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(71) Applicant (for all designated States except AT, US): **NOVARTIS AG** [CH/CH]; Lichtstrasse 35, CH-4056 Basel (CH).

(71) Applicant (for AT only): **NOVARTIS PHARMA GMBH** [AT/AT]; Brunner Strasse 59, 1235 Vienna (AT).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **GREMLICH, Hans-Ulrich** [CH/CH]; Buchenrain 3, CH-4106 Therwil (CH). **KNEUER, Rainer** [DE/DE]; Königsbergerstrasse 3, 79539 Lörrach (DE).

(74) Agent: **BOURGAREL, Denis**; Novartis AG, Corporate Intellectual Property, CH-4002 Basel (CH).

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(54) Title: NON-INVASIVE METHOD FOR ASSESSING GASTRIC MOTILITY

(57) Abstract: The present invention relates to the field of optical molecular imaging. More specifically, the invention provides a probe for use in a method for assessing the rate of gastric emptying and/or gastro-intestinal passage in an animal, said probe consisting of a non-nutrient semisolid meal comprising a fluorochrome, and, optionally, a pharmaceutically acceptable vehicle. In a preferred embodiment, the probe comprises protected beads of copolymers of polystyrene and polyethylene glycol, such as TentaGel™ Beads, loaded with 1% Cy5.5. The invention further relates to the use of the probe for assessing gastric motility and a method for screening modulators of gastric and/or gastro-intestinal motility.



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NON-INVASIVE METHOD FOR ASSESSING GASTRIC MOTILITY

The present invention relates to the field of optical molecular imaging. More specifically, the invention provides a probe for use in a method for assessing the rate of gastric emptying in an animal, said probe consisting of a non-nutrient semi-solid meal comprising a fluorochrome, and, optionally a pharmaceutically acceptable vehicle. In a preferred embodiment, the probe comprises protected beads of copolymers of polystyrene and polyethylene glycol, such as TentaGelTM Beads, loaded with 1% Cy5.5. The invention further relates to the use of the probe for assessing gastric motility and a method for screening modulators of gastric motility.

Non-invasive *in vivo* imaging with light photons represents an intriguing avenue for studying molecular and physiological processes in the intact organism. As hemoglobin and water, the major absorbers of visible and infrared light, have their lowest absorption coefficient in the near-infrared (NIR) region of the electromagnetic spectrum around 600 – 1100 nm, the NIR window is ideally suited for *in vivo* applications. Depth of penetration for NIR light in tissue is several centimeters allowing for whole body imaging of small animals as well as investigation of superficial tissue in larger species including humans. Examples are the detection of tumors, their characterization by visualization of molecular events such as enzymatic activities or drug receptor interactions or the study of bone formation in rodents. In these studies, the high sensitivity of optical techniques has been exploited to monitor fluorescent reporters at low tissue concentrations. Alternatively, NIR imaging could be used to assess the physiological functions of organs using non-targeted labels.

Classically, gastric emptying is studied in laboratory animals using invasive methods, frequently requiring postmortem analyses: the rate of emptying is determined by measuring the gastric content or a marker thereof [1]. Few alternative methods based on non-invasive readouts have been proposed, such as x-ray measurements monitoring the passage of radio-opaque tracers [4] or a modified breath test [5, 6]. In the latter case, the amount of an isotope labeled metabolite (CO₂) of an orally administered substrate is determined in the exhaled air. The measure, however, is affected by a cascade of physiological and metabolic processes, gastric passage being only one of them and, hence, constitutes an indirect measure of gastric emptying. Not surprisingly, the use of these two techniques in small animal studies has been very limited.

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Furthermore, classical approaches to determine gastric emptying in laboratory rodents are invasive techniques that might require chronic surgery and in most of the cases lead to the death of the animal at the end of the experiment. This has several major implications: an animal provides only one gastric emptying value for a fixed time point. Hence, time-course or comparative studies have to be performed in different animals, which increase the degree of variability associated to inter-individual differences. Studies using classical techniques are limited to transversal comparisons of single measurements and, therefore, the number of animals needed is relatively high.

Thus, there is a need in the art for *in vivo* optical imaging probes to assess gastric emptying and/or gastro-intestinal passage that overcome the drawbacks of the classical approaches. Especially, there is a need to provide a non-invasive method that is sensitive and allows repetitive measurements on the same animal.

It has been found that gastric emptying and/or gastro-intestinal passage can be assessed using non-invasive near-infrared fluorescence (NIRF) technique with results in excellent accordance with data derived from classical approaches. The pattern of gastric emptying and/or gastro-intestinal passage can be monitored over time; the same animal can be used in several experiments and for comparative studies, in which gastric emptying and/or gastro-intestinal passage can be measured before and after treatment. In addition, the animals are not subjected to any surgery, which might compromise the validity of the results.

The method of the invention displays good reproducibility and sensitivity allowing the detection of both accelerated and delayed gastric emptying pattern and thus offers the potential to characterize molecular targets and pathways involved in physiological regulation and pharmacological modulation of gastric emptying.

DETAILED DESCRIPTION OF THE INVENTION

The instant application thus provides an optical imaging probe for use in a method for assessing the rate of gastric emptying and/or gastro-intestinal passage in an animal, said probe consisting of a non-nutrient semi-solid meal comprising a fluorochrome, and, optionally, other pharmaceutically acceptable vehicles or excipients.

As used herein, a "fluorochrome" includes fluorochromes that fluoresce in the near-infrared region (in the range of 600-1100 nm), e.g., after excitation in the far-red range of visible light wavelengths. Specific examples include the cyanine dyes, such as Cy5TM,

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Cy5.5TM and Cy7TM (Amersham Biosciences, Piscataway, NJ), ALEXA FLUOR® 680, ALEXA FLUOR® 700, ALEXA FLUOR® 750 (Molecular Probes, Eugene), IRDye38TM, IRDye80TM, IRDye80TM (LiCor, Lincoln, NE), NIR-1 and IC5-OSu (Dojindo, Kumamoto, Japan); FAR-Blue, FAR-Green Onem and FAR-Green Two (Innosense, Giacomini, Italy), ADS 790-NS and ADS 821-NS (American Dye Source, Montreal, Canada), Atto680 (Atto-Tec, Siegen, Germany), DY-680, DY-700, DY-730, DY-750, DY-782 (Dyomics, Jena, Germany), EVOBlue (Evotec, Hamburg, Germany) and indocyanine green and its analogs and derivatives (Akorn et al.) and LaJolla BlueTM (Diatron). One of skill in the art would appreciate that a large number of fluorochromes with different chemical and optical properties can be used to produce the probe of the invention. In one specific embodiment, said fluorochrome is selected among the group consisting of the following: Cy5.5, Cy7, Cy 7.5, Alexa Fluor 680, Alexa Fluor 750.

In one embodiment, said non-nutrient semi-solid meal comprises or consists of said fluorochrome chemically linked to beads.

As used herein, the term "chemically linked" means "connected by any attractive force between atoms to allow the combined aggregate to function as a unit". This includes, but not limited to, chemical bonds such as covalent bonds (e.g., polar or non-polar), non covalent bonds such as ionic bonds, metallic bonds and bridge bonds, and hydrophobic and van-der-Waals interactions.

The fluorochromes are chemically bound to non-nutrient particles appropriate for gastric environment. Preferably, the particles should be stable at low pH and should not stick to the gastric surface. The size of the particles should allow normal pyloric evacuation during gastric emptying. For example, in mice, particles of less than 1 mm can pass throughout the pylorus during normal emptying process. In a further embodiment, the beads have a particle size ranging from 100-500 μm , preferably between 150-250 μm .

Particles with reactive amino alkyl groups are preferably used for the synthesis of fluorescent conjugates. For example, appropriate particles can be selected among the group consisting of polystyrene-polyethylene glycol beads such as beads made of copolymers of cross linked polystyrene matrix on which polyethylene glycol is grafted (for example, TentagelTM Beads), controlled pore glass and polystyrene beads. In order to mimic the texture of a semisolid meal, the particles or beads can be suspended in a viscous solution, such as 0.5% methylcellulose.

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According to a preferred embodiment, said probe consists of cyanine dyes, such as Cy5.5, covalently bound to polystyrene-polyethylene glycol beads such as TentaGel™ Beads.

The particles are reacted with fluorochrome in 0.1-100% molar ratios (calculated on particles surface free reactive groups). The molar ratios will be preferably determined in order to provide optimal fluorescence activity. According to a preferred embodiment, beads with reactive amino groups, for example polystyrene-polyethylene beads such as TentaGel™ Beads, are loaded with between 0.8 and 1.2% fluorochromes such as cyanine dyes, for example Cy5.5, preferably around 1.0%.

In order to generate a protected form suitable for long-term storage, it is preferable to cap the remaining free reactive groups of the particles after loading with fluorochromes. As an example, polystyrene-polyethylene glycol beads-cyanine dyes conjugates are treated by capping the remaining amino groups by addition of acetic acid anhydride to generate a protected form. At the end of the reaction, the obtained particles have no remaining free amino groups. Following this procedure, the protected particles show no decomposition for months when stored in the dark.

Another aspect of the invention pertains to the use of an optical imaging probe as defined above, for assessing gastric emptying and/or gastro-intestinal passage in an animal.

In yet another aspect, the invention provides a kit comprising the optical imaging probe as above-defined and instructions for the performance of an assay for assessing gastric emptying and/or gastro-intestinal passage, more specifically, the kit is for use in a method for assessing the rate of gastric emptying and/or gastro-intestinal passage in an animal as defined hereafter.

Another aspect of the invention is directed to a method for assessing the rate of gastric emptying and/or gastro-intestinal passage in an animal, comprising:

- a) providing the optical imaging probe as defined above,
- b) administering said probe by oral gavage to said animal,
- c) determining the fluorescent activity corresponding to the anatomical location of the stomach and/or intestine at different time points by using a near-infrared fluorescence

(NIRF) imaging system, wherein the fluorescent signal intensity measured as a function of time determines the amount of intragastric and/or intrainestinal probe as a function of time; thereby assessing the rate of gastric emptying and/or gastro-intestinal passage in said animal.

Considering the depth of penetration for near-infrared light in tissue being several centimeters, small animals are preferably used in the method. In a preferred embodiment, said animal is a non-human mammal, for example a rodent, and more preferably a small rodent selected among the group of rats, mice, rabbits, guinea pigs and hamsters.

Preferably, prior to the probe administration, the animals are fasted for a few hours, resulting in an almost complete emptying of the stomach. The amount of probe administered to the animal by oral gavage will depend of different parameters, including the species, the weight of the animal and the composition of the probe. In a specific embodiment, in mice, between 200 mg kg⁻¹ to 500 mg kg⁻¹ of fluorescent probes can be administered.

For carrying out the method of the invention, any convenient near-infrared fluorescence (NIRF) imaging system can be used according to the invention. Preferably, non-invasive imaging system is used. In a specific embodiment, a laser beam generated by a laser diode or a pulsed laser is used, and the fluorescence light emitted from the animal is detected by an appropriate detector, e.g. a charge-coupled device (CCD) camera or a photo-multiplier tube. The obtained NIRF Images are obtained at different time points and quantitatively analyzed on a region-of-interest basis. More specifically, the spatially integrated fluorescent activity corresponding to the anatomical location of the stomach (Region Of Interest, ROI) is determined for the different experimental points. In a preferred embodiment, the ROI is defined from the fluorescent area recorded immediately after the oral gavage of the fluorescent probes. The signals are then treated using software available in the Art to allow quantitative analysis. Especially, images can be corrected for heterogeneities of the laser diode excitation profile by dividing the measured fluorescence signal by a reference signal recorded at an excitation wavelength for each pixel, for example according to the following formula:

$$FRI(t) = S_{720\text{ nm}}^{corr}(x, y; t) = \frac{S_{720\text{ nm}}(x, y; t)}{S_{660\text{ nm}}(x, y; t)}, \quad [1]$$

where $FRI(t) = S_{720nm}^{corr}(x, y; t)$ represents the corrected fluorescence signal at 720 nm for pixel (x,y) at time t, $S_{720nm}(x, y; t)$ the measured fluorescence signal at 720 nm and $S_{660nm}(x, y; t)$ the reference signal at the excitation wavelength of 660 nm.

The gastric emptying and/or gastro-intestinal passage can then be determined as a function of time t GE(t) from the changes in total fluorescent activity in the ROI, for example, in % according to the following formula:

$$GE_{\%}(t) = 100 \times \left(1 - \frac{FRI(t)}{FRI(0)} \right) \quad [2]$$

where $FRI(t)$ and $FRI(0)$ represent the corrected fluorescence intensities (Eq. [1]) measured at times t and zero, respectively. In equation [2], we assumed that the FRI activity is a direct measure for the amount of beads in the ROI.

The method of the invention offers the potential either to characterize molecular targets and pathways involved in physiological and pharmacological modulation of gastric emptying, or to identify a compound that modulates gastrointestinal motility in an animal.

Thus, the invention also relates to a method of identifying a compound that modulates gastrointestinal motility in an animal, said method comprising:

- a) administering a test compound to a test animal;
 - b) assessing the rate of gastric emptying and/or gastro-intestinal passage in said test animal by the method of the invention as defined above; and,
 - c) comparing the rate of gastric emptying between the test animal and a control animal to which no candidate compound has been administered to;
- wherein a significant difference in gastric emptying between the control and test animals is indicative that said test compound is a compound capable of modulating gastrointestinal motility in said animal.

As used herein, the term "a compound that modulates gastrointestinal motility" refers to any compound that alters gastric or gastro-intestinal motility either by decreasing (e.g. clonidine) or by increasing (e.g. cisapride) gastric or gastro-intestinal motility.

To identify a test compound that is capable of modulating gastrointestinal motility in the method above, one would measure or determine the rate of gastric emptying and/or gastrointestinal passage in the absence of the administration of the candidate substance (the control animal). In a specific embodiment, the control animal can be the same animal as the test animal, the rate of gastric emptying being first assessed before the administration of the compound to the animal and then assessed after or with the administration of the compound. A test compound, which increases the rate of gastric emptying and/or gastro-intestinal passage relative to that observed in its absence, is indicative of a candidate substance being a prokinetic agent with ability to stimulate or increase the rate of gastric emptying and/or gastro-intestinal passage. Conversely, the test compound may be identified as one which slows the rate of gastric emptying. Such compounds may be useful in the treatment or amelioration of disorders which manifest diarrhea or too rapid a rate of gastric emptying.

The test compound may be screened among peptides, polypeptides, proteins, antibodies or nucleic acids, or any other synthetic or natural products. Compounds selected among those being structurally related to other known modulators of gastric emptying are preferred. More specifically, the compounds may be screened among those that are structurally related to existing prokinetic agents.

Other features and advantages of the invention will become apparent from the following examples.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. TentaGel™ beads loaded with Cy5.5 (1.0%), suspended in methylcellulose 0.5% aqueous solution (400 mg kg^{-1} , 0.1 ml), were administered orally at time $t=0$ and FRI images were recorded at time points $t=0$, 30, 60, 120, and 180 minutes after administration of the probe. At $t = 180 \text{ min}$, two spatial distinct areas of FRI signals could be detected: stomach (lower area) and small intestine (upper area). The fluorescence intensity in the ROI (in percent % of the initial signal) declined with time (A) reflecting increasing gastric emptying $\text{GE}_{\%}(t)$ (B).

Figure 2. Reproducibility of gastric emptying pattern measured by NIRF imaging in mice. To determine the reproducibility of the NIRF measurements, the rate of gastric emptying was determined in three consecutive experiments in the same group of animals. For each time point, the integrated fluorescence intensity covering the anatomical location of the stomach was used to calculate gastric emptying over a 3-hour period. Each data point represents the mean $\pm$ s.e.m. of 8 – 10 animals.

Figure 3. Effects of cisapride, tegaserod and clonidine on gastric emptying in mice. Accelerated gastric emptying was observed for the 5HT_4 agonist tegaserod (A, B) while the α_2 inhibitor clonidine has clearly inhibitory effects (C). Each data point represents the mean $\pm$ s.e.m. of 4 – 13 animals.

EXAMPLES

METHODS***Animals***

Adult male Balb/C mice (20 - 25 g; Charles River, France) were used. Animals were housed in groups with up to six animals per cage under standard conditions and with food and water *ad libitum*. All procedures were in accordance with the Swiss animal welfare laws and approved under the animal research license BS 1827.

Synthesis and stability of fluorescence conjugates

Particles with reactive amino alkyl surface groups were used for the synthesis of the fluorescent conjugates. Controlled pore glass, TentaGel™ beads or polystyrene beads were reacted with the amino reactive Cy5.5 succinimidyl ester in 0.1-100 % molar ratios (calculated on particles surface free amino groups) for 2 h in N,N-dimethylformamide. Labeled beads were obtained as blue (controlled pore glass) or blue-green to black solids (TentaGel™, polystyrene beads) after several washings. Relative fluorescence spectra of labeled beads were recorded on a SPEX Fluorolog spectrometer equipped with a cooled R928 detector (1 mm slit) (Jobin Yvon Inc., Edison, NJ, USA).

The blue-green color of the TentaGel™ beads (1% loading with Cy5.5) changed to yellow-green after several days, indicating a partial decomposition of the chromophor on the beads. For that reason, a protected form of the chromophor (protected conjugate) was prepared. TentaGel™ beads were reacted with the amino reactive Cy5.5 succinimidyl ester in a 1% molar ratio, as described above. At the end of the reaction, the remaining free amino groups were capped by addition of five equivalents of acetic acid anhydride. The absence of free amino groups after this treatment has been proved using the trinitrobenzenesulfonic acid test [3]. Following these procedures, protected conjugate (protected TentaGel™ beads loaded with 1% Cy5.5) was obtained as stable blue solid.

Stability of the protected conjugate at low pH was assessed by determining time-related changes in the fluorescent spectra of a sample maintained at pH=2.0 for up to 23 h. The protected conjugate was suspended in 0.01 N HCl (pH ~ 2.0) and the relative fluorescence spectra at 15 min and 3, 6 and 23 h were recorded on a SPEX Fluorolog spectrometer, as described above.

Drugs and chemicals

Controlled pore glass (Aminopropyl-CPG-1400 Å, particle size 120-200 mesh, capacity 0.04 mmol/g; Fluka Chemie AG, Buchs, Switzerland), TentaGel™ beads (TentaGel MB-NH₂, particle size 200 - 250 µm, capacity 0.2 - 0.3 mmol/g; Rapp Polymere GmbH, Tübingen, Germany), polystyrene beads (Polystyrene AM-NH₂, particle size 160 - 200 µm, capacity 0.8 - 1.2 mmol g⁻¹, Rapp Polymere GmbH) and the fluorescent dye Cy5.5 succinimidyl ester (Amersham Biosciences) were used for the synthesis of fluorescent conjugates. Tegaserod [5-methoxy-indol-3-carboxaldehyd-amino-(pentyl-amino) methylenhydrazon-hydrogenmaleinat; Novartis Pharma AG, Basel, Switzerland] was dissolved in 10% (v:v) 1-methyl-2-pyrrolidon in normal saline. Cisapride [cis-4-amino-5-chloro-N-(1-(3-(p-fluorophenoxy)propyl)-3-methoxy-4-piperidyl)-o-anisamide; Novartis Pharma AG] was dissolved in 1% (v:v) lactic acid in distilled water. Clonidine hydrochloride [2-(2,6-dichloroanilino)-2-imidazoline hydrochloride; Novartis Pharma AG] was dissolved in physiological saline solution. All solutions were freshly prepared before each experiment.

Experimental protocols for in vivo determination of gastric emptying.

Prior to the experiments, animals were fasted for 4 to 5 hours, with water available *ad libitum* resulting in an almost complete emptying of the stomach in mice [1]. To avoid circadian variations, fasting was always started between 8:00 and 9:00 am and all measurements were carried out between 12:30 and 4:30 pm.

The fluorescent probe was administered by oral gavages (400 mg kg⁻¹ of protected conjugate suspended in 0.5% methylcellulose in water, 0.1 ml per animal). NIRF measurements were performed immediately after the administration of the probe (time 0) and at different time-points thereafter. For NIRF imaging, the animals were temporarily anaesthetized with isoflurane (1.5% vapor concentration in nitrous oxide:oxygen 2:1; Forene®, Abbott, Baar, Switzerland). Anesthesia was induced in a chamber and maintained during the imaging time with a facemask. For gastric emptying measurements, animals were placed in right lateral position exposing the left side (where the stomach is localized) to the NIR excitation/detection system. The spatially integrated fluorescent activity corresponding to the anatomical location of the stomach (region of interest = ROI) was determined at 0, 30, 60, 120 and 180 min after the administration of the fluorescent probe; in some cases an additional imaging data set was collected at 90 min. At each time point, the imaging procedure lasted about 5 minutes; in-between measurements, animals were returned to their

home cage and were left undisturbed until the next measurement time point. In order to ensure the same positioning at each time point of gastric emptying measurements, *i.e.* to minimize co-registration errors, the anaesthetized mice were placed in a positioning device. The positioning device consisted of wax and its shape was adapted to the individual contour of each animal prior to an experiment. In addition, the ROI was marked with black dots on the skin of the animals for the reproducible illumination with the laser beam (Fig. 1). The ROI was defined from the fluorescent area recorded immediately after the oral gavage of the labeled beads (time 0).

For each animal a maximum five experiments were conducted with the treatment regimen varied in a randomized manner. Between two consecutive experiments, animals were allowed to recover for at least three days.

Validation and pharmacological studies:

In a first set of experiments the reproducibility of the technique and the normal gastric emptying pattern was determined by carrying out three identical experiments in the same non-treated animals (N = 8 – 10).

In a second set of experiments the pharmacological modulation of gastric emptying was studied following oral administration of the 5-HT₄ receptor agonists tegaserod (0.03 mg kg⁻¹ or 0.1 mg kg⁻¹, N = 7 – 8) and cisapride (0.3 mg kg⁻¹ or 1.0 mg kg⁻¹, N = 8) or following intraperitoneal administration of the (2 receptor agonist clonidine (0.03 mg kg⁻¹ or 0.1 mg kg⁻¹, N = 4 – 5). Test compounds or the appropriate vehicles (N = 6 – 13) were administered 15 min before the administration of the fluorescent probe (0.1 ml).

Determination of gastric emptying and statistical analysis

For each individual animal, the gastric emptying in %, GE%(t), was determined as a function of time t from the changes in total fluorescent activity in the respective ROI, according to

$$GE_{\%}(t) = 100 \times \left(1 - \frac{FRI(t)}{FRI(0)} \right) \quad [2]$$

where $FRI(t)$ and $FRI(0)$ represent the corrected fluorescence intensities (Eq. [1]) measured at times t and zero, respectively. In equation [2], we assumed that the FRI activity is a direct measure for the amount of beads in the ROI.

For each individual animal, the gastric half-emptying time, $T_{1/2}$, defined by $GE_{\%}(T_{1/2}) = 0.5 \cdot GE_{\%}(0)$ was determined by non-linear regression analysis (sigmoidal Boltzmann fit) followed by interpolation / extrapolation. In 8 out of 105 experiments, an extrapolation of $T_{1/2}$ values could not be performed due to the low incremental increase in gastric emptying with time. These experiments (5 with vehicle, 2 with cisapride and 1 with clonidine) were excluded from data analysis.

All results are expressed as mean $\pm$ s.e.m. (N = number of animals). Differences between treatment groups were assessed using analysis of variance (ANOVA) followed, if necessary, by a multiple comparison test (Dunnett's or Student-Newman-Keuls method, as appropriate). P values < 0.05 were considered statistically significant.

NIRF imaging system

For NIRF imaging, a custom tailored spectroscopic imaging system has been used (multi-wavelength near-infrared imaging system) [2]. The laser beam generated by a laser diode emitting at 660 nm with a power of 35 mW (Sharp Corporation, Osaka, Japan) was dispersed by a lens system to a circular diameter of 4 cm at an object distance of 40 cm. The fluorescent light emitted from the sample (mouse) was detected by a charge-coupled device (CCD) camera (Hamamatsu Photonics K. K., Hamamatsu City, Japan) equipped with a focusing lens system (macro lens 60 mm, 1:2.8; Nikon, Tokyo, Japan). The CCD features low noise and low dark signal enabling both the detection of low intensity light as well as the use of long integration times. The matrix size of the images is 532 $\times$ 256 pixels. A liquid crystal tunable filter (LCTF, Cambridge Research & Instrumentation Inc., Woburn, Massachusetts, USA) was used for selection of the detection wavelength (720 nm). Data acquisition (i.e. integration) times ranged from 3 to 16 s depending on the intensity of the fluorescence signal. The data acquisition process was controlled by a PC using a LabView-based imaging software (B. Schattka, National Research Council, Winnipeg, Canada). An anatomical reference image was obtained at the excitation wavelength (660 nm) with diffuse illumination of the whole animal.

Image analysis

Images were analyzed using the BioMap 2.4 software (M. Rausch, Novartis Institutes for BioMedical Research, Basel). The camera-offset signal was removed by automatic baseline correction prior to quantitative analysis. For this purpose, the value of the lowest peak in the intensity histogram of the image was subtracted from all image pixels. Thereafter, images were corrected for inhomogeneities of the laser diode excitation profile by dividing the fluorescence signal by a reference signal recorded at the excitation wavelength for each pixel, according to

$$FRI(t) = S_{720\text{ nm}}^{\text{corr}}(x, y; t) = \frac{S_{720\text{ nm}}(x, y; t)}{S_{660\text{ nm}}(x, y; t)}, \quad [1]$$

where $FRI(t) = S_{720\text{ nm}}^{\text{corr}}(x, y; t)$ represents the corrected fluorescence signal at 720 nm for pixel (x,y) at time t, $S_{720\text{ nm}}(x, y; t)$ the measured fluorescence signal at 720 nm and $S_{660\text{ nm}}(x, y; t)$ the reference signal at the excitation wavelength of 660 nm. For illustration purposes, the FRI images were superimposed to the corresponding anatomical image. The FRI images were quantitatively analyzed on a region of interest (ROI) basis.

RESULTS

Preparation and characterization of the fluorescent sensor

TentaGel™ beads, polystyrene beads and controlled pore glass beads conjugated with the cyanine dye Cy5.5 as fluorochrome were stable and showed maximal fluorescence emission at 710 - 720 nm, except for controlled pore glass beads that showed more variable emission spectra (data not shown). A fluorochrome loading of 1% was optimal for the fluorescence properties of the different conjugates, fluorescence intensities decreased with higher Cy5.5 loadings. Because of their physico-chemical properties and easy handling, TentaGel™ beads with a 1.0% Cy5.5 loading were selected for further characterization. The blue-green color of the TentaGel™ beads (1% loading) changed to yellow-green after several days, indicating a partial decomposition of the fluorochrome. For that reason, protected TentaGel™ beads loaded with 1.0% Cy5.5 (the protected conjugate) were prepared. The protected conjugate was obtained as blue solid, which shows no decomposition (as monitored by color change) for months when stored in the dark. The emission spectrum of the protected conjugate was virtually identical to that of the beads-dye conjugate before the capping of the free amino groups, with a maximal emission at 720 nm.

Moreover, the protected conjugate was stable at low pH (~2.0) for up to 23 h, as demonstrated by the lack of changes in the emission spectrum (data not shown).

Visualization of gastric emptying in vivo by non-invasive near-infrared fluorescence reflectance imaging

Gastric emptying was monitored as reduction of the integrated corrected fluorescence intensity $FRI(t)$ in the ROI corresponding to the anatomical location of the stomach over time. A series of representative images have been obtained from an animal administered with TentaGel™ beads-dye conjugate in 0.5% methylcellulose (400 mg kg^{-1} , 0.1 ml, p.o.) at time 0 (Data not shown). $FRI(t)$ values decreased as a function of time reflecting the progressive reduction of the number of labeled beads in the stomach and thus gastric emptying (Fig. 1 A-B).

Repetitive imaging in the same animal revealed good quantitative reproducibility of gastric emptying measurements. In three consecutive experiments the mean $T_{1/2}$ values amounted to $115 \pm 18 \text{ min}$, $107 \pm 15 \text{ min}$ and $124 \pm 13 \text{ min}$ (mean $\pm$ s.e.m., $N = 8 - 10$ animals), respectively. At the end of the 3-hour experimental period, $FRI_{(180\text{min})}$ amounted to $42.2 \% \pm 5.6 \%$, $37.0 \% \pm 7.2 \%$ and $42.0 \% \pm 8.0 \%$ of the initial value corresponding to the respective gastric emptying rates of $GE_{\%(180\text{min})} = 57.8 \%$, 63.0% and 58.0% (Fig. 2). The coefficients of variation for intra-individual differences, *i.e.* the day-to-day variability amounted to 0.32 ± 0.05 and 0.30 ± 0.05 for $T_{1/2}$ and $GE_{\%(180\text{min})}$, respectively (mean $\pm$ s.e.m., $n = 3$ experiments in $N = 8 - 10$ animals). The coefficients of variation for inter-individual differences, *i.e.* the variability between the mice within one experiment, amounted to 0.39 ± 0.10 and 0.34 ± 0.04 for $T_{1/2}$ and $GE_{\%(180\text{min})}$, respectively (mean $\pm$ s.e.m., $N = 8 - 10$ animals in $n = 3$ experiments).

The 5-HT₄ agonists tegaserod (0.1 mg kg^{-1}) and cisapride (0.3 mg kg^{-1} or 1 mg kg^{-1}) significantly accelerated gastric emptying as reflected by the shortened gastric half-emptying times ($T_{1/2} = 44 \text{ min} - 69 \text{ min}$) and the increased gastric emptying rates ($GE_{\%(180\text{min})} = 70 \% - 83 \%$) (Fig. 3A, Table 1). The α_2 adrenergic agonist clonidine (0.1 mg kg^{-1}) significantly prolonged (doubled) gastric half-emptying times and reduced (halved) gastric emptying rates (Fig. 3B, Table 1).

Table 1 Effects of cisapride, tegaserod and clonidine on gastric emptying rate and gastric half-emptying time ($T_{1/2}$). (Data represent the mean $\pm$ s.e.m., N = number of animals, *: $P < 0.05$ vs. respective vehicle group)

<i>Treatment</i>	<i>Dose (mg kg⁻¹)</i>	<i>N</i>	<i>Gastric Emptying (% in 180 min)</i>	<i>T_{1/2} (min)</i>
Vehicle		13	52.3 $\pm$ 5.5	113 $\pm$ 13
Cisapride	0.3	8	70.0 $\pm$ 5.1 *	69 $\pm$ 16 *
	1.0	8	82.8 $\pm$ 4.3 *	57 $\pm$ 8 *
Vehicle		11	51.9 $\pm$ 3.7	106 $\pm$ 14
Tegaserod	0.03	7	56.8 $\pm$ 5.9	87 $\pm$ 20
	0.1	8	73.8 $\pm$ 7.7 *	44 $\pm$ 6 *
Vehicle		6	69.9 $\pm$ 2.4	67 $\pm$ 11
Clonidine	0.03	5	55.4 $\pm$ 6.3	102 $\pm$ 27
	0.1	4	28.8 $\pm$ 10.4 *	222 $\pm$ 20 *

DISCUSSION

It is shown here that NIRF imaging in combination with fluorescently labeled particles can be used to visualize gastrointestinal functions such as gastric emptying *in vivo*. One fluorochrome used in the examples, Cy5.5, belongs to the cyanine-type dyes extensively used in biomedicine and has been previously used as an enzyme-responsive reporter to measure cathepsin B enzyme activity *in vivo*. Conjugation with different carriers did not alter the fluorescent characteristics of the dye. TentaGel™ beads-, controlled pore glass- or polystyrene beads-dye conjugates were stable and showed similar fluorescent activity when excited. Loading with different concentrations of Cy5.5 dye showed a maximal fluorescent activity with a 1.0% loading. The further increase in loading up to 10% resulted in a reduced activity, most probably due to internal quenching. The observed tendency of the chromophore to decompose with time was prevented by capping the free amino groups at the end of the dye loading reaction. The protected conjugate was very stable, even in acidic solutions mimicking the intragastric environment. No changes in the emission spectrum were observed when the protected conjugate was maintained at a pH ~2.0 up to 23 h. The time-dependent reduction of integrated fluorescence intensity in the mouse stomach can therefore be attributed to transport of beads out of the stomach rather than label decomposition and lost of fluorescence activity. Hence, temporal changes of the integrated fluorescence intensity are a measure of gastric emptying.

The suitability of the technique to monitor gastric emptying *in vivo* is demonstrated by three facts: i) the signal derived from the fluorescent marker can be detected and analyzed easily; ii) measurements are reproducible among different experiments carried out under similar conditions, giving a consistent pattern of gastric emptying; and iii) the technique has high enough sensitivity as to detect both the stimulation and the inhibition of the normal emptying pattern.

The sensitivity of the technique for studying pharmacological effects was evaluated using the gastroprokinetic 5-HT₄ agonists cisapride and tegaserod as well as the inhibiting α_2 adrenergic agonist clonidine. The results demonstrated that the NIRF technique is able to detect the dose-related stimulatory and inhibitory drug effects on gastric emptying and, hence, constitutes an attractive tool for drug discovery and development.

Limitations of NIRF imaging are caused by the light absorption and scattering of tissue compromising accurate spatial localization of the fluorescent source and quantification of the fluorescence activity. The intensity values measured depend on both the intensity of the fluorescence source (the number of labeled beads) and its depth of with regard to the

surface of the animal. Increasing the distance that the light photons have to propagate through tissue will attenuate and disperse the signals detected. Nevertheless, semi-quantitative image analysis is only feasible assuming reproducible tissue geometry, *i.e.* the region of interest has to be located at the same position with respect to the animal's surface throughout the imaging study. However, in lack of external references, the very exact repositioning of the animals is difficult and co-registration errors might affect the quantitative measures of gastric emptying by inclusion of anatomical regions adjacent to the stomach.

Nevertheless, these limitations are suggested of minor importance since the present findings are in accordance with data derived from other approaches. In basal conditions, we observed gastric half-emptying times ($T_{1/2}$) of a semi-solid meal of 70 min – 120 min, which are in line with the $T_{1/2}$ values of 20 min and 150 min of respective liquid and solid meals measured with an adapted breath test in mice. The breath test is based in the production of labeled CO_2 after the ingestion of a labeled substrate (usually with either ^{13}C or a radioactive isotope). In addition, the gastric emptying rate of a solid nutrient meal in mice has been reported to be 30 % to 60 % at 2 hours following the meal as compared to 40 % to 70 % determined at 3 hours following a non-nutrient semi-solid meal in our study. It should however be mentioned that using classical approaches in non-anaesthetized mice, gastric half-emptying times and gastric emptying rates of non-nutrient semi-solid meals have also been reported to be respective 30 minutes and 40 % – 60 % within 15 to 30 minutes. The discrepancy to the present data might be due to the repeated anesthesia used in our study. High doses of isoflurane have been shown to cause a prolongation of gastric half-emptying times in man and animals.

The pharmacological validation of the technique provided evidence that the 5-HT₄ receptor agonist, tegaserod (Zelnorm®), has gastroprokinetic properties. Doses of 0.03 and 0.1 mg kg⁻¹ stimulated gastric emptying, consistent with preliminary evidences in dogs, rats and mice. Maximum efficacy was observed at a dose of 0.1 mg kg⁻¹ and was comparable to that of cisapride at a dose of 1.0 mg kg⁻¹, suggesting a higher potency of tegaserod as compared to cisapride. This observation suggests that tegaserod has therapeutic potential for the treatment of pathophysiological states with impaired gastric emptying, such as diabetic gastroparesis or post-operative gastric ileus, or states in which gastroprokinetic drugs might contribute to a general symptom improvement, such as functional dyspepsia.

In summary, these results demonstrate that *in vivo* NIRF imaging in combination with the fluorescent probes of the invention is an appropriate technique for monitoring gastric emptying.

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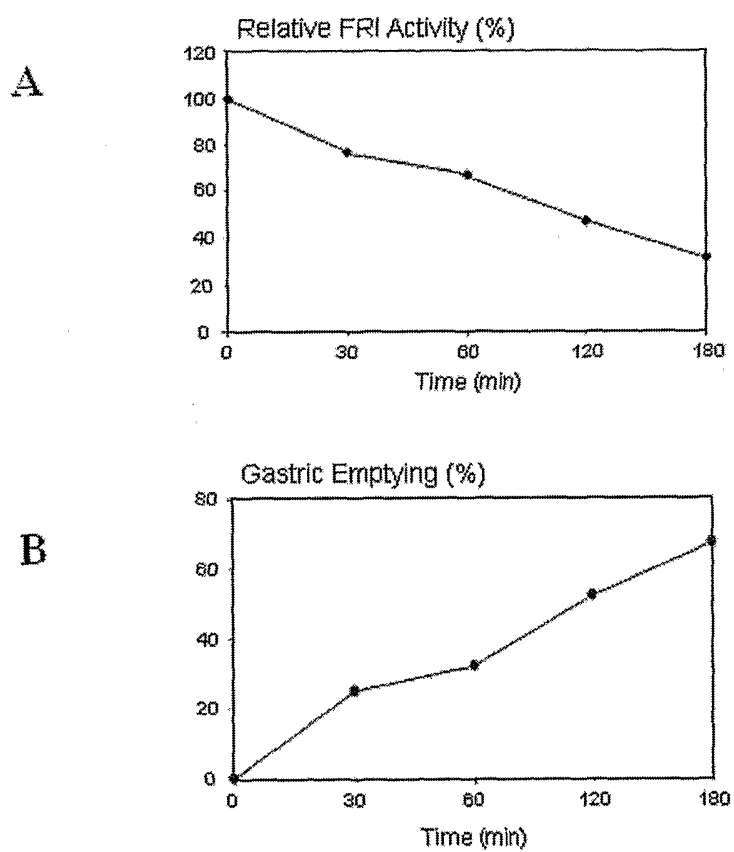
CLAIMS

1. An optical imaging probe for use in a method for assessing the rate of gastric emptying and/or gastro-intestinal passage in an animal, said probe consisting of a non-nutrient semi-solid meal comprising a fluorochrome and, optionally, a pharmaceutically acceptable vehicle.
2. The probe according to Claim 1, wherein said non-nutrient semi-solid meal comprises said fluorochrome chemically linked to beads
3. The probe according to Claim 2, wherein said particles have a size ranging from 100-500 μm , preferably between 150-250 μm .
4. *The probe according to any of Claims 2-3, wherein said particles are selected among the group consisting of polystyrene-polyethylene glycol beads, controlled pore glass and polystyrene beads.*
5. The probe according to any of Claims 2-4, wherein said beads are suspended in a viscous suspension, such as 0.5% methylcellulose solution.
6. The probe according to any of Claims of 1-5, wherein said fluorochrome has absorption and emission maximum between 600 and 1100 nm
7. The probe according to any of Claims of 1-6, wherein said fluorochrome is selected among the group consisting of the following: Cy5.5, Cy7, Cy 7.5, Alexa Fluor 680, Alexa Fluor 750.
8. The probe according to any of Claims 1-7, wherein said probe consists of cyanine dyes, such as Cy5.5, covalently bound to polystyrene-polyethylene glycol beads.
9. The probe according to Claim 8, wherein said cyanine dyes-bead conjugates is treated by capping the remaining amino groups by addition of acetic acid anhydride to generate a protected form.
10. The probe according to Claim 8 or 9, wherein the beads are loaded with between 0.8 and 1.2% cyanine dyes, preferably around 1.0% cyanine dyes.
11. A method for assessing the rate of gastric emptying and/or gastro-intestinal passage in an animal, comprising:
 - a. providing the optical imaging probe according to any of Claims 1-10,
 - b. administering said probe by oral gavage to said animal,

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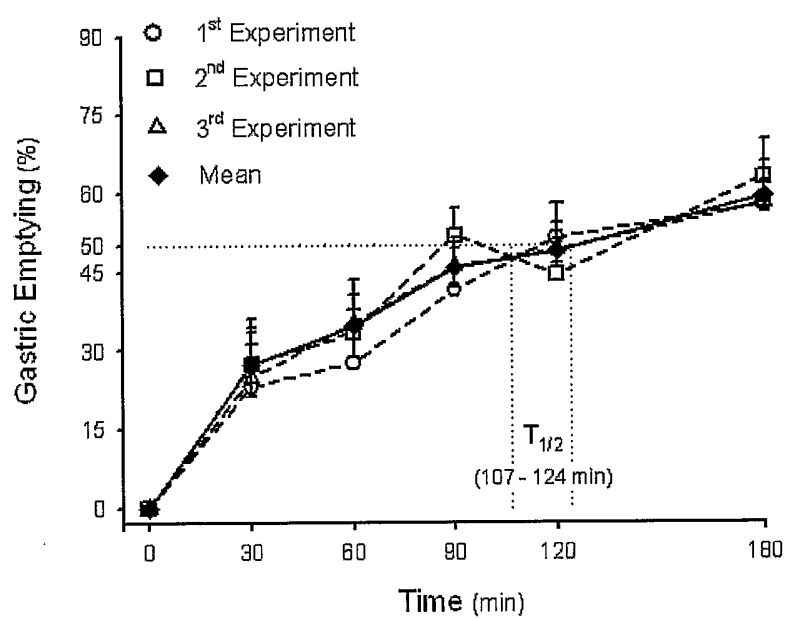
- c. determining the fluorescent activity corresponding to the anatomical location of the stomach and/or the intestine at different time points by using a near-infrared fluorescence imaging system, wherein the fluorescent signal intensity measured as a function of time determines the amount of intragastric and/or intrainstestine probe as a function of time; thereby assessing the rate of gastric emptying and/or gastrointestinal passage in said animal.
12. The method according to Claim 11, wherein said animal is a non-human.
13. The method according to Claim 11 or 12, wherein said animal is a non-human mammal, for example a rodent, and more preferably a small rodent selected among the group of rats, mice, rabbits, guinea pigs and hamsters.
14. A method of identifying a compound that modulates gastrointestinal motility in an animal, said method comprising:
- a. administering a candidate compound to a test animal;
 - b. assessing the rate of gastric emptying in said test animal by the method according to any of Claims 11-13; and,
 - c. comparing the rate of gastric emptying between the test animal and a control animal to which no candidate compound has been administered to wherein a significant difference in gastric emptying between the control and test animals is indicative that said candidate compound is a compound capable of modulating gastrointestinal motility in said animal.
15. A use of a probe according any of Claims 1-10, for assessing gastric emptying and/or gastro-intestinal passage in an animal.
16. The use according to Claim 15, wherein said animal is non-human mammal, for example a rodent, and more preferably a small rodent selected among the group of rats, mice, rabbits, guinea pigs and hamsters.
17. A kit comprising the probe according to any of Claims 1-10 and instructions for the performance of an assay for determining gastric and/or gastro-intestinal motility.

Fig. 1



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Fig. 2



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Fig. 3

