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(54) Title: INDOCYANINE GREEN FORMULATIONS AND METHODS FOR INTRAOPERATIVE LOCALIZATION OF RECTAL TUMORS

(57) Abstract: The present invention relates to an imaging composition for use in localization, e.g., intraoperative localization, of a tumor, in particular a gastrointestinal or colon tumor, e.g., in intraoperative localization of a rectal tumor during laparoscopic anterior resection, which comprises particles each comprising a phospholipid, wherein a near infrared (NIR) fluorescent probe is non-covalently linked to said particle.



INDOCYANINE GREEN FORMULATIONS AND METHODS FOR INTRAOPERATIVE LOCALIZATION OF RECTAL TUMORS

TECHNICAL FIELD

[0001] The present invention relates to an imaging composition for intraoperative localization of tumors, in particular gastrointestinal or colon tumors, e.g., rectal tumors, and to a method of use.

BACKGROUND ART

[0002] Rectal adenocarcinoma consist about a third of all colorectal cancers, and resection of such tumors entails complete removal of the tumor without affecting the sphincter apparatus (incontinence). Laparoscopic anterior resection (LAR) is commonly implied in the treatment of resectable rectal cancer. This treatment confers the known benefit of laparoscopic with similar oncological outcomes (Bonjer *et al.*, 2015; Fleshman *et al.*, 2015). However, the use of laparoscopic surgery has depleted surgeon from their tactile perception. In the case of rectal tumors, this may pose a problem in determination of resection borders. Usually, oncologically acceptable resection is defined as 2 cm distal to the tumor. Increasing this distance may impair sphincter function or post-operative quality of life, whereas decreasing these borders may compromise oncological outcomes.

[0003] Tumor identification in the rectum is problematic, especially during laparoscopic surgery (Corbitt, 1992; Hoffman *et al.*, 1994; Wexner *et al.*, 1995). The confined spaces in the pelvis, in addition to the mesorectal encasement of all but the rectal anterior wall, contribute to this difficulty. Common solutions for tumor localization are somewhat limited. Pre-operative tattooing of the tumor is not always visible due to mesorectal encasement (Feingold *et al.*, 2004). Intraoperative rectoscopy is cumbersome and does not mark the extra-luminal wall. Intraoperative ultra-sound is operator dependent and limited by intra-luminal air (Hyung *et al.*, 2004). Clipping of the tumor mandates pre-operative colonoscopy and use of X-ray for intraoperative localization. Thus, there is a clinical need to develop a tool that can easily and accurately help the surgeon to localize the tumor during the operation.

[0004] Near-infrared (NIR) imaging seems specifically appropriate for this indication. Human tissue contains no auto-florescence in the NIR range, and florescence emission can penetrate 5-10 mm, which can enable tumor localization through the rectal wall.

[0005] Up-to-date, the only United States Food and Drug Administration (FDA) and European Medicines Agency (EMA) human approved NIR molecule is indocyanine green (ICG), a water soluble tricarbocyanine dye having excellent safety profile. ICG is widely used in the clinic for determination of cardiac output, hepatic function and liver blood flow, inspection of retinal and choroidal vessels (Dzurinko *et al.*, 2004), and diagnosis of burn depth (Still *et al.*, 2001). Unfortunately, ICG's elimination is mostly hepatic, through a variety of uptake and efflux transporters (Bax *et al.*, 1980; Huang and Vore, 2001; Portnoy *et al.*, 2012), with negligible non-hepatic elimination (Bax *et al.*, 1980; Cherrick *et al.*, 1960). In the context of colorectal surgery, ICG was used for assessment of anastomotic perfusion (Hellan *et al.*, 2014) and to map rectal sentinel node (Cahill *et al.*, 2012).

[0006] Liposomes are a very attractive delivery form because they are physically and chemically well-characterized structures that can be delivered through almost all routes of administration, and are biocompatible. Utilization of ICG-loaded liposomes in biological systems was recently described by Sandanaraj *et al.* (2010) and Proulx *et al.* (2010).

[0007] International Publication No. WO 2012/032524 discloses a liposomal formulation for detection of tumors in the gastrointestinal track, wherein at least one NIR fluorescent probe such as ICG and at least one active agent, e.g., a peptide, polypeptide or protein, are non-covalently bound to the outer surface of phospholipid-based particles, i.e., passively adsorbed to said phospholipid-based particles. The utilization of this preparation was only by oral delivery.

[0008] International Publication No. WO 2016/128979 discloses an imaging composition for use in imaging the urinary pathways, more particularly for intraoperative identification of ureters, wherein the composition comprises particles each comprising (a) a phospholipid, wherein a NIR fluorescent probe, e.g., a cyanine dye such as ICG, is either adsorbed to or embedded within said particle. The liposomal particles shown are, in fact, nanoparticles having at least one dimension (such as width) that is preferably in the range of 30-60 nm.

[0009] The entire contents of all published patent applications cited herein are hereby incorporated by reference in their entirety as if fully disclosed herein.

SUMMARY OF INVENTION

[0010] The aim of the study described herein was to evaluate the feasibility to intraoperatively localize, i.e., image the borders (boundaries) of, colorectal tumors using liposomal ICG, considering that tumoral blood vessels are fenestrated due to the aberrant angiogenesis, and assuming that liposomes in an appropriate size will thus extravasate and consequently give a specific staining only in tumors. The model used was the mice model recently described in Zigmond *et al.* (2011), wherein colonic carcinoma cell line are endoscopically injected in the rectum, and mice are followed for tumor development until tumor occupies 30-50% of rectal lumen. The liposomal ICG formulation used was similar to that utilized in the International Publication No. WO 2016/128979, and consisted of Phospholipon[®] 75 particles to which ICG is non-covalently bound; however, in order to take the advantage of the enhanced permeability and retention effect described for particles and cancer (Matsumura and Maeda, 1986), the size of the liposomes prepared was substantially bigger, more specifically about 100 nm, so as to meet the imaging requirements for tumor imaging. As postulated, this size would afford penetration of ICG only in "leaky vessels" like the peri-tumoral capillaries, and the retention effect would also be increased due to the size limitation on further diffusion.

[0011] It has now been found, in accordance with the present invention, that a liposomal ICG formulation as described hereinabove is highly effective in localizing rectal tumors, wherein the emission intensity of the ICG from said tumor upon excitation at a proper wavelength is measured about 12 hours after systemic administration of the formulation. These findings should assist a surgeon determining the distal extent of rectal resection, thus minimizing positive margins and re-resections.

[0012] In one aspect, the present invention thus relates to a method for localization, e.g., intraoperative localization, of a tumor in a subject in need thereof, said method comprising: (i) systemically administering to said subject an imaging composition comprising particles each independently comprising a phospholipid, wherein a NIR fluorescent probe is non-covalently bound to said particle, i.e., either adsorbed to or embedded within said particle; and (ii) quantitatively or qualitatively measuring the emission intensity of said NIR fluorescent probe from said tumor upon excitation at a proper wavelength, thereby imaging the borders of said tumor. In a particular such aspect, the method disclosed herein is used

for localization, e.g., intraoperative localization, of a gastrointestinal or colon tumor, e.g., for intraoperative localization of a rectal tumor during laparoscopic anterior resection.

[0013] In certain embodiments, the imaging composition administered according to the method of the invention comprises particles each independently comprising a lecithin, e.g., Phospholipon[®] 75 (mainly composed of a phospholipid mixture), wherein a cyanine dye such as ICG is either adsorbed to or embedded within said particle.

[0014] In another aspect, the present invention provides an imaging composition as defined above, i.e., an imaging composition comprising particles each independently comprising a phospholipid, wherein a NIR fluorescent probe is non-covalently bound to said particle, i.e., either adsorbed to or embedded within said particle, for use in localization, e.g., intraoperative localization, of a tumor such as a gastrointestinal or colon tumor, e.g., for intraoperative localization of a rectal tumor during laparoscopic anterior resection.

[0015] In a further aspect, the present invention relates to use of an imaging composition as defined above for the preparation of a medicament for localization, e.g., intraoperative localization, of a tumor.

[0016] In particular embodiments, the imaging composition referred to in each one of the aspects above comprises phospholipid-based particles each having a size in a range of 90-120 nm, e.g., 90-95 nm, 95-100 nm, 100-105 nm, 105-110 nm, 110-115 nm, or 115-120 nm, preferably about 100 nm.

BRIEF DESCRIPTION OF DRAWINGS

[0017] **Fig. 1** shows that liposome size is inversely related to sonication time. As sonication time increase (all other parameters without change, see Material and Methods), liposome size decreases. Shown is one representative experiment out of three.

[0018] **Fig 2** shows Cryo TEM images of 30-60nm and 80-100nm liposomal ICG obtained by the large scale preparation, before (two left panels) and after (two right panels) lyophilization.

[0019] **Fig. 3** shows liposomal ICG stability after preparation of solution. The stability of the signal intensity was checked for various size liposomes.

[0020] **Fig. 4** shows that optimal tumor to background ratio (TBR) varies by time post inoculation. For this analysis, background was defined as proximal rectum. Data represent two different experiments ($n=4$ for each group), for the 12 hours group, $n=9$.

[0021] **Figs. 5A-5B** show typical demonstration of rectal tumor by liposomal ICG. **(5A)** Tumor under visible light. **(5B)** *In vivo* NIR imaging of tumor. White arrow - tumor; asterisk - draining lymph nodes (LN); on H&E analysis shows mainly inflammatory infiltrate (data not shown).

DETAILED DESCRIPTION OF THE INVENTION

[0022] The present invention provides a method for intraoperative localization of a tumor by systemic administration of a pharmaceutically acceptable imaging composition comprising biocompatible and stable nanoparticles that are fluorescent in the NIR range, more particularly, biocompatible and stable phospholipid-based nanoparticles, in the form of liposomes or micelles, to which a NIR fluorescent probe, as the sole active agent, is non-covalently linked. The term "biocompatible" as used herein with respect to the phospholipid-based particles composing said imaging composition means that these particles are made of compounds suitable for administration to humans; and the term "stable" as used herein means that said particles are both physically and chemically stable, i.e., can be stored for a substantial period of time (e.g., weeks, months or years), and are not chemically degraded under physiological conditions for a period of time longer than about 30, 45, 60, 75, 90, 105, or 120 minutes.

[0023] In one aspect, the present invention relates to a method for localization, e.g., intraoperative localization, of a tumor in an subject in need thereof, said method comprising: (i) systemically administering to said subject an imaging composition containing a NIR fluorescent probe; and (ii) quantitatively or qualitatively measuring the emission intensity of said NIR fluorescent probe from said tumor upon excitation at a proper wavelength, thereby imaging the borders of said tumor, wherein said imaging composition comprises particles each independently comprising a phospholipid, i.e., liposomes or micelles, wherein said NIR fluorescent probe is non-covalently bound to said particle, i.e., either adsorbed to the outer surface of said particle or embedded within said particle.

[0024] The term "tumor" as used herein refers to a solid tumor, i.e., to any neoplastic tissue (benign or malignant) that forms a discrete mass containing leaky blood vessels, e.g., cancers of the brain, ovary, breast, prostate, gastrointestinal, colon, rectum, pancreas, and kidney, or benign tumors that induce neo-angiogenesis as part of their

growth/development, e.g., benign tumors (such as polyps) of the duodenum, colon and rectum.

[0025] The term “subject” as used herein with respect to the method of the present invention refers to any mammal, e.g., a human (i.e., individual).

[0026] In a particular such aspect, the method of the present invention is used for localization, e.g., intraoperative localization, of a gastrointestinal or colon benign or malignant tumor, e.g., for intraoperative localization of a rectal (colorectal) tumor during laparoscopic anterior resection.

[0027] The term “near infrared (NIR) fluorescent probe” as used herein refers to any fluorescent probe having an absorption and fluorescence spectrum in the NIR region. Examples of such fluorescent probes include, without being limited to, cyanine dyes such as indocyanine green (ICG), Cy5, Cy5.5, Cy5.18, Cy7 and Cy7.18; IRDye 78, IRDye 680, IRDye 750, IRDye 800 phosphoramidite (LI-COR Biosciences), DY-681, DY-731, DY-781 (Dyomics GmbH), or Alexa Fluor dyes such as Alexa Fluor[®] 610, Alexa Fluor[®] 633, Alexa Fluor[®] 647, Alexa Fluor[®] 660, Alexa Fluor[®] 680, Alexa Fluor[®] 700 and Alexa Fluor[®] 750. In particular embodiments, the NIR fluorescent probe contained within the imaging composition administered according to the method of the invention is the cyanine dye ICG, which is currently the only US FDA-approved NIR molecule. According to the present invention, the NIR-fluorescent probe is non-covalently linked to the phospholipid-based particle, i.e., either adsorbed to or embedded within said particle, and the probe molecule thus stays intact.

[0028] Administration of the imaging composition can be carried out by any suitable systemic administration route, e.g., intravenously (IV), intraarterially, intramuscularly, intraperitoneally, intrathecally, intrapleurally, intratracheally, or subcutaneously, taking into consideration *inter alia* the type and general location of the tumor to be localized, and as deemed appropriate by the practitioner. In certain embodiments, the composition is administered IV.

[0029] The method of the present invention is used for localization of a solid tumor, e.g., a gastrointestinal or colon benign or malignant tumor, in a subject undergoing a medical or surgical operation for the resection of said tumor, e.g., for intraoperative localization of a colorectal tumor in a subject undergoing laparoscopic anterior resection. According to this method, intraoperative localization of the tumor is carried out by visualization of fluorescence and/or by measurement of the emission intensity of the NIR fluorescent probe

from said tumor upon excitation at a proper wavelength, a sufficient period of time, e.g., 10-18 hours, e.g., about 12, 12.5, 13, 13.5, or 14 hours, after systemic administration of the imaging composition which may be carried out as single-shot or repetitive administration. The phrase "measuring the emission intensity of said NIR fluorescent probe" in step (ii) of said method refers to either quantitative measurement of the emission intensity of said fluorescent probe or qualitative measurement, i.e., detection, of said probe. The term "proper wavelength" with respect to the NIR fluorescent probe means any wavelength in the NIR region that would be suitable for excitation of the NIR fluorescent probe, and preferably the particular wavelength(s) at which the maximum emission intensity peak of the NIR fluorescent probe is observed.

[0030] In certain embodiments, the imaging composition administered according to the method of the present invention as defined in any one of the embodiments above comprises a phospholipid-based particles, wherein said phospholipid is selected from a lecithin such as egg or soybean lecithin, or a derivative thereof, e.g., a lecithin having polyethylene glycol (PEG) chains; a phosphatidylcholine such as egg phosphatidylcholine; a hydrogenated phosphatidylcholine; a lysophosphatidylcholine; dipalmitoylphosphatidylcholine; distearoylphosphatidylcholine; dimyristoylphosphatidylcholine; dilauroylphosphatidylcholine; a glycerophospholipid such as phosphatidylglycerol, phosphatidylserine, phosphatidylethanolamine, lysophosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol phosphate, phosphatidylinositol bisphosphate and phosphatidylinositol triphosphate; sphingomyelin; cardiolipin; a phosphatidic acid; a glycolipid such as a glyceroglycolipid, e.g., a galactolipid and a sulfolipid, a glycosphingolipid, e.g., a cerebroside (a glucocerebroside and a galactocerebroside), and a glycosylphosphatidylinositol; a plasmalogen; a phosphosphingolipid such as a ceramide phosphorylcholine, a ceramide phosphorylglycerol, and a ceramide phosphorylethanolamine; or a mixture thereof.

[0031] In particular non-limiting embodiments, the imaging composition administered according to the method of the invention comprises phospholipid-based particles, wherein said phospholipid is a commercially available product such as Phospholipon[®] 50, Phospholipon[®] 75, Phospholipon[®] 85G or Phospholipon[®] 90G, essentially consisting of soybean lecithins and phospholipids; Phospholipon[®] 80H or Phospholipon[®] 90H, essentially consisting of hydrogenated soybean lecithins and phospholipids; Phospholipon[®] E25, Phospholipon[®] E35 or Phospholipon[®] E, essentially consisting of egg yolk lecithins

and phospholipids; and Phospholipon[®] LPC20, Phospholipon[®] LPC25 or Phospholipon[®] LPC65, essentially consisting of partially hydrolyzed soybean lecithins (all of Lipoid). In a more particular embodiment, the phospholipid composing the phospholipid-based particles is Phospholipon[®] 50, i.e., a soybean lecithin with about 45% phosphatidylcholine and about 10 to about 18% phosphatidylethanolamine, or Phospholipon[®] 75, i.e., a soybean lecithin with about 75% phosphatidylcholine.

[0032] In certain embodiments, the imaging composition administered according to the method of the present invention as defined in any one of the embodiments above comprises phospholipid-based particles, wherein said phospholipid is admixed with one or more, e.g., two, three or four, nonphosphorous-containing molecules. Non-limiting examples of suitable nonphosphorous-containing molecules include fatty amines such as octylamine, laurylamine, N-tetradecylamine, hexadecylamine, stearylamine, oleylamine, tallowamine, hydrogenated tallowamine, and cocoamine; fatty acids; fatty acid amides; esters of fatty acid such as isopropyl myristate, hexadecyl stearate, and cetyl palmitate; cholesterol; cholesterol esters; diacylglycerols; or glycerol esters such as glycerol ricinoleate.

[0033] The phospholipid-based particles composing the imaging composition of the present invention are negatively charged and have zeta potential of $\geq |10|$ mV (absolute value), and their size is preferably about 80 nm or more. In order to prevent uptake by the reticuloendothelial system and hence increase circulating time of the particles, a PEGylated phospholipid can be admixed with the phospholipid composing the particle.

[0034] In certain embodiments, the imaging composition administered according to the method of the present invention as defined in any one of the embodiments above comprises a phospholipid-based particles, wherein said phospholipid is optionally admixed with one or more nonphosphorous-containing molecules as defined above, and thus further admixed with one or more, e.g., two, three or four, PEGylated phospholipids. Examples of suitable PEGylated phospholipids include, without being limited to, PEGylated dipalmitoyl phosphatidylethanolamine (DPPE-PEG), PEGylated palmitoyl oleoyl phosphatidylethanolamine (POPE-PEG), PEGylated dioleoyl phosphatidylethanolamine (DOPE-PEG) and PEGylated distearoyl phosphatidylethanolamine (DSPE-PEG), preferably 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[polyethyleneglycol 2000] (PEG-DSPE-2000). In a particular embodiment, the phospholipid composing the phospholipid-based particles is optionally admixed with one or more nonphosphorous-

containing molecules as defined above, and further admixed with PEG-DSPE-2000, wherein said particles each comprises up to 15% by weight of PEG-DSPE-2000.

[0035] In certain embodiments, the imaging composition administered according to the method of the present invention comprises a phospholipid-based particles, wherein said phospholipid is optionally admixed with one or more nonphosphorous-containing molecules and/or one or more PEGylated phospholipids as defined above, and said particles each comprises said NIR fluorescent probe in a weight ratio that enables the highest fluorescent signal after administration of said imaging composition, e.g., about 2%, 4%, 6%, 8%, 10%, 12%, 14%, 16%, 18%, 20% or more, by weight of said NIR fluorescent probe, depending on the actual performance.

[0036] In particular embodiments, the imaging composition administered according to the method of the invention comprises phospholipid-based particles, wherein said phospholipid is Phospholipon[®] 50 or Phospholipon[®] 75, and said particles each comprises, e.g., about 5% to about 20% but preferably about 10% to about 20% by weight, ICG, either adsorbed to or embedded within said particle.

[0037] The phospholipid-based particles composing the imaging composition administered according to the method of the invention are, in fact, nanoparticles. The term "nanoparticles" as used herein refers to materials and structures or particles having a uniform shape, e.g., spherical or elongated, or a variety of shapes, wherein each particle has at least one dimension (such as width) which is a micron or smaller in size, e.g., in the range of 80-200 nanometers, but preferably in the range of 90-140, e.g., 90-95 nm, 95-100 nm, 100-105 nm, 105-110 nm, 110-115 nm, 115-120 nm, 120-125 nm, 125-130 nm, 130-135 nm, or 135-140 nm, although other dimensions (such as length) may be longer than a micron. In certain embodiments, the size of the particles composing the imaging composition is in a range of 90-120 nm, e.g., 90-95 nm, 95-100 nm, 100-105 nm, 105-110 nm, 110-115 nm, or 115-120 nm, preferably about 100 nm.

[0038] Example 1 hereinafter shows the efficacy of a composition comprising liposomal-based particles at about 100 nm size, each comprising Phospholipon[®] 75 wherein ICG is either adsorbed to or embedded within said particle, vs. a composition comprising free ICG, in imaging a colorectal tumor induced in C57Bl mice. As shown, the liposomes extravasate and retain in the tumor with excellent tumor to background ratio, wherein the best time point for tumor imaging was about 12 hours post intravenous administration of the liposomal composition (in earlier time points background fluorescence impaired the

ability to localize tumor borders; and in 24 hours tumor fluorescence intensity diminished). The tumor localized is clearly discerned from the normal surrounding bowel, wherein visibility is demonstrated from the outside, simulating the situation during laparoscopic anterior resection. Moreover, the liposomes had also concentrated in lymph nodes draining the primary tumor, and this raises the possibility of further using the liposomal ICG as an estimation tool for the lymphadenectomy extent in rectal resection.

[0039] As laparoscopic anterior resection gains more acceptance, tools that may help the surgeon shorten operative time and improve surgical outcomes will become increasingly needed (Ris *et al.*, 2015). Tumor localization within the rectum and determination of distal (inferior) border may improve remnant rectal function post operatively and assist at proper oncological border. NIR imaging is appropriate for this aim due to tissue penetrance. In addition, NIR imaging technology is available for intraoperative use, both as part of the robotic system and as part of conventional laparoscopic equipment. However, the lack of validated fluorescent dyes for intraoperative use impairs the widespread use of NIR imaging systems.

[0040] The issue of ICG specificity for tumoral imaging was raised before (Tummers *et al.*, 2015), and it was concluded that free ICG identifies both inflammatory and calcified lesions. The study disclosed herein shows that liposomal ICG affords localization of the draining lymph nodes. Of note, the specific lymph nodes identified was not involved by tumor, but by inflammatory infiltrate. Again, the non-specific targeting of the liposome does not discriminate between inflammatory and tumor associated capillaries.

[0041] In another aspect, the present invention provides an imaging composition as disclosed above, i.e., an imaging composition comprising particles each independently comprising a phospholipid as defined in any one of the embodiments above, wherein a NIR fluorescent probe, as the sole active agent, is either adsorbed to or embedded within said particle, for use in localization, e.g., intraoperative localization, of a tumor. In a particular such aspect, the invention provides an imaging composition as disclosed above, for localization, e.g., intraoperative localization, of a gastrointestinal or colon benign or malignant tumor, e.g., for intraoperative localization of a rectal tumor during laparoscopic anterior resection.

[0042] In a further aspect, the present invention relates to use of an imaging composition as defined in any one of the embodiments above for the preparation of a medicament for localization, e.g., intraoperative localization, of a tumor, more particularly localization,

e.g., intraoperative localization, of a gastrointestinal or colon benign or malignant tumor, e.g., a rectal tumor during laparoscopic anterior resection.

[0043] In certain embodiments, the imaging composition of the present invention comprises particles each comprising a phospholipid, i.e., liposomes or micelles, wherein said NIR fluorescent probe is either adsorbed to said particle or embedded within said particle. Particular such imaging compositions comprise particles each comprising Phospholipon[®] 50 or Phospholipon[®] 75, wherein ICG is either adsorbed to or embedded within said particle.

[0044] Phospholipid-based particles, e.g., liposomes or micelles, having a NIR fluorescent probe either adsorbed to or embedded within for use in the imaging composition of the present invention can be prepared according to any procedure and/or technique known in the art, e.g., as described in WO 2012/032524 and/or in the experimental section herein. For example, small unilamellar liposomes can be prepared by high-energy sonication of a phospholipid or a mixture of phospholipids as defined herein, and a NIR fluorescent probe binding can then be performed by incubating the liposomes prepared with a solution of the fluorescent probe. The adsorbed quantity of the NIR fluorescent probe may be calculated by measuring the optical density of the solution obtained after filtering the sample to thereby remove all the liposomes. When preparing those liposomal particles, solutions containing various concentrations of the fluorescent probe might be used, aimed at preparing liposomal particles comprising as high concentration of the fluorescent probe as possible, without causing aggregation of the particles or decreasing the fluorescent signal.

[0045] The imaging composition disclosed herein is a pharmaceutically acceptable composition. Such imaging compositions may be prepared by conventional techniques, e.g., as described in Remington: The Science and Practice of Pharmacy, 19th Ed., 1995. The composition can be prepared, e.g., by uniformly and intimately bringing the active ingredient, i.e., the particles composing the imaging composition as defined above, into association with a liquid carrier. In certain embodiments, the imaging composition is in the form of a liquid, e.g., an injectable liquid, and may further include pharmaceutically acceptable fillers, carriers, diluents or adjuvants, and other inert ingredients and excipients. In other embodiments, the imaging composition is in the form of a powder, which disperses, i.e., reconstitutes, well upon contact with an injectable liquid. Imaging

compositions in the form of a powder can be prepared by any suitable method known in the art, e.g., by lyophilization (freeze drying) or spray drying.

[0046] Imaging compositions as disclosed herein can be formulated for any suitable parenteral route of administration, e.g., for intravenous, intraarterial, intramuscular, intraperitoneal, intrathecal or subcutaneous administration, but they are preferably formulated for intravenous administration. The imaging composition may be in the form of a sterile injectable aqueous solution or suspension, e.g., in a non-toxic parenterally acceptable diluent or solvent, and may be formulated according to the known art using suitable dispersing, wetting or suspending agents. Acceptable vehicles and solvents that may be employed include, without limiting, water, Ringer's solution and isotonic sodium chloride solution. The dosage administered as well as the duration and rate of administration will be determined as deemed appropriate by the practitioner.

[0047] The detection of NIR emission from the tumor localized according to the method of the invention may be carried out utilizing any suitable means, i.e., an appropriate intraoperative NIR imaging system like, without being limited to, Mini-Fluorescence-Assisted Resection and Exploration (FLAIR)TM imaging system or a robotic system like the *da Vinci*TM surgical system (Intuitive Surgical).

[0048] Unless otherwise indicated, all numbers expressing, e.g., the NIR fluorescent probe weight ratio in the imaging composition, or the size of the liposomal particles comprised within said composition, as used in this specification are to be understood as being modified in all instances by the term "about". Accordingly, unless indicated to the contrary, the numerical parameters set forth in this specification are approximations that may vary by up to plus or minus 10% depending upon the desired properties to be obtained by the present invention.

[0049] The invention will now be illustrated by the following non-limiting Examples.

EXAMPLES

Materials and Methods

Liposomal ICG preparation

[0050] ICG was purchased from Acros Organics (Geel, Belgium). Phospholipon[®] 75 was obtained from Lipoid (Steinhausen, Switzerland). 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was purchased from Avanti (Alabaster, USA). All other reagents

were from Sigma-Aldrich (Rehovot, Israel). Liposomes were prepared by sonication using the Adaptive Focused Acoustics™ technology, which delivers controlled energy precisely and accurately to a sample tube while maintaining temperature control, and therefore enables controlling liposome size mainly by time of sonication. In particular, Phospholipon® 75 5% was dispersed in 2 mM phosphate buffer with 9.3% sucrose and stirred using a magnetic stirrer at room temperature for 40 minutes or until all solid material dissolved. All preparations were performed under Argon. 100-nm liposomes were then prepared by sonication using S220 Focused-ultrasonicators (Covaris M-series miniTubes) under the following conditions: peak incident power=40W, duty factor=50%, cycles per burst=1000, duration=180 seconds, bath temperature=20°C, power mode=frequency sweeping, degassing mode=N/A, volume=1 ml. ICG was dissolved in double-distilled water (DDW) to 3.2 mM ICG stock solution, and binding to liposomes was performed by adding ICG stock solution to liposomes in a ratio of 1:5 (ICG:liposomes). The dispersion was then incubated under mild agitation at 5°C for 24 hours in the dark.

Liposomal ICG scale up preparation

[0051] For experiments in large animals and in humans, larger quantities of the imaging formulation are needed. Therefore, upscaling preparation experiments were conducted as follows.

[0052] Liposomal ICG were prepared in a large scale by sonication. In particular, Phospholipon® 75 5% was dispersed in 2 mM phosphate buffer with 9.3% sucrose and stirred using a magnetic stirrer at room temperature for 40 minutes or until all solid material dissolved. All preparations were performed under Nitrogen. 50/100-nm liposomes were then prepared by sonication using Ultrasonic cell crusher SKL-750 (SYCLON), probe of 1.1 cm diameter defined as probe no. 10 under the following conditions: For 30-60nm: 100 min, amplitude: 80%, ON: 2 sec, OFF: 1 sec volume=200 ml. For 80-100nm: 35 min, amplitude: 80%, ON: 2 sec, OFF: 1 sec volume=200 ml. During the sonication process, the liposomes were cooled in an ice bath.

[0053] ICG was dissolved in DDW to 3.2 mM ICG stock solution, and binding to liposomes was performed by adding ICG stock solution to liposomes in a ratio of 1:5 (ICG:liposomes). The dispersion was then incubated under mild agitation at room temperature for 24 hours in the dark. The liposomal ICG dispersion was placed in 1L

round bottom flask and cooled by liquid nitrogen till freezing. The frozen sample in the flask was lyophilized for 48 hours (LABCONCO, FREE ZONE 2.5. -43°C, less than 1 mBar. For animal experiments, the obtained powder was dispersed in 2 mM phosphate buffer by mixing with vortex for 30 seconds by keeping the liposomes and ICG original concentrations. Typically, 1 g powder is dispersed in 7.3 g buffer.

Liposome characterization

[0054] Liposome size measurements were performed using a Zetasizer Nano-S (Malvern Instruments, Worcestershire, United Kingdom). The aqueous dispersions were measured after dilution to 0.005%. For the dynamic light scattering measurements, the refractive index for the liposomes was taken as 1.45 and the absorbance for the liposomal-ICG was taken as 0.3. For microscopic analysis liposome samples were diluted with phosphate buffer to 1% and the nanoparticles were then imaged using cryo-transmission electron microscopy (TEM) as previously described (Portnoy *et al.*, 2011).

[0055] Liposomal ICG stability was examined after dissolution in phosphate buffered saline (PBS). Fluorescence intensity was determined after different time points, while the liposomal solution was kept in the dark overnight at 4°C.

Animals

[0056] 6-7 weeks C57Bl mice were purchased from Harlan laboratories (Rehovot, Israel) and kept in the animal facility of the Tel-Aviv Sourasky Medical Center. The mice had free access to food (a standard diet) and water, and were maintained on a 12/12-h automatically-timed light/dark cycle. Mice were endoscopically injected with 1×10^5 Murine MC38 colon cancer line cells as previously described (Zigmond *et al.*, 2011). Mice were followed daily for clinical signs, and colonoscopy to stage tumor was done at 14 and 21 days post inoculation. When rectal tumors occupied about 30-50% of rectal lumen as determined by an experienced endoscopist, imaging experiments were conducted. The animal study protocol was approved by the Institutional Animal Care and Use Committee and the procedures followed were in accordance with the institutional guidelines.

Imaging experiments

[0057] Mice were inoculated (IV injection) with 8 mg/kg of ICG (present in its liposomal powder form) and subjected to laparotomy and rectal excision. Fluorescence intensity was calculated with ImageJ software after determination of region of interest (ROI). Both maximal and average intensities were calculated and analyzed.

[0058] Whole body florescence was measured in IVIS system, and organs resected were *ex-vivo* subjected to Typhoon FLA 9500 biomolecular imager.

Example 1. Liposomal ICG is effective in imaging rectal tumors

Liposome preparation and characterization

[0059] Using the sonication parameters for the small scale described above, we obtained liposomes at about 100 nm size. By Intensity: Z-Average- 96nm, peak- 121nm, pdi-0.287; by Volum: Z-Average- 96nm, peak- 59nm, pdi-0.287 (evaluation was performed by the Zetasizer Nano-S). **Fig. 1** shows the dependence of liposome size on duration of sonication, wherein the liposome size presented are the Z-average results obtained from each measurement.

[0060] The measured sizes for the liposomes obtained by the scale-up preparation process are as follows:

For 30-60nm preparation. Before lyophilization: by Intensity: Z-Average- 80nm, peak- 110nm, pdi-0.302; by Volum: Z-Average- 80nm, peak- 69nm, pdi-0.302. ***After lyophilization:*** by Intensity: Z-Average- 61nm, peak- 68nm, pdi-0.124; by Volum: Z-Average- 61nm, peak- 51nm, pdi-0.124.

For 80-100nm preparation. Before lyophilization: by Intensity: Z-Average- 101nm, peak- 127nm, pdi-0.271; by Volum: Z-Average- 101nm, peak- 94nm, pdi-0.271. ***After lyophilization:*** by Intensity: Z-Average- 86nm, peak- 106nm, pdi-0.217; by Volum: Z-Average- 86nm, peak- 74nm, pdi-0.217.

[0061] The cryo-TEM microscopy of the liposomes obtained at the large scale preparation showed spherical liposomes (**Fig. 2**) with size distribution as expected according to the size measurements. We evaluated liposome stability after preparation. As found (**Fig. 3**), the fluorescence intensity was kept for 24 hours. Of note, if the lyophilized liposomes are not dissolved, their stability is longer (about 2 weeks).

Optimal tumor to background signal

[0062] In order to determine the best time point to image rectal tumors in the context of tumor to background ratio (TBR) we performed analysis of the TBR in different time points. For the purpose of this analysis, the proximal rectum was defined as a background. As shown in **Fig. 4**, the best time point for tumor imaging is 12 hours post inoculation. In

earlier time points, background fluorescence impairs the ability to localize tumor borders; and in 24 hours, tumor fluorescence intensity also diminished.

[0063] **Fig. 5** is a typical demonstration of tumor imaging using liposomal ICG. **Fig. 5A** shows tumor under visible light, and **Fig. 5B** shows tumor under NIR imaging. The tumor is clearly discerned from the normal surrounding bowel. Of note, the rectum is closed and visibility is demonstrated from the outside, which simulates the situation during operation. The figure further shows the draining lymph nodes in the retroperitoneum, near the inferior vena cava. Histological analysis of this lymph nodes shows inflammatory infiltrate but not tumor involvement (data not shown).

Discussion

[0064] As laparoscopic anterior resection gains more acceptance, tools that can help the surgeon shorten operative time and improve surgical outcomes will become increasingly needed (Ris *et al.*, 2015). Tumor localization within the rectum and determination of distal (inferior) border may improve remnant rectal function post operatively and assist at proper oncological border. NIR imaging is appropriate for this aim due to tissue penetrance. In addition, NIR imaging technology is available for intraoperative use, both as part of the robotic system and as part of conventional laparoscopic equipment. However, the lack of validated fluorescent dyes for intraoperative use impairs the widespread use of NIR imaging systems.

[0065] The approach presented herein was to formulate ICG in liposome. In the context of tumor imaging, it affords more specificity: the increased size of the liposome will extravasate from the circulation only if blood vessels have enough inter-endothelial space. This is a common characteristic of tumoral capillaries. This approach is also expected to gain easier regulatory approval, as both liposome constituents are approved for human use.

[0066] We here report the preparation of ICG-based liposome from a commercially available and human approved phospholipid. The liposome is stable in solution when dissolved even 24 hours prior to injection.

[0067] When tested *in vivo*, the liposomal ICG gained access to both tumor and normal appearing bowel. In fact, we had to determine the best time point in which background signal is washed out. This was found to be around 12 hours. It is possible that bowel signal stems from liposomal ICG within vessels, and not from extravasation of the dye. Experiments in similar liposome for ureteral imaging showed that the liposome is able to

adhere to endothelial cells, and yields fluorescence signal even without extravasation. Further experiments would address this question.

[0068] The issue of ICG specificity for tumoral imaging was raised before (Tummers *et al.*, 2015), and it was concluded that free ICG identifies both inflammatory and calcified lesions. Here we show that liposomal ICG affords localization of the draining lymph nodes. Of note, the specific lymph nodes identified in this study were not involved by tumor, but by inflammatory infiltrate. Again, the non-specific targeting of the liposome does not discriminate between inflammatory and tumor associated capillaries.

[0069] To conclude, we show here a method for preparation of liposomal ICG which enables tumor imaging. The liposomes extravasate and retain in the tumor with excellent tumor to background ratio. Tumor imaging with NIR has the potential to serve as extra-luminal imaging due to NIR penetrance.

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CLAIMS

1. An imaging composition for use in intraoperative localization of a tumor, said composition comprising particles each independently comprising a phospholipid, wherein a near infrared (NIR) fluorescent probe is either adsorbed to or embedded within said particle.
2. The imaging composition of claim 1, wherein said NIR fluorescent probe is a cyanine dye, IRDye 78, IRDye 680, IRDye 750, IRDye 800 phosphoramidite, DY-681, DY-731, DY-781, or an Alexa Fluor dye.
3. The imaging composition of claim 2, wherein said cyanine dye is indocyanine green (ICG), Cy5, Cy5.5, Cy5.18, Cy7, or Cy7.18; or said Alexa Fluor dye is Alexa Fluor 610, Alexa Fluor 633, Alexa Fluor 647, Alexa Fluor 660, Alexa Fluor 680, Alexa Fluor 700, or Alexa Fluor 750.
4. The imaging composition of claim 3, wherein said NIR fluorescent probe is ICG.
5. The imaging composition of any one of claims 1 to 4, wherein said phospholipid is a lecithin or a PEGylated derivative thereof, a phosphatidylcholine, a hydrogenated phosphatidylcholine, a lysophosphatidylcholine; dipalmitoylphosphatidylcholine, distearoylphosphatidylcholine, dimyristoylphosphatidylcholine, dilauroylphosphatidylcholine, a glycerophospholipid; sphingomyelin; cardiolipin, a phosphatidic acid, a glycolipid, a plasmalogen, a phosphosphingolipid, or a mixture thereof.
6. The imaging composition of claim 5, wherein said lecithin is egg lecithin, or soybean lecithin; said phosphatidylcholine is egg phosphatidylcholine; said glycerophospholipid is phosphatidylglycerol, phosphatidylserine, phosphatidylethanolamine, lysophosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol phosphate, phosphatidylinositol bisphosphate, or phosphatidylinositol triphosphate; said glycolipid is glyceroglycolipid, a glycosphingolipid, or a glycosylphosphatidylinositol; or said phosphosphingolipid is a ceramide phosphorylcholine, a ceramide phosphorylglycerol, or a ceramide phosphorylethanolamine.

7. The imaging composition of claim 6, wherein said glyceroglycolipid is a galactolipid, or a sulfolipid; or said glycosphingolipid is a cerebroside (a glucocerebroside and a galactocerebroside).
8. The imaging composition of any one of claims 1 to 7, wherein said phospholipid is admixed with one or more nonphosphorous-containing molecules each independently is a fatty amine, a fatty acid, a fatty acid amide, an ester of a fatty acid, cholesterol, a cholesterol ester, a diacylglycerol, or a glycerol ester.
9. The imaging composition of claim 8, wherein said fatty amine is octylamine, laurylamine, N-tetradecylamine, hexadecylamine, stearylamine, oleylamine, tallowamine, hydrogenated tallowamine, or cocoamine; said ester of a fatty acid is isopropyl myristate, hexadecyl stearate, or cetyl palmitate; or said glycerol ester is glycerol ricinoleate.
10. The imaging composition of any one of claims 1 to 9, wherein said phospholipid is admixed with one or more PEGylated phospholipids.
11. The imaging composition of claim 10, wherein said PEGylated phospholipid is PEGylated dipalmitoyl phosphatidylethanolamine (DPPE-PEG), PEGylated palmitoyloleoyl phosphatidylethanolamine (POPE-PEG), PEGylated dioleoyl phosphatidylethanolamine (DOPE-PEG), or PEGylated distearoyl phosphatidylethanolamine (DSPE-PEG), preferably 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[polyethyleneglycol 2000] (DSPE-PEG-2000).
12. The imaging composition of claim 1, wherein said imaging composition comprises particles each comprising Phospholipon[®] 50 or Phospholipon[®] 75, wherein ICG is either adsorbed to or embedded within said particle.
13. The imaging composition of claim 12, wherein said particles comprise about 10% to about 20% by weight ICG.
14. The imaging composition of claim 1, wherein the size of said particles is in a range of 90-120 nm, preferably about 100 nm.

15. The imaging composition of claim 1, wherein said composition is in the form of a liquid, preferably injectable liquid, or a powder capable of dispersing within an injectable liquid.
16. The imaging composition of any one of claims 1 to 15, for use in intraoperative localization of a gastrointestinal or colon tumor.
17. The imaging composition of claim 16, for use in intraoperative localization of a rectal tumor during laparoscopic anterior resection.
18. A method for intraoperative localization of a tumor in a subject in need thereof, said method comprising:
- (i) systemically administering to said subject an imaging composition according to any one of claims 1 to 15; and
 - (ii) quantitatively or qualitatively measuring the emission intensity of said NIR fluorescent probe from said tumor upon excitation at a proper wavelength, thereby imaging the borders of said tumor.
19. The method of claim 18, wherein the size of said particles is in a range of 90-120 nm, preferably about 100 nm.
20. The method of claim 18 or 19, wherein step (ii) is carried out 10-18 hours, preferably 12-14 hours, after step (i).
21. The method of claim 18 or 19, wherein said imaging composition comprises particles each comprising Phospholipon[®] 50 or Phospholipon[®] 75, wherein ICG is either adsorbed to or embedded within said particle.
22. The method of claim 21, wherein said particles comprise about 10% to about 20% by weight ICG.
23. The method of any one of claims 18 to 22, for intraoperative localization of a gastrointestinal or colon tumor, such as a rectal tumor during laparoscopic anterior resection.
24. Use of an imaging composition according to any one of claims 1 to 15 for the preparation of a medicament for intraoperative localization of a tumor.

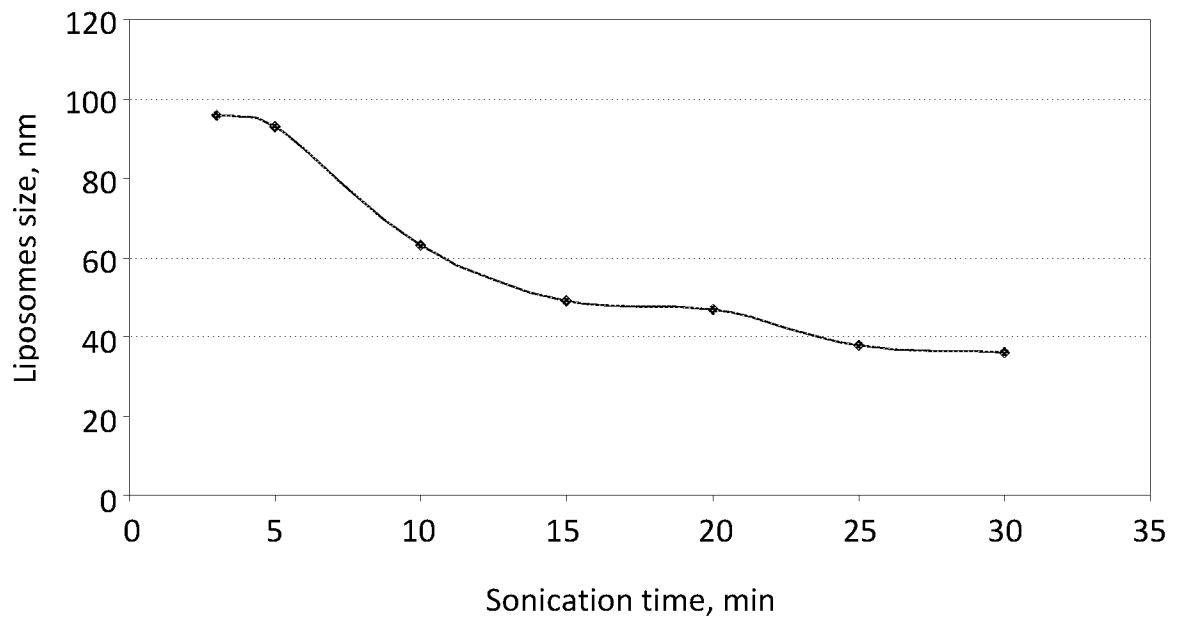
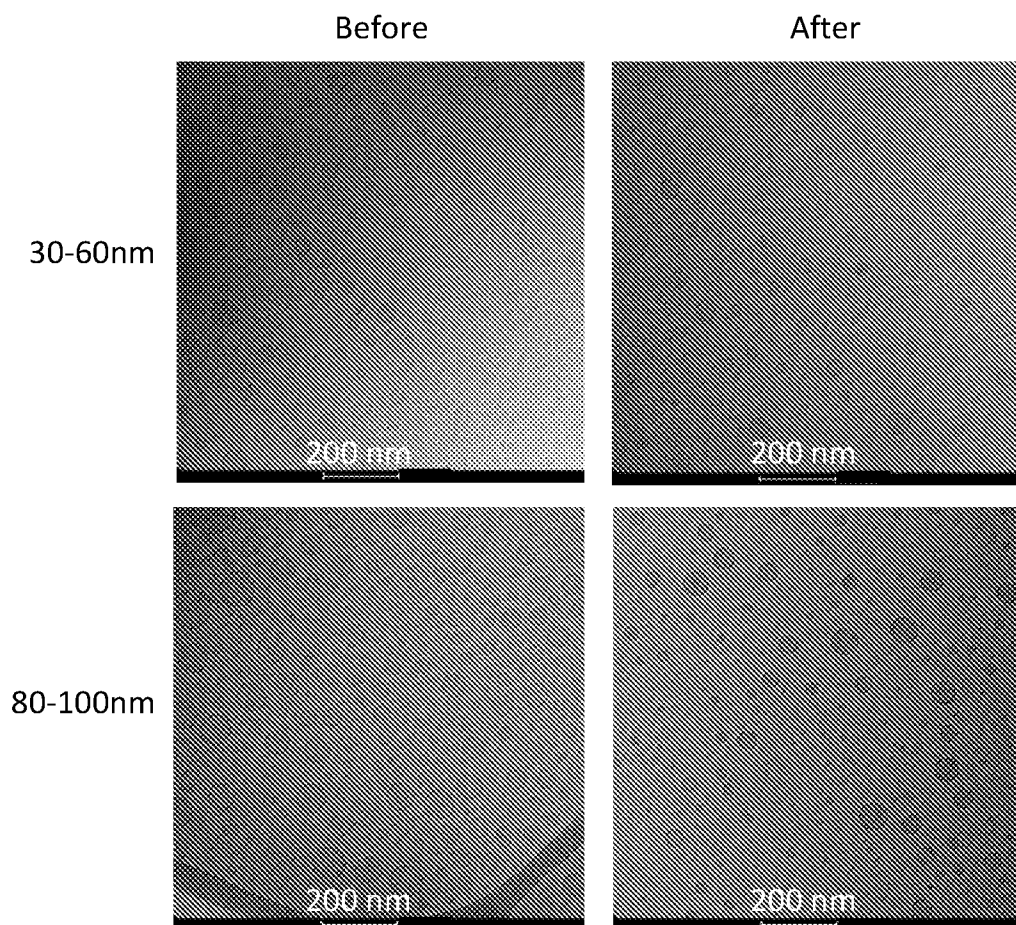
Fig. 1**Fig. 2**

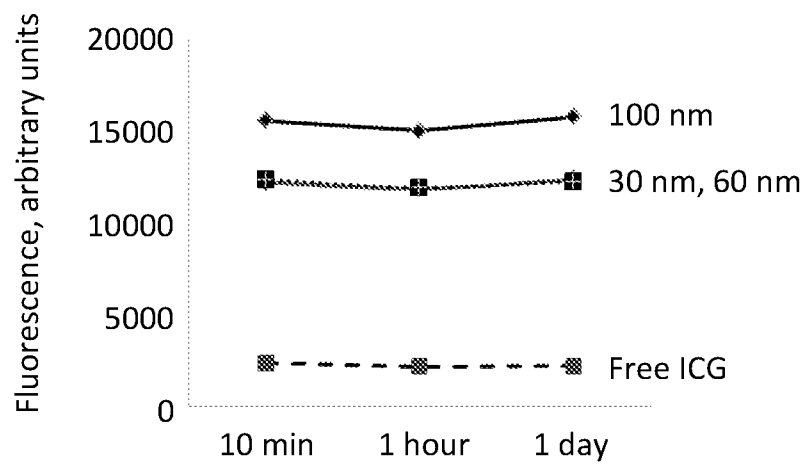
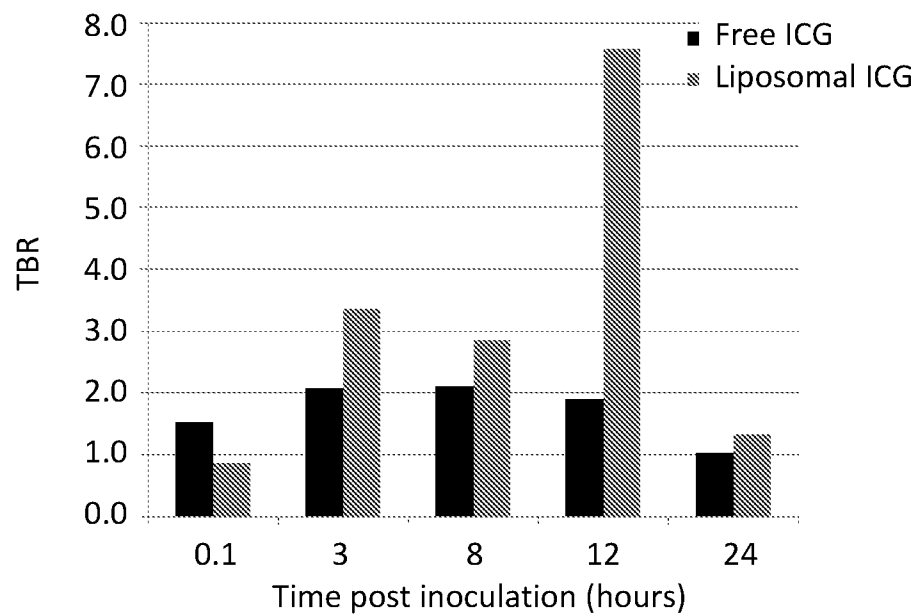
Fig. 3**Fig. 4**

Fig. 5A

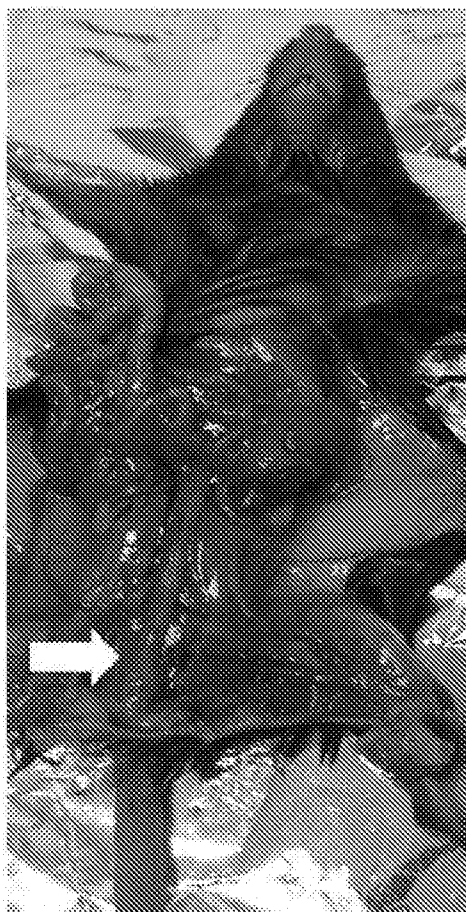
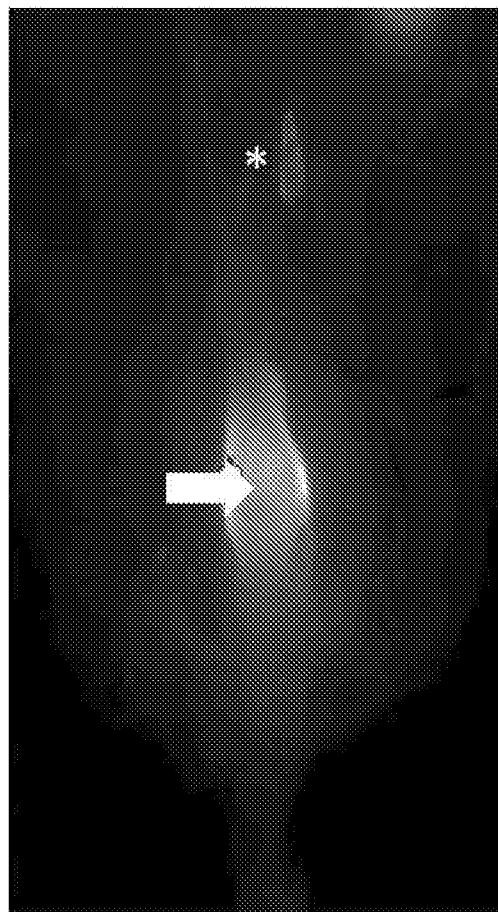


Fig. 5B



INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2017/050431

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K49/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SUGANAMI AKIKO ET AL: "Liposomally formulated phospholipid-conjugated indocyanine green for intra-operative brain tumor detection and resection", INTERNATIONAL JOURNAL OF PHARMACEUTICS, vol. 496, no. 2, 23 October 2015 (2015-10-23), pages 401-406, XP029343982, ISSN: 0378-5173, DOI: 10.1016/J.IJPHARM.2015.10.001 abstract figures page 403, right-hand column, paragraph 3.3 - page 405, right-hand column ----- -/--	1-24



Further documents are listed in the continuation of Box C.



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Date of the actual completion of the international search

25 July 2017

Date of mailing of the international search report

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Authorized officer

Dullaart, Anwyn

INTERNATIONAL SEARCH REPORT

International application No

PCT/IL2017/050431

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO 2015/169843 A1 (GLÜER CLAUS-CHRISTIAN [DE]; PENATE MEDINA TUULA [DE]; PENATE MEDINA OU) 12 November 2015 (2015-11-12) paragraph [0071] example 4 -----	1-24
X	WO 2012/032524 A1 (YISSUM RES DEV CO [IL]; MAGDASSI SHLOMO [IL]; LAZAROVICI PHILIP [IL];) 15 March 2012 (2012-03-15) cited in the application examples -----	1-24
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X	EP 2 579 027 A1 (UNIV CHIBA NAT UNIV CORP [JP]) 10 April 2013 (2013-04-10) examples paragraph [0087] ----- -/--	1-24

INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2017/050431

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	S. T. PROULX ET AL: "Quantitative Imaging of Lymphatic Function with Liposomal Indocyanine Green", CANCER RESEARCH, vol. 70, no. 18, 7 September 2010 (2010-09-07), pages 7053-7062, XP055270410, & 103RD ANNUAL MEETING OF THE AMERICAN-ASSOCIATION-FOR-CANCER-RESEARCH; CHICAGO, IL, USA; MARCH 31 -APRIL 04, 2012 ISSN: 0008-5472, DOI: 10.1158/0008-5472.CAN-10-0271 abstract page 7055, right-hand column, paragraph 1 figures page 7056, left-hand column, last paragraph - page 7059 page 7061	1-24
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/IL2017/050431

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