Title: IMAGEABLE LIPID-OIL-WATER NANOEMULSION THERAPEUTIC DELIVERY SYSTEM

Abstract: A stable lipid-oil-water nanoemulsion associated with an imaging agent and a therapeutic agent is useful for both imaging and treating cells characterized by aberrant lipid metabolism and/or cellular hyperproliferation, such as cancer cells, in an exquisitely specific and selective manner compared to non-transformed cells.
Imageable Lipid-OH-Water Nanoemulsion Therapeutic Delivery System

Field of the Invention

This invention relates to therapeutic and diagnostic agents, and more particularly to a lipid-oil-water nanoemulsion suitable for introduction into a patient and capable of promoting both active and selective imaging and therapeutic agent concentration into cells characterized by aberrant lipid metabolism, including but not limited to tumor cells, and imaging and treatment of a primary or metastatic tumor formed by such cells.

Background of the Invention

Response Criteria In Solid Tumors (RECIST), a set of unified anatomic imaging criteria for measuring tumor response to anticancer therapy based on a simplification of diverse but accepted international protocols, has been used in clinical oncology for nearly a decade. While voluntary, the use of RECIST is recommended for US National Cancer Institute-sponsored trials for the evaluation of experimental drugs in clinical trials from Phase II and beyond. Evaluation of response of the phase I clinical trials is rendered more difficult by an escalating dose, diverse patients with advanced disease and uncertainty as to imaging or other assay tools by which to measure response other than by survival. RECIST uses x-ray, computer aided tomography (CT), and magnetic resonance imaging (MRI) to extract linear measurements that are proposed to be an adequate substitute for two- or three-dimensional methods. Measurement and response is determined by comparative measurements of the most recent imaging results to measurements taken at time of diagnosis or at baseline prior to entering a clinical trial. Staging of disease may also taking into account alternative imaging techniques such as positron emission tomography (PET) using radiolabelled glucose, ultrasound, or imaging using radiolabelled antibodies which target tumor specific antigens. A complete response is disappearance of all target lesions, while a
partial response is a 30% decrease in the sum of the longest diameter of target lesions. Progressive disease is a 20% increase in the sum of the longest diameter of target lesions and/or the appearance of new tumors. Stable disease is considered to be small changes that do not meet the above criteria. To limit of detectability can affect interpretation of results as swelling of tumors can occur leading to an enlarged image even as tumor cells are responding to drug. Attempts to improve RECIST methodology include validating for clinical trial acceptable methods to incorporate volumetric three-dimensional anatomical imaging and the use of dynamic contrast imaging.

Historically, anatomical imaging has been fundamental to the evaluation of patients receiving drugs that are cytotoxic and expected to shrink tumors. It has been assumed not only that early determination of tumor shrinkage may potentially spare patients from experiencing those debilitating side effects accompanying chemotherapy but also that tumor shrinkage would correlate well with longer term survival. What has emerged in retrospective studies, however, is not only that tumor shrinkage may not always correlate well with longer term survival but also that metastatic disease as well as local relapse can contribute to disease-related death. Additionally, not all patients may respond to a drug at the same rate or in the same way. Furthermore, the emergence of new drugs with novel mechanisms of action has made the use of anatomical imaging questionable as an indicator for tumor response to drug. For example, where a drug has been found to be cytostatic, in some cases patients who would have been characterized under RECIST as having stable disease or a slowed trajectory of disease progression have gone on to achieve longer term survival than those patients receiving cytotoxic agents who would be considered responders under RECIST. In short, some drugs are able to help cancer be "managed" so that patients bearing tumors may nevertheless enjoy longer life expectancy and better life quality.
An additional confounding consideration for RECIST is that with some targeted therapies, the drug-related mechanism of tumor cell death can influence what appears in an anatomical image and vary with time of treatment. For example, the dissolution and shrinkage of tumors is part of the final steps in a complicated cascade of cellular and subcellular local and immune system changes as a response to therapy. Under some circumstances, tumor swelling, edema, and inflammation can occur, phenomena which may suggest tumor enlargement or growth on an anatomical image. Such effects make the timing of taking the image, the kinetics of these effects, and the resolution of these effects critical. While the use of contrast agents and additional imaging methods may help better elucidate both what is occurring within the tumor and the kinetics thereof, in many situations an imaging center may lack the expertise, equipment, or resources to accurately reach the proper interpretation of the tumor image presented. Cancer specific necrosis, apoptosis, or autophagy, or a combination of these and the precise impact of the mechanism of cell death on the nature of an image obtained by the various imaging modalities is unknown. It has been suggested that mechanisms of cell death may be linked to the dying cell's metabolic state and location relative to tumor vasculature, as well as the influence of a variety of signal transduction ligands. Hence, a simple linear measurement of the longest tumor diameter is less likely to provide an adequate picture of intratumoral events. Using the current RECIST criteria, it is possible that patients who are in fact responding to a particular drug may show tumor enlargement on imaging by CT or related methods and thus be mistakenly withdrawn from study if imaged too early. Indeed, in a worst-case scenario, a patient could be denied a lifesaving therapeutic without real cause.

Collectively, these issues indicate an urgent need for a more accurate ability to understand the benefit of therapeutic or investigational therapeutic agents, or lack thereof, early in the course of treatment through the use of imaging. Knowing when to continue to
treat or to change a patient's therapeutic regimen is fundamental to optimizing cancer care. Any improved imaging technology should preferably use methodology and equipment that is or can be made widely available to generate results which can be standardized and validated as a predictor of longer term survival. Such an ability to accurately measure disease response to chemotherapy will most likely require an integration of imaging modalities and the use of biomarkers. Biomarkers related to a drug's mechanism of action may thus prove to be more valuable than disease-related markers, which may be highly variable among patients and stages of disease.

Medical imaging technologies have undergone explosive growth over the last few decades in the availability of methods and modalities as well as in the collection and interpretation of results. Imaging is consequently assuming an increasingly important role in both clinical trials and in the treatment of cancer patients. It has been stated in the art that molecular targeted imaging agents are expected to broadly expand the capabilities of conventional anatomical imaging methods, potentially allowing clinicians not only to see where tumors may be located but also to visualize the expression and activity of specific molecules (e.g. proteases or protein kinase) and biological processes (e.g., cancer metabolism, metastasis, angiogenesis, and apoptosis) that influence tumor behavior and/or response to therapy. (See Weissleder R (2006). Molecular imaging in cancer. Science 312:1168-71, herein incorporated by reference.) It is anticipated that, upon validation through clinical studies, the use of molecular targeted imaging will be accepted as a biomarker, and perhaps ultimately as a surrogate endpoint, by the US FDA and worldwide regulatory agencies for clinical trials. This would result in smaller clinical trials requiring fewer patients, earlier go/no decisions for investigational drugs, a better understanding of dose-response relationships, faster regulatory approval for new drugs, and earlier use in clinical care in a manner that can be optimized for each patient.
The desired standard in the clinical practice of cancer imaging will thus most likely involve the use of targeted molecular imaging technology to accurately diagnose, manage, and treat many types of cancer. Targeted molecular imaging of cancer in combination with an anatomical imaging modality permits a physician to characterize a cancer mass' biological properties accurately and ultimately to select the treatment most likely to be effective based on the tumor's characteristics. Conventional imaging modalities, which often use additional contrast agents administered to the patient to enhance the quality of the images generated; these contrast agents often differ by the imaging modality used, and each contrast agent demonstrates different inherent strengths and limitations, such as toxicity. Additionally, through the use of molecular imaging technologies which exploit cancer's unique physiology, such as PET which uses small amounts of a variety of radioactive compounds to accurately and rapidly gauge the severity and spread of cancer by revealing the metabolic activity of cancer cells versus normal cells, a physician is now able to see within days of treatment through such cancer imaging how the tumor was affected by the therapy. Appropriate radiolabelled agents include 18-fluorodeoxyglucose (FDG) to measure metabolic activity of cancer cells versus normal cells, or radiolabeled nucleotides to measure cellular proliferation. A physician interpreting a PET image thereby gains visual and/or semi-quantitative information about treatment effectiveness much more quickly than through other conventional imaging modalities. Since responsive and nonresponsive disease can now theoretically be quickly differentiated, this is an important advance, as patients should not be detained from a potentially more effective treatment where one should be considered to exist. Further, patients experiencing a decreased quality of life as a result of being administered an ineffective drug should not have to endure a painful and disruptive side effect profile. Therefore earlier detected changes in cellular energy metabolism assessed by FDG PET are increasingly being relied upon to predict responses than are changes in tumor size. Indeed, it
has been reported for diverse indications that a significant decrease in cancer energy metabolism as detected by FDG PET correlates extremely well with longer term survival. Still, PET imaging has its shortfalls: the technique requires exposure to radiation, and some drugs may alter the ability for the radiolabeled agent to be taken up intracellularly without killing the tumor cells at a rate that is consistent with what appears on image. Additionally, false positives can occur on inflammation and invasion of tumors with infiltrating lymphocytes, or even on healing and fibrosis. Given these shortfalls, the ability to utilize molecular targeted ligands that are specific for cancer cells is therefore extremely important so as to enable distinguishing true disease response or progression from artifact.

As for other molecular targeted imaging approaches, measuring disruption of tumor blood flow and perfusion by MRI is another possible approach by which a physician may be able to see within days of treatment how tumors have been affected by the therapy. Finally, in the related field of radioimmunotherapy, molecularly-targeted isotopes can be delivered to specific targets within a cancer cell, concentrating cell-killing doses of radiation directly to a tumor mass, thereby decreasing radiation exposure to healthy tissue.

Distribution within tumors and their microenvironment of drugs and imaging agents is not been well understood. Higher doses of even the most potent drugs are often given to drive diffusion and encourage drug accumulation within a tumor mass. However, increasing drug concentration in a tumor mass does not always lead to increased intracellular drug concentration or the ability to reach an intracellular target organelle. Some drugs have been shown to increase concentration in a tumor mass by conjugation to polymers, liposome encapsulation, or solid lipid nanoparticle formulation. However, such conjugation or encapsulation may ultimately inhibit cellular uptake and consequently misdirect distribution. Higher dosing to drive tumor penetration by diffusion is also likely to increase the occurrence or intensity of side effects and contribute to the high morbidity of cancer treatment. It is
therefore desirable to devise a drug delivery technology that would improve drug penetration through tumor tissue and increase its concentration within tumor cells to the appropriate target organelle while simultaneously presenting an increased safety and efficacy profile. While diverse approaches have been explored for the passive accumulation of drugs into tumors, however, few technologies are available for the promotion of active tumor cell uptake. This is especially the case with targeted molecular imaging.

Lipid-soluble drugs readily penetrate cell membranes and may be transported through cells. Cells characterized by hyperproliferation, such as cancer cells, generally exhibit an aberrant lipid metabolism, marked by their greatly-increased preferential uptake of lipids and fatty acids. Indeed, it is not uncommon for cancer patients to present with low or depleted levels of serum lipids, including cholesterol. Upregulation of lipid receptors as well as changes in plasma membrane lipid raft compositions have been suggested to be associated with diverse solid tumor cell types and increased lipid uptake. Thus, with respect to cancer, lipids have been found to play essential roles in membrane structure, growth and metastasis, signal transduction, and transport processes.

Indeed, lipids themselves have been to be of anticancer benefit. Ceramide is a sphingolipid that activates stress kinases such as p38 and c-JUN N-terminal kinase (JNK). In one study, it was reported that, although chronic myelogenous leukemia (CML)-derived K562 cells resisted killing by short chain C2-ceramide, longer chain C6-ceramide promoted apoptosis in these cells. (See Nica AF, Tsao CC, Watt JC, Jiffar T, Kurinna S, Jurasz P, Konopleva M, Andreeff M, Radomski MW, and Ruvolo PP (2008). Ceramide promotes apoptosis in chronic myelogenous leukemia-derived K562 cells by a mechanism involving caspase-8 and JNK. Cell Cycle 7:3362-70, herein incorporated by reference.) Additionally, in US Patent 5773431 to Javitt, herein incorporated by reference, 26-aminocholesterol and the related compound 27-hydroxycholesterol were demonstrated to have selective and potent
cell-killing effects on L1210 mouse leukemia cells, K-12 rat colon adenocarcinoma cells, and HCT-8 human colon carcinoma cells.

Caused by the characteristic leaky features of tumor vasculature, the phenomenon known as the enhanced permeability and retention (EPR) effect for lipid particles, liposomes, synthetic nanoparticles, and other macromolecular agents is universal in solid tumors and has been explored for more selective targeting and tumor mass accumulation of, for example, polymer-conjugated anticancer drugs. The use of encapsulating agents such as polymeric vesicles, liposomes, and solid polymeric or lipid nanoparticles, each prepared in a variety of compositions, formulations, and structures and administered under a variety of physiological conditions, has been reported in the prior art. However, these agents permit only passive accumulation in tumors. Moreover, these agents are often rapidly cleared from the circulation by extensive co-accumulation in the reticuloendothelial system, and often the use of these agents is associated with cytotoxicity and heightened immunoresponses.

Studies have shown that multiple receptor types and subtypes, transport proteins, and lipid raft phenomena are likely to be involved in lipid uptake into cancer cells, even among patients with the same disease but altered genetics. While lipid-based nanoparticles or HDL- or LDL-based drug carriers that mimic the natural receptor ligand and increase targeting and drug uptake into cancer cells have been prepared with various therapeutic agents, however, optimized formulations for the promotion of active tumor cell drug uptake, especially agents for the imaging of cancer, have been lacking.

Despite existing for almost a century, fluorescent dyes have relatively only recently been explored for their capability to act as targeted molecular imaging agents for cancer. While many classes of highly fluorescent organic compounds are known, over the past two decades dyes from the difluoro-boraindacene (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) family (BODIPY) have been recognized as a photostable substitute for fluorescein.
BODIPY's inherent photophysical properties, including excellent thermal and photochemical stability relative to fluorescein; high fluorescence quantum yield; negligible triplet-state formation and nanosecond excited-state lifetimes; intense absorption profile and large absorption coefficients; narrow emission profiles; good solubility; relative insensitivity to changes in polarity and pH; and large range of colors have all added to the general attractiveness of these fluorophore materials. Several new BODIPY-based dyes have thus been designed for and used as biological labelling reagents, with most current labelling studies involving the use of commercial materials bearing an anchor with which to attach the dye to the biological host.

3,3'-dioctadecyloxacarbocyanine perchlorate (DiO) is a lipophilic carbocyanine green fluorescent dye which is weakly fluorescent in aqueous solutions but becomes highly fluorescent and reasonably photostable when incorporated into cell membranes. DiO has been used as in neuronal and lymphocyte tracking studies and has recently been described as being useful as part of an organic, solvent-free, and specific method for the in vitro fluorescence confocal imaging of cancer cells using nanocrystals of DiO and perylene. (See Koichi Baba K, Kasai H, Masuhara A, Oikawa H, and Nakanishi H (2009). Organic solvent-free fluorescence confocal imaging of living cells using pure nanocrystal forms of fluorescent dyes. *Jpn J Appl Phys* 48: 117002, herein incorporated by reference.)

Eosin Y, an acidic fluorescent red dye, is a tetrabromo derivate of fluorescein used to stain basic parts of the cell, such as cytoplasm, collagen, red blood cells, and muscle fibers, for examination under the microscope. Eosin is most often used as a counterstain to Hematoxylin in H&E (Hematoxylin and Eosin) staining. For staining, Eosin Y is typically used in concentrations of 1 to 5 percent weight by volume, dissolved in water or ethanol; a small concentration (0.5 percent) of acetic acid usually gives a deeper red stain to the tissue.
Similarly, Erythrosine B, the disodium salt of 2,4,5,7-tetraiodofluorescein, is used as a cherry-pink biological stain and a radiopaque medium with a maximum absorbance at 530 nm in an aqueous solution. Because Erythrosine B is identical in structure to Eosin Y except that iodine is substituted for the bromines at each position on Eosin Y, Erythrosine B is often used as a substitute for Eosin Y staining.

Finally, the stain Rose Bengal (4,5,6,7-tetrachloro-2',4',5',7' -tetraiodofluorescein) is a nontoxic dye whose sodium salt is commonly used in eye drops to stain damaged conjunctival and corneal cells and thereby identify damage to the eye. This fluorescent stain has also been studied for its separate property as having a therapeutic effect on metastatic melanoma. In one study, 26 target lesions in eleven metastatic melanoma patients were directly injected with 10% w/v Rose Bengal in saline at a dose of 0.5 ml/cc lesion volume, with twenty-eight additional untreated lesions observed for potential bystander effect. The treatment was well tolerated and an objective response was observed in twelve target lesions, with no cancer progression observed in seven lesions over at least three months of observation. Response rate was dose dependent, increasing to 69% after higher dose injections. Nontarget lesions exhibited a 27% objective response rate that increased to 44% in patients exhibiting a positive response of target lesions. It was concluded that intralesional Rose Bengal could benefit patients with metastatic melanoma. (See Thompson JF, Hersey P, and Wachter E (2008). Chemoablation of metastatic melanoma using intralesional Rose Bengal. Melanoma Res 18:405-1 1, herein incorporated by reference.)

As an alternative to fluorescent stains or dyes, labelled cholesterol-derived sterols have been formulated in the past to be used to study the metabolism and intracellular localization of cholesterol, including a variety of photoreactive, spin-labelled, and fluorescent cholesterol analogues. (See, e.g., Gimpl G and Gehrig-Burger K (2007). Cholesterol reporter molecules. Biosci. Rep. 27:335-358, herein incorporated by reference.) Although none of
these analogues possess all of the properties of the parental cholesterol molecule, these molecules have been reported to localize in cholesterol-enriched microdomains. Photoreactive cholesterol probes such as cholesteryl diazoacetate; 3a-azido-5-cholestone; 3a-(4-azido-3-iodosalicylic)-cholest-5-ene; p-azidophenacyl 3α-hydroxy-5β-cholan-24-ate; 25-azidonorcholesterol; sterols with diazoacetate, aryltriazirines or fluorodiazirine attached at C-22 or C-24; [3H]22-(p-benzoylphenoxy)-23,24-bisnorcholan-5-en-3p-ol; and [3H]6-azi-5α-cholestanol (i.e., photocholesterol) and [3H]7-azi-5α-cholestanol have been used to visualize the nicotinic acetylcholine receptor and oxytocin receptors and may be used to identify and analyze other putative cholesterol binding proteins at the molecular level. Spin-labelled cholesterol analogues having a nitroxide spin-label attached at the C-3 or C-25 position (e.g., 3β-doxy-5α-cholestane) provide information about the structure of biological membranes, including the orientation, distribution and transbilayer movements of cholesterol in liposomes and biological membranes, by using nuclear magnetic and electron spin resonance spectroscopy. Finally, to study the distribution and dynamics of cholesterol in membranes and cells, researchers can select among fluorescent cholesterol analogues, which are distinguished between those analogues which possess intrinsic fluorescence (e.g., dehydroergosterol and cholestatrienol) and those in which a fluorophore or photoreactive group is attached (e.g., NBD-cholesterol, BODIPY-cholesterol, and dansyl-cholestanol).

Such technology has also been used to analyze the potential efficacy of cholesterol-utilizing agents in the treatment of cancer. Thus, in light of the fact that receptors for vasoactive intestinal peptide (VIP) are overexpressed in human breast cancer, but that sterically stabilized liposomes (SSL) with VIP non-covalently associated on their surface were unable to actively target to breast cancer in rats in situ, most probably due to dissociation of non-covalently associated VIP from SSL, researchers covalently inserted VIP into BODIPY-cholesterol-labelled SSL and then incubated rat breast cancer tissue sections in situ.
vitro with the liposomes. The labelling technique verified that significantly more VIP-SSL were attached to rat breast cancer tissues, thereby indicating that SSL with covalently attached VIP can be actively targeted to rat breast cancer tissues. (See Dagar S, Sekosan M, Lee BS, I. Rubinstein I, and Önyiiksel H (2001). VIP receptors as molecular targets of breast cancer: implications for targeted imaging and drug delivery. J Control. Release 74:129-134, herein incorporated by reference.)

Furthermore, to determine the transport of imaging probes attached to cholesterol and introduced via a liposomal formulation in order to evaluate the intracellular distribution and kinetics of small molecular cargo that might be attached to cholesterol or phospholipids, researchers compared BODIPY-cholesteryl ester and NBD-cholesterol to NBD-phosphatidylcholine and NBD-phosphatidylethanolamine using a liposomal formulation. It was discovered that the rate and transfer efficiency were NBD-cholesterol > BODIPY-cholesteryl ester > NBD-phosphatidylcholine > NBD-phosphatidylethanolamine. While in the absence of polyethylene glycol (PEG) within the liposome, the transfer rate decreased, NBD-cholesterol, delivered by liposomes with an average diameter of 100 nm, localized in the perinuclear region and lipid storage droplets in a mostly time-constant manner with transfer observed in as little as five minutes, suggesting a unidirectional mode of entry. (See Kheirolomoom A and Ferrara KW (2007). Cholesterol transport from liposomal delivery vehicles. Biomaterials 28:431 1-4120, herein incorporated by reference.) This study thus further highlights the desirability of utilizing labelled sterols to determine the efficacy of an appropriate drug delivery platform.

US Patents 4684479 and 5215680 to D’Arrigo, herein incorporated by reference, describe the formation of gas- or air-filled lipid coated microbubbles (LCMs), and methods of production and use thereof, to be used as imaging agents for ultrasound methods and for drug delivery. The production process for LCMs is based on simple mechanical shaking of an
aqueous suspension of nonionic lipids, such as saturated glycerides and cholesterol esters, of specific chain lengths and in a fixed ratio. In all cases, the majority of lipids added (99%) flocculate or precipitate with additional loss of material on filtration with yields less than 1%. Still, these artificial LCMs were found to be very long-lived, lasting over 6 months in vitro. Also, despite their low filtration yields, LCMs are sufficiently small and pliable enough to pass across the fenestrated capillary walls of tumor-tissue microcirculation. Of particular note is the highly-selective, temperature-dependent, apparently saturable uptake of LCMs into rodent brain tumor cells as well as into spontaneous tumors in dogs, likely mimicking cancerous cells' natural uptake of certain lipids. To date, however, there has not been an efficacious formulation of a targeted molecular imaging agent utilizing LCM technology which presents minimal to nonexistent levels of adverse side effects upon patient administration.

Additionally, production methods are complex and small changes in conditions can result in significant differences in particle size and performance features. Typical approaches include high-shear homogenization and ultrasound, high-pressure homogenization, hot homogenization, cold homogenization, and solvent emulsification evaporation. Sterilization by aseptic filtration or radiation may also be complex. Thus, attempts to increase drug payload and solubility of lipids in LCMs with addition of a drug in ethanol and aqueous solution followed by high-shear homogenization result in the formation of poorly-stable non-gas-containing particles that contain little if any drug.

by reference.) Stability was achieved by modification of conditions of high shear homogenization. To date, however, there has not been an efficacious formulation of a targeted molecular imaging agent using lipid-oil-water nanoemulsion technology which presents minimal to nonexistent levels of adverse side effects upon patient administration.

Administering imaging agents with an appropriate delivery vehicle that simultaneously promotes accumulation and cellular internalization in a cancer mass but limits accumulation in healthy tissues to promote accurate imaging of the cancer mass is highly desirable. With more efficient delivery, systemic and healthy tissue concentrations of imaging agents may be reduced while achieving the same or better imaging results with fewer or diminished side effects. Further, a delivery vehicle that would not be limited to a single type of cancer but would allow for selective accumulation into a cancer mass and cellular internalization into diverse cancer cell types would be especially desirable and allow for safer more effective treatment of cancer. A delivery vehicle that would also allow for elevated loading capacity for the imaging agent would be a significant advance in the art.

Concomitant with these goals is the similar need to deliver therapeutic agents into the tumor mass and thereupon into tumor cells and target organelles therein. Just as with the administration of imaging agents, administering therapeutic agents with an appropriate delivery vehicle that simultaneously promotes accumulation and cellular internalization in a tumor mass but limits accumulation in healthy tissues is also highly desirable. With more efficient delivery, systemic and healthy tissue concentrations of therapeutic agents may be reduced while achieving the same or better therapeutic results with fewer or diminished side effects. Such delivery of agents with inherent degrees of tumor cell selectivity would offer additional advantages. Further, a delivery vehicle that would not be limited to a single tumor type but would allow for selective accumulation into a tumor mass and cellular internalization into diverse cancer cell types would be especially desirable and allow for safer
more effective treatment of cancer. A delivery vehicle that would also allow for elevated loading capacity for the therapeutic agent would be a significant advance in the art.

It has further been suggested in the art that labelled nanoparticles may act as biomarkers that suggest that a particular anticancer therapeutic agent shall be efficacious in a particular target cell type. For example, one team of researchers performed cell viability experiments and confocal fluorescence microscopy using 3-mercaptopropionic acid-capped gold nanoparticles to determine whether these nanoparticles could enhance the delivery of daunorubicin to drug-resistant leukemia K562/ADM cells. Both fluorescence microscopy and electrochemical studies demonstrated the enhancement effect of the nanoparticles in delivering daunorubicin to these resistant cells, as observed through growth inhibition of the cells. The study concluded that the use of such functionalized nanoparticles merited further exploration as a novel strategy to inhibit multidrug resistance in targeted tumor cells and as a sensitive method for the early diagnosis of certain cancers. (See Li J, Wang X, Wang C, Chen B, Dai Y, Zhang R, Song M, Lv G, and Fu D (2007). The enhancement effect of gold nanoparticles in drug delivery and as biomarkers of drug-resistant cancer cells. ChemMedChem 2:374-8, herein incorporated by reference.)

Overall, then, a major challenge in the treatment of cancer patients is the validation of biomarkers that can be utilized both to monitor benefits of drug treatment as well as to predict which patients would be most likely to benefit from any given drug or cocktail therapy. The ability to measure this increased uptake with a lipid-oil-water nanoemulsion that may also be used to deliver drug presents an opportunity to directly link a biomarker phenomenon to predicting the likelihood of therapeutic benefit. There is therefore a clear need for a stable, easily-prepared, biocompatible, efficacious formulation of targeted molecular imaging agents with simultaneous delivery of therapeutic agents, which would promote selective uptake into cancer cells to permit more accurate and more efficacious imaging of cancer.
Objects of the Invention and Industrial Applicability

Consequently, it is an object of the present invention to provide a targeted molecular imaging agent to be used for the imaging of diseases, conditions, and syndromes characterized by aberrant lipid metabolism, such as cancer, which exhibits rapid and increased selective and preferential uptake into tumor cells.

It is a further object of the present invention to provide a targeted molecular imaging agent to be used for the imaging of diseases, conditions, and syndromes characterized by aberrant lipid metabolism, such as cancer, which causes minimal side effects upon administration.

It is a still further object of the present invention to provide a targeted molecular imaging agent to be used for the imaging of diseases, conditions, and syndromes characterized by aberrant lipid metabolism, such as cancer, which is easily manufactured at the least possible cost and is capable of being stored for the longest possible period.

It is a still further object of the present invention to provide a targeted molecular imaging agent to be used for the imaging of diseases, conditions, and syndromes characterized by aberrant lipid metabolism, such as cancer, which permits a physician to select a more efficacious treatment modality for a cancer patient.

It is a still further object of the present invention to provide a targeted molecular imaging agent to be used for the imaging of diseases, conditions, and syndromes characterized by aberrant lipid metabolism, such as cancer, which can also deliver therapeutic agents to their intended molecular targets within a tumor mass.

It is a still further object of the present invention to provide a targeted molecular imaging agent to be used for the imaging of diseases, conditions, and syndromes characterized by aberrant lipid metabolism, such as cancer, which acts as a biomarker for
diseased, injured, or mutated tissue, cells, and/or at least one organelle thereof, that are amenable to delivery of therapeutic agents by the nanoemulsion.

Summary of the Invention

To achieve these objects, the present invention broadly provides a stable lipid-oil-water nanoemulsion that demonstrates dramatically increased and selective uptake and concentration into the cytosol of diseased, injured, or mutated cells of warm-blooded animals, including humans, compared to healthy cells. This nanoemulsion is useful for selectively and preferentially imaging a diseased, injured, or injuriously mutated tissue, cell, or at least one organelle thereof, and treating the disease, injury, or injurious mutation. In a preferred embodiment of the present invention, the disease, injury, or mutation is characterized by aberrant lipid metabolism, and in a more preferred embodiment of the present invention, the disease is cancer. The lipid-oil-water nanoemulsion is comprised of lipid particles as hereinafter defined, uniformly dispersed through homogenization in an aqueous phase capable of being selectively and preferentially internalized within a diseased cell, including a cancer cell; an effective amount of at least one pharmaceutically-acceptable imaging agent associated with the lipid nanoemulsion; an effective amount of at least one pharmaceutically-acceptable therapeutic agent associated with the lipid nanoemulsion; and a pharmaceutically-acceptable carrier or excipient therefor. The lipid particles each comprise at least one non-bilayer-forming lipid, among which the imaging agent is physically or chemically integrated. Such suitable lipid particles have been found to enhance significantly the targeted delivery and concentration of an associated payload into diseased cells characterized by aberrant lipid metabolism, including cancer cells. Moreover, the nanoemulsion exhibits exceptional physical and chemical stability for an extended duration of time, thereby greatly facilitating prepackaging of the nanoemulsion in stable, ready-to-administer forms.

In one embodiment of the present invention, the imaging agent may be a fluorescent
dye, such as, but not limited to, BODIPY, DiO, Rose Bengal, Eosin Y, and Erythrosine B. In another embodiment of the present invention, the imaging agent may be a labelled sterol, such as, but not limited to, BODIPY-cholesterol, NBD-cholesterol, and dansyl-cholesterol, and their associated cholesteryl esters. In yet another embodiment of the present invention, the imaging agent is radiolabeled, such as, but not limited to, $^{14}$C-cholesterol. Upon administration of the nanoemulsion either to a patient in vivo or obtained from patient biopsy in vitro, the demonstration of increased uptake into cancer cells associated with a particular tumor type upon use of an imaging modality such as x-ray, CT, PET, or MRI, without limitation, or lack thereof, is expected to be a useful indicator of the potential benefits to be achieved by delivering a chemotherapeutic agent known to be efficacious in treating that tumor type. Indeed, the exquisite specificity of the nanoemulsion for both tumor mass and tumor cell uptake allows the nanoemulsion to act as a biomarker for the treatment of those targeted diseased, injured, or mutated cells that form the cancerous mass. Furthermore, any component of the nanoemulsion of the present invention (e.g., the lipid particles or the labelled moiety) may act as an antigen, or conversely as an antibody, which may be detected through immunologic-based imaging modalities, such as immunofluorescence.

The at least one pharmaceutically-acceptable therapeutic agent is to be present in an amount effective to kill or at least suspend the growth of the hyperproliferated cells while simultaneously providing the capability of imaging the tumor mass. In a preferred embodiment of the present invention, the therapeutic agent is a microtubule-interacting agent. Suitable microtubule-interacting agents include, but are not limited to, taxanes, such as, for example, paclitaxel; epothilones; vinca alkaloids, such as, for example, vincristine; eleutherobins; discodermolide; dolastatins; colchicine; combrestatins; phomopsin A; halichondrin B; spongistatin 1; sarcodictyins; laulimalides; and derivatives, analogs, congeners, and combinations of each of the aforementioned agents thereof. It may be
possible that the imaging agent itself demonstrates some therapeutic effect, such as where the imaging agent is Rose Bengal and the target cell is a metastatic melanoma cell. Similarly, it may be that a lipid used to form the nanoemulsion demonstrates some therapeutic effect, such as, but not limited to, ceramide when used with CML cells or 26-amincholesterol.

In a still further aspect of the present invention, there is provided a method of preparing the nanoemulsion disclosed herein, comprising the steps of:

a) mixing the at least one non-bilayer-forming lipid with an effective amount of at least one imaging agent and at least one therapeutic agent to yield a lipid portion;

b) adding the lipid portion to an aqueous phase to yield a dispersion; and

c) agitating the dispersion under high-shear conditions sufficient to disperse the aforementioned lipid therethrough to form a lipid nanoemulsion comprised of lipid particles.

**Detailed Description of the Invention**

The present invention is generally directed to a stable lipid-oil-water nanoemulsion that demonstrates dramatically increased and selective uptake and concentration into the cytosol of diseased, injured, or mutated cells in warm-blooded animals compared to healthy cells. Such warm-blooded animals include those of the mammalian class, such as humans, horses, cattle, domestic animals including dogs and cats, and the like, subject to disease, injury, mutation, and other pathological conditions and syndromes. This nanoemulsion is useful for selectively and preferentially imaging a diseased, injured, or injuriously mutated tissue, cell, or at least one organelle thereof, and treating the disease, injury, or injurious mutation. In a preferred embodiment of the present invention, the disease, injury, or mutation is characterized by aberrant lipid metabolism, and in a more preferred embodiment of the present invention, the disease is cancer. The nanoemulsion is comprised of lipid particles as hereinafter defined, uniformly dispersed through homogenization in an aqueous phase capable of being selectively and preferentially internalized within a cell characterized by
aberrant lipid metabolism, including a cancer cell; an effective amount of at least one pharmaceutically-acceptable imaging agent associated with the lipid nanoemulsion; an effective amount of at least one pharmaceutically-acceptable therapeutic agent associated with the lipid nanoemulsion; and a pharmaceutically-acceptable carrier or excipient therefor, thereby making the lipid particles particularly well-suited for the selective delivery to and effective concentration within such diseased cells and tissues as tumorous ones.

The lipid particles of the nanoemulsion are structured to facilitate both elevated passive accumulation and active internalization into diseased cells and tissues, including tumor cells and tissues. The lipid particles are taken into these cells through active metabolic uptake as they passively accumulate in the vascular area of the diseased tissue. Thus, the lipid particles of the present invention provide a delivery vehicle selectively and preferentially targeted for uptake and internalization by cells characterized by aberrant lipid metabolism, including tumor and cancer cells. "Internalization" as used herein means that the lipid particles are actively taken up by the cell.

The lipid particles of the nanoemulsion are exceptionally physically and chemically stable over an extended period of time and hence experience minimal loss of imaging or therapeutic capability due to undesirable precipitation, aggregation, or insolubility that is typically exhibited in delivery systems in the prior art. Moreover, these lipid particles display other favorable characteristics including controlled release; enhanced drug stability; positive drug loading capacity; better compatibility with hydrophobic drugs; relatively low biotoxicity; and low organic solvent content. The lipid particles are also relatively simple and convenient to prepare and to administer.

As used herein, the term "lipid particle" is meant to encompass any lipid-containing structures, typically nanosized, which are at least substantially-intact particles forming part of a nanoemulsion. The term "substantially-intact" means that the particles maintain their shape
in the absence of a membrane, as contrasted with a liposome. The lipid particles are comprised of at least one non-bilayer-forming lipid.

A lipid bilayer structure or arrangement is typically formed by certain kinds of lipids having a hydrophilic end (polar head region) and a hydrophobic end (nonpolar tail region), including amphipathic molecules such as phospholipids, which exhibit the ability and/or tendency to self-organize into two opposing layers of lipid molecules in aqueous solution. The two opposing layers of lipid molecules are arranged so that their hydrophobic ends face one another to form an oily core, while their hydrophilic ends face the aqueous solutions on either side of the bilayer structure. In the present invention, the term "non-bilayer-forming lipid" encompasses a lipid that lacks such ability and/or tendency to form a lipid bilayer structure or arrangement in an aqueous environment. Examples of non-bilayer-forming lipids include lipids that are no more than weakly polar, preferably lipids that are substantially non-polar or neutral. The more-preferred lipids in the present invention are neutral lipids.

The lipid particles used in the nanoemulsion of the present invention are distinguishable from the gas-containing microbubbles described in U.S. Patent Nos. 4684479 and 5215680, and are also structurally distinguishable from liposomes, such as those described, for example, in U.S. Patent Nos. 6565889 and 6596305, all herein incorporated by reference. In particular, the lipid particles are formed by a mixture of non-bilayer-forming lipids that are physiologically acceptable and at least substantially free from the presence of charged or polar lipids, including, for example, phospholipids. Suitable examples of non-bilayer-forming lipids include those selected from glycerol monoesters of saturated and unsaturated carboxylic acids; glycerol monoesters of saturated aliphatic alcohols; sterol aromatic acid esters; sterols; terpenes; bile acids; alkali metal salts of bile acids; sterol esters of aliphatic acids; sterol esters of sugar acids; esters of sugar acids; esters of aliphatic alcohols; esters of sugars; esters of aliphatic acids; sugar acids; saponins; sapogenins;
glycerol; glycerol di-esters of aliphatic acids; glycerol tri-esters of aliphatic acids; glycerol diesters of aliphatic alcohols; glycerol triesters of aliphatic alcohols; and combinations thereof.

In an embodiment of the present invention, the lipid particles to be used to form the nanoemulsion are prepared by first forming a mixture of a select group of non-bilayer-forming lipids which provides the lipid particles with a size described hereinafter that facilitates high internalization levels when applied to targeted diseased tissues and cells. The lipid mixture generally comprises:

a) at least one first member selected from the group consisting of glycerol monoesters of carboxylic acids containing from about 9 to 18 carbon atoms and aliphatic alcohols containing from about 10 to 18 carbon atoms;

b) at least one second member selected from the group consisting of sterol aromatic acid esters;

c) at least one third member selected from the group consisting of sterols, terpenes, bile acids and alkali metal salts of bile acids;

d) at least one optional fourth member selected from the group consisting of sterol esters of aliphatic acids containing from about 1 to 18 carbon atoms; sterol esters of sugar acids; esters of sugar acids and aliphatic alcohols containing from about 10 to 18 carbon atoms, esters of sugars and aliphatic acids containing from about 10 to 18 carbon atoms; sugar acids, saponins; and sapogenins; and

e) at least one optional fifth member selected from the group consisting of glycerol, glycerol di- or tri-esters of aliphatic acids containing from about 10 to 18 carbon atoms and aliphatic alcohols containing from about 10 to 18 carbon atoms.

While the lipid mixture described above only includes the presence of members (a) through (c), it is more preferred to incorporate members (d) and/or (e) because the long-term
stability and uniformity of size of the lipid particles are theoretically enhanced by the presence of these two optional members.

In a preferred embodiment of the present invention, the five members (including the two optional members) making up the lipid mixture forming the lipid particles of the present invention are combined in a weight ratio of (a):(b):(c):(d):e of (1-5):(0.25-3):(0.25-3):(0-3):(0-3), respectively.

While the first member of the lipid mixture has been described as including glycerol monoesters of saturated carboxylic acids containing from about 10 to 18 carbon atoms, it is contemplated that glycerol monoesters of mono- or polyunsaturated carboxylic acids containing from about 9 to 18 carbon atoms, such as but not limited to the 9-carbon oleic or elaidic acids, are also useful in the construction of the lipid mixture.

It will be understood that the proportions of the members of the lipid mixture may vary depending on several factors, including, but not limited to, the type of cells and/or tissues being targeted for delivery, the structure and desired dosage of any payload to be encapsulated within or otherwise associated with the nanoemulsion, the pharmaceutically-acceptable carrier used, the mode of administration, the presence of other excipients or additives, and so forth. Furthermore, factors that enable the lipid particles to be selectively internalized by targeted diseased tissues and cells include not only the composition of the lipid mixture and the structure of the resulting lipid particles but also the size and molecular weight of the particles as described hereinafter.

The lipid particles of the present invention maintain a desirable particle size distribution, preferably where a major portion of the particles have a mean average particle size ranging from about 0.02 to 0.2 μm, preferably 0.02 to 0.1 μm, with varying minor amounts of particles falling above or below the range and some lipid particles only ranging up to about 200 nm. The particle size ranges attainable in the lipid particles of the present
invention further lead to enhanced physical and chemical stability over an extended period of
time, and substantial reduction in undesirable agglomeration and drug precipitation.
Furthermore, this range is particularly suitable for the treatment of cancer; larger particles
may be appropriate for other uses (e.g., targeting of other types of cells or tissues). The range
provided herein will be determined in part by the lipid mixture employed and the type and
amount of the therapeutic agent added.

In one embodiment of the present invention, the imaging agent associated with the
nanoemulsion of the present invention may be a fluorescent dye, such as, but not limited to,
BODIPY, DiO, Eosin Y, and Erythrosine B. In another embodiment of the present invention,
the imaging agent may be a labelled sterol, such as, but not limited to, BODIPY-cholesterol,
NBD-cholesterol, and dansyl-cholesterol, and their associated cholesteryl esters. In yet
another embodiment of the present invention, the imaging agent is radiolabelled, such as but
not limited to $^{14}$C-cholesterol. Upon administration of the nanoemulsion either to a patient in vivo or obtained from patient biopsy in vitro, the demonstration of increased uptake into
cancer cells associated with a particular tumor type upon use of an imaging modality such as
x-ray, CT, PET, or MRI, without limitation, or lack thereof, is expected to be a useful
indicator of the potential benefits to be achieved by delivering a chemotherapeutic agent
known to be efficacious in treating that tumor type. Indeed, the exquisite specificity of the
nanoemulsion for both tumor mass and tumor cell uptake allows the nanoemulsion to act as a
biomarker for the treatment of those targeted diseased, injured, or mutated cells that form the
cancerous mass.

The at least one pharmaceutically-acceptable therapeutic agent, for which the lipid
particles have an enhanced loading capacity, should be present in a pharmaceutically-
sufficient amount to kill or at least suspend the growth of the hyperproliferated cells while
simultaneously providing the capability of imaging the tumor mass. The capacity of the
nanoemulsion for elevated internalization levels within a target cell, coupled with a high loading capacity of the particles for the therapeutic agent, provides a potent vehicle for treatment of these target cells, as well as imaging through detection of the imaging agent, by delivering an effective amount of the therapeutic agent to such targets, thereby inducing a therapeutically-beneficial effect, including stopping growth, inducing differentiation, or killing the cell. The lipid particles of the present invention hence not only enhance delivery of the therapeutic agent to the diseased cells and tissues but also reduce the amount of the therapeutic agent needed to achieve the desired efficacy, especially as compared to delivery systems in the prior art.

The therapeutic agents employed in the present invention may be uncharged or charged, nonpolar or polar, natural or synthetic, and so on. The term "therapeutic agent" as used herein includes any substance including, but not limited to, drugs, hormones, vitamins, nutrients, substances, and the like, that affect microtubule production, structure, association, function, and destruction, and thus are useful in prevention and treatment of a disease, condition, syndrome, characterized by cellular hyperproliferation as a subset of aberrant lipid metabolism, or symptoms thereof, including cancer. Thus, the therapeutic agents useful in the present invention include all types of drugs, lipophilic polypeptides, cytotoxins, oligonucleotides, cytotoxic antineoplastic agents, antimetabolites, hormones, and radioactive molecules, which affect microtubule production, structure, association, function, and destruction. The term "oligonucleotides" includes both antisense oligonucleotides and sense oligonucleotides, (e.g., nucleic acids conventionally known as vectors). Oligonucleotides may be "natural" or "modified" with regard to subunits or bonds between subunits.

In a still more preferred embodiment, the therapeutic agent is a microtubule-interacting agent. The microtubule-interacting agent may be selected from a group consisting of taxanes, such as, for example, paclitaxel, docetaxel, cephalomannine baccatin-III, 10-
deacetyl baccatin III, deacetylpaclitaxel, and deacetyl-7-epipaclitaxel; vinca alkaloids, such as, for example, vincristine, vinblastine, vinorelbine, vindesine, and analogs thereof; epothilones; eleutherobins; discodermolide; dolastatins; colchicine; combrestatins; phomopsin A; halichondrin B; spongistatin 1; sarcodictyins; laulimalides; derivatives, analogs, congeners, and combinations of each of the aforementioned agents thereof; and similar drugs or substances known to exhibit such microtubule-interacting activity.

The imaging agent associated with the nanoemulsion may itself be of therapeutic benefit upon delivery to the cell, based upon the properties of the imaging agent as well as the type of cell targeted. For example, should the imaging agent be Rose Bengal, therapeutic benefit would be expected to be demonstrated in a metastatic melanoma cell. Similarly, it may be that a lipid used to form the nanoemulsion demonstrates some therapeutic effect, such as ceramide when used with CML cells or 26-aminocholesterol.

The nanoemulsion of the present invention exhibits long-term physical and chemical stability, allowing such compositions to be conveniently pre-packaged into stable, ready-to-administer dosage forms and thereby eliminating the need for the bedside dilution and formulation prior to administration typically associated with similar compositions in the prior art. The nanoemulsion of the present invention exhibits desirable drug and emulsion stability over an extended time period (e.g., at least 14 days at about 30°C and at least 12 months at 4°C). The nanoemulsion of the present invention contains lipid particles in an amount of from about 0.1 µg/mL to 1000 µg/mL, preferably from about 10 µg/mL to 800 µg/mL, and most preferably from about 200 µg/mL to 600 µg/mL. Typical concentrations of the therapeutic agent based on the total volume of the nanoemulsion may be at least 0.001% w/v, preferably 0.001% to 90% w/v, and more preferably from about 0.1% to 25% w/v. The amount of therapeutic agent potentially present in the nanoemulsion may range from about 0.001 µg/mL to 1000 µg/mL, preferably from about 0.1 µg/mL to 800 µg/mL, and more
preferably from about 60 µg/mL to 400 µg/mL.

The nanoemulsion of the present invention may further include emulsion-enhancing agents selected from a plant-based fat source, a solvent, a surfactant, or combinations thereof. The emulsion-enhancing agents have been found, individually or in combination, to enhance the stability and maintain the small particle size properties of the lipid particles theoretically by reducing or minimizing undesirable precipitation or aggregation of the lipid particles, thereby positively influencing and facilitating the active uptake of the lipid particles into the cancer cells. The emulsion-enhancing agents should also improve the physical and chemical stability and drug-carrying capacity of the nanoemulsion of the present invention.

In a preferred embodiment of the present invention, the plant-based fat sources may be produced, derived, or modified from natural, chemical, or recombinant means. Examples of such fat sources include vegetable-derived fatty acids generally in the form of vegetable oil, such as, for example, soybean oil, flaxseed oil, hemp oil, linseed oil, mustard oil, rapeseed oil, canola oil, safflower oil, sesame oil, sunflower oil, grape seed oil, almond oil, apricot oil, castor oil, corn oil, cottonseed oil, coconut oil, hazelnut oil, neem oil, olive oil, palm oil, palm kernel oil, peanut oil, pumpkin seed oil, rice bran oil, walnut oil, and mixtures thereof. The more preferred vegetable oil is soybean oil.

The vegetable oil is generally present in amounts sufficient to permit higher surface tension in the nanoemulsion, which in turn increases the probability of hydrophobic interactions with the plasma membranes of the target cell, or receptors thereupon. The plant-based fat source may be present in amounts of from about 0.001% v/v to 5.0% v/v, more preferably from about 0.005% v/v to 4.0% v/v, and most preferably from about 0.01% v/v to 2.5% v/v.

In another preferred embodiment of the present invention, the surfactants are those selected from non-ionic surfactants. Examples of non-ionic surfactants include sorbitan
esters and mixtures thereof, such as fatty-acylated sorbitan esters and polyoxyethylene
derivatives thereof, and mixtures thereof including, but not limited to, Poloxamer compounds
(188, 182, 407 and 908), Tyloxapol, Polysorbate 20, 60 and 80, sodium glycolate, sodium
dodecyl sulfate and the like, and combinations thereof. More preferred non-ionic surfactants
are detergent polysorbates, such as, for example, Tween®-80.

The surfactant is generally present in amounts sufficient to increase the kinetic
stability of the nanoemulsion by stabilizing the interface between the hydrophobic and
hydrophilic components of the nanoemulsion and keeping the hydrophobic components from
coalescing, such that, once formed, the nanoemulsion does not significantly change in
storage. The surfactant may be present in amounts of from about 0.01% w/v to 4.0% w/v,
more preferably from about 0.1% w/v to 3.0% w/v, and most preferably from about 0.2% w/v
to 2.5% w/v.

In yet another preferred embodiment of the present invention, the solvents include any
pharmacologically-acceptable water-miscible diluents or solvents such as, for example, polar
protic and polar aprotic solvents. Such solvents are preferably selected from 1,3-butanediol;
dimethyl sulfoxide; alcohols such as methanol, butanol, benzyl alcohol, isopropanol, and
ethanol; and the like. A more preferred solvent is benzyl alcohol.

The solvent is generally present in amounts sufficient to control the extent of the
aggregation of non-ionic surfactants in the nanoemulsion. The solvent may be present in
amounts of from about 0.001% v/v to 99.9% v/v, more preferably 0.005% v/v to 80% v/v,
and most preferably from about 0.005% v/v to 70% v/v.

The composition of the present invention does not modify or alter the underlying
pharmacological activity or chemical properties of the imaging agent and therapeutic agent
but simply enhances their delivery to and internalization into the diseased cell or tissue,
including cancerous cells or tissue, to impart imaging and/or therapeutic benefits. Examples
of teachings related to the use of taxanes as therapeutic agents in treating cancer are
disclosed, for example, in U.S. Patent Nos. 6346543; 6384071; 6387946; 6395771; 6403634;
and 6500858, each incorporated herein by reference.

Generally, the nanoemulsion of the present invention is prepared by combining the
lipid particles with the therapeutic agent and thoroughly mixing the same. The lipid mixture
may be mixed with a surfactant in combination with a plant-based fat source prior to mixing
with the imaging agent, which themselves may be mixed with a water-miscible solvent for
dissolution. The lipid particle-imaging agent combination is then mixed with water,
preferably purified water. The resulting mixture is then subjected to high shear forces
typically produced in standard conventional shear-intensive homogenizing mixers or
homogenizers to produce a nanoemulsion comprising the lipid particles dispersed within the
aqueous phase. Sufficient high shear forces can be produced with a suitable shear-intensive
homogenizing mixer or homogenizer such as Microfluidizer® Fluid Materials Processors
marketed by Microfluidics of Newton, MA. The resulting nanoemulsion may be further
treated to yield a more purified form, which may be used for administration to warm-blooded
animals, including humans.

In some instances, it may be desirable to remove unduly large particles from the
mixing process, so as to maintain the particle size distribution within a desired range.
Suitable filtration systems, such as those from Millipore Corporation of Waltham,
Massachusetts, are available for this purpose. The selection of a suitable filter system
therefore may be a factor in controlling the particle size distribution of the lipid particles
within a desirable range and is within the routine skill of the skilled artisan. Alternatively,
the nanoemulsion may be processed through dialysis to remove the impurities, with the
resulting dialysate retained for pharmaceutical use. Dialysis is a preferred method of
removing any non-particulated lipid mixture components, drugs, and/or solvents and
achieving any desired buffer exchange or concentration. Dialysis membrane nominal molecular weight cutoffs of 5000 to 500000 can be used, with a molecular weight of 10000 to 300000 being preferred. The lipid particles produced as described, when purified such as by dialysis to remove non-particulated drug, may be characterized to determine the extent to which the lipid particles may be internalized in targeted cells, such as, for example, C₆ glioma cells.

The compositions of the present invention may further include a pharmaceutically-acceptable carrier or excipients. Examples of pharmaceutically-acceptable carriers are well known in the art and include those conventionally used in pharmaceutical compositions, such as, but not limited to, antioxidants, buffers, chelating agents, flavorants, colorants, preservatives, absorption promoters to enhance bioavailability, antimicrobial agents, and combinations thereof. The amount of such additives depends on the properties desired, which can readily be determined by one skilled in the art.

The nanoemulsion of the present invention may routinely contain salts, buffering agents, preservatives, and compatible carriers, optionally in combination with other therapeutic ingredients. When used in medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically-acceptable salts may conveniently be used to prepare pharmaceutically-acceptable salts thereof and are not excluded from the scope of the invention. Such pharmacologically- and pharmaceutically-acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, palicylic, p-toluene sulfonic, tartaric, citric, methane sulfonic, formic, malonic, succinic, naphthalene-2-sulfonic, and benzene sulfonic. Also, pharmaceutically-acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts of the carboxylic acid group.

Improved imaging of cancer through the use of the nanoemulsion of the present
invention is especially contemplated, including imaging of primary tumors prior to or concurrent with surgical removal, or radiological or other chemotherapeutic treatment of a primary tumor. The nanoemulsion of the present invention is useful in such cancer types as primary or metastatic melanoma, lymphoma, sarcoma, lung cancer, liver cancer, Hodgkin's and non-Hodgkin's lymphoma, leukemias, uterine cancer, cervical cancer, bladder cancer, kidney cancer, colon cancer, and adenocarcinomas such as breast cancer, prostate cancer, ovarian cancer, and pancreatic cancer.

For imaging and therapeutic applications, the nanoemulsion can be administered directly to a patient when combined with a pharmaceutically-acceptable carrier. This method may be practiced by administering the therapeutic agent alone or in combination with an effective amount of another therapeutic agent, which may or may not be a second microtubule-interacting agent. When not a microtubule-interacting agent, this second agent may be, but is not limited to, a cytostatic agent, a folic acid inhibitor, an alkylating agent, a topoisomerase inhibitor, a tyrosine kinase inhibitor, a podophyllotoxin, an antitumor antibiotic, a chemotherapeutic agent, an apoptosis-inducing agent, and combinations thereof. Such therapeutic agents may further include metabolic inhibition reagents. Many such therapeutic agents are known in the art. The combination treatment method provides for simultaneous, sequential, or separate use in treating such conditions as needed to amplify or ensure patient response to the treatment method.

The methods of the present invention may be practiced using any mode of administration that is medically acceptable, and produces effective levels of the active compounds without causing clinically unacceptable adverse effects. Although formulations specifically suited for parenteral administration are preferred, the compositions of the present invention can also be formulated for inhalational, oral, topical, transdermal, nasal, ocular, pulmonary, rectal, transmucosal, intravenous, intramuscular, subcutaneous, intraperitoneal,
intrathoracic, intrapleural, intrauterine, intratumoral, or infusion methodologies or administration, in the form of aerosols, sprays, powders, gels, lotions, creams, suppositories, ointments, and the like. If such a formulation is desired, other additives well-known in the art may be included to impart the desired consistency and other properties to the formulation.

Those skilled in the art will recognize that the particular mode of administering the imaging and therapeutic agent depends on the particular agent selected; whether the administration is for treatment, diagnosis, or prevention of a disease, condition, syndrome, or symptoms thereof; the severity of the medical disorder being treated or diagnosed; and the dosage required for therapeutic efficacy. For example, a preferred mode of administering an anticancer agent for treatment of leukemia would involve intravenous administration, whereas preferred methods for treating skin cancer could involve topical or intradermal administration.

As used herein, "effective amount" refers to the dosage or multiple dosages of the imaging agent and/or therapeutic agent at which the desired imaging and/or therapeutic effect is achieved. Generally, an effective amount of the imaging agent and/or therapeutic agent may vary with the activity of the specific agent employed; the metabolic stability and length of action of that agent; the species, age, body weight, general health, dietary status, sex and diet of the subject; the mode and time of administration; rate of excretion; drug combination, if any; and extent of presentation and/or severity of the particular condition being treated. The precise dosage can be determined by an artisan of ordinary skill in the art without undue experimentation, in one or several administrations per day, to yield the desired results, and the dosage may be adjusted by the individual practitioner to achieve a desired imaging and/or therapeutic effect or in the event of any complication. Importantly, when used to treat cancer, the dosage amount of the therapeutic agent used should be sufficient to inhibit or kill tumor cells while leaving normal cells substantially unharmed.
The imaging agent and/or therapeutic agent included in the nanoemulsion of the present invention can be prepared in any amount desired up to the maximum amount that can be solubilized by, suspended in, or operatively associated with the given lipid particles. The amount of the imaging agent or therapeutic agent may range from 0.001 μg/mL to 1000 μg/mL, preferably from about 0.1 μg/mL to 800 μg/mL, and more preferably about 300 μg/mL.

Generally, the lipid particles will be delivered in a manner sufficient to administer an effective amount to the patient. The dosage amount may range from about 0.1 mg/kg to 175 mg/kg, preferably from about 1 mg/kg to 80 mg/kg, and more preferably 5 mg/kg to 60 mg/kg. The dosage amount may be administered in a single dose or in the form of individual divided doses, such as from one to four or more times per day. In the event that the response in a subject is insufficient at a certain dose, even higher doses (or effective higher doses by a different, more localized delivery route) may be employed to the extent of patient tolerance. Multiple doses per day are contemplated to achieve appropriate systemic or targeted levels of the imaging agent and/or therapeutic agent.

In another aspect of the present invention, there is provided a method of preparing the nanoemulsion of the present invention. The lipid mixture is incorporated into the imaging agent and/or therapeutic agent in amounts such that, upon processing with an aqueous phase, the composition forms a lipid nanoemulsion comprising a dispersion of lipid particles wherein the dispersed phase of lipid particles are present in the form of macromolecules or clusters of small molecules on the nanoscale order of particle size. In preparing the nanoemulsion of the present invention, the lipid mixture and the imaging agent and/or therapeutic agent are combined with an aqueous phase comprising water, preferably filtered water. The resulting mixture is processed to form lipid particles having a mean average particle size range typically, but not always, in the range of up to 200 nm, a size particularly
suited for the treatment of cancer, with larger particles appropriate for other uses. The range obtained will in part be affected by the lipid mixture employed, the type and amount of the therapeutic and imaging agent added to the lipid mixture, and the technique used to produce the lipid particles.

The nanoemulsion of the present invention can be made using conventional dispersion-producing techniques or processes known in the art. Such techniques include, but are not limited to, high-shear homogenization, ultrasonic agitation or sonication, high-pressure homogenization, solvent emulsification/evaporation, and the like. In one embodiment of the present invention, the lipid particles may be prepared through conventional high-pressure homogenization techniques using a suitable high-pressure homogenizer. Homogenizers of suitable sizes are commercially available. High-pressure homogenizers are generally designed to push a fluid through a narrow gap spanning about a few microns at high pressure, typically from about 100 to 2000 bar. The pressurized fluid accelerates over a very short distance to a very high velocity of over 1000 km/hr. Pressurized fluids containing the lipid mixture encounter very high-shear stress and cavitation forces, effectively disrupting and comminuting the lipid mixture into particles in the submicron range. As previously discussed, a major portion of the lipid particles should have a mean average particle size ranging from about 0.02 to 0.2 µm, preferably 0.02 to 0.1 µm, with varying minor amounts of particles falling above or below the range, especially with some lipid particles ranging up to about 200 nm.

In another particular method of preparing the lipid particles, the lipid mixture may be mixed with a plant-based fat source, such as a vegetable oil, and a surfactant, such as a non-ionic surfactant, to yield a lipid phase. The imaging agent and/or therapeutic agent may be mixed with a solvent, such as a water-miscible solvent, to yield a labelling/therapeutic phase. The lipid and labelling/therapeutic phases are thereafter mixed and blended together in the
presence of an aqueous phase, preferably through sonication. The resulting mixture is thereafter homogenized under high-shear forces to produce the corresponding nanoemulsion of the present invention. The nanoemulsion may then be filtered through a 0.2 µ membrane, sterilizing and/or removing impurities such as unused lipid materials, excess therapeutic or imaging agent, and so on, to yield a purified form suitable for delivery as a nanoemulsion to warm-blooded animals, including humans, in need of imaging and/or treatment.

The following examples are provided to facilitate understanding of the nanoemulsion of the present invention.

**EXAMPLE 1**

**PROCESS FOR FORMULATION OF NANOEMULSION WITH 26-AMINOCHOLESTEROL**

10 g of soybean oil was combined with 8 g of Tween-80HX (supplied from NOF) in a 500 mL round bottom flask. 200mg of lipids were then added, followed by 100mg of 26-aminocholesterol compound and 2mL of ethanol. The mixture was stirred and heated to 40-50°C until the solids had dissolved. 480mL of water was added, and the resulting milky white emulsion was stirred vigorously for fifteen minutes.

The mixture was then poured into the microfluidizer and processed through 11 discrete passes at 18kpsi. The outflow temperature of each discrete pass was controlled with external cooling and maintained from 15-20°C. After the eleventh pass, the product was collected and vacuum filtered through a 0.45µm filter, followed immediately by vacuum filtration through a 0.22µm filter. Both filtrations were fast and complete, and no visible residues were observed on either filter. The batch showed a particle size of \( N = 28, V = 36 \) and \( I = 156 \), where \( N \) mean number measurement, \( V \) means volume measurement, and \( I \) means intensity measurement. The product was stored refrigerated, with a sample also held at room temperature. Visually the samples can be described as translucent emulsions.
Nearly two months later, both the room temperature and refrigerated samples were refiltered through a 0.22 µm filter. Both samples filtered quickly with no visible residues on the filter membranes. Visually the samples had not changed, and the particle size was N = 33, V = 53, l = 174, effectively unchanged.

**EXAMPLE 2**

**SENSITIVITY OF CANCER CELLS TO NANOEMULSION CONTAINING 26-AMINOCHOLESTEROL**

**Objective**

The objective of this investigation was to assess sensitivity of BXPC3 human pancreatic cancer and H460 human non small lung carcinoma cancer cells to 26-aminocholesterol containing emulsiphan solution.

**Materials and Methods**

**Materials**

All materials were obtained through normal distribution channels from the manufacturer stated.

- Costar® 96 well plate, Corning Costar Corporation, Cambridge, MA, cat. no. 3598
- Fisher Scientific cat no.07-200-628 FLUOstar OPTIMA, BMG LABTECH, Offenburg, Germany
- RPMI 1640 Tissue culture medium. Mediatech. Fisher Scientific cat. no. MT-10040-CV
- Fetal Bovine Serum (FBS) Fisher Scientific cat. no. MTT3501 1CV
- Penicillin and Steptomycin. Fisher Scientific cat. no. MT 30-009-CI
Tumor Cell Lines

Two human tumor cell types, BXPC3 human pancreatic cancer cells and H460 non-small lung carcinoma cells, were used in this investigation. The BXPC3 and H460 cells were originally obtained from American Type Cell Culture (ATCC). All tumor cells were maintained at 37°C in a humidified 5% CO₂ atmosphere in T75 tissue culture flasks containing 20mL of Roswell Park Memorial Institute (RPMI) 1640 containing 2mM L-glutamine, 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (100IU/mL penicillin and 100µg/mL streptomycin). The tumor cells were split at a ratio of 1:5 every 4-5 days by trypsinization and resuspended in fresh medium in a new flask. Cells were harvested for experiments at 70-90% confluency.

Measurement of Growth Inhibition by Nanoemulsion Containing 26-aminocholesterol

Nanoemulsion containing 26-aminocholesterol was formulated as above at a concentration of 200 mg/L by Dr. Frank Gibson of Cornerstone Pharmaceuticals, Inc., Cranbury, NJ. Cells were plated at a density of 2000 cells per well in 100 µL of growth medium in all wells of a 96-well plate. A control plate for background signal and 0% growth was prepared by seeding the top four rows of a plate with 2000 cells/well and the bottom four rows with growth medium only. After 24 hours, the whole control plate was read using the CellTiter-Glow assay kit (Promega) which measures ATP concentration using a luciferase-based luminescence assay. Preliminary experiments showed linearity of assay response to cell number within the range of cell concentrations used.

The following dilutions of the nanoemulsion containing 26-aminocholesterol were made in RPMI media containing 10% FBS: 1:2, 1:5, 1:10, 1:100, 1:500, and 1:1000. 100 µL of each dilution was added to the cells. After exposure to the test articles for 72 hours, the number of viable cells in each well was determined using the CellTiter-Glow assay kit (Promega) according to manufacturer specifications.
Data from luminescence readings was copied onto EXCEL spreadsheets, and the percent of viable cells relative to control (i.e., in the absence of 26-aminocholesterol) was calculated, using the following equation:

\[
\% \text{ number of viable cells} = \frac{\text{mean luminescence of the test article}}{\text{mean luminescence untreated}} \times 100\%
\]

**Results**

Treatment of cancer cells with nanoemulsion containing 26-aminocholesterol resulted in complete cell kill in both cell lines tested up to 1:100 dilution (Table 1).

**Table 1. % viable cells resulting from treatment of cells with 26-aminocholesterol nanoemulsion**

<table>
<thead>
<tr>
<th>Dilution</th>
<th>H460 % viable cells</th>
<th>BXPC-3 % viable cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R1</td>
<td>R2</td>
</tr>
<tr>
<td>NT</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
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The average luminescence for each cell line at different dilutions was calculated by taking the mean of two different experiments having four replicates each. The presumed concentrations of the nanoemulsion containing 26-aminocholesterol are shown in ( ).

**Conclusion**

The nanoemulsion containing 26-aminocholesterol resulted in a significant cell kill at the dilutions tested.
It will be understood that the present invention is directed to a delivery system in the form of a composition for delivering imaging and therapeutic agents, including anticancer agents, for treating cancerous cells and tissues. Accordingly, all anticancer agents are within the scope of the present invention, as well as all diseased tissues and cells exhibiting cellular hyperproliferation and aberrant lipid metabolism and elevated uptake of lipids, including cancer cells, which may be treated by such therapeutic agents.

The foregoing discussion discloses and describes merely exemplary embodiments of the present invention. One skilled in the art will readily recognize from such discussion, and from the accompanying claims, that various changes, modifications and variations can be made therein without departing from the spirit and scope of the invention as defined in the following claims. Furthermore, while exemplary embodiments have been expressed herein, others practiced in the art may be aware of other designs or uses of the present invention. Thus, while the present invention has been described in connection with exemplary embodiments thereof, it will be understood that many modifications in both design and use will be apparent to those of ordinary skill in the art, and this application is intended to cover any adaptations or variations thereof. It is therefore manifestly intended that this invention be limited only by the claims and the equivalents thereof.
The invention to be claimed is:

1. A stable lipid-oil-water nanoemulsion simultaneously useful for selectively and preferentially imaging a diseased, injured, or injuriously mutated tissue, cell, or at least one organelle thereof, and treating the disease, injury, or injurious mutation.

2. The nanoemulsion of claim 1, wherein the tissue, cell, or at least one organelle thereof, is found in a warm-blooded animal, including humans.

3. The nanoemulsion of claim 1, wherein the nanoemulsion comprises:
   a lipid-oil-water nanoemulsion containing at least one non-bilayer-forming lipid, or analog thereof, capable of being preferentially and selectively concentrated on or within a diseased, injured, or mutated tissue, cell, or at least one organelle thereof;
   an effective amount of at least one pharmaceutically-acceptable imaging agent encapsulated within, incorporated into, or associated with the lipid nanoemulsion;
   an effective amount of at least one pharmaceutically-acceptable therapeutic agent encapsulated within, incorporated into, or associated with the lipid nanoemulsion;
   and a pharmaceutically-acceptable carrier or excipient therefor,
wherein said nanoemulsion is produced by processing through homogenization and sterilization.

4. The nanoemulsion of claim 3, wherein the sterilization is accomplished through use of radiation, solvents, endotoxin removal means, or pasteurization.

5. The nanoemulsion of claim 1, wherein the diseased, injured, or injuriously mutated cells exhibit changes in lipid processing.

6. The nanoemulsion of claim 5, wherein the cells are capable of forming tumors.

7. The nanoemulsion of claim 6, wherein the tumors are malignant.

8. The nanoemulsion of claim 3, wherein the at least one non-bilayer-forming lipid, or analog thereof, may be of therapeutic benefit.
9. The nanoemulsion of claim 7, wherein the lipid is ceramide.

10. The nanoemulsion of claim 3, wherein the therapeutic agent is, without limitation, a drug, prodrug, protein, nucleic acid, carbohydrate, hormone, vitamin, nutrient, or other substance shown to produce a desired therapeutic effect on a disease, condition, syndrome, or symptom thereof.

11. The nanoemulsion of claim 10, wherein the disease, condition or syndrome is characterized by cellular hyperproliferation.

12. The nanoemulsion of claim 11, wherein the disease, condition, or syndrome is cancer.

13. The nanoemulsion of claim 3, wherein the effective amount of the therapeutic agent is sufficient to produce a desired therapeutic effect.

14. The nanoemulsion of claim 3, wherein the therapeutic agent is radioactive.


16. The nanoemulsion of claim 1, wherein the nanoemulsion is taken up into the cell in an endosome.

17. The nanoemulsion of claim 16, wherein the endosome is taken up actively.

18. The nanoemulsion of claim 17, wherein the endosome is taken up by phagocytosis or pinocytosis.
19. The nanoemulsion of claim 15, wherein the endosome is taken up passively.

20. The nanoemulsion of claim 16, wherein the endosome is degraded upon cellular uptake such that the therapeutic agent is distributed within the cell.

21. The nanoemulsion of claim 3, wherein the amount of the therapeutic agent is at least 0.001% by weight based on the total volume of the nanoemulsion.

22. The nanoemulsion of claim 21, wherein the amount of the therapeutic agent is present from about 0.1% to 90% by weight based on the total volume of the nanoemulsion.

23. The nanoemulsion of claim 3, where the non-bilayer forming lipid forms lipid particles.

24. The nanoemulsion of claim 23, wherein the lipid particles are comprised of a mixture of non-bilayer forming-lipids.

25. The nanoemulsion of claim 23, wherein the lipid particles are present in an amount of from about 0.1 µg/mL to 1000 µg/mL.

26. The nanoemulsion of claim 25, wherein the lipid particles are present in an amount of from about 10 µg/mL to 800 µg/mL.

27. The nanoemulsion of claim 26, wherein the lipid particles are present in an amount of from about 200 µg/mL to 600 µg/mL.

28. The nanoemulsion of claim 23, wherein the average particle size of the lipid particles is up to 0.2 µm.

29. The nanoemulsion of claim 28, wherein the average particle size of the lipid particles is from about 0.02 to 0.2 µm.

30. The nanoemulsion of claim 3, wherein the non-bilayer-forming lipid is no more than weakly polar.

31. The nanoemulsion of claim 30, wherein the non-bilayer-forming lipid is substantially non-polar or neutral.
32. The nanoemulsion of claim 31, wherein the non-bilayer-forming lipid is neutral.

33. The nanoemulsion of claim 1, wherein the nanoemulsion is stably dispersed in an aqueous phase for at least seven days.

34. The nanoemulsion of claim 33, wherein the nanoemulsion is stably dispersed in an aqueous phase for at least fourteen days.

35. The nanoemulsion of claim 34, wherein the nanoemulsion is stably dispersed in an aqueous phase for at least one month.

36. The nanoemulsion of claim 35, wherein the nanoemulsion is stably dispersed in an aqueous phase for at least six months.

37. The nanoemulsion of claim 36, wherein the nanoemulsion is stably dispersed in an aqueous phase for at least one year.

38. The nanoemulsion of claim 3, further comprising emulsion-enhancing agents.

39. The nanoemulsion of claim 38, wherein the emulsion-enhancing agents are selected from the group consisting of a surfactant, a plant-based fat source, a solvent, and combinations thereof.

40. The nanoemulsion of claim 39, wherein the surfactant is a non-ionic surfactant.

41. The nanoemulsion of claim 40, wherein the non-ionic surfactant may be linear or branch-chained.

42. The nanoemulsion of claim 40, wherein the non-ionic surfactant may be of uniform composition.

43. The nanoemulsion of claim 40, wherein the non-ionic surfactant may be derived from co-polymers.

44. The nanoemulsion of claim 43, wherein the polymers have a molecular weight
between 500 and 7500.

45. The nanoemulsion of claim 44, wherein the polymers have a molecular weight between 1000 and 5000.

46. The nanoemulsion of claim 45, wherein the polymers have a molecular weight between 1200 and 4000.

47. The nanoemulsion of claim 43, wherein the polymers form an intracellular scaffold susceptible to enzymatic or environmental condition-based cleavage within the cell or at least one organelle thereof.

48. The nanoemulsion of claim 40, wherein the non-ionic surfactant is a detergent polysorbate.

49. The nanoemulsion of claim 48, wherein the detergent polysorbate is Tween®-80.

50. The nanoemulsion of claim 40, wherein the non-ionic surfactant is a sorbitan ester.

51. The nanoemulsion of claim 50, wherein the sorbitan ester is selected from the group consisting of fatty-acylated sorbitan esters and polyoxyethylene derivatives thereof, and mixtures thereof.

52. The nanoemulsion of claim 39, wherein the surfactant is present in an amount sufficient to prevent the nanoemulsion from deteriorating in storage.

53. The nanoemulsion of claim 52, wherein the surfactant is present in amounts of from about 0.01% w/v to 4.0% w/v.

54. The nanoemulsion of claim 53, wherein the surfactant is present in amounts of from about 0.1% w/v to 3.0% w/v.

55. The nanoemulsion of claim 39, wherein the solvent is a water-miscible solvent.
56. The nanoemulsion of claim 55, wherein the water-miscible solvent is selected from the group consisting of a polar solvent and combinations thereof.

57. The nanoemulsion of claim 56, wherein the solvent is selected from the group consisting of a polar aprotic solvent, a polar protic solvent, and combinations thereof.

58. The nanoemulsion of claim 57, wherein the polar aprotic solvent is dimethyl sulfoxide or dimethylamine.

59. The nanoemulsion of claim 57, wherein the polar protic solvent is an alcohol.

60. The nanoemulsion of claim 59, wherein the alcohol is selected from the group consisting of methanol, ethanol, propanol, isopropanol, butanol, benzyl alcohol, and combinations thereof.

61. The nanoemulsion of claim 39, wherein the solvent is present in an amount sufficient to control the extent of aggregation of the surfactant.

62. The nanoemulsion of claim 61, wherein the solvent is present in amounts of from about 0.001% v/v to 99.9% v/v.

63. The nanoemulsion of claim 62, wherein the solvent is present in amounts of from about 0.005% v/v to 80% v/v.

64. The nanoemulsion of claim 39, wherein the plant-based fat source is produced or derived from natural sources.

65. The nanoemulsion of claim 39, wherein the plant-based fat source is produced or derived by chemical means.

66. The nanoemulsion of claim 39, wherein the plant-based fat source is produced or modified by recombinant means.

67. The nanoemulsion of claim 39, wherein the plant-based fat source is suitably stable for pharmaceutical use at room temperature.

68. The nanoemulsion of claim 67, wherein the nanoemulsion is stable at room
temperature for at least one month.

69. The nanoemulsion of claim 68, wherein the nanoemulsion is stable at room temperature for at least six months.

70. The nanoemulsion of claim 69, wherein the nanoemulsion is stable at room temperature for at least one year.

71. The nanoemulsion of claim 39, wherein the plant-based fat source is suitably stable for pharmaceutical use upon storage in a refrigerator.

72. The nanoemulsion of claim 71, wherein the nanoemulsion is stable in storage for at least three days.

73. The nanoemulsion of claim 72, wherein the nanoemulsion is stable in storage for at least one month.

74. The nanoemulsion of claim 73, wherein the nanoemulsion is stable in storage for at least six months.

75. The nanoemulsion of claim 74, wherein the nanoemulsion is stable in storage for at least one year.

76. The nanoemulsion of claim 39, wherein the plant-based fat source is suitably stable for pharmaceutical use upon storage in a freezer.

77. The nanoemulsion of claim 76, wherein the nanoemulsion is stable in storage for at least one week.

78. The nanoemulsion of claim 77, wherein the nanoemulsion is stable in storage for at least one month.

79. The nanoemulsion of claim 78, wherein the nanoemulsion is stable in storage for at least six months.

80. The nanoemulsion of claim 79, wherein the nanoemulsion is stable in storage for at least one year.
81. The nanoemulsion of claim 39, wherein the plant-based fat source is vegetable oil.

82. The nanoemulsion of claim 81, wherein the vegetable oil is, without limitation, soybean oil, flaxseed oil, hemp oil, linseed oil, mustard oil, rapeseed oil, canola oil, safflower oil, sesame oil, sunflower oil, grape seed oil, almond oil, apricot oil, castor oil, corn oil, cottonseed oil, coconut oil, hazelnut oil, neem oil, olive oil, palm oil, palm kernel oil, peanut oil, pumpkin seed oil, rice bran oil, walnut oil, and combinations and/or specific fractions thereof.

83. The nanoemulsion of claim 81, wherein the vegetable oil is leguminous.

84. The nanoemulsion of claim 83, wherein the vegetable oil is soybean oil.

85. The nanoemulsion of claim 39, wherein the plant-based fat source is present in an amount sufficient to permit higher surface tension in the nanoemulsion, thereby increasing the probability of hydrophobic interactions with the plasma membrane of a target cell.

86. The nanoemulsion of claim 85, wherein the plant-based fat source is present in amounts of from about 0.001% v/v to 5.0% v/v.

87. The nanoemulsion of claim 86, wherein the plant-based fat source is present in amounts of from about 0.005% v/v to 4.0% v/v.

88. The nanoemulsion of claim 3, wherein the at least one non-bilayer-forming lipid is selected from the group consisting of glycerol monooesters of carboxylic acids, aliphatic alcohols, sterol aromatic acid esters, sterols, terpenes, bile acids, alkali metal salts of bile acid, sterol esters of aliphatic acids, sterol esters of sugar acids, esters of sugar acids, esters of aliphatic alcohols, esters of sugars, esters of aliphatic acids, sugar acids, saponins, sapogenins, glycerol, glycerol di-esters of aliphatic acids, glycerol tri-esters of aliphatic acids, glycerol di-esters of aliphatic alcohols, and glycerol tri-esters of aliphatic alcohols, and combinations thereof.
89. The nanoemulsion of claim 3, wherein the at least one non-bilayer-forming lipid is in the form of a lipid mixture comprising:
   a) at least one first member selected from the group consisting of glycerol monoesters of carboxylic acids containing from about 9 to 18 carbon atoms and aliphatic alcohols containing from about 10 to 18 carbon atoms;
   b) at least one second member selected from the group consisting of sterol aromatic acid esters; and
   c) at least one third member selected from the group consisting of sterols, terpenes, bile acids and alkali metal salts of bile acids.

90. The nanoemulsion of claim 89, wherein the glycerol monoesters of carboxylic acids are saturated.

91. The nanoemulsion of claim 89, wherein the glycerol monoesters of carboxylic acids are unsaturated.

92. The nanoemulsion of claim 89, wherein the lipid mixture has a weight ratio of (a):(b):(c) of (1-5):(0.25-3):(0.25-3) based on the total weight of the nanoemulsion.

93. The nanoemulsion of claim 89, wherein the lipid mixture further comprises:
   d) at least one fourth member selected from the group consisting of sterol esters of aliphatic acids containing from about 1 to 18 carbon atoms; sterol esters of sugar acids; esters of sugar acids and aliphatic alcohols containing from about 10 to 18 carbon atoms; esters of sugars and aliphatic acids containing from about 10 to 18 carbon atoms; sugar acids, saponins; and sapogenins; and
   e) at least one fifth member selected from the group consisting of glycerol; glycerol di- and tri-esters of aliphatic acids containing from about 10 to 18 carbon atoms; and aliphatic alcohols containing from about 10 to 18 carbon atoms.

94. The nanoemulsion of claim 93, wherein the lipid mixture has a weight ratio of
(a);(b);(c);(d);(e) of (1-5):(0.25-3):(0.25-3):(0.25-3):(0.25-3) based on the total weight of the nanoemulsion.

95. The nanoemulsion of claim 1, wherein the nanoemulsion acts as a biomarker for diseased, injured, or mutated tissue, cells, and/or at least one organelle thereof, that are amenable to delivery of the therapeutic agent by the nanoemulsion.

96. A method of preparing the nanoemulsion of claim 3, comprising the steps of:
   a) mixing the at least one non-bilayer-forming lipid with an effective amount of the at least one imaging agent and the at least one therapeutic agent to yield a lipid portion;
   b) adding the lipid portion to an aqueous phase to yield a dispersion; and
   c) agitating the dispersion under high-shear conditions to sufficiently disperse the at least one non-bilayer-forming lipid therethrough to form a lipid nanoemulsion comprised of lipid particles.

97. The method of claim 96, further comprising the step of adding an emulsion-enhancing agent to the nanoemulsion, wherein the emulsion-enhancing agent is selected from the group consisting of a surfactant, a solvent, a plant-based fat source, and combinations and/or specific fractions thereof.

98. The method of claim 97, further comprising the step of mixing the surfactant with the at least one non-bilayer-forming lipid prior to the step of mixing with the at least one imaging agent and the at least one therapeutic agent.

99. The method of claim 97, further comprising the step of mixing the plant-based fat source with the at least one non-bilayer-forming lipid prior to the step of mixing with the at least one imaging agent and at least one therapeutic agent.

100. The method of claim 97, further comprising the step of mixing the solvent with the at least one non-bilayer forming lipid prior to the step of mixing with the at least one imaging agent and the at least one therapeutic agent.
101. The method of claim 96, wherein the agitating step comprises processing the lipid mixture by a fluid homogenization process.

102. The method of claim 101, wherein the fluid homogenization process is selected from the group consisting of high-shear homogenization, ultrasound homogenization, high-pressure homogenization, membrane extrusion, sonication, fluidization, and combinations thereof.

103. The method of claim 96, further comprising sterilizing the nanoemulsion.

104. The method of claim 103, wherein the sterilization is accomplished through use of radiation, solvents, endotoxin removal means, or pasteurization.

105. The method of claim 96, further comprising processing the nanoemulsion by aseptic filtration.

106. The method of claim 105, wherein the processing step comprises passing the nanoemulsion through a 0.2 µm membrane.

107. The method of claim 96, wherein the therapeutic agent is complexed, conjugated, or otherwise rendered lipophilic to facilitate lipid portion formation.
INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 11/00312

A. CLASSIFICATION OF SUBJECT MATTER

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According to International Patent Classification (IPC) or to both national classification and IPC

B. MINIMUM DOCUMENTATION SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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Documented searched other than minimum documentation to the extent that such documents are included in the fields searched

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>US 2005/0079131 A1 (Lanza et al.) 4 April 2005 (14.04.2005), especially, claim 18, para [0002], [0010], [0014], [0015], [0019], [0020], [0025], [0027], [0028], [0030], [0036], [0042], [0043], [0045], [0046], [0049], [0051], [0067], [0072], [0073], [0079], [0084], [0090], [0100], [0101], [0102], [0104], [0107], [0112], [0114], [0122] and [0123].</td>
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<td>US 2003/075205 A1 (Jorgensen et al.) 18 September 2003 (18.09.2003), especially, para [0043], [0090], [0115], [0124], [0181] and [0200].</td>
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<td>US 2003/0026831 A1 (Lakkaraju et al.) 6 February 2003 (06.02.2003), especially, para [0003], [0028], [0039] and [0398].</td>
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<td>US 2006/0222696 A1 (Okada et al.) 5 October 2006 (05.10.2006), especially, para [0030] and [0371].</td>
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<td>US 2006/0240092 A1 (Brestenkamp et al.) 26 October 2006 (26.10.2006), especially, para [0042].</td>
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<td>US 2009/0047608 A1 (Manoharan et al.) 1 October 2009 (01.10.2009), especially, para [0030], [0341] and [0342].</td>
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* Special categories of cited documents:
  "A" document defining the general state of the art which is not considered to be of particular relevance
  "E" earlier application or patent but published on or after the international filing date
  "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  "O" document referring to an oral disclosure, use, exhibition or other means
  "P" document published prior to the international filing date but later than the priority date claimed

Further documents are listed in the continuation of Box C.

Date of the actual completion of the international search
21 May 2011 (21.05.2011)

Date of mailing of the international search report
06 Jun 2011

Name and mailing address of the ISA/US
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Authorized officer: Lee W. Young
PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

Form PCT/ISA/6.10 (second sheet) (July 2009)
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<td>US 2007/01281 17 A1 (Bettinger et al.) 7 June 2007 (07.06.2007), especially, para [0009] and [0055].</td>
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