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TÍTULO SOLICITADO:

"UN NANOCOMPLEJO MICELAR"

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RESEARCH

APODERADO:

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TRÁMITE:

REMISION DE INFORMACION

JUAN PABLO REYES VILLAMIZAR, mayor de edad, identificado como aparece al pie de mi firma, abogado en ejercicio, titular de la tarjeta profesional número 92.189 del Consejo Superior de la Judicatura, obrando en calidad de apoderado de la sociedad **LABORATORIOS LEGRAND S.A.**, muy respetuosamente me permito formular la siguiente

I. PETICIÓN

Sírvase negar el beneficio patentario a la solicitud contenida en el expediente NC2016-0004921, por no cumplir con lo dispuesto en la Decisión 486 de 2000, con base en los fundamentos que se expondrán a continuación.

II. COMPLEMENTO DE INFORMACION

La solicitud NC2016-0004921, referida a nanocomplejos micelares con un agente encapsulado y a un método para formar los mismos, no cumple los requisitos materiales para otorgársele el privilegio de patente de invención, tal como puede determinarse al valorar los siguientes argumentos:

3.1. El capítulo reivindicatorio carece de claridad y concisión debido a que no caracteriza debidamente el nanocomplejo —pues no presenta los compuestos de la micela, ni define el agente encapsulado— ni su método de obtención —pues no presenta las condiciones técnicas del mismo—. En el presente capítulo reivindicatorio se emplean numerosas reivindicaciones para definir el polímero-flavonoide sin lograrlo de forma precisa y concisa. Adicionalmente, se observan caracterizaciones mediante: resultados a alcanzar, vías de administración, dosificación del agente activo y enfermedades en las que se puede emplear el nanocomplejo; tales caracterizaciones de ningún modo pueden considerarse claras o concisas.

El grupo de 36 reivindicaciones presenta numerosos defectos en cuanto a claridad y concisión se refiere, pues no define de forma inequívoca el nanocomplejo de interés, ni el agente encapsulado y/o los componentes de dicha micela.

La reivindicación principal se refiere a un nanocomplejo sin definir inequívocamente sus componentes —la micela y el agente encapsualdo—; en el caso del conjugado polímero-, no se definen claramente cuáles son el polímero hidrofílico y el flavonoide de interés, de manera que la reivindicación principal no define con claridad y concisión el nanocomplejo de interés:

« 1. Un nanocomplejo micelar que comprende una micela y un agente encapsulado dentro de la micela, dicha micela que comprende un conjugado polímero- flavonoide, en donde el polímero está enlazado al anillo B de dicho flavonoide; donde dicho polímero es un polímero hidrofílico; donde dicho flavonoide es seleccionado a partir del grupo que consta de flavones, isoflavonas, flavanos, proantocianidinas y antocianidinas; donde dichos flavanos son seleccionados a partir del grupo que consta de (-)-epicatequina, (+)-epicatequina, (-)-catequina, (+)-catequina, galato de epicatequina, epigalocatequina, galato de

epigallocatequina, Fisetinidol, Galocatequina, galato de Galocatequina, Mesquitol y Robinetinidol, elagitanino, galotanino, oolongteanino, florotanino, tanino, teacitrina, teadibenzotropolona, teaflavina, teanaftoquinona, tearubiginas, teasinensina y mezclas de los mismos.»

Por su parte, la reivindicación 2 se refiere al enlace del polímero, sin embargo, tal característica no es clara ni concisa:

«2. El nanocomplejo micelar de conformidad con la reivindicación 1, caracterizado porque por lo menos un flavonoide está enlazado al polímero.»

En la reivindicación 7 se menciona que el polímero hidrofílico puede estar incluido en casi cualquier grupo funcional de la química orgánica —como por ejemplo alquilos o alcoholes— lo cual es poco preciso:

«7.El nanocomplejo micelar de conformidad con la reivindicación 1, caracterizado porque el polímero hidrofílico comprende monómeros seleccionados a partir del grupo que consta de acrilamidas, alquilos, oxazolinas, alquenilos, minas, ácidos acrílicos, metacrilatos, dioles, oxiranos, alcoholes, aminas, anhídridos, ésteres, lactonas, carbonates, ácidos carboxílicos, acrilatos, hidroxilos, fosfatos, tereftalato, amidas y éteres.»

Las reivindicaciones 8 y 9 continúan definiendo el polímero hidrofílico de la micela, lo cual demuestra la falta de concisión del capítulo reivindicatorio pues aún en estas reivindicaciones existe una carencia de concisión para reivindicar tal polímero:

«8. El nanocomplejo micelar de conformidad con la reivindicación 1, caracterizado porque el polímero hidrofílico es seleccionado a partir del grupo que consta de poliacrilamida, poli(N-isopropilacrilamida), poli(oxazolina), polietilenimina, ácido poli(acrílico), polimetacrilato, poli(etilen glicol), óxido de poli(etileno), alcohol poli(vinílico), poli(vinilpirrolidinona), poliéteres, poli(alilamina), polianhídridos, poli((3-amino éster), poli(butilen succinato), policaprolactona, policarbonato, polidioxanona, poli(glicerol), ácido poliglicólico, ácido poli(3-hidroxipropiónico), poli(2-hidroxietil metacrilato), poli(N- (2-hidroxipropil)metachlamida), ácido poliláctico, ácido poli(iactic-co-glicólico), poli(orto ésteres), poli(2-oxazolína), ácido poli(sebácico), poli(tereftalato-co-fosfato) y copolímeros de los mismos.»

«9.El nanocomplejo micelar de conformidad con la reivindicación 1, caracterizado porque el polímero hidrofílico es un polisacárido seleccionado a partir del grupo que consta de ácido hialurónico, dextrano, pululano, quitosán, celulosa, amilosa, almidón, gelatina, carragenano, ciclodextrina, dextran sulfato, Ficoll, gelano, goma guar, pectina, polisacarosa, pululano, escleroglucano, xantano, xiloglucano y alginato.»

Las reivindicaciones 11 y 12 tratan de definir el agente activo de interés, sin embargo fallan en hacerlo de una forma clara y concisa:

«11.El complejo micelar de conformidad con cualquiera de las reivindicaciones anteriores, caracterizado porque el agente terapéutico es un agente quimioterapéutico seleccionado a partir del grupo que consta de agentes alquilantes, antraciclinas, disruptores citoesqueléticos, epotilonas, inhibidores de histona deacetilasa, inhibidores de topoisomerasa I, inhibidores de topoisomerasa II, inhibidores de quinasa, anticuerpos monoclonales, conjugados de anticuerpo-fármaco, análogos de nucleótido, análogos de precursor, antibióticos de péptido, agentes en base a platino, retinoides, alcaloides de la vinca, citocinas, anti-metabolitos, y derivados de alcaloides de la vinca, y otros citotóxicos.»

«12.El complejo micelar de conformidad con la reivindicación 11, caracterizado porque el agente quimioterapéutico es seleccionado a partir del grupo que consta de Actinomicina, Afatinib, ácido transretinoico total, Axitinib, Azacitidina, Azatioprina, Bevacizumab, Bleomicina, Bosutinib, Bortezomib, Carboplatino, Capecitabina, Cetuximab, Cisplatino, Clorambucilo, Crizotinib, Ciclofosfamida, Citarabina, Dasatinib, Daunorubicina, Docetaxel, Doxifluridina, Doxorubicina, Epirubicina, Epotilona A (C₂₆H₃₉NO₆S), Epotilona B (C₂₇H₄₁NO₆S), Epotilona C (C₂₆H₃₉NO₅S), Epotilona D (C₂₇H₄₁NO₅S), Epotilona E (C₂₆H₃₉NO₇S), Epotilona F (C₂₇H₄₁NO₇S), Erlotinib, Etopósido, Fluorouracilo, Fostamatinib, Gefitinib, Gemcitabina, Hidroxiurea, Idarubicina, Imatinib, Irinotecan, Lapatinib, Lenvatinib, Mecloretamina, Mercaptopurina, Metotrexato, Mitoxantrona, Nilotinib, Oxaliplatin, Paclitaxel, Panitumumab, Pazopanib, Pegaptanib, Pemetrexed, Ranibizumab, Regorafenib, Ruxolitinib, Sorafenib, Sunitinib, Trastuzumab, Tenipósido, Tioguanina, Tofacitinib, Topotecan, Valrubicina, Vemurafenib, Vinblastina, Vinchistina, Vindesina, Vinorelbina.»

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La reivindicación 13 define el tamaño de la micela con múltiples rangos entre las dimensiones de 30 y 300 nm, lo cual le resta concisión y adicionalmente se estaría refiriendo a resultados a alcanzar:

«13.El nanocomplejo micelar de conformidad con cualquiera de las reivindicaciones anteriores, caracterizado porque el nanocomplejo micelar tiene un tamaño en el rango de 30 hasta 300 nm, 50 hasta 300 nm, 100 hasta 300 nm, 30 hasta 50 nm, 30 hasta 100 nm, 30 hasta 150 nm, 150 hasta 300 nm, 200 hasta 300 nm, 250 hasta 300 nm, 100 hasta 150 nm, 100 hasta 200 nm, 100 hasta 250 nm, 130 hasta 180 nm, o 130 hasta 250 nm.»

Las reivindicaciones 14 y 15 definen la micela por la eficiencia y el porcentaje de carga que puede tener la misma, lo cual se refiere a un resultado a alcanzar y no podría considerarse adecuado para definir la materia objeto de la protección:

«14.El nanocomplejo micelar de conformidad con cualquiera de las reivindicaciones anteriores, caracterizado porque la eficiencia de carga del agente presente dentro de la micela que es mayor a 30%, mayor a 35%, mayor a 40%, mayor a 45%, mayor a 50%, mayor a 55%, mayor a 60%, mayor a 65%, mayor a 70%, mayor a 75%, o 80%.»

«15.El nanocomplejo micelar de conformidad con cualquiera de las reivindicaciones anteriores, caracterizado porque el contenido de carga del agente presente dentro de la micela está en el rango de 1 hasta 10 % p/p, 5 hasta 25 % p/p, 20 hasta 45 % p/p, 30 hasta 50 % p/p, 35 hasta 50 % p/p, 40 hasta 50 % p/p, 45 hasta 50 % p/p, 30 hasta 35 % p/p, 30 hasta 40 % p/p o 30 hasta 45 % p/p.»

La reivindicación 16 se refiere a un método para obtener las micelas y se describen los pasos de agregar el agente en un solvente y permitir el automontaje de la micela, pero falla en definir los parámetros técnicos de los pasos a seguir — que además son ampliamente conocidos—:

«16.Un método para formar un nanocomplejo micelar puede comprender una micela y un agente encapsulado dentro de la micela, el método que comprende las etapas de:

a. agregar el agente en un solvente adecuado a un conjugado polímero-flavonoide, en donde el polímero está enlazado al anillo B del flavonoide; y

b. permitir el automontaje de una micela que comprende el conjugado polímero-flavonoide y el encapsulado del agente dentro de la micela para formar de esta manera el nanocomplejo micelar.»

La reivindicación 21 se refiere nuevamente al polímero del conjugado sin concisión ni claridad:

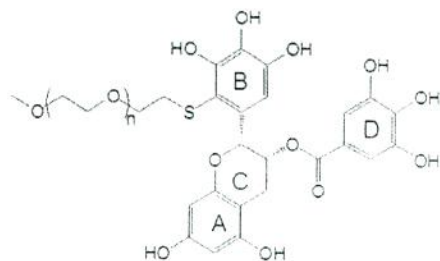
«21.El conjugado polímero-flavonoide de conformidad con la reivindicación 20, caracterizado porque el polímero es seleccionado a partir del grupo que consta de a polisacárido, poliacrilamida, poli(N-isopropilacrilamida), poli(oxazolina), polietilenimina, ácido poli(acrílico), polimetacrilato, polifetilen glicol), óxido de poli(etileno), alcohol poli(vinílico), poli(vinilpirrolidinona), poliéteres, poli(alilamina), polianhídridos, poli (β- amino éster), poli(butilen succinato), policaprolactona, policarbonato, polidioxanona, poli(glicerol), ácido poliglicólico, ácido poli(3-hidroxipropiónico), poli(2-hidroxietilmetacrilato), poli(N-(2-hidroxipropil) metacrilamida), ácido poliláctico, ácido poli(lactic-co- glicólico), poli(orto esteres), poli(2-oxazolina), ácido poli(sebácico), poli(tereftalato-co- fosfato) y copolímeros de los mismos.»

La reivindicación 22 se refiere nuevamente al flavonoide del conjugado, sin claridad ni concisión:

«22. El conjugado polímero-flavonoide de conformidad con cualquiera de las reivindicaciones 20 a 21, caracterizado porque el flavonoide es seleccionado a partir del grupo que consta de (-)-epicatequina, (+)-epicatequina, (-)-catequina, (+)-catequina, galato de epicatequina, epigalocatequina, galato de epigalocatequina, Fisetinidol, Galocatequina, galato de Galocatequina, Mesquitol y Robinetinidol, elagitanino, galotanino, oolongteanino, florotanino, tanino, teacitrina, teadibenzotropolona, teaflavina, teanaftoquinona, tearubiginas, teasinensina y mezclas de los mismos.»

La reivindicación 25 define el polímero-flavonoide de forma confusa y muy amplia:

«25. El conjugado polímero-flavonoide de conformidad con la reivindicación 24, caracterizado porque el conjugado tiene la siguiente fórmula:



en donde n está en el rango de 20 a 910.»

Las reivindicaciones 26, 28 y 29 definen un método para formar el conjugado mediante una adición nucleofílica, en las cuales no se definen condiciones de tal reacción, ni tampoco los reactivos allí involucrados:

«26. Un método para formar el conjugado polímero-flavonoide de conformidad con cualquiera de las reivindicaciones 20 a 25 que comprende la etapa de conjugar el flavonoide con dicho polímero a través de adición nucleofílica bajo condiciones básicas, en donde el polímero tiene un grupo nucleofílico libre.»

«28. El método de conformidad con la reivindicación 26 o 27, que comprende además la etapa de conducir la etapa de conjugación en un solvente que evita sustancialmente la agregación de dicho flavonoide.»

«29. El método de conformidad con cualquiera de las reivindicaciones 26 a 28, que comprende además la etapa de agregar un agente depurador para evitar la oxidación hbCte-mediada del grupo nucleofílico para incrementar de este método la eficiencia de la etapa de conjugación.»

Las reivindicaciones 32, 33 y 34 definen el nanocomplejo micelar mediante la ruta de administración del nanocomplejo, lo cual no es una característica técnica sino un uso que puede tener:

«33. El método de conformidad con la reivindicación 32, caracterizado porque dicho nanocomplejo micelar es adecuado para la administración por vía parenteral, mediante inhalación por aspersión, por vía tópica, rectal, nasal, bucal, vaginal, a través de un depósito implantado, mediante inyección, por vía subdérmica, intraperitoneal, transmucosa, oral o en una preparación oftálmica.»

«34. El método de conformidad con la reivindicación 32 o 33, donde dicho nanocomplejo micelar es adecuado para la administración parenteral comprende subcutánea, intracutánea, intravenosa, intramuscular, intraarticular, intraarterial, intrasinovial, intraesternal, intratecal, intralesión y mediante inyección intracraneal o técnicas de infusión.»

La reivindicación 35 define la dosis del agente que transporta el nanocomplejo micelar, lo cual no se puede considerar como una característica que defina tal composición:

«35. El método de conformidad con cualquiera de las reivindicaciones 32 a 34, caracterizado porque el agente presente en dicho nanocomplejo micelar es administrado en una dosis de 1 a 80 mg/kg por día.»

La reivindicación 36 define las enfermedades en las que puede usarse los nanocomplejos, lo cual definitivamente no constituye un elemento técnico que defina la invención:

«36. El método de conformidad con cualquiera de las reivindicaciones 32, donde dicho medicamento es adecuado para un cáncer seleccionado a partir del grupo que consta de carcinoma adrenocortical, linfoma relacionado con SIDA, cáncer anal, cáncer de apéndice, astrocitoma grado I (anaplásico), astrocitoma grado II, astrocitoma grado III, astrocitoma grado IV, tumor teratoideo/rabdoide del sistema nervioso central, carcinoma de célula basal, cáncer de vejiga, cáncer bronquial, carcinoma bronquioalveolar, linfoma de Burkitt, cáncer cervical, cáncer de colon, cáncer colorectal, craneofaringioma, linfoma de célula T cutáneo, cáncer endometrial, cáncer uterino endometrial, ependimoblastoma, ependimoma, cáncer esofágico, estesioneuroblastoma, sarcoma de Ewing, tumor de célula germinal extracraneal, tumor de célula germinal extragonadal, cáncer de ducto biliar extrahepático, histiocitoma fibroso, cáncer de vesícula biliar, cáncer gástrico, tumor carcinoide gastrointestinal, tumor del estroma gastrointestinal, tumor trofoblástico gestacional, glioma, cáncer de cabeza y cuello, cáncer de corazón, cáncer hepatocelular, colangiocarcinoma de Hilar, linfoma de Hodgkin, cáncer hipofaríngeo, melanoma infraocular, tumor de células de los islotes, sarcoma de Kaposi, histiocitosis de células de Langerhans, cáncer de laringe, cáncer de labio, linfoma, macroglobulinemia, histiocitoma fibroso maligno, meduloblastoma, meduloepitelioma, melanoma, carcinoma de célula de Merkel, mesotelioma, neoplasia endocrina, mieloma múltiple, micosis fungoide, mielodisplasia, neoplasias mielodisplásicas/mieloproliferativas, trastornos mieloproliferativos, cáncer de cavidad nasal, cáncer nasofaríngeo, neuroblastoma, linfoma no-Hodgkin, cáncer oral, cáncer orofaríngeo, osteosarcoma, carcinoma de célula clara ovárico, cáncer epitelial ovárico, tumor de célula germinal de ovario, papilomatosis, cáncer de seno paranasal, cáncer paratiroideo, cáncer de pene, cáncer faríngeo, tumor parenquimal pineal, pineoblastoma, tumor de pituitaria, neoplasma de célula plasmática, blastema pleuropulmonar, linfoma de sistema nervioso central primario, cáncer de próstata, cáncer rectal, cáncer de célula renal, cáncer del tracto respiratorio con cambios en cromosoma 15, retinoblastoma, rabdomiosarcoma, cáncer de glándula salival, síndrome de Sezary, cáncer de intestino delgado, carcinoma de tejido blando, carcinoma de células escamosas,

cáncer escamoso de cuello, tumor neuroectodérmico primitivo supratentorial, cáncer testicular, cáncer de garganta, carcinoma tímico, timoma, cáncer de tiroides, cáncer de pelvis renal, cáncer uretral, sarcoma uterino, cáncer vaginal, cáncer vulvar, macroglobulinemia de Waldenstrom, y tumor de Wilms.»

Como se ha demostrado, el capítulo reivindicatorio referido a un nanocomplejo micelar y a un complejo polímero-flavonoide, no cumple la función de definir de forma clara y concisa la materia objeto de protección, pues su alcance es demasiado amplio.

En el caso del método de obtención de las micelas, no se revelan las condiciones técnicas de cada paso sino que se definen los pasos del procedimiento en términos muy generales y en el tipo de adición sintética. Por último, el capítulo reivindicatorio caracteriza al nanocomplejo mediante resultados a alcanzar lo cual no brinda información precisa de lo que realmente comprende la solicitud.

De acuerdo a lo anterior, la solicitud falla en cumplir con lo dispuesto por el Artículo 30 de la D. 486 que precisa lo siguiente: *“Las reivindicaciones definirán la materia que se desea proteger mediante la patente. Deben ser claras y concisas y estar enteramente sustentadas por la descripción.»*

Así las cosas, la materia de las reivindicaciones, y en general la solicitud en estudio no puede ser objeto de patente pues incumple las normas mínimas de presentación, como son la claridad y concisión.

3.2. En el estado de la técnica se describen conjugados de un polímero-flavonoide:

- i) WO 2006/124000 enseña conjugados de un agente que contiene un polímero y un flavonoide, en donde el flavonoide puede ser galato de epigallocatequina, además menciona que el oligómero de catequina puede tener más de dos monómeros y que el polímero puede ser ácido hialurónico o polietilenglicol;**
- ii) WO 2009/054813 revela un conjugado con un polímero y un flavonoide, como galato de epigallocatequina;**
- iii) WO 2011/112156 expone un conjugado de un polímero, como polietilenglicol, y con doxorrubicina, con un diámetro entre 192 y 19 nm.**

i) La publicación internacional WO 2006/124000 A1, titulada "**Aldehyde conjugated flavonoid preparations**", de Zhuo Lang et al., publicada en 2006-11-23, se refiere a un conjugado para entregar un aldehído y un flavonoide —siendo conjugados mediante el anillo A del flavonoide—, y a su método de fabricación:

« [0009] In one aspect, there is provided a conjugate of a delivery agent containing a free aldehyde and a flavonoid, having the delivery agent conjugated at the C6 and/or the C8 position of the A ring of the flavonoid.

[0010] In another aspect, there is provided a delivery vehicle comprising the conjugate described herein.

[0011] In a further aspect, there is provided a method of conjugating a delivery agent having a free aldehyde group in the presence of acid to a flavonoid, comprising reacting the delivery agent with the flavonoid in the presence of an acid catalyst.

[0012] In yet a further aspect, a method of delivering a catechin-based flavonoid to a subject comprising administering to the subject the conjugate or the delivery vehicle described herein.

[0048] Conjugation of a delivery agent to a flavonoid can provide a composition that is suitable for administration to a subject by incorporating the flavonoid into a particular vehicle formed with the delivery agent, and can allow for administration of higher concentrations of flavonoids than can be obtained through diet. The delivery agent can provide stability to the composition, resulting in a composition that is metabolized or degraded more slowly, and which thus may have a longer half-life in the body than the unconjugated flavonoid alone. For example, the delivery agent may be of such a nature that the flavonoid is incorporated into a composition that enhances the water-solubility of the flavonoid, which can avoid uptake by the reticuloendothelial system and subsequent clearance by the kidneys, resulting in a longer half-life in the body. Conjugation of other delivery agents may protect the flavonoid from enzyme degradation.»

En cuanto al flavonoide, la publicación WO 2006/124000 menciona que puede ser (-)-epicatequina, (+)-epicatequina, (-)-catequina, (+)-catequina, galato de epicatequina, epigallocatequina, galato de epigallocatequina:

«[0050] The flavonoid may be any flavonoid from the general class of molecules derived from a core phenylbenzyl pyrone structure, and includes flavones, isoflavones, flavonols, flavanones, flavan-3-ols, catechins, anthocyanidins and chalcones. In a particular embodiment the flavonoid is a catechin or a catechin-based flavonoid. A catechin, or a catechin-based flavonoid is any flavonoid that belongs to the class generally known as catechins (or flavan-3-ol derivatives), and includes catechin and catechin derivatives, including epicatechin, epigallocatechin, catechin, epicatechin gallate and epigallocatechin gallate, and including all possible stereoisomers of catechins or catechin-based flavonoids. In particular embodiments, the catechin-based flavonoid is (+)-catechin or (-)-epigallocatechin gallate. (-)- Epigallocatechin gallate (EGCG) is thought to have the highest activity among the catechin-based flavonoids, possibly due to the trihydroxy B ring and gallate ester moiety at the C3 position of this flavonoid.

(...)

Claim 4. The delivery vehicle of claim 3 wherein the flavonoid is a catechin-based flavonoid that is (-)-epicatechin, (-)-epigallocatechin, (+)-catechin, (-)-epicatechin gallate or (-)-epigallocatechin gallate.»

La mencionada anterioridad WO 2006/124000, también revela que el agente de entrega puede ser aldehído o cualquiera que se pueda convertir en aldehído en presencia de ácido y que puede ser dimérico o con más unidades de catequina:

«[0051] The delivery agent is any chemical group or moiety that contains a free aldehyde or group, or a functional group that can be converted to a free aldehyde group in the presence of acid, for example an acetal group. The delivery agent is capable of being formed into a delivery vehicle, thus allowing for the incorporation of a conjugated flavonoid into the delivery vehicle without compromising the biological or pharmacological properties of the flavonoid. As well, the delivery agent should be biocompatible, and may be biodegradable in some embodiments.

[0056] The oligomer of the catechin-based flavonoid may be of 2 or more monomeric units linked together. In certain embodiments, the catechin-based flavonoid oligomer has from 2 to 100 flavonoid monomer units, from 10 to 100, from 2 to 80, from 10 to 80, from 2 to 50, from 10 to 50, from 2 to 30, from 10 to 30, from 20 to 100, from 30 to 100 or from 50 to 100 monomeric units.»

Más adelante menciona que el aldehído puede ser polietilenglicol, o ácido hialurónico:

«[0058] The polymer may be chosen based on its particular characteristics and its ability to form certain types of delivery vehicles. For example, the polymer may be an aldehyde-terminated poly (ethylene glycol), or it may be hyaluronic acid derivatized with an aldehyde group, or a derivative of such polymers. Alternatively, the polymer may be a phenoxymethyl(methylhydrazono) dendrimer (PMMH), for example, cyclotriphosphazene core PMMH or thiophosphoryl core PMMH. The polymer may also be any biological polymer, modified to contain a free aldehyde group or a group that is convertible to an aldehyde in the presence of acid, for example an aldehyde-modified protein, peptide or nucleic acid. In one particular embodiment the polymer is an aldehyde-terminated poly (ethylene glycol) (PEG-CHO). In another particular embodiment, the polymer is aldehyde-derivatized hyaluronic acid, hyaluronic acid conjugated with aminoacetylaldehyde diethylacetal, or either of the aforementioned hyaluronic acid polymers derivatized with tyramine.»

ii) Por su parte, la publicación internacional WO 2009/054813 A1, titulada **“Method of delivering an anti-cancer agent to a cell”**, de Ying Jackie et al., publicada en 2009-04-30, se refiere a un conjugado de un agente anti cáncer y un conjugado que comprende un aldehído libre y un flavonoide, unidos por el anillo A y en donde el aldehído puede ser polietilenglicol o ácido hialurónico:

« (abstract) There is provided a delivery vehicle comprising an anti-cancer agent together with a conjugate of a delivery agent containing a free aldehyde and a flavonoid, having the delivery agent conjugated at the C6 and/or the C8 position of the A ring of the flavonoid. The resulting delivery vehicles may be used to deliver an anti-cancer agent to a cell.

[0032] In order to increase the availability of beneficial flavonoid compounds, the conjugation of flavonoids to various delivery agents through a free aldehyde group on the delivery agent to the A ring of the flavonoid allows for modification of the physical properties of the flavonoid without disrupting the polyphenol structure of

the flavonoid, while augmenting the biological and pharmacological properties of the flavonoid.

[0044] The polymer may be chosen based on its particular characteristics and its ability to form certain types of delivery vehicles. For example, the polymer may be an aldehyde-terminated poly (ethylene glycol), or it may be hyaluronic acid derivatized with an aldehyde group, or a derivative of such polymers. Alternatively, the polymer may be a phenoxymethyl(methylhydrazono) dendrimer (PMMH), for example, cyclotriphosphazene core PMMH or thiophosphoryl core PMMH. The polymer may also be any biological polymer, modified to contain a free aldehyde group or a group that is convertible to an aldehyde in the presence of acid, for example an aldehyde-modified protein, peptide, polysaccharide or nucleic acid. In one particular embodiment the polymer is an aldehyde-terminated poly (ethylene glycol) (PEG-CHO). In another particular embodiment, the polymer is aldehyde-derivatized hyaluronic acid, hyaluronic acid conjugated with aminoacetylaldehyde diethylacetal, or either of the aforementioned hyaluronic acid polymers derivatized with tyramine.»

Además, revela el método de conjugación del polímero y el flavonoide y menciona que el oligómero basado en catequinas y flavonoides puede ser usado para asociar el agente anticáncer antes de formar el nanocomplejo micelar; también menciona que el flavonoide puede ser (-)-epicatequina, (+)-epigallocatequina, (+)-catequina, galato de epicatequina, epigallocatequina, galato de epigallocatequina:

« [0048] To conjugate the polymer and the catechin-based flavonoid, the polymer and the catechin-based flavonoid may be separately dissolved in a suitable solvent. The polymer with the free aldehyde is added, for example by dropwise addition, to the solution containing the catechin-based flavonoid, in the presence of an acid. The reaction is allowed to go to completion. Following the conjugation reaction, excess unreacted polymer or catechin-based flavonoid can be removed from the conjugated composition, for example by dialysis or by molecular sieving.
(...)

[0060] By conjugating PEG-CHO to a catechin-based flavonoid, a conjugate is formed that has strong self-assembly tendencies. In one embodiment, PEG is conjugated to a monomer of a catechin-based flavonoid, to form a PEG-flavonoid. The delivery vehicle is formed together with non-conjugated catechin-based flavonoids and the anti-cancer agent. Thus, the central core contains relatively high concentrations of a flavonoid and the anti-cancer agent, while the external shell of the micellar nanocomplex comprises the conjugated PEG-monomeric flavonoid, and is assembled in a two-step process. In a particular embodiment, the central core is oligomeric EGCG (OEGCG) and the external core is made up of conjugated PEG-EGCG.
(...)

[0062] This embodiment of the delivery vehicle is well suited to deliver the anticancer agent. Since the catechin-based flavonoids have a rigid, multi-ring core structure, these molecules associate well with anti-cancer agents such as proteins and nucleic acids, as well as other molecules containing ring structures, likely by stacking of the catechin rings with the ring or rings on the anti-cancer agent. Thus, an oligomeric catechin-based flavonoid can be used to associate with the anti-cancer agent prior to assembly in the micellar nanocomplex.
(...)

3. The delivery vehicle of claim 2 wherein the flavonoid is a catechin-based flavonoid that is (-)-epicatechin, (-)-epigallocatechin, (+)-catechin, (-)-epicatechin gallate or (-)-epigallocatechin gallate.»

iii) La publicación Internacional WO 2011/112156 A1, titulada “**Anti-cancer agent delivery vehicles capable of improved loading**”, de Kurisawa Motoichi et al., publicada en 2006-12-21, se refiere a un conjugado de una parte química y al menos un flavonoide que puede estar en forma dimérica o monomérica, en donde el flavonoide puede ser flavona, isoflavona, flavonoles, flavanonas, catequinas, antocianidinas y chalconas, y en donde el agente está conjugado en el anillo A, pero que podría unirse al anillo B:

«There is provided a conjugate of a delivery agent containing a chemical moiety and at least one flavonoid. The flavonoid exists in a monomeric form or dimeric form before conjugation and remains in the monomeric form or dimeric form after conjugation. Preferably, the conjugate comprises two flavonoids. The delivery agent is conjugated at the C6 and/or the C8 position of the A ring of the flavonoid. An anti-cancer agent delivery vehicle comprising an anti-cancer agent and the conjugate is also provided.

(...)

The flavonoid may be any flavonoid from the general class of molecules derived from a core phenylbenzyl pyrone structure, and includes flavones, isoflavones, flavonols, flavanones, flavan-3-ols, catechins, anthocyanidins and chalcones. In a particular embodiment, the flavonoid is a catechin or a catechin-based flavonoid. A catechin, or a catechin-based flavonoid is any flavonoid that belongs to the class generally known as catechins (or flavan-3-ol derivatives), and includes catechin and catechin derivatives, including epicatechin, epigallocatechin, catechin, epicatechin gallate and epigallocatechin gallate, and including all possible stereoisomers of catechins or catechin-based flavonoids.

In particular embodiments, the catechin-based flavonoid is (-)-epicatechin, (-)-epigallocatechin, (+)-catechin, (-)-epicatechin gallate, or (-)-epigallocatechin gallate. (-)-epigallocatechin gallate (EGCG) is thought to have the highest activity among the catechin-based flavonoids, possibly due to the trihydroxy B ring and gallate ester moiety at the C3 position of this flavonoid. In addition, EGCG is particularly chosen to form the conjugate because of its high activity and by conjugation with, say, PEG (to be discussed in subsequent paragraphs), the resultant PEG-EGCG conjugate forms a stable composition that is metabolised or degraded more slowly, and which thus may have a longer half-life in the body. Furthermore, the EGCG moiety is capable of forming interactions, such as hydrophobic interaction, π - π stacking interaction or other physical or chemical bond, with the drug molecule to thereby encapsulate or confine the drug molecule within the inner core of the delivery vehicle.

(...)

Preferably, the delivery agent is conjugated at the C6 and/or the C8 position of the A ring of the flavonoid. While the C6 and C8 positions are most preferred, it will be apparent to a person skilled in the art that other conjugation positions of the flavonoid (e.g. at other rings) are also possible. » [Resumen, página 8, ln. 10 a 31 y página 11, ln 9 a 11]

También revela el conjugado Doxorubicina con PEG-EGCG, en donde el vehículo es una micela. Veamos:

«Example 1: Formation of Delivery Vehicle Comprising Doxorubicin and PEG-EGCG Conjugates

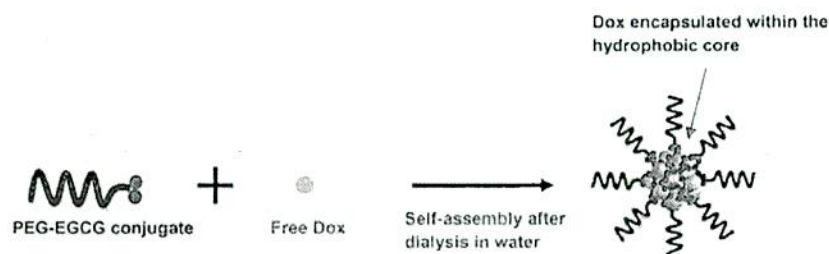
In one embodiment, the drug molecule is doxorubicin ("Dox" for short). FIG 1 shows a scheme illustrating the self-assembly of PEG-EGCG conjugates loaded with Dox to form the delivery vehicle.¹

In this embodiment, the delivery vehicle is in the form of a micelle. It is based on the self-assembly of PEG-EGCG conjugates into micelles in aqueous environment, stemming from the hydrophilic nature of the PEG chain of the PEG-EGCG conjugate. Due to the similarity in the multi-ring structures of the EGCG moiety and Dox, the EGCG moiety is designed to encapsulate Dox through the hydrophobic interactions and π - π stacking interactions. Dox was successfully encapsulated within the delivery vehicles by simple mixing and dialysis. Advantageously, It is found that the delivery vehicles demonstrate exceptionally high Dox loading in comparison to other delivery vehicles systems (e.g. polymeric micellar systems) known in the prior art. Dynamic light scattering (DLS) measurements also showed that the dimensions of the delivery vehicles are ideal for passive tumor targeting via EPR effect.

In this example, the delivery vehicle was prepared by dialysis of a mixture of PEG-EGCG conjugates and Dox dissolved in organic solvent dimethylformamide (DMF) against distilled water. Different concentrations of PEG-EGCG conjugates (0, 0.5, 1, 3, 6, 12, 24 mg/ml, respectively) were used to encapsulate a fixed concentration of Dox at 12 mg/ml. During the dialysis, the gradual replacement of the organic solvent with water triggered the self-assembly of the delivery vehicles and the entrapment of Dox within the assembled structures. As shown in FIG. 2, precipitates were obtained during the dialysis when PEG-EGCG concentrations of 0, 0.5 and 1 mg/ml were used, indicating ineffective delivery vehicle formation. In contrast, with PEG-EGCG concentrations of 3, 6, 12 and 24 mg/ml, homogeneous solutions were achieved, demonstrating the successful encapsulation of Dox within the delivery vehicles. This example indicated that a suitable ratio of Dox to PEG-EGCG conjugates should be carefully selected in order for the Dox/PEG-EGCG delivery vehicles to self-assemble effectively.» [página 14 a 15]

Adicionalmente, la WO 2011/112156 describe la síntesis de los conjugados PEG-EGCG especificando todas condiciones técnicas, revela la eficiencia y el contenido

¹ Fig.1, WO 2011/112156, PCT/SG2011/000100



de carga del agente presentes dentro de la micela y en lo que se refiere a tamaño del vehículo, menciona que pueden estar entre 78,5 nm y 192 nm:

«Synthesis of PEG-EGCG Conjugate: The aldehyde-terminated PEG (0.35 g) and EGCG (0.65 g) were separately dissolved in a mixture of acetic acid, water and dimethyl sulfoxide (DMSO). The reaction was started with the dropwise addition of the PEG-CHO solution at 20°C (pH 2) under a nitrogen atmosphere for 48 h. The resulting products were dialyzed (molecular weight cut-off (MWCO) = 3500) and lyophilized to give the PEG-EGCG conjugate. **Synthesis of Delivery Vehicle:** Dox HCl (12 mg, 21 μ mol) was dissolved in 1 ml of DMF. Triethylamine (29 μ l, 210 pmol) was then added to the solution to neutralize the hydrochloride. Next, PEG-EGCG conjugates (1 ml) with different concentrations dissolved in DMF were added to the solution, and the resulting mixtures were vortexed for 20 min. Each sample solution was then transferred to dialysis tubes with a MWCO of 3500 Da. The tubes were dialyzed against distilled water for 2 days, with the water replaced every 12 h. The sample solutions were lyophilized to obtain solid micelles.

Determination of Drug Loading: The absorbance of the sample solutions was measured at 480 nm using a Hitachi U2810 UV-Vis spectrophotometer after the dried samples were dissolved in DMF. The amount of Dox in each sample was estimated by comparing to the Dox standards. Drug loading was calculated from the amount of Dox in the delivery vehicles, and the weight of the dried delivery vehicles samples. **Determination of Delivery Vehicle Size:** The size of the delivery vehicle samples was determined by DLS using a Brookhaven 90Plus Particle Size Analyzer. Each sample solution was prepared using distilled water to give 10 μ g/ml of Dox before conducting the DLS measurements at 25°C.

Results: characteristics of the delivery vehicle The characteristics of the delivery vehicle of Example 1 are summarized in Table 1 below. Drug loading is defined as the weight percentage of Dox in the delivery vehicle, while drug loading efficiency is defined as the weight percentage of the entrapped Dox compared to the free Dox introduced initially. The samples with initial PEG-EGCG concentrations of 3 and 6 mg/ml showed an extraordinarily high drug loading of 85 and 68 wt%, respectively. The highest Dox loading achieved (85 wt%) corresponded to more than 4 times the average Dox loading (10-20 wt%) in the block copolymeric delivery vehicles developed by other groups (H. M. Aliabadi et al, Expert Opinion on Drug Delivery, 2006, 3, 139-162). Without being limited to any particular mechanism it is hypothesized that such a high drug loading is possibly due to the strong hydrophobic interaction and/or π - π stacking interactions between the EGCG moiety and Dox, which would possibly enhance the encapsulation of Dox in the self-assembly process, although the EGCG moiety was much smaller than the hydrophobic chain of other PEG block copolymers reported. The size of the delivery vehicles ranged from 78.5 to 192.4 nm (see Table 1).

The size increased as the drug loading increased, but decreased with increasing initial PEG-EGCG concentration. The increase in size should be correlated to the encapsulation efficiency since the EGCG moiety played a key role in encapsulating Dox. Thus, it is expected that not only the size, but also the drug release profile and the stability of delivery vehicles, which are important aspects for any targeted drug delivery system, could be controlled by the initial PEG-EGCG concentration introduced. The nanometer dimensions of the delivery vehicles (less than 200 nm) conferred the ability of targeting tumor tissue via EPR effect. At the same time, they enabled prolonged blood residence time, as they helped to avoid opsonization and renal clearance of delivery vehicles.

Samples	Initial PEG-EGCG concentration (mg/ml)	Initial Dox concentration (mg/ml)	Average hydrodynamic diameter (nm)*	Drug loading efficiency (wt%)	Drug loading (wt%)
1	3	12	192	60	85
2	6	12	129	77	68
3	12	12	115	77	49
4	24	12	79	79	35

*Determined by DLS measurements.

Table 1. Characteristics of PEG-EGCG Conjugates Loaded with Dox in Accordance with an Embodiment of the Present Invention

»

[página 14 - 17]

3.3. Las características técnicas esenciales de la presente solicitud, han sido anticipadas en el arte previo, por lo tanto, los conjugados y/o procedimientos para su obtención no son novedosos ni inventivos.

Teniendo en cuenta lo descrito en el anterior numeral, la materia reclamada sería obvia puesto que los documentos WO2006/124000, WO2009/054813 y WO2011/112156 publicados anteriormente a la fecha de presentación de la solicitud en estudio, se relacionan con complejos micelares que contienen conjugados polímeros-flavonoides, como derivados de catequinas. Veamos:

17

Rv.	Exp. NC2016/0004921	WO 2006/124000	WO 2009/054813	WO 2011/112156
1	Un nanocomplejo micelar que comprende una micela y un agente encapsulado dentro de la micela, dicha micela que comprende un conjugado polímero- flavonoide,	SI [todo]	SI [todo]	SI [todo]
	en donde el polímero está enlazado al anillo B de dicho flavonoide;	NO	NO	SI
	donde dicho polímero es un polímero hidrofílico;	SI [0058]	SI [0044]	SI [Ex. 1]
	donde dicho flavonoide es seleccionado a partir del grupo que consta de flavones, isoflavonas, flavanos, proantocianidinas y antocianidinas; donde dichos flavanos son seleccionados a partir del grupo que consta de (-)-epicatequina, (+)-epicatequina, (-)-catequina, (+)-catequina, galato de epicatequina, epigallocatequina, galato de epigallocatequina, Fisetinidol, Galocatequina, galato de Galocatequina, Mesquitol y Robinetinidol, elagitanino, galotanino, oolongteanino, florotanino, tanino, teacitrina, teadibenzotropolona, teaflavina, teanaftoquinona, tearubiginas, teasinensina y mezclas de los mismos .	SI [0050] y (R. 4)s	SI, (R. 3)	SI, (p. 8)
16	Un método para formar un nanocomplejo micelar puede comprender una micela y un agente encapsulado dentro de la micela, el método que comprende las etapas de:	SI [0011]	SI	SI, Ex. 1 y 2
	a. Agregar el agente en un solvente adecuado a un conjugado polímero- flavonoide, en donde el polímero está enlazado al anillo B del flavonoide; y	SI [0011]	SI [0048]	
	b. permitir el automontaje de una micela que comprende el conjugado polímero-flavonoide y el encapsulado del agente dentro de la micela para formar de esta manera el nanocomplejo micelar.	SI, [0011]	SI [0060] Fig. 1	
20	Un conjugado polímero-flavonoide que comprende un polímero enlazado al anillo B de un flavonoide.	NO	NO	SI
21	El conjugado polímero-flavonoide de conformidad con la reivindicación 20, caracterizado porque el polímero es	SI	SI	SI

seleccionado a partir del grupo que consta de:			
a. polisacárido, poliacrilamida, poli(N-isopropilacrilamida), poli(oxazolina), polietilenimina, ácido poli(acrílico), polimetacrilato, polifetilen glicol), óxido de poli(etileno), alcohol poli(vinílico), poli(vinilpirrolidinona), poliéteres, poli(alilamina), polianhídridos, poli(J3- amino éster), poli(butilen succinato), policaprolactona, policarbonato, polidioxanona, poli(glicerol), ácido poliglicólico, ácido poli(3-hidroxipropiónico), poli(2-hidroxietil metacrilato), poli(A/(2-hidroxipropil)metacrilamida), ácido poliláctico, ácido poli(lactic-co- glicólico), poli(orto esteres), poli(2-oxazolina), ácido poli(sebácico), poli(tereftalato-co- fosfato) y copolímeros de los mismos.	SI [0058]	SI [0044]	SI

Teniendo en cuenta la información consignada en el anterior cuadro comparativo, consideramos que la materia que busca ser protegida por la solicitud colombiana en trámite, carece de los requisitos de claridad, concisión, novedad y nivel inventivo; también es importante destacar que si bien es cierto los documentos WO2006/124000, WO2009/054813 revelan que la unión del polímero se realiza mediante el anillo A, el documento WO2011/112156 revela que el enlace puede ser realizado mediante el anillo B, por tanto, se considera que éste último es capaz de afectar la novedad de la solicitud.

Rogamos se analicen los argumentos expuestos con el rigor y detenimiento habituales y que en consecuencia se decrete, además de la negación de la solicitud en comento, el archivo del expediente que la contiene

IV. PRUEBAS

Ruego que se tenga como tal, sin perjuicio de aquellas que el Despacho considere oficiosamente, las siguientes:

- La publicación Internacional WO 2006/124000 A1, titulada "**Aldehyde conjugated flavonoid preparations**", de *Zhuo Lang*, publicada en 2006-11-23, portada, página 3, 7 – 9 y 39.
- La publicación Internacional WO 2009/054813 A1, titulada "**Method of delivering an anti-cancer agent to a cell**", de *Ying Jackie*, publicada en 2009-04-30, portada, página 6, 7, 9, 10, 13 y 28.
- La publicación internacional WO 2011/112156 A1, titulada "**Anti-cancer agent delivery vehicles capable of improved loading**", de *Kurisawa Motoichi*, publicada en 2006-12-21, portada, página 8 (ln. 10 a 31), página 11 (ln 9 a 11), y página 14 a 17

Señor Superintendente,



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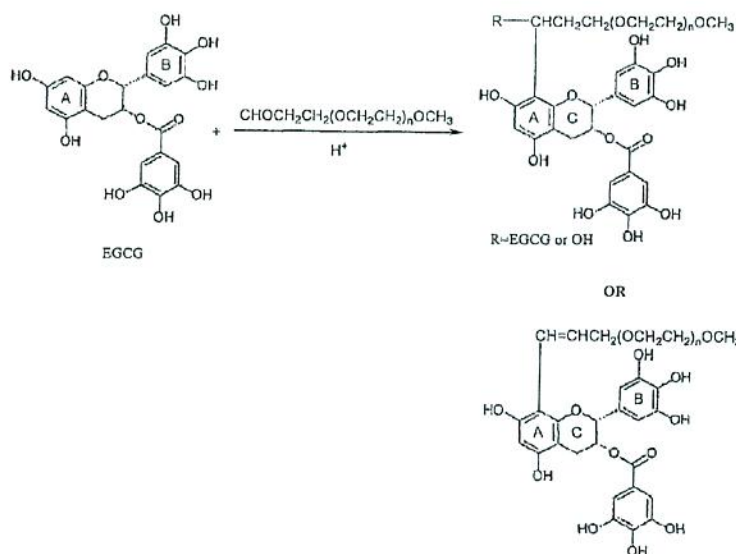
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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SI, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: ALDEHYDE CONJUGATED FLAVONOID PREPARATIONS



(57) Abstract: There is provided a method of conjugating a polymer containing a free aldehyde group with a flavonoid in the presence of an acid catalyst, such that the polymer is conjugated to the C6 or C8 position of the flavonoid A ring. The resulting conjugates may be used to form delivery vehicles to deliver high doses of flavonoids, and may also be used as delivery vehicles to deliver an additional bioactive agent.

WO 2006/124000 A1



Declarations under Rule 4.17:

- *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
- *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*
- *of inventorship (Rule 4.17(iv))*

Published:

- *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

ALDEHYDE CONJUGATED FLAVONOID PREPARATIONS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims benefit and priority from U.S. provisional patent application No. 60/682,801, filed on May 20, 2005, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

[0002] The present invention relates generally to preparations of flavonoids, and particularly to delivery agent-conjugated flavonoids and/or delivery agent-conjugated oligomers of flavonoids.

BACKGROUND OF THE INVENTION

[0003] Flavonoids are one of the most numerous and best-studied groups of plant polyphenols. The flavonoids consist of a large group of low-molecular weight polyphenolic substances naturally occurring in fruits and vegetables, and are an integral part of the human diet. Dried green tea leaves can contain as much as 30% flavonoids by weight, including a high percentage of flavonoids known as catechins (flavan-3-ol derivatives or catechin-based flavonoids), including (-)-epicatechin, (-)-epigallocatechin, (+)-catechin, (-)-epicatechin gallate and (-)-epigallocatechin gallate.

[0004] In recent years, these green tea catechins have attracted much attention because they have been recognized to have biological and pharmacological properties, including antibacterial, antineoplastic, anti-thrombotic, vasodilatory, antioxidant, anti-mutagenic, anti-carcinogenic, hypercholesterolemic, antiviral and anti-inflammatory properties, which have been demonstrated in numerous human, animal and *in vitro* studies (Jankun J., *et al. Nature* **387**, 561 (1997); Bodoni A. *et al. J. Nutr. Biochem.* **13**, 103-111 (2002); Nakagawa K. *et al. J. Agric. Food Chem.* **47**, 3967-3973 (1999)). These biological and pharmacological properties are potentially beneficial in preventing diseases and protecting the stability of the genome. Many of the beneficial effects of catechins are thought to be linked to the antioxidant actions of the catechins (Terao J., *et al. Arch. Biochem. Biophys.* **308**, 278-284 (1994)). Among the catechins, (-)-epigallocatechin gallate (EGCG), which is a major component of green tea, is thought to have the highest activity, possibly due to the trihydroxy B ring and the

gallate ester moiety at the C3 position (Isemura M., *et al. Biofactors* **13**, 81-85 (2000); Ikeda I., *et al. J. Nutr.* **135**, 155 (2005); Lill G., *et al. FEBS Letters* **546**, 265-270 (2003); Sakanaka S. and Okada Y. *J. Agric. Food Chem.* **52**, 1688-1692 (2004); Yokozawa T., *et al., J. Agric. Food Chem.* **48**, 5068-5073 (2000)).

[0005] In general, the activity half-life of flavonoids is limited to a few hours inside the body; metabolism of these compounds has not yet been established. Despite the favorable anti-oxidation and anti-cancer properties of the catechins including EGCG, it is impractical to achieve a therapeutic level of this compound in the body by directly ingesting a large amount of green tea, due to the inherent volume constraint. That is, in order to obtain a therapeutic or pharmacological benefit from flavonoids through diet alone, it would be necessary to ingest an amount of food and beverage that is larger than is practical to consume. Moreover, pro-oxidant activity has been reported for several flavonoids including EGCG, making ingesting crude green tea directly a less effective means of delivering EGCG (Yen G. C., *et al. J. Agric. Food Chem.* **45**, 30-34 (1997); Yamanaka N., *et al. FEBS Lett.* **401**, 230-234 (1997); Roedig-Penman A. and Gordon M. H. *J. Agric. Food Chem.* **1997**, **45**, 4267-4270).

[0006] On the other hand, a relatively high-molecular fraction of extracted plant polyphenols (procyanidins) and synthetically oligomerized (+)-catechin and rutin have been reported to exhibit enhanced physiological properties such as antioxidant and anti-carcinogenic activity compared to low-molecular weight flavonoids, (Zhao J., *et al. Carcinogenesis*, 1999, **20**, 1737-1745; Ariga T. and Hamano M. *Agric. Biol. Chem.* **54**, 2499-2504 (1990); Chung J. E., *et al. Biomacromolecules* **5**, 113-118 (2004); Kurisawa M., *et al. Biomacromolecules* **4**, 1394-1399 (2003); Hagerman A. E., *et al. J. Agric. Food Chem.* **46**, 1887 (1998)) and without pro-oxidant effects (Hagerman A. E., *et al. J. Agric. Food Chem.* **46**, 1887 (1998); Li C. and Xie B. *J. Agric. Food Chem.* **48**, 6362 (2000)). However, neither naturally occurring nor synthesized high molecular weight flavonoids are expected to be absorbed and transported to other tissues after ingestion, since these compounds are typically large, form strong complexes with proteins and are resistant to degradation (Zhao J., *et al. Carcinogenesis*, 1999, **20**, 1737-1745).

[0007] In cases of flavonoids consumed via oral intake of foods and beverages,

the flavonoids may play a role as antioxidants to protect the digestive tract from oxidative damage during digestion. However, flavonoids can be expected to remain only in the digestive tract and thus their beneficial physiological activities are not likely to be utilized to other tissues. Moreover, their strong hydrophobicity as well as their tendency to form complexes with proteins makes parenteral delivery of these compounds difficult.

[0008] Given the beneficial nature of these compounds, it is desirable to find methods of delivery that would allow for larger quantities to be consumed, or would provide for the use of catechin-based flavonoids in contexts in which they are not normally found, potentially providing increased consumption and/or exposure to the catechin-based flavonoids, thereby increasing the potential to receive the pharmacological benefit of these compounds.

SUMMARY OF THE INVENTION

[0009] In one aspect, there is provided a conjugate of a delivery agent containing a free aldehyde and a flavonoid, having the delivery agent conjugated at the C6 and/or the C8 position of the A ring of the flavonoid.

[0010] In another aspect, there is provided a delivery vehicle comprising the conjugate described herein.

[0011] In a further aspect, there is provided a method of conjugating a delivery agent having a free aldehyde group in the presence of acid to a flavonoid, comprising reacting the delivery agent with the flavonoid in the presence of an acid catalyst.

[0012] In yet a further aspect, a method of delivering a catechin-based flavonoid to a subject comprising administering to the subject the conjugate or the delivery vehicle described herein.

[0013] Other aspects and features of the present invention will become apparent to those of ordinary skill in the art upon review of the following description of specific embodiments of the invention in conjunction with the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] In the figures, which illustrate, by way of example only, embodiments of

the present invention,

[0015] Figure 1A is a schematic depiction of the oligomerization of (-)-epigallaocatechin gallate (EGCG) to yield oligomeric (-)-epigallaocatechin gallate (OEGCG);

[0016] Figure 1B is a schematic depiction of the conjugation of poly(ethylene glycol) (PEG) and EGCG to yield the PEG-epigallaocatechin gallate conjugate (PEG-EGCG);

[0017] Figure 1C is a schematic depiction of the conjugation of PEG and OEGCG to yield poly(ethylene glycol)-oligomeric epigallaocatechin gallate conjugate (PEG-OEGCG);

[0018] Figure 2A is a schematic depiction of a micellar nanocomplex system comprising the self-assembled OEGCG/protein complex surrounded by PEG-EGCG;

[0019] Figure 2B is a schematic depiction of a micellar nanocomplex of a PEG-OEGCG/protein complex;

[0020] Figure 3 is UV-VIS spectra of EGCG, OEGCG, PEG-EGCG, and PEG-OEGCG in an aqueous solution;

[0021] Figure 4 is a DSC thermodiagram for EGCG, OEGCG, PEG-EGCG, PEG-OEGCG, and PEG;

[0022] Figure 5 is plot of ζ -poteintial of PEG, EGCG, PEG-EGCG, OEGCG, and PEG-OEGCG in PBS;

[0023] Figure 6 is UV-VIS spectra of DPPH solutions treated with EGCG, OEGCG, PEG-EGCG, and PEG-OEGCG, measured at 519 nm;

[0024] Figure 7 is a graph depicting XO inhibition activity of EGCG, OEGCG, PEG-EGCG, PEG-OEGCG, and Allopurinol (n=8);

[0025] Figure 8 is a graph depicting uPA inhibition activity of EGCG, OEGCG, PEG-EGCG, and PEG-OEGCG (n=8);

[0026] Figure 9 is a graph showing the effect of OEGCG and protein

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concentration on the size of the micellar nanocomplex;

[0027] **Figure 10** is a graph showing the effect on size of the micellar nanocomplex upon PEG-EGCG addition, at varying concentrations of PEG;

[0028] **Figure 11** shows the ζ -potential of the various components in PBS;

[0029] **Figure 12** is a graph indicating the size of the micellar nanocomplex formed in the presence or absence of OEGCG [a: BSA; b: BSA+PEG; c: BSA+PEG-EGCG; d: (BSA+PEG-EGCG)+BSA; e: PEG-EGCG; f: PEG-EGCG+BSA; g: PEG-EGCG in DMSO; h: (BSA+OEGCG)+PEG-EGCG; i: ((BSA+OEGCG)+PEG-EGCG)+BSA];

[0030] **Figure 13A** is a TEM image of the OEGCG/protein, PEG-EGCG micellar nanocomplex;

[0031] **Figure 13B** indicates the size distribution of the OEGCG/protein, PEG-EGCG micellar nanocomplex as measured by light scattering;

[0032] **Figure 14** is a graph indicating the size of the complex formed with DNA [○: OEGCG + DNA; ■: EGCG + DNA];

[0033] **Figure 15** is a graph showing the size of the complex formed in the various samples [a: (BSA+OEGCG)+PEG-EGCG; b: OEGCG+PEG-EGCG; c: (OEGCG+PEG-EGCG)+BSA; d: OEGCG+PEG; e: (OEGCG+PEG)+BSA];

[0034] **Figure 16** is a graph showing the size of the complex formed in the various samples [a: BSA; b: BSA+PEG-OEGCG; c: b after ultrasonication; d: BSA+OEGCG; e: (BSA+OEGCG)+PEG-OEGCG; f: e after ultrasonication];

[0035] **Figure 17** is a graph showing the effect of pH on PEG-OEGCG complexation;

[0036] **Figure 18** is a graph showing the size of the complex formed in the various samples [a: PEG-OEGCG in distilled water; b: EGCG+PEG-OEGCG in distilled water; c: OEGCG+PEG-OEGCG in distilled water; d: BSA+PEG-OEGCG in distilled water; e: (BSA+EGCG)+PEG-OEGCG in distilled water; f: (BSA+OEGCG)+PEG-OEGCG in distilled water; g: f after replaced in PBS];

[0037] Figure 19 is a representation of the synthesis method of hyaluronic acid-aminoacetylaldehyde diethylacetal conjugate;

[0038] Figure 20 is a representation of the synthesis method of hyaluronic acid-EGCG conjugate;

[0039] Figure 21 is a schematic depiction of synthesis of an hyaluronic acid-tyramine-EGCG (HA-Tyr-EGCG) hydrogel;

[0040] Figure 22A is a graph showing the amount of FITC-BSA released from various HA-Tyr-EGCG hydrogels;

[0041] Figure 22B is a graph showing the amount of FITC-BSA released from various HA-Tyr-catechin hydrogels;

[0042] Figure 23A is a graph showing the superoxide scavenging activity of various hyaluronic acid-EGCG (HA-EGCG) conjugates;

[0043] Figure 23B is a graph showing the xanthine oxidase inhibition activity of various HA-EGCG conjugates; and

[0044] Figure 24 is a graph showing the urokinase inhibition activity of an HA-EGCG conjugate.

DETAILED DESCRIPTION

[0045] It is generally desirable to find ways to readily increase the concentration of flavonoids in the body and to improve effective delivery of such flavonoids to various tissues in the body.

[0046] In order to increase the availability of beneficial flavonoid compounds, the inventors have found that conjugation of flavonoids to various delivery agents through a free aldehyde group on the delivery agent to the A ring of the flavonoid allows for modification of the physical properties of the flavonoid without disrupting the polyphenol structure of the flavonoid, while augmenting the biological and pharmacological properties of the flavonoid.

[0047] That is, the aldehyde-mediated conjugation of a delivery agent to the

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flavonoid results in attachment of the delivery agent at the C6 and/or C8 position of the flavonoid A ring, and does not disrupt or affect the B and C rings of the flavonoid or the various hydroxyl groups on the flavonoid.

[0048] Conjugation of a delivery agent to a flavonoid can provide a composition that is suitable for administration to a subject by incorporating the flavonoid into a particular vehicle formed with the delivery agent, and can allow for administration of higher concentrations of flavonoids than can be obtained through diet. The delivery agent can provide stability to the composition, resulting in a composition that is metabolized or degraded more slowly, and which thus may have a longer half-life in the body than the unconjugated flavonoid alone. For example, the delivery agent may be of such a nature that the flavonoid is incorporated into a composition that enhances the water-solubility of the flavonoid, which can avoid uptake by the reticuloendothelial system and subsequent clearance by the kidneys, resulting in a longer half-life in the body. Conjugation of other delivery agents may protect the flavonoid from enzyme degradation.

[0049] Thus, there is presently provided a method of conjugating a delivery agent to a flavonoid comprising reacting the delivery agent with the flavonoid in the presence of an acid catalyst, the delivery agent having a free aldehyde group, or a group that is able to be converted to a free aldehyde group in the presence of acid.

[0050] The flavonoid may be any flavonoid from the general class of molecules derived from a core phenylbenzyl pyrone structure, and includes flavones, isoflavones, flavonols, flavanones, flavan-3-ols, catechins, anthocyanidins and chalcones. In a particular embodiment the flavonoid is a catechin or a catechin-based flavonoid. A catechin, or a catechin-based flavonoid is any flavonoid that belongs to the class generally known as catechins (or flavan-3-ol derivatives), and includes catechin and catechin derivatives, including epicatechin, epigallocatechin, catechin, epicatechin gallate and epigallocatechin gallate, and including all possible stereoisomers of catechins or catechin-based flavonoids. In particular embodiments, the catechin-based flavonoid is (+)-catechin or (-)-epigallocatechin gallate. (-)-Epigallocatechin gallate (EGCG) is thought to have the highest activity among the catechin-based flavonoids, possibly due to the trihydroxy B ring and gallate ester moiety at the C3 position of this flavonoid.

[0051] The delivery agent is any chemical group or moiety that contains a free aldehyde or group, or a functional group that can be converted to a free aldehyde group in the presence of acid, for example an acetal group. The delivery agent is capable of being formed into a delivery vehicle, thus allowing for the incorporation of a conjugated flavonoid into the delivery vehicle without compromising the biological or pharmacological properties of the flavonoid. As well, the delivery agent should be biocompatible, and may be biodegradable in some embodiments.

[0052] The following discussion refers to an embodiment in which the flavonoid is a catechin-based flavonoid and in which the delivery agent is a polymer. However, it will be understood that the aldehyde condensation reaction between an aldehyde-containing chemical group and a flavonoid is applicable to conjugation of any delivery agent having a free aldehyde group, including following acid treatment of the delivery agent, to any flavonoid, as described above.

[0053] Thus, in one embodiment the method involves conjugation of a polymer containing a free aldehyde group or a group that is able to be converted to a free aldehyde group in the presence of acid to a catechin-based flavonoid.

[0054] The catechin-based flavonoid may be a single monomeric unit of a catechin-based flavonoid or it may be an oligomer of one or more catechin-based flavonoids. An oligomer of the catechin-based flavonoid tends to have amplified levels of the biological and pharmacological properties associated with catechin-based flavonoids, and may even have reduced pro-oxidant effects that are sometimes associated with monomeric catechin-based flavonoids.

[0055] Oligomers of catechin-based flavonoids are known, including oligomers prepared through enzyme-catalyzed oxidative coupling and through aldehyde-mediated oligomerization. An aldehyde-mediated oligomerization process results in an unbranched oligomer that has defined linkages, for example through CH-CH₃ bridges linked from the C6 or C8 position on the A ring of one monomer to the C6 or C8 position on the A ring of the next monomer, including in either possible stereoconfiguration, where applicable. For example, **Figure 1A** depicts oligomeric (-)-epigallocatechin gallate (OEGCG) produced from an aldehyde-mediated oligomerization method, which is connected through C6-C8 linkages of (-)-

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epigallocatechin gallate monomers.

[0056] The oligomer of the catechin-based flavonoid may be of 2 or more monomeric units linked together. In certain embodiments, the catechin-based flavonoid oligomer has from 2 to 100 flavonoid monomer units, from 10 to 100, from 2 to 80, from 10 to 80, from 2 to 50, from 10 to 50, from 2 to 30, from 10 to 30, from 20 to 100, from 30 to 100 or from 50 to 100 monomeric units.

[0057] The polymer may be any polymer having a free aldehyde group prior to conjugation with the catechin-based flavonoid, or having a group that is converted to an aldehyde group in the presence of acid, for example an acetal group. Furthermore, it will be understood that the polymer should be non-toxic, biocompatible and suitable for pharmacological use. The polymer may also have other desirable properties, for example, the polymer may have low immunogenicity, and it may be biodegradable or non-biodegradable depending on the desired biological application of the composition, for example, for controlled release of catechin-based flavonoids or other bioactive agents at a particular site in a body.

[0058] The polymer may be chosen based on its particular characteristics and its ability to form certain types of delivery vehicles. For example, the polymer may be an aldehyde-terminated poly (ethylene glycol), or it may be hyaluronic acid derivatized with an aldehyde group, or a derivative of such polymers. Alternatively, the polymer may be a phenoxymethyl(methylhydrazono) dendrimer (PMMH), for example, cyclotriphosphazene core PMMH or thiophosphoryl core PMMH. The polymer may also be any biological polymer, modified to contain a free aldehyde group or a group that is convertible to an aldehyde in the presence of acid, for example an aldehyde-modified protein, peptide or nucleic acid. In one particular embodiment the polymer is an aldehyde-terminated poly (ethylene glycol) (PEG-CHO). In another particular embodiment, the polymer is aldehyde-derivatized hyaluronic acid, hyaluronic acid conjugated with aminoacetylaldehyde diethylacetal, or either of the aforementioned hyaluronic acid polymers derivatized with tyramine.

[0059] The free aldehyde group on the polymer allows for the conjugation of the polymer in a controlled manner to either the C6 or the C8 position of the A ring, or both, of the flavonoid structure, thus preventing disruption of the flavonoid structure,

particularly the B and C rings of the flavonoid, and thus preserving the beneficial biological and pharmacological properties of the flavonoid.

[0060] The polymer is conjugated to the catechin-based flavonoid via a reaction of the aldehyde group of the polymer with the C6 and/or the C8 position of the A ring of the catechin-based flavonoid, as shown in **Figure 1B** and **Figure 1C**.

[0061] The conjugate is synthesized using acid catalysis of a condensation of the aldehyde group of the polymer with the catechin-based flavonoid, or using acid to convert a functional group on the polymer to a free aldehyde prior to condensation of the aldehyde group with the catechin-based flavonoid.

[0062] To conjugate the polymer and the catechin-based flavonoid, the polymer and the catechin-based flavonoid may be separately dissolved in a suitable solvent. The polymer with the free aldehyde is added, for example by dropwise addition, to the solution containing the catechin-based flavonoid, in the presence of an acid. The reaction is allowed to go to completion. Following the conjugation reaction, excess unreacted polymer or catechin-based flavonoid can be removed from the conjugated composition, for example by dialysis or by molecular sieving.

[0063] The ratio of catechin-based flavonoid to polymer may be varied, so that there is only one polymer moiety attached to the catechin-based flavonoid portion of the polymer, or so that there is a catechin-based flavonoid portion attached at more than one position on the polymer, or so that the catechin-based flavonoid portion has two polymer portions attached, one at either of the C6 and C8 positions of the catechin-based flavonoid.

[0064] The ratio of polymer to catechin-based flavonoid in the final composition can be controlled through the ratio of starting reagents. For example, when the molar ratio of polymer moiety to catechin-based flavonoid moiety is about 1, a single polymer moiety will be attached to a single catechin-based flavonoid moiety (either monomeric or oligomeric may be used). However, at higher concentrations of polymer, for example at a 10:1 molar ratio of polymer to catechin-based flavonoid, a composition having a tri-block structure of polymer-flavonoid-polymer may be obtained.

PE-3	0.27	0.5	1:20	9.5 ^a (0.03:0.4:0.57)	2	N ₂	7900	6800	1.2
PE-4	0.35	0.65	1:20	12.3 ^b (0.17:0.22:0.61)	2	N ₂	7800	6600	1.2
PE-5	PEG-CHO(II) 0.55	0.5	1:20	12.3 ^c (0.03:0.13:0.24: 0.6)	2	N ₂	12200	10200	1.2
							12500	10600	1.2

^aa mixture of acetic acid, ethanol and H₂O, volume ratio in parenthesis, ^ba mixture of acetic acid, DMSO and H₂O, volume ratio in parenthesis, ^ca mixture of acetic acid, ethanol, DMSO and H₂O, volume ratio in parenthesis, ^dmolecular weight was measured by SEC after acetylation.

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Table 3. Synthesis of PEG conjugates with oligomeric EGCG

sample	PEG-CHO (g)	OEGCG (g)	Molar ratio (PEG- CHO:OEGCG)	Sovent (ml)	Time (d)	Temp. (°C)	atmosphere	Yield (%)	Mw ^d	Mn ^d	Mw/Mn ^d
PO-1	PEG-CHO(I) 0.19	0.01 (OE-1)	10:1	2.5 ^a (0.03:0.4:0.57)	6	20	Air	7	10300	9000	1.1
PO-2	0.28	0.1 (OE-1)	1:1	3.9 ^a (0.02:0.62:0.36)	14	20	Air	42	18000	17100	1.1
PO-3	0.61	0.3 (OE-2)	1:1	16.3 ^b (0.18:0.15:0.67)	2	20	N ₂	75	10100	8800	1.1
PO-4	PEG-CHO(II) 1.0	0.21 (OE-3)	1:1	20.8 ^c (0.02:0.09:0.48:0.41)	1	50	N ₂	64	10000	9000	1.1
									15900	13200	1.2

^aa mixture of acetic acid, ethanol and H₂O, volume ratio in parenthesis, ^ba mixture of acetic acid, DMSO and H₂O, volume ratio in parenthesis, ^ca mixture of acetic acid, ethanol, DMSO and H₂O, volume ratio in parenthesis, ^dmolecular weight was measured by SEC after acetylation.

Table 4. Free radical scavenging activity

Sample	DPPH scavenging activity IC ₅₀ (μM)	Superoxide radical scavenging activity IC ₅₀ (μM)
EGCG	7.3±0.6 ^a	2.9±0.3 ^a
OEGCG	4.9±0.5 ^a	1.1±0.2 ^a
PEG-EGCG	6.2±0.3 ^a	0.9±0.1 ^a
PEG-OEGCG	3.8±0.8 ^a	0.4±0.1 ^a
Vitamin C	44.7±0.4	30.3±0.3
BHT	>>250.0	>>250.0

^a: EGCG moiety concentration of samples, n=3.

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WHAT IS CLAIMED IS:

1. A conjugate of a delivery agent containing a free aldehyde and a flavonoid, having the delivery agent conjugated at the C6 and/or the C8 position of the A ring of the flavonoid.
2. The conjugate of claim 1 wherein the delivery agent is a polymer and wherein the flavonoid is a catechin-based flavonoid.
3. A delivery vehicle comprising the conjugate of claim 1 or claim 2.
4. The delivery vehicle of claim 3 wherein the flavonoid is a catechin-based flavonoid that is (-)-epicatechin, (-)-epigallocatechin, (+)-catechin, (-)-epicatechin gallate or (-)-epigallocatechin gallate.
5. The delivery vehicle of claim 3 or claim 4 wherein the flavonoid is monomeric.
6. The delivery vehicle of claim 3 or claim 4 wherein the flavonoid is oligomeric.
7. The delivery vehicle of any one of claims 3 to 6 wherein the polymer is aldehyde-terminated poly (ethylene glycol), aldehyde-derivatized hyaluronic acid, hyaluronic acid aminoacetylaldehyde diethylacetal conjugate, aldehyde-derivatized hyaluronic acid-tyramine, hyaluronic acid aminoacetylaldehyde diethylacetal conjugate-tyramine, cyclotriphosphazene core phenoxymethyl(methylhydrazono) dendrimer or thiophosphoryl core phenoxymethyl(methylhydrazono) dendrimer.
8. The delivery vehicle of claim 7 wherein the polymer is aldehyde-terminated poly (ethylene glycol) and the delivery vehicle is a micellar nanocomplex.
9. The delivery vehicle of claim 8 wherein the micellar nanocomplex comprises aldehyde-terminated poly (ethylene glycol) conjugated to an oligomeric catechin-based flavonoid.
10. The delivery vehicle of claim 8 wherein the micellar nanocomplex comprises an inner core containing an oligomeric catechin-based flavonoid and an outer shell containing the conjugate, the conjugate comprising aldehyde-terminated poly

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(54) Title: METHOD OF DELIVERING AN ANTI-CANCER AGENT TO A CELL

(57) Abstract: There is provided a delivery vehicle comprising an anti-cancer agent together with a conjugate of a delivery agent containing a free aldehyde and a flavonoid, having the delivery agent conjugated at the C6 and/or the C8 position of the A ring of the flavonoid. The resulting delivery vehicles may be used to deliver an anti-cancer agent to a cell.

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(□) Herceptin alone, or (○) PBS control.

DETAILED DESCRIPTION

[0024] Flavonoids are known to have a variety of biological properties, including having anti-cancer effects, inhibiting growth of cancer cells. Delivery vehicles comprising flavonoids were previously described in published international application WO 2006/124000 and published US application 2008/102052.

[0025] Surprisingly, the inventors have found that the combination of the flavonoid delivery vehicle and bioactive anti-cancer agent has a synergetic anti-cancer effect, greater than the combined effects of each of the flavonoid delivery vehicle and bioactive anti-cancer agent when used alone. Thus, such delivery vehicles loaded with an anti-cancer agent provide an effective way of delivering anti-cancer agents to a cell, taking advantage of the synergistic effect between the anti-cancer activity of the flavonoid portion of the delivery vehicle and the anti-cancer effect of the anti-cancer agent.

[0026] The inventors developed a delivery vehicle containing an anti-cancer agent such as a protein, nucleic acid or drug, by self-assembly using flavonoids and conjugates of flavonoids such as EGCG. The delivery vehicles were formed by self-assembly. For example, delivery vehicles were synthesised by (i) two-step self-assembly process involving assembly of oligomeric (-)-epigallocatechin-3-O-gallate (OEGCG) and the anti-cancer agent, and then assembly of the pre-formed OEGCG-anti-cancer agent complex with a conjugate of poly(ethylene glycol) (PEG) and EGCG (PEG-EGCG); and (ii) one-step self-assembly process involving assembly of a conjugate of PEG and OEGCG (PEG-OEGCG) and the anti-cancer agent. Both delivery vehicles formed as stable and highly oriented micellar complexes loaded with the anti-cancer agent via spontaneous self-assembly in a mild aqueous solution. The resulting micellar nanocomplexes characteristically display a PEG outer shell, with an inner core comprised of OEGCG-anti-cancer agent complex.

[0027] Since the formation of a micellar nanocomplex is mainly driven by hydrophobic interaction and hydrogen bonding, the delivery vehicles described herein can be used to load a wide variety of anti-cancer agents for improved delivery to a cell.

[0028] The delivery vehicles are designed to take advantage of the enhanced permeability and retention (EPR) effect, and to avoid reticuloendothelial system (RES) uptake when administered *in vivo*, providing for selective accumulation at cancer sites. Ideally, the delivery vehicles preserve the stability of both the flavonoid molecules and the anti-cancer agent from loss of activity and from degradation during delivery, exerting a synergistic therapeutic effect when delivered to a cell. The delivery vehicle may mask the activity of the flavonoid and the anti-cancer agent activity by sequestering these moieties within the inner core of the delivery vehicle until successfully delivered and released at the cell, restoring the respective activities only upon dissociation of the delivery vehicle at the site of a cell.

[0029] To demonstrate the synergistic therapeutic effects by the delivery vehicle, the anti-cancer protein Herceptin (trastuzumab) was loaded into the micellar complex, as described in Example 1 below. The micellar complex kept its integrity and showed good stability in the presence of serum without size change as a function of time, and the protein was safely protected from proteolysis within the micellar complex. A greater inhibition was demonstrated with the Herceptin-loaded delivery vehicle as compared to the individual components (OEGCG, PEG-EGCG and Herceptin).

[0030] Since the formation of the delivery vehicle is mainly driven by hydrophobic interaction, it can be dissociated by hydrophobic competitors, such as amphiphilic molecules. Thus, the delivery vehicle may be dissociated by bio-amphiphilic molecules (such as plasma membrane lipids) to release the contained therapeutic flavonoid and anti-cancer compounds when the delivery vehicle accumulates at the targeted cell site.

[0031] Thus, there is provided a method of delivering an anti-cancer agent to a cell. A delivery vehicle containing a flavonoid and an anti-cancer agent is used, as previously described in published international application WO 2006/124000 and published US application 2008/102052, and as described below. The delivery vehicle is then contacted with the cell to which the anti-cancer agent is to be delivered.

Flavonoid Conjugates and Delivery Vehicles

[0032] In order to increase the availability of beneficial flavonoid compounds, the conjugation of flavonoids to various delivery agents through a free aldehyde group on

the delivery agent to the A ring of the flavonoid allows for modification of the physical properties of the flavonoid without disrupting the polyphenol structure of the flavonoid, while augmenting the biological and pharmacological properties of the flavonoid.

[0033] That is, the aldehyde-mediated conjugation of a delivery agent to the flavonoid results in attachment of the delivery agent at the C6 and/or C8 position of the flavonoid A ring, and does not disrupt or affect the B and C rings of the flavonoid or the various hydroxyl groups on the flavonoid.

[0034] Conjugation of a delivery agent to a flavonoid can provide a composition that is suitable for administration to a subject by incorporating the flavonoid into a particular vehicle formed with the delivery agent, and can allow for administration of higher concentrations of flavonoids than can be obtained through diet. The delivery agent can provide stability to the composition, resulting in a composition that is metabolized or degraded more slowly, and which thus may have a longer half-life in the body than the unconjugated flavonoid alone. For example, the delivery agent may be of such a nature that the flavonoid is incorporated into a composition that enhances the water-solubility of the flavonoid, which can avoid uptake by the reticuloendothelial system and subsequent clearance by the kidneys, resulting in a longer half-life in the body. Conjugation of other delivery agents may protect the flavonoid from enzyme degradation.

[0035] A delivery agent may be conjugated to a flavonoid by reacting the delivery agent with the flavonoid in the presence of an acid catalyst, the delivery agent having a free aldehyde group, or a group that is able to be converted to a free aldehyde group in the presence of acid.

[0036] The flavonoid may be any flavonoid from the general class of molecules derived from a core phenylbenzyl pyrone structure, and includes flavones, isoflavones, flavonols, flavanones, flavan-3-ols, catechins, anthocyanidins and chalcones. In a particular embodiment the flavonoid is a catechin or a catechin-based flavonoid. A catechin, or a catechin-based flavonoid is any flavonoid that belongs to the class generally known as catechins (or flavan-3-ol derivatives), and includes catechin and catechin derivatives, including epicatechin, epigallocatechin, catechin,

epicatechin gallate and epigallocatechin gallate, and including all possible stereoisomers of catechins or catechin-based flavonoids. In particular embodiments, the catechin-based flavonoid is (+)-catechin or (-)-epigallocatechin gallate. (-)-epigallocatechin gallate (EGCG) is thought to have the highest activity among the catechin-based flavonoids, possibly due to the trihydroxy B ring and gallate ester moiety at the C3 position of this flavonoid.

[0037] The delivery agent is any chemical group or moiety that contains a free aldehyde or group, or a functional group that can be converted to a free aldehyde group in the presence of acid, for example an acetal group. The delivery agent is capable of being formed into a delivery vehicle, thus allowing for the incorporation of a conjugated flavonoid into the delivery vehicle without compromising the biological or pharmacological properties of the flavonoid. As well, the delivery agent should be biocompatible, and may be biodegradable in some embodiments.

[0038] The following discussion refers to an embodiment in which the flavonoid is a catechin-based flavonoid and in which the delivery agent is a polymer. However, it will be understood that the aldehyde condensation reaction between an aldehyde-containing chemical group and a flavonoid is applicable to conjugation of any delivery agent having a free aldehyde group, including following acid treatment of the delivery agent, to any flavonoid, as described above.

[0039] Thus, the reaction may involve conjugation of a polymer containing a free aldehyde group or a group that is able to be converted to a free aldehyde group in the presence of acid to a catechin-based flavonoid.

[0040] The catechin-based flavonoid may be a single monomeric unit of a catechin-based flavonoid or it may be an oligomer of one or more catechin-based flavonoids. As stated above, conjugation of a polymer to a flavonoid results in augmentation of the flavonoid's biological or pharmacological properties. As well, an oligomer of the catechin-based flavonoid tends to have amplified or augmented levels of the biological and pharmacological properties associated with catechin-based flavonoids, and may even have reduced pro-oxidant effects that are sometimes associated with monomeric catechin-based flavonoids. Thus, the catechin-based flavonoid is an oligomerized catechin-based flavonoid having amplified or augmented

flavonoid properties.

[0041] Oligomers of catechin-based flavonoids are known, including oligomers prepared through enzyme-catalyzed oxidative coupling and through aldehyde-mediated oligomerization. An aldehyde-mediated oligomerization process results in an unbranched oligomer that has defined linkages, for example through carbon-carbon linkages such as CH-CH₃ bridges linked from the C6 or C8 position on the A ring of one monomer to the C6 or C8 position on the A ring of the next monomer, including in either possible stereoconfiguration, where applicable. Thus, the CH-CH₃ linkage may be between the C6 position of the A ring of one monomer and either of the C6 or C8 position of the next monomer or it may be between the C8 position of the A ring of the first monomer and either of the C6 or C8 position of the next monomer.

[0042] The oligomer of the catechin-based flavonoid may be of 2 or more monomeric units linked together. In certain embodiments, the catechin-based flavonoid oligomer has from 2 to 100 flavonoid monomer units, from 10 to 100, from 2 to 80, from 10 to 80, from 2 to 50, from 10 to 50, from 2 to 30, from 10 to 30, from 20 to 100, from 30 to 100 or from 50 to 100 monomeric units.

[0043] The polymer may be any polymer having a free aldehyde group prior to conjugation with the catechin-based flavonoid, or having a group that is converted to an aldehyde group in the presence of acid, for example an acetal group. Furthermore, it will be understood that the polymer should be non-toxic, biocompatible and suitable for pharmacological use. The polymer may also have other desirable properties, for example, the polymer may have low immunogenicity, and it may be biodegradable or non-biodegradable depending on the desired biological application of the composition, for example, for controlled release of catechin-based flavonoids and the anti-cancer agent at a particular site in a body.

[0044] The polymer may be chosen based on its particular characteristics and its ability to form certain types of delivery vehicles. For example, the polymer may be an aldehyde-terminated poly (ethylene glycol), or it may be hyaluronic acid derivatized with an aldehyde group, or a derivative of such polymers. Alternatively, the polymer may be a phenoxymethyl(methylhydrazono) dendrimer (PMMH), for example, cyclotriphosphazene core PMMH or thiophosphoryl core PMMH. The

polymer may also be any biological polymer, modified to contain a free aldehyde group or a group that is convertible to an aldehyde in the presence of acid, for example an aldehyde-modified protein, peptide, polysaccharide or nucleic acid. In one particular embodiment the polymer is an aldehyde-terminated poly (ethylene glycol) (PEG-CHO). In another particular embodiment, the polymer is aldehyde-derivatized hyaluronic acid, hyaluronic acid conjugated with aminoacetylaldehyde diethylacetal, or either of the aforementioned hyaluronic acid polymers derivatized with tyramine.

[0045] The free aldehyde group on the polymer allows for the conjugation of the polymer in a controlled manner to either the C6 or the C8 position of the A ring, or both, of the flavonoid structure, thus preventing disruption of the flavonoid structure, particularly the B and C rings of the flavonoid, and thus preserving the beneficial biological and pharmacological properties of the flavonoid.

[0046] The polymer is conjugated to the catechin-based flavonoid via a reaction of the aldehyde group of the polymer with the C6 and/or the C8 position of the A ring of the catechin-based flavonoid.

[0047] The conjugate is synthesized using acid catalysis of a condensation of the aldehyde group of the polymer with the catechin-based flavonoid, or using acid to convert a functional group on the polymer to a free aldehyde prior to condensation of the aldehyde group with the catechin-based flavonoid.

[0048] To conjugate the polymer and the catechin-based flavonoid, the polymer and the catechin-based flavonoid may be separately dissolved in a suitable solvent. The polymer with the free aldehyde is added, for example by dropwise addition, to the solution containing the catechin-based flavonoid, in the presence of an acid. The reaction is allowed to go to completion. Following the conjugation reaction, excess unreacted polymer or catechin-based flavonoid can be removed from the conjugated composition, for example by dialysis or by molecular sieving.

[0049] The ratio of catechin-based flavonoid to polymer may be varied, so that there is only one polymer moiety attached to the catechin-based flavonoid portion of the polymer, or so that there is a catechin-based flavonoid portion attached at more than one position on the polymer, or so that the catechin-based flavonoid portion has

two polymer portions attached, one at either of the C6 and C8 positions of the catechin-based flavonoid.

[0050] The ratio of polymer to catechin-based flavonoid in the final composition can be controlled through the ratio of starting reagents. For example, when the molar ratio of polymer moiety to catechin-based flavonoid moiety is about 1, a single polymer moiety will be attached to a single catechin-based flavonoid moiety (either monomeric or oligomeric may be used). However, at higher concentrations of polymer, for example at a 10:1 molar ratio of polymer to catechin-based flavonoid, a composition having a tri-block structure of polymer-flavonoid-polymer may be obtained.

[0051] A conjugate of a polymer containing a free aldehyde and a catechin-based flavonoid, having the polymer conjugated at the C6 and/or the C8 position of the A ring of the flavonoid is also contemplated.

[0052] Conjugation of the polymer also allows for the incorporation of catechin-based flavonoids into various compositions or vehicles. By selection of the particular polymer containing a free aldehyde group based on the physical properties of the polymer, it is possible to incorporate flavonoids into a variety of different vehicle types, allowing for the delivery of high concentrations of flavonoids in different contexts to various targeted areas of the body.

[0053] Thus, the conjugate resulting from the above-described reaction may be formed into a delivery vehicle, depending on the nature of the polymer portion of the conjugate. The delivery vehicle may be used to deliver the catechin-based flavonoid to a body, including a particular targeted site in a body, depending on the nature of the delivery vehicle.

Anti-cancer Agents

[0054] An anti-cancer agent is included in the delivery vehicle, which is then contacted with a cell to which the anti-cancer agent is to be delivered. Thus, there is provided a delivery vehicle comprising a catechin-based flavonoid conjugated to a polymer through a free aldehyde group on the polymer, the delivery vehicle further comprising an anti-cancer agent.

[0055] The anti-cancer agent may be any agent that has an anti-cancer effect on a cell, including an anti-tumour effect, such as a cytotoxic, apoptotic, anti-mitotic anti-angiogenesis or inhibition of metastasis effect. The anti-cancer effect is intended to include inhibition or reduction of tumour cell growth, inhibition or reduction of carcinogenesis, killing of tumour cells, or inhibition or reduction of carcinogenic or tumorigenic properties of a cell, including a tumour cell.

[0056] An anti-cancer agent includes a protein, a nucleic acid, a small molecule or a drug. An anti-cancer agent that is a protein may be a peptide, an antibody, a hormone, an enzyme, a growth factor, or a cytokine. An anti-cancer agent that is a nucleic acid may be single stranded or double stranded DNA or RNA, a short hairpin RNA, an siRNA, or may comprise a gene encoding an anti-cancer product. Also included in the scope of anti-cancer agent is a chemotherapeutic agent or an angiogenesis inhibitor. The anti-cancer agent may be an antibody, including a monoclonal antibody, directed against a tumour cell-surface marker, an immunoregulatory peptide, a cytokine or a growth factor. The anti-cancer agent may be Herceptin (trastuzumab) or TNP470.

Formation of Delivery Vehicle Containing Anti-cancer Agent

[0057] In one particular embodiment, the delivery vehicle is a micellar nanocomplex, which is suitable for parenteral delivery of a catechin-based flavonoid and an anti-cancer agent to a cell, including a cell located at a particular site within a body of a subject.

[0058] To form the delivery vehicle containing the anti-cancer agent, the polymer is chosen to have properties that allow it to assemble with the catechin-based flavonoid portion of the composition, protecting the flavonoid from the solution environment. If a suitable solvent is chosen in which the polymer portion of the conjugate is soluble and is more soluble than the catechin-based flavonoid, the conjugate will self-assemble, excluding the solution from the flavonoid core, thus allowing for assembly of micellar complexes.

[0059] In a particular embodiment of the micellar nanocomplex delivery vehicle, the polymer chosen is aldehyde-terminated PEG, or a derivative thereof. PEG is a polymer widely used as a pharmacological ingredient, and possesses good

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hydrophilic, non-toxic, non-immunogenic and biocompatibility characteristics with low biodegradability.

[0060] By conjugating PEG-CHO to a catechin-based flavonoid, a conjugate is formed that has strong self-assembly tendencies. In one embodiment, PEG is conjugated to a monomer of a catechin-based flavonoid, to form a PEG-flavonoid. The delivery vehicle is formed together with non-conjugated catechin-based flavonoids and the anti-cancer agent. Thus, the central core contains relatively high concentrations of a flavonoid and the anti-cancer agent, while the external shell of the micellar nanocomplex comprises the conjugated PEG-monomeric flavonoid, and is assembled in a two-step process. In a particular embodiment, the central core is oligomeric EGCG (OEGCG) and the external core is made up of conjugated PEG-EGCG.

[0061] Formation of this two-step assembly of the delivery vehicle results in temporary partial or complete masking of the biological activities of the oligomeric flavonoid that is incorporated into the core of the delivery vehicle, as well as protecting the anti-cancer agent from degradation prior to delivery to the cell. For example, while assembled into core of the delivery vehicle, the augmented properties of the oligomerized EGCG are less available, due to physical interactions with other molecules in the assembled core portion of the delivery vehicle. Upon release from the delivery vehicle, for example by fusion of the vehicle with a cellular phospholipid membrane, the components of the delivery dissociate, unmasking the biological properties of the oligomeric catechin-based flavonoid, and releasing the anti-cancer agent for delivery into the cell.

[0062] This embodiment of the delivery vehicle is well suited to deliver the anti-cancer agent. Since the catechin-based flavonoids have a rigid, multi-ring core structure, these molecules associate well with anti-cancer agents such as proteins and nucleic acids, as well as other molecules containing ring structures, likely by stacking of the catechin rings with the ring or rings on the anti-cancer agent. Thus, an oligomeric catechin-based flavonoid can be used to associate with the anti-cancer agent prior to assembly in the micellar nanocomplex.

[0063] The concentration of the anti-cancer agent is chosen depending on the total

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WHAT IS CLAIMED IS:

1. A delivery vehicle comprising an anti-cancer agent and a conjugate of a delivery agent containing a free aldehyde and a flavonoid, having the delivery agent conjugated at the C6 and/or the C8 position of the A ring of the flavonoid.
2. The delivery vehicle of claim 1 wherein the delivery agent is a polymer and wherein the flavonoid is a catechin-based flavonoid.
3. The delivery vehicle of claim 2 wherein the flavonoid is a catechin-based flavonoid that is (-)-epicatechin, (-)-epigallocatechin, (+)-catechin, (-)-epicatechin gallate or (-)-epigallocatechin gallate.
4. The delivery vehicle of any one of claims 1 to 3 wherein the flavonoid is monomeric or wherein the flavonoid is oligomeric.
5. The delivery vehicle of claim 4 wherein the flavonoid is oligomeric and is oligomerized through: (i) enzyme-catalyzed oxidative coupling; (ii) aldehyde-mediated oligomerization; or (iii) a carbon-carbon linkage between the C6 or C8 position on the A ring of a first monomeric unit to the C6 or C8 position on the A ring of a second monomeric unit.
6. The delivery vehicle of claim 5 wherein the flavonoid is oligomerized via a carbon-carbon linkage between the C6 position on the A ring of the first monomeric unit to the C8 position on the A ring of the second monomeric unit or wherein the flavonoid is oligomeric (-)-epigallocatechin gallate (OEGCG) oligomerized through carbon-carbon C6-C6, C6-C8, C8-C6 or C8-C8 linkages of (-)-epigallocatechin gallate monomers.
7. The delivery vehicle of claim 1 wherein the polymer is aldehyde-terminated poly(ethylene glycol), aldehyde-derivatized hyaluronic acid, hyaluronic acid aminoacetylaldehyde diethylacetal conjugate, aldehyde-derivatized hyaluronic acid-tyramine, hyaluronic acid aminoacetylaldehyde diethylacetal conjugate-tyramine, cyclotriphosphazene core phenoxymethyl(methylhydrazono)dendrimer or thiophosphoryl core phenoxymethyl(methylhydrazono)dendrimer.

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FIGURE 1

(57) Abstract: There is provided a conjugate of a delivery agent containing a chemical moiety and at least one flavonoid. The flavonoid exists in a monomeric form or dimeric form before conjugation and remains in the monomeric form or dimeric form after conjugation. Preferably, the conjugate comprises two flavonoids. The delivery agent is conjugated at the C6 and/or the C8 position of the A ring of the flavonoid. An anti-cancer agent delivery vehicle comprising an anti-cancer agent and the conjugate is also provided.

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FIG. 1 shows a scheme illustrating the self-assembly of poly ethylene glycol/(-)-epigallocatechin gallate (PEG-EGCG) conjugates loaded with doxorubicin;

FIG. 2 demonstrates the formation of PEG-EGCG conjugates loaded with doxorubicin at varying initial PEG-EGCG concentrations. The numeral reference
5 below each bottle sample represents the initial PEG-EGCG concentration in mg/ml;

FIG. 3 shows the electrospray ionization time-of-flight mass spectra of (a) PEG and (b) PEG-EGCG, respectively;

FIG. 4 shows a scheme illustrating the self-assembly of PEG-EGCG conjugates
10 loaded with cisplatin and/or herceptin; and

FIG. 5 shows the in-vivo HER2-overexpressed human breast cancer cell (BT-474) xenograft to Athymic Nude-Foxn1nu (n-6) with PEG-EGCG conjugates loaded with herceptin.

FIG. 6 shows confocal images of Cy3-labeled negative control siRNA (red)
15 counterstained with a nuclei stain, DAPI (Blue) 24 hours after siRNA transfection. siRNA concentration was 50nM. The overlay images show that the siRNA was localized only in the cytoplasm and not in the nuclei. (A) No PEG-EGCG; (B) 1.5µg/ml PEG-EGCG; (C) 3.0µg/ml PEG-EGCG; (D) 6.0µg/ml and (E) PEG alone.

FIG. 7 shows delivery of 200nM siRNA to pGL3 DNA transfected cells using
20 PEG-EGCG. Ratio of firefly to *renilla* luciferase activities were normalized against cell population transfected only with pGL3 DNA. Control refers to pGL3 DNA transfected cells treated with siRNA alone. siPORT™ is a polyamine-based transfection agent used as a positive control. Luciferase activities were measured 48 hours after transfection without change of medium. Data are means of 3
25 experiments; S.D. is shown.

FIG. 8 shows fluorescence observation of GFP downregulation 48 hours after GFP-22 siRNA transfection. The microscopic images on the right shows the downregulation of GFP compared to the control microscopic images on the left. siPORT™ is a polyamine-based transfection agent used as a positive control. (A)

No siRNA transfection; (B) siPORT™ + GFP-22 siRNA; (C) PEG-EGCG + control siRNA; (D) PEG-EGCG + GFP-22 siRNA. Figure 8 E & F show flow cytometric analysis of cells 48 hours after siRNA transfection. 1000 to 1200 cell events were counted. (E) PEG-EGCG + control siRNA; (F) PEG-EGCG + GFP-22 siRNA.

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DETAILED DESCRIPTION

The present invention relates generally to anti-cancer agent delivery vehicles capable of improved drug loading.

The present inventors have in previous attempts developed anti-cancer agent
10 delivery vehicles and methods of the delivery. In WO 2006/124000, conjugates of a delivery agent containing a free aldehyde and a flavonoid where the delivery agent is conjugated at the C6 and/or the C8 position of the A ring of the flavonoid have been provided. In WO 2009/054813, delivery vehicles comprising an anti-cancer agent and a conjugate of a delivery agent containing a free aldehyde and a
15 flavonoid, where the delivery agent is conjugated at the C6 and/or the C8 position of the A ring of the flavonoid have been provided. The contents of both WO 2006/124000 and WO 2009/054813 are herein incorporated by way of reference.

Surprisingly, the inventors have found that the combination of a delivery vehicle containing a chemical moiety and at least one flavonoid, and a bioactive anti-
20 cancer agent has a synergistic anti-cancer effect, greater than the combined effects of each of the delivery vehicle and bioactive anti-cancer agent when used alone. Thus, such delivery vehicles loaded with an anti-cancer agent provide an effective way of delivering anti-cancer agents to a cell, taking advantage of the synergistic effect between the anti-cancer activity of the flavonoid portion of the
25 delivery vehicle and the anti-cancer effect of the anti-cancer agent. It is believed that the synergistic effect and therefore a higher anti-cancer agent loading is due to the flavonoid remaining in its original monomeric form or dimeric form after conjugation with the chemical moiety.

In accordance with one aspect of the invention, there is provided a conjugate of
30 a delivery agent containing a chemical moiety and at least one flavonoid, wherein

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In certain embodiments, the flavonoid may be EGCG containing carboxyl-terminated group, amine-terminated group, succinimide-terminated group, or any other group suitable for conjugating with the chemical moiety.

Preferably, the delivery agent contains a chemical moiety selected from the

5 group consisting of a polymer having a free aldehyde group or a functional group capable of being converted to an aldehyde group, a polymer having a free carboxyl group or a functional group capable of being converted to a carboxyl group, a polymer having a free amine group or a functional group capable of being converted to an amine group, a polymer having a free succinimide group or a

10 functional group capable of being converted to a succinimide group, and a mixture thereof. In certain embodiments, the polymer is a carboxyl-terminated poly(ethylene glycol) or succinimide-terminated poly(ethylene glycol) which can react with amine-terminated EGCG to form the conjugate. In alternative

embodiments, the polymer is an amine-terminated poly(ethylene glycol) which can

15 react with carboxyl-terminated EGCG or succinimide-terminated EGCG to form the conjugate.

The delivery agent is capable of being formed into a delivery vehicle, thus allowing for the incorporation of a conjugated flavonoid into the delivery vehicle without compromising the biological or pharmacological properties of the flavonoid.

20 As well, the delivery agent should be biocompatible, and may be biodegradable in some embodiments.

The following discussion refers to an embodiment in which the flavonoid is a catechin-based flavonoid and in which the delivery agent is an aldehyde-terminated polymer. However, it will be understood that the aldehyde

25 condensation reaction between an aldehyde-containing chemical group and a flavonoid is applicable to conjugation of any delivery agent having a functional group described above, including following acid treatment of the delivery agent, to any flavonoid.

Thus, the reaction may involve conjugation of a polymer containing a free

30 aldehyde group or a group that is able to be converted to a free aldehyde group, for example, in the presence of an acid to a catechin-based flavonoid.

The catechin-based flavonoid may be used in its monomeric form or dimeric form and it is important to retain the monomeric form or dimeric form of the flavonoid during conjugation so that the conjugation does not result in the formation of oligomers of the flavonoid. As stated above, conjugation of a polymer
5 to a flavonoid results in augmentation of the flavonoid's biological or pharmacological properties.

The polymer may be any polymer having a free aldehyde group prior to conjugation with the catechin-based flavonoid, or having a group that is converted to an aldehyde group in the presence of an acid, for example an acetal group.
10 Furthermore, it will be understood that the polymer should be non-toxic, biocompatible and suitable for pharmacological use. The polymer may also have other desirable properties. For example, the polymer may have low immunogenicity, and it may be biodegradable or non-biodegradable depending on the desired biological application of the composition, for example, for controlled
15 release of catechin-based flavonoids and the anti-cancer agent at a particular site in a body.

The polymer may be chosen based on its particular characteristics and its ability to form certain types of delivery vehicles. For example, the polymer may be an aldehyde-terminated poly(ethylene glycol). Alternatively, the polymer may be an
20 aldehyde-derivatized hyaluronic acid, hyaluronic acid aminoacetylaldehyde diethylacetal conjugate, aldehyde-derivatized hyaluronic acid-tyramine, hyaluronic acid aminoacetylaldehyde diethylacetal conjugate-tyramine, cyclotriphosphazene core phenoxymethyl(methylhydrazono) dendrimer, or thiophosphoryl core phenoxymethyl(methylhydrazono) dendrimer. The polymer may also be any
25 biological polymer, modified to contain a free aldehyde group or a group that is convertible to an aldehyde in the presence of an acid, for example an aldehyde-modified protein, peptide, polysaccharide or nucleic acid. In one particular embodiment, the polymer is an aldehyde-terminated poly(ethylene glycol) (PEG-CHO). PEG is particularly chosen to form the conjugate because PEG is a
30 polymer widely used as a pharmacological ingredient, and possesses good hydrophilic, non-toxic, non-immunogenic and biocompatibility characteristics with low biodegradability.

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platinum, carboplatin, gemcitabine, paclitaxel, docetaxel, etoposide, vinorelbine, topotecan, or irinotecan. Enzymes inducing apoptosis may include TRAIL-R1, TRAIL-R2 or FasL. Nucleic acid anti-cancer agents may include plasmid DNA (encoding therapeutic proteins) or antisense oligonucleotides (ODNs) or small
5 interfering RNA (siRNA). ODN is a short single stranded DNA, which is complementary to the transcribed mRNA (sense sequence), and hence can bind to this mRNA and prevent it from being translated into the protein inside the cells. Alternatively, ODNs can be targeted to bind to a splicing site of a pre-mRNA, and aimed to modify the exon end of the mRNA. siRNA, on the other hand, is a short
10 double or single stranded RNA, which assembles into endoribonuclease-containing complexes upon entering cells, also known as RNA-induced silencing complex (RISC). These siRNA molecules then unwind inside the RISC, making the complex activated to recognize and splice its mRNA target strands in the cells, and thus down-regulating the expression of the target protein. Similarly micro-RNA
15 capable of inducing apoptosis may be initiated by a specific micro-RNA. The micro-RNA may include: MiR-15, MiR-16, MiR-99a/let7c/MiR-125b2 or other suitable pro-apoptotic micro-RNA.

The biological activity of the anti-cancer agent is temporarily partially or completely masked while incorporated into the present delivery vehicles, making
20 them less available while the anti-cancer agent is assembled in the delivery vehicle, meaning that the anti-cancer agent is not able to exert anti-cancer activity or interact with other molecules in a bioactive manner while contained in the delivery vehicle, and is also protected from activity of other molecules. Upon release of the anti-cancer agent from the delivery vehicle, the biological properties
25 of the anti-cancer agent are once again available, and the anti-cancer agent is able to exert an anti-cancer effect once delivered to the cell.

In one embodiment an anti-cancer agent is loaded into the conjugate or delivery agent. The anti-cancer agent may be loaded or encapsulated in the conjugate or anti-cancer delivery vehicle through hydrophobic interactions and/or π - π stacking
30 interactions with the flavonoid.

The drug loading of the anti-cancer agent in the delivery agent of the invention is preferably more than other known loading of drugs in delivery agents. Drug loading is defined as the weight percentage of the anti-cancer agent in the delivery vehicle, while drug loading efficiency is defined as the weight percentage of the entrapped anti-cancer agent compared to the free anti-cancer agent introduced initially, as described in the examples. The drug loading may be increased by 1.5 to 5 times the reported range of drug loading. Preferably the drug loading is increased by 1.75 to 4.25 times the reported range of drug loading. In the case of antibodies and siRNA where drug loading is not so well established we have demonstrated that a polypeptide and a nucleic acid is not only able to be successfully loaded into the delivery agent but can also be successfully delivered *in vivo* and *in vitro*.

The ratio of drug to conjugate can range from 6:1 to 1:6. In particular embodiment's ratios of doxorubicin to PEG/EGCG were successful at ratios of 6:1, 4:1, 2:1 1:1, and 1:2. Preferably the amount of anti-cancer agent is greater than the amount of conjugate provided the anti-cancer agent is able to form an interaction with the flavonoid.

EXAMPLES

(-)-Epigallocatechin gallate (EGCG), a main ingredient of green tea, exhibits numerous biological and pharmacological effects. In the following examples, conjugates of poly(ethylene glycol) with EGCG were synthesized using aldehyde-mediated condensation by an acid catalyst.

Example 1: Formation of Delivery Vehicle Comprising Doxorubicin and PEG-EGCG Conjugates

In one embodiment, the drug molecule is doxorubicin ("Dox" for short). **FIG. 1** shows a scheme illustrating the self-assembly of PEG-EGCG conjugates loaded with Dox to form the delivery vehicle. In this embodiment, the delivery vehicle is in the form of a micelle. It is based on the self-assembly of PEG-EGCG conjugates into micelles in aqueous environment, stemming from the hydrophilic nature of the PEG chain of the PEG-EGCG conjugate. Due to the similarity in the multi-ring structures of the EGCG moiety and Dox, the EGCG moiety is designed to

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encapsulate Dox through the hydrophobic interactions and π - π stacking interactions. Dox was successfully encapsulated within the delivery vehicles by simple mixing and dialysis. Advantageously, It is found that the delivery vehicles demonstrate exceptionally high Dox loading in comparison to other delivery vehicles systems (e.g. polymeric micellar systems) known in the prior art. Dynamic light scattering (DLS) measurements also showed that the dimensions of the delivery vehicles are ideal for passive tumor targeting via EPR effect.

In this example, the delivery vehicle was prepared by dialysis of a mixture of PEG-EGCG conjugates and Dox dissolved in organic solvent dimethylformamide (DMF) against distilled water. Different concentrations of PEG-EGCG conjugates (0, 0.5, 1, 3, 6, 12, 24 mg/ml, respectively) were used to encapsulate a fixed concentration of Dox at 12 mg/ml. During the dialysis, the gradual replacement of the organic solvent with water triggered the self-assembly of the delivery vehicles and the entrapment of Dox within the assembled structures. As shown in FIG. 2, precipitates were obtained during the dialysis when PEG-EGCG concentrations of 0, 0.5 and 1 mg/ml were used, indicating ineffective delivery vehicle formation. In contrast, with PEG-EGCG concentrations of 3, 6, 12 and 24 mg/ml, homogeneous solutions were achieved, demonstrating the successful encapsulation of Dox within the delivery vehicles. This example indicated that a suitable ratio of Dox to PEG-EGCG conjugates should be carefully selected in order for the Dox/PEG-EGCG delivery vehicles to self-assemble effectively.

Experimental

Materials: Aldehyde-terminated PEG (PEG-CHO, Mw 5000) was purchased from NOF Co., Japan. EGCG was purchased from Kurita Ltd., Japan. Doxorubicin hydrochloride (Dox-HCl) was obtained from Boryung Pharmaceuticals Inc., Korea.

Synthesis of PEG-EGCG Conjugate: The aldehyde-terminated PEG (0.35 g) and EGCG (0.65 g) were separately dissolved in a mixture of acetic acid, water and dimethyl sulfoxide (DMSO). The reaction was started with the dropwise addition of the PEG-CHO solution at 20°C (pH 2) under a nitrogen atmosphere for 48 h. The resulting products were dialyzed (molecular weight cut-off (MWCO) = 3500) and lyophilized to give the PEG-EGCG conjugate.

Synthesis of Delivery Vehicle: Dox·HCl (12 mg, 21 μ mol) was dissolved in 1 ml of DMF. Triethylamine (29 μ l, 210 μ mol) was then added to the solution to neutralize the hydrochloride. Next, PEG-EGCG conjugates (1 ml) with different concentrations dissolved in DMF were added to the solution, and the resulting mixtures were vortexed for 20 min. Each sample solution was then transferred to dialysis tubes with a MWCO of 3500 Da. The tubes were dialyzed against distilled water for 2 days, with the water replaced every 12 h. The sample solutions were lyophilized to obtain solid micelles.

Determination of Drug Loading: The absorbance of the sample solutions was measured at 480 nm using a Hitachi U2810 UV-Vis spectrophotometer after the dried samples were dissolved in DMF. The amount of Dox in each sample was estimated by comparing to the Dox standards. Drug loading was calculated from the amount of Dox in the delivery vehicles, and the weight of the dried delivery vehicles samples.

Determination of Delivery Vehicle Size: The size of the delivery vehicle samples was determined by DLS using a Brookhaven 90Plus Particle Size Analyzer. Each sample solution was prepared using distilled water to give 10 μ g/ml of Dox before conducting the DLS measurements at 25°C.

Results: characteristics of the delivery vehicle

The characteristics of the delivery vehicle of Example 1 are summarized in **Table 1** below. Drug loading is defined as the weight percentage of Dox in the delivery vehicle, while drug loading efficiency is defined as the weight percentage of the entrapped Dox compared to the free Dox introduced initially. The samples with initial PEG-EGCG concentrations of 3 and 6 mg/ml showed an extraordinarily high drug loading of 85 and 68 wt%, respectively. The highest Dox loading achieved (85 wt%) corresponded to more than 4 times the average Dox loading (10–20 wt%) in the block copolymeric delivery vehicles developed by other groups (H. M. Aliabadi *et al*, *Expert Opinion on Drug Delivery*, 2006, **3**, 139-162). Without being limited to any particular mechanism it is hypothesised that such a high drug loading is possibly due to the strong hydrophobic interaction and/or π - π stacking interactions between the EGCG moiety and Dox, which would possibly enhance

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the encapsulation of Dox in the self-assembly process, although the EGCG moiety was much smaller than the hydrophobic chain of other PEG block copolymers reported.

The size of the delivery vehicles ranged from 78.5 to 192.4 nm (see **Table 1**).
5 The size increased as the drug loading increased, but decreased with increasing initial PEG-EGCG concentration. The increase in size should be correlated to the encapsulation efficiency since the EGCG moiety played a key role in encapsulating Dox. Thus, it is expected that not only the size, but also the drug release profile and the stability of delivery vehicles, which are important aspects
10 for any targeted drug delivery system, could be controlled by the initial PEG-EGCG concentration introduced. The nanometer dimensions of the delivery vehicles (less than 200 nm) conferred the ability of targeting tumor tissue via EPR effect. At the same time, they enabled prolonged blood residence time, as they helped to avoid opsonization and renal clearance of delivery vehicles.

Samples	Initial PEG-EGCG concentration (mg/ml)	Initial Dox concentration (mg/ml)	Average hydrodynamic diameter (nm)*	Drug loading efficiency (wt%)	Drug loading (wt%)
1	3	12	192	60	85
2	6	12	129	77	68
3	12	12	115	77	49
4	24	12	79	79	35

15 *Determined by DLS measurements.

Table 1. Characteristics of PEG-EGCG Conjugates Loaded with Dox in Accordance with an Embodiment of the Present Invention

The electrospray ionization time-of-flight (ESI-TOF MS) mass spectra of (a)
20 PEG and (b) PEG-EGCG, respectively, are shown in **FIG. 3**. The m/z difference between two clusters of peaks was observed as a PEG repeating unit of 44 for both PEG and PEG-EGCG taking account of a charge of +5 calculated from 0.2 m/z difference between each peak (e.g. (1037.26-1028.43) x 5 = 44.15). However,