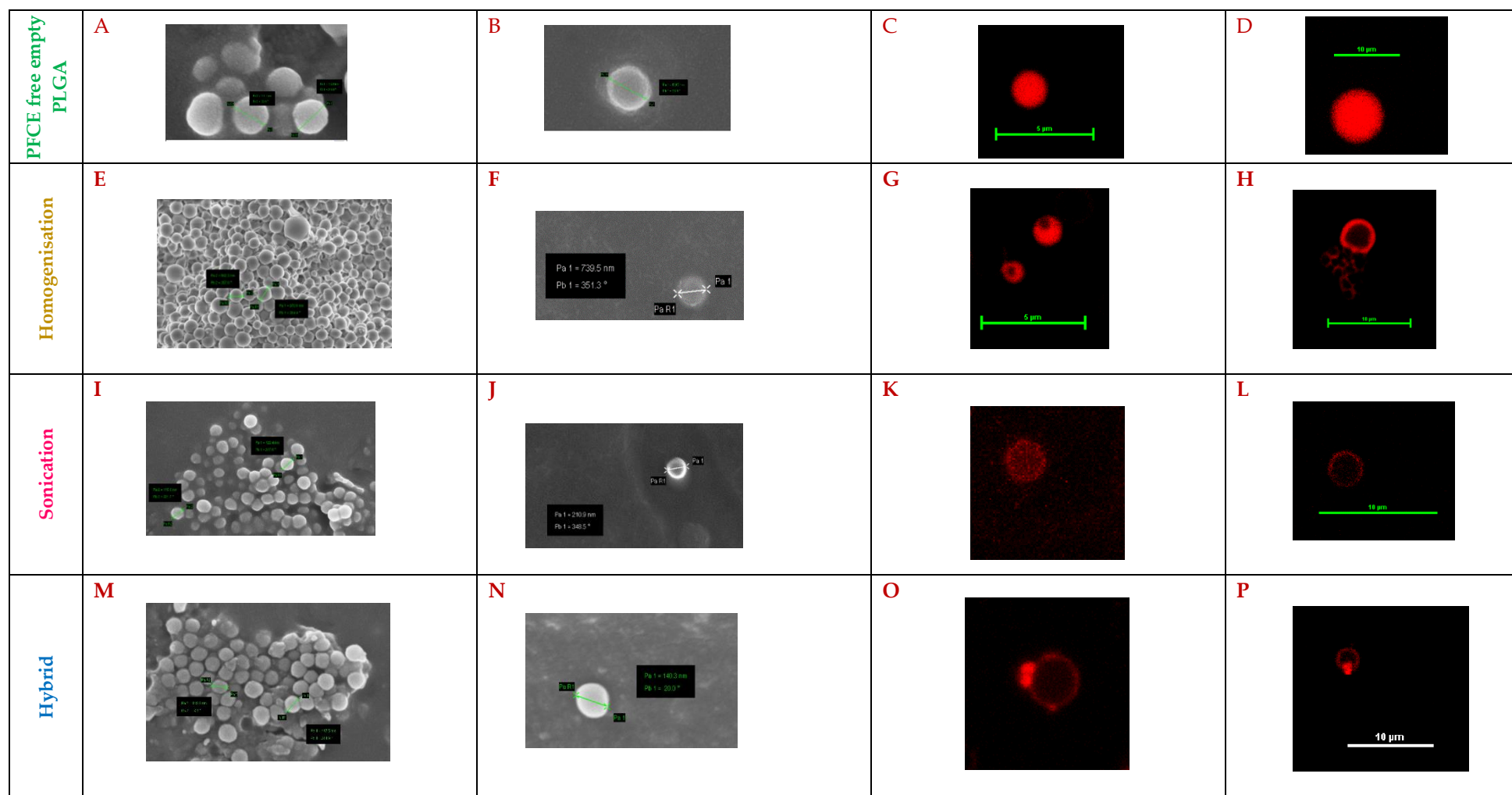


Figure 6. Comparative analysis of PLGA samples using SEM and confocal microscopy: Unencapsulated PLGA samples (SEM images A & B, confocal images C & D), samples prepared by homogenization (SEM images E & F, confocal images G & H), samples prepared by sonication (SEM images I & J, confocal images K & L), and samples prepared by a hybrid method (SEM images M & N, confocal images O & P) (PLGA with a MW of 7,000-17,000 and PVA with a MW of 9,000 – 10,000 (LL))

4. Discussion

4.1 General Mechanism of homogenisation and sonication

Homogenisation using an ultraturrax, or a toothed disc dispersing machine, consists of a rotating rotor and a stationary stator with vertical slots (*figure 9*). The rotor is located inside the stator. The machine works by forcing solutions through the gap between the rotor and stator, where high shear and turbulence occurs. This causes the particles in the solution to be disrupted. The disruption of the particles leads to the formation of droplets, which can either coalesce with other droplets to form aggregates or stabilize with stabilizer molecules in the solution (such as PVA) to form stable emulsion [23]. The coalescence and disruption of the droplets continue until a stable emulsion is formed. The rotation of the rotor also creates a vacuum that draws the liquid in and out, facilitating the sample circulation. When samples undergo circular motion within the apparatus, the rotor accelerates the sample tangentially, directing it through the stator slots (*figure 9*). This action results in pronounced velocity gradients adjacent to the slots, generating substantial hydrodynamic stresses that ensure efficient dispersion [24]. The efficiency of the emulsion formation using an homogeniser depends on several factors, such as the gap width, rotor radius, flow rate, rotational speed, etc [25]. Sample viscosity is also an important parameter for rotor stator homogenization. Research indicates that the viscosity of samples plays a significant role in the disruption process and the quality of emulsions produced, with varying outcomes observed across samples of low to high viscosity [24-26].

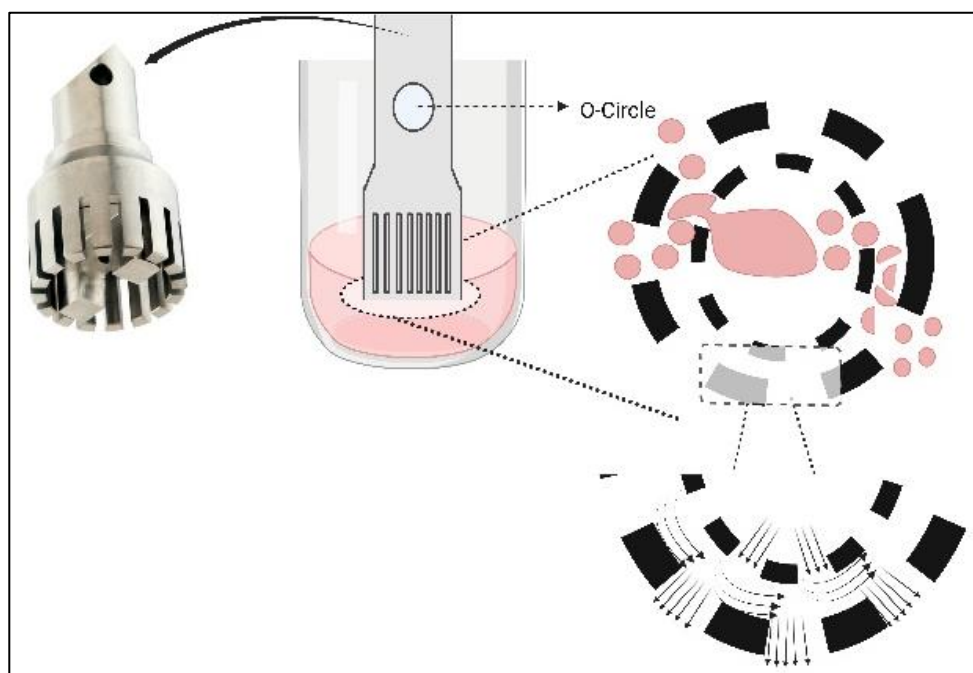


Figure 9. Mechanistic operations involved in homogenization, where a rotor-stator mechanism is utilized to facilitate the breakdown of larger particles

Sonication involves inserting a probe (sometimes referred to as “horn”) into a sample (*figure 10*) for the effective dissipation of energy. The probe vibrates rapidly, thereby transferring its ultrasonic energy to the sample. Sonication works on the principle of sonochemistry - the effects of intense sound waves traveling through a liquid. This process involves the interaction of sound with matter through acoustic cavitation, and to induce the cavitation process, intense sound waves, specifically ultrasound waves, are produced in a liquid [27]. The waves consist of alternating cycles of compression and expansion that leads to the formation, growth, and collapse of bubbles in the liquid. The bubbles collapse very rapidly, generating high temperatures (approximately 5,500°C) and pressures inside them. The

sonochemistry of two immiscible liquids, such as oil and water, stems from the ability of ultrasound to emulsify liquids. Ultrasonic compression and expansion stress liquid surfaces, overcoming the cohesive forces that hold a large droplet together, causing the droplet to burst into smaller ones sending shock waves through the liquid, and this results in microscopic droplets of one liquid being suspended in the other and eventually, the liquids are emulsified [27]. In recent years, the use of ultrasound in the emulsification process has increased mainly because of its energetic efficiency, low cost of production, ease of handling and better control over components [28]. The formulation of an emulsion using ultrasound depends on several factors, including time, amplitude, sample volume and viscosity, surfactant type, among other relevant variables.

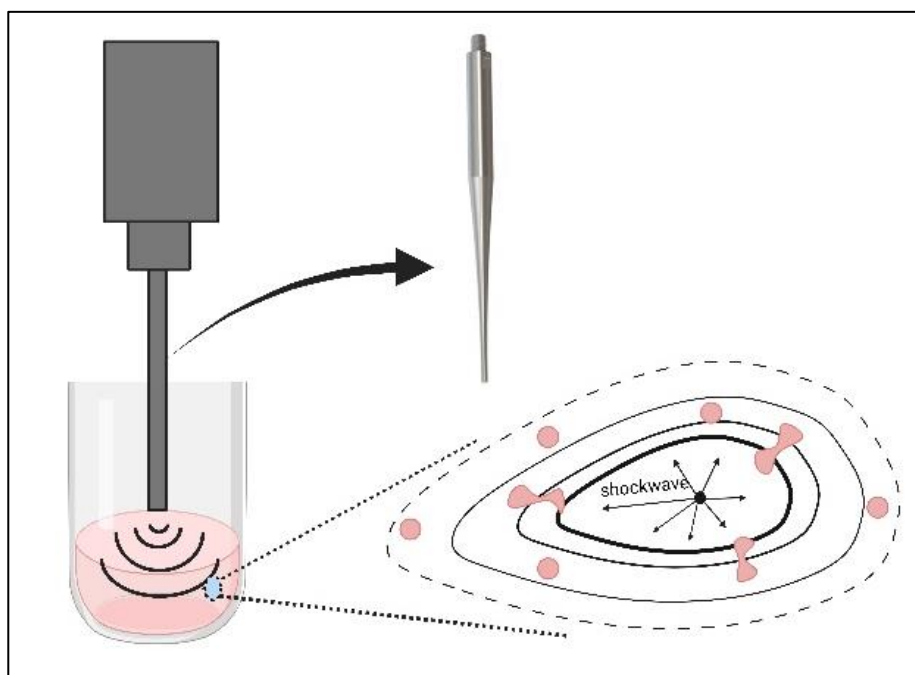


Figure 10. The process of sonication involves the use of a probe to facilitate the breakdown of particles

4.2 Comparative Analysis of NP Formulations: Polymer & Surfactant MW and Preparation Techniques

In the comparative analysis of NP formulations, the MW of the surfactant emerged as a critical factor influencing NP characteristics such as size, PDI, and LC. This influence was particularly pronounced during homogenization and sonication processes. During homogenization, a higher MW of PVA ranging from 13,000 to 23,000 Da, when combined with polymers of varying MWs, resulted in the formation of larger NPs with greater heterogeneity and enhanced PFCE loading. Conversely, a lower MW of PVA (9,000 – 10,000 Da) yielded smaller, more uniform NPs with reduced PFCE content. In sonication, this trend was reversed; higher MW PVA led to NPs with lower PFCE loading, while lower MW PVA produced NPs with higher PFCE content.

The observed differences in NP properties can be attributed to the controlled variation in the preparation technique and the MW of the surfactant and polymer. The viscosity of the solutions, influenced by the MW of the polymer and surfactant, likely contributed to these differences. According to the Mark-Houwink equation (3) [31].

$$[\eta] = KM^a \quad (3)$$

where η is intrinsic viscosity,

K and α are constants that depend on the particular polymer-solvent system, and

M is the MW

The direct correlation between intrinsic viscosity and MW suggests that a higher MW corresponds to increased intrinsic viscosity. Consequently, formulations with higher MWs for both polymer and surfactant (HH combination) exhibited higher overall solution viscosity compared to those with lower MWs (LL combination). The behaviour of the HH and LH combinations was similar for both homogenization and sonication, with larger NP sizes, higher PDI, and better LC observed in homogenization, and higher LC in sonication. Conversely, the LL and HL combinations displayed smaller NP sizes, lower PDI, and lower LC in homogenization, with higher LC in sonication. The surfactant's MW appeared to have a more pronounced effect on the formulation than the polymer's MW. The surfactant was prepared in a 25 ml solution, while the polymer was dissolved in a 3 ml concentrated DCM polymer solution. It is possible that the surfactant's viscosity had a greater impact than the polymer's, resulting in similar behaviour between the LH and HH combinations and between the HL and LL combinations.

The differential results can also be explained by the energy mechanisms employed by the two techniques on samples with varying viscosities. In homogenization, increased solution viscosity (HH & LH) may have hindered dispersion and promoted NP coalescence, leading to aggregates with larger sizes and higher PDI values. The high LC observed could be due to the larger particle sizes and the presence of diverse NP populations, as evidenced by SEM and confocal studies. In contrast, the HL and LL combinations produced NPs with an average size of 300 nm and a PDI below 0.2, indicative of more efficient emulsification and dispersion in lower viscosity samples, resulting in reduced LC due to smaller particle sizes. A notable distinction between homogenization and sonication is also the temporal aspect of the processes. PFCE, with its high density of 1.48 g/cm³, tends to separate rapidly from the emulsion unless quickly stabilized. Homogenization requires sequential processing through a rotor-stator system for emulsification, while sonication utilizes rapid energy delivery through shock waves. Consequently, the process of homogenization necessitates a prolonged period to attain a stable emulsion. This protracted time frame, in conjunction with factors related to intrinsic viscosity, augments the propensity for phase separation of PFCE. Such a separation propensity heightens the potential for an emulsification process that is inefficient.

The LC trends of sonication process were opposite to those in homogenization. This reversal may be due to the efficient energy transfer by ultrasonic waves in lower viscosity samples, facilitating better solubility of PFCE with DCM under sonication conditions. This phenomenon may be attributed to the efficient energy transfer facilitated by ultrasonic waves in samples with lower viscosity. Conversely, samples with higher viscosity may have experienced reduced energy transfer efficiency [32] [31]. As discussed in Section 3.8, PFCE demonstrated solubility with DCM under sonication, which could have been more effective for samples with lower viscosity that received greater energy transfer to the PFCE. The PFCE dissolved in the DCM polymer matrix during sonication might have been temporarily encapsulated within the polymer matrix upon DCM evaporation, with the remaining PFCE forming the core of the NPs. This encapsulation process could explain the speckled shell structure observed in sonication NPs, as revealed by confocal studies.

The hybrid technique, which combines mechanical emulsion using an Ultraturrax (T10) for primary emulsion creation and subsequent secondary emulsification via sonication, demonstrated stability in particle size and PDI. However, the NPs produced had markedly low LC percentages and variable ζ values. Notably, a significant loss of PFCE was observed during the washing steps of the hybrid technique, as delineated in *Supplementary Table S6*, which indicates a pronounced reduction in PFCE content from the first to the third wash. This loss was unique to the hybrid technique when compared to the other methods at equivalent wash stages. The confocal studies suggest that this phenomenon may be attributed to the formation of a thin PLGA shell, which potentially facilitated the more rapid

loss of PFCE from the NPs. Additionally, this fragile structural integrity could also elucidate the considerable variation in ζ observed across all groups (HH, HL, LH, LL) within the hybrid technique. Altering one step in the formation process appears to significantly impact the amount of contrast agent loaded and the formulation of the NPs themselves.

4.3 Observational Analysis of Morphological Characteristics

SEM analysis (figure 6) was performed to study the external morphology of the NPs. The size discrepancy observed between the homogenization-derived NPs shown in figures 6E & 6F and the sizes determined by DLS may stem from multiple factors, including sedimentation and a high PDI. Considering the nanometric dimensions of the particles, sedimentation is anticipated to be a gradual phenomenon. Moreover, a high PDI can result in a wider range of particle sizes, potentially leading to inconsistencies in DLS measurements. These observations imply that other variables may be at play, influencing the DLS outcomes and warranting additional investigation to elucidate the determinants of the noted size disparities [29, 30]. DLS analysis also demonstrated a population of 5 μm particles consistently in homogenisation batches but the number average was very small compared to the ~300 nm particles produced in the formulation. The NPs, which were not encapsulated with PFCE as shown in figures 6A & 6B, and those formulated via sonication (figures 6I & 6J) and the hybrid method (figures 6M & 6N), presented slightly reduced dimensions compared to the measurements obtained through DLS. This observed discrepancy may be linked to the hydration shell that envelops the NPs during DLS analysis, a result of using DI water to resuspend the NPs. The hydrodynamic diameter, as gauged by DLS, accounts for the Brownian motion of the particles in suspension, factoring in the associated water molecules, which may lead to an increased apparent size of the NPs.

Observations from confocal microscopy studies indicated that the distribution of PFCE within PLGA NPs varied according to the preparation technique employed. In the homogenization process, as depicted in figures 6G & 6H, the PFCE-PLGA NPs exhibited a core-shell structure, with PFCE at the core encased by a substantial PLGA shell. Conversely, the sonication process resulted in a less pronounced PLGA shell, as shown in figures 6K & 6L. The surface displayed a speckled pattern of Nile red fluorescence with varying intensities, surrounding a darker region presumably indicative of the PFCE core, thus forming a core-speckled shell morphology. The dimensionality of the particles, extending into the micrometre scale, presents a challenge in accurately ascertaining the internal architecture of the NPs. Previous literature has documented that NPs formulated through similar fabrication processes and utilizing comparable polymer-surfactant MW profiles exhibit a multicore distribution of PFCE in the PLGA [17, 19]. The hybrid technique revealed a distinct morphology, characterized by a slender PLGA outer layer enveloping the PFCE core, as observed in figures 6O & 6P. Additionally, small bead-like structures were noted on the surface. The observed particles obtained using hybrid technique may have resulted from the aggregation of NPs into microparticles, as evidenced by the particle sizes detected in the micrometre range.

4.4 *In vitro* studies - cytotoxicity & protein corona studies

Cytotoxicity assays conducted on fibroblasts, detailed in Figure 8, substantiate the biocompatibility of the NPs. Remarkably, NPs from all three techniques exhibited no toxicity at two different concentrations (10 and 20 μg). Additionally, protein corona studies evaluated the NPs' biocompatibility in terms of their behaviour with components of biological fluids, utilizing albumin and plasma proteins. When NPs encounter proteins in the biological environment, they can attach to its surface and form a protein corona around them [33]. One simple way to detect the protein corona is to measure the size of the NPs, since proteins have a size range of 5 to 50 nm [34]. If the NPs' size changes after exposure to proteins, it may indicate the presence of proteins on their surface. Significant increase in particle sizes were observed in the PLGA empty system across all three temporal measurements, as illustrated in

figures 7A & 7B. This pattern was consistent with the NPs produced through homogenization (figures 7C and 7D), sonication (figures 7E and 7F), and hybrid techniques (figures 7G and 7H). Initially, NPs formulated via the hybrid method exhibited no significant binding with BSA at the zero-hour time point, as their particle sizes were nearly identical. However, over time, a substantial increase in particle size was observed. Furthermore, when exposed to plasma proteins, these NPs displayed significant enlargement at all three measured time points. The increase in particle size noted at each of the three-time intervals suggests that the NPs quickly attract proteins to their surface, which may lead to faster recognition and subsequent clearance by the reticuloendothelial system after they are administered. This occurrence is a recognized characteristic of nano-formulated drugs [35, 36]. To mitigate this, the implementation of a 'stealth' coating around the NPs is a prevalent approach to improve their physiological interactions [37].

5. Conclusion

In this study, PLGA NPs encapsulating PFCE were formulated as potential ^{19}F -MRI contrast agents utilizing three distinct techniques: homogenization, sonication, and a hybrid approach. Concurrently, the MW of both PLGA and PVA was systematically varied. Comprehensive characterization of these NPs revealed significant alterations in size, PDI, ζ , and LC % contingent upon the formulation technique and the polymer-surfactant MW combination.

The homogenization process yielded NPs with the broadest size range and the highest variability in PDI. The MW of PVA was a critical determinant of NP characteristics, with higher MWs leading to larger, more heterogeneous, and more heavily loaded NPs. ζ measurements displayed inconsistency, indicating the influence of additional factors beyond MW and preparation method. The sonication technique produced NPs with intermediate sizes and PDI values, situated between those obtained via homogenization and the hybrid technique. Notably, this method achieved the highest LC %, suggesting efficient PFCE encapsulation. The absence of clear trends in size, PDI, or ζ across varying MW combinations pointed to a uniform energy distribution during sonication. The hybrid technique consistently generated the smallest NPs with the most uniform size and PDI. However, LC % and ζ values exhibited variability, potentially due to the intricacies of the dual emulsification process. A significant loss of PFCE during washing steps was observed, which may be indicative of the NPs' structural integrity. SEM confirmed the spherical morphology of the NPs, while confocal microscopy was instrumental in elucidating the distribution of PFCE within the NPs. Cytotoxicity assays affirmed the non-toxic nature of all formulated NPs across the three techniques for fibroblasts. Protein corona studies suggested a propensity for rapid uptake by the reticuloendothelial system post-injection, which could be mitigated by surface modification with polymers such as polyethylene glycol to reduce protein corona formation.

Each formulation technique exhibited unique advantages and limitations, which can be strategically exploited to tailor NP properties for specific applications. The selection of MW for PVA and PLGA was pivotal in modulating the NPs' attributes, underscoring the significance of technique choice and material properties in NP fabrication. These findings pave the way for further optimization in NP design, particularly for targeted drug delivery and biomedical applications.

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Supplementary information

The Art of Preparations of PFCE Encapsulated PLGA Nanoparticles : Formulation and optimisation using High Speed Homogenization and Ultrasonication

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Table S1: T test and ANOVA test results for the size parameter of PLGA -PFCE NPs

Particle Size	T test			ANOVA test
	Homogenisation & Sonication	Homogenisation & Hybrid	Sonication & Hybrid	
H _{PLGA} H _{PVA} (HH)	***	***	***	Yes (P < 0.001)
H _{PLGA} L _{PVA} (HL)	***	***	***	Yes (P < 0.001)
L _{PLGA} H _{PVA} (LH)	***	***	*	Yes (P < 0.001)
L _{PLGA} L _{PVA} (LL)	***	***	**	Yes (P < 0.001)

Table S2: T test and ANOVA test results for the PDI parameter of PLGA -PFCE NPs

Poly Dispersity Index (PDI)	T test			ANOVA test
	Homogenisation & Sonication	Homogenisation & Hybrid	Sonication & Hybrid	
H _{PLGA} H _{PVA} (HH)	***	***	-	Yes (P < 0.001)
H _{PLGA} L _{PVA} (HL)	-	**	**	Yes (P < 0.01)
L _{PLGA} H _{PVA} (LH)	***	***	-	Yes (P < 0.001)
L _{PLGA} L _{PVA} (LL)	-	-	-	No

Table S3: T test and ANOVA test results for the zeta potential parameter of PLGA -PFCE NPs

Zeta Potential (ζ)	T test			ANOVA test
	Homogenisation & Sonication	Homogenisation & Hybrid	Sonication & Hybrid	
H _{PLGA} H _{PVA} (HH)	***	***	*	Yes (P < 0.001)
H _{PLGA} L _{PVA} (HL)	***	**	***	Yes (P < 0.001)
L _{PLGA} H _{PVA} (LH)	-	-	**	No
L _{PLGA} L _{PVA} (LL)	*	-	*	Yes (P < 0.01)

Table S4: T test and ANOVA test results for the loading capacity of PLGA -PFCE NPs

Loading Capacity (LC %)	T test			ANOVA test
	Homogenisation & Sonication	Homogenisation & Hybrid	Sonication & Hybrid	
H _{PLGA} H _{PVA} (HH)	*	*	**	Yes (P < 0.01)
H _{PLGA} L _{PVA} (HL)	***	***	***	Yes (P < 0.001)
L _{PLGA} H _{PVA} (LH)	-	*	*	Yes (P < 0.05)
L _{PLGA} L _{PVA} (LL)	***	***	***	Yes (P < 0.001)

Table S5: Removal of DCM from the NP formulation by rotary and overnight evaporation, samples prepared using PLGA 502H and PVA 9,000 - 10,000, formulation by sonication technique

	Evaporation of Organic solvent	Size (nm)	PDI	ζ (mV)	LC (wt/wt%)
1.	Rotary	241 $\pm$ 10	0.11 $\pm$ 0.01	-24.0	44 $\pm$ 10
2.	Overnight	251 $\pm$ 10	0.15 $\pm$ 0.02	-23.0	50 $\pm$ 4

Table S6: Centrifugation condition of force, time and temperature and their effect on size, PDI and LC % studied using the samples prepared using PLGA 502H and PVA 9,000 - 10,000, formulation by sonication technique

	Parameter	Limit	Size (nm)	PDI	LC (wt/wt%)
1.	Force (g)	4k – 20k			
2.	Time (min)	20 – 60			
3.	Temperature (°C)	4 – 18			

Table S7: PFCE solubility in 1 ml solvent with 10 μ L PFCE

1.	Methanol	<i>not soluble</i>
2.	Hexane	<i>soluble</i>
3.	Dichloromethane (DCM)	<i>not soluble</i> <i>temporary solubility on application of ultrasonic energy</i>
4.	Chloroform (CHCl ₃)	<i>not soluble</i>
5.	Acetone	<i>not soluble</i>
6.	Tetrahydrofuran (THF)	<i>not soluble</i>
7.	Ethyl acetate	<i>not soluble</i> <i>temporary solubility on application of ultrasonic energy</i>
8.	Diethyl ether	<i>soluble</i>
9.	Pentane	<i>soluble</i>
10.	Acetonitrile	<i>not soluble</i>
11.	Dimethyl sulfoxide (DMSO)	<i>not soluble</i>
12.	Water (H ₂ O)	<i>not soluble</i>
	Others	
13.	H ₂ O+ NaCl	<i>not soluble</i>
14.	H ₂ O+NaOH	<i>not soluble</i>
	Eutectic mixture (different ratios with PFCE)	

15.	PEG ₂₀₀ +NaCl	<i>not soluble</i>
16.	Glycerol+ NaCl	<i>not soluble</i>
17.	H ₂ O+NaClO ₄	<i>not soluble</i>
18.	Thymol + Menthol	<i>not soluble</i>
19.	Diethyl ether + H ₂ O+NaOH	<i>PFCE soluble in ether but H₂O layer separated out</i>
20.	H ₂ O+NaCl+PEG ₂₀₀	<i>not soluble</i>
21.	H ₂ O+NaOH+PEG ₂₀₀	<i>not soluble</i>
22.	H ₂ O+NaOH+Glycerol	<i>Temporary Solubility on application of heat gun</i>

Table S8: Loss of loading capacity in hybrid preparation system for the 4 groups

Group	1 st wash	3 rd wash
HH	53 ± 19	14 ± 2
HL	49 ± 13	29 ± 6
LH	28 ± 3	12 ± 2
LL	60 ± 11	13 ± 3

3. Practical Aspects

Foam formation after five minutes of sample preparation was one of the challenges encountered while using ultraturrax. Therefore, selecting an appropriate container for ultraturrax was crucial. To minimize the discrepancy between ultraturrax and sonication processes, the sample volume was fixed at 30 mL. The T25 dispersing tool was suitable for samples ranging from 1 mL to 2 L, with a speed variation from 3,000 rpm to 25,000 rpm and a shaft length of 160 mm. The immersion of samples up to the O-ring (around 50mm from bottom) in the shaft ensured a better emulsion by facilitating the effective mixing of samples in the solution. A glass tube with a diameter of 14 cm and a length of 18.5 cm was chosen to balance the glassware for this sample volume. The speed was set at 13,000 rpm, as higher speeds would result in more foam formation and sample overflow during the emulsion. For sonication, a 100 mL beaker was adequate for the effective immersion of the probe and the prevention of foam overflow. Foam produced by ultraturrax was always much higher than sonication.

Preparation of samples with homogenization as a pre-emulsion				
Preparation	Size	PDI	Zeta potential	Loading Capacity
Homogenisation	365 ± 50	0.26 ± 0.01	-24.0	37
Homogenization followed by sonication	250 ± 10	0.05 ± 0.01	-28.0	45

Chapter 4

Article

Central Composite Design of Experiment Based Optimization of Key Formulation Parameters for PFCE-Encapsulated PLGA Nanoparticles

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Abstract

Physicochemical properties, such as particle size, homogeneity, charge, and drug loading, can be markedly influenced by different formulation parameters during the nanoparticle (NP) preparation process. Selecting the appropriate parameter conditions for NP formulation via the traditional single-parameter approach is a demanding process that often requires significant investment of time, effort, and financial resources, and it may not always yield successful outcomes. The statistical analysis based on central composite design (CCD) has garnered widespread interest across various scientific disciplines due to its ability to elucidate the influence of parameters, construct mathematical models, and forecast the optimal combination of factors while reducing the number of required experiments. In this study, we used a three-factor, five-level CCD design to optimize the preparation of poly(lactic-co-glycolic acid) (PLGA) NPs to encapsulate a fluorinated crown ether (perfluoro-15-crown-5-ether, PFCE), a contrast agent that can enhance the signal intensity and contrast of ^{19}F fluorine magnetic resonance imaging (^{19}F MRI), formulated using sonication. After preliminary trials, the surfactant concentration, PFCE volume, and sonication time were selected as independent variables to study effect on particle size (PS), polydispersity index (PDI), zeta potential (ζ), loading capacity, signal to noise ratio (SNR) and relaxation time T_1 and T_2 . The optimized model of the formulation of PLGA-PFCE NPs was thus validated.

1. Introduction

1.1 Fluorine Molecule Encapsulation in a Biocompatible PLGA Carrier System

Poly (lactic-co-glycolic acid) (PLGA) has been extensively researched as a carrier system for various hydrophobic and hydrophilic drugs. The ability to modify the polymer adds an advantage, making it one of the most studied carrier systems for drug delivery platforms [1]. Perfluorinated (PFC) molecules, known for their simultaneous hydrophobic and lipophobic nature, have been used as a contrast agent for Fluorine 19 magnetic resonance imaging (^{19}F MRI), necessitating a carrier system for effective action [2]. Current platforms under study mainly include lipids and polymers. The formulation of PFC into nanocarriers, like any drug into a carrier system, involves various factors that influence the outcome, some of which are controllable while others are not. In the simple PLGA-PFC system, factors that can affect the outcome include the type and ratio of PLGA, the type and concentration of surfactant, the type and amount of solvent, the temperature and speed of emulsification, the size and distribution of NPs, the stability and dispersibility of the nanocarriers, and the loading and release of the ^{19}F contrast agent, as shown in the fishbone diagram (figure 1). Several process variables/factors influence the physicochemical properties of nanocarriers, such as size, uniformity, and drug loading efficiency. These can affect the quality and performance of the ^{19}F MRI contrast agents, including signal intensity, signal to noise ratio (SNR), spatial resolution, specificity and sensitivity, and toxicity or biocompatibility.

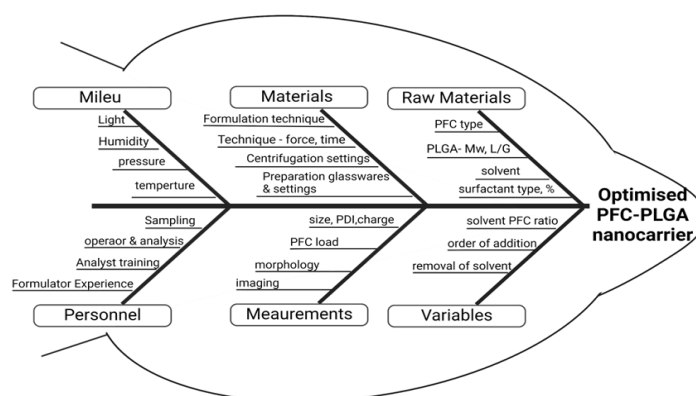


Figure 1. An optimization study of PFC formulation using Ishikawa/fishbone diagram to identify the critical parameters.

1.2 Experimental Overview – Introduction to Design of experiment (DoE)

The separate control variable method, a common method to optimize process parameters in NP preparation, adjusts one variable at a time (known as ‘one-factor-at-time’ or OFAT method) and keeps other parameters constant to understand the experimental result or observation (known as the ‘response’) [3]. To study the effect of a factor (also known as the ‘input variable’) on the response, two or more values of the factor are used, referred to as levels or settings. The input variables are referred to as independent variables. A flow chart like a fishbone diagram (figure 1) can be used to identify potential factors. However, the OFAT can give reliable results as long as the mixture of different variables does not affect each other/there is no interaction between the factors. Compared to the OFAT approach, the computer-assisted statistical design of experiment (DoE) optimization approach allows the development of a factorial design by varying all levels simultaneously to determine the relationship/interactions between different controllable factors (Xs) affecting a process and the behavior/output of that process (Ys), also being able to study interaction effects between the input variables.

Statistical experimental design, along with the basic ideas underlying DoE, was born in the 1920s from the work of Sir Ronald Aylmer Fisher. Regression analysis is about determining how changes in the independent variables are associated with changes in the dependent variable [4]. A regression model is a statistical model that measures how one dependent variable change with one or more independent variables and forecasts the future values of the dependent based on the current values of the independent variables. The relationship between the variable and the response is referred to as the response surface, which can be discrete or continuous. Response surface methodology (RSM), introduced by Box and Wilson, is based on experimental results to fit empirical models whose purpose is to link response values and input process parameters.

A variety of DoE methodologies, including Plackett–Burman, fractional factorial design, and full factorial design, Central Composite Design (CCD) and the Box–Behnken Design (BBD) are accessible to the experimental designer (figure 2) [3]. The selection of the appropriate DoE technique is contingent on the problem under investigation and the objectives of the experiment. In the field of pharmaceuticals, RSM techniques, specifically the CCD and the BBD, are predominantly used for system analysis. The BBD approach, when considering a three-variable experimental space x - y - z (as shown in figure 2), involves conducting experimental runs at intermediate points, excluding the edges of the space, thereby forming a response surface with three levels. On the other hand, the Box-Wilson Central Composite Design, often referred to as CCD, is composed of three components: factorial points, axial or star points, and centre points, necessitating a five-level design (refer figure 2). In addition to the factorial points located at the corners of the experimental space, the axial or star points are symmetrically positioned on the axes of the coordinate system (figure 2), relative to the central point at a distance α from the design centre, thereby considering runs at extreme levels. Several studies demonstrated that CCD had superior performance in terms of prediction and model fitting [5, 6].

In the exploration of PLGA systems with various drugs, several studies have utilized the DoE approach. The CCD method was employed to study the optimized system for the encapsulation of flurbiprofen in PLGA nanospheres for ocular application [7]. A three-factor, five-level system was used to investigate the effects of drug concentration, surfactant concentration, and pH value of the aqueous phase on the size, ζ , and entrapment efficiency. Similarly, the CCD method was also used to study the encapsulation of fluorometholone for the treatment of eye inflammatory disorders [8, 9], as well as the encapsulation of cilnidipine [9], riluzole [10], and ovalbumin protein [11]. These studies primarily involved investigating the concentration of PLGA polymer, surfactant concentration, drug concentration, and, in some cases, the pH of the solution. The main focus was to study their effects on size, PDI, ζ , and encapsulation efficiency. The specifics of these studies vary. Similarly, numerous studies have reported the use of the BB model. For instance in one study, for the encapsulation of bovine serum albumin, five independent factors were evaluated: surfactant concentration, PLGA concentration, drug

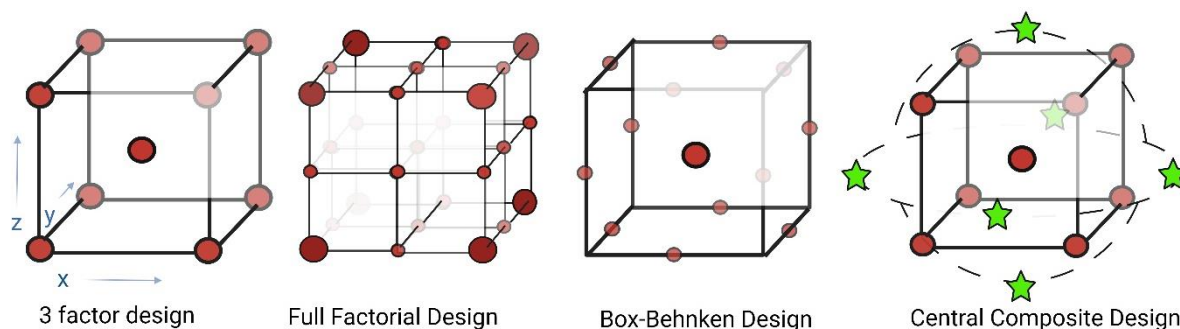


Figure 2 .Comparative Analysis of Experimental Design Methods: Full Factorial, Box-Behnken, and Central Composite Designs

concentration, polymer-drug ratio, and preparation time to understand their effect on the size of the PLGA NPs [12], while in another study a three-factor design was used to assess its encapsulation [13]. The encapsulation of sildenafil citrate was determined using a three-factor BB design [14], lorazepam with a four-factor design [15], curcuminoid with a three-factor design [16, 17], astaxanthin with a four-factor design [18], coffee with a four-factor design [19], topotecan with a three-factor design [20], and curcumin and paclitaxel together with a three-factor design [21]. Similar to the CCD, these studies primarily focused on the concentration of the drug and surfactant. Other factors that were varied include the examination of the volume ratio of drug to polymer or the study of the preparation time.

Our research involved utilizing perfluoro 15 crown 5 ether (PFCE) to examine its encapsulation within the PLGA polymer matrix. We opted for CCD over the OFAT method due to its ability to yield more precise conclusions with fewer experiments. To explore the optimal formulation condition, we utilized RSM to assess the relationship between factors and responses with a minimal number of experiments and employed CCD for result analysis. To the best of our knowledge, no prior CCD studies have been conducted for the optimization of PFCE-loaded PLGA NPs to understand the most optimal conditions for producing NPs with desired traits including lower size and PDI, higher ζ and loading capacity. This was accomplished by conducting 20 independent experiments using a solvent evaporation emulsification technique. The particle size, PDI, Zeta potential (ζ), loading capacity, signal to noise ratio (SNR) and relaxation time T_1 and T_2 were then measured for each formulation.

2. Material and Methods

Resomer® RG 502 H, acid terminated, lactide: glycolide 50:50, Mw 7,000-17,000, Polyvinyl alcohol (PVA) (average Mw 9,000-10,000, 80% hydrolysed), deuterium oxide, were purchased from Sigma-Aldrich. Dichloromethane (DCM, CH_2Cl_2 , Mw 84,93 g/mol) used to prepare the NPs, was obtained from CarloErba (Milan, Italy). The contrast agent-loaded into the NPs is perfluoro-15-crown-5-ether; 98% (PFCE) ($\text{C}_{10}\text{F}_{20}\text{O}_5$, Mw 580,07) and was supplied by abcr GmbH (Karlsruhe, Germany).

2.1 PLGA Polymer preparation

2.1.1. Sonication

PFCE encapsulated PLGA NPs was formulated by PFCE/O/W single emulsion solvent evaporation method as described by Swider *et al.* [22] with slight modifications. The process of formulating PFCE-loaded PLGA via sonication is depicted in Figure 3. In summary, a mixture of 900 μ l PFCE and 3 ml DCM, containing 90 mg of PLGA polymer, was manually stirred using a pipette to form the PFCE/O phase. This mixture was then incorporated into 25 ml of a 2% *w/v* PVA water solution and sonicated using a Sonicator (Qsonica, Newtown, CT, US) with a 6 mm sonicator tip for 3 minutes at 40% amplitude. This process was conducted in an ice bath to create the PFCE/O/W phase. the organic DCM solvent was eliminated by magnetic stirring (using IKA® RCT basic ikamag™, Staufen, Germany at 400 rpm) for overnight evaporation, facilitating the solidification of NPs. Following this, the NPs were separated by centrifugation (high speed micro-centrifuge, D3024R, Scilogex, Rocky Hill, CT, USA) at 8,000 g for 30 min at 4°C. To remove PVA and any unformed polymers from the NP formulation, the NPs were then rinsed with deionised (DI) water and each rinse was followed by a centrifugation step. The NPs were then frozen with liquid nitrogen, lyophilized (using a Freeze dryer - FreeZone, Labconco, Kansas City, MO, USA) for 15-20h, and preserved at -20°C.

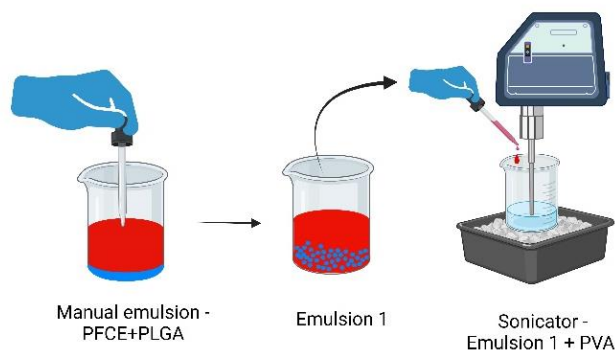


Figure 3: Formulation of PFCE-Encapsulated PLGA NPs via double emulsion solvent evaporation method utilizing ultrasonic sonicator

2.2 Characterization of NPs

2.2.1. Size, polydispersity index (PDI) and Zeta Potential (ζ)

The particle size (size) and PDI of NPs were measured using dynamic light scattering (DLS) technique on the Zetasizer Nano (DLS Zetasizer Nano S90, Malvern Instruments (Worcestershire, UK)). PDI was measured to determine the distribution/ uniformity of particle size. Small PDI values approaching zero indicate monodisperse particles while large values (>0.4) indicate heterogenous particles. Size and PDI of NPs were measured by dispersing the formulation in DI water at 25°C and an angle of 90°. ζ was determined using the same instrument in a capillary zeta cell and the measurements were performed at 25°C. All samples were measured in triplicate and the average values and standard deviation of the measurements were calculated.

2.2.2. Loading Capacity and relaxation time (T_1 & T_2) Measurements

PFCE load content and T_1 and T_2 relaxation time of PLGA NPs was determined by Fluorine-19 nuclear magnetic resonance spectroscopy (^{19}F -NMR) using Varian Mercury plus 500, Crawley, UK. 5 mg of lyophilized NPs were dissolved in deuterium oxide (D_2O) to achieve a concentration of 10 mg/mL. Trifluoroacetic acid (TFA) at a concentration of 0.1 M was added from the stock solution to serve as an internal standard. The quantity of perfluorocarbon (PFCE) was determined by calculating the integration ratio of the PFCE peak to the TFA peak. Fluorine contents were calculated according to the equation 1:

$$\text{Loading capacity (wt/wt\%)} = \frac{\text{Mass of PFCE in gram}}{\text{Mass of NPs in gram}} * 100 \quad (1)$$

2.2.3 ¹⁹F MRI Signal to noise ratio (SNR) acquisition and data processing

All MRI scans were performed on a 9.4T ultra shielded MR scanner (Bruker Avance Neo) with a 25mm resonator tunable to both ¹H (linear) and ¹⁹F (quadrature) located in the Institute for Molecular Cardiology at Heinrich Heine University (HHU), Düsseldorf. 25 mg of the lyophilised sample was resuspended in deionised water for the measurements. ¹⁹F scans of phantoms were acquired with RARE sequence (Rapid acquisition with relaxation enchantment), size = 128x128, FOV = 30x30 mm², slice thickness = 3mm, TE = 9.78ms, TR = 2500, Avg = 60, RARE factor = 32, Bandwidth = 15151,5 Hz

2.3. Statistical analysis

A three-factor, five-level CCD statistical design was employed to optimize the process and formulation parameters in preparation of PLGA-PFCE NPs and evaluate main effects, interaction effects, and quadratic effects of the process parameters on the prescription attributes assessed included the particle size (PS), PDI, ζ , loading capacity, SNR and relaxation time T_1 and T_2 . The design was used to explore quadratic response surfaces and constructing second-order polynomial models and contour plots to predict responses with Design Expert (Version 8.0.3, Stat-Ease Inc., Minneapolis, MN). Variance analysis (ANOVA) was used to verify the polynomial equation generated after analysis, and then determine the significance of the regression coefficient. Finally, 2D and 3D plots were generated to provide a graphical representation of the model. CCD method utilizes a combination of variables at low, medium, and high levels, along with axial points, to achieve the desired outcome. The selected variables were studied at five levels of coded values as $-\alpha$, -1, 0, 1, and $+\alpha$. The value for alpha (± 1.682) was calculated to fulfil both rotatability and orthogonality in the design. Three factors, forming the independent variables, were utilized to optimize the formulation, and the dependent variables were employed to analyze the outcomes. These are detailed in *table 1*. The experimental design comprised 20 runs, as depicted in *table 2*. Quantitative effect of independent variables at different levels on the dependent response was investigated by using the polynomial equations generated by the model. RSM was used for experimental design, and CCD was used for optimization.

Table 1. Overview of the selected three independent (X_1 , X_2 , X_3) and seven dependent variables ($Y_1, Y_2, Y_3, Y_4, Y_5, Y_6, Y_7$) chosen for the CCD model

Variables	Unit	Levels				
		Low axial (- α)	Low (- 1)	Central (0)	High (+ 1)	High axial (+ α)
Independent						
X_1 = Sonication time	min	1	3	6	9	11.05
X_2 = PVA	w/v %	1	2	3	4	4.7
X_3 = PFCE volume	μ l	95.46	300	600	900	1104.5
Dependent		Constraints				
Y_1 = Particle Size (PS)	(nm)	Minimise				
Y_2 = Polydispersity Index (PDI)	-	Minimise				
Y_3 = Zeta Potential (ζ)	(mV)	Maximise				
Y_4 = Loading Capacity	(w/w %)	Maximise				
Y_5 = Signal to noise ratio (SNR)		Maximise				
Y_6 = T_1 Relaxation Time	(ms)	-				
Y_7 = T_2 Relaxation Time	(ms)	-				

2.3.1. Contour plots and response surface plots

To understand the main and the interaction effects of two variables, response surface plots were used as a function of two factors at a time, maintaining the third factor at fixed level. In each plot the interaction of two variables was investigated at the same time, while the third one was in its middle level value. These plots were generated by calculating the values for one variable while systematically varying a second variable, under the constraint of a fixed (Y) value. Subsequently, the contour maps (2D) and response surface maps (3D) were generated by the software to understand the relationships between variables and their interactions. Contour plots are a diagrammatic representation of the values of the response. The RSM and contour plots can be obtained by employing the regression equation, and the impact of each variable on the response variables can be studied effectively.

Table 2. Overview of the runs for the optimisation of the nanosystem using the CCD modal

Run	Time (min) - Factor X ₁		PVA (%v/v) - Factor X ₂		PFCE (μL) - Factor X ₃	
	X ₁	Notation	X ₂	Notation	X ₃	Notation
1.	6	0	3	0	600	0
2.	11.05	+ α	3	0	600	0
3.	6	0	3	0	600	0
4.	6	0	3	0	95.46	- α
5.	9	+ 1	2	- 1	900	+ 1
6.	9	+ 1	4	+ 1	300	- 1
7.	3	- 1	2	- 1	300	- 1
8.	3	- 1	2	- 1	900	+ 1
9.	6	0	3	0	600	0
10.	6	0	3	0	600	0
11.	1	- α	3	0	600	0
12.	6	0	3	0	1104.5	+ α
13.	3	- 1	4	+ 1	300	- 1
14.	6	0	3	0	600	0
15.	6	0	3	0	600	0
16.	9	+ 1	4	+ 1	900	+ 1
17.	6	0	1	- α	600	0
18.	6	0	4.7	+ α	600	0
19.	3	- 1	4	+ 1	900	+ 1
20.	9	+ 1	2	- 1	300	- 1

Table 3. Comparative analysis of experimental and predictive data from the CCD. The observed values are the values obtained from the experiment, and the predicted values are the values obtained from the software predicting the values from the input variables

Independent Variables				Dependent Variables													
Run	X ₁	X ₂	X ₃	Y ₁		Y ₂		Y ₃		Y ₄		Y ₅		Y ₆		Y ₇	
				Observed /(Obs)	Predicted /(Pre)	Obs	Pre	Obs	Pre	Obs	Pre	Obs	Pre	Obs	Pre	Obs	Pre
1.	0	0	0	265 ± 4	274	0.11 ± 0.00	0.17	-31.0	-28.0	49	42	72	77	783.4	785.3	267.6	257.4
2.	+ α	0	0	318 ± 4	306	0.27 ± 0.01	0.26	-29.0	-29.0	34	44	62	61	780.4	779.8	259.9	254.0
3.	0	0	0	270 ± 4	274	0.19 ± 0.02	0.17	-29.0	-28.0	52	42	82	77	806.9	785.3	272.8	257.3
4.	0	0	- α	248 ± 4	234	0.21 ± 0.01	0.24	-26.0	-26.0	31	38	39	45	800	797.4	273.8	262.5
5.	+ 1	- 1	+ 1	274 ± 4	284	0.22 ± 0.02	0.24	-27.0	-28.0	45	42	68	77	778.2	777.4	254.2	248.9
6.	+ 1	+ 1	- 1	304 ± 3	305	0.27 ± 0.03	0.25	-25.0	-25.0	47	42	51	54	802.9	794.5	256.4	264.9
7.	- 1	- 1	- 1	177 ± 2	189	0.15 ± 0.00	0.13	-23.0	-29.0	28	27	50	53	783.1	784.0	246.3	244.6
8.	- 1	- 1	+ 1	190 ± 3	252	0.12 ± 0.01	0.16	-25.0	-25.0	40	47	62	65	769.0	785.5	258.7	252.8
9.	0	0	0	297 ± 4	274	0.23 ± 0.04	0.17	-30.0	-28.0	53	42	84	77	781.4	785.3	257.3	257.4
10.	0	0	0	267 ± 5	274	0.16 ± 0.00	0.17	-28.0	-28.0	24	42	67	77	777.1	785.3	228.3	257.4
11.	- α	0	0	216 ± 1	209	0.06 ± 0.00	0.10	-30.0	-27.0	43	54	69	72	789.7	778.7	261.6	263.9
12.	0	0	+ α	275 ± 5	271	0.20 ± 0.04	0.17	-26.0	-26.0	47	38	85	80	786.2	777.4	262.4	270.0
13.	- 1	+ 1	- 1	217 ± 3	220	0.16 ± 0.01	0.15	-29.0	-27.0	44	48	89	79	776.2	785.0	263.1	272.0
14.	0	0	0	272 ± 1	274	0.15 ± 0.01	0.17	-27.0	-28.0	42	42	86	77	790.8	785.3	268.4	257.4
15.	0	0	0	260 ± 11	274	0.15 ± 0.02	0.17	-25.0	-28.0	34	42	72	77	770.2	785.3	249.2	257.4
16.	+ 1	+ 1	+ 1	262 ± 7	286	0.09 ± 0.00	0.13	-31.0	29.0	19	21	87	84	762.2	769.3	261.2	265.5
17.	0	- α	0	293 ± 6	268	0.22 ± 0.03	0.21	-32.0	-31.0	11	15	79	78	792.4	780.6	225.2	231.5
18.	0	+ α	0	288 ± 1	296	0.13 ± 0.04	0.13	-29.0	-30.0	21	15	104	106	774.3	774.6	279.3	269.3
19.	- 1	+ 1	+ 1	281 ± 5	275	0.11 ± 0.01	0.12	-31.0	-31.0	14	24	104	110	770.1	768.6	279.8	277.0
20.	+ 1	- 1	- 1	276 ± 6	295	0.30 ± 0.02	0.30	-33.0	-33.0	27	18	73	66	775.2	784.8	238.8	244.2

Notes X₁= Sonication time ; X₂ = PVA ; X₃ = PFCE volume ; Y₁ = Particle Size ; Y₂= polydispersity index (PDI), Y₃= Zeta Potential (ζ), Y₄= Loading Capacity, Y₅= Signal to noise ratio (SNR), Y₆= T₁ Relaxation Time, Y₇= T₂ Relaxation Time

3. Results and Discussion

3.1 Analysis of Variable Parameters

In simple linear regression, the relationship between the dependent and independent variables is assumed to be linear. However, as previously stated, some processes exhibit a non-constant relationship where the effect of one independent variable on the dependent variable may change based on the level of another independent variable. This indicates an interaction between the independent variables that collectively impact the dependent variable. In such scenarios, it is essential to employ a polynomial interaction model. A model that incorporates both polynomial and interaction terms - specifically, a model with terms up to the second degree - can effectively capture these complex relationships. This is exemplified by the model presented as *equation 2* [23].

$$Y_i = \beta_0 + \beta_1 X_{i1} + \beta_2 X_{i1}^2 + \beta_3 X_{i2} + \beta_4 X_{i2}^2 + \beta_5 X_{i1} X_{i2} + u \quad (2)$$

where Y_i = the response of the variable

β_0 = the y-intercept of regression equation (value of y when all other parameters are set to 0)

$\beta_1 X_{i1}, \beta_3 X_{i2}$ – Linear term

$\beta_2 X_{i1}^2, \beta_4 X_{i2}^2$ – Quadratic term

$\beta_5 X_{i1} X_{i2}$ – Interaction Terms

u = random error

The results from the regression analysis, which is a statistical method used to examine the relationship between a dependent variable and one or more independent variables, show a response surface model for the different response variables. The regression analysis presents first a coefficient value, that signifies how much the mean of the dependent variable changes given a one-unit shift in the independent variable, while holding other variables in the model constant, that allows to assess the effect of each variable in isolation from the others. The sign of a coefficient apprises of a positive or negative correlation between each independent variable and the dependent variable. A positive coefficient indicates that as the value of the independent variable increases, the mean of the dependent variable also tends to increase, and a negative coefficient suggests that independent variables have inverse relationship to dependent variable.

The regression analysis then calculates the t -statistic and p -value for each regression coefficient in the model. The t -statistics, also known as the t -values, are calculated by dividing the coefficient by the standard error (Std. Error). They measure the number of standard deviations by which the coefficient differs from zero. The larger the absolute value of the t -statistics, the stronger the evidence that the coefficients are not zero. The p -value for the t -statistic in a regression model denoted by $Pr(>|t|)$ tests the null hypothesis that the coefficient is equal to zero (no effect). The null hypothesis typically states that the regression coefficient of an independent variable is zero, implying no effect of the independent variable on the dependent variable. A low p -value suggests that the null hypothesis is implausible and follows that the independent variable has a significant influence on the dependent variable [24]. A conventional significance level is 0.05, the lower the p -value is, the stronger the evidence against the null hypothesis and the higher the confidence that the result is not a random occurrence [25]. The intercept (often called the “*constant*”) in a regression model represents the mean value of the response variable when all of the predictor variables in the model are equal to zero.

The subsequent step in the analysis is based on the multiple R -squared and adjusted R -squared values that show how well the model fits the data [26, 27]. R -squared value is a statistical measure that gives information about the proportion of the variance in the dependent variable that can be predicted from the independent variables. In short, the R -squared values indicate how well the model fits the data, by measuring the proportion of the variation in the response variable that is explained by the model. While the R -squared value increases as we add more predictors (like X_1, X_2, X_3) to our model, the adjusted R -squared compensates for the addition of predictors. It only increases if the new predictor improves the

model more than would be expected by chance, and it can decrease if the predictor improves the model less than expected by chance. It is always lower or equal to the R -squared value. The closer these values are to 1, the better our model is at making predictions. F -statistic associated with R -squared and adjusted R -squared values is a value that indicates if the model provides a better fit to the data than a model with no independent variables. In other words, it gives information if the independent variables we have included in our model, those that one assumes can affect the outcome, are actually helpful in making accurate predictions. It does it by comparing the amount of the variance between groups in the data (which reflects the effect of the independent variables) to the variance within groups (which is attributable to random error). If the means of these groups are significantly different, as indicated by the $Pr(>F)$ value, it suggests that the independent variables have a significant effect. The p -values of the F -statistics, $Pr(>F)$ indicate whether the model is significantly better than the null model that assumes no relationship between the response variable and the predictor variables.

The analysis of variance (ANOVA) table delineates the distribution of the degree of freedom (Df), sum of squares (Sum Sq), mean squares (Mean Sq), F -values, and p -values for the disparate constituents of the model [27, 28]. ANOVA results provide statistical test that breaks down the total variability in the data into different components. The degree of freedom (Df) is used to calculate the sum of squares and mean squares which in turn helps in the calculation of F -value. The first order (FO), two-way interaction (TWI), and pure quadratic (PQ) effects are scrutinized independently, in addition to the residuals. FO (X_1, X_2, X_3) symbolizes the primary effects of the independent variables. TWI (X_1, X_2, X_3) symbolizes the two-way interaction effects of the factors and PQ (X_1, X_2, X_3) symbolizes the quadratic effects of the factors. The lack of fit test juxtaposes the pure error with the lack of fit error and evaluates whether the model sufficiently encapsulates the curvature in the data. Residuals are the discrepancies between the observed (actual) and predicted values. F -value in an ANOVA analysis gives information about the differences between the means of the groups are significant or if they are likely due to chance. The lack of fit test evaluates whether the model fits the data well. The p -values of the lack of fit tests indicate whether there is evidence that the model does not fit the data well, by comparing the lack of fit mean square with the pure error mean square. Pure Error is a measure of the variability in the data that cannot be explained by the model. It represents the random variation or noise in the data. The smaller the pure error, the better the model fits the data. A non-significant lack of fit (p -value > 0.05) insinuates that the model adequately represents the data.

The relationship between two parameters at the centre level of the third parameter is shown visibly in the plots. The important part in regression analysis is the contour plots, and 3D plots were used to determine the relationship between the independent variables and response named as response surface plots [29]. A contour plot is a graphical technique that displays the relationship between two independent variables and a response variable in a two-dimensional format by representing a 3-dimensional surface by plotting constant z slices, called contours, on a 2-dimensional format. The X and Y values are displayed along the x - and y -axes, while contour lines and bands represent the z value. Contour lines connect points that have the same response value, and coloured contour bands represent ranges of the response values. The final part is the comparing the values obtained by the experiment and that the model predicts for the given values of the variables.

For the optimization of PLGA-PFCE NPs, CCD was used as the DoE. The three most effective independent variables found by running initial experiments were sonication time (3–12 min) and percentage of PVA (2 – 4%) in emulsification, and PFCE (300–900 μ L) volume. All responses observed were fitted to linear, second order and quadratic models, and were evaluated in expressions of statistically significant coefficients p -values and R -squared values. In order to graphically show the relationship and interactions between the variables and the response, contour plots and three-dimensional surface plots were used in this study. NPs were organized using the optimal formulation, and resultant experimental values of the responses were quantitatively compared with the predicted values for calculating the percentage of the predicted error. Predicted error is the variation between the

experimental value and the predicted value per predicted value. Validation of the optimization procedure was demonstrated for predicted errors lower than 5%.

3.2. Response variable: Size

The results from the regression analysis for the dependent variable (size) and independent variables X_1 (Time), X_2 (PVA), and X_3 (PFCE) are shown in *table 4*. The model is a second-degree polynomial that includes linear, quadratic, and interaction terms. The coefficients table shows the estimated values, standard errors (Std. Error), t -values, and p -values of each term in the model. The intercept 274.19 that represents the expected mean value of 'sizes' when all independent variables (X_1 , X_2 , X_3) are zero and it is very significant. The coefficients of the model represent the effect of each factor on the response variable. Here X_1 and X_3 has a positive effect on sizes (coefficient = 28.9878 and 11.0427 respectively), meaning that as X_1 / X_3 increases, sizes also increase. A small p -value (< 0.05) indicates that one can reject the null hypothesis. X_1 , X_3 and X_1 : X_3 are very significant predictors as they have p -values less than 0.01. Interaction effects occur when the effect of one variable depends on the value of another variable. The estimate for X_1 : X_3 is -18.5000, This suggests that the effect of X_1 on size is not constant but varies with the level of X_3 , and vice versa. Specifically, for each unit increase in the product of X_1 and X_3 , the sizes variable decreases by 18.5000, assuming all other variables are held constant. From the regression analysis quadratic second-order polynomial model was fitted with the equations 3 and 4 as follows is:

$$Y_1 = \beta_0 + \beta_1 X_1 + \beta_3 X_3 + \beta_5 (X_1 * X_3) \quad (3)$$

$$\text{or} \quad \text{Size} = 274.19 + 28.98 \text{ time} + 11.04 \text{ PFCE} - 18.50 (\text{time} * \text{PFCE}) \quad (4)$$

Table 4 : The coefficients table for the dependent variable size that gives quantitative relation among the DoE factor and the responses.

	Coefficient	Std. Error	t value	Pr(> t)	
(Intercept)	274.1956	6.6631	41.1512	1.720e-12	***
Time (X_1)	28.9878	4.4289	6.5452	6.512e-05	***
PVA (X_2)	8.2514	4.4216	1.8661	0.091587	.
PFCE (X_3)	11.0427	4.4214	2.4976	0.031579	*
X_1 : X_2	-5.2500	5.7767	-0.9088	0.384824	
X_1 : X_3	-18.5000	5.7767	-3.2025	0.009451	**
X_2 : X_3	-1.9250	5.7767	-0.3332	0.745839	
X_1^2	-5.9843	4.3314	-1.3816	0.197174	
X_2^2	2.6911	4.3045	0.6252	0.545842	
X_3^2	-7.7093	4.3036	-1.7914	0.103495	

Significant values are marked with an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Regarding 'size', the model explains about 87% of the variation in the response variable, as indicated by the multiple R -squared value presented in Table 5. After adjusting for the number of predictors, the adjusted R -squared value is 75.99%, indicating that about 75.99% of the variability in 'size' can be explained by the predictors. The F -value of 17.4687 and the extremely small p -value (0.0002662) (table 6) for FO (X_1 , X_2 , X_3) suggest that at least one of the linear effects is statistically significant, as already observed with the X_1 / X_3 variable. The F -value of 3.7310 and the p -value of 0.0492517 (table 6) for TWI (X_1 , X_2 , X_3) suggest that at least one of these interaction effects is statistically significant at the 0.05 level (validates the X_1 : X_3 interaction). The F -value of 1.8405 and the p -value of 0.2035908 (table 5) suggest that these quadratic effects are not statistically significant at the 0.05 level. F -value associated with lack of fit is 2.3557, and the p -value is 0.1843623, which is greater than 0.05, suggesting that the lack of fit is not statistically significant, indicating the adequacy of the model. This implies that the influence of the independent variables on the dependent variable is substantial compared to random error, and at least one of the independent variables significantly affects the dependent variable. The sum of squares for

the pure error is 795.6, and the mean square is 159.1 that suggests that the model fits the data reasonably well. Table 13A (contour) and 13B (3D plot) presents a response surface plot for 'size', illustrating a curvilinear regression between 'time' and 'PFCE'. A curvilinear effect indicates a relationship between two or more variables that is not linear but forms a curve when graphed. As evident from the surface plot, an increase in both 'time' and 'PFCE' volume leads to an increase in particle size. Upon examining table 3, which juxtaposes the experimentally derived values of all variables with those predicted by the model, it is evident that the model's estimations of sizes align closely with the actual measurements in the majority of instances.

Overall, for the size, the model is significant and explains 87% of the variation in size. The significant effects are the linear effect of PVA (X_2), the linear effect of PFCE (X_3), and the interaction effect of time and PFCE ($X_2:X_3$). Increasing time and PFCE increases size, but the interaction effects are negative, which means that the effect of time on size depends on the level of PFCE, and vice versa, and that the effect of PFCE on size is not linear.

Table 5. Summary of the regression analysis for all the output parameters.

	Variable	Multiple R-squared	Adjusted R-squared	F-statistic (on 9 and 10 Df)	p-value
1.	Size	0.8736	0.7599	7.680	0.0019
2.	PDI	0.8028	0.6254	4.524	0.0136
3.	Zeta Potential (ζ)	0.7127	0.4541	2.756	0.0650
4.	Loading Capacity	0.6642	0.3620	2.198	0.1179
5.	Signal to Noise Ratio (SNR)	0.8714	0.7557	7.531	0.0020
6.	T_1 relaxation time	0.3536	-0.2282	0.607	0.7668
7.	T_2 relaxation time	0.5215	0.0908	1.211	0.3826

Table 6. Analysis of Variance table (ANOVA) for size

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
FO (X_1, X_2, X_3)	3	13990.5	4663.5	17.4687	0.0002662
TWI (X_1, X_2, X_3)	3	2988.1	996.0	3.7310	0.0492517
PQ (X_1, X_2, X_3)	3	1474.0	491.3	1.8405	0.2035908
Residuals	10	2669.6	267.0		
Lack of fit	5	1874.1	374.8	2.3557	0.1843623
Pure error	5	795.6	159.1		

3.3 Response variable: polydispersity index (PDI)

For the PDI variable output from the coefficients suggests that the all the 3 independent variables having significant effect, very highly significance ($p < 0.001$) for the time (X_1) effect and significant PVA and PFCE ($p < 0.04$) linear effects (table 7). No significant quadratic or interaction effect were observed in PDI (full data is given in the supplementary). The coefficient for X_1 is 0.0474522, which means that for every unit increase in X_1 , the response PDI increases by 0.0474522 units, assuming X_2 and X_3 are held constant. From the multiple R squared value (table 5), 80.28% of the variability in PDI can be explained by the input independent variable. The goodness of fit given by adjusted R-squared value is 62.54%. F-value of 4.524 with associated $p < 0.01$ value signifying there is at least one predictor variable that significantly influences the outcome variable. From the ANOVA results from table 8 for PDI, as already implied from the coefficient table 7, first order effects (FO (X_1, X_2, X_3)) are very significant ($p < 0.001$), whereas 2-way interactions and quadratic interactions do not contribute to the variability in the response. The lack of fit test suggests that the model fits the data reasonably well, as the p -value is not significant ($0.633614 > 0.05$). The model explains a substantial portion of the variability in PDI, but there is still some unexplained variability as can be understood from the residuals. The models predictions

are reasonably close to the actual PDI (table 3). Overall, for PDI the model is significant and explains 80% of the variation in PDI. The significant effect is the linear effect of time (X_1), the linear effect of PVA (X_2), and the linear effect of PFCE (X_3). Increasing the time results in an increase in PDI, while there is an inverse effect observed with PVA and PFCE.

The regression analysis yielded a quadratic second-order polynomial model, which is represented by the equations 5 and 6:

$$Y_2 = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 \quad (5)$$

$$PDI = 0.166 + 0.047 \text{ time} - 0.023 \text{ PVA} - 0.022 \text{ PFCE} \quad (6)$$

Table 7 . Model Coefficients for PDI

	Coefficient	Std. Error	t value	Pr(> t)	Significance
(Intercept)	0.1668077	0.0150443	11.0878	6.122e-07	***
Time (X_1)	0.0474522	0.0099997	4.7454	0.0007857	***
PVA (X_2)	-0.0230498	0.0099833	-2.3088	0.0435936	*
PFCE (X_3)	-0.0229269	0.0099828	-2.2966	0.0445075	*

Significant values are marked with an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

3.4. Response variable: Zeta potential (ζ)

The interaction effects of the independent variables were the main contributors to the variability in ζ (table 9). Specifically, the interactions of time and PVA, and of PVA and PFCE, had a significant positive (1.4) and negative (-2.17) impact on ζ , respectively. No linear or quadratic effects of the independent variables were observed, which was consistent with the ANOVA results (table 8), that showed only the two-way interaction factor to be significant ($p < 0.01$ for TWI(X_1 , X_2 , X_3)). The lack of fit test suggested a reasonable fit of the model to the data, as the p -value was not significant ($0.52937 > 0.05$). The model accounted for about 71% of the variation in the response, as indicated by the R -squared of 0.7127 (table 5). However, the model was only slightly better than the null model, as the p -value of the F -statistic was 0.06501. Figure 13C & 13D shows the response surface plot for the factors time and PVA, and for the factors PVA and PFCE. In general, for ζ , the model is significant and explains 71% of the variation in ζ . The significant effects are the interaction effect of time and PVA ($X_1:X_2$), the interaction effect of PVA and PFCE ($X_2:X_3$) which means that the effect of time on ζ depends on the level of PVA and effect of PVA on ζ depends on the PFCE volume.

Based on regression analysis, a quadratic second-order polynomial model has been formulated, which is expressed by equations (7) and (8)

$$Y_3 = \beta_0 + \beta_4 (X_1:X_2) + \beta_6 (X_2:X_3) \quad (7)$$

$$\zeta = -27.83 + 1.44 (\text{Time} \times \text{PVA}) - 2.17 (\text{PVA} \times \text{PFCE}) \quad (8)$$

Table 9. Model Coefficients for Zeta Potential (ζ)

	Coefficient	Std. Error	t-value	Pr(> t)	Significance
(Intercept)	-27.8346816	0.7285975	-38.2031	3.602e-12	***
Time (X_1)	-0.5469509	0.4842863	-1.1294	0.285094	
PVA (X_2)	0.2177439	0.4834938	0.4504	0.662061	
PFCE (X_3)	-0.0031424	0.4834679	-0.0065	0.994942	
$X_1:X_2$	1.4487500	0.6316695	2.2935	0.044745	*
$X_2:X_3$	-2.1737500	0.6316695	-3.4413	0.006318	**

Significant values are marked with an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

3.5. Response variable: Loading Capacity and Signal to noise ratio (SNR)

The significant terms in the model for the loading capacity are the interaction term between X_2 and X_3 , and the quadratic term of X_2 (table 10). The loading capacity gave effect with interaction part and the quadratic part with no effect on the linear part. From the ANOVA analysis (table 8), only the quadratic part (PQ (X_1 , X_2 , X_3)) was of significance. The model is moderately significant and explains about 66% of the variation in loading capacity. The lack of fit test indicates that the model is adequate to explain the effect. For the loading capacity, the model is not very significant overall, as the p -value of the F -statistic is 0.1179 (table 5), which is greater than 0.05. This means that the model is not much better than the null model that assumes no relationship between the predictor variables and the response variable. In summary, for loading capacity, the model is marginally significant and explains 66% of the variation in loading capacity.

From the data obtained through regression analysis, a quadratic second-order polynomial model has been established, with its equation 9 and 10:

$$Y_4 = \beta_0 + \beta_6(X_2:X_3) + \beta_8(X_2^2) \quad (9)$$

$$\text{Loading Capacity} = 42.34 - 11.09 (PVA \times PFCE) - 9.6 PVA^2 \quad (10)$$

Table 10 . Model Coefficients for Loading Capacity

	Coefficient	Std. Error	t value	Pr(> t)	Significance
(Intercept)	42.340454	4.688270	9.0311	4.011e-06	***
PVA (X_2)	0.030406	3.111113	0.0098	0.992394	
PFCE (X_3)	-0.167601	3.110947	-0.0539	0.958096	
$X_2:X_3$	-11.095000	4.064571	-2.7297	0.021205	*
X_2^2	-9.604370	3.028681	-3.1711	0.009968	**

Significant values are marked with an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

For the SNR, the significant terms in the model are the linear effects of X_2 and X_3 , the interaction effect of X_1 and X_2 , and the quadratic effects of X_2 and X_3 (table 11). These terms have positive or negative impacts on SNR, depending on their signs. This variable exhibited the most significant influence comparing all the dependent variables: a linear relationship with PVA and PFCE, a two-level interaction between time and PFCE, and a quadratic relationship involving both PVA and PFCE. The interaction effects are shown in table 13I & 13J. ANOVA analysis confirmed the significance at 3 levels of the significance (table 8). Lack of fit shows that the model is sufficient. The analysis of variance table 8 shows that all the sources of variation (first-order, two-way interaction, and pure quadratic) are significant, indicating that they contribute to the variability in the response. The model is highly significant and explains about 87% of the variation in SNR, as indicated by the F -test, the p -value, and the R -squared value (table 5). The p -value of the lack of fit test is not significant (table 8), which means that the model fits the data reasonably well. Altogether, for the SNR the model is significant and explains 87% of the variation in SNR. The significant effects are the linear effect of PVA (X_2) and the linear effect of PFCE (X_3), the interaction effect of time and PVA ($X_1:X_2$) and the quadratic effect of PVA (X_2^2) and the quadratic effect of PFCE (X_3^2).

The regression analysis led to the formulation of a quadratic second-order polynomial model, which is characterized by the ensuing equation 11 and 12:

$$Y_5 = \beta_0 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 (X_1:X_2) + \beta_8 (X_2^2) \quad (11)$$

$$\text{SNR} = 77.77 + 8.28 PVA + 10.40 PFCE - 9.56(PVA \times PFCE) + 5.17 PVA^2 \quad (12)$$

Table 11. Model Coefficients for Signal to Noise Ratio (SNR)

	Estimate	Std. Error	t value	Pr(> t)	Significance
(Intercept)	77.44839	3.35088	23.1128	5.196e-10	***
Time (X ₁)	-3.37056	2.22728	-1.5133	0.1611444	
PVA (X ₂)	8.28958	2.22363	3.7279	0.0039236	**
PFCE (X ₃)	10.40510	2.22351	4.6796	0.0008681	***
X ₁ : X ₂	-9.56375	2.90510	-3.2921	0.0081218	**
X ₂ ²	5.17140	2.16471	2.3890	0.0380265	*
X ₃ ²	-5.24312	2.16426	-2.4226	0.0359023	*

Significant values are marked with an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

3.6. Response variable: T_1 and T_2 relaxation time

For the T_1 variable, the second-order polynomial regression model, which includes interaction and quadratic terms for the predictor variables Time, PVA, and PFCE, yields coefficients that suggest the model is not significant, accounting for only 35% of the variability in T_1 . None of the terms in the model are significant (table given in the supplementary S5), indicating that none of the predictor variables have a significant effect on T_1 . For the T_2 variable, PVA primarily influences the outcomes (table 12). The response surface analysis for T_2 relaxation time indicates that the linear effect of X_2 . However, other effects do not show significance. The model accounts for approximately 52% of the variance in T_2 , but it does not provide an adequate fit, as suggested by the F -test and p -value (table 5). The analysis of variance (table 8) indicates that no variation sources, be it first-order, two-way interaction, or pure quadratic—are significant. Collectively, for T_1 relaxation time the model is not significant ($p = 0.117$), and the model is not sufficient to give best fit data, explains 35% of the variation in T_1 relaxation time and none of the effects are significant. Additionally, the model does not exhibit any lack of fit. For T_2 relaxation time a R -squared value of 52% and a p -value for F -statistics of 0.38 mean that the model explains very little of the variation in the response variable (T_2 have a liner effect on PVA (X_2)), but there is no evidence that the model does not fit the data (p -value of lack of fit is 0.78).

A quadratic second-order polynomial model was derived from the regression analysis, as encapsulated by the subsequent equation 13 and 14 :

$$Y_7 = \beta_0 + \beta_2 X_2 \quad (13)$$

$$T_2 \text{ relaxation time} = 257.36 + 11.238 \text{ PVA} \quad (14)$$

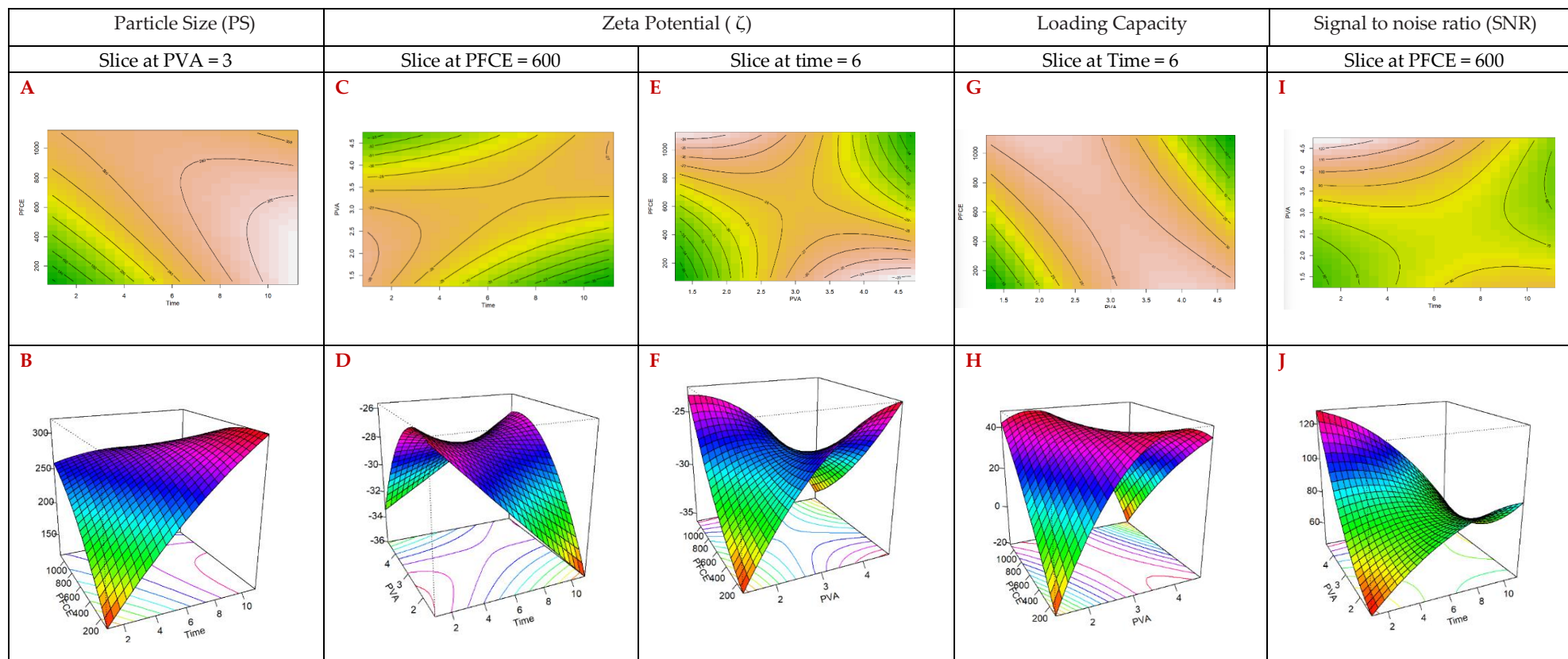
Table 12. Model Coefficients for T_2 relaxation time

	Coefficient	Std. Error	t value	Pr(> t)	Significance
(Intercept)	257.36218	5.80026	44.3708	8.129e-13	***
PVA (X ₂)	11.23872	3.84903	2.9199	0.0153	*

Significant values are marked with an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

The table 3 shows the observed and predicted values of all the variables for each run of the experiment. The predicted values are obtained by plugging in the values of the explanatory variables into the fitted model. The agreement between the observed values and the values predicted by the equation confirms the statistical significance of the model and its adequate precision in predicting the optimum condition in the domain of levels chosen for the independent variables. The difference between the observed and predicted values is the residual, which measures the discrepancy between the model and the data.

Table 13. The 2D contour plots and 3D surface plots illustrate the following relationships: (A) The impact of time and PFCE on particle size, with a corresponding 3D plot in (B). The influence of time and PVA (C), and PVA and PFCE (E) on ζ , with their respective 3D plots in (D) and (F). (G) The effect of PVA and PFCE on loading capacity, with a corresponding 3D plot in (H). (I) The impact of time and PVA on the signal-to-noise ratio, with a corresponding 3D plot in (J).



4. Discussion

We investigated the effects of time of sonication, PVA%, and PFCE content on the properties of PFCE encapsulated PLGA NPs. To the best of our knowledge, this is the first study that investigated the effects of PFCE content and the interaction and quadratic effects of the predictor variables on the properties of PFCE encapsulated PLGA NPs. Table 14 shows the results of a response surface analysis for seven response variables: size, PDI, ζ , loading capacity, Signal to Noise Ratio (SNR), T_1 relaxation time, and T_2 relaxation time for the second-order polynomial that includes the linear, interaction, and quadratic terms of the predictors. The predictor variables are Time (X_1), PVA (X_2), and PFCE (X_3). The table 14 shows the coefficients of the model terms, the adjusted R -squared values, the p -values of the R -squared values, p -values of F statistics and the p -values of the lack of fit tests for each response variable. The coefficients indicate the direction and magnitude of the effect of each term on the response variable.

Table 14. Response Surface Analysis of Time, PVA, and PFCE on different outcomes of PFCE encapsulated PLGA NPs.

		Size	PDI	ζ	Loading Capacity	SNR	T_1	T_2
Linear	Time (X_1)	+++	+++					
	PVA (X_2)		-			++		+
	PFCE (X_3)	+	-			+++		
Interaction	Time:PVA ($X_1:X_2$)			+		--		
	PVA:PFCE ($X_1:X_3$)			--	-			
	Time:PFCE ($X_2:X_3$)	--						
Quadratic	(Time) ² (X_1^2)							
	(PVA) ² (X_2^2)				--	+		
	(PFCE) ² (X_3^2)					-		
R-squared %		87 %	80%	71 %	66 %	87 %	35%	52%
p -value of F -statistics ()		0.001	0.013	0.065	0.117	0.002	0.76	0.38
p -value for lack of fit		0.184	0.63	0.52	0.44	0.41	0.44	0.78

+++/- $p < 0.001$, ++/- $p < 0.01$, +/- $p < 0.05$

Our results showed that time of sonication had a significant linear effect on the size and PDI of the NPs, with longer sonication time leading to larger and more heterogeneous particles. PVA % also had a significant linear effect on PDI, SNR, and T_2 relaxation time, with higher PVA % resulting in lower PDI, higher SNR, and increasing T_2 relaxation time. Our research corroborates certain aspects of earlier studies that have observed the impact of sonication time and PVA % on the size and PDI of PLGA NPs [20, 30, 31]. These studies explained that longer sonication time can cause particle aggregation and charge development, leading to larger and more heterogeneous particles. They also explained that higher PVA % can increase the viscosity of the solution, which can slow down the evaporation of the organic solvent after the emulsification process, leading to smaller and more homogeneous particles [30, 31]. Our results revealed that ζ was influenced by the interaction effects of the predictor variables, but not by their linear effects. This implies that the charge of the NPs was not determined by single parameters alone. The interaction effects were as follows: time and PVA had a positive effect on ζ , while PVA and PFCE had a negative effect. These findings highlight the importance of considering the interaction and quadratic effects of the predictor variables on the response variables, as they can have a significant impact on the final outcome of the NPs physicochemical characteristics.

PFCE content had a significant linear effect on size, PDI, and SNR, with higher PFCE content leading to bigger, more homogeneous, and more contrast-loaded particles. In addition, we observed significant interaction and quadratic effects of the predictor variables on some of the response variables. For example, time of sonication and PFCE content had a significant negative interaction effect on size, indicating that the effect of sonication time on size was dependent on the level of PFCE content, and vice versa. Similarly, PVA % and time of sonication had a significant negative interaction effect on SNR, suggesting that the effect of PVA % on SNR was dependent on the point of time in sonication, and vice versa. Moreover, PVA % and PFCE content had significant quadratic effects on SNR, implying that the effects of these variables on these responses were not linear, but curved.

A notable finding in our study was the discrepancy between the loading capacity and the SNR factors. These factors reflect the same concept – the amount of PFCE in the NPs. Loading capacity was measured by NMR, while SNR was measured by FMRI using phantom studies. However, as shown in *table 14*, they exhibited different dependencies on the predictor variables. This prompted us to investigate the loading capacity data obtained by NMR. We usually calculated the loading capacity using an internal standard (TFA) resuspended in deuterated water and measured the T_1 and T_2 relaxation times on the same sample (data presented in supplementary information). We realized that the loading capacity value was affected by various factors, such as the time/days after the sample analysis (*supplementary information table S7*), the time/days after the sample analysis (*supplementary information table S8*), and the resuspension of the samples in the deuterated solvent. The findings indicate that the stability of NP batches may fluctuate over time, exhibiting no uniform trend and potentially being influenced by external factors. Therefore, we caution the reader about these aspects.

For T_2 relaxation time a R -squared value of 52% and a p -value for F -statistics of 0.38 mean that the model explains very little of the variation in the response variable (T_2 have a liner effect on PVA (X_2)), but there is no evidence that the model does not fit the data (p -value of lack of fit is 0.78). This may suggest that the model is underfitting the data, meaning that it is too simple and does not capture the complexity of the data. It might be beneficial to consider additional predictors or incorporate higher-order terms, although in our situation, this may not be optimal since the 2nd order effects are already accounted for in the CCD. Alternatively, adopting a different model type could enhance the fit. This could also indicate that the dependent variable is not influenced further by the independent variables within the established constraints. As indicated by the R -squared value, and p -value of F -statistics, the data from NMR – the loading capacity, T_1 and T_2 relaxation times – had the lowest R -squared value and highest p -value of F -statistics. This could be attributed to the measurement itself or the statistical model.

5. Conclusion

This study employed a central composite design (CCD) to investigate various parameters affecting PFCE encapsulated PLGA NPs. The independent variables included sonication time, PFCE volume, and surfactant percentage. Our model assessed the linear, interaction, and quadratic effects on outcomes such as size, polydispersity index (PDI), zeta potential (ζ), loading capacity, signal-to-noise ratio (SNR), and T_1 and T_2 relaxation times. The results revealed that our statistical model is robust, explaining a significant portion of the variability in NP size, PDI, ζ , and SNR. Specifically, it accounts for 87% of the size variation, 80% of the PDI variation, 71% of the ζ variation, and 87% of the SNR variation. Although the loading capacity model was less conclusive, it still explained 66% of the variation. The T_1 relaxation time model did not demonstrate significance, while the T_2 relaxation time model had limited explanatory power with an R -squared value of 52%.

In summary, the study underscores the critical influence of sonication time, PFCE volume, and surfactant percentage on the characteristics of PLGA NPs. This research offers crucial insights that not only lay the groundwork for enhancing NP formulations in biomedical contexts, particularly for improving MRI contrast agents, but also emphasize the optimization of NP preparation conditions. Such optimization is pivotal for facilitating validation and scaling up processes during industrial production.

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7. References

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Central Composite Design of Experiment Based Optimization of Key Formulation Parameters for PFCE-Encapsulated PLGA Nanoparticles

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Table S1: Model Coefficients for PDI

	Coefficient	Std. Error	t value	Pr(> t)	Significance
(Intercept)	0.1668077	0.0150443	11.0878	6.122e-07	***
Time (X ₁)	0.0474522	0.0099997	4.7454	0.0007857	***
PVA (X ₂)	-0.0230498	0.0099833	-2.3088	0.0435936	*
PFCE (X ₃)	-0.0229269	0.0099828	-2.2966	0.0445075	*
X ₁ : X ₂	-0.0176250	0.0130429	-1.3513	0.2063722	
X ₁ : X ₃	-0.0216250	0.0130429	-1.6580	0.1283109	
X ₂ : X ₃	-0.0133750	0.0130429	-1.0255	0.3293098	
X ₁ ²	0.0041311	0.0097795	0.4224	0.6816564	
X ₂ ²	0.0010313	0.0097188	0.1061	0.9175926	
X ₃ ²	0.0132336	0.0097167	1.3619	0.2031071	

Table S2 :Model Coefficients for Zeta Potential

	Coefficient	Std. Error	t value	Pr(> t)	Significance
(Intercept)	-27.8346816	0.7285975	-38.2031	3.602e-12	***
Time (X ₁)	-0.5469509	0.4842863	-1.1294	0.285094	
PVA (X ₂)	0.2177439	0.4834938	0.4504	0.662061	
PFCE (X ₃)	-0.0031424	0.4834679	-0.0065	0.994942	
X ₁ : X ₂	1.4487500	0.6316695	2.2935	0.044745	*
X ₁ : X ₃	0.1237500	0.6316695	0.1959	0.848605	
X ₂ : X ₃	-2.1737500	0.6316695	-3.4413	0.006318	**
X ₁ ²	0.0202374	0.4736235	0.042	0.966759	
X ₂ ²	-1.0076061	0.4706832	-2.1407	0.057963	
X ₃ ²	0.4903401	0.4705850	1.0420	0.321957	

Table S3: Model Coefficients for Loading Capacity (LC)

	Coefficient	Std. Error	t value	Pr(> t)	Significance
(Intercept)	42.340454	4.688270	9.0311	4.011e-06	***
Time (X ₁)	-2.985051	3.116212	-0.9579	0.360694	
PVA (X ₂)	0.030406	3.111113	0.0098	0.992394	
PFCE (X ₃)	-0.167601	3.110947	-0.0539	0.958096	
X ₁ : X ₂	0.792500	4.064571	0.1950	0.849315	
X ₁ : X ₃	0.972500	4.064571	0.2393	0.815734	
X ₂ : X ₃	-11.095000	4.064571	-2.7297	0.021205	*
X ₁ ²	2.389303	3.047601	0.7840	0.451214	
X ₂ ²	-9.604370	3.028681	-3.1711	0.009968	**
X ₃ ²	-1.457525	3.028049	-0.4813	0.640633	

Table S4: Model Coefficients for Signal to Noise Ratio (SNR)

	Estimate	Std. Error	t value	Pr(> t)	Significance
(Intercept)	77.44839	3.35088	23.1128	5.196e-10	***
Time (X_1)	-3.37056	2.22728	-1.5133	0.1611444	
PVA (X_2)	8.28958	2.22363	3.7279	0.0039236	**
PFCE (X_3)	10.40510	2.22351	4.6796	0.0008681	***
$X_1: X_2$	-9.56375	2.90510	-3.2921	0.0081218	**
$X_1: X_3$	-0.20125	2.90510	-0.0693	0.9461367	
$X_2: X_3$	4.71625	2.90510	1.6234	0.1355601	
X_1^2	-3.94503	2.17824	-1.8111	0.1002204	
X_2^2	5.17140	2.16471	2.3890	0.0380265	*
X_3^2	-5.24312	2.16426	-2.4226	0.0359023	*

Table S5: Model Coefficients for T1 relaxation time

	Coefficient	Std. Error	t value	Pr(> t)	Significance
(Intercept)	785.32446	5.35273	146.7147	<2e-16	***
Time (X_1)	0.35409	3.55787	0.0995	0.9227	
PVA (X_2)	-1.79835	3.55205	-0.5063	0.6236	
PFCE (X_3)	-5.93918	3.55186	-1.6721	0.1254	
$X_1: X_2$	2.19000	4.64064	0.4719	0.6471	
$X_1: X_3$	-2.19000	4.64064	-0.4719	0.6471	
$X_2: X_3$	-4.46250	4.64064	-0.9616	0.3589	
X_1^2	-2.17501	3.47953	-0.6251	0.5459	
X_2^2	-2.72185	3.45793	-0.7871	0.4495	
X_3^2	0.72605	3.45721	0.2100	0.8379	

Table S6: Model Coefficients for T2 relaxation time

	Coefficient	Std. Error	t value	Pr(> t)	Significance
(Intercept)	257.36218	5.80026	44.3708	8.129e-13	***
Time (X_1)	-2.94929	3.85534	-0.7650	0.4619	
PVA (X_2)	11.23872	3.84903	2.9199	0.0153	*
PFCE (X_3)	2.20803	3.84882	0.5737	0.5789	
$X_1: X_2$	-1.66375	5.02863	-0.3309	0.7476	
$X_1: X_3$	-1.11375	5.02863	-0.2215	0.8292	
$X_2: X_3$	-0.78875	5.02863	-0.1569	0.8785	
X_1^2	0.56720	3.77045	0.1504	0.8834	
X_2^2	-2.45936	3.74704	-0.6563	0.5264	
X_3^2	3.14349	3.74626	0.8391	0.4210	

Stability study of PLGA- PFCE NPs

Table S7 : Stability of the loading capacity over a 9-week period for two batches of DoE at – 20°C

	DoE 7	DoE 16
1 st week	52.66	83.39
2 nd week	75.37	97.56
3 rd week	59.04	47.23
4 th week	55.77	67.17
5 th week	59.28	60.55
6 th week	89.90	95.75
7 th week	48.79	56.58
8 th week	92.66	71.17
9 th week	73.94	77.92

Table S8. The DoE samples, which were preserved in the sample suspension contains trifluoroacetic acid (TFA) and then evaluated after periods of 1, 2 and 5 days

Name	Days	DoE 7	DoE 16
Take 1	1	39	52
Take 2	2	31	53
Take 3	5	30	35

Chapter 5

Article

Strategic Enhancement of PLGA Nanoparticles: PEG and Fluorine Additions for PFCE Encapsulation

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Abstract

Magnetic Resonance Imaging (MRI) has revolutionized medical imaging by offering a non-invasive technique that eschews ionizing radiation, making it particularly user-friendly. The clarity of MRI images can be significantly improved with the use of contrast agents. In this context, our research has focused on the encapsulation of perfluorocarbon, specifically perfluoro-15-crown-5-ether (PFCE), within a poly(lactic-co-glycolic acid) (PLGA) system. The synthesis of PLGA was achieved using a novel cerium-based catalyst, which also played a pivotal role in the creation of PLGA-polyethylene glycol (PLGA-PEG) and PLGA fluorine derivatives. Our study delved into the effects of PEGylation on the protein corona, as well as the fluorination of PLGA. We conducted a thorough assessment of the physicochemical properties of all formulations, including size, polydispersity index (PDI), zeta potential (ζ), and loading capacity. The introduction of a novel cerium-based catalyst for the ring-opening polymerization of PLGA has been a cornerstone of our research, enabling the preparation of various PLGA-based formulations. The encapsulation efficiency of the newly synthesized Ce-based PLGA with PFCE was evaluated and found to be promising for practical applications. We investigated two PEGylation strategies—full substitution and a 1:1 blend of PLGA and PLGA-PEG—and assessed their impact on the physicochemical properties of the resulting NP formulations. The empirical data indicated that a PEGylation ratio of 1:1 PLGA-PEG to PLGA was superior, enhancing the NP properties compared to pristine PLGA, particularly in terms of protein corona behavior. Concurrently, synthetic efforts were directed towards PLGA fluorine and its amalgamation with PLGA-PEG to exploit the potential synergistic effects. Cytotoxicity assays conducted on the formulated nanoparticles with fibroblast cultures affirmed their non-toxic nature. This comprehensive study not only advances our understanding of PLGA-based systems but also opens new avenues for the development of effective MRI contrast agents.

1. Introduction

Perfluorocarbons (PFCs) are organic fluorocarbons that are biologically inert molecules and have been studied to be used as a contrast agent (CA) for fluorine magnetic resonance imaging (^{19}F MRI). Fluorine, possessing the highest electronegativity value, these classes of molecules have several unique properties including exceptional thermal, chemical and oxidative stability, bio-inertness and among the nuclear resonance active molecules ^{19}F appeared to be promising because of unambiguous detection and acceptable *in vivo* acquisition times [1]. With the establishment that fluorine molecules can be used in the MRI as CA in addition to the conventionally used proton, together they can overcome several drawbacks of the H MRI technique including localization ambiguity and fluorine agent ability to give a second colour/hot spot images can also help in the more precise quantitative analysis. The possibility to use fluorine also opened doors to study an alternative for the currently used gadolinium based contrast agents, some of which were found to cause fibrosis of kidney cells and gadolinium ions accumulation in brain [2] especially the linear chelated ones. This prompted scientists to find alternative CAs that have more advanced imaging performance and better safety profiles.

With all the advantages of the above-mentioned class of PFCs, one of the most challenging properties of them is that most of them are simultaneously hydrophobic and lipophobic. This requires that they have to be used with a delivery system in order to improve their stability. Several systems including lipophilic, polymeric nanoparticles, or inorganic based carrier systems are currently being used for the encapsulation of fluorinated agents for fMRI to increase ^{19}F CA biocompatibility and solubility [3]. Encapsulation inside polymeric nanoparticles (NPs) like poly(lactic-co-glycolic) acid (PLGA) gives the

advantages like apparent solubility and stability of the encapsulated molecules, possibility to further functionalize the NPs and they are biocompatible and biodegradable. PLGA is a synthetic linear copolymer composed of lactic and glycolic acid monomers polymerized at different ratios.

It should also be noted that the size of NPs changes as they are passing through the biological environment. For instance, protein corona, is one of the key factors that changes the NP size after exposure. After administration of NPs into a biological fluid, NPs immediately tend to interact with blood proteins leading to the formation of a “protein corona” [4]. Hydrophobic NPs adhered to proteins like opsonins are susceptible to recognition by macrophages that lead to phagocytosis, reducing the half-life of NPs and facilitating the removal of the NP from the body [5]. A well-studied strategy to circumvent this scenario is increasing the hydrophilicity of the system through the PEGylation of the polymer. Amphiphilic block polymers like Poly(ethylene glycol)-Poly(lactic-co-glycolic acid) (PEG-PLGA) is one of the most studied techniques to camouflage the NP surface and prevent the formation of the protein corona. PEG and its derivatives with high water solubility and excellent biocompatibility have been approved by FDA for biomedical application [6, 7].

PLGA is a commercially available polymer made using ring opening polymerisation (ROP) reaction, a form of chain-growth polymerization that occurs via ionic reactions amongst the cyclic monomers thus opening its ring system to form a longer polymer chain [8]. In the synthesis of PLGA, lactide and glycolide monomers upon heating is opened up by a lewis acid catalysts like tin (II) bis (2-ethylhexanoate) (SnOct_2) in the presence of alcohol as a co-initiator. SnOct_2 is a commercial catalyst (food and drug administration (FDA) approved threshold concentration of tin should be less than 20 ppm for biomedical applications [9]) preferred by the industries because of their low cost and high-performance polymerization at the high temperatures needed for melting the monomers [10]. To meet the biomedical requirement of less than 20 ppm of tin in the final polymer, the synthesis must use either a low amount of catalyst or a solvent based purification step to remove the extra catalyst based on current good manufacturing practice [10]. Despite their great effectiveness, the genetic toxicity of tin compounds, as demonstrated for PLGA microsphere using the comet test in mesenchymal stem cells and human umbilical vein endothelial cells [11] is a scientific challenge for producing nontoxic biomaterials. Moreover, in the product specification document issued by sigma aldrich for the commercial PLGA polymer (Resomer® 502H), presence of tin in a concentration less than or equal to 200 ppm is indicated.

ROP in the presence of suitable metal complexes is a well-established method for synthesizing biocompatible polymers with precise control over their structure, properties, and purity. In our laboratory, we have employed cerium trichloride heptahydrate ($\text{CeCl}_3 \cdot 7\text{H}_2\text{O}$) as an inexpensive, water-tolerant, eco-sustainable, and non-toxic catalyst [12-14] for the synthesis of PLGA. Moreover, the toxicity of this catalyst was extremely lower, and less risky for the health than that of the PLGA syntheses using tin-based one. This polymer was used for the formulation of PLGA NPs for the encapsulation of perfluorocarbon-15-crown-5-ether (PFCE). The use of PFCE as a contrast agent is particularly advantageous due to the unambiguous identification it provides, owing to the presence of 20 fluorine atoms in a consistent chemical environment. Additionally, we synthesized poly(ethylene glycol)-block-poly(lactide-glycolide) copolymer (PLGA-PEG) using the same catalyst to improve protein corona behaviour. Furthermore, we modified PLGA with a fluorine end cap to create heptadecafluoro-1-nonanol-block-poly(L-lactide-co-glycolide) copolymer (PLGA-F), leveraging fluorophilic interactions to better retain the PFC within the NPs.

2. Materials and Methods

2.1. Materials

PLGA Resomer® RG 502H, Poly (D, L-lactide-co-glycolide), (acid terminated, lactide: glycolide 50:50, Mw 7,000-17,000) was purchased from Sigma-Aldrich. Lactide and glycolide PLGA, Poly (D, L-lactide-co-glycolide), (Sn-based, ester terminated, lactide:glycolide 46:54, Mw 20,000), PLGA, (Ce-based, ester terminated, lactide:glycolide 45:55, Mw 17,600), PEG₂₀₀₀-PLGA, Poly (ethylene glycol)-Poly (D, L-lactide-co-glycolide), (Ce-based, ester terminated, lactide: glycolide 42:58, Mw 10,000), C₉F₁₇-PLGA, Heptadecafluoro-1-nonanol - Poly (D, L-lactide-co-glycolide), (Ce-based, ester terminated, lactide:glycolide 42:58, Mw 17,388), were kindly provided by an organic chemistry research group in ChIP (Chemistry Interdisciplinary Project, University of Camerino, Camerino). Poly (vinyl alcohol) (PVA; Mw 9,000-10,000; 80% hydrolyzed) obtained from Sigma-Aldrich. The solvent dichloromethane (DCM, CH₂Cl₂, Mw 84.93 g/mol) used to prepare the NPs, was obtained from CarloErba (Milano). Perfluoro-15-crown-5-ether; 98% (PFCE) (C₁₀F₂₀O₅, Mw 580.07) was supplied by abcr GmbH. Results are presented as mean values ± standard deviation (SD).

2.3 Nanoparticle formulation

2.3.1. Sonication

PFCE encapsulated PLGA NPs were formulated by PFCE-O/W double emulsion solvent evaporation method as described in [15] using PLGA to obtain NPs. PFCE loaded PLGA formulated by probe sonicator. Briefly, PFCE (900 µl) and 3ml DCM containing 90 mg PLGA polymer were taken together and mixed manually using a pipette and this mixture was immediately added into 25 ml of 2 % w/v PVA water solution and sonicated (Qsonica with 6mm sonicator's tip) in an ice bath for 3.15 minutes at 40% amplitude. Then the emulsion was stirred at room temperature overnight for solvent removal and NP solidification.

2.3.2. Washing and characterisation

Once the DCM was evaporated off, the NPs were distributed to eppendorfs to be washed at first to settle the NPs and then further wash them 3 times using centrifuge – 8,000g, 35 minutes and 4°C. After they are washed, the NP solution was freeze dried using nitrogen and then lyophilised. The lyophilised particles were later used for further characterisation.

2.4 Characterization of NPs

2.4.1. Size and Zeta Potential Characterization

The particle size (size) and polydispersity index (PDI) of prepared NPs were measured using dynamic light scattering (DLS) technique on the Zetasizer Nano (DLS Zetasizer Nano S90, Malvern Instruments (Worcestershire, UK). Size and PDI of prepared NPs were measured by dispersing the formulation in deionised (DI) water at 25°C and an angle of 90. Zeta potential (ZP) was determined using the same instrument in a capillary zeta cell the measurements were performed at 25°C. For statistical analysis all samples were measured in triplicate and the average values and standard deviation of the measurements were calculated.

2.4.2. Loading Capacity Measurements

PFCE load content and encapsulation efficiency of PLGA NPs was determined by Fluorine-19 nuclear magnetic resonance spectroscopy (19F-NMR). Lyophilized nanoparticles were dissolved in D₂O containing 0.1M trifluoroacetic acid (TFA) as internal standard. The amount

of PFCE was calculated by the integration ratio between PFCE peak to TFA peak. Fluorine contents were calculated according to the **Formula 1**.

$$\text{Loading capacity in weight percentage} = \frac{\text{Mass of PFCE (gram)}}{\text{Mass of nanoparticles in gram}} * 100 \quad (1)$$

2.4.3. *In vitro* Analysis

2.4.3.1 Protein Corona Study

Investigations into the protein corona were performed utilizing bovine serum albumin (BSA) sourced from Sigma Aldrich and plasma procured from human volunteers, with the latter isolated via blood centrifugation. NP concentrations were established at 1.5 mg/ml, while BSA concentrations were sustained at 20 mg/ml, yielding an NP to protein ratio of 1:13. Temporal analysis was conducted at three junctures: immediately (0 hours), 2 hours, and 24 hours post-incubation. BSA in isolation manifested peaks at 10 nm and 100 nm within the dynamic light scattering (DLS) spectrum, culminating in a z-average of approximately 13 nm. This denoted a reduced z-average size when considering the combined presence of BSA and NPs. To discern the influence of BSA on the nanosystem, emphasis was placed on the peak mean intensity (PMI) of the first peak, indicative of the NP size exclusively. Comparative analyses of NP size were conducted using baseline measurements obtained from NPs resuspended in Phosphate Buffer Solution (PBS) prior to exposure to bovine serum albumin (BSA) and plasma proteins.

2.4.3.2 Cytotoxicity studies

The metabolic activity of viable cells was determined by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay. NCTC clone 929 [L cell] (connective mouse tissue) (ATCC) fibroblasts were plated at the density of 5000 cells/well in 96 culture plates and were grown until 80% of confluence. Cultures were incubated for 24 h with empty (control) or PFCE loaded PLGA NPs samples at concentrations at 5 to 10 µg/mL in complete medium. Then, cells were incubated with 20 µL/well of CellTiter 96 Aqueous One Solution Reagent (Promega Italia, Milano, Italy) for 2 h in a humidified, 5% CO₂ atmosphere. The quantity of formazan product is directly proportional to the number of living cells in the culture. The colored formazan was measured by reading the absorbance at 490 nm using a 96-well plate reader.

2.5. Statistical data analysis

A paired student t-test was used to evaluate significant differences between the properties of the prepared nanoparticle formulations. The level of significance accepted was at $p < 0.05$.

3. Results and Discussion

3.1 Commercial-PLGA, Tin based and Novel Cerium- based NPs

The NPs were prepared by emulsion evaporation method. After the preparation, the NPs were lyophilised and analysed by the DLS to analyse the size, the PDI, and the zeta potential (ζ). The NMR gave quantitative information about the PFCE that was present in the NPs and, in particular, its concentration expressed in LC (wt/wt%). The NPs from commercial PLGA which are based on the Tin (Sn) catalyst, whose characteristics include broad Mw between 7,000 and 17,000 Da, 50:50 lactide: glycolide ratio, and an acid end group (Table 1) gave NPs with size around 250 nm, PDI was 0.16, the

ZP was -22 mV and the LC was 50 ± 4 (table 2). For experimenting a new route of synthesis of the PLGA using a Ce-based catalyst the ROP for the synthesis of the PLGA was conducted under microwaves irradiation that speeded up the reaction (figure 1) and provided a polymer close to the range of Mw of the commercial PLGA (17,600 Da, ester terminated, lactide: glycolide 45:55). The preparation of the PLGA (Ce-based) NPs resulted in batch-to-batch reproducibility and provided particles with dimensions 300 nm, PDI around 0.27, negative ζ and a higher loading capacity of 59%. The comparison graphs with the commercial and synthesized polymer for the nanofomulations are shown in figure 2.

Table 1. Chemical characteristics of commercial and Ce-synthesised PLGA, synthesized PLGA PEG and PLGA-F

Polymer	Manufacturer	Mw (kDa)	L/G ^b	End cap
Commercial PLGA 502H	Sigma Alrich	7,000 – 17,000	50/50	Acid
Ce-synthesised PLGA	In-house	17,600	45/55	Ester
PLGA-PEG	In-house	10,000	42/58	Ester
PLGA -F (Heptadecafluoro-1-Nonanol)	In-house	17,400	42/58	F

Comparing the two formulations together (table 2) Ce based gave the higher particle size, PDI, lower ζ and a higher loading capacity although statistically the loading capacity was not significant. Polymeric nanoformulations physicochemical characteristics are particularly dependent the polymer molecular weight, end groups hydrophilicity/hydrophobicity depending on the ratio of lactide/glycolide ratio, polymer end group to name a few [16-25]. Although Ce had Mw that are close to the commercial ones, Kumskova *et. al.* [25] have demonstrated that how even a 5,000 Da change in Mw can give different PLGA particle size, PDI, or ζ . This was correlated to different viscosity of the different Mw PLGA. The polymer solution viscosity depends on the polymer concentration and Mw [17]. Higher Mw PLGA polymers produced more viscous droplets in the emulsion, which resisted further fragmentation and resulted in less size variation and lower PDI of NPs. This could explain the higher size and PDI for the Ce based formulations. In contrast, lower Mw PLGA polymers formed less viscous droplets that underwent more emulsion-induced breakage and yielded smaller NPs with lower average size [21]. However, there are some literatures that reports lowest molecular weight polymer having larger NP size [16]. The authors explain that this is due to the lower viscosity and higher solubility of the lower Mw polymers, which result in faster solvent diffusion and larger droplets during the emulsification process or dependent on other parameters like the pH of solution used or used drug etc .

Table 2. Comparison of commercial and cerium synthesised PLGA

Name	Size (nm)	PDI	Zeta Potential (mV)	Loading Capacity (wt/wt %)
Commercial PLGA	251 ± 30	0.16 ± 0.03	-22 ± 4	50 ± 4
Ce synthesized	299 ± 40	0.27 ± 0.04	-26 ± 2	59 ± 13

One major difference between the polymer were the end group – the commercial one had an acid and the in house was with an ester terminated end group. This could have the reason for the difference in ζ for the 2 formulations. The charge characteristics of NP surfaces are predominantly influenced by the nature of the polymer termini [17, 18, 21, 24]. Carboxylic terminations are known to confer a pronounced negative charge due to the facile ionization of the proton. In contrast, the hydrolytic cleavage of ester linkages [21], along with the density of polymer chains per unit mass [17], may lead to varying of surface potential values. From the findings of Jahan *et al.* [18], who investigated the influence of acid and ester end groups on plain PLGA and reported that the end groups had significantly affected the drug encapsulation efficiency of antibody modified PLGA. In the studies of Blanco *et al.* [24], acid terminated PLGA had better encapsulation of protein drug compared to the ester terminated PLGA and Banderas *et al.* [16] showed Cannabinoid derivate had a better encapsulation in PLGA with acid terminated compared to ester terminated PLGA. These studies show the importance of PLGA end group for drug encapsulation. Although PFC is a unique drug unlike the drugs mentioned, one of the parameters in the slight difference in the loading capacity behaviour could be attributed to the end group of PLGA given the Mw and L/G is almost similar. From these, it is clear that polymer characteristics can significantly control the formulation outcome. From the results it can be concluded that Ce based PLGA polymer represented a valid alternative for the PLGA synthesis and, subsequently, for the NPs preparation.

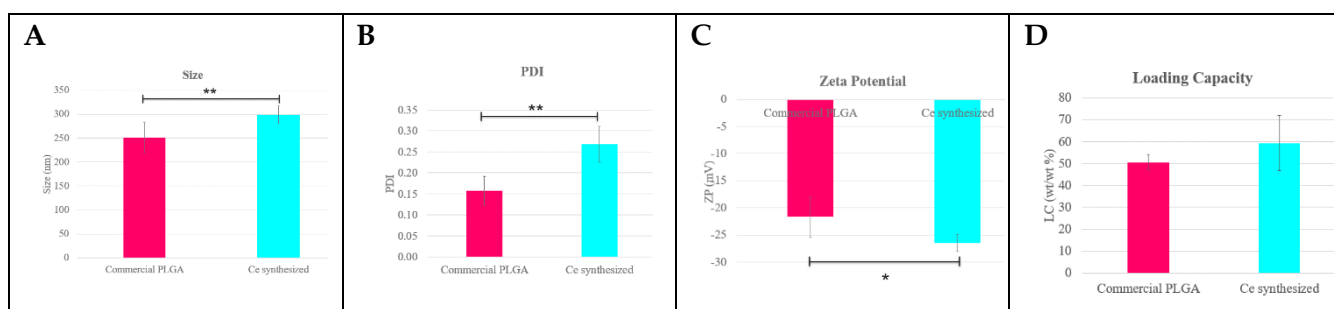


Figure 2: Graphical Comparison of Commercial and Cerium-Synthesized PLGA: Size (A), Polydispersity Index (B), Zeta Potential (C), and Loading Capacity (D). Significant values are marked with an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

3.2 PLGA PEG

The efficiency of the Ce-catalyst was also tested not only in the synthesis of the PLGA but also in the functionalization of the PLGA polymer. In particular, it was utilised for the PEGylation and the fluorination of the PLGA. The purpose of the PEG chains is to create a barrier layer to block the adhesion of opsonins present in the blood serum, so that the particles can remain camouflaged or invisible to

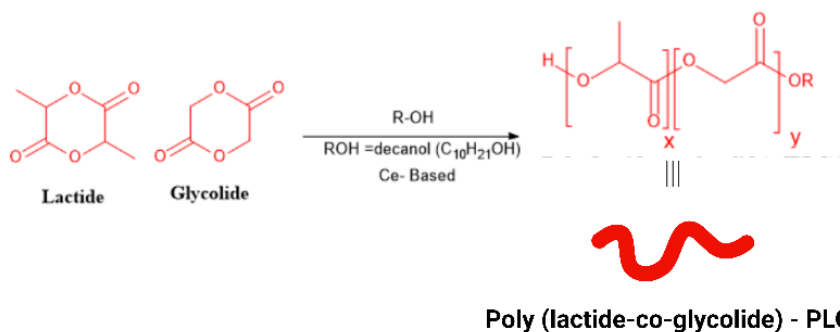


Figure 1: PLGA obtained by the ROP of lactide and glycolide using the cerium-based catalyst. (Mw is 17,600 Da, ester terminated, lactide: glycolide ratio 45:55)

phagocytic cells [4]. Research has shown that the circulation time of NPs with surface-adsorbed PEG is shorter than that of NPs with covalently bound PEG chains [26]. To ensure this, a promising technique consists of the use of a copolymer of PEG with another biodegradable polymer, which in our case is PLGA. The PEGylation of the PLGA was achieved by Ce-catalysed the reaction with the lactide, glycolide, and the methoxy PEG (2kDa) (methoxy-PEG₂₀₀₀) as an alcoholic co-initiator, to produce a PLGA which had bound to only one side the PEG, thus attaining the diblock copolymer PEG₂₀₀₀-PLGA/ PEG-PLGA as shown in *figure 2*. The methoxy-PEG₂₀₀₀ is characterized by an alcoholic functional group at one terminus and a methoxy group at the opposite end. The alcoholic moiety facilitates the initiation of the reaction and the subsequent attachment of monomers, while the methoxy group serves to selectively terminate the reaction on one side. Utilization of this copolymer in nanoparticle synthesis aims to minimize recognition by the reticuloendothelial system (RES), thereby preventing the formation of a protein corona upon administration.

It is important to note that for effective blocking or repulsion of proteins to occur, the surface coating layer needs to exceed a minimum layer thickness which is depended on the PEG molecular weight, surface chain density, and conformation [26]. Most research indicates that a surface PEG chain molecular weight of at least 2000 Da [27, 28] or greater is considered sufficient for reducing recognition by immune cells required to achieve RES avoidance characteristics although most studied range is between 1000 to 5,000 Da for PLGA system [29-41]. In addition coverage of PEG chains in the NPs play a role – low coverage (mushroom modal) can lead to inefficient protection whereas a high coverage (brush modal) lead to decreases the mobility of the PEG chains and thus decreases the steric hindrance properties of the PEG layer [42]. Therefore, it is essential to balance these parameters while formulating a nanosystem.

Initially, PLGA-PEG NPs (1:0) was prepared, directly utilizing the synthesised polymers for formulations. A recognized method for varying PEG content involves blending PLGA with varying ratios of PLGA-PEG. In our pursuit to modulate the PEG content within the NPs, we employed a balanced mixture, consisting of a 1:1 ratio of PEG₂₀₀₀-PLGA to PLGA, to formulate the NPs. We then characterized the NPs for their size, PDI, ζ , and LC, and repeated the preparation two or three times to ensure its reproducibility (*figure 4*). The DLS results showed that the 1:1 PEGylated NPs were more stable than the 1:0 PEGylated NPs. Both the size and PDI of the PLGA-PEG NPs were larger than those of the plain PLGA NPs. The size of the NPs is influenced by the PEGylating through its effects on the hydrophobicity of the polymer matrix [38]. The 1:0 PEGylated NPs had twice the size of the 1:1 PEGylated NPs, likely due to their higher tendency to aggregate, as indicated by their PDI. The ζ of both PEGylation levels was similar (~ -25 mV). These findings of increased size with increased PEG content were consistent with the previous studies by Kirby *et al.* [40], Spek *et al.* [41] and Xu *et al.* [39], who explained this phenomenon by the increased viscosity of the polymer solutions used for NP formulation. PEGylation generally lowers the absolute value of the zeta potential of NPs, making them less charged and more neutral [38, 39]. However, the extent of this change depends on the specific conditions of PEGylation, such as the shielding effect, as demonstrated by Spek *et al.*, who reported that there is no change in the zeta potential of PLGA NPs with PEG content ranging from 0 to 15% (w/w) which agreed with our case, where the zeta potential didn't change and was -25 mV for both PEGylation levels. The PEGylated PLGA at 1:1 gave LC 55% similar to observed for commercial PLGA/Ce based PLGA, showing PEGylation at 1:1 did not influence of loading of the CA but when the percentage of PEG was increased (1:0), the LC significantly reduced, reaching to 12%. This could be attributed to the aggregation of NP (seen by the PDI of 0.41).

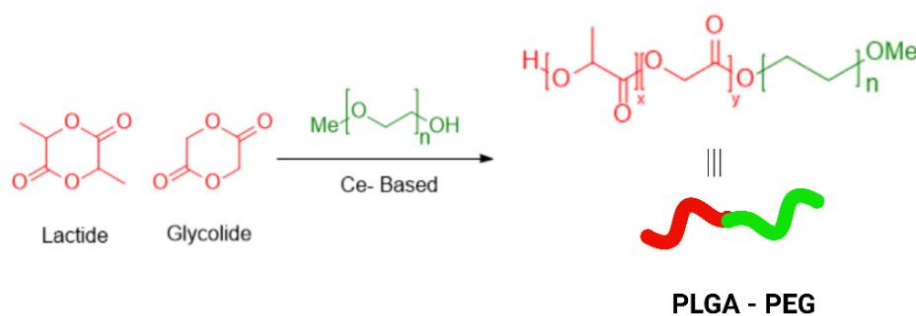


Figure 3. The ROP with Ce-based catalyst for the preparation of PLGA – PEG from lactide, glycolide and methoxy PEG ($M_w = 2000$ Da) to produce polymers with characteristics of M_w 10,000, ester terminated, lactide: glycolide ratio 42:58

We observed an interesting phenomenon when we washed the PEGylated NPs. As shown in *table 3* and *figure 5*, the particle sizes increased markedly after washing, especially for the 1:0 PEGylation. The particle sizes for the 1:1 PEGylation rose from 320 nm (no wash) to 390 nm (SD = 40) after three washes, while the particle sizes for the 1:0 PEGylation soared from 330 nm (no wash) to 700 nm after three washes. This could be attributed to the hydration of the PEG layer on the NP surface, which enhanced the aggregation of the NPs into clusters at higher PEGylation. The PDI values for the 1:1 PEGylation changed slightly from 0.24 (no wash) to 0.28 (three washes), whereas the PDI values for the 1:0 PEGylation followed the same trend as the particle sizes, increasing from 0.20 (no wash) to 0.41 (three washes).

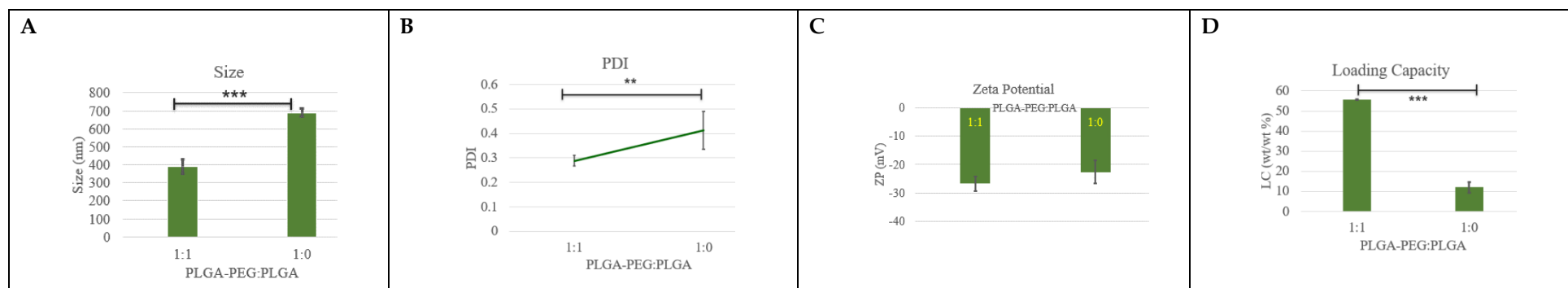
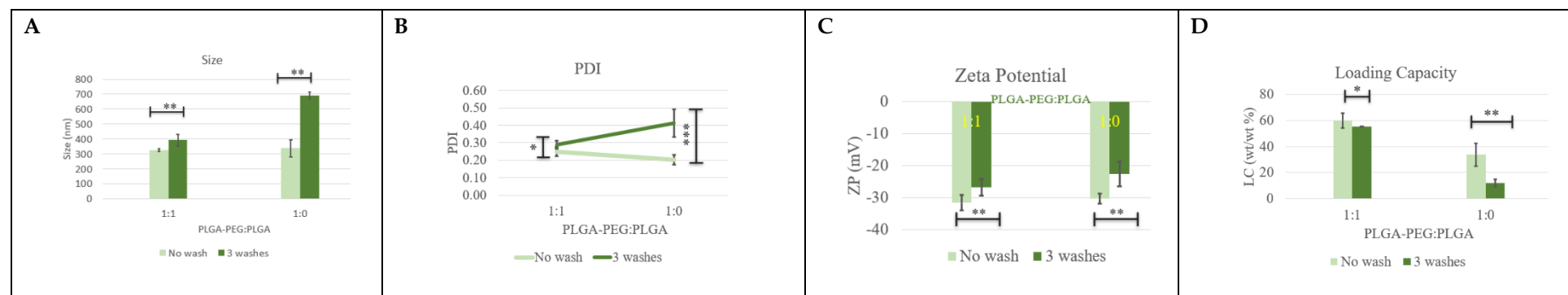
In the context of diblock copolymer systems such as PLGA-PEG, the PEG segment is predisposed to align with the external aqueous phase during the emulsification process, thereby forming the nanoparticle's outermost layer [43]. This orientation is believed to endow PEG with its 'stealth' characteristics. Due to its hydrophilic nature, PEG can attract and bind water molecules along its chain, leading to the expansion of the PEG molecule as the bound water molecules increase the overall volume of the chain [44, 45]. This expansion may influence the molecule's stability. Nanoparticle aggregation is contingent upon the equilibrium between attractive and repulsive forces; disruption of this balance can induce aggregation. PEG is recognized for reducing the attractive forces between nanoparticles by introducing a steric hindrance that deters close proximity between them and enhances repulsive forces through alterations in surface charge or hydration layers [46]. Post-lyophilization, we assessed the physicochemical attributes of the nanoparticles. Post-lyophilization, we assessed the physicochemical attributes of the NPs. Both the 1:0 and 1:1 PEGylated NPs exhibited stability in the absence of washing ('no wash' data from *table 3*). The washing process, which exposes PEG to water and removes PVA surfactant, could also potentially contribute to aggregation. Additionally, the observed aggregation may be ascribed to the nanoparticles' instability under severe lyophilization conditions—a situation that could be mitigated by employing PVA as a cryoprotectant, as suggested by various studies [47, 48]. Notably, severe aggregation was solely detected in the 1:0 PEGylated NPs after washes, which may additionally be correlated with the higher outer PEG density. While PEG coatings are generally recognized for their protective role against NP aggregation, our findings indicate that the efficacy of PEG coatings is dependent on specific PEG properties and formulation parameters.

During the washing stages, both PEGylated NP formulations exhibited shifts in ζ . Specifically, the 1:0 formulation saw a change from -32 mV to -27 mV, and the 1:1 formulation from -30 mV to -22 mV. These shifts highlight the influence of PEG exposure on the PLGA-PEG NP surface. Initially, the NP surface is shrouded with surfactants, polymers that did not form NPs, or unencapsulated PFC, which limits

the mobility of the outer PEG chains. Consequently, the measured surface charge may not accurately reflect the true charge of the NP surface. The washing process reveals the actual NP surface by removing these layers. Since PEG is neutrally charged and, in this instance, ester-terminated, the rise in ζ post-washing could be due to the increased visibility of the PEG ester chains on the surface. The loading capacity for the 1:1 PEGylation remained stable after three washes, unlike the 1:0 PEGylation, which experienced a significant drop in loading capacity values from 34 *wt/wt%* to 12 *wt/wt%*. This substantial decrease in LC for the 1:0 formulation might be associated with NP aggregation, as suggested by a PDI of 0.4.

Table 1. Assessment of Size, PDI, Zeta Potential (ζ), and loading capacity for 1:1 and 1:0 PEGylated PLGA

	Substitution	Washes	Size (nm)	PDI	ζ (mV)	Loading Capacity (wt/wt %)
1.	1:1	No wash	325 $\pm$ 8	0.24 $\pm$ 0.02	-32 $\pm$ 2	60 $\pm$ 6
		3 washes	392 $\pm$ 40	0.28 $\pm$ 0.02	-27 $\pm$ 3	55 $\pm$ 1
2.	1:0	No wash	337 $\pm$ 56	0.20 $\pm$ 0.02	-30 $\pm$ 2	34 $\pm$ 9
		3 washes	690 $\pm$ 24	0.41 $\pm$ 0.07	-22 $\pm$ 4	12 $\pm$ 3

**Figure 3.** Comparative Analysis of Size (A), PDI (B), ζ (C), and loading capacity (D) for PLGA with 1:1 and 1:0 PEGylated PLGA. Significant values are marked with an asterisk (* p < 0.05, ** p < 0.01, *** p < 0.001)**Figure 4.** Assessment of Washing Impact on NPs: Evaluating Size (A), PDI (B), ζ (C), and loading capacity (D) of 1:1 and 1:0 PEGylated PLGA Before and After Three Wash Cycles. Significant values are marked with an asterisk (* p < 0.05, ** p < 0.01, *** p < 0.001)

3.2.1 PLGA protein corona

The research delineated the protein corona's formation, emphasizing the contrast between PEGylated and non-PEGylated nanoparticles. PEGylation has been extensively studied. 1:0 PEGylation from our study was taken to study the protein corona interaction. Bovine serum albumin (BSA) and plasma proteins were employed to assess the NPs' response. Protein adherence to the NPs' surface is expected to result in an augmented particle size, detectable via dynamic light scattering (DLS) [34-36]. The NPs' dimensions were gauged prior to and subsequent to protein exposure at three intervals: immediately, 2 hours, and 24 hours post-exposure to BSA and plasma proteins (*figure 6*). The ratio of NPs to protein was maintained at 1:13 for all particles. The PLGA empty system (PLGA devoid of PFC) exhibited notable interactions at each interval (*figure 6A & B*), akin to the sonication-derived NPs (*figure 6C & D*). Conversely, the PEGylated particles displayed distinct behaviour. No substantial dimensional alterations were observed immediately post-exposure or at 24 hours as indicated in *figure 6E & F*. A significant size increase was only noted at 2 hours, attributed to the transient formation of a soft corona.

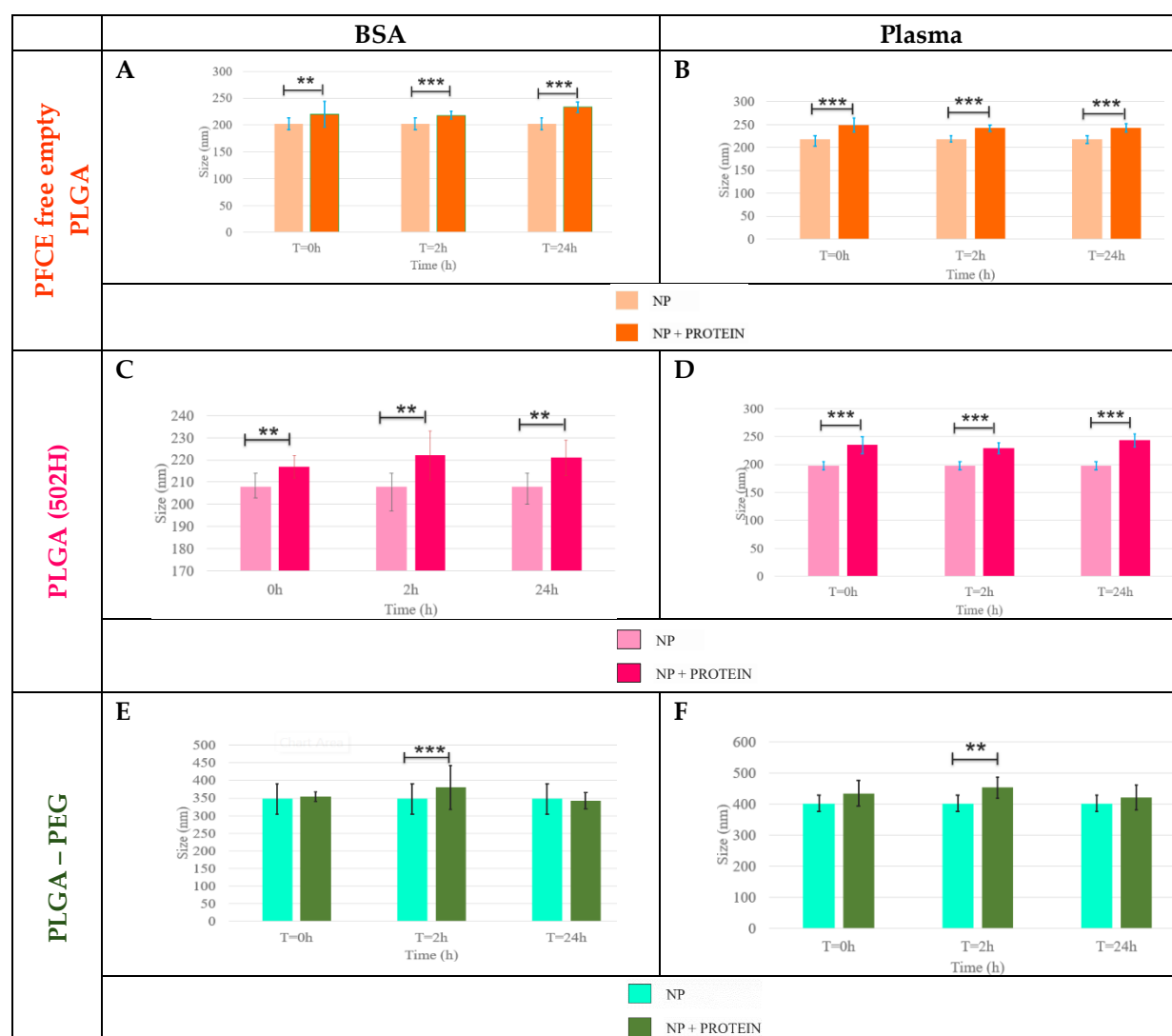


Figure 6. Analysis of Protein Corona Formation on PLGA Variants: Empty PLGA (A & B), Sigma-Aldrich PLGA 502H (C & D), and PLGA-PEG (E & F). Significant values are marked with an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Upon exposure to biological fluids, NPs first attract and bind proteins that are present in large quantities. Subsequently, these proteins are displaced by others that have a stronger affinity for the NP surfaces, a phenomenon recognized as the Vroman effect [49]. The protein corona, based on its affinity, is categorized into ‘hard corona’ and ‘soft corona.’ The hard corona consists of a stable, equilibrium layer of proteins that adhere strongly to the NP surface. These proteins have a high affinity and bind irreversibly, characterized by a low dissociation constant, and they form quickly after administration. This hard corona is more stable and closely connected to the nanoparticles, playing a crucial role in how the nanoparticles interact with nearby cells [50]. In contrast, the ‘soft corona’ is composed of proteins with lower affinity, exhibiting a more dynamic nature, engaging in swift exchanges with the proteins in the surrounding medium, and demonstrating lower rates of association [50, 51]. Nevertheless, further investigation into the effects of soft corona formation in the PEGylated is warranted to elucidate its implications for biocompatibility.

3.3 PLGA F

Fluorination of PLGA was explored as a method to potentially enhance the PFCE-loading efficiency of NPs. The strategy involved using an alcohol derivative of fluorine, specifically heptadecafluoro-1-nonanol, in place of the traditional alcohol co-initiator. This choice was based on selecting a fluorine alcohol with the highest fluorine content, containing 17 fluorine atoms. Consequently, the Ce-based ROP utilized lactide and glycolide with the selected fluorine alcohol to synthesize a polymer with a Mw of 17,388 Da and a lactide to glycolide ratio of 42:58 (figure 7). Fluorination levels were verified by F-NMR to be at 20%.

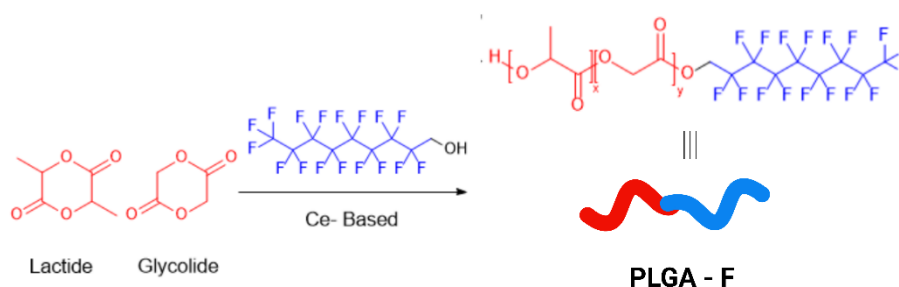


Figure 7. ROP for the fluorine -PLGA (F17-PLGA) synthesis (Mw 17,388Da, heptadecafluoro-1-nonanol as co-initiator, Ce-based, lactide: glycolide ratio 42:58)

Fluorine-containing molecules exhibit unique interactions that allow them to form a distinct phase, characterized by simultaneous hydrophobic and lipophobic properties. Introducing fluorine into the polymer system may enhance fluorophilic interactions, a concept explored in various studies [52-59] which could be advantageous for retaining PFCs. To understand the behaviour of fluorinated PLGA, we examined the encapsulation of PFCE within NPs. Although the fluorination of polylactic acids has been extensively researched [52, 53, 57, 59], there are limited studies on PLGA fluorination. Previous attempts, such as those by Neri *et al.* [60], involved fluorinating PLGA polymers with different fluorinated amine ligands to produce F3-PLGA and F9-PLGA, containing three and nine fluorine atoms, respectively. These modified PLGAs demonstrated improved loading capacities for fluorine-containing drugs. Another study by Zerrillo *et al.* [61], involved fluorinating PEGylated PLGA with trifluoroacetic acid for osteoarthritis imaging applications, where the fluorine content in trifluoroacetic acid was sufficient for FMRI imaging.

We hypothesized that the incorporation of PFCE into NPs could be enhanced by the potential fluorophilic interactions between the copolymer and PFCE, leading to more effective retention when using fluorinated PLGA (PLGA-F). A formulation with 20% fluorine was developed to observe the polymer's behaviour in NP form. Full substitution (20%) was used to assess this behaviour. Particle sizes were measured at 450 nm before washing and increased to approximately 500nm post-washing (*table 7, 1*), with a PDI ranging from 0.5 to 0.6. Such aggregation behaviour is consistent with previous findings in fluorinated polymeric systems, a phenomenon termed "hydrophobic aggregation-induced signal attenuation" [58, 62]. It has been shown that when the ^{19}F content in the molecular structure exceeds 10 *wt.%*, it results in the hydrophobic aggregation of fluorinated segments leading to the non-detection of fluorine signals due to aggregation. In an aqueous environment, the segments of the polymers that contain fluorine are prone to aggregate. This tendency to come together could result in the suboptimal creation of nanosystems, thereby affecting the encapsulation process of PFCE.

A negative zeta potential was observed, as expected from the alcohol end group. Without washing, the loading capacity reached up to 70 *wt/wt %* on average, albeit with a high standard deviation. However, post-washing, the PFC loading was found to decrease, which could be attributed to imprecise measurements due to aggregation or the high fluorine concentration causing the fluorinated PLGA to segregate without retaining PFCE.

3.4 Formulation of PLGA – F & PLGA PEG (F_{17} -PLGA-PEG)

Aggregation was noted upon reaching a fluorine concentration of 20%, prompting a reduction in concentration to augment hydrophilicity through the introduction of polyethylene glycol (PEG). A 50:50 experimental blend of poly(lactic-co-glycolic acid)-fluorine (PLGA-F) and poly(lactic-co-glycolic acid)-polyethylene glycol (PLGA-PEG) was subjected to testing (*table 7, 2*). This blend maintained the PEG ratio consistent with that utilized in protein corona investigations. Subsequent particles demonstrated an increase in size but a decrease in aggregation relative to PLGA-F in isolation, as evidenced in *table 7.2*. The zeta potential was presumably diminished due to the presence of the PEG exterior layer. Variability in particle PDI was introduced following particle washing, resulting in an elevated standard deviation (0.33 ± 0.10). It is noteworthy that the loading capacity experienced a reduction after the washing process, and when benchmarked against standard PLGA, no enhancements were discernible. This underscores the necessity for further system optimization.

Table 7. Evaluation of Size, PDI, Zeta Potential, and loading capacity for fluorinated plga (F_{17} PLGA) and formulation combining PLGA PEG and fluorinated PLGA (F_{17} -PLGA-PEG)

	Substitution	Washes	Size (nm)	PDI	Zeta Potential (mV)	Loading Capacity (wt/wt %)
1.	F_{17} -PLGA	No wash	444 ± 98	0.52 ± 0.10	-32 ± 1	71 ± 20
		3 washes	493 ± 90	0.59 ± 0.11	-27 ± 2	56 ± 5
2.	F_{17} -PLGA-PEG	No wash	338 ± 54	0.29 ± 0.08	-29 ± 1	52 ± 11
		3 washes	418 ± 94	0.33 ± 0.10	-18 ± 4	34 ± 15

3.5 Cytotoxicity studies

Cytotoxicity assessments with fibroblasts were conducted using two concentrations of NPs, 5 and 10 μg . The study encompassed PLGA empty, commercial PLGA (PLGA 502H), and Ce-based PLGA, as well as PLGA-PEG and PLGA-F. All variants were found to be non-toxic (figure 8).

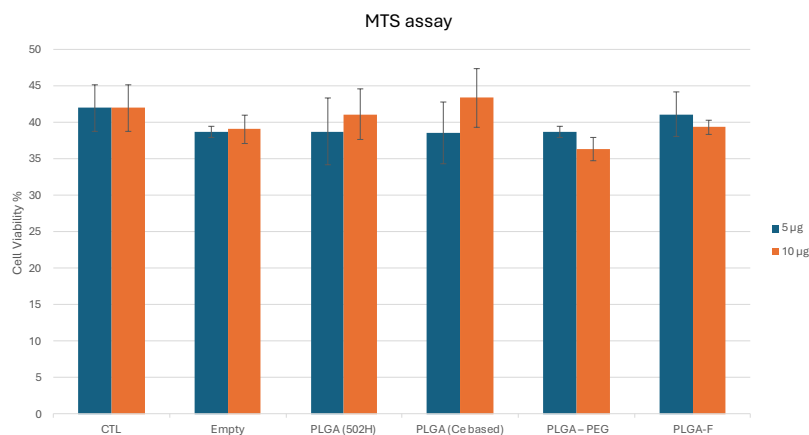


Figure 8. Biocompatibility studies determined by MTS assay in fibroblasts cells. Values are expressed as mean $\pm$ S.D. ($n = 3$)

4. Conclusion

In this study, a novel CE-based catalyst was introduced for the ring-opening polymerization of PLGA, facilitating the preparation of PLGA, PLGA-PEG, and PLGA-F formulations. The newly synthesized Ce-based PLGA was investigated for its encapsulation efficiency with PFC and was deemed suitable for use. We evaluated formulations with ratios of 1:1 and 1:0 for PLGA-PEG to PLGA based on physicochemical properties including size, PDI, ζ , and loading capacity. The formulation with a 1:1 PEGylation ratio was selected for further exploration of protein corona behavior and was found to enhance the properties compared to unmodified PLGA. The interaction at the 2-hour mark warrants additional investigation. Attempts to prepare PLGA fluorine and combine PLGA-F with PLGA-PEG were also made. This exhaustive research not only propels the comprehension of PLGA-based systems forward but also paves the way for the innovation of efficacious FMRI contrast agents.

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Chapter 6

Conclusion and Future Perspectives

1. Summary

This research delves into the increasingly popular topic of perfluorocarbons (PFCs) for medical applications.

Chapter 1 *Introduction to Perfluorocarbons*: Chapter 1 provides a general introduction to PFCs, highlighting their role as contrast agents in fluorine-19 magnetic resonance imaging (^{19}F -MRI) and the necessity for an effective drug delivery system, such as poly(lactic-co-glycolic acid) (PLGA), to utilize PFCs efficiently. The overarching goal of the thesis is outlined in this chapter.

Chapter 2 *Nanotechnology as a Versatile Tool for ^{19}F -MRI Agent's Formulation: A Glimpse into the Use of Perfluorinated and Fluorinated Compounds in Nanoparticles*: Chapter 2 presents a literature survey of state-of-the-art PFCs and their delivery systems currently employed as contrast agents. This chapter details the drawbacks in the use of gadolinium-based contrast agents, and explores the growing interest in PFCs. A comprehensive review of the currently utilized PFCs, their properties, and the characteristics of an ideal contrast agent are discussed. Subsequently, various carrier systems, including polymeric, liposomal, micellar, and metallic systems for PFC delivery are examined. Our study synthesizes the majority of literature on PFC updates up to the year 2023.

Chapter 3 *The Art of Preparations of PFCE Encapsulated PLGA Nanoparticles : Formulation and optimisation using High Speed Homogenization and Ultrasonication*: Chapter 3 focuses on the formulation of PFC nanoparticles (NPs), specifically perfluoro-15-crown-5-ether (PFCE) with PLGA. This section investigates various formulation aspects, including the use of two different molecular weights of PLGA and the surfactant polyvinyl alcohol (PVA). Comparisons between probe sonication and homogenization were conducted. The structure and physicochemical properties of the formulated nanosystems were studied. The findings reveal that the preparation technique and polymer-surfactant molecular weight combination significantly influence NP size, Polydispersity Index (PDI), zeta potential (ζ), and Loading Capacity.

Chapter 3 delves into the Phase 1 optimization of NPs, focusing on the profound impact that various techniques have on the physicochemical properties of formulations. Despite the common practice of selecting techniques based on laboratory availability, the outcomes significantly influence the properties of the NPs. In this polymeric system, the employment of varied molecular weights of PLGA polymer and surfactant led to findings that necessitated analysis beyond simple metrics such as size, PDI, charge or loading capacity. To formulate preliminary conclusions, it was imperative to utilize a suite of comprehensive methods. These included scattering techniques for analyzing populations, scanning electron microscopy for surface morphology, and confocal microscopy for internal structure assessment.

The **chapter 3** attempts to consolidate findings on several aspects:

- I. The influence of two molecular weights of PLGA polymer.
- II. The effect of two molecular weights of surfactant.
- III. The choice of techniques, namely sonication and homogenization.

The study compared three NP formulation techniques: homogenization, sonication, and a hybrid method. Homogenization resulted in the largest size (up to 500nm) and most variable NP PDI (0.1 – 0.5), sonication produced intermediate-sized NPs with high loading capacity, indicating successful PFCE encapsulation, and the hybrid method created the smallest and most uniform NPs, albeit with variable loading capacity and ζ . Structural analysis confirmed the spherical shape and distribution of PFCE within the NPs. Cytotoxicity tests confirmed the NPs' safety, and protein corona studies suggested a quick systemic uptake, pointing to surface modification as a means to prolong circulation time. The findings emphasize the significance of selecting the appropriate technique and molecular weight (MW) of materials for NP design, providing valuable insights for enhancing NP-based drug delivery systems.

Chapter 4 *Central Composite Design of Experiment Based Optimization of Key Formulation Parameters for PFCE-Encapsulated PLGA Nanoparticles:* Chapter 4 approaches the formulation from a statistical perspective. A thorough investigation of the formulation parameters, including the percentage of surfactant, sonication time, and volume of PFCE, is conducted using central composite design. The impact of these parameters on particle size, PDI, ζ , loading capacity, signal-to-noise ratio, and T_1 and T_2 relaxation times is analysed.

In **Chapter 3**, the investigation of various facets necessitated numerous experimental iterations and a substantial commitment of time for Phase 1 optimization. This culminated in the implementation of a statistical methodology for Phase 2 optimization, which is examined in **Chapter 4**. Three parameters identified as critical during Phase 1 were selected for further study: the percentage of surfactant (ranging from 2 to 4%), the volume of PFCE (ranging from 300 to 900 μL), and the sonication time (ranging from 3 to 9 minutes). In our series of 20 experiments, we examined both first-order and second-order effects on the outcomes. A significant advantage of these experiments was the capacity to reveal the second-order effects within the formulations, demonstrating the simultaneous influence of two factors. This insight is crucial, as it is common for experiments to be erroneously designed under the presumption that outcomes are governed by a single variable. Our findings indicated that certain parameters (for example zeta potential) displayed exclusively second-order effects, with no linear effects detected. This underscores the importance of a statistical approach to problem-solving, which also offers significant time savings. As of March 2024, there were scarcely any formulation studies investigating PFC formulations using a statistical approach. A notable gap is the need for a statistical expert knowledgeable in the different available designs and capable of performing computational studies, particularly when creating 2D and 3D plots. However, this is a learnable skill and should be considered an essential tool for formulators tasked with optimizing several parameters concurrently.

The investigation in **chapter 4** utilized a central composite design (CCD) to explore the impact of various parameters on the characteristics of PFCE-encapsulated PLGA NPs. The study meticulously analyzed the influence of sonication time, PFCE volume, and surfactant concentration on the NP's size, PDI, ζ , loading capacity, SNR, and relaxation times. The robust statistical model employed provided substantial insight into the factors affecting NP behavior, accounting for a significant percentage of the observed variability in key properties. Notably, the model explained 87% of the size variation, 80% of the PDI, 71% of the ζ , and 87% of the SNR. While the loading capacity and T_1 relaxation time models were less definitive, the T_2 relaxation time model offered some explanatory power. These findings highlight the pivotal role of the examined variables in tailoring PLGA NPs for enhanced performance as MRI contrast agents.

Chapter 5 *Strategic Enhancement of PLGA Nanoparticles: PEG and Fluorine Additions for PFCE Encapsulation:* Chapter 5 discusses the synthesis of modified PLGA and their application for encapsulation of PFCE. A biocompatible cerium (Ce) catalyst is utilized for the ring-opening polymerization (ROP) of PLA, which was previously studied, thus the ROP of PLGA is examined and assessed for its capacity to load the contrast agent PFCE. The PLGA is then modified with polyethylene glycol (PEG) and fluorine. PEGylation is performed to enhance biocompatibility, and fluorination is conducted with the aim of improving the retention of PFC within the nanosystem. **Chapter 5** continues to explore the encapsulation of PFCE, but with a focus on polymers synthesized in-house. While commercially available polymers are more convenient to use, their fundamental properties are often not well-defined. For instance, the molecular weight is typically provided as a broad range, reflecting the polydisperse nature of polymers produced industrially, which does not guarantee a monodisperse population. This polydispersity can affect the outcome of experiments, leading to non-reproducible results—not due to experimental error, but because of inherent variability in the polymer properties. The commonly used tin catalyst is known to be toxic at concentrations greater than 20 ppm, and according to the safety data sheet from Sigma Aldrich, the tin content is reported to be less than or equal to 200 ppm. These two critical factors motivated our decision to synthesize PLGA in-house and

subsequently functionalize these polymers for further study. The state-of-the-art catalyst was evaluated for its ring-opening polymerization (ROP) capabilities, and the physicochemical properties of the resulting polymer indicated its potential suitability for PFC encapsulation.

Functionalization of PLGA with polyethylene glycol (PEGylation) was undertaken as a direct consequence of observations made in **Chapter 2**. When PLGA alone was used for formulation, a significant increase in particle size was noted upon exposure to proteins, suggesting that the particles might be readily recognized by the reticuloendothelial system in the body and removed from circulation, being identified as a foreign entity. PEGylation has been studied extensively and found to mitigate this behaviour, prompting us to investigate the PEGylation of PLGA. Indeed, the behaviour of PEGylated PLGA was found to differ markedly from that of unmodified PLGA. The optimization of fluorination is still ongoing and requires further study. **Chapter 5** introduced a pioneering Ce-based catalyst for the ring-opening polymerization process, streamlining the synthesis of various PLGA formulations, including PLGA, PLGA-PEG, and PLGA-F. The encapsulation efficiency of the novel PLGA with PFC was assessed and confirmed as effective. We explored two PEGylation ratios, 1.0 and 1:1 of PLGA-PEG to PLGA, evaluating their physicochemical attributes such as particle size, PDI, ζ , and loading capacity. The study favoured a 1:1 PEGylation for in-depth analysis of protein corona formation, which showed improved characteristics over the non-PEGylated PLGA. Notably, the 2-hour interaction point emerged as a critical area for further study. Additionally, the research ventured into combining PLGA-F with PLGA-PEG and PLGA fluorine to investigate potential synergistic effects.

2. Future Perspectives

The potential of PLGA-based matrices in medical applications is vast, with possibilities for creating polymers tailored for specific uses or degradation rates. The approval of PLGA by the FDA and EMA for a variety of medical applications is one of its key benefits and gives PLGA-based materials significant advantages for clinical trials and applications. Owing to its exceptional mechanical characteristics, robust biodegradability, and biocompatibility, PLGA is acknowledged as an apt system for the controlled release of pharmaceuticals, peptides, proteins, and various biomolecules. Generally speaking, the monomer composition, molecular weight, and characteristic viscosity of PLGA all have a significant impact on its characteristics. These factors also have an impact on the drug-loading capacity (in our case the contrast agent PFC), when PLGA is used as a drug-delivery matrix. As a result, when selecting various PLGA types, researchers should take their physicochemical characteristics into account. Functionalization of NPs surfaces can enhance targeted delivery to diseased tissues while minimizing impact on healthy cells, thereby improving disease diagnosis and treatment. However, the translation of these advancements into clinical practice necessitates further research. The dynamic field of NPs presents both opportunities and challenges, with current technologies like *in vivo* imaging facilitating the study of NPs behaviour. Despite progress, discrepancies in research findings underscore the complexity of biological interactions and the need for refined analytical methods. Bridging the gap between fundamental research and product development of PLGA NPs is crucial, as is understanding the interaction between PLGA and drugs to optimize therapeutic efficacy. Future studies should focus on elucidating the mechanisms governing stability, and bioavailability within PLGA systems.

2.1 Advancing Clinical Translation: Navigating the Complexities of PFC Delivery Systems

PFC molecules are purely synthetic molecules consisting of majorly carbon and fluorine atoms. Some newly explored ideas include the use of fluorinated molecules in gene therapy and the utilization of nano phase shift droplets in ultrasound imaging. Despite the dual hydrophobic and lipophobic characteristics of PFCs posing challenges for *in vivo* administration, these properties facilitate their effortless translocation across cell membranes. This ability enhances the transmembrane delivery of biomolecules such as genes and proteins, thereby positioning PFCs as potential gene therapy vectors [1-4]. PFCs, traditionally used as contrast agents in ultrasound imaging, were predominantly limited to microparticle forms. The microscale dimensions of these particles are essential for achieving effective acoustic properties that reflect sound waves, thereby generating a signal. However, this microscale size

confines their use to specific applications such as cardiac or vascular imaging and is associated with a brief half-life. The emergence of PFC phase-shift nanodroplets represents a significant advancement. These nanodroplets are capable of transitioning from a liquid to a gaseous state through a process known as acoustic droplet vaporization (liquid droplets are converted into gas bubbles). This transition facilitates their penetration into tumor tissues, attributable to the high compressibility of PFCs, which also serves to extend their half-life [5, 6]. Furthermore, these phase-shift nanodroplets hold potential for applications in photothermal therapy and concurrent drug delivery.

The scope of PFCs' applications continues to expand beyond MRI imaging. PFC also have found application in computed tomography [7], fluorescence imaging [8, 9], photoacoustic imaging [10], radiation therapy [11-13], sonodynamic therapy (drugs that become cytotoxic upon exposure to ultrasound) [14, 15], high-intensity focused ultrasound therapy [16, 17], chemotherapy [18-21], combination therapy, [22, 23] *etc.* PFC research, despite its extensive history and global development efforts, faces challenges in clinical translation, with only a few formulations achieving regulatory approval. The inherent properties of PFCs, such as human biocompatibility and remarkable stability, contrast with the clinical setbacks primarily caused by the carrier systems used for delivery rather than the PFCs themselves. Historical instances, including the withdrawal of FDA-approved formulations like Fluosol-DA and Oxycyte due to adverse inflammatory responses, underscore the criticality of understanding the physicochemical interactions within the carrier systems [24]. The scientific community has extensively explored various PFC carrier systems, from traditional nanoemulsions to innovative liposomes, NPs, and micelles, alongside alternative fluorine molecule synthesis and diverse therapeutic applications. Yet, research gaps persist in fundamental areas such as optimal carrier system development techniques, appropriate surfactant selection and concentration, and the need for specialized surfactants for effective fluorine encapsulation.

Further investigation is required to determine the maximum PFC load a carrier system can sustain, the tunability of this load, the ideal PFC quantity for diagnostic imaging, and the stability of different PFC system forms across applications. Moreover, the mechanisms that retain PFCs within the carrier system and their potential inflammatory effects upon introduction to the body warrant thorough examination. Recognizing that "The Dose Makes the Poison," it is imperative to establish the optimal dosing not only of PFCs but also of the entire PFC-carrier system for safe and effective clinical use. This complex endeavour calls for a multidisciplinary approach, uniting chemists, biologists, statisticians, physicians, and clinicians to address these pivotal questions. Our study hopes to contribute to this collective knowledge, offering insights and foundational principles that may guide future formulators towards the successful clinical implementation of PFC-based contrast agents, thereby advancing the field of effective PFC drug delivery systems.

2.2 Enhancing Our System

In our research, we advocate for advancements to augment the safety and efficacy of NP systems. In refining our system, we propose several enhancements. We propose a shift from using chlorinated solvents such as dichloromethane (DCM), which carry potential health risks, to more benign alternatives like ethyl acetate. The 'PEG dilemma' – the immune response triggered by repeated doses of PEG-containing treatments leading to the production of anti-PEG antibodies – necessitates exploration of innovative polymers [25-27]. These antibodies, particularly IgM and IgG, expedite the clearance of PEGylated NPs, diminishing treatment effectiveness and potentially causing hypersensitivity reactions. A study by Grenier *et al.* [28] highlighted that even a single dose of PEG-PLGA NPs could initiate the production of anti-PEG IgM, thereby increasing NP clearance through the activation of the complement system. This reaction not only curtailed the circulation time of PEG-PLGA NPs after subsequent administrations but also led to an enhanced clearance of PEGylated liposomes. To address these challenges, we suggest investigating alternative polymers [27] such as polyphosphoesters, polysaccharides, polyacrylamide, poly(N-vinyl-2-pyrrolidone), poly(2-oxazoline), or

poly(2-ethyl-2-oxazoline), which may offer promising pathways to mitigate immune responses and improve NP circulation time in future studies.

3. Conclusion

This dissertation conducts an in-depth analysis of the formulation process for PLGA NPs containing PFCE, offering a detailed exploration of the individual parameters influencing the formulation. The study examines the impact of formulation techniques, the molecular weight of PLGA, and surfactant concentration—each analysed individually. Additionally, factors such as surfactant percentage, volume of PFC, and sonication duration are investigated through a central composite design to determine their effects on the physicochemical properties of the NPs. The study further innovates by modifying PLGA with a novel Ce-based catalyst and integrating PEG and fluorine to enhance its properties. While some PFC based formulations in the developmental phase and clinical trials continue, the journey of PFCs from the laboratory to clinical practice remains challenging. Nonetheless, the relentless pursuit over decades and the integration of new technologies in PFC delivery systems offer a beacon of hope for regulatory approval. The insights garnered from this research are poised to underpin future breakthroughs in NP-based biomedical applications. With a focus on the clinical translation of these findings, there is a strong belief that many PFC products will soon transition from promising concepts to tangible medical solutions.

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List of Abbreviations

^{19}F MRI/FMRI	Fluorine-19 Magnetic Resonance Imaging
^{19}F NMR	Fluorine-19 Nuclear Magnetic Resonance
^1H MRI/HMRI	Proton Magnetic Resonance Imaging
AFM	Atomic Force Microscopy
C NMR	Carbon-13 Nuclear Magnetic Resonance
CA	Contrast Agent
CLSM	Confocal Laser Scanning Microscopy
CM	Confocal Microscopy
CrM	Correlation Microscopy
C-SEM	Scanning Cryo-Electron microscopy
CT	Computed Tomography
C-TEM	Cryo-Transmission Electron Microscopy
DLS	Dynamic Light Scattering
EDX	Energy-Dispersive X-Ray Spectroscopy
EGDMA	Ethylene Glycol Dimethylacrylate
EMA	European Medicines Agency
EPR	Electron Paramagnetic Resonance Spectrum
FC	Flow Cytometry
FDA	The Food and Drug Administration
Fe_3O_4	Iron Oxide/Magnetite
FI	Fluorescence Imaging
FITC	Fluorescein Isothiocyanate
FLAME	Fluorine Accumulated Silica NP for MRI Contrast Enhancement
FM	Fluorescence Microscopy
FTIR	Fourier Transform Infrared Spectroscopy
GBCAs	Gadolinium Based Contrast Agents
Gd(III)/Gd $^{3+}$	Gadolinium-III
GPC	Gel Permeation Chromatography
HNMR	Proton Nuclear Magnetic Resonance
HNT	Halloysite Nanotube
HPLC	High-Performance Liquid Chromatography
mPEG	Monodisperse Poly (ethylene-glycol)
MR	Magnetic Resonance
MRI	Magnetic Resonance Imaging
MRS	Magnetic Resonance Spectroscopy
NIR	Near Infrared
NIRS	Near Infrared Spectroscopy and Imaging
NMR	Nuclear Magnetic Resonance
NP	Nanoparticle
PAI	Photoacoustic Imaging
PEG	Poly (ethylene-glycol)
PET	Positron Emission Tomography
PFC	Perfluorocarbon
PFCE	Perfluoro-15-crown-5 ether
PFOB	Perfluorooctyl Bromide
PFP	Perfluoropropane
PFPE	Perfluoropolyether
PFTB	Perfluoro-tert-butanol
PLGA	Poly (lactic-co-glycolic acid)
PTT	Photothermal Therapy
RF	Radiofrequency

ROS	Reactive Oxygen Species
SANS	Small Angle Neutron Scattering
SEM	Scanning Electron Microscope
SD	Standard Deviation
SLS	Static Light Scattering
SNR	Signal/Contrast-to-Noise Ratio
STEM	Scanning Transmission Electron Microscope
TEM	Transmission Electron Microscope
TFEA	2,2,2-trifluoroethyl Acrylate
TGA	Thermogravimetric Analysis
US	Ultrasound
UV-Vis	UV/Vis Absorption Spectra
XPS	X-Ray Photoelectron Spectroscopy
XRD	X-Ray Diffraction
ZP	Zeta Potential

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