**Centro de Investigación en Alimentación y Desarrollo, A.C.**

# EVALUACIÓN DE LA ACTIVIDAD ANTICANCERÍGENA DE NANOPARTÍCULAS DE MAGNETITA FUNCIONALIZADAS CON α-TOCOFERIL SUCCINATO

---

Por:

Aracely Angulo Molina

TESIS APROBADA POR LA:

COORDINACIÓN DE NUTRICIÓN

Como requisito para obtener el grado de:

DOCTOR EN CIENCIAS

Hermosillo, Sonora Diciembre del 2013

## APROBACIÓN

Los miembros del comité designado para revisar la tesis de Aracely Angulo Molina, la han encontrado satisfactoria y recomiendan que sea aceptada como requisito parcial para obtener el grado de Doctor en Ciencias.

Dr. Jesús Hernández López
Director de Tesis

Dr. Julio Reyes Leyva
Co-director de Tesis

Dra. Verónica Mata Haro
Asesora

Dra. Silvia Y. Moya Camarena
Asesora

Dr. Aurelio López Malo
Asesor

## DECLARACION INSTITUCIONAL

Se permiten y agradecen las citas breves del material contenido en esta tesis sin permiso especial del autor, siempre y cuando se dé el crédito correspondiente. Para la reproducción parcial o total de la tesis con fines académicos, se deberá contar con la autorización escrita del director del Centro de Investigación en Alimentación y Desarrollo A.C. (CIAD, A.C.).

La publicación en comunicaciones científicas o de divulgación popular de los datos contenidos en esta tesis, deberá dar créditos a CIAD, A.C., previa aprobación escrita del manuscrito en cuestión del director de tesis.

Dr. Pablo Wong González
Director General del CIAD, A.C.

## DEDICATORIA

A cada uno de los integrantes de mi familia y amigos. Los que están cerca, los que están lejos y los que ya no están. Los quiero.

**Aracely**

## AGRADECIMIENTOS

Al Consejo Nacional de Ciencia y Tecnología, CONACYT.

Al Centro de Investigación en Alimentación y Desarrollo, A.C.

Al proyecto SEP-CONACYT No. 154602 (Fondo de Investigación Científica Básica).

A la Dra. Gloria Yépiz, Coordinadora del Posgrado del Centro de Investigación en Alimentación y Desarrollo, A.C.

A Laura García, Argelia Marín, Verónica Araiza y Héctor Galindo de la Dirección de Posgrado, así como Aurora Vidal del área de videoconferencia del Centro de Investigación en Alimentación y Desarrollo, A.C.

A la fundación Carrillo-Angulo de Puebla.

Al personal y estudiantes del Centro de Investigación Biomédica de Oriente (CIBIOR) del IMSS, Metepec, Puebla.

A los nanotecnólogos Dra. Teresa Palacios y Dr. Miguel Méndez, del Laboratorio de Nanotecnología de la UDLAP.

A los estudiantes, técnicos e investigadores del Laboratorio de Inmunología de la Dirección de Nutrición del Centro de Investigación en Alimentación y Desarrollo, A.C.

Al Centro de Nanociencias y Nanotecnología (CNYN) de la UNAM, de Ensenada, BCN.

A los estudiantes becarios, tesistas, directivos, profesores, personal de vigilancia y de videoconferencias de la Universidad de las Américas Puebla (UDLAP).

A los estudiantes becarios del Instituto Tecnológico de Tlaxcala (ITT).

Al Dr. Carlos Escamilla y Dr. Francisco Collazo del Bioterio Jean Claude Bernard de la Benemérita Universidad Autónoma de Puebla (BUAP).

Al MC Fidel Pacheco y MC Iracema Valeriano del Centro de Alta Tecnología de la Universidad Popular Autónoma del Estado de Puebla (UPAEP).

Al Dr. Octavio Villanueva del Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ).

Al Dr. Salomón Hernández de la Universidad Panamericana.

Al personal del laboratorio de patología del ISSSTEP y del Hospital del Niño Poblano (HNP).

A los centros de radiodiagnóstico del Hospital Betania, Radiodiagnóstico Calderón y del Hospital veterinario UPAEP en Puebla.

Al Dr. Marcus Textor del Swiss Federal Institute of Technology Zurich (ETH).

A la Dra. Ofelia Olivero del National Institute of Health (NIH) de Estados Unidos.

A los coautores de los artículos y anexos presentados en este trabajo.

A los profesores de cursos a distancia y presenciales que participaron en mi formación doctoral:
Dra. Ana María Calderón de la Barca, Dra. Juana María Meléndez, Dra. Teresa Gollas Galván, Dra. Herlinda Soto, Dr. Alfonso Gardea, Dr. Francisco Vargas, Dr. Jesús Hernández, Dr. Ramón Pacheco, Dra. Verónica Mata, Dr. Julio Reyes, Dra. Verónica Vallejo, Dra. Lilián Flores, Dr. Gerardo López y Dra. Virginia Sedeño.
Trabajar a distancia fue un reto compartido.

Gracias a la Dra. Verónica Mata, Dra. Silvia Moya y Dr. Aurelio López Malo, integrantes del comité de tesis por el apoyo constante al desarrollo de este

proyecto. Gracias a todo el comité por su guía, crítica constructiva, infraestructura, paciencia, ejemplo y amistad.

Y especialmente al Dr. Jesús Hernández y al Dr. Julio Reyes, director y co-director de esta tesis, gracias por el voto de confianza, amistad y paciencia.

**Gracias a todos por ser parte del primer proyecto de Doctorado en Ciencias, opción a Distancia del CIAD.**

**Aracely**

Este trabajo se realizó en las instalaciones del Laboratorio de Inmunología del Centro de Investigación en Alimentación y Desarrollo (CIAD) A. C., en el Centro de Investigación Biomédica de Oriente (CIBIOR) del IMSS, así como el Laboratorio de Nanotecnología de la UDLAP, bajo la dirección del Dr. Jesús Hernández y el Dr. Julio Reyes Leyva.

## CONTENIDO

Resumen ..... xi

Introducción General ..... 1

Integración del Trabajo de Investigación ..... 10

Hipótesis ..... 14

Objetivo General ..... 15

Objetivos Específicos ..... 15

Capítulo I ..... 16
El Papel del Alfa Tocoferil Succinato (α-TOS) como un Agente Anticancerígeno Potencial

Capítulo II ..... 27
Nanopartículas de Magnetita Funcionalizadas con α-Tocoferil Succinato (α-TOS) Promueve la Muerte Selectiva de Células de Cáncer de Cérvix

Capítulo III ..... 57
Nanopartículas de Magnetita Funcionalizadas con α-Tocoferil Succinato: Distribución *in vivo* y Actividad antitumoral en un Modelo de Melanoma

Anexos ..... 89

Anexo I ..... 90

**CONTENIDO** (continuación)

Riesgos Ambientales de la Nanotecnología: Evaluando la Ecotoxicidad de Nanomateriales

Anexo II ..... 120
Nutrición y Biotecnología Alimentaria. Bases para la Sustentabilidad Social

Anexo III ..... 144
Presentación en Congresos y Estancias

# RESUMEN

Las nanopartículas de magnetita (Nps) poseen propiedades físicas y químicas que les permite funcionar como una plataforma para proteger y acarrear principios activos a través de la funcionalización a su superficie. El alfa-tocoferil succinato (α-TOS), un análogo de la vitamina E, induce de manera selectiva la muerte de una amplia variedad de células tumorales con efectos mínimos o nulos en células normales. Un problema de este análogo es la pérdida de su bioactividad por la susceptibilidad a las enzimas esterasas presentes en algunas células tumorales. La susceptibilidad del α-TOS a las esterasas puede evitarse a través de su funcionalización a Nps. Además, el proceso de funcionalización se ha asociado a efectos antitumorales significativos con menores dosis del principio activo funcionalizado. Ambas aplicaciones no han sido descritas para análogos de vitamina E en cáncer de cérvix resistente y melanoma. El objetivo de este trabajo fue evaluar la actividad anticancerígena de α-TOS funcionalizado a Nps en modelos *in vitro* e *in vivo*. Se sintetizaron y caracterizaron las nanopartículas funcionalizadas con α-TOS (α-TOS-Nps). Se obtuvieron Nps de 15 nm con forma esférica irregular. Los análisis de espectroscopía de energía dispersiva y de difracción electrónica de área seleccionada confirmaron la cristalinidad de la magnetita ($Fe_3O_4$). La espectroscopía de infrarrojo confirmó la presencia de material orgánico en las α-TOS-Nps después de la funcionalización. La carga de α-TOS fue 8.14% con una eficiencia de atrapamiento del 31.4%. En la evaluación *in vitro* se observó un efecto citotóxico selectivo de α-TOS-Nps dosis y tiempo dependiente de 24-72 h ($p<0.05$) en las células de cáncer de cérvix (SiHa) resistentes al α-TOS, sin efectos en células normales. Para la evaluación *in vivo* se estableció un modelo tumoral de melanoma de células B16F0 trasplantadas en ratones desnudos Balb/c. El modelo se confirmó a las dos semanas con un tumor sólido bien delimitado y pseudoencapsulado con interior reblandecido de color negro con áreas necróticas y hemorrágicas. Al microscopio se identificó neoplasia

maligna de patrón sólido, células pleomórficas poligonales o redondas de núcleo redondo hipercromático. La cromatina se observó irregular y de grumos gruesos. Después del establecimiento del tumor los ratones fueron tratados intratumoralmente (i.t.) por 2 semanas con 0.075, 0.150, 1 y 2 mg de α-TOS-Nps. Se observó una disminución significativa del volumen tumoral a los 10 días después de iniciado el tratamiento con 0.75 mg y 2 mg de α-TOS-Nps ($p<0.05$), sin efectos tóxicos aparentes. Aunque no se observaron diferencias del patrón tumoral por ultrasonido y rayos X con los diferentes tratamientos, si se observó por histología un incremento notable de la necrosis tumoral a mayores dosis de α-TOS-Nps. Adicionalmente con la tinción azul de Prusia se observaron agregados de α-TOS-Nps a las dosis más altas en los tumores y se determinó su biodistribución en bazo, hígado, piel, pulmón, riñón e intestino sin daño tisular aparente en los órganos analizados. Los efectos observados con la aplicación *in vitro* e *in vivo* de α-TOS-Nps sugieren que la funcionalización de α-TOS a nanopartículas de magnetita tiene un uso potencial biomédico para mejorar la actividad antitumoral de este análogo en cáncer de cérvix y melanoma.

Palabras clave: alfa-tocoferil succinato, análogos de vitamina E, nanopartículas de magnetita, funcionalización, cáncer

## ABSTRACT

The iron oxide nanoparticles (Nps) possess exceptional physical and chemical properties that make them potential drug carriers. Nps can be coated and functionalized with bioactive ligands bound to the shell. Alpha-tocopheryl succinate (α-TOS), a vitamin E analogue, selectively kills a wide range of human cancer cells with no or low toxic effects for nonmalignant cells. However, a problem with α-TOS is its vulnerability to esterases in some cancer cells. The susceptibility of α-TOS to high levels of esterases could be protected by the conjugation of α-TOS with Nps. Additionally, functionalization has been associated with antitumor effects using minor doses of the drugs. These application has not been described for vitamin E analogues in cervix cancer and melanoma. In this work, we functionalized Nps with α-TOS (α-TOS-Nps) to evaluate its anticancer activity *in vitro* and *in vivo*. The nanoparticles were prepared and characterized. Electronic microscopy studies revealed sphere-like nanoparticles with a 15 nm average size. Inorganic chemical composition and magnetite crystalline phase was confirmed by energy dispersive X ray spectroscopy and selected area electron diffraction respectively. Organic and functional groups were analyzed by Fourier transform infrared spectroscopy. The load of α-TOS in the magnetite nanoparticles was estimated in 8.14% with an entrapment efficiency of 31.4%. The *in vitro* evaluation shows that α-TOS-Nps selectively affected the viability of cervical cancer cells, a resistant cell line, in a dose and time dependent way at 24-72 h ($p<0.05$) without toxic effects for nonmalignant cells. For *in vivo* evaluation, a melanoma model in female BALB/c nude mice was established. The model was confirmed two weeks later; a solid tumor formation was observed. Those tumors became large and grew quickly once they were palpable. Histological analysis revealed dermic tumor proliferation, some areas were highly pigmented with numerous necrotic areas with small hemorrhagic foci. Pleomorphic cells were also observed, it was characterized by rounded or polygonal cells with oval and hyperchromatic

nuclei. The chromatin was irregular and granular. The mice were i.t. treated with α-TOS-Nps (0.075, 0.150, 1 or 2 mg) for two weeks. A significant difference in the tumor growth was observed after 10 days of treatment with 0.75 and 2 mg of α-TOS-Nps ($p<0.05$), not apparently toxic effect in the mice was observed. Although there were not ultrasonography and X Ray changes in the pattern of the tumors, an increase of necrotic cell death and loss of viability in the melanoma tumor growth was observed in all the evaluated doses of α-TOS-Nps. Additionally, Prussian blue staining indicated the presence of larger aggregates inside of tumors in the higher doses of α-TOS-Nps and the biodistribution was evaluated as well. α-TOS-Nps was detected in spleen, liver, skin, kidney and gastrointestinal tract without apparently toxic effect in major organs. In conclusion, the *in vitro* and *in vivo* effects observed suggest that the functionalization of α-TOS with magnetite nanoparticles improve its bioactivity in cervix cancer and melanoma with a potential use in biomedical applications for the development of new cancer therapies.

Key words: alpha-tocopheryl succinate, vitamin E analogue, magnetite nanoparticles, functionalization, cancer

# INTRODUCCION GENERAL

El α-tocoferil succinato (α-TOS), uno de los análogos más representativos de la vitamina E, induce de manera selectiva la muerte de una amplia variedad de células tumorales *in vitro* e *in vivo* con efectos mínimos o nulos en células normales. Este análogo semisintético se deriva de la sustitución del grupo hidroxilo del carbono 6 del anillo cromanol del α-tocoferol por un succinato en la misma posición. El succinato es la molécula responsable de sus cualidades anticancerígenas, ya que se requiere que esté intacta para ejercer su bioactividad.

A diferencia del α-tocoferol, el α-TOS tiene potentes propiedades anticancerígenas generadas por su efecto en la desestabilización de la mitocondria, a través de la producción de especies reactivas de oxígeno (ERO). Las células tumorales tienen una defensa antioxidante deficiente, lo que promueve el aumento de la producción de ERO estimulada por α-TOS. Además, este análogo actúa de forma más eficiente a pH ácido, y las células tumorales tienen como característica un ambiente intracelular más ácido que las células normales. Ambos fenómenos favorecen la bioactividad selectiva de α-TOS y promueven la activación de la apoptosis por la vía mitocondrial. Por ello, al α-TOS se le considera dentro del grupo de los mitocanos, agentes capaces de inducir la muerte de células cancerígenas por la vía mitocondrial. Adicionalmente, el α-TOS es capaz de suprimir el número de tumores, disminuir el volumen tumoral, inhibir la metástasis, así como la angiogénesis.

Actualmente, gran parte de los tratamientos anticancerígenos como la radioterapia y la quimioterapia no distinguen entre células normales y anormales. Además, la gran mayoría presentan una alta toxicidad hacia células normales. En este sentido, dada la selectividad y alta bioactividad de α-TOS

por células tumorales, se le considera un agente anticancerígeno muy prometedor. Sin embargo, un problema de α-TOS es su vulnerabilidad hacia las enzimas esterasas. Varios reportes muestran que su bioactividad se ve afectada en células tumorales de cáncer de cérvix u ovario con altos niveles de esterasas. De ahí que se estén buscando formulaciones específicas para proteger o incrementar la bioactividad de este análogo.

En años recientes la utilización de nanoplataformas para acarrear, liberar e incrementar la bioactividad de principios activos en sitios específicos ha tomado gran auge. A nivel nanoescala algunos materiales tienen propiedades ópticas, magnéticas y mecánicas únicas que diversifican e incrementan las posibilidades de utilizarlos a la vez como agentes terapéuticos y de diagnóstico de cáncer. A este tipo de sustancias se les llama agentes teragnósticos. La posibilidad de funcionar de una u otra forma, o incluso de ambas depende de sus propiedades fisicoquímicas. De ahí la importancia de su caracterización, la cual permite conocer su composición química, su concentración, la estabilidad, biodisponibilidad, forma, tamaño, solubilidad, agregación y otras propiedades físicas que pueden influir en la interacción celular, así como con otras sustancias. Aunado a ello se debe considerar que a escala nanométrica las nanopartículas poseen una gran área superficial para conjugación o acoplamiento con diferentes agentes; éstos pueden unirse covalentemente, o pueden adsorberse o encapsularse en nanopartículas de 1-100 nm.

Las nanopartículas pueden modificarse superficialmente con diferentes recubrimientos para generar nanoplataformas multifuncionales que permiten no sólo transportar un medicamento, sino también modular la captura celular, la internalización y la especificidad tumoral. Por otra parte, se ha observado que ciertos nanomateriales cuando se administran *in vivo* pueden acumularse de forma preferencial en el tumor, fenómeno denominado retención pasiva intratumoral. Entre estos nanomateriales se encuentran las nanopartículas de óxidos metálicos como los de magnetita ($Fe_3O_4$). Las nanopartículas de

magnetita (Nps) poseen propiedades físicas y químicas excepcionales que les permite funcionar como una plataforma para proteger, acarrear y liberar principios activos a través de la funcionalización o acoplamiento de la molécula bioactiva a su superficie.

De gran importancia es que las Nps han mostrado biocompatibilidad y ya se aplican para estudios biomédicos de imagenología y de terapia hipertérmica. Las Nps pueden recubrirse *in situ* durante la nucleación y el proceso de crecimiento que ocurre en la reacción de síntesis. Además, también pueden ser recubiertas después de su producción, lo que amplía y diversifica sus aplicaciones.

Por ejemplo, cuando las Nps se sintetizan por el método de coprecipitación se generan Nps con grupos OH en su superficie. Estos grupos OH se pueden aprovechar para la reacción de silanización, que consiste en recubrir a la Nps con agentes silanos como el trimetoxisilano. Al recubrir las Nps con el agente silano (Nps silanizadas) quedan grupos amino expuestos que pueden utilizarse posteriormente para reacciones de acoplamiento de biomoléculas, proceso conocido como funcionalización. Posteriormente, a las Nps silanizadas se le puede unir por enlace covalente el ligando de interés mediante una reacción de condensación. Esta reacción se da entre un carboxilo libre del principio activo y el grupo amino libre del silano, generándose así un enlace amida.

Considerando que en la estructura del α-TOS las esterasas atacan el enlace éster entre el succinato y el α-tocoferol, su funcionalización a Nps silanizadas podría protegerlo del ataque enzimático evitando el reconocimiento. Esto puede ocurrir tanto por un efecto estérico como por la formación del enlace amida resistente a las esterasas. Con ello se favorecería que la molécula α-TOS permanezca intacta por más tiempo y pueda ejercer su acción.

Además, hay reportes que muestran que la funcionalización mejora la endocitosis y la respuesta biológica de otros principios activos *in vivo* a menores

dosis, aunado a una menor toxicidad. Las reacciones adversas asociadas a las altas dosis y la falta de selectividad de los agentes quimioterapéuticos actuales es uno de los retos más importantes en los últimos años para mejorar la calidad de vida del paciente con cáncer.

Por ello, debido a la aplicación potencial de las nanopartículas de magnetita para proteger o mejorar la respuesta anticancerígena de α-TOS, aunado a la búsqueda de terapias antitumorales alternativas con dosis bajas y menores efectos tóxicos, en este trabajo se evaluó la actividad anticancerígena *in vitro* e *in vivo* de nanopartículas de magnetita funcionalizadas con α-TOS.

El estudio se dividió en tres etapas, la primera donde se realizó la síntesis, funcionalización y caracterización de las nanopartículas. La 2da etapa fue la evaluación *in vitro*. En la 3ra etapa se realizó la evaluación *in vivo* en un modelo de cáncer murino.

En la primera etapa se utilizó el método de coprecipitación para la síntesis de las Nps de magnetita; posteriormente las Nps se silanizaron y se funcionalizaron con el análogo α-TOS (α-TOS-Nps). Después se realizó la caracterización morfométrica y físico-química. Los estudios de microscopía electrónica de transmisión y de barrido mostraron nanopartículas de 15 nm con forma esférica irregular y algunos agregados.

La funcionalización fue confirmada a través de técnicas espectroscópicas. Para la parte inorgánica se utilizó espectroscopía de energía dispersiva (EDS) y de difracción electrónica de área seleccionada (SAED). Con ellas se confirmó la estructura básica y cristalina de la magnetita ($Fe_3O_4$) con alta estabilidad la cual no se vio afectada por los diferentes procesos de recubrimiento. La espectroscopía de infrarrojo con trasformada de Fourier (IR-TF) confirmó la presencia de material orgánico en las α-TOS-Nps. Se detectaron los picos característicos de los grupos funcionales esperados por el acoplamiento del análogo de vitamina E, como los enlaces C-H de la cadena fitil, C=O del

succinato y del enlace C=N del grupo amida formado entre el grupo carboxilo extremo terminal del α-TOS y los grupos amino de la Np silanizada. El enlace amida confirmó la funcionalización. Además, con el análisis termogravimétrico se determinó una carga del 8.14% de α-TOS en las nanopartículas de magnetita, con una eficiencia de atrapamiento del 31.4% de la vitamina agregada en la reacción inicial de funcionalización.

La segunda etapa consistió en la evaluación *in vitro*. Para ello, después de la caracterización de las nanopartículas se procedió a realizar el ensayo de viabilidad/citotoxicidad del MTT en células SiHa, una línea celular de cáncer de cérvix no susceptible al α-TOS con un alto contenido en esterasas. Así mismo se utilizó una línea de fibroblastos normales. En el estudio se evaluó el efecto por separado de las Nps y de α-TOS, así como de las nanopartículas funcionalizadas α-TOS-Nps. Se encontró que las α-TOS-Nps fueron citotóxicas en dosis y tiempo dependiente de 24-72 h ($p<0.05$) con una $IC_{50}$ de 65.29 µg/mL y sin efectos significativos en células normales (fibroblastos) a las concentraciones evaluadas. En cambio tanto las Nps como la vitamina sola no afectaron a ninguna de las líneas celulares. Considerando que uno de los fines de la funcionalización fue generar un enlace amida entre la Np y la vitamina para hacerla resistente al ataque de las esterasas, se puede inferir que la funcionalización mantuvo la integridad de la vitamina y su bioactividad. Así mismo, los resultados demostraron que la funcionalización con nanopartículas de magnetita no afectaron la especificidad de la vitamina hacia células tumorales ni su biocompatibilidad en células normales.

Adicionalmente, para conocer la biodistribución intracelular de α-TOS-Nps, éstas se marcaron con fluoresceína (α-TOS-Nps-Fluor). Se observó mediante microscopía confocal que las nanopartículas empezaron a acumularse alrededor del núcleo a partir de las 24 h, siendo más evidente a las 48 h. Para las 72 h se observó la acumulación intranuclear de α-TOS-Nps-Fluor en las células muertas, las cuales fueron identificadas por citomorfología y con ioduro

de propidio. También se evaluó si las nanopartículas inducían apoptosis en la células SiHa de 24-72h con una dosis de 80 µg/mL. Para ello se buscó la presencia de ADN fragmentado con el fluorocromo naranja de acridina. Se observó un mayor número de células en apoptosis a dosis altas y con los tratamientos más prolongados con α-TOS-Nps. Esto demostró que al menos uno de los mecanismos de acción de las α-TOS-Nps para ejercer su capacidad anticancerígena está mediada por apoptosis.

En la tercera etapa se realizó la evaluación *in vivo* para conocer si la funcionalización mejoraba la bioactividad de α-TOS y si con ello se requerían dosis más bajas del tratamiento. Así, se procedió al establecimiento de un modelo tumoral de melanoma en ratones Balb/c desnudos nu/nu. Para la elección de este modelo se consideró que:

a) Existen reportes sobre la susceptibilidad del melanoma al α-TOS en modelos con ratones nu/nu; b) El melanoma ocupa el tercer lugar en incidencia de cáncer de piel con un 7.9%, es altamente metastásico y es la causa del 75% de muertes por cáncer de piel; c) Su incidencia en México se ha incrementado en un 20% en adultos en edad productiva, apareciendo a edades más tempranas, además se cree que hay un importante sub-registro de casos; d) Los tratamientos para melanoma son muy limitados; e) No existe mucha información sobre medicamentos contra el melanoma acoplados a nanopartículas; f) Reportes reciente muestran que las nanopartículas se internalizan y bio-distribuyen con mayor rapidez y eficiencia en células tumorales, observándose además un aumento en la retención pasiva intratumoral del principio activo; g) En varios modelos tumorales se ha observado que se requieren menores dosis de agentes anticancerígenos cuando están acoplados a nanopartículas; h) Una de las metas de las terapias anticancerígenas es la reducción de las dosis de los tratamientos y que generalmente conlleva a una disminución de los efectos adversos; h) No se

conoce si el efecto antitumoral del α-TOS *in vivo* en un modelo de melanoma se incrementa cuando está funcionalizado a la magnetita.

Por ello, y dado que los resultados *in vitro* indicaron que la actividad antitumoral de α-TOS se ve incrementada y se requieren menores dosis, se propuso evaluar y comparar el efecto antitumoral de α-TOS-Nps y sus constituyentes por separado (α-TOS o las Nps solas) en un modelo *in vivo* de melanoma murino. Para establecer el modelo, se inocularon células B16F0 de melanoma en el costado derecho de ratones nu/nu. El modelo se confirmó a las dos semanas por la presencia de un tumor sólido bien delimitado y pseudoencapsulado con interior reblandecido de color negro con áreas necróticas y hemorrágicas.

Con rayos X se identificó una imagen radiodensa de comportamiento maligno mientras que por ultrasonido se identificó una imagen de bordes regulares, elíptica con zona hiperecoica homogénea que delimitaba al tumor. Por histología y con la tinción de H&E se observó al microscopio un patrón sólido con manto amplio y células pleomórficas, medianas, redondas y poliédricas de núcleo redondo y cromatina irregular de grumos gruesos. Por inmunohistoquímica, se detectó el antígeno tumoral HMB45 específico de melanoma. Los estudios de imagenología, citomorfología y la presencia del antígeno tumoral para identificar el origen melanocítico confirmaron el establecimiento del modelo.

Una vez establecido el melanoma, 6 grupos de 4 ratones macho nu/nu se trataron intratumoralmente por 2 semanas con diferentes dosis de α-TOS-Nps, Nps y α-TOS. Durante el tratamiento se monitoreó el volumen tumoral, el peso de los animales y signos de toxicidad, así como cambios ultrasonográficos y de rayos X, además de los antígenos tumorales Ki-67 y S-100. Se midió la longitud (L) y el ancho del tumor (A) en milímetros por ultrasonografía y con caliper. Se aplicó la fórmula: Volumen tumoral=$(L \times A^2)/2$. Se observó una disminución

significativa del volumen tumoral con las dosis de 0.75 y 2 mg de α-TOS-Nps ($p<0.05$) a los 10 días post tratamiento. Se observó un efecto sinérgico de la actividad antitumoral de α-TOS cuando está funcionalizado a las Nps, pero no cuando se aplica sólo, un efecto similar al observado *in vitro*. No se encontraron diferencias en los patrones de malignidad ultrasonográfica ni de rayos X de los tumores. Sin embargo, la evaluación histológica indica que a mayores dosis de α-TOS-Nps se induce una mayor necrosis intratumoral, que se refleja en una disminución del volumen tumoral o de su peso. Estos son efectos deseables previos a una cirugía, ya que se facilita la remoción completa del tumor y también se asocia a una mayor sobrevida. Además, no se observó infiltración tumoral a tejidos aledaños en los animales tratados con α-TOS-Nps, en contraste con la infiltración de tejido musculoesquelético y de tejido adiposo observada con los tratamientos por separado de α-TOS o Nps puras. En cuanto a los antígenos tumorales Ki-67 y S-100, la preservación del tejido tumoral no fue la óptima para realizar la recuperación antigénica.

La biodistribución se determinó con la tinción de azul de Prusia en los cortes histológicos del tumor y de los órganos extraídos. Esta prueba fue positiva tanto para los tumores tratados con Nps como con α-TOS-Nps en todas las dosis aplicadas. Se observaron agregados intratumorales de mayor tamaño en los animales tratados con Nps que en aquellos tratados con α-TOS-Nps. Se detectaron las α-TOS-Nps en tumor, bazo, hígado, piel, pulmón, riñón e intestino. Se observó una positividad mayor en bazo e hígado. Esto pudo deberse a que esos órganos tienen reservas de hierro y fisiológicamente tienen una mayor capacidad de captar y acumular el hierro de la magnetita ($Fe_3O_4$) presente en el núcleo de las α-TOS-Nps. Además hay reportes indicando que α-TOS por si solo se acumula principalmente en hígado. Lo anterior puede favorecer el incremento de α-TOS-Nps en este órgano. No se observaron lesiones histológicas de toxicidad en los órganos positivos.

En conclusión, en este trabajo se describe la síntesis y caracterización de nanopartículas de magnetita funcionalizadas con α-TOS y su evaluación *in vitro* e *in vivo*. La caracterización es de suma importancia para empezar a reconocer cuáles propiedades fisicoquímicas pueden relacionarse a los efectos biológicos de este tipo de nanopartículas. Los efectos observados con la aplicación *in vitro* e *in vivo* de α-TOS-Nps sugieren que la funcionalización de α-TOS a nanopartículas de magnetita protege la acción anticancerígena de α-TOS *in vitro* e incrementa su bioactividad *in vivo*. La nanoplataforma de magnetita tiene un uso potencial biomédico para el desarrollo de nuevas terapias anticancerígenas basados en su funcionalización con α-TOS.

## INTEGRACION DEL MANUSCRITO DE TESIS

La información derivada de esta investigación se sintetizó en capítulos y anexos. Los capítulos están conformados por tres artículos en inglés. Así mismo, se incluye un apartado de la producción académica en anexos integrados por dos capítulos de libros y el listado de las participaciones en congresos.

Capítulos

**Capítulo I "El Papel del Alfa Tocoferil Succinato (α-TOS) como un Agente Anticancerígeno Potencial".**

En este artículo de revisión se describe el estado del arte de los análogos de vitamina E, representados por alfa-tocoferil succinato y su papel como un agente anticancerígeno. Se presenta un panorama general de las características estructurales de los análogos, las perspectivas y aplicaciones clínicas. Se describen tanto los efectos observados *in vitro* e *in vivo* en diferentes líneas celulares y modelos animales. También se describen las pruebas biológicas utilizadas para su evaluación, así como los posibles mecanismos de acción de cada uno de los efectos, principalmente la apoptosis inducida por la vía mitocondrial. Finalmente, se mencionan las nuevas formulaciones para mejorar la solubilidad, la acción terapéutica y su uso potencial como agente anticancerígenos selectivos.

Artículo aceptado 21 de Octubre del 2013, FI 2.78. Taylor & Francis Group.
Cita: Aracely Angulo-Molina, Julio Reyes-Leyva, Aurelio López-Malo, Jesús Hernández. The role of alpha tocopheryl succinate (α-TOS) as a potential anticancer agent. Nutrition and Cancer: An International Journal.

**Capítulo II "Nanopartículas de Magnetita Funcionalizadas con α-Tocoferil Succinato (α-TOS) Promueve la Muerte Selectiva de Células de Cáncer de Cérvix".**

En este artículo original se describe la síntesis y caracterización fisicoquímica de nanopartículas de magnetita funcionalizadas con el análogo de vitamina E, el α-tocoferil succinato. Se detallan las características fisicoquímicas que pueden estar asociadas al incremento en la resistencia del análogo y su actividad anticancerígena en células de cáncer de cérvix, detallando aquellas que pueden relacionarse a su bioactividad. Además se describen los resultados de la evaluación *in vitro* en células tumorales no susceptibles al α-tocoferil succinato y en células normales, su localización intracelular y el efecto en la viabilidad dosis-tiempo dependiente.

Artículo en revisión enviado el 25 de Julio del 2013, FI 2.17. Springer.
Cita: Aracely Angulo-Molina, Miguel Ángel Méndez-Rojas, Teresa Palacios-Hernández, Oscar Edel Contreras-López, Gustavo Alonso Hirata-Flores, Juan Carlos Flores-Alonso, Saúl Merino-Contreras, Olivia Valenzuela, Jesús Hernández, Julio Reyes-Leyva. Magnetite nanoparticles functionalized with α-tocopheryl succinate (α-TOS) promote selective cervical cancer cell death. Journal of Nanoparticle Research.

**Capítulo III "Nanopartículas de Magnetita Funcionalizadas con α-Tocoferil Succinato: Distribución *in Vivo* y Actividad antitumoral en un Modelo de Melanoma**".

En este artículo original se describe el efecto antitumoral y la biodistribución de nanopartículas de magnetita funcionalizadas con α-tocoferil succinato en un modelo tumoral de melanoma establecido en ratones inmunosuprimidos nu/nu. Las nanopartículas se caracterizaron por TEM, dispersión dinámica de la luz, efecto Tyndall y actividad magnética. La actividad antitumoral *in vivo* se evaluó por ultrasonografía, rayos X, cambios en el volumen tumoral e histología.

También se evaluaron signos de toxicidad. La biodistribución se evaluó a través de la detección del hierro de la magnetita con la tinción de Azul de Prusia analizando cortes histológicos del tumor y de órganos como el bazo, hígado, riñón, cerebro, estómago, tubo digestivo y piel.

Artículo enviado. Diciembre del 2013

Cita: Aracely Angulo-Molina, Salomón Hernández, Carlos Escamilla, Francisco Collazo, Teresa Palacios-Hernández, Miguel Ángel Méndez, Oscar Edel Contreras-López, Gustavo Hirata-Flores, Julio Reyes-Leyva, Jesús Hernández. Magnetite nanoparticles functionalized with α-tocopheryl succinate: *in vivo* distribution and tumor suppressing activity in melanoma model.

Anexos

**Anexo I "Riesgos Ambientales de la Nanotecnología: Evaluando la Ecotoxicidad de Nanomateriales**

En este capítulo de libro en inglés se discuten las propiedades físicas y químicas de nanomateriales de uso común y cómo esas propiedades pueden tener implicaciones en la salud humana y un efecto potencial como contaminantes ambientales. Además, se presenta un panorama de los nanomateriales que pueden ser utilizados en productos comerciales actuales y futuros. Así mismo, se discute su impacto ecológico y sobre la salud humana considerando las propiedades que le permiten atravesar las barreras biológicas, su bioacumulación y las alteraciones metabólicas que pueden generar reacciones tóxicas a corto y a largo plazo. Finalmente se exponen las pruebas *in vitro* e *in vivo* utilizadas para su evaluación.

Capítulo de libro aceptado Junio del 2013 y en proceso de publicación. Editorial John Wyley & Sons, NY.

Cita: Méndez-Rojas MA, Sánchez-Salas JL, Angulo-Molina A and Palacios-Hernández TJ. "Environmental risks of nanotechnology: Evaluating the ecotoxicity of nanomaterials". In: Kharisov BI, Kharissova O, Dias RH. Nanomaterials for Environmental Protection. John Wiley & Sons, NY. In press.

**Anexo 2 "Nutrición y Biotecnología Alimentaria. Bases para la Sustentabilidad Social".**

En este capítulo de libro se describe cómo la nutrición y la biotecnología aplicada al desarrollo y mejora de productos alimenticios pueden intervenir en aspectos básicos de la sustentabilidad social. Se inicia con una descripción general del desarrollo de las ciencias nutricionales, el impacto que ha tenido el conocimiento del ADN y la nutrigenómica. Se describe el papel de la biotecnología alimentaria en el desarrollo de alimentos transgénicos, alimentos funcionales, nutracéuticos y "nuevos alimentos". Se discute su aplicación en el desarrollo sostenible de la agricultura, la pesca y la actividad forestal, así como de las industrias alimentarias. Finalmente se proponen alternativas como los modelos de traspatio para la producción familiar de alimentos para combatir la inseguridad alimentaria. El libro cuenta con el prólogo del Premio Nobel de Química, Mario Molina.

Libro publicado en Octubre del 2012.
Cita: Ortega Regules AE, Angulo Molina A, Lozada Ramírez JD. Nutrición y biotecnología alimentaria, bases para la sustentabilidad social. En: Asili, N. Vida sustentable, la experiencia de un sueño compartido. Publicaciones UDLAP. 2012, 1ra edic. Pág. 408-426, ISBN 978-607 7690-12-2.

**Anexo III "Presentaciones en Congresos".** En este anexo se incluye un listado de los trabajos presentados en modalidad poster y/o presentación oral en congresos nacionales e internacionales durante la realización del proyecto. Se incluyen además las estancias académicas de investigación y capacitación.

## HIPÓTESIS

La funcionalización de α-TOS a nanopartículas de magnetita aumenta su potencial anticancerígeno.

# OBJETIVOS

## Objetivo General

Evaluar la capacidad anticancerígena del α-TOS funcionalizado a nanopartículas de magnetita en modelos de cáncer *in vitro* e *in vivo*.

## Objetivos Específicos

1. Sintetizar nanopartículas de magnetita de alta estabilidad y funcionalizarlas con α-TOS.
2. Caracterizar la composición orgánica, mineral y la morfometría de las nanopartículas en las diferentes etapas de síntesis y funcionalización.
3. Determinar si la funcionalización protege la actividad anticancerígena del α-TOS y mantiene su selectividad mediante su evaluación *in vitro* en células normales y en células resistentes al α-TOS.
4. Establecer un modelo tumoral *in vivo* en ratones inmunosuprimidos para la evaluación anticancerígena de las nanopartículas funcionalizadas.
5. Evaluar la biodistribución de las nanopartículas funcionalizadas en un modelo tumoral *in vivo*.

# Capítulo I

# El Papel del Alfa Tocoferil Succinato (α-TOS) como un Agente Anticancerígeno Potencial

Aracely Angulo-Molina, Julio Reyes-Leyva, Aurelio López-Malo, Jesús Hernández. The role of alpha tocopheryl succinate (α-TOS) as a potential anticancer agent. Nutrition and Cancer: An International Journal. Taylor & Francis Group. Aceptado.

## RESUMEN

En años recientes los esfuerzos para mejorar las terapias contra el cáncer se han enfocado en el desarrollo de nuevos agentes anticancerígenos como los mitocanos. Estos agentes, incluyen a los análogos de vitamina E (AVE). El alfa-tocoferil succinato (α-TOS) es el análogo más representativo de los AVE el cual es capaz de inhibir tanto la proliferación celular como inducir la muerte de células tumorales a través de la apoptosis por la vía mitocondrial. Los estudios *in vitro* e *in vivo* han demostrado la selectividad del α-TOS para inducir la muerte de células tumorales por estas vías, con efectos mínimos o nulos en células normales. De ahí su potencial aplicación clínica. Esta revisión presenta un panorama general del α-TOS en el tratamiento contra el cáncer, las perspectivas actuales y las aplicaciones clínicas.

*Nutrition and Cancer*, 00(00), 1–10
Copyright © 2014, Taylor & Francis Group, LLC
ISSN: 0163-5581 print / 1532-7914 online
DOI: 10.1080/01635581.2014.863367

Routledge
Taylor & Francis Group

# The Role of Alpha Tocopheryl Succinate (α-TOS) as a Potential Anticancer Agent

**Aracely Angulo-Molina**
*Departamento de Ciencias Químico Biológicas, Universidad de las Américas Puebla, Puebla, México; Centro de Investigación Biomédica de Oriente Instituto Mexicano del Seguro Social, Puebla, México; Laboratorio de Inmunología, Centro de Investigación en Alimentación y Desarrollo, Sonora, México*

**Julio Reyes-Leyva**
*Centro de Investigación Biomédica de Oriente Instituto Mexicano del Seguro Social, Puebla, México*

**Aurelio López-Malo**
*Departamento de Ciencias Químico Biológicas, Universidad de las Américas Puebla, Puebla, México*

**Jesús Hernández**
*Laboratorio de Inmunología, Centro de Investigación en Alimentación y Desarrollo, Sonora, México*

**In recent years, efforts to improve cancer therapy have focused on developing new anticancer agents, such as mitocans. These agents include vitamin E analogues and suppress cancer by inducing apoptosis by targeting mitochondria. Alpha tocopheryl succinate (α-TOS) is the most effective form of vitamin E analogues causing inhibition of proliferation and apoptosis of cancer cells. Both *in vitro* and *in vivo* studies have demonstrated that α-TOS selectively kills tumor cells with little or no effect on normal cells. Treatment with α-TOS shows great promise for future clinical applications, as it causes cell death, at least in part, by selectively inducing apoptosis by mitochondrial destabilization. This review presents an overview of perspectives on α-TOS and the potential uses of α-TOS in cancer treatment and other clinical applications.**

## INTRODUCTION

Recent efforts to improve cancer therapy have focused on developing new drugs and additional strategies to inhibit cancer cell growth. These agents include, among others, vitamin E analogues (VEAs) (1–5), which are best represented by α-TOS. VEAs are considered, within the mitocans, to be anticancer compounds that act by selectively destabilizing the mitochondria of cancer cells (3, 6, 7). α-TOS and alpha tocopheryloxiacetic acid (α-TEA) are 2 of the most significant of the reported VEAs. α-TOS has been shown to kill tumor cells at treatment doses that have little or no effect on normal cells, whereas many of the established chemotherapeutic agents (e.g., doxorubicin and cisplatin) kill not only tumor but also normal cells, resulting in serious side-effects (3, 4). Notably, however, adverse effects have been attributed to vitamin E. Previous reports using meta-analysis have described that high-doses of vitamin E supplements may increase mortality (7, 8). This is a controversial issue, though others have found no adverse effects using a similar methodology (9). According to a recent review, more information is needed to confirm the benefits of vitamin E in animal and human health (10). In this review, we describe a group of anticancer agents, focusing on α-TOS, and discuss their potential uses in cancer therapy.

## α-TOS IN CANCER

α-TOS, one of the most important VEAs, is a redox-silent and semisynthetic compound, derived by substitution of the hydroxyl group on the chroman head of α-tocopherol with a succinyl group. Unlike α-tocopherol, which functions as an antioxidant, α-TOS has potent antineoplastic properties (11). One limitation to treatment with α-TOS is its vulnerability to esterases, complicating its oral use (12, 13). Recently, an analog of α-TOS that is resistant to esterase attack, α-TEA, was synthesized and has been shown to suppress human carcinoma cells in several experimental models (12, 14–16). α-TEA has an acetic acid moiety linked to the phenolic oxygen at carbon 6 of the chroman head of RRR-α-tocopherol by an ether linkage, yielding

Submitted 3 January 2013; revised 11 October 2013; accepted in final form 21 October 2013.
Address correspondence to Jesús Hernández, Laboratorio de Inmunología, Centro de Investigación en Alimentación y Desarrollo A.C. Carretera a La Victoria km 0.6 C.P. 83304, Hermosillo, Sonora, México. E-mail: jhdez@ciad.mx

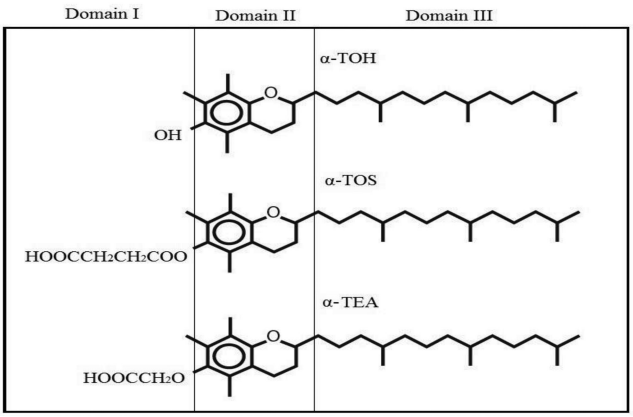



FIG. 1. Structure and scheme of functional domains in the vitamin E analogues. Alpha tocopheryl succinate ($\alpha$-TOS) and alpha tocopheryloxiacetic acid ($\alpha$-TEA) are semisynthetic derivatives of vitamin E. $\alpha$-TOS differs from vitamin E ($\alpha$-TOH) in that the OH on $C_6$ of the phenolic ring of the chroman head has been replaced by a succinic acid. $\alpha$-TEA has an acetic acid moiety in the same position attached via an ether bond is not prone to hydrolysis by esterases. There are 3 major domains, each responsible for a separate function. Domain I, or the functional domain, determines if the compound is redox active or redox inactive. Domain II, or the signaling domain, is involved in modulation of signaling pathways, such as the protein phosphatase 2A/protein kinase C pathway. Domain III, or the hydrophobic domain, is responsible for docking of the compounds in biological membranes and lipoproteins. Adapted from Hahn et al. (17) and Tomasetti and Neuzil (19).

a stable, nonhydrolyzable entity, and this latter structure may be partly responsible for the anticancer properties because it is not susceptible to esterases (Fig. 1) (16–19). The anticancer activity in human breast cancer and mouse mammary cancer cell lines of novel tocopheryl-based derivatives was reported recently. These compounds have incorporated fluorine at the chroman head and/or ether linkage between the chroman head and the phytyl tail of RRR-alpha tocopherol (20). In a similar way, it was shown that modifications of domain II of tocotrienols by electrophilic substitution reactions can selectively improve their anticancer properties in vitro and in vivo (21). These reports are coincident in accomplishing structural modifications of vitamin E that give rise to new drug candidates for cancer treatment. In addition, the importance of vitamin E compounds (tocopherols and totrienols) as adjuvants during cancer therapy were discussed previously by Ling et al. (22).

The main strategies to treat cancer, besides surgery, have been radiotherapy and chemotherapy. These treatments damage cells at DNA level during replication or cell division and induce cell death, but they usually do not distinguish between malignant and normal types of proliferating cells, causing unwanted toxicity to normal tissues (1, 18, 19). Another effect of chemotherapeutic drugs is the induction of proliferation arrest and apoptosis; however, some cancer cells escape drug toxicity and become resistant. VEAs as $\alpha$-TOS and $\alpha$-TEA, may circumvent these issues (13, 23–25). Moreover, VEAs have been shown to suppress tumor growth in several preclinical animal models, including mice with experimental breast, lung, prostate, and colon carcinomas, as well as mesotheliomas (4, 23).

Because of its selectivity for cancer cells and low toxicity to nonmalignant cells, the ester analogue $\alpha$-TOS has significant clinical potential (26). This has led to studies of the role of $\alpha$-TOS in cancer prevention and treatment. In addition, it has been demonstrated that $\alpha$-TOS is a potent growth inhibitor of various cancer cell types in vitro. $\alpha$-TOS induced high levels of apoptosis in at least 50 types of cancer cells tested thus far from different species (human, murine, and avian) and tissue types (breast, prostate, lung, stomach, ovary, lymphoma, colon, and mesothelium) (4, 18, 23, 27, 28). For example, $\alpha$-TOS inhibits the growth of human monoblastic leukemia cells, avian lymphoid cells, murine EL4 T lymphoma cells, and murine B-16 melanoma cells in vitro, hamster buccal pouch tumor cells in vivo, and human gastric and breast cancer cells in vitro and in vivo (29). Further investigations demonstrated that $\alpha$-TOS is a potent inducer of apoptosis in a wide range of human cancer cells of both epithelial and lymphoid origin (4, 29–35). It was demonstrated that the level of apoptosis induced by $\alpha$-TOS varied from 30% to 60% in different malignant cells (50 $\mu$M during 12 h of exposure). This action of $\alpha$-TOS was observed in multiple cancer cell lines and involved lysosomal and mitochondrial destabilization and caspase-3 activation. $\alpha$-TOS also limited tumor growth in a colorectal cancer xenograft model when mice received intraperitoneal (i.p.) injections (50 $\mu$L) of 200 mM $\alpha$-TOS (31). Wu et al. (29) reported that treatment with 20 $\mu$g/mL $\alpha$-TOS for 48 h induced apoptosis in 90% of a population of SGC-7901 human stomach cancer cells. This activity was selective for malignant cells (29). In general $\alpha$-TOS induce less than 5% apoptosis in normal cells (4). Selectivity of $\alpha$-TOS is due to the fact that it acts more efficiently at a low pH, a common characteristic of cancer cells. $\alpha$-TOS is a weak acid with low p$K$a value. Therefore, at neutral pH of normal interstitial tissue, the majority of $\alpha$-TOS exists in the charged and deprotonated state. In contrast, the tumor microenvironment is acidic, causing protonation of $\alpha$-TOS and facilitating its free diffusion into the cell (32, 36).

In mice, the benzopyrene-induced forestomach carcinoma model, higher doses of $\alpha$-TOS (200 mg/kg body weight) suppressed the number and volume of tumors. In nude mouse models, $\alpha$-TOS suppressed colon cancer metastases to the liver and mammary tumor metastases to the lungs, which further strengthens and extends the prospects for $\alpha$-TOS as an anticancer drug (reviewed in Ref. 4). Oral administration of $\alpha$-TOS in rodents was inefficient, suggesting that most of the $\alpha$-TOS was hydrolyzed before entering the blood stream. Indeed, when $\alpha$-TOS is given orally, it is absorbed by the intestinal villi and immediately hydrolyzed into free, redox active $\alpha$-TOH which is secreted in chylomicrons into the mesenteric lymphatics and subsequently into the blood stream (19). To avoid hydrolysis of $\alpha$-TOS by intestinal esterases it has been administered by intraperitoneal or intravenous routes (11, 33 Zhao 35). The provitamin activity of $\alpha$-TOS occurs in the blood stream where it

is associated with circulating lipoproteins and delivered to the tumor microvasculature and induces apoptosis. However, a proportion of α-TOS is eventually cleared by hepatocytes with high esterase levels that cleave α-TOS to the redox active α-TOH, with vitamin activity.

To avoid the possibility of hydrolysis in the gut, the nonhydrolyzable ether form of α-TOS, α-TEA, was synthesized. Like α-TOS, α-TEA has generated interest as a cancer therapeutic because of its selective toxicity toward tumor cells and its ability to suppress tumor growth in various rodent and human xenograft models (17, 37, 38). Hahn et al. (12) reported the efficacy of dietary α-TEA in the clinically relevant MMTV-PyMT mouse model of spontaneous breast cancer. This mouse model of cancer closely resembles human disease. They found an 80% reduction in spontaneous metastases mediated by apoptosis and showed the ability of orally administered α-TEA in the diet to delay both tumor onset and metastatic progression (12).

In preclinical studies with syngeneic transplantable mouse mammary cancer, it has been demonstrated that α-TEA can reduce tumor burden and inhibit lung metastases when delivered by aerosol or in the diet. This result has also been observed in xenograft models using immune compromised mice transplanted with human ovarian, breast, or prostate cancer cells and in spontaneous breast cancer models (12, 14, 38). Cell culture studies have shown that α-TEA induces human ovarian, prostate, and breast cancer cells to undergo DNA synthesis arrest and apoptosis and that α-TEA-induced apoptosis involves activation of Fas/Fas ligand and c-Jun NH2-terminal kinase (JNK) proapoptotic pathways, as well as suppression of Akt, FLIP, and surviving anti-apoptotic/pro-survival factors (16). Recently, it was reported that in addition to its direct cytotoxic effects and antitumor effect, α-TEA treatment may activate the immune response (14, 17). Table 1 summarizes the most recent experiments evaluating growth inhibition and increases in survival using animal models; in most of them apoptosis of cancer cells was the principal effect of α-TEA treatment.

## α-TOS AND APOPTOGENIC PROPERTIES

In many instances, growth inhibition following terminal differentiation or anticancer drug treatment results in apoptosis. Apoptosis, or programmed cell death, is an active and physiological process characterized by a series of morphological and biological alterations including condensation of the cytoplasm, loss of membrane microvilli, segmentation of the nucleus, and extensive degradation of chromosomal DNA into oligomers of 180 bp. Apoptosis is an innate and evolutionary conserved process in which cells deactivate, disassemble, and degrade their own components and structures in a coordinated and characteristic manner. Apoptotic cell recognition is an event that involves a number of receptors acting either simultaneously or in isolation (39, 40).

Because α-TOS was discovered to be one of the most effective forms of vitamin E capable of inhibiting cell proliferation and cell death in murine melanoma cells in culture, several publications have shown that α-TOS produces similar effects on a variety of human and rodent tumor cell lines without affecting the proliferation of most normal cells in vitro (15, 23, 33, 35, 41–43). Previous studies have also shown that α-TOS inhibits tumor cell growth by a variety of mechanisms, including DNA synthesis arrest, cell cycle blockade, induction of apoptosis, inhibition of tumor cell proliferation and differentiation, as well as inhibition of angiogenesis (24, 29, 44–46). This analogue kills cells via apoptosis and affects expression of genes involved in cell proliferation and cell death in a sub-apoptotic manner during the cell cycle (23). The cell cycle is controlled by numerous mechanisms ensuring correct division. The transition from one cell cycle phase to another occurs in an orderly fashion and is regulated by different cellular proteins. These proteins are the cyclin-dependent kinases, a family of serine/threonine protein kinases that are activated at specific points of the cell cycle (47). α-TOS can inhibit proliferation of cancer cells by inhibition of cyclin A binding to the transcription factor E2F, suggesting an effect on cell cycle progression (48). In addition, α-TOS can inhibit proliferation and trigger apoptosis of malignant cells in vitro and in vivo via effects on the multi-complex transcription factor nuclear factor-kappa B (NF-κB). NF-κB has an important role in regulation of the immune and inflammatory responses and also exerts antiapoptotic activities (4, 34). The suppression of nuclear NF-κB activation by α-TOS induces secretion and activation of transforming growth factor (TGF)-β, enhanced expression of TGF-β type II receptors, and enhanced cell surface expression of Fas (CD95) in various cancer cell lines of human and murine carcinomas, including breast, cervical, endometrial, prostate, colon, lung, and lymphoid. Activation of the extrinsic cell death pathways is initiated by ligation of death receptors, which include Fas (17, 19, 43, 48–50). In this sense, α-TOS is also implicated to play a role in nonmitochondrial signaling involved in apoptosis activation.

The critical roles of these signaling pathways for α-TOS and mitocan-induced apoptosis have been demonstrated by various functional knockout approaches, including the following: 1) blocking antibodies to TGF-β ligands and Fas receptor; 2) chemical inhibition of TGF-β ligand activation and caspase activity; 3) antisense blockage of TGF-β receptor II, TGF-β1 ligand and c-Jun; and 4) dominant negative blockage of c-Jun (6, 19, 51, 52). As different types of cancers are complex and can differ considerably in their DNA mutations, it will be very unlikely to cure cancer with drugs targeted to only a few gene products or single pathways involved in tumor survival (53).

The importance of mitocans, such as VEAs, as anticancer agents that target mitochondria to trigger apoptosis is that mitochondrial function is a universal cellular requirement. Mitochondria are unique organelles essential for life and death of eukaryotic cells (4, 6); thus, mitochondria are prime targets and transmitters of apoptosis that, if selectively activated in cancer cells, would provide an effective treatment for a variety of

TABLE 1
Effects of vitamin E analogues in cancer models

| Animal model | Inoculated cell line or tumor inducer | Duration of treatment and dose | Treatment effect on tumor | Reference |
|---|---|---|---|---|
| Nude mouse | MDA-MB-231 breast cancer human cells | 2 wk; 150 mg/kg/day $\alpha$-TOS in sesame oil | 80–90% tumor dormancy | Malafa et al., 2000 |
| Female Kunming mouse | Forestomach tumor using benzopirene | 4 wk; 200 mg/kg $\alpha$-TOS in oil twice a weck | ~85% tumor growth inhibition | Wu et al., 2001 |
| Nude mouse | HCT116 colon cancer human cells | 10 days; 100 mg/kg $\alpha$-TOS en DMSO every third day | ~75% tumor growth inhibition | Neuzil et al., 2001 |
| Nude mouse | B16F10 melanoma murine cells | 2 wk; 100 mg/kg/day $\alpha$-TOS in sesame oil | 80–90% tumor dormancy, inhibition of liver metastases | Malafa et al., 2002 |
| Nude mouse | CT26 colon cancer human cells | 2 wk; 100 mg/kg/day $\alpha$-TOS in DMSO | ~75% inhibition of liver metastasis | Barnett et al., 2002 |
| Nude mouse | B16F10 murines melanoma cells | 2 wk; 150 mg/kg/day $\alpha$-TOS in sesame oil | ~70% tumor growth inhibition | Weber at al., 2002 |
| Nude mouse | HCT116 colon cancer human cells | 10 days; 50 mg/kg $\alpha$-TOS in DMSO every third day plus 20 $\mu$g hrTRAIL | ~70% tumor growth inhibition | Weber at al., 2002 |
| Nude mouse | Ist-Mes2 mesothelioma human cells (xenograft s.c.) | 2 wk; 100 mg/kg $\alpha$-TOS en DMSO every 2$^{nd}$ day | >90% tumor growth inhibition | Stapelberg et al., 2005 |
| Nude Mouse | MDA-MB-435 FL breast cancer cells | 4.5 wk; 36 $\mu$g de $\alpha$-TEA every day in aerosol | ~60% tumor growth inhibition | Zhang et al., 2004 |
| Nude mouse | 4T1 breast cancer | 4 wk; 4 mg $\alpha$-TOS or $\alpha$-TEA i.p. every 4 days o every day oral or 5.5 mg $\alpha$-TEA in diet. | 60% reduction in tumor size by $\alpha$-TOS by i.p. or $\alpha$-TEA by both i.p. and orally; inhibition of lung metastasis by $\alpha$-TEA | Lawson et al., 2004 |
| Mouse C57BL/6 | 3LLD122 Lung cancer murine cells | 3 wk; 200 mg/kg $\alpha$-TOS in ethanol or 200 mg/kg vesiculated $\alpha$-TOS | 70% tumor growth inhibition | Ramanathapuran et al., 2006 |
| Mouse transgenic c-neu | Spontaneous breast carcinoma | 3 wk; 15 $\mu$mol $\alpha$-TOS in corn oil every third day | 30% reduction in volume tumor and ~50% inhibition of angiogenesis in tumor | Dong et al., 2007 |
| Nude mouse | LNCa Prostate cancer cells | 2 wk of 7 wk in total; 100 mg/kg $\alpha$-TOS in sesame oil every day | ~70% reduction in original tumor size | Basu et al., 2007 |
| Mouse transgenic c-neu | Spontaneous breast carcinoma | 3 wk; 15 $\mu$mol of $\alpha$-TOS or 5 $\mu$mol a-TOS- LTVSPWY in oil every 3–4 days | 50% reduction in tumor size by 15 $\mu$mol $\alpha$-TOS or 70% by 5 $\mu$mol peptide conjugate | Wang et al., 2007 |

*(Continued on next page)*

TABLE 1
Effects of vitamin E analogues in cancer models *(continued)*

| Animal model | Inoculated cell line or tumor inducer | Duration of treatment and dose | Treatment effect on tumor | Reference |
|---|---|---|---|---|
| Nude mouse | JHU-022 prostate cancer | 3-wk pretreatment with α-TOS liposome (1.0 mg in DMSO/mouse by i.p. injection) on alternate days | Average tumor weight was lower in the α-TOS treated group at day 55 | Gu et al., 2008 |
| Mouse transgenic MMTV-PyMT | Spontaneous breast cancer | 9 wk, 2 mg of oral α-TEA per day starting at 6 weeks of age until 15 weeks of age | Oral α-TEA resulted in a 50% reduction of the average number of tumors | Hahn et al., 2009 |
| Mouse transgenic FVB/N | Breast cancer | 2 wk 15μM α-TOS liposome and 15μM TAM liposome every 2 day | α-TOS and α-TAM suppressed breast carcinoma by 90–100% | Turánek et al., 2009 |
| Nude mouse | Human bladder cancer cells | 4 wk, 150 mg/kg of α-TOS in DMSO alone or in combination with paclitaxel daily by i.p. injection | α-TOS, paclitaxel and combination treatments suppressed tumor growth to 61.0%, 63.3%, and 33.1%, respectively | Kanai et al., 2010 |
| Nude mouse | HCT116 colorectal carcinoma cells | 4 wk, 15 μmol of α-TOS or 1–2 μmol of mitochondrially targeted α-TOS (MitoVES) in oil every 3–4 days | MitoVES, applied at 10-fold lower concentration than α-TOS, suppressed the growth of colorectal carcinomas | Dong et al., 2011 |

different tumors and could be used for efficient therapy of many different cancers (1–4, 15, 54).

Several mechanisms have been suggested to explain how α-TOS works, mostly involving mitochondrial destabilization throughout ROS production and apoptosis. α-TOS stimulates the production of ROS and causes retardation of cell growth in malignant, but not in normal cells. It has also been reported that α-TOS acts as a Bcl-2 analogue homology-3 (BH3) mimetic because it interacts with the BH3 domain of the Bcl-2 family proteins, disrupting the interaction between Bak, Bcl-xL, and Bcl-2 in prostate cancer cells. Another report suggested that α-TOS induces translocation of Bax into the mitochondria in breast cancer cells, although the mechanism of this process was not determined (reviewed in Ref.55). These results led to the proposal that ROS production induced dimerization of Bax, followed by its mitochondrial mobilization (56–58), perhaps helping to explain the events occurring in α-TOS-treated cells (4, 57). However, α-TOS leads to elevated formation and accumulation of ROS that induces the intrinsic, mitochondria-dependent proapoptotic pathways (1, 17, 59, 60). However, the precise mechanisms of mitochondrial translocation and/or activation of apoptogenic Bcl-2 family proteins triggered by α-TOS remain unclear.

Biochemical evidence supports the notion that α-TOS interferes with the ubiquinone (UbQ)-binding site(s) of the mitochondrial complex II, impairing electron transfer flowing along the redox chain and stimulating ROS production (6, 15, 23, 26, 59–61). Neuzil et al. (54) proposed a model for the molecular mechanism of apoptosis initiation by α-TOS. In this model, there are 2 roles for α-TOS: The first model uses α-TOS to inhibit oxidative respiration at the level of complex II and the second involves α-TOS binding to Bcl-2 and Bcl-xL, allowing Bax to form mitochondrial membrane channels (54). Thus, α-TOS impairs the transfer of electrons along the redox chain. This leads to the generation of ROS, such as superoxide anion radicals. ROS then contributes to the oxidation of the cysteine residues on Bax monomers to form disulfide bridges. The dimerization modifies the conformation of Bax, so that the

4C/Art

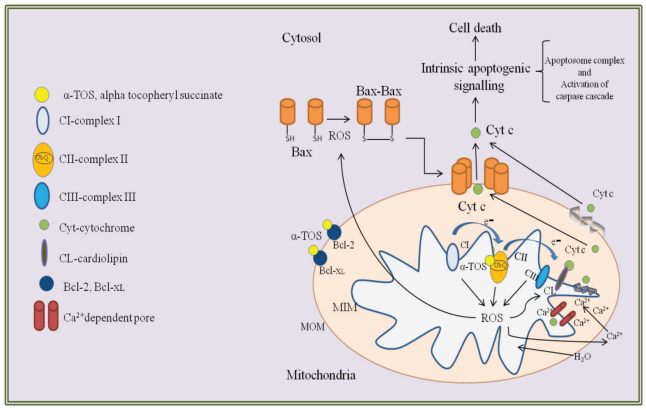



FIG. 2. Molecular mechanism for apoptosis initiation by alpha tocopheryl succinate ($\alpha$-TOS). In this model there are 2 roles for $\alpha$-TOS: the major role, in which $\alpha$-TOS interferes with the ubiquinone (UbQ)-binding site (s) of the mitochondrial complex II impairing electron transfer flowing along the redox chain, resulting in high levels of reactive oxygen species (ROS), thus, activating apoptogenic signaling; and an auxiliary role, which involves its interaction with pro-apoptotic proteins as Bcl-2 and anti-apoptotic proteins such as Bcl-xL, allowing Bax to form mitochondrial membrane multimeric channels. Mitochondrial apoptotic regulators such as Cyt c dissociated from CL via ROS translocate to the cytosol, activating caspases that result in apoptosis. In addition to ROS, $\alpha$-TOS stimulates rapid $Ca^{2+}$ entry to the cells, facilitating the opening of a non-specific $Ca^{2+}$ dependent pore in MIM. This is followed by the influx of water and ions causing rupture of MOM and the release of Cyt c, increasing the pool of free Cyt c. UbQ = ubiquinone binding site of CII; MIM = mitochondrial inner membrane; MOM = mitochondrial outer membrane. Adapted from Godvadze et al. (23) and Ralph and Neuzil (25). (color figure available online).

mitochondria-docking motif is exposed and the dimers merge in the mitochondrial outer membrane (MOM), forming a channel. ROS also oxidizes cardiolipin (CL) within the mitochondrial inner membrane (MIM). This allows the dissociation of cytochrome c (Cyt c), which escapes via the Bax channel from the MOM into the cytoplasm. $\alpha$-TOS occupies the BH3 domains of Bcl-2 and Bcl-xL and prevents Bax from forming inactive oligomers with Bcl-2 and Bcl-xL, thereby increasing the pool of available Bax for dimerization and formation of MOM channels, thus promoting induction of apoptosis (19). On the other hand, under certain circumstances ROS induces the mitochondrial permeability transition (MPT) due to the opening of a nonspecific pore in the MIM (23). MPT occurs as a sudden change in permeability of the mitochondrial membranes when exposed to high levels of calcium ($Ca^{2+}$). The pore opening is $Ca^{2+}$ dependent and can be facilitated by ROS. The opening of MPT pores in the MIM causes an influx of water and mitochondrial swelling, rupture of MIM and MOM, and the release of intermembrane proteins such as Cyt c; in addition it promotes caspase activation and apoptosis (23) (Fig. 2).

Other researchers have investigated how the molecular structure affects the anticancer effect of the vitamin E analogue. Birringer et al. (60) tested how modifications of the vitamin E molecule may influence its apoptogenic activity. They tested a number of newly synthesized VEAs on malignant cell lines and found that analogues of $\alpha$-TOS with a lower number of methyl substitutions on the aromatic ring were less active than $\alpha$-TOS. Methylation of the free succinyl carboxyl group on $\alpha$-TOS and $\delta$-tocopheryl succinate completely prevented the apoptogenic activity of the parent compounds. $\alpha$-tocotrienol failed to induce apoptosis, whereas succinylated $\gamma$-tocotrienol was more apoptogenic (60). These findings have shown that modifications of different functional moieties of the vitamin E molecule can enhance apoptogenic activity. The presence of the succinyl group confers proapoptotic properties to $\alpha$-TOS, as the cell killing activity of $\alpha$-TOS requires the compound to be intact. Some types of malignant cells appear to be unable to significantly hydrolyze the ester due to the absence of relevant esterases that are present in normal cells, including hepatocytes and intestinal epithelial cells (62). This attribute may be one of the factors that make $\alpha$-TOS selectively toxic to malignant cells. The basic structure of $\alpha$-TOS has the potential of compromising its anticancer efficacy in vivo in that the ester linkage hydrolyzed by cellular esterases loses its anticancer properties (13); for example, $\alpha$-TOS is less effective than $\alpha$-TEA as an anticancer agent in human ovarian cancer cells in which cellular esterases hydrolyze the ester linkage or when it is delivered orally, presumably because of inactivation by intestinal esterases (18, 30). In turn, $\alpha$-TEA is not hydrolyzed by cellular esterases and can be useful for oral administration. This idea was supported when Hahn et al. (12) reported the efficacy of dietary $\alpha$-TEA in vivo. They showed that oral $\alpha$-TEA inhibited the growth of both a transplanted (4T1) and a spontaneous MMTV-PyMT mouse model of breast cancer (12). In this sense, it was demonstrated that $\alpha$-TEA is not sensitive to attack by intestinal esterases.

Other vitamin E analogues, such as $\alpha$-TEA, are directly cytotoxic to tumor cells via a mechanism that includes mitochondrial depolarization and generation of ROS leading to apoptotic cell death (13, 63), similar to the mechanism reported for $\alpha$-TOS. Recently, it was reported that oral $\alpha$-TEA therapy has immunostimulatory activities. Hahn et al. (13) demonstrated that $\alpha$-TEA therapy inhibits the growth of established breast tumors and prolongs survival in an animal model of breast cancer. $\alpha$-TEA increased the frequencies of activated $CD4^+$ and $CD8^+$ T cells in the tumor microenvironment and induced a tumor-specific cytotoxic lymphocyte response. $\alpha$-TEA treatment also modulated the intratumoral cytokine and chemokine milieus and increased intratumoral interferon-$\gamma$ levels, but they decreased interleukin-4 levels, suggesting a shift toward a T cell-mediated T helper type 1 response. These results may prove useful in designing combined immunotherapy strategies for breast cancer (13, 17).

## α-TOS FORMULATIONS

Recently, there has been an increased interest in the development of special formulations to protect or improve the anticancer activity of VEAs, such as $\alpha$-TOS and other drugs (30, 64, 65). The limitation of $\alpha$-TOS as an anticancer agent is its susceptibility to the action of esterases. $\alpha$-TOS is ineffective as an

anticancer agent in cancer cells with high levels of esterases or when orally administered, presumably because of its susceptibility to attack by intestinal esterases (12, 18, 66). Another significant limitation of using α-TOS and other VEAs is their low solubility in aqueous solvents (11, 64). The hydrophobic character and low solubility of α-tocopherol and other VEAs predetermine their drug formulations (64).

Different research groups have been working to avoid the susceptibility of VEAs to esterases and to improve their clinical efficacy (11, 41, 64, 67). Development of an optimal delivery system for α-TOS needs to focus on the preparation of formulations of VEAs that would be stable during long-term storage, retain their biological activity, and be useful for clinical application. Importantly, new formulations have enhanced cytotoxicity as well as the reduced side effects (65). In this context, some examples of VEAs that have a hydrophobic character dictating the formulation and the administration route are described. The initial in vivo studies used formulations of α-TOS in ethanol, DMSO, or vegetable oil emulsions by intravenous or i.p. routes. Moreover, these routes are largely restricted to mouse tumor models, with little clinical relevance (4, 61). Vesiculated forms of α-TOS have been tested as suitable formulations for human application (64). Ramatnathapuran et al. (11) evaluated a vesiculated α-TOS (Vα-TOS). Unlike α-TOS, which is only soluble in inorganic solvents, Vα-TOS is hydrophilic and more soluble. This formulation avoids the toxicity associated with inorganic solvents, such as DMSO or ethanol, commonly used to solubilize α-TOS for parenteral administration, making Vα-TOS better suited for long-term use in humans. Vα-TOS is produced by adding NaOH and sonication in a buffered saline to form a colloidal suspension, where Vα-TOS arises spontaneously. Importantly, Vα-TOS retains the anticancer properties of α-TOS (11). For example, experiments performed to compare the growth-inhibitory and tumoricidal properties of vesicle forms Vα-TEA and Vα-TOS on the murine breast cancer cell line 4T1 demonstrated that the exposure to these analogues for 24 h killed 4T1 tumor cells in a dose-dependent manner with similar efficacy (67). Treatment of cells with 20 μg/mL of Vα-TEA or Vα-TOS caused 67% of the cell death, which increased to 99% and 100% when treated with 40 and 80 μg/mL of the drug, respectively. In this work, the efficacy of Vα-TEA and Vα-TOS as a single treatment modality was compared when given by i.p. injection or oral gavage to control the growth of established 4T1 tumors. Both compounds inhibited the growth of tumors when given i.p. In contrast, when given by oral gavage, only the esterase-resistant Vα-TEA was able to suppress the growth of tumors and reduce the metastasis (67). These results indicate that the Vα-TOS hydrolysis caused by esterases was not avoided.

Other strategies include liposomes, nanoparticles, and different routes of administration. In recent years, there has been more interest in using nanoparticle formulations that serve as controlled release delivery. Nanomedicine can help to improve the efficacy of new formulations because this science considers the size effect and new properties observed at nanoscale (65). Favorable pharmacokinetic characteristics of nanoparticles include long systemic circulation time, enhanced tumor permeability, accumulation and retention, improved therapeutic efficacy with reduced therapeutic dosage, reduced toxicity, and controlled delivery combination of anticancer agents (65, 68–70). Liposomes and nanoparticles served as controlled release carriers and biocompatible solubilizing vehicles for α-TOS. In addition, liposomes represent the most advanced versatile nanodelivery system for drug formulation. Liposomes are lipid membranous vesicles that can eliminate or suppress organ specific toxic side effects of various drugs (30). α-TOS and other VEAs could be easily incorporated into the lipid bilayers to produce liposomes of different particle size distribution and surface modification, affecting their half-life, toxicity, organ distribution, and targeting to cancer cells (64).

Liposomal formulation with embedded drugs offers several advantages, including improving the solubility of hydrophobic drugs. Turanek et al. (64) developed lyophilized liposomal formulation of both α-TOS and alpha-tocopheryl maleamide (α-TAM) to solve the problem of neurocytotoxicity of free α-TAM as well as the low solubility of both drugs. For the in vivo assay, transgenic FVB/N c-neu mice with spontaneous breast carcinomas were treated by injection i.p. with liposomal α-TOS and α-TAM at 400 mg/kg or 40 mg/kg per dose, respectively, administered on Day 0, 4, 7, and 13. The Berlin test of general toxicity was used as the method to evaluate potential toxic effects of liposomes in normal mice. Typical symptoms of toxicity include motor disorders, respiratory problems, apathy, behavioral changes, and loss of body mass. The liposomes were not toxic, neither were the liposomal preparations of both α-TOS and α-TAM; however, α-TOS and α-TAM did suppress breast carcinomas in the c-neu mice by 90 and 100%. This is especially encouraging in the case of α-TAM, which is extremely toxic when applied as a solution in DMSO (64).

There is an interesting derivative of α-TOS, the α-tocopheryl polyethylene glycol succinate (TPGS), that has been used as an effective surfactant and an efficient emulsifier for synthesis of nanoparticles of biodegradable polymers (71). It has also shown important anticancer activity when it was used to enhance the bioavailability of poorly absorbed drugs for cancer treatment or in combination with chemotherapeutic drugs such as doxorubicin and cisplatin (65). Recently, TPGS has been under intensive investigation in the construction of nanostructures and micelles for biomedical applications such as imagenology and thermotherapy during cancer treatment (65, 71). Nanoparticles are attracting considerable interest because they can be used in many biomedical applications. We are currently trying to protect the α-TOS hydrolysis caused by esterases by coupling this analogue with magnetite nanoparticles (manuscript in preparation). Q1

It is also possible to design modified VEAs to target cancer cells that overexpress certain receptors, such as the receptor

tyrosine kinase erbB2. For example, the newly synthesized $\alpha$-TOS-LTVSPWY conjugate efficiently killed breast cancer cells with high levels of the receptor tyrosine kinase erbB2 (4). The major problem associated with high expression of erbB2 is auto-phosphorylation of the receptor and the ensuing activation of growth signaling pathways and pro-angiogenic and anti-apoptotic mechanisms. When Wang et al. (46) used the $\alpha$-TOS conjugate to peptide at 5 $\mu$mol, it reduced the initial volume of breast carcinomas in the c-neu transgenic mouse (with spontaneous erbB2-high tumors) by $\approx$70%, more effective than $\alpha$-TOS alone at 15 $\mu$mol. In this work the $\alpha$-TOS-LTVSPWY induced higher level of apoptosis in erbB2-overexpressing cells than $\alpha$-TOS (46).

In other studies, $\alpha$-TOS was targeted specifically to the MIM by tagging it with the positively charged triphenylphosphonium group TTP+ (6, 15). At least 7 compounds mitochondrially targeted (MitoVES) were evaluated and the one with superior activity was labeled as $MitoVE_{11}S$ (6). This study was based on modeling and theoretical considerations that suggested that $\alpha$-TOS tagged with a cationic group such as TTP+ could preferentially interact with CII and have a greater apoptogenic activity than the untagged $\alpha$-TOS. In fact, they reported that $MitoVE_{11}S$ affected the cancer cells based on its strong interaction with the binding site of UbQ to the CII, increasing ROS production and consequently increasing the apoptosis (6). Additional findings support to the mitochondrially targeted MitoVES as a promising candidate for cancer therapy (15). Finally, $\alpha$-TOS used as an anti-cancer molecule show important anti-cancer properties in vitro and in vivo. Designing liposomes, vesicles, or conjugates to target cancer cells carrying $\alpha$-TOS can be useful in the development of more effective cancer therapies.

## CONCLUSIONS

This review focuses on the recent advances on the use of $\alpha$-TOS as an anticancer agent, which has a great promise for future clinical applications. There is a trend suggesting that deaths from cancers are increasing, and the antitumoral property of $\alpha$-TOS gives some hope in the design and finding of efficient anti-cancer drugs. In the last decade, $\alpha$-TOS has been successfully tested in vitro and in vivo with different types of cancer. The information discussed above suggests this analogue inhibits the proliferation of rodent and human cancer cells with little or no effect on normal cells. The exact mechanism by which they induce apoptosis is not completely known. Most likely, it involves a combination of membrane destabilizing activity and deregulation of signaling pathways in the mitochondria. The main disadvantage of $\alpha$-TOS is their very low solubility in the aqueous environment and their susceptibility to esterase attack. Additional studies are necessary for their use in preclinical and clinical trials; new formulations and preparation of delivery systems must be investigated. However, $\alpha$-TOS represents a novel compound that holds substantial promise as future anticancer drugs.

## ACKNOWLEDGMENTS

This work has been funded by home institution funds.

## REFERENCES

1. Rohlena J, Dong LF, and Neuzil J: Targeting the mitochondrial electron transport chain complexes for the induction of apoptosis and cancer treatment. *Curr Pharm Biotechnol* **14,** 377–389, 2013.
2. Morrison BJ, Andera L, Reynolds BA, Ralph SJ, and Neuzil J: Future use of mitocans against tumour-initiating cells? *Mol Nutr Food Res* **53,** 147–153, 2009.
3. Rodriguez-Enriquez S, Marin-Hernandez A, Gallardo-Perez JC, Carreno-Fuentes L, and Moreno-Sanchez R: Targeting of cancer energy metabolism. *Mol Nutr Food Res* **53,** 29–48, 2009.
4. Zhao Y, Neuzil J, and Wu K: Vitamin E analogues as mitochondria-targeting compounds: from the bench to the bedside? *Mol Nutr Food Res* **53,** 129–139, 2009.
5. Tomasetti M, Strafella E, Staffolani S, Santarelli L, Neuzil J, et al.: alpha-Tocopheryl succinate promotes selective cell death induced by vitamin K3 in combination with ascorbate. *Br J Cancer* **102,** 1224–1234, 2010.
6. Dong LF, Jameson VJ, Tilly D, Cerny J, Mahdavian E, et al.: Mitochondrial targeting of vitamin E succinate enhances its pro-apoptotic and anti-cancer activity via mitochondrial complex II. *J Biol Chem* **286,** 3717–3728, 2011.
7. Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG, and Gluud C: Antioxidant supplements for prevention of mortality in healthy participants and patients with various diseases. *Cochrane Database Syst Rev* **3,** CD007176, 2012.
8. Miller ER 3rd, Pastor-Barriuso R, Dalal D, Riemersma RA, Appel LJ, et al.: Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. *Ann Intern Med* **142,** 37–46, 2005.
9. Berry D, Wathen JK, and Newell M: Bayesian model averaging in meta-analysis: vitamin E supplementation and mortality. *Clin Trials* **6,** 28–41, 2009.
10. Cardenas E and Ghosh R: Vitamin E: A dark horse at the crossroad of cancer management. *Biochem Pharmacol* **86,** 845–852, 2013.
11. Ramanathapuram LV, Hahn T, Graner MW, Katsanis E, and Akporiaye ET: Vesiculated alpha-tocopheryl succinate enhances the anti-tumor effect of dendritic cell vaccines. *Cancer Immunol Immunother* **55,** 166–177, 2006.
12. Hahn T, Fried K, Hurley LH, and Akporiaye ET: Orally active alpha-tocopheryloxyacetic acid suppresses tumor growth and multiplicity of spontaneous murine breast cancer. *Mol Cancer Ther* **8,** 1570–1578, 2009.
13. Hahn T, Jagadish B, Mash EA, Garrison K, and Akporiaye ET: alpha-Tocopheryloxyacetic acid: a novel chemotherapeutic that stimulates the antitumor immune response. *Breast Cancer Res* **13,** R4, 2011.
14. Hahn T, Bradley-Dunlop DJ, Hurley LH, Von-Hoff D, Gately S, et al.: The vitamin E analog, alpha-tocopheryloxyacetic acid enhances the anti-tumor activity of trastuzumab against HER2/neu-expressing breast cancer. *BMC Cancer* **11,** 471, 2011.
15. Rodriguez-Enriquez S, Hernandez-Esquivel L, Marin-Hernandez A, Dong LF, Akporiaye ET, et al.: Molecular mechanism for the selective impairment of cancer mitochondrial function by a mitochondrially targeted vitamin E analogue. *Biochim Biophys Acta* 1817, 1597–1607, 2012.
16. Shun MC, Yu W, Park SK, Sanders BG, and Kline K: Downregulation of epidermal growth factor receptor expression contributes to alpha-TEA's proapoptotic effects in human ovarian cancer cell lines. *J Oncol* 824571, 2010. Q2
17. Hahn T, Polanczyk MJ, Borodovsky A, Ramanathapuram LV, Akporiaye ET, et al.: Use of anti-cancer drugs, mitocans, to enhance the immune responses against tumors. *Curr Pharm Biotechnol*, **14**: 357–376, 2013.
18. Anderson K, Simmons-Menchaca M, Lawson KA, Atkinson J, Sanders BG, et al.: Differential response of human ovarian cancer cells to induction of apoptosis by vitamin E succinate and vitamin E analogue, alpha-TEA. *Cancer Res* **64,** 4263–4269, 2004.

19. Tomasetti M and Neuzil J: Vitamin E analogues and immune response in cancer treatment. *Vitam Horm* **76,** 463–491, 2007.
20. Chen W, Park SK, Yu W, Xiong A, Sanders BG, et al.: Synthesis and screening of novel vitamin E derivatives for anticancer functions. *Eur J Med Chem* **58,** 72–83, 2012.
21. Behery FA, Akl MR, Ananthula S, Parajuli P, Sylvester PW, et al.: Optimization of tocotrienols as antiproliferative and antimigratory leads. *Eur J Med Chem* **59,** 329–341, 2013.
22. Ling MT, Luk SU, Al-Ejeh F, and Khanna KK: Tocotrienol as a potential anticancer agent. *Carcinogenesis* **33,** 233–239, 2012.
23. Gogvadze V, Norberg E, Orrenius S, and Zhivotovsky B: Involvement of Ca2+ and ROS in alpha-tocopheryl succinate-induced mitochondrial permeabilization. *Int J Cancer* **127,** 1823–1832, 2010.
24. Malafa M and Neitzel L: Vitamin E succinate promotes breast cancer tumor dormancy. *The Journal of Surgical Research* **93,** 163–170, 2000.
25. Ralph SJ and Neuzil J: Mitochondria as targets for cancer therapy. *Mol Nutr Food Res* **53,** 9–28, 2009.
26. Dong LF, Freeman R, Liu J, Zobalova R, Marin-Hernandez A, et al.: Suppression of tumor growth in vivo by the mitocan alpha-tocopheryl succinate requires respiratory complex II. *Clin Cancer Res* **15,** 1593–1600, 2009
27. Fariss MW, Fortuna MB, Everett CK, Smith JD, Trent DF, et al.: The selective antiproliferative effects of alpha-tocopheryl hemisuccinate and cholesteryl hemisuccinate on murine leukemia cells result from the action of the intact compounds. *Cancer Res* **54,** 3346–3351, 1994.
28. Liu B, Wu K, and Zhao D: Study on vitamin E succinate inducing apoptosis of human gastric carcinoma cell. *Wei Sheng Yan Jiu* **29,** 234–236, 2000.
29. Wu K, Zhao Y, Liu BH, Li Y, Liu F, et al.: RRR-alpha-tocopheryl succinate inhibits human gastric cancer SGC-7901 cell growth by inducing apoptosis and DNA synthesis arrest. *World J Gastroenterol* **8,** 26–30, 2002.
30. Kline K, Lawson KA, Yu W, and Sanders BG: 2007. Vitamin E and cancer. *Vitam Horm* **76,** 435–461.
31. Neuzil J, Weber T, Schroder A, Lu M, Ostermann G, et al.: Induction of cancer cell apoptosis by alpha-tocopheryl succinate: molecular pathways and structural requirements. *FASEB J* **15,** 403–415, 2001.
32. Kozin SV, Shkarin P, and Gerweck LE: The cell transmembrane pH gradient in tumors enhances cytotoxicity of specific weak acid chemotherapeutics. *Cancer Res* **61,** 4740–4743, 2001.
33. Barnett KT, Fokum FD, and Malafa MP: Vitamin E succinate inhibits colon cancer liver metastases. *J Surg Res* **106,** 292–298, 2002.
34. Dalen H and Neuzil J: Alpha-tocopheryl succinate sensitises a T lymphoma cell line to TRAIL-induced apoptosis by suppressing NF-kappaB activation. *Br J Cancer* **88,** 153–158, 2003.
35. Ramanathapuram LV, Kobie JJ, Bearss D, Payne CM, Trevor KT, et al.: alpha-Tocopheryl succinate sensitizes established tumors to vaccination with nonmatured dendritic cells. *Cancer Immunol Immunother* **53,** 580–588, 2004.
36. Neuzil J: Vitamin E succinate and cancer treatment: a vitamin E prototype for selective antitumour activity. *British Journal of Cancer* **89,** 1822–1826, 2003.
37. Lawson KA, Anderson K, Simmons-Menchaca M, Atkinson J, Sun L, et al.: Comparison of vitamin E derivatives alpha-TEA and VES in reduction of mouse mammary tumor burden and metastasis. *Exp Biol Med (Maywood)* **229,** 954–963, 2004.
38. Shun MC, Yu W, Gapor A, Parsons R, Atkinson J, et al.: Pro-apoptotic mechanisms of action of a novel vitamin E analog (alpha-TEA) and a naturally occurring form of vitamin E (delta-tocotrienol) in MDA-MB-435 human breast cancer cells. *Nutr Cancer* **48,** 95–105, 2004.
39. Ameisen JC: Response: programmed cell death. *Science* **274,** 20c–21c, 1996.
40. Favaloro B, Allocati N, Graziano V, Di Ilio C, and De Laurenzi V: Role of apoptosis in disease. *Aging (Albany NY)* **4,** 330–349, 2012.
41. Ramanathapuram LV, Hahn T, Dial SM, and Akporiaye ET: Chemo-immunotherapy of breast cancer using vesiculated alpha-tocopheryl succinate in combination with dendritic cell vaccination. *Nutr Cancer* **53,** 177–193, 2005..
42. Prasad KN, Kumar B, Yan XD, Hanson AJ, and Cole WC: Alpha-tocopheryl succinate, the most effective form of vitamin E for adjuvant cancer treatment: a review. *J Am Coll Nutr* **22,** 108–117, 2003.
43. Tomasetti M, Rippo MR, Alleva R, Moretti S, Andera L, et al.: Alpha-tocopheryl succinate and TRAIL selectively synergise in induction of apoptosis in human malignant mesothelioma cells. *Br J Cancer* **90,** 1644–1653, 2004.
44. Basu A, Grossie B, Bennett M, Mills N, and Imrhan V: Alpha-tocopheryl succinate (alpha-TOS) modulates human prostate LNCaP xenograft growth and gene expression in BALB/c nude mice fed two levels of dietary soybean oil. *Eur J Nutr* **46,** 34–43, 2007.
45. Kanai K, Kikuchi E, Mikami S, Suzuki E, Uchida Y, et al.: Vitamin E succinate induced apoptosis and enhanced chemosensitivity to paclitaxel in human bladder cancer cells in vitro and in vivo. *Cancer Sci* **101,** 216–223, 2010.
46. Wang XF, Birringer M, Dong LF, Veprek P, Low P, et al.: A peptide conjugate of vitamin E succinate targets breast cancer cells with high ErbB2 expression. *Cancer Res* **67,** 3337–3344, 2007.
47. Vermeulen K, Van Bockstaele DR, and Berneman ZN: The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif* **36,** 131–149, 2003.
48. Weber T, Lu M, Andera L, Lahm H, Gellert N, et al.: Vitamin E succinate is a potent novel antineoplastic agent with high selectivity and cooperativity with tumor necrosis factor-related apoptosis-inducing ligand (Apo2 ligand) in vivo. *Clin Cancer Res* **8,** 863–869, 2002.
49. Zhang S, Lawson KA, Simmons-Menchaca M, Sun L, Sanders BG, Kline K, et al.: Vitamin E analog alpha-TEA and celecoxib alone and together reduce human. *Breast Cancer Res Treat* **87,** 111–121, 2004.
50. Gu X, Song X, Dong Y, Cai H, Walters E, et al.: Vitamin E succinate induces ceramide-mediated apoptosis in head and neck squamous cell carcinoma in vitro and in vivo. *Clin Cancer Res* **14,** 1840–1848, 2008.
51. Israel K, Yu W, Sanders BG, and Kline K: Vitamin E succinate induces apoptosis in human prostate cancer cells: role for Fas in vitamin E succinate-triggered apoptosis. *Nutr Cancer* **36,** 90–100, 2000.
52. Kline K, Yu W, and Sanders BG: Vitamin E: mechanisms of action as tumor cell growth inhibitors. *J Nutr* **131,** 161S–163S, 2001.
53. Krieger N, Bassett MT, and Gomez SL: Breast and cervical cancer in 187 countries between 1980 and 2010. *Lancet* **379,** 1391–1392, 2012.
54. Neuzil J, Wang XF, Dong LF, Low P, and Ralph SJ: Molecular mechanism of 'mitocan'-induced apoptosis in cancer cells epitomizes the multiple roles of reactive oxygen species and Bcl-2 family proteins. *FEBS Lett* **580,** 5125–5129, 2006.
55. Hail N Jr. and Lotan R: Cancer chemoprevention and mitochondria: targeting apoptosis in transformed cells via the disruption of mitochondrial bioenergetics/redox state. *Mol Nutr Food Res* **53,** 49–67, 2009.
56. Yu W, Jia L, Park SK, Li J, Gopalan A, et al.: Anticancer actions of natural and synthetic vitamin E forms: RRR-alpha-tocopherol blocks the anticancer actions of gamma-tocopherol. *Mol Nutr Food Res* **53,** 1573–1581, 2009.
57. Neuzil J, Tomasetti M, Zhao Y, Dong LF, Birringer M, et al.: Vitamin E analogs, a novel group of "mitocans," as anticancer agents: the importance of being redox-silent. *Mol Pharmacol* **71,** 1185–1199, 2007.
58. Berridge MV, Herst PM, and Lawen A: Targeting mitochondrial permeability in cancer drug development. *Mol Nutr Food Res* **53,** 76–86, 2009.
59. Neuzil J, Dyason JC, Freeman R, Dong LF, Prochazka L, et al.: Mitocans as anti-cancer agents targeting mitochondria: lessons from studies with vitamin E analogues, inhibitors of complex II. *J Bioenerg Biomembr* **39,** 65–72, 2007.
60. Birringer M, Eytina JH, Salvatore BA and Neuzil J: Vitamin E analogues as inducers of apoptosis: structure-function relation. *Br J Cancer* **88,** 1948–1955, 2003.
61. Neuzil J, Dong LF, Ramanathapuram L, Hahn T, Chladova M, et al.: Vitamin E analogues as a novel group of mitocans: anti-cancer agents

that act by targeting mitochondria. *Mol Aspects Med* **28,** 607–645, 2007.

62. Zhao Y, Li R, Xia W, Neuzil J, Lu Y, et al.: Bid integrates intrinsic and extrinsic signaling in apoptosis induced by alpha-tocopheryl succinate in human gastric carcinoma cells. *Cancer Lett* **288,** 42–49, 2010.
63. Dong LF, Swettenham E, Eliasson J, Wang XF, Gold M, et al.: Vitamin E analogues inhibit angiogenesis by selective induction of apoptosis in proliferating endothelial cells: the role of oxidative stress. *Cancer Res* **67,** 11906–11913, 2007.
64. Turanek J, Wang XF, Knotigova P, Koudelka S, Dong LF, et al.: Liposomal formulation of alpha-tocopheryl maleamide: in vitro and in vivo toxicological profile and anticancer effect against spontaneous breast carcinomas in mice. *Toxicol Appl Pharmacol* **237,** 249–257, 2009.
65. Mi Y, Zhao J, and Feng SS: Targeted co-delivery of docetaxel, cisplatin and herceptin by vitamin E TPGS-cisplatin prodrug nanoparticles for multimodality treatment of cancer. *J Control Release* **169,** 185–192, 2013.
66. Dong LF, Grant G, Massa H, Zobalova R, Akporiaye E, et al.: alpha-Tocopheryloxyacetic acid is superior to alpha-tocopheryl succinate in suppressing HER2-high breast carcinomas due to its higher stability. *Int J Cancer* **131,** 1052–1058, 2012.
67. Hahn T, Szabo L, Gold M, Ramanathapuram L, Hurley LH, et al.: Dietary administration of the proapoptotic vitamin E analogue alpha-tocopheryloxyacetic acid inhibits metastatic murine breast cancer. *Cancer Res* **66,** 9374–9378., 2006
68. Mu L and Feng SS: Vitamin E TPGS used as emulsifier in the solvent evaporation/extraction technique for fabrication of polymeric nanospheres for controlled release of paclitaxel (Taxol). *J Control Release* **80,** 129–144, 2002.
69. De Teresa JM, Marquina C, Serrate D, Fernández-Pacheco R, Morellon L, et al.: From magnetoelectronic to biomedical applications based on the nanoscale properties of advanced. *Int J Nanotechnology* **2**(2), 3–22, 2005.
70. Nguyen TK and Green LAW: Functionalisation of nanoparticles for biomedical applications. *Nano Today* **5,** 213–230, 2010.
71. Chandrasekharan P, Maity D, Yong CX, Chuang KH, Ding J, et al.: Vitamin E (D-alpha-tocopheryl-co-poly(ethylene glycol) 1000 succinate) micelles-superparamagnetic iron oxide nanoparticles for enhanced thermotherapy and MRI. *Biomaterials* **32,** 5663–72, 2011.

# Capítulo 2

# Nanopartículas de magnetita funcionalizadas con α-tocoferil succinato (α-TOS) promueve la muerte selectiva de células de cáncer de cérvix

Magnetite nanoparticles functionalized with α-tocopheryl succinate (α-TOS) promote selective cervical cancer cell death. Aracely Angulo-Molina, Miguel Ángel Méndez-Rojas, Teresa Palacios- Hernández, Oscar Edel Contreras-López, Gustavo Alonso Hirata-Flores, Juan Carlos Flores-Alonso, Saúl Merino-Contreras, Olivia Valenzuela, Jesús Hernández, Julio Reyes-Leyva. Journal of Nanoparticle Research. En revision.

## RESUMEN

El α-tocoferil succinato (α-TOS) induce selectivamente la muerte por apoptosis en células tumorales, pero es sensible a las enzimas esterasas presentes en las células de cáncer de cérvix. En este trabajo, funcionalizamos nanopartículas de magnetita con α-TOS (α-TOS-Nps) para mejorar sus resistencia y actividad anticancerígena. Las nanopartículas se prepararon con el método de reducción-coprecipitación; la superficie de las nanopartículas se silanizó y se conjugó con el α-TOS. La composición química se analizó por espectroscopía de dispersión de energía de rayos X; los grupos funcionales se analizaron por espectroscopía de infra-rojo; la morfología, el tamaño y la estructura cristalina se analizaron por microscopía electrónica de transmisión, así como por difracción electrónica del área seleccionada. La carga de α-TOS se estimó por termogravimetría. La actividad biológica se evaluó en células no malignas (fibroblastos), así como en células de cáncer de cérvix por medio del ensayo colorimétrico del MTT. La internalización se analizó por microscopía confocal. Los análisis revelaron nanopartículas de forma esférica irregular con 15 nm de diámetro cristalinas y de alta estabilidad, con los constituyentes orgánicos e inorgánicos esperados. Se encontró que las nanopartículas fueron internalizadas en el núcleo y afectaron selectivamente la viabilidad de las células de cáncer de cérvix en una forma dosis y tiempo dependiente y no afectaron las células normales. En conclusión, la funcionalización de α-TOS a nanopartículas de magnetita preservó la actividad anticancerígena de α-TOS en células de cáncer de cérvix no susceptibles al α-TOS.

CENTRO DE INVESTIGACION
EN ALIMENTACION Y DESARROLLO, A.C.

CIAD

July 25, 2013

**Professor Mihail C. Roco**
**Editor**
***Journal of Nanoparticle Research***

**Dear Prof. Roco:**

Attached, please find a manuscript entitled "Magnetite nanoparticles functionalized with α-tocopheryl succinate (α-TOS) promote selective cervical cancer cell death" that we are submitting to be considered for publication in *Journal of Nanoparticle Research.*

I hereby declare that there is no conflict of interest involved. There was no financial arrangement or funding for this research by private or public companies. The information in this manuscript has not been submitted to any other source of publication and all the authors have read and approved its final version. The authors have disclosed any conflict of interest related to this article.

Thank you,

Sincerely,

Jesús Hernández, Ph.D.
Laboratorio de Inmunología, CIAD, A.C.
Hermosillo, Sonora. México
Phone and Fax: +52 662 280 0010
e-mail: jhdez@ciad.mx

# Magnetite nanoparticles functionalized with α-tocopheryl succinate (α-TOS) promote selective cervical cancer cell death

Aracely Angulo-Molina[a,b*], Miguel Ángel Méndez-Rojas[a], Teresa Palacios-Hernández[a,c], Oscar Edel Contreras-López [d], Gustavo Alonso Hirata-Flores[d], Juan Carlos Flores-Alonso[e], Saul Merino-Contreras[f], Olivia Valenzuela[g], Jesús Hernández[b], Julio Reyes-Leyva[e*]

[a] Universidad de las Américas Puebla (UDLAP), Puebla, México.

[b] Laboratorio de Inmunología, Centro de Investigación en Alimentación y Desarrollo, A.C. Hermosillo, Sonora, México

[c] Universidad Popular Autónoma del Estado de Puebla (UPAEP), Puebla, México.

[d] Universidad Nacional Autónoma de México, Centro de Nanociencias y Nanotecnología (CNYN), Ensenada, BCN, México.

[e] Centro de Investigación Biomédica de Oriente, Instituto Mexicano del Seguro Social, Metepec, Puebla, México.

[f] Benemérita Universidad Autónoma de Puebla (BUAP), Puebla, México.

[g] Departamento de Ciencias Químico-Biológicas, Universidad de Sonora, Hermosillo, Sonora, México.

* Corresponding Author:

- Aracely Angulo Molina, Universidad de las Américas Puebla, Departamento de Ciencias de la Salud, Oficina SL-305-A Ex-Hda. de Sta. Catarina Mártir, San Andrés Cholula 72820, Puebla, México. Phone: +(52)-222-229-2000, EXT 4335; Fax: +(52)-222-229-24-19; Email: aracely.angulo@udlap.mx

- Julio Reyes-Leyva, Centro de Investigación Biomédica de Oriente, Instituto Mexicano del Seguro Social, Metepec, Puebla, México. Phone: +52 244 444 0122; Email: julio.reyes@imss.gob.mx

**ABSTRACT**

α-tocopheryl succinate (α-TOS) selectively induces apoptosis in cancer cells but it is sensitive to esterases present in cervical cancer cells. In this work, we functionalized magnetite nanoparticles with α-TOS (α-TOS-Nps) to enhance its resistance and anticancer activity. The nanoparticles were prepared by a reduction-coprecipitation method; their surface was silanized and conjugated to α-TOS. Chemical composition was analyzed by energy dispersive X ray spectroscopy, functional groups were determined by Fourier transform infrared spectroscopy; morphology, size and crystal structure were analyzed by scanning electron microscopy, transmission electron microscopy and selected area electron diffraction; α-TOS load on nanoparticles was estimated by thermogravimetric analysis. The biological activity of α-TOS-Nps was evaluated in nonmalignant fibroblastes and cervical cancer cells by means of the colorimetric viability test and the intracellular localization was identified by confocal laser scanning microscopy. Our results demonstrated functionalization of magnetite nanoparticles with α-TOS. Electronic microscopy studies revealed sphere-like nanoparticles with a 15 nm average size. The characterization results support the nanoparticles formation by mineral and organic constituents detection respectively with high stability. The α-TOS-Nps were internalized in the nucleus and selectively affected the viability of cervical cancer cells in a dose and time dependent way. In normal cells α-TOS-Nps were biocompatible. In conclusion, the functionalization magnetite nanoparticles protected the anticancer activity of α-TOS in non sensitive cancer cells.

**Keywords:** magnetite nanoparticles; α-tocopheryl succinate; cancer; biomaterials.

## Introduction

Iron oxide nanoparticles possess exceptional physical and chemical properties, which led to their potential use in biomedical applications such as drug carrier and drug release of conventional chemotherapeutic agent in modern cancer therapies (Baba et al. 2012; Kim et al. 2006). In particular, magnetite nanoparticles (Nps) offer higher biocompatibility than other crystalline phases of other magnetic iron oxide nanoparticles such as maghemite (Baba et al. 2012), and are widely used for magnetic resonance imaging (MRI) (Hultman et al. 2008; Amstad et al. 2009), cell and tissue targeting (Min et al. 2011; Mohapatra et al. 2007) or hyperthermia therapy (Baba et al. 2012). Furthermore, their surface can be modified with functional molecules to obtain bioactive core-shell nanocarriers and more effective drug delivery systems (Rivas et al. 2012; Ghotbi and bin Hussein 2012; Zhang et al. 2002). The utility

57 of a nanoscale delivery system is based on their potential to enhance drug delivery, higher accumulation in the target
58 area and drug delivery efficacy into tumor tissues, biocompatibility, higher chemical stability and reducing non
59 specific toxicity (Ghotbi and bin Hussein 2012; Nguyen and Luke 2010; Mohapatra et al. 2007). The characteristics
60 of iron oxide nanoparticles are crucial for medical purposes. Different nanoparticles have already been used as
61 carrier systems for pharmaceutical drugs in the past, and recently they have attracted attention as a carrier system for
62 bioactive food components such as vitamin E.

63 One of the most important vitamin E analogues, α-tocopheryl succinate, has shown to selectively kill tumor cells
64 (Neuzil et al. 2001). This analogue is an esterified derivative of α-tocopherol (α-TOH), which suppressed cell growth
65 in a wide range of human cancer cells such as prostate, breast, lung, endometrial, leukemia, lymphoma, colon, and
66 melanoma (Dong et al. 2012; Kanai et al. 2010; Tomasetti et al. 2010; Gu et al. 2008; Anderson et al. 2004; Malafa
67 et al. 2002; Neuzil et al. 2001). α-TOS selectively kills cancer cells without toxic effects or low toxicity for
68 nonmalignant cells; by these characteristics this analogue is considered an agent with significant clinical potential
69 (Neuzil et al. 2001; Anderson et al. 2004). However, a problem with α-TOS is its vulnerability to esterases in
70 cervical and ovarian cancer cells. In these cell lines α-TOS is less effective than other vitamin E analogues, because
71 the endogenous esterases can hydrolyze the succinate moiety of α-TOS converting it into α-TOH, which is an
72 ineffective agent (Dong et al. 2012; Anderson et al. 2004). In recent years, there has been increased interest in the
73 development of special formulations or multidrug combinations to improve the anticancer activity of vitamin E
74 analogues such α-TOS but in cervical cancer the reports are scarce (Kanai et al. 2010; Ma et al. 2010a; Tomasetti et
75 al. 2010).

76 The susceptibility of α-TOS to high levels of esterases present in cancer cells could be protected by the conjugation
77 of α-TOS to a carrier or drug delivery platform. In this sense, iron oxide Nps possess exceptional physical and
78 chemical properties that make of them potential drug carriers (Chen et al. 2012; Amstad et al. 2011; Nguyen and
79 Luke 2010; Amstad et al. 2009; Mahmoudi et al. 2009; Gupta and Wells 2004; Zhang et al. 2002). Nps can be coated
80 with cross-linker molecules as silanes and subsequently functionalized with bioactive ligands covalently bound to the
81 silane shell. Nps functionalization is becoming important approach for many applications especially in the
82 biomedical field (Amstad et al. 2011; Nguyen and Luke 2010; Zhang et al. 2002). For example, functionalized Nps
83 are internalized by endocytosis and can interact with cell membranes resulting in enhanced response and low toxicity
84 (Mohapatra et al. 2007; Gupta and Wells 2004; Zhang et al. 2002).

In this study, we described the synthesis and characterization of magnetite Nps functionalized with α-TOS. We also show that α-TOS-Nps achieved effective cytotoxic activity in cervical cancer cells without side effects in non-malignant cells. Thus, the efficacy of α-TOS as anticancer drug was enhanced *in vitro* by means of its functionalization on magnetite Nps.

## Materials and methods

### Materials

The vitamin E analogue α-TOS, ferric chloride hexahydrate, (3-Aminopropyl) trimethoxysilane (APTMS), N-Hydroxysuccinimide (NHS), N,N′-Diisopropylcarbodiimide (NDC), triethylamine (TEA), fluorescein isothiocianate (FITC), sodium sulfite ($Na_2SO_3$) were analytical grade and purchased from Sigma-Aldrich. Other reagents were Toluene ($C_7H_8$), ammonium hydroxide ($NH_3OH$) and absolute ethanol ($CH_3CH_2OH$) from RBM, hydrochloric acid (HCl) from Meyer and bencyl alcohol ($C_6H_5CH_2OH$) from JT Baker. All the chemicals were used as received without further purification.

### Synthesis and functionalization of nanoparticles

The Nps were prepared by a reduction-coprecipitation method as previously reported using ferric chloride ($FeCl_3{\cdot}6H_2O$) as the precursor material but with some modifications (Qu et al. 1999). Briefly, the precursor was partially reduced to the ferrous ion by $Na_2SO_3$ before alkalinizing with ammonia and subsequently a black precipitate was formed. The precipitate, was washed in absolute ethanol, centrifuged and dried at 60$^o$ C overnight to remove adsorbed water. In order to functionalize the surface of Nps was chemically modified with the polymer spacer (3-Aminopropyl) trimethoxysilane (APTMS) obtaining silanized Nps with exposed amino groups on the Nps surfaces as previously described (Zhang and Zhang 2005; Zhang et al. 2002). It was expected that silanized Nps with the amino groups exposed could provide the medium for their α-TOS functionalization through a chemical reaction between amino and carboxyl group in α-TOS. Thus, the functionalization was carried out by adding 100 mg of silanized Nps to 6.6 mL of an ethanolic mixture of 10 mM α-TOS, 10 mL of 15mM N-Hydroxysuccinimide (NHS),

113 10 mL of 75mM N,N′-Diisopropylcarbodiimide (NDC) solution and triethylamine (TEA) (Zhang et al. 2002). The
114 pH was adjusted to 9 and after incubation under stirring at 50$^{o}$ C for 4 h the suspension was centrifuged and the
115 precipitate (labeled as α-TOS-Nps) was sonicated for 5 min. Afterwards, α-TOS-Nps was washed with benzyl
116 alcohol and deionized water and dried in an oven at 60$^{o}$ C overnight.
117
118 To study cell internalization, some α-TOS-Nps were further conjugated to fluorescein isothiocianate (FITC)
119 following essentially the same procedure described above and labeled as α-TOS-Nps-FITC. Briefly, 100 mg of Nps-
120 αTOS were added to an ethanolic solution of 31.9 mL 15mM FITC, 10 mL 15mM NHS, 10 mL 75mM NDC and
121 TEA. The pH was adjusted to 9 and after incubation at 37$^{o}$ C for 4 h, the mixture was centrifuged and the precipitate
122 (α-TOS-Nps-FITC) was washed and dried in an oven at 60$^{o}$ C overnight. α-TOS-Nps-FITC were kept in the dark
123 until further use. Before each experiment, the nanoparticles were dispersed by pulsed sonication to reduce particle
124 agglomeration to the minimum.
125
126 Characterization of nanoparticles
127
128 All synthesized and functionalized Nps were characterized by various analytical techniques. Chemical composition
129 was analyzed by energy dispersive X ray spectroscopy (EDS) using a Thermo Scientific Super Dry II Instrument.
130 The functional groups were determined by Fourier Transform Infrared Spectroscopy (FTIR) with a Varian Scimitar
131 FTIR-800 Instrument equipped with an ATR detector. FTIR analyses were performed on gently grinded samples and
132 each recorded spectrum resulted after averaging 16 scans in the 400-4000 $cm^{-1}$ region at a resolution of 4 $cm^{-1}$.
133 Morphology was analyzed by Scanning Electron Microscopy (SEM) imaging using a JEOL JSM5300 microscope
134 operating with electron beam energy of 15 keV. Transmission Electron Microscopy (TEM) observations and
135 Selected Area Electron Diffraction (SAED) analyses were carried out with a JEOL JEM2010 microscope operated
136 with an electron beam energy of 200 keV. The α-TOS load and shell surrounding on the magnetite nanoparticles
137 were estimated by Thermogravimetric Analysis (TGA). The samples were heated in a Netzsch TGA apparatus at 30-
138 500$^{o}$ C a rate of 20 K/min. The analysis was performed under a flow of $N_2$ (60 mL/min). For drug load determination
139 was considered: a) the difference between mass loss in thermal profiles; b) all iron oxide nanoparticles were in the
140 form of magnetite ($Fe_3O_4$); c) nanoparticles were completely oxidized at 500$^{o}$C; d) the ratio of silanized Nps and α-

TOS was 3.86:1 in the final reaction and e) percent of each component in the samples (Rutnakornpituk et al. 2009). Finally, with these considerations the drug loading and entrapment efficiency were determined as:

$$\text{Drug loading} = (\text{Weight of drug in nanoparticles} / \text{Weight of nanoparticles}) \times 100$$

$$\text{Entrapment efficiency} = (\text{Weight of drug add} / \text{Weight of loaded drug}) \times 100$$

Cell culture

The human cervical cancer cell line SiHa (ATCC No. HTB-35) and the nonmalignant mouse fibroblasts cell line (ATCC No. CCL-1) used in these experiments were provided by Dr. Verónica Vallejo (Centro de Investigación Biomédica de Oriente, Puebla, México) and Dr. Verónica Mata (Centro de Investigación en Alimentación y Desarrollo, Sonora, México), respectively. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 1% penicillin-streptomycin and 1% glutamine at 37$^{o}$ C in a humidified atmosphere with 5% $CO_2$.

Cellular viability

The effect on cell viability/cytotoxicity of the Np, α-TOS and α-TOS-Nps was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) colorimetric assay. Fibroblasts and cancer cells were plated at a density of 1 x $10^4$ cells/well in a 96-wells plate and incubated in a humidified 5% $CO_2$ at 37$^{o}$ C. After 24 h of culture to allow cell attaching, the medium in the wells was replaced with fresh medium containing different concentrations of Nps, α-TOS or α-TOS-Nps (0, 2.5, 5, 10, 20, 40, and 80 µg/mL). The nanoparticles were previously sterilized by filtration through 0.45 µm Millex GV filter units (Millipore), and incubated for 24, 48 and 72 h. After this time, the medium containing unbound nanoparticles was removed and MTT solution (5 mg/mL in PBS pH 7.4) was added. After a further incubation at 37$^{o}$ C in the dark for 4 h, 100 µL of acidified isopropanol were added to each well and the absorbance was monitored in a microplate reader at a wavelength of 550 nm. All the tests were performed by triplicate, untreated cells were considered as controls and the cell viability was calculated as:

$$\%\ \text{Cell viability} = (\text{Absorbance of sample well}/\text{absorbance of control well}) \times 100$$

171

172 The 50% inhibiting concentration ($IC_{50}$), defined as the concentration required for 50% inhibition of cell growth in
173 comparison with control sample, was determined by curve fitting of the cell viability data (Ma et al. 2010b).

174

175 Cellular internalization

176

177 To study cellular internalization of functionalized Nps, SiHa cervical cancer cells were seeded at a density of 1.5 x
178 $10^5$ cells per well in an 8-well plastic Lab-TekII Chamber slides (Nalge Nunc Inc). After 24 h the medium was
179 replaced with fresh medium containing α-TOS-Nps-FITC at 0, 5, 40, and 80µg/mL concentrations. Chamber slides
180 were incubated at 37$^o$ C, 5% $CO_2$ for 72 h. After this, the medium was removed and the cells were washed twice with
181 PBS and fixed with 1:1 (vol/vol) methanol-acetone solution by 30 min followed by washing in PBS-Tween 0.05%.
182 Cells were counterstained with propidium iodide (PI) for 5 min. All the samples were viewed with a Nikon D-
183 Eclipse C1 confocal laser scanning microscope. The FITC and PI were excited using 488 and 533 nm wavelength
184 lasers, respectively.

185

186 Statistical analysis

187

188 Results were expressed as mean values ± SEM in triplicate. The program GraphPad Prism 5 was used for the
189 calculation of viability curves. Statistical significance was analyzed using SPSS software (v. 13; SPSS Inc.,
190 Chicago). The data were analyzed by analysis of variance (ANOVA) and Scheffe post hoc tests at a 0.05 level of
191 significance.

192

193 **Results and Discussion**

194

195 Firstly, the uncoated Nps, silanized Nps and α-TOS-Nps were characterized with various methods. Figure 1 shows
196 representative SEM, TEM and SAED images obtained from the nanoparticles. SEM analysis, for all kinds of
197 nanoparticles, reveals an agglomerated-like structure with grain dimensions in the range of 4-6 µm (Figure 1a, 1d
198 and 1g). However, TEM observation on single grains reveals a nanostructured material basically formed by sphere-

like nanoparticles with a 15 nm average size (Figure 1b, 1e and 1h). Electron diffraction of a single crystallite is shown in Figure 1c and its indexation corresponds to the Fd-3m space group of the magnetite structure. The interplanar spacings associated to the indexes are in agreement with the standard JCPDS card No.19-0629 of $Fe_3O_4$. SAED analysis on all observed nanoparticles reveals the crystal structure of the iron oxide (Figure 1c, 1f, and 1i). No appreciable changes in morphology and crystallinity were observed on the Nps after coupling with APTMS or α-TOS, respectively.

Surface modification of Nps was confirmed by an EDS analysis. The analysis of the EDS spectra of pure Nps reveals the characteristic X-ray line for oxygen and iron (Figure 2a), the elemental constituents of the magnetite compound. The small peak observed below 0.5 keV is associated to carbon and corresponds to the supporting carbon tape used to hold the samples. Once the Nps were treated with APTMS ($C_6H_{17}NO_3Si$), an X-ray line related to silicon Si-Kα became evident (Figure 2b) and can be related to surface silanization of the magnetite Nps. Any X-ray emission from nitrogen atoms can be enveloped together with the X-ray peak associated to carbon; the Kα bands for nitrogen and carbon are < 0.1 keV split apart and are located at the lower detection limit (less sensitive region) of the EDS system, which makes them difficult to discern. Silicon X-ray signal was also observed on functionalized Nps with α-TOS (Figure 2c), as expected because they still contain the silane moiety. From the EDS analysis, it can be concluded that Nps surfaces are now chemically modified with APTMS and can be further functionalized with α-TOS.

In order to evaluate the molecular coupling between α-TOS and silanized Nps, FTIR spectroscopy was performed. A characteristic FTIR spectrum for α-TOS is shown in Figure 3a. Relevant peaks related to α-TOS chemical structure are found around 2915 $cm^{-1}$, that consisted in saturated C-H stretching vibrations, characteristic of the phytyl chain. At 1747 and 1701 $cm^{-1}$ C=O asymmetric stretching from the succinate moiety is observed; peaks around the region of 1450-1360 $cm^{-1}$ due to C-H bending vibration of $–CH_2$ and $–CH_3$ groups, and between 1200-1300 $cm^{-1}$ peaks related to C-C and C-C-H stretching. The FTIR spectrum from uncoated Nps ($Fe_3O_4$ nanoparticles before chemically modification of their surfaces) presents less peaks as shown in Figure 3c which is in agreement with other reports and expected for a pure crystalline metal oxide. A broad and very small shoulder appears around 3400-3200 $cm^{-1}$, which is related to hydroxyl groups present on the magnetite surface. The strong peak that was around 500-600 $cm^{-1}$, is typically associated to Fe-O stretching vibrations (Mohapatra et al. 2007; Kim et al. 2006).

Figure 3b shows a FTIR spectrum from the α-TOS-Nps. The relevant features in this spectrum are the peaks at 2976 and 2889 $cm^{-1}$ related to the phytyl chain (C-H stretching vibrations); at 1700 $cm^{-1}$ (C=O stretching from the

succinate carbonyl groups); a peak around 1640 $cm^{-1}$ (C=N stretching from the amide group); a set of peaks around 1527 $cm^{-1}$ (C-H aromatic ring), 1458, 1366 and 1256 $cm^{-1}$ (C-H bending); a peak around 1142 $cm^{-1}$ related to a Si-O vibrational stretching from the preliminary modification of the surface with APTMS; and finally the strong peak at 500-600 $cm^{-1}$ already associated to the Fe-O stretching vibration. Based in these results can be conclude that Nps were correctly functionalized with α-TOS.

TGA was performed to estimate the amount of α-TOS loaded in α-TOS-Nps. The thermograms of magnetite nanoparticles showed an initial and continuous weight loss in all the samples between 0-300$^{o}$ C (Figure 4). In the range of ~0-120$^{o}$ C the weight loss may be attributed to desertion of physically adsorbed water molecules (Mohapatra et al. 2007). In the range of ~290-475$^{o}$ C the weight loss in uncoated Nps (Figure 4a) was less and comparatively weaker in contrast with silanized Nps and α-TOS-Nps (Figure 4b-c). The values of weight loss ranged from 290$^{o}$ C to 475$^{o}$ C in the samples (Figure 4a-c) represent the most important decomposition of organic components coupled to nanoparticle surfaces (Rutnakornpituk et al. 2009). By comparing the difference in mass loss measured with TGA between the samples and considering these values corresponding to 99.52% of the vitamin E analogue in α-TOS-Nps, the α-TOS loaded was estimated in 8.14% (153 $\mu mol \cdot g^{-1}$ iron oxide) with an entrapment efficiency of 31.4%.

Following the successful synthesis and functionalization of α-TOS-Nps, the effects of α-TOS-Nps in cervical cancer cells (SiHa) and in non-malignant cells (fibroblastes) were evaluated. First, the effect of Nps, α-TOS and α-TOS-Nps on the cell morphology was evaluated with phase contrast microscopy at 24, 48 (data not shown) and 72 h (Figure 5) Fibroblastes and SiHa cells exposed to either Nps or α-TOS maintained their normal morphology at the highest doses evaluated (80 μg/mL) after 72 h. Indeed, treated cells were indistinguishable from untreated control cells (Figure 5d-f). Treatment with α-TOS-Nps induced drastic morphological changes suggestive of cell death in cervical cancer cells. The damage was more evident at 72 h after treatment (Figure 5g-h). In contrast, fibroblasts remained unchanged. Some fibroblastes with abnormal morphology were seen only at the highest α-TOS-Nps concentration at 72 h.

In order to prove that *in vitro* effects of α-TOS-Nps in cervical cancer cells are due to the internalization of α-TOS-Nps, the intra-cellular distribution of α-TOS-Nps was studied using α-TOS-Nps conjugated to fluorescein (α-TOS-Nps-FITC) observed at different Z-stage images under confocal microscopy. Clear accumulation of α-TOS-Nps-FITC (green) is observed in the nucleus (Figure 6b-d). Cells were counterstained with PI, which is excluded from viable cells and only stains dead cells (red). This result was observed in all concentrations evaluated after 72 h. These

255 data suggest that α-TOS-Nps can be internalized in cervical cancer cells and by this way may exert their toxic
256 effects. Similar changes in cells treated with magnetite nanoparticles loaded with different drugs have been reported
257 (Zhang et al. 2002).

258 Finally, dose and time response studies on the viability of SiHa and fibroblastes cells were conducted. These assays
259 were realized to confirm that α-TOS-Nps achieved effective citotoxicity effect in resistant cancer cells without side
260 effects in non-malignant cells. Figure 7 shows viability curves of SiHa and fibroblastes cells with all the treatments
261 at 24, 48 and 72 h. Data reveals that cells remained viable with Np or α-TOS in all the concentrations and times
262 evaluated. Importantly, these results suggest that α-TOS-Nps was cytotoxic only in SiHa cells in a dose and time
263 dependent manner; the inhibitory effect of α-TOS-Nps was significantly different from the values of the control at
264 48-72h in SiHa cells ($p<0.05$). In contrast, a very low cytotoxicity to fibroblast cells was observed, even at relatively
265 high concentrations. The highest concentration of 80 μg/mL of α-TOS-Nps did not result in a significant difference
266 in citotoxicity between the control, and even Nps or α-TOS ($p<0.05$). The $IC_{50}$ for α-TOS-Nps in SiHa cells at 72 h
267 exposure was 65.29 μg/mL, corresponding to 5.37 μg/mL of α-TOS loaded on the surface of nanoparticles. Taking
268 these results together suggested that functionalized α-TOS-Nps protect α-TOS from cellular esterase as previously
269 hypothesized, and their cytotoxic effects on SiHa cells can be maintained.

270 In this study, magnetite nanoparticles were synthesized and functionalized with α-TOS, in order to increase the anti-
271 cancer property of this vitamin E analogue, because it is usually inactivated by cellular esterases. We proposed that
272 the functionalization of Nps with α-TOS protects the selective anticancer activity in a cervical cancer cell line
273 without side effects in non-malignant cells. SiHa cells, a malignant human cervical cell line, were chosen because it
274 was reported as non-sensitive to α-TOS due to the high esterases content (Anderson et al. 2004). Our results in SiHa
275 cells acquire relevance because cervical cancer is the second most common cancer in women worldwide and
276 therapeutic drugs for the metastatic stage of cervical cancer are limited (Ma et al. 2010a; Anderson et al. 2004).

277 The magnetite nanoparticles were synthesized with the co-precipitation method, one of the most conventional and
278 economic methods for obtaining iron oxide nanoparticles (Wu et al. 2008; Qu et al. 1999). Then, the nanoparticles
279 were prepared with the silane APTMS due to its advantages in biocompatibility as well as for the high density of
280 surface functional endgroups that allows the attachment of other biomolecules (Shen et al. 2004; Zhang et al. 2002;
281 Zhang and Zhang 2005). Importantly, during the synthesis and functionalization, the nanoparticles were
282 characterized to evaluate and guarantee the process. It is important to properly and precisely characterize

nanoparticles to ensure the reproducibility of the results and to understand and relate to biological effects of nanoparticles (Vippola et al. 2009). Thus, size and morphology, key elements in the bioactivity of nanoparticles, were evaluated with TEM and SEM. The images indicated sphere-like nanoparticles with a 15 nm average size. Some moderately agglomerated nanoparticles as well as separated nanoparticles were present in the samples. Agglomeration can be a problem that limits cell penetration of Nps and α-TOS-Nps, but it was solved with sonication for 5 min before each *in vitro* assay. More details about chemical structure were obtained from SAED analysis, which revealed that all of the observed nanoparticles had the crystalline structure of the magnetite (Cai and Wan 2007). No appreciable changes in morphology and crystallinity were observed on the Nps after coupling with APTMS or α-TOS, respectively. These results indicate that the silanization and functionalization process did not affect the Nps.

Results from EDS and FTIR analyses confirmed that Nps surfaces were chemically modified with APTMS and supported further functionalization with α-TOS. During silanization, the silane group of APTMS is attached to the magnetite surface through Si-O-Fe bonds (Zhang et al. 2002). α-TOS functionalization can be carried out through the chemical condensation of the carboxylic group of α-TOS with the $-NH_2$ groups on the aminosilane moiety attached to the magnetite surface after silanization, forming a functional amide group (Figure 8c). Based in this principle, magnetite Nps have been successfully functionalized with: folic acid, polyethylene glycol, polyethylene glycol-folic acid or folic acid-fluorescent conjugates (Mohapatra et al. 2007; Zhang et al. 2002; Zhang and Zhang 2005).

TGA was used to confirm and to estimate the α-TOS load and shell surrounding on the magnetite nanoparticles. Thermal degradation profiles permit to identify changes in sample weight by dehydration and decomposition of physically and chemoabsorbed molecules (Choy et al. 2010; Amstad et al. 2009; Rutnakornpituk et al. 2009). α-TOS-Nps were summited to TGA to determine their weight loss in comparison with uncoated Nps and silanized Nps. A continuous weight loss in all the samples was observed in the temperature interval of 0-300$^{o}$ C attributed to dehydration of the magnetite samples. This finding is consistent with other works (Rutnakornpituk et al. 2009; Perez-Gonzalez et al. 2011). Furthermore, it was considered that the weight loss up to ≈300$^{o}$ C may be attributed to decomposition of physically and chemoadsorbed organic components as silanol and α-TOS molecules that require higher energy to dissociate from the particle surface as well (Choy et al. 2010; Mohapatra et al. 2007; Daou et al. 2006). Thus, in the interval of ≈300-500$^{o}$ C the weight loss in uncoated Nps was less and comparatively much weaker than the silanized Nps and α-TOS-Nps (Figure 5) (Mohapatra et al. 2007). This result supports the presence

311 of organic components in silanized Nps and α-TOS-Nps surface but not in uncoated Nps. The organic components
312 were lost when the samples were heated within this temperature interval (Mohapatra et al. 2007; Choy et al. 2010).
313 In this way, the α-TOS shell surrounding the magnetite nanoparticles was estimated in 8.14% with an entrapment
314 efficiency of 31.4%. Some similar considerations have been used to confirm the organic contents in nanocapsules
315 with TGA (Choy et al. 2010). In other works, TGA has been used coupled to a FTIR to confirm the presence and
316 quantify the amount of organic coated agents on iron oxide nanoparticles (Amstad et al. 2009). In the present study,
317 FTIR and TGA were separately used to estimate and additionally support the presence of bound molecules on the
318 particle surface.
319 In this work, the characterization was important because there is a lack of knowledge about Nps functionalized with
320 vitamins. Small changes in parameters as size, shape and surface chemistry can dramatically influence
321 biocompatibility and the cytotoxic effect. We functionalizated Nps with α-TOS, a vitamin E analogue, because the
322 Nps have been used alone or in combination to enhance the efficiency of other anticancer drugs (Chen et al. 2009).
323 Previous works have shown that α-TOS is inefficient in cervical cancer cells that express esterases abundantly (Dong
324 et al. 2011; Anderson et al. 2004). α-TOS is vulnerable to esterases, which converts it to vitamin E. α-TOS differs
325 from vitamin E in the hydroxyl group at carbon 6 of the phenolic ring that has been replaced by a succinic acid
326 residue linked by an ester bond (Figure 8a), this change make α-TOS sensitive to hydrolytic cleavage by esterases. In
327 this work, was hypothesized that coupling to Nps protects α-TOS against esterases attack. A sketch of the different
328 steps for magnetite Nps functionalization with α-TOS is illustrated in Figure 8b-c.
329 Our results showed that α-TOS-Nps recovered their cytotoxic activity on cervical cancer cells; therefore we
330 hypothesized that coupling to Nps protects α-TOS against esterase attack. The evaluation of viability in fibroblasts
331 and cancer cells allows contrasting the biological effects on two kinds of cells that are not sensitive to α-TOS. The
332 viability/citotoxicity effect was determined by the colorimetric MTT assay, a common method used to evaluate
333 nanomaterials. After the treatments, the cells are exposed to MTT salt, and the viable cells transform this salt to
334 formazan, which can be quantified by absorbance at 570 nm (Huang et al. 2012; Mahmoudi et al. 2009; Gupta and
335 Wells 2004). Our results confirmed that both viability and morphology of SiHa cells was not affected by α-TOS or
336 uncoated Nps. We observed that the viability of SiHa cells treated with α-TOS was enhanced in a doses dependent
337 manner at 24 h (10-80 μg/mL), provably due to α-TOH nutritional effect. These results confirmed SiHa cells are not
338 sensitive to α-TOS treatment at doses used in previous studies (Anderson et al. 2004). Importantly, neither α-TOS

339 nor Nps alone showed toxicity to non-malignant fibroblastes in agreement with previous reports (Anderson et al.
340 2004; Kim et al. 2006). However, it was reported that fibroblastes treated with surface modified Nps showed loss in
341 viability of about 25-50 % (Gupta and Wells 2004). Although this result can be contradictory, the cells were treated
342 with 250 μg/mL magnetite nanoparticles, a dose three times higher and different physicochemical characteristic than
343 in our work and this could be the reason for the different obtained results.
344 Notably, α-TOS-Nps selectively affected the cancer cells in a dose-and time-dependent way at 48-72 h and
345 fibroblastes exhibited a non significant toxicity (<20%) at the highest applied concentration of α-TOS-Nps at 72 h
346 exposure. Non toxicity and biocompatibility of magnetite nanocarriers in normal cells are crucial characteristics for
347 medical purposes (Ghotbi and bin Hussein 2012). The low toxicity of α-TOS-Nps in fibroblasts is important because
348 a serious problem encountered when the structure of anticancer compounds has been modified is the loss of
349 selectivity for cancer cells and side effects in normal cells (Turanek et al. 2009). In this sense, α-TOS-Nps was
350 selective in cancer cells and biocompatible in non malignant cells.
351 Anderson et al (2004) reported that 5-10 μg/mL of α-TOS induced selectively 50% of cell apoptosis in human breast,
352 prostate and colon cancer cells but not in cervical and ovarian cancer cells. In the present work we calculated that
353 $IC_{50}$ for α-TOS-Nps was 65.29μg/mL after 72 h treatment; considering the load drug results with TGA, this value
354 corresponds to 5.37μg/mL of α-TOS, a very close dose previously reported (Anderson et al. 2004). This may be
355 indicative of the death promotion at α-TOS doses that have been used in sensitive cancer cells in others works.
356 The mechanism by which α-TOS-Nps can affect the viability in resistant cervical cancer cells remains unclear. It is
357 generally believed that the inhibitory effects of α-TOS are not mediated by its antioxidant property (Gogvadze et al.
358 2010; Kline et al. 2001). The ester linkage that attaches succinic acid to vitamin E eliminates the hydroxyl moiety
359 (Figure 8a), which mediates vitamin E´s classical antioxidant properties (Gogvadze et al. 2010). Previous studies
360 have shown that α-TOS inhibits selectively cancer cells growth by apoptosis (Gogvadze et al. 2010; Anderson et al.
361 2004; Neuzil et al. 2001). In cancer cells the antioxidant defenses are decreased and α-TOS has the property to
362 induce the accumulation of reactive oxygen species (ROS) leading to apoptosis and cell death (Gogvadze et al.
363 2010). Magnetite Nps are considered biocompatible, however it was recently reported that induce oxidative stress
364 and apoptosis in lung epithelial cells treated with 15 and 20 μg/mL of Nps after 24 h exposure (Ramesh et al. 2012).
365 Although these results are in contradiction with the reports about magnetite biocompatibility (Chen et al. 2012; Min
366 et al. 2011; Hultman et al. 2008; Kim et al. 2006) in some way they may provide an insight about the synergic effect

observed when Nps are functionalized with α-TOS. Thus, it is intriguing whether the iron of internalized α-TOS-Nps by itself can catalyze the production of ROS via the redox activity in addition to the effect of functionalized α-TOS. It is impossible to consider if the oxidative stress contributed to α-TOS-Nps effect however, further investigation is required to know the process implicated in α-TOS-Nps bioactivity.

In this work was demonstrated that magnetite Nps were successfully synthesized and functionalized with α-TOS. The results show that the functionalization improves the anti-cancer activity in resistant cervical cancer cells and it is possible that these findings may be extended to *in vivo* studies. Therefore, these results are believed to be useful in the anticancer carrier and drug delivery material design as a promising alternative to conventional chemotherapy to other malignancies where α-TOS is hydrolyzed losing its bioactivity. Further studies are needed in order to corroborate the specific anticancer bioactivity of these functionalized nanoparticles.

In conclusion, we demonstrated that sphere-like magnetite nanoparticles functionalized with α-TOS, one of the most important vitamin E analogues with anticancer activity, are biocompatible in normal cells and bioactive for resistant cervical cancer cells. To the best of our knowledge, this is the first report about how the functionalization can protect the bioactivity of α-TOS in a resistant cervical cancer line. Collectively, these data indicate that the α-TOS-Nps formulation may be used in other α-TOS non sensitive cancer cells.

**Acknowledgments** The authors are grateful to Francisco Ruiz for TEM support, Ma. Iracema Valeriano Arreola and Fidel Pacheco for TGA analysis, personal from CIBIOR for technical assistance. This study was supported by the SEP-CONACYT (Fondo de Investigación Científica Básica) grant No. 154602. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

**Conflict of interest** The authors declare that there is no conflict of interest.

**References**

Amstad E, Textor M, Reimhult E (2011) Stabilization and functionalization of iron oxide nanoparticles for biomedical applications. Nanoscale 3 (7):2819-2843. doi:10.1039/c1nr10173k

Amstad E, Zurcher S, Mashaghi A, Wong J, Textor M, Reimhult E (2009) Surface functionalization of single superparamagnetic iron oxide nanoparticles for targeted magnetic resonance imaging. Small (Weinheim an der Bergstrasse, Germany) 5 (11):1334-1342. doi:10.1002/smll.200801328

Anderson K, Simmons-Menchaca M, Lawson KA, Atkinson J, Sanders BG, Kline K (2004) Differential response of human ovarian cancer cells to induction of apoptosis by vitamin E Succinate and vitamin E analogue, alpha-TEA. Cancer research 64 (12):4263-4269. doi:10.1158/0008-5472.can-03-2327

398 Baba D, Seiko Y, Nakanishi T, Zhang H, Arakaki A, Matsunaga T, Osaka T (2012) Effect of magnetite nanoparticles
399 on living rate of MCF-7 human breast cancer cells. Colloids and surfaces B, Biointerfaces 95:254-257.
400 doi:10.1016/j.colsurfb.2012.03.008
401 Cai W, Wan J (2007) Facile synthesis of superparamagnetic magnetite nanoparticles in liquid polyols. Journal of
402 colloid and interface science 305 (2):366-370. doi:http://dx.doi.org/10.1016/j.jcis.2006.10.023
403 Chen B, Liang Y, Wu W, Cheng J, Xia G, Gao F, Ding J, Gao C, Shao Z, Li G (2009) Synergistic effect of magnetic
404 nanoparticles of Fe3O4 with gambogic acid on apoptosis of K562 leukemia cells. International journal of
405 nanomedicine 4:251
406 Chen D, Tang Q, Li X, Zhou X, Zang J, Xue WQ, Xiang JY, Guo CQ (2012) Biocompatibility of magnetic
407 Fe(3)O(4) nanoparticles and their cytotoxic effect on MCF-7 cells. International journal of nanomedicine
408 7:4973-4982. doi:10.2147/ijn.s35140
409 Choy JH, Shin J, Lim SY, Oh JM, Oh MH, Oh S (2010) Characterization and stability analysis of zinc oxide
410 nanoencapsulated conjugated linoleic acid. Journal of food science 75 (6):N63-68. doi:10.1111/j.1750-
411 3841.2010.01676.x
412 Daou TJ, Pourroy G, Bégin-Colin S, Grenèche JM, Ulhaq-Bouillet C, Legaré P, Bernhardt P, Leuvrey C, Rogez G
413 (2006) Hydrothermal Synthesis of Monodisperse Magnetite Nanoparticles. Chemistry of Materials 18
414 (18):4399-4404. doi:10.1021/cm060805r
415 Dong LF, Grant G, Massa H, Zobalova R, Akporiaye E, Neuzil J (2012) alpha-Tocopheryloxyacetic acid is superior
416 to alpha-tocopheryl succinate in suppressing HER2-high breast carcinomas due to its higher stability.
417 International journal of cancer Journal international du cancer 131 (5):1052-1058. doi:10.1002/ijc.26489
418 Dong LF, Jameson VJ, Tilly D, Cerny J, Mahdavian E, Marin-Hernandez A, Hernandez-Esquivel L, Rodriguez-
419 Enriquez S, Stursa J, Witting PK, Stantic B, Rohlena J, Truksa J, Kluckova K, Dyason JC, Ledvina M,
420 Salvatore BA, Moreno-Sanchez R, Coster MJ, Ralph SJ, Smith RA, Neuzil J (2011) Mitochondrial targeting
421 of vitamin E succinate enhances its pro-apoptotic and anti-cancer activity via mitochondrial complex II. The
422 Journal of biological chemistry 286 (5):3717-3728. doi:10.1074/jbc.M110.186643
423 Ghotbi MY, bin Hussein MZ (2012) Controlled release study of an anti-carcinogenic agent, gallate from the surface
424 of magnetite nanoparticles. Journal of Physics and Chemistry of Solids 73 (7):936-942.
425 doi:http://dx.doi.org/10.1016/j.jpcs.2012.02.031
426 Gogvadze V, Norberg E, Orrenius S, Zhivotovsky B (2010) Involvement of Ca2+ and ROS in alpha-tocopheryl
427 succinate-induced mitochondrial permeabilization. International journal of cancer Journal international du
428 cancer 127 (8):1823-1832. doi:10.1002/ijc.25204
429 Gu X, Song X, Dong Y, Cai H, Walters E, Zhang R, Pang X, Xie T, Guo Y, Sridhar R, Califano JA (2008) Vitamin
430 E succinate induces ceramide-mediated apoptosis in head and neck squamous cell carcinoma in vitro and in
431 vivo. Clinical cancer research : an official journal of the American Association for Cancer Research 14
432 (6):1840-1848. doi:10.1158/1078-0432.ccr-07-1811
433 Gupta AK, Wells S (2004) Surface-modified superparamagnetic nanoparticles for drug delivery: preparation,
434 characterization, and cytotoxicity studies. NanoBioscience, IEEE Transactions on 3 (1):66-73.
435 doi:10.1109/tnb.2003.820277
436 Huang S, Li C, Cheng Z, Fan Y, Yang P, Zhang C, Yang K, Lin J (2012) Magnetic Fe3O4@mesoporous silica
437 composites for drug delivery and bioadsorption. Journal of colloid and interface science 376 (1):312-321.
438 doi:10.1016/j.jcis.2012.02.031
439 Hultman KL, Raffo AJ, Grzenda AL, Harris PE, Brown TR, O'Brien S (2008) Magnetic resonance imaging of major
440 histocompatibility class II expression in the renal medulla using immunotargeted superparamagnetic iron
441 oxide nanoparticles. ACS nano 2 (3):477-484. doi:10.1021/nn700400h
442 Kanai K, Kikuchi E, Mikami S, Suzuki E, Uchida Y, Kodaira K, Miyajima A, Ohigashi T, Nakashima J, Oya M
443 (2010) Vitamin E succinate induced apoptosis and enhanced chemosensitivity to paclitaxel in human
444 bladder cancer cells in vitro and in vivo. Cancer Science 101 (1):216-223. doi:10.1111/j.1349-
445 7006.2009.01362.x
446 Kim DH, Lee SH, Im KH, Kim KN, Kim KM, Shim IB, Lee MH, Lee YK (2006) Surface-modified magnetite
447 nanoparticles for hyperthermia: Preparation, characterization, and cytotoxicity studies. Current Applied
448 Physics 6, Supplement 1 (0):e242-e246. doi:http://dx.doi.org/10.1016/j.cap.2006.01.048
449 Kline K, Yu W, Sanders BG (2001) Vitamin E: mechanisms of action as tumor cell growth inhibitors. The Journal of
450 nutrition 131 (1):161S-163S
451 Ma Y, Huang L, Song C, Zeng X, Liu G, Mei L (2010a) Nanoparticle formulation of poly(ε-caprolactone-co-
452 lactide)-d-α-tocopheryl polyethylene glycol 1000 succinate random copolymer for cervical cancer
453 treatment. Polymer 51 (25):5952-5959. doi:http://dx.doi.org/10.1016/j.polymer.2010.10.029

454 Ma Y, Zheng Y, Liu K, Tian G, Tian Y, Xu L, Yan F, Huang L, Mei L (2010b) Nanoparticles of Poly(Lactide-Co-
455 Glycolide)-d-a-Tocopheryl Polyethylene Glycol 1000 Succinate Random Copolymer for Cancer Treatment.
456 Nanoscale research letters 5 (7):1161-1169. doi:10.1007/s11671-010-9620-3
457 Mahmoudi M, Simchi A, Milani AS, Stroeve P (2009) Cell toxicity of superparamagnetic iron oxide nanoparticles.
458 Journal of colloid and interface science 336 (2):510-518. doi:10.1016/j.jcis.2009.04.046
459 Malafa MP, Fokum FD, Mowlavi A, Abusief M, King M (2002) Vitamin E inhibits melanoma growth in mice.
460 Surgery 131 (1):85-91
461 Min JH, Kim ST, Lee JS, Kim K, Wu JH, Jeong J, Song AY, Lee K-M, Kim YK (2011) Labeling of macrophage cell
462 using biocompatible magnetic nanoparticles. Journal of Applied Physics 109 (7):07B309-303
463 Mohapatra S, Mallick SK, Maiti TK, Ghosh SK, Pramanik P (2007) Synthesis of highly stable folic acid conjugated
464 magnetite nanoparticles for targeting cancer cells. Nanotechnology 18 (38):385102
465 Neuzil J, Weber T, Gellert N, Weber C (2001) Selective cancer cell killing by alpha-tocopheryl succinate. British
466 journal of cancer 84 (1):87-89. doi:10.1054/bjoc.2000.1559
467 Nguyen TKT, Luke AWG (2010) Functionalisation of nanoparticles for biomedical applications. Nano Today 5.
468 doi:10.1016/j.nantod.2010.05.003
469 Perez-Gonzalez T, Rodriguez-Navarro A, Jimenez-Lopez C (2011) Inorganic Magnetite Precipitation at 25 °C: A
470 Low-Cost Inorganic Coprecipitation Method. J Supercond Nov Magn 24 (1-2):549-557.
471 doi:10.1007/s10948-010-0999-y
472 Qu S, Yang H, Ren D, Kan S, Zou G, Li D, Li M (1999) Magnetite Nanoparticles Prepared by Precipitation from
473 Partially Reduced Ferric Chloride Aqueous Solutions. Journal of colloid and interface science 215 (1):190-
474 192. doi:10.1006/jcis.1999.6185
475 Ramesh V, Ravichandran P, Copeland CL, Gopikrishnan R, Biradar S, Goornavar V, Ramesh GT, Hall JC (2012)
476 Magnetite induces oxidative stress and apoptosis in lung epithelial cells. Molecular and cellular
477 biochemistry 363 (1-2):225-234. doi:10.1007/s11010-011-1174-x
478 Rivas J, Bañobre-López M, Piñeiro-Redondo Y, Rivas B, López-Quintela MA (2012) Magnetic nanoparticles for
479 application in cancer therapy. Journal of Magnetism and Magnetic Materials 324 (21):3499-3502.
480 doi:http://dx.doi.org/10.1016/j.jmmm.2012.02.075
481 Rutnakornpituk M, Meerod S, Boontha B, Wichai U (2009) Magnetic core-bilayer shell nanoparticle: A novel
482 vehicle for entrapmentof poorly water-soluble drugs. Polymer 50 (15):3508-3515.
483 doi:http://dx.doi.org/10.1016/j.polymer.2009.06.015
484 Shen X-C, Fang X-Z, Zhou Y-H, Liang H (2004) Synthesis and characterization of 3-aminopropyltriethoxysilane-
485 modified superparamagnetic magnetite nanoparticles. Chemistry Letters 33 (11):1468-1469
486 Tomasetti M, Strafella E, Staffolani S, Santarelli L, Neuzil J, Guerrieri R (2010) alpha-Tocopheryl succinate
487 promotes selective cell death induced by vitamin K3 in combination with ascorbate. British journal of
488 cancer 102 (8):1224-1234. doi:10.1038/sj.bjc.6605617
489 Turanek J, Wang XF, Knotigova P, Koudelka S, Dong LF, Vrublova E, Mahdavian E, Prochazka L, Sangsura S,
490 Vacek A, Salvatore BA, Neuzil J (2009) Liposomal formulation of alpha-tocopheryl maleamide: in vitro
491 and in vivo toxicological profile and anticancer effect against spontaneous breast carcinomas in mice.
492 Toxicology and applied pharmacology 237 (3):249-257. doi:10.1016/j.taap.2009.01.027
493 Vippola M, Falck GC, Lindberg HK, Suhonen S, Vanhala E, Norppa H, Savolainen K, Tossavainen A, Tuomi T
494 (2009) Preparation of nanoparticle dispersions for in-vitro toxicity testing. Human & experimental
495 toxicology 28 (6-7):377-385. doi:10.1177/0960327109105158
496 Wu W, He Q, Jiang C (2008) Magnetic iron oxide nanoparticles: synthesis and surface functionalization strategies.
497 Nanoscale research letters 3 (11):397-415. doi:10.1007/s11671-008-9174-9
498 Zhang Y, Kohler N, Zhang M (2002) Surface modification of superparamagnetic magnetite nanoparticles and their
499 intracellular uptake. Biomaterials 23 (7):1553-1561
500 Zhang Y, Zhang J (2005) Surface modification of monodisperse magnetite nanoparticles for improved intracellular
501 uptake to breast cancer cells. Journal of colloid and interface science 283 (2):352-357.
502 doi:http://dx.doi.org/10.1016/j.jcis.2004.09.042
503
504

505

506

507 **Figure legends.**

508

509 **Fig. 1 SEM, TEM and SAED images of magnetite nanoparticles before and after functionalization.** Magnetite
510 nanoparticles uncoated (Nps); magnetite nanoparticles coupled with APTMS (Silanized Nps); and magnetite
511 nanoparticles functionalized with α-TOS (α-TOS-Nps).

512

513 **Fig. 2 Energy Dispersive Spectroscopy.** $Fe_3O_4$ uncoated nanoparticles (a); after treatment with APTMS (Silanized
514 Nps) (b); and functionalized α-TOS-Nps (c).

515

516 **Fig. 3. FTIR Spectroscopy:** a) α-TOS, b) α-TOS-Nps, and c) uncoated Nps.

517

518 **Fig. 4. TGA thermogram**. The values represent the mass loss ranged between 290ºC to 475ºC for: a) uncoated Nps,
519 b) silanized Nps and c) α-TOS-Nps.

520

521 **Fig. 5**. **Fibroblastes and SiHa cells morphology**. The cells were exposed for 72 h to 0-80 µg/mL of Nps (uncoated
522 magnetite), α-TOS (α-tocopheryl succinate) and magnetite functionalized with α-TOS (α-TOS-Nps). All he pictures
523 were taken at the highest dose (80µg/mL). The cells treated with Nps or α-TOS alone look biocompatible. The
524 cervical cancer cells (SiHa) look unhealthy only in α-TOS-Nps treatment and damaged cells due the presence of α-
525 TOS-Nps. Control was untreated cells. All treatments were dissolving in culture media. Phase contrast microscopy,
526 Bar=50 µm

527

528 **Fig. 6. Localization of α-TOS-Nps in cervical cancer cells.** Image of an optical section taken from cells after 72 h
529 incubation with α-TOS-Nps-FITC at 40 µg/mL. a) Phase contrast image, b) nucleus cell staining with PI, c) Presence
530 of α-TOS-Nps-FITC into the nucleus, d) overlaying images. Bar= 10 µm

531

532 **Fig. 7**: **Cell viability after 24-72 h.** Fibroblastes (non malignant cells) and SiHa cells (cervical cancer cell) were
533 treated with Nps, α-TOS or α-TOS-Nps at different doses (0-80 µg/mL) at 24, 48 and 72 h. The Nps, α-TOS and α-
534 TOS-Nps are biocompatible in normal cells. The SiHa cells are non sensitive to α-TOS or Nps alone, but the cells

535 become susceptible at α-TOS-Nps ($IC_{50}$=65.29 g/mL**)** and the viability is affected by the dose and time dependent. α-
536 TOS-Nps affect the viability only in cancer cells and not in normal cells.

537

538 **Fig. 8**. **Schemes of functionalization of α-TOS on the Nps surface.** a) Structure of Vitamin E (α-TOH) and alpha
539 tocopheryl succinate (α-TOS); b) schemes of the simplified silanization and c) functionalization of silanizated Nps
540 with α-TOS. APTMS (3-aminopropyltrimetoxysilane), α-TOS-Nps (magnetite nanoparticles coupled with alpha-
541 tocopheryl succinate; NDC (N,N′-Diisopropylcarbodiimide), NHS (N-Hydroxysuccinimide).

542

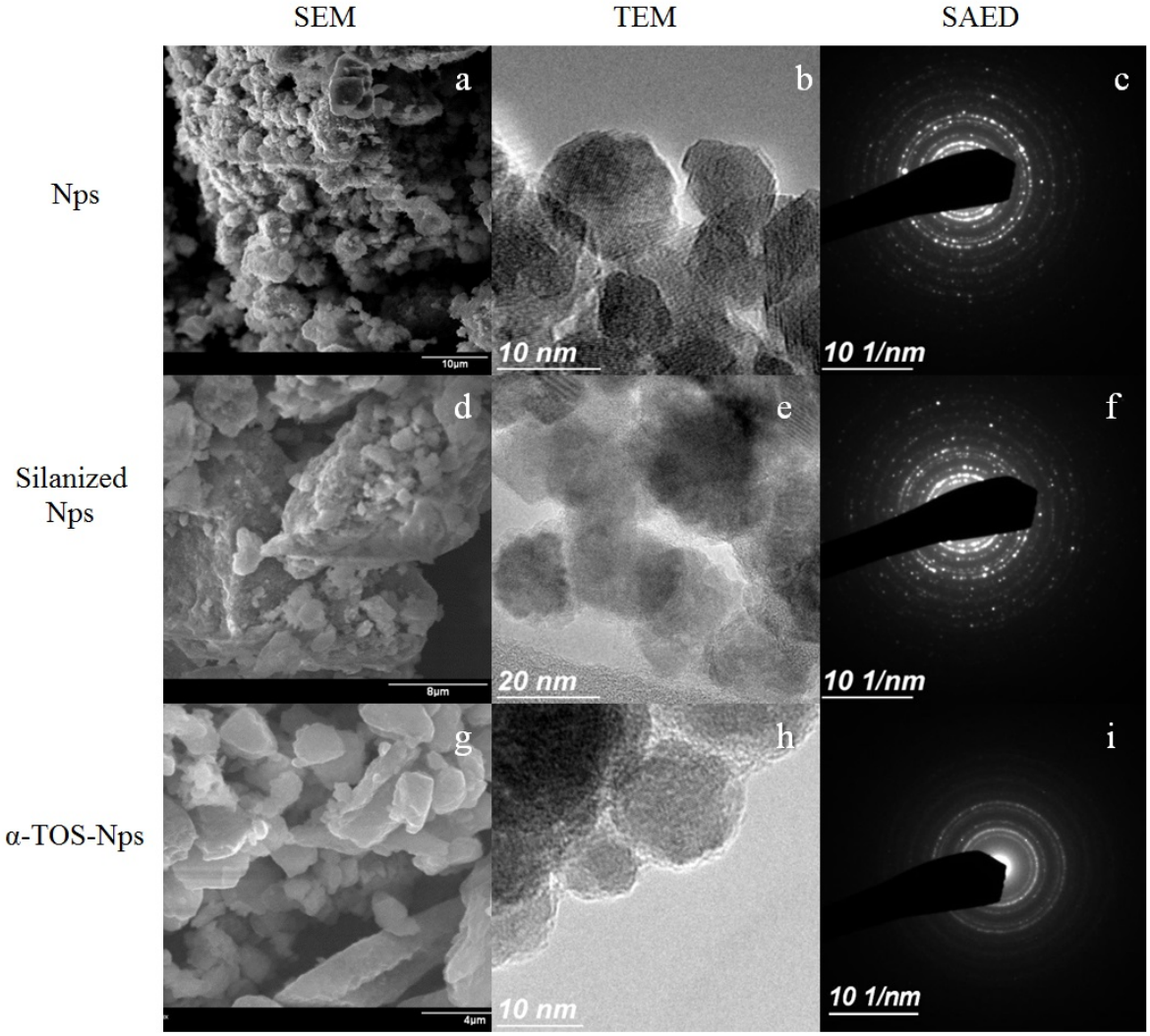

SEM
TEM
SAED
Nps
Silanized Nps
α-TOS-Nps
a
b
c
d
e
f
g
h
i
10 nm
10 1/nm
20 nm
10 1/nm
10 nm
10 1/nm

**Figure 1**.

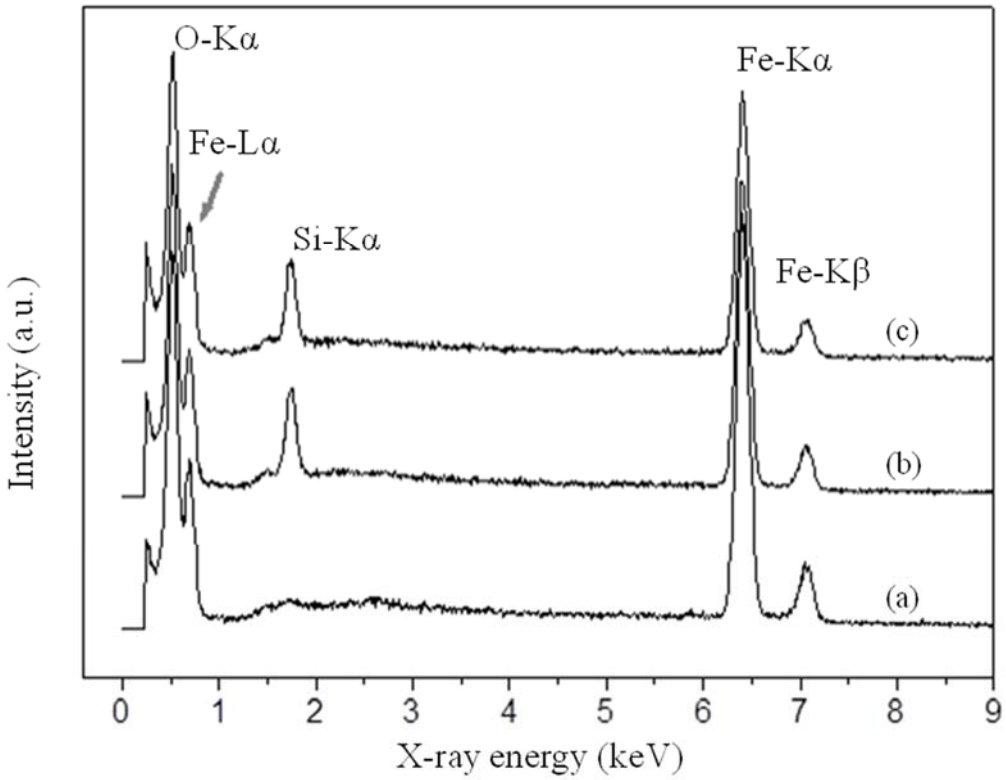

O-Kα
Fe-Lα
Si-Kα
Fe-Kα
Fe-Kβ
(c)
(b)
(a)
Intensity (a.u.)
0
1
2
3
4
5
6
7
8
9
X-ray energy (keV)

**Figure 2**.

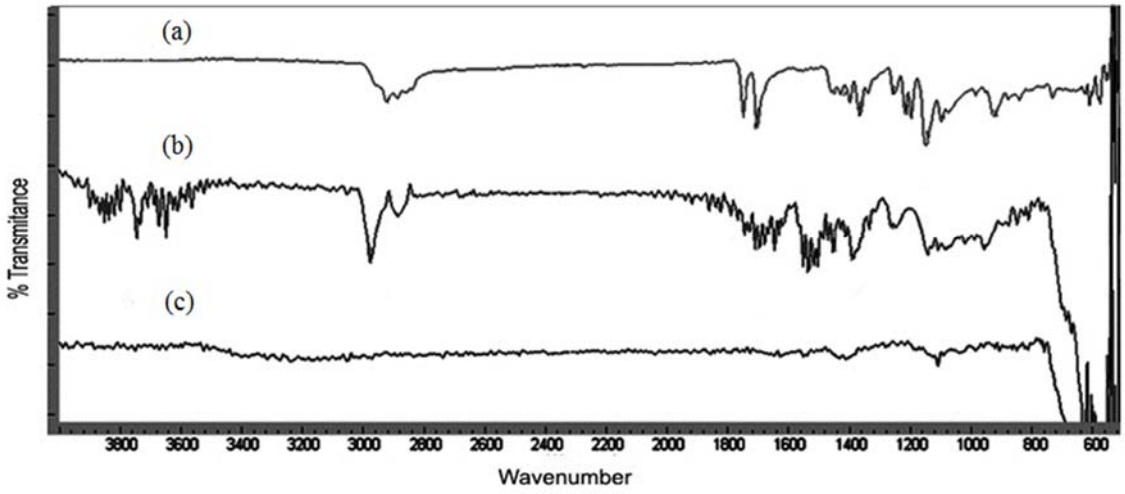

(a)
(b)
(c)
% Transmitance
3800
3600
3400
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
Wavenumber

**Figure 3.**

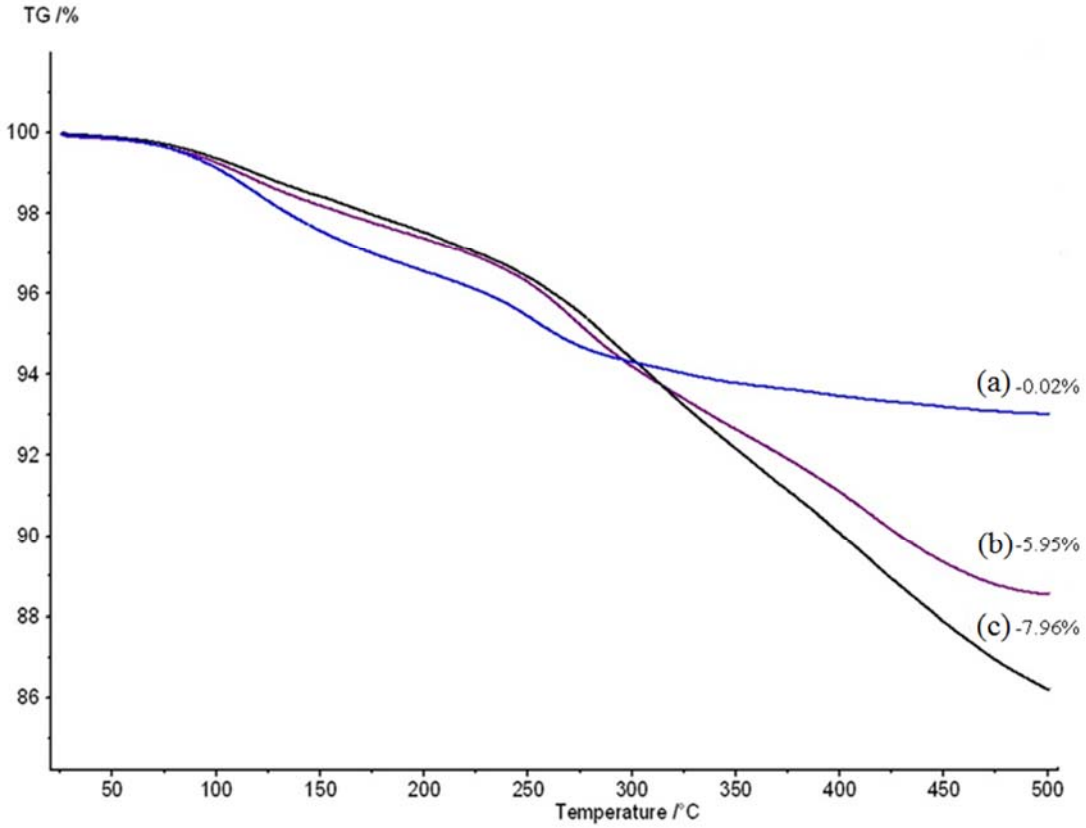

TG /%
100
98
96
94
92
90
88
86
50
100
150
200
250
300
350
400
450
500
Temperature /°C
(a) -0.02%
(b) -5.95%
(c) -7.96%

**Figure 4.**

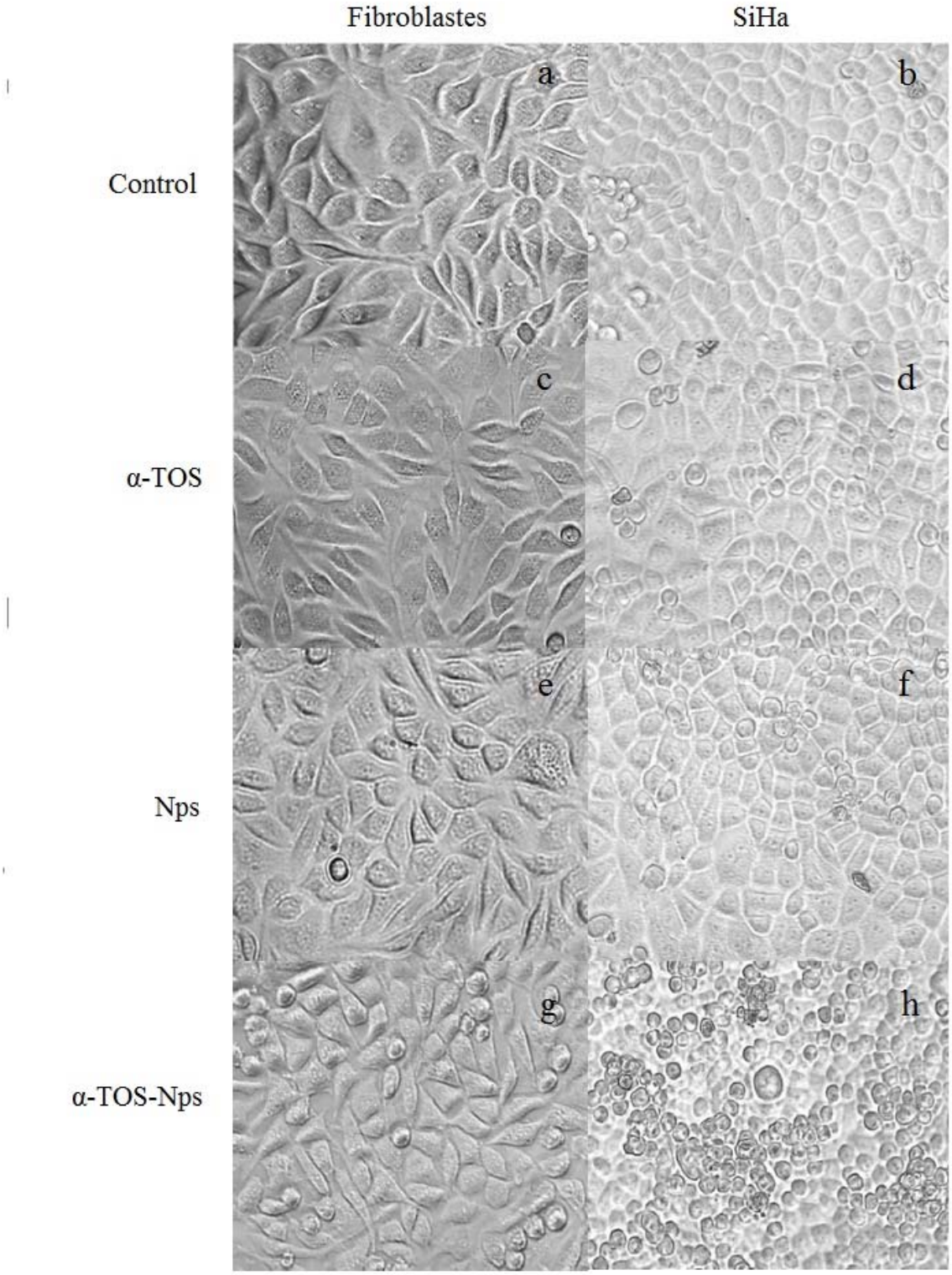



**Figure 5**.

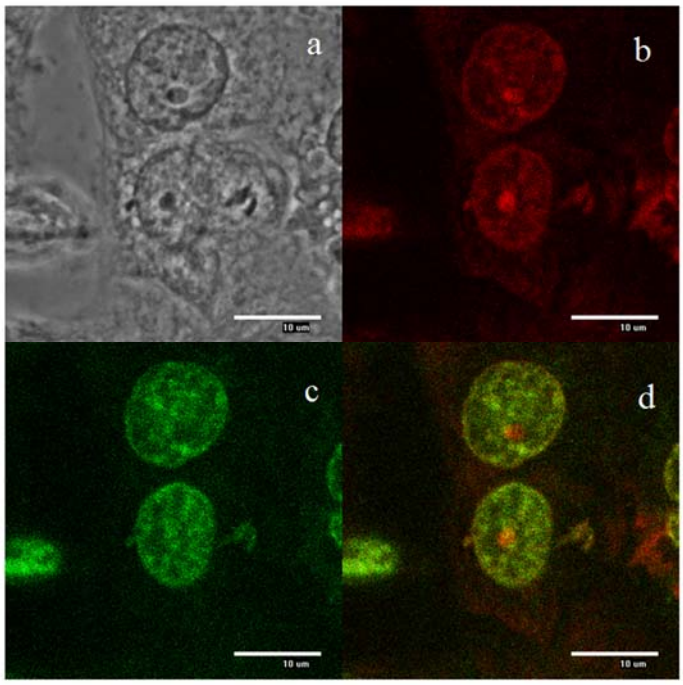
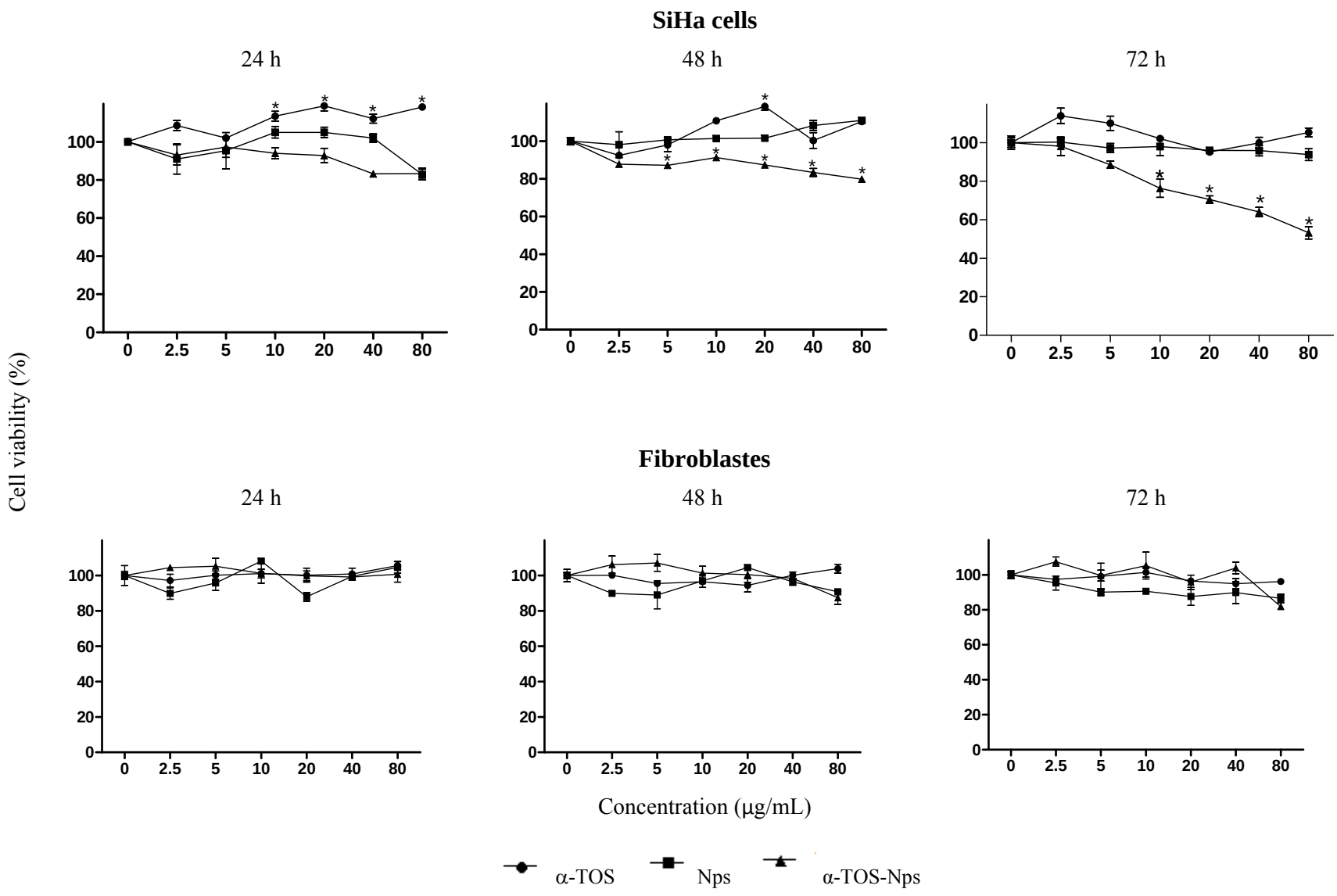



**Figure 6.**

**Figure 7.**


SiHa cells
24 h
48 h
72 h
Fibroblastes
24 h
48 h
72 h
Cell viability (%)
0
20
40
60
80
100
0
2.5
5
10
20
40
80
Concentration (µg/mL)
α-TOS
Nps
α-TOS-Nps

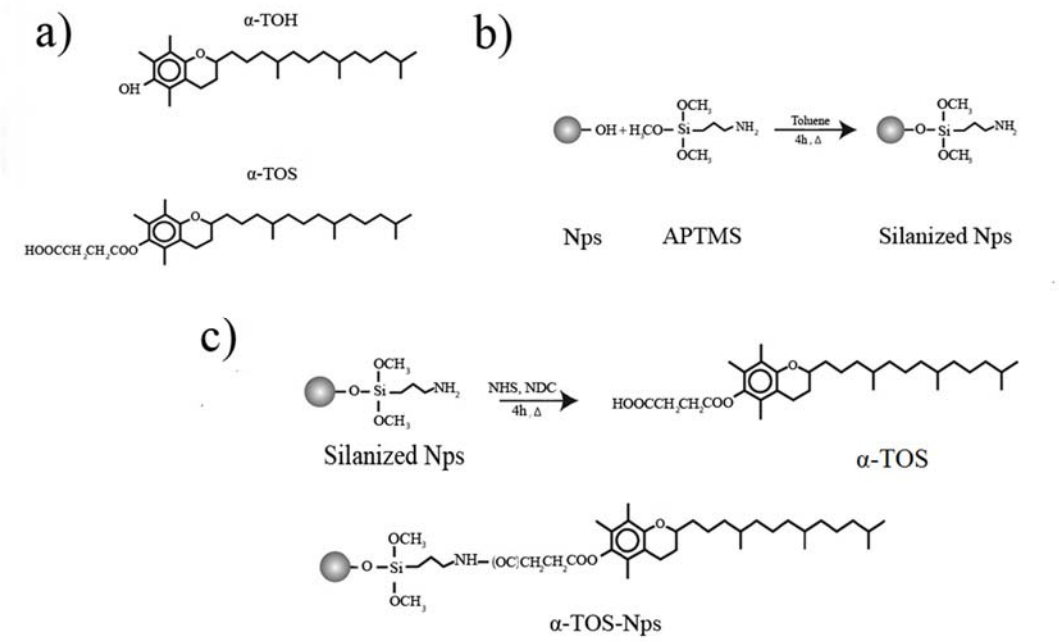

a)
α-TOH
OH
α-TOS
HOOCCH2CH2COO
b)
Toluene
4h, Δ
Nps
APTMS
Silanized Nps
c)
NHS, NDC
4h, Δ
Silanized Nps
α-TOS
α-TOS-Nps

**Figure 8**.

# Capítulo III

# Nanopartículas de Magnetita Funcionalizadas con alfa-Tocoferil Succinato: Distribución *in vivo* y Actividad Antitumoral en un Modelo de Melanoma

Magnetite nanoparticles functionalized with α-tocopheryl succinate: *in vivo* distribution and tumor suppressing activity in melanoma model. Aracely Angulo-Molina, Miguel Ángel Méndez-Rojas, Salomón Hernández-Gutierrez, Carlos Escamilla-Weinmann, Francisco Collazo-Ramos, Teresa Palacios-Hernández, Oscar Edel Contreras-López, Gustavo Hirata-Flores, Alberto Delgado-Velazquez, Julio Reyes-Leyva, Jesús Hernández.

## RESUMEN

La funcionalización de nanopartículas de magnetita ha recibido mucha atención dado su uso potencial con fines biomédicos. Nosotros encontramos recientemente que la actividad anticancerígena *in vitro* del α-tocoferil succinato se mantiene en células capaces de inactivarlo, cuando este análogo está funcionalizado a las nanopartículas de magnetita. Sin embargo se desconoce cuál es su efecto antitumoral y su biodistribución *in vivo*. Por ello, en este trabajo se estableció un modelo de melanoma en ratones desnudos inoculando células de melanoma B16F0 el cual fue confirmado por imagenología, histología y el antígeno HMB45 específico de melanoma. El modelo se estableció a las dos semanas, observándose un tumor sólido bien delimitado y pseudoencapsulado de características malignas. Una vez establecido el melanoma, 6 grupos de 3-4 ratones nu/nu se trataron intratumoralmente cada 3er día por 2 semanas con diferentes dosis de Nps, α-TOS y α-TOS-Nps. Se utilizó un grupo control sin tratamiento; un grupo tratado con Nps pura (2 mg); un grupo tratado con α-TOS (2 mg) y cuatro grupos tratados con 0.075, 0.150, 1 mg o 2 mg de α-TOS-Nps respectivamente. Durante el tratamiento se monitoreó el volumen tumoral, el peso de los animales y signos de toxicidad. Se observó una disminución significativa del volumen tumoral con las dosis de 0.75 y 2 mg de α-TOS-Nps ($p<0.05$) a los 10 días post tratamiento, además de un efecto sinérgico de la actividad antitumoral de α-TOS cuando está funcionalizado a las Nps, un efecto similar al observado *in vitro*. No se encontraron diferencias en los patrones de malignidad ultrasonográfica ni de rayos X de los tumores. Sin embargo, la evaluación histológica indica que a mayores dosis de α-TOS-Nps se induce una mayor necrosis intratumoral coagulativa y licuefactiva, que se refleja en una disminución del volumen tumoral. Estos son efectos deseables previos a una cirugía, ya que se facilita

la remoción completa del tumor y también se asocia a una mayor sobrevida. Además, no se observó infiltración tumoral a tejidos aledaños en los animales tratados con α-TOS-Nps, en contraste con la infiltración de células tumorales en el tejido musculoesquelético y en tejido adiposo observada con los tratamientos por separado de α-TOS o Nps puras. Las nanopartículas se distribuyeron en bazo, hígado, piel, pulmón, riñón e intestino. No se observaron alteraciones citomorfológicas de toxicidad en los órganos evaluados sugiriendo biocompatibilidad. Los efectos observados con la aplicación *in vivo* de α-TOS-Nps sugieren que la funcionalización de α-TOS a nanopartículas de magnetita puede tener un uso potencial para el mejoramiento de la actividad antitumoral de este análogo en melanoma.

# Magnetite Nanoparticles Functionalized with α-Tocopheryl Succinate: In Vivo Distribution and Tumor Suppressing Activity in Melanoma Model

*Aracely Angulo Molina1, 2*, Miguel Ángel Méndez Rojas1, Salomón Hernández Gutiérrez3, Carlos Escamilla Weinmann4, Francisco Collazo Ramos4, Teresa Palacios Hernández1, 5, Oscar Edel Contreras López6, Gustavo Hirata Flores6, Alberto Delgado Velazquez7, Julio Reyes Leyva8, Jesús Hernández2**

1Health Sciences Department, School of Sciences, Universidad de las Américas Puebla (UDLAP), Ex-Hda. Sta. Catarina Mártir, San Andrés Cholula, Puebla, 72820, México, 2Immunology Department, Centro de Investigación en Alimentación y Desarrollo A.C. (CIAD), Km 0.6 Carretera a La Victoria, Hermosillo, Sonora, 83304, México, 3School of Medicine, Universidad Panamericana (UP), Augusto Rodin 498 Col. Insurgentes Mixcoac, Benito Juárez, México, D.F., 03920, México, 4Bioterio Jean Claude Bernard, Benemérita Universidad Autónoma de Puebla (BUAP), Puebla, 72000, México, 5Universidad Popular Autónoma del Estado de Puebla (UPAEP), 21 Sur 1103, Barrio Santiago, Puebla, 72410, México, 6Center for Nanoscience and Nanotechnology-Universidad Autónoma de México, Km 107 Carretera Tijuana-Ensenada, Ensenada,

B.C, 22860, México, 7Pathology Department, Hospital de Cardiología del Centro Médico Nacional Siglo XXI, IMSS, Av. Cuauhtémoc 330 Col. Doctores C.P. 06725, México, DF, 8Centro de Investigación Biomédica de Oriente (CIBIOR), IMSS, HGZ No. 5, Km 4.5 Carretera Federal Atlixco-Metepec, Metepec, Puebla, 42730, México.

**ABSTRACT** The *in vivo* anticancer activity of the vitamin E analogue, alpha tocopheryl succinate (α-TOS), when is functionalized to magnetite nanoparticles (Nps) is unknown. In this study, we evaluated the tumor suppressing activity and biodistribution of magnetite nanoparticles functionalized with α-TOS (α-TOS-Nps) by using a melanoma model in BALB/c nude mice. The endotoxin level, size distribution and Z potential of the nanoparticles were determined. The mice were intra-tumoral treated with α-TOS-Nps, α-TOS or Nps alone at different doses. The *in vivo* effects were evaluated by ultrasonography and histopathology; the biodistribution was evaluated with Prussian blue staining. A chemotherapeutic efficacy in the melanoma tumor, characterized by large necrotic areas, was observed in all the evaluated doses; a significant less volume tumor was observed ten days postreatment with α-TOS-Nps, in contrast with control groups. α-TOS-Nps were detected in tumor, spleen, liver, skin, kidney, and gastrointestinal tract without apparent toxic effect in major organs. These results suggest that functionalization of α-TOS to magnetite nanoparticles may prove a better potential anticancer agent for future applications.

**KEYWORDS**: α-tocopheryl succinate, nanoparticles, magnetite, biodistribution, cancer.

One of the most important vitamin E analogues, α-tocopheryl succinate (α-TOS), has been shown to selectively kill cancer cells. This analogue is an esterified derivative of α-

tocopherol (α-TOH, Figure 1), which suppressed cell growth in a wide range of human cancer cells such as melanoma (Tomasetti and Neuzil, 2007; Zhao et al, 2009). Alpha-TOS selectively kills cancer cells and has low toxicity for nonmalignant cells; because of these characteristics, this analogue is considered a promising chemical agent for potential clinical treatment of cancer (Kanai et al, 2010; Zhao et al, 2010). In recent years, there has been a growing interest in the development of special formulations or multidrug combinations to improve the anticancer activity of vitamin E analogues (Turánek et al, 2009; Kanai et al, 2010), such as the use of magnetite ($Fe_3O_4$) nanoparticles as carriers. This nanoparticles have the potential to be used as novel biomedical devices and in applications such as supports for drug delivery, imaging and diagnosis, and tissue engineering (Thanh and Green, 2010; Cochran et al, 2013). These nanoparticles are considered to be biocompatible and non-cytotoxic (Babba et al, 2012, Alexiou et al, 2008). We have previously reported that the anticancer activity of the vitamin E analogue, α-TOS, is enhanced *in vitro* when it is linked to the surface of magnetite nanoparticles (Angulo et al, submitted), although its biodistribution is unknown. *In vivo* systems are extremely complicated, and interactions between nanoparticles depend of several factors such as surface charge, which can change size of nanoparticles by inducing aggregation in aqueous dispersion (Thomas et al, 2013). The biological components, such as proteins and cells, may affect the biodistribution as well (Laurent et al, 2011; Haglund et al, 2009). When nanomaterials come into contact with biological systems, a nanobiointerface is formed. In the interface occurring dynamic physicochemical interactions, kinetics and thermodynamic exchanges between the surfaces of nanomaterials and biological components affecting the interactions of the nanoparticles with the cells. Small changes

in parameters can dramatically influence bioactivity (Jimbow et al, 2013). That is why we have to know the physical-chemical characteristics of the nanomaterials to understand their behavior in *vitro* and *in vivo.*

In order to be considered for biomedical applications, nanoparticle tissue distribution has to be evaluated (Kim et al, 2006). Our previous *in vitro* results suggest that the anticancer activity of α-TOS can be protected and enhanced when it is chemically bound to magnetite nanoparticles (labeled as α-TOS-Nps), but the *in vivo* effects and biodistribution of this nanoparticles are still unknown. Usually, biodistribution is assessed using transmission electron microscopy (TEM) which may be expensive and difficult due to sample processing. Prussian blue staining is a simple technique that can be used to visually indicate the presence of iron (from $Fe_3O_4$ nanoparticles) inside the cells (Haglund et al, 2009; Zhu et al, 2012).

Malignant melanoma has the most significant effect on human health and carries the highest risk of mortality and metastasis (Speroni et al, 2009; Jimbow et al, 2013). Given the global large number of skin cancer related deaths, and the need for a new and more effective treatment that may lead to an improvement of patient conditions and to the reduction of side effects, an *in vivo* melanoma model was selected to test the anticancer activity of α-TOS-Nps. We proposed that this nanoparticles can affect the tumor growth and to be mobilized from melanoma tumors to other organs using the new blood vessels from the tumor to systemic circulation. The present study shows the antitumor effect and the biodistribution of nanoparticles repeatedly applied by i.t. injection in a mouse melanoma model during two weeks, to contribute to our understanding for future potential *in vivo* biomedical applications of α-TOS-Nps.

87 **RESULTS AND DISCUSSION**

88 **Characterization.** Aqueous dispersion of the nanoparticles in PBS had a positive
89 Tyndall effect, confirming that they form a colloidal suspension as expected for α-TOS-
90 Nps (Figure 2A). The Tyndall effect occurs in colloidal systems because the laser beam
91 is scattered by small particles dispersed in the solvent.

92 Transmission electron microscopy observations show spherical shaped morphology with
93 an average size of 15 nm in diameter (Figure 2B). Particle size plays an important role in
94 drug transport and delivering into the cells; nanoparticles with sizes below 200 nm
95 facilitate the uptake of the particles (Thanh and Green, 2010). High-resolution TEM
96 analysis reveals that each nanoparticle is a magnetite-phase single nanocrystallite. PBS
97 dispersions of the nanoparticles prepared were characterized by dynamic light scattering
98 (DLS) showing a hydrodynamic size distribution in the range from 190 to 1100 nm, with
99 a mean value of 550 nm (Figure 1D) and a Zeta potential value of +42 mV. Zeta potential
100 is an indicator of surface charge, which determines particle stability in the dispersion and
101 redispersibility of the nanoparticles (Feng et al, 2007). The observed value for α-TOS-
102 Nps is characteristic for a stable aqueous dispersion. The surface charge also has an
103 important role in the induction of biological effects. The positively or negatively charged
104 nanoparticles display bioactivity in more low doses than the neutral nanoparticles.
105 Additionally, positively charged particles tend to adhere nonspecifically to cells. In
106 contrast, strong negative charged particles result in increased liver uptake (Schlachter et
107 al, 2011).

108 By other way, the average hydrodynamic size obtained from the particle size
109 distribution analysis was larger than that observed with TEM. The observed size

distribution may be relationated with spontaneous aggregation and agglomeration. Particles with sizes between 6 to 15 nm maintain their superparamagnetic properties (Tartaj et al, 2003; Gupta and Gupta, 2005). In the absence of any external magnetic field, the magnetic dipole interactions between particles can induce spontaneous aggregation (Mahmoudi et al, 2009). Aggregation is a common problem for this type of nanoparticles, even after chemical modification of their surfaces. Some moderately agglomerated nanoparticles as well as separated nanoparticles were found in the tissue samples. Agglomeration can be a problem before and after exposure of nanoparticles in cellular and *in vivo* systems (Amstad et al, 2011). In this study, this issue was solved by sonication during 5 min before each treatment using an ultrasonic processor.

Importantly, nanoparticle activity and toxicity depend not only on their chemical and structural properties but also on their size and surface properties (Hanini et al 2011). Particle size is a very important parameter for characterizing the physicochemical properties of nanoparticles. When the size is reduced nanoparticles possess a large surface area with more atoms on the surface, which are not bonded on one side, and are indeed more active than the atoms residing inside. An increase in surface area therefore leads to an increase in surface reactivity. Even more, nanoparticles with large size will usually be taken up by liver, spleen and other parts of reticuloendothelial system (RES) *in vivo*. In contrast, nanoparticles with size less that 100 nm in diameter and uniform size distribution are preferred for tumor targeting (Brannon-Peppas et al, 2004).

The bacterial endotoxin in α-TOS-Nps was <1 EU in agreement with FDA set limits for drug formulation. The bacterial endotoxin or lipopolysaccharide (LPS) is a membrane component of all Gram-negative bacteria. The administration of products contaminated

133 with bacterial endotoxin can cause fever, shock, and even death. Accordingly, the FDA
134 sets limits on the number of endotoxin units (EU) that may be present in a drug or device
135 product. *Limulus amoebocyte lysate* (LAL) is the extract from amoebocytes of the
136 horseshoe crab (*Limulus polyphemus)*, which reacts with bacterial endotoxin. Detection
137 of the products of this reaction is an effective way for quantifying the EU present in a
138 nanoparticle formulation (Neun et al, 2011). The nanoparticles evaluated in this study
139 showed appropriated characteristics for biomedical use.

140 **Melanoma model.** We used melanoma model because even though it accounts for less
141 than five percent of skin cancer cases, melanoma is responsible for the majority of skin
142 cancer deaths. Based on a desire to develop a clinically useful anticancer nanocomposite,
143 we used this model to determine the effect of α-TOS when it is coupled with magnetite
144 nanoparticles.

145 The melanoma model employed in this work was evaluated by using X-ray (Figure 3
146 A). X-ray imaging showed a nodular mass with regular border, oval or lobular
147 morphology with heterogeneous density and nodular calcifications. Additionally, to
148 confirm the establishment of the melanoma and to evaluate the effect of α-TOS-Nps, the
149 tumors were dissected and prepared for histology staining with H&E and
150 immunohistochemical staining to detect the HMB45 antigen. The tumor growing in the
151 flank of the mice showed a moderate cellular pleomorphic form and it was characterized
152 by rounded or polygonal cells with oval and hyperchromatic nucleus. Cells were disposed
153 in acinus and mitotic activity was moderate (Figure 3B). There were areas with numerous
154 necrotic areas with small hemorrhagic foci. Slides were incubated with monoclonal
155 antibody HMB45, which is a specific melanoma biomarker, that recognize a 100-kD

156 glycoprotein (gp100) originally found in pre- and early-stage (immature) melanosomes.
157 The presence of the antigen indicates active melanosome formation and thus melanocytic
158 differentiation. Tumor antigen HMB45 was positive confirming for melanoma (Figure
159 3C). Tumor volumes average value of 222.25 ±98.32 $mm^3$ by day 14 after inoculation of
160 the B16F10 cells.

161 **Treatment effects.** The nude mice were divided in seven groups: untreated, α-TOS (2
162 mg), pure Nps (2 mg) and α-TOS-Nps in 0.075, 0.150, 1 and 2 mg doses. The body weight
163 and tumor growth were monitored each third day (Quintana et al, 2008). Throughout the
164 treatment period, body weight was measured (Figure 4), and mice were monitored for
165 clinical signs of toxicity. There was no mortality and no important clinical signs of
166 toxicity.

167 The ultrasonography was used on the onset and at the end of the study (Figure 5). With
168 this tool, the entire tumor can be surveyed without affecting the tumor itself. Medical
169 ultrasound images are produced by passing an electrical current through a piezoelectric
170 ceramic probe (transducer) that expands and contracts to produce sound waves when
171 electrically excited. After reflection from tissue, part of the ultrasound energy returns to
172 the transducer, which-produces an electrical impulse that is converted into the image (Lee
173 et al, 2012). The tumors showed similar ultrasonographic characteristics with all the
174 treatments. A non-cystic solid tumor, with regular borders, and irregular and nodular
175 calcifications was observed in all the treatments. The echogenicity was heterogeneous and
176 hyperechogenic disperse semi-nodules were also observed at the onset and the end of the
177 study. There were numerous necrotic areas with small hemorrhagic foci corroborated with
178 histological observations. Ultrasonography also allowed serial measurements of the same

179 tumor over time during treatments regimens. With this tools, it was possible to evaluate
180 and to accurately determinate tumor growth (Figure 6).

181 After the treatments, mice injected with all the doses of α-TOS-Nps had smaller tumor
182 volume (2320.2 $mm^3$, 2005.9 $mm^3$ and 1258.1 $mm^3$ for low, medium and high doses of
183 α-TOS-Nps respectively compared with the control mice 3738.6 $mm^3$). The only
184 significant differences were observed in 0.75 and 2 mg of α-TOS-Nps at the 24 day (10
185 days postreatment, Figure 6). The tumor volume in α-TOS treatment alone was not
186 smaller (4568 $mm^3$) than the control at the end of the study. Mice treated with the vitamin
187 E analogue alone showed the largest tumor volume among all experimental groups. This
188 result was corroborated with caliper measurements and ultrasonography (Figure 5C), and
189 it was unexpected, as it has been reported that melanoma is susceptible to α-TOS
190 treatments both *in vitro* and *in vivo* experiments (Malafa et al, 2002). One reason for this
191 discrepancy may be related to the establishment of melanoma, the onset of treatments and
192 the inoculated cells. In the Malafa study (2002), they applied treatments with α-TOS alone
193 two days after the melanoma cell inoculation. This procedure allowed a significant
194 inhibition of the growth of the tumor (367 mm3 vs 2350 $mm^3$ in untreated group). In
195 contrast, in our work the treatments were injected two weeks after tumor cells were
196 inoculated. Probably, the time period was not long enough to inhibit the observed rapid
197 growth, Nps alone also exerted modest activity, but in this case an important cancer cell
198 infiltration in normal cells was detected, an effect that was not observed in none of the
199 treatments with α-TOS-Nps.

200 The histologic evaluation shows that in higher doses of α-TOS-Nps a higher
201 intratumoral necrosis is induced. In fact, a higher proportion of liquefactive necrosis was

202 histologic confirmed (Figure 7 D-G). Necrosis is associated to a loss of the tumoral
203 viability induced by chemotherapeutic agent. This could be reflected in a diminution of
204 tumoral volume and tumor weight. This effect is desirable previous to surgery because
205 facilitate the complete tumor removal and is related with a longer survival. Additionally,
206 aggregates nanoparticles in tumors were observed (Figure 8). Histological images showed
207 the formation of dose dependent aggregates of nanoparticles. In some cases, it was noticed
208 that some of the nanoparticles were endocytosed at the inoculation site. Nanoparticles that
209 do not dissolve but remain active have the potential to stress the cell or tissue as well. The
210 aggregates nanoparticles could be affecting in this way the tumor. We founded a large
211 necrotic areas inside the tumor and this effect was more evident in highest concentration
212 of α-TOS-Nps where more aggregated nanoparticles were observed. If the aggregates of
213 Nps were in part the cause of the observed effects needs to be evaluated. Importantly,
214 apparently toxic effects in the animals and tissues around of the aggregates was not
215 observed.

216 **Distribution.** Prussian blue staining was evaluated as an alternative to detect $Fe_3O_4$
217 nanoparticles in tumor and other major organs. Nanoparticle detection was possible for
218 all applied doses. We proposed that these nanoparticles can be mobilized from tumors to
219 other organs using the new blood vessels from the tumor to systemic circulation.
220 Additionally, our results clearly indicated that α-TOS-Nps were distributed in different
221 organs. Nanoparticles were found in tumors in doses dependent manner and in small
222 amounts in spleen, liver, skin, kidney, and gastrointestinal tract as revealed by the Prussian
223 blue staining (Figure 9). This staining detects ferric ions deposits in tissue when it reacts
224 with the soluble ferrocyanide to form a hydrated ferric ferrocyanide complex *in situ*,

225 which is insoluble. This substance is visualized under light microscopy as blue or purple
226 deposits within the cells. Intratumoral injected nanoparticles were biodistributed and
227 mainly found in spleen and liver, in agreement to other works (Schlachter et al, 2011).
228 None of the studied organs showed either direct evidence of excessive iron accumulation
229 or tissue alterations suggestive of iron overload. In contrast, dose dependent magnetite
230 accumulation in tumors was observed. Some studies indicate that smaller nanoparticles
231 show a more widespread organ distribution. The nanomaterials in systemic circulation
232 can be taken up by reticuloendothelial system, this results in entrapment of nanoparticles
233 mainly in organs with a high content of macrophages such as liver and spleen. In this
234 study the highest concentration of nanoparticles were observed in these organs with
235 Prusian blue. Although the sequestration of nanoparticles occurred in these organs was
236 possible to decrease tumor growth with the administered dose. This may be in relation
237 with the modification of the surface area by coating with the vitamin E analogue. In this
238 study the uptake as percentage of administered dose was not determinated.

239 Tissue distribution is mainly influenced by particle size; whereby larger nanoparticles
240 (> 50 nm) tend to rely on passive targeting, such as uptake by the reticuloendotelial
241 system; the smaller nanoparticles (<50 nm) benefit from slower opsonization and
242 clearance of the reticuloendotelial system. Additionally, biodistribution depends on
243 properties such as surface morphology and surface charge. Positively charged particles
244 tend to adhere nonspecifically to cells. In contrast, strong negative charged particles result
245 in increased liver uptake (Schlachter et al, 2011). No evidence of nanoparticles in brain
246 tissues treated with Prussian blue staining was detected. The results suggest that the i.t.
247 applied nanoparticles did not disrupt the blood brain barrier´s (BBB) permeability,

although further studies should be done to obtain conclusive data on this aspect. No abnormal histopathological gross lesions were observed in the organs of treated groups. All the observed organs did not shown apparent toxicity effects. More advanced and sophisticated kinetic models are required to take the differential tissue distribution of α-TOS-Nps in count, in order to delineate the detailed behavior of nanoparticles in different organs. Taken together, repeated administration of nanoparticles did not cause any apparent ultrasonographic changes, and in our study, the nanoparticles were able to mobilize and penetrate into different organs without apparently altering their morphology.

**CONCLUSIONS** Although no changes in malignancy characteristics were observed under X ray and ultrasound tumor images, the chosen doses of α-TOS-Nps protocol produced a growth tumor delay in melanoma model and larger necrotic areas in all the evaluated dose. Tumor growth affected with α-TOS-Nps treatments did not induce any apparent significant toxic effect in major organs. Further studies are clearly needed, additionally, detailed quantification of ferric ions and the biodistributions from systemic administrations and other administration ways need to be performed to compare and evaluate the behavior of α-TOS-Nps *in vivo*.

## EXPERIMENTAL SECCTION

**Chemicals.** The following materials were analytical grade, used as received without further purification and purchased from SIGMA-Aldrich: α-TOS Ferric chloride ($FeCl_3 \cdot 6H_2O$), $Na_2SO_3$, NH3OH, EtOH, (3-Aminopropyl) trimethoxysilane (APTMS), N-Hydroxysuccinimide (NHS), N,N′-Diisopropylcarbodiimide (NDC),and triethylamine (TEA), benzyl alcohol, potassium ferrocyanide trihydrate, $K_4Fe$ [CN] 6.3H2O, and HCl

271 **Preparation of α-TOS-Nps.** Spherical shaped magnetite nanoparticles ($Fe_3O_4$) functionalized
272 with vitamin E analogue α-TOS were prepared using a previously described and established co-
273 precipitation method with some modifications (Angulo-Molina et al.,*submitted*). Ferric chloride
274 ($FeCl_3 \cdot 6H_2O$) was used as precursor material (Qu et al. 1999). Briefly, the precursor was partially
275 reduced to the ferrous ion using $Na_2SO_3$ before alkalinizing with ammonium hydroxide, yielding
276 a black precipitate. Then, the precipitate was washed with absolute ethanol, centrifuged and dried
277 at 60$^o$C overnight under vacuum. Then, the nanoparticles surface was silanized and functionalized
278 with α-TOS following published methods previously described for other vitamins (Zhang and
279 Zhang 2005; Zhang et al. 2002). In a typical preparation, the functionalization was carried out by
280 adding 100 mg of silanized Nps to 6.6 mL of an ethanolic mixture of 10 mM α-TOS, 10 mL of
281 15mM N-Hydroxysuccinimide (NHS), 10 mL of 75mM N,N′-Diisopropylcarbodiimide (NDC)
282 solution and triethylamine (TEA) (Zhang et al. 2002). The pH was adjusted to 9 and after
283 incubation under stirring at 50$^o$ C for 4 h the suspension was centrifuged. The α-TOS-
284 functionalized nanoparticles (α-TOS-Nps), were washed with benzyl alcohol and deionized water
285 and dried in an oven at 60$^o$C overnight under vacuum. The nanoparticles were sonicated and
286 redispersed in sterile PBS before use.

287 **Characterization of nanoparticles.** The α-TOS-Nps were characterized by various analytical
288 techniques. The cristallinity of the Nps was analyzed by Transmission Electron Microscopy
289 (TEM) using a JEOL Model JEM2010 microscope operated at 200 kV. TEM samples were
290 prepared by placing one drop of a diluted suspension of nanoparticles in water on a carbon-coated
291 grid and allowing the solvent to evaporate at room temperature. Additionally, aqueous dispersions
292 of the nanoparticles were characterized by dynamic light scattering (DLS) to obtain the
293 hydrodynamic size distribution and Zeta potential (Nanotrac-Wave system, Microtrac, Inc.,
294 Montgomeryville, PA, USA). In brief, α-TOS-Nps were prepared using 1 mg/ml in PBS at a
295 temperature of 25$^o$C. Then, the nanoparticles were sonicated to prevent the aggregation and to
296 produce a uniform colloidal suspension. The Tyndall effect was tested to determine the formation

297 of an aqueous colloidal suspension using a laser beam. The magnetic response was evaluated by
298 exposing the nanoparticles to a strong magnetic field generated by a permanent ceramic Nd
299 magnet.

300 **Endotoxins.** The detection and quantification of endotoxin, in nanoparticle preparations were
301 based on the end-point chromogenic LAL assay (QLC-1000$^{TM}$), following the manufacture
302 instructions. This method utilizes a modified *Limulus amebocyte* lysate and a synthetic color
303 producing substrate to detect endotoxin chromogenically. Briefly, a sample is mixed with the LAL
304 supplied in the test kit and incubated at 37°C (±1°C) for 10 minutes. A substrate solution is then
305 mixed with the LAL-sample and incubated at 37°C (±1°C) for 6 minutes. The reaction is stopped
306 with a stop reagent. If endotoxin is present in the sample, a yellow color will develop. The
307 absorbance of the sample lies in the 405-410 nm range. Since this absorbance is directly
308 proportional to the amount of endotoxin present, the concentration of endotoxin is calculated from
309 a calibration curve.

310 **Cellular Culture.** The mice melanoma cancer cell line B16F0 (ATCC CRL 6322) was growth
311 and plated on 100 mm cell culture plate dishes with complete Dulbecco's modified Eagle medium
312 (DMEM), supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO, USA) plus antibiotic-
313 antimycotic 100 U/ml penicillin, 100 µl/ml streptomycin (GIBCO, USA), 1% glutamine and were
314 incubated at 37 °C and 5% $CO_2$ in a humidified atmosphere.

315 **Nude mice assay.** In order to establish the melanoma model, adherent B16F0 cells were
316 detached with 1 min trypsin-EDTA and were harvested by centrifugation and washed once with
317 PBS. The tumorigenicity assay with B16F0 cells was performed by subcutaneous injection of 8
318 week-old BALB/Cannes nu/nu male SPF mice with 1 x $10^5$ cells that were resuspended in 0.2 ml
319 of PBS. The mice were obtained from the Instituto Nacional de la Nutrición Salvador Zubirán,
320 kept in microisolation boxes, fed with NubLab (Mexico), and allowed filtered and sterilized water
321 ad libitum. The injection sites were observed regularly for development and progression of
322 tumors. Tumor volume was determined as: Volume = (Length x Width$^2$)/2. All animals used in

323 this study were maintained under standards established by the guidelines for animal care and use
324 of NOM-039 and Norma Official Mexicana NOM-062-ZOO-1999. Tumor growth was monitored
325 by measuring the tumor length (L) and width (W) using a diagnostic X-ray apparatus (General
326 Electric Medical Systems Monitrol/15) operating at 30 kV and 100 mA, and an Ultrasound
327 Transducer (General Electric Logiq 400 Pro) operating at 11 MHz).

328 **Treatments.** The treatments were applied two weeks after tumor injection, when the tumor was
329 established and became clearly palpable. Additionally, we evaluated the tumor by X-ray imaging
330 and ultrasonography at the beginning and at the end of the study. The mice were anesthetized by
331 a ketamine/xilacine solution via i.p. during the imaging session. Mice were randomized in seven
332 groups (n=3 per group): untreated, α-TOS, pure Nps and α-TOS-Nps in 0.075, 0.150, 1 and 2 mg
333 doses and received the treatments dissolved in PBS each third day by i.t. injection. Untreated
334 control mice received a corresponding volume of vehicle (PBS). Animal weights and tumor
335 growth were determined in 3–4 day intervals, until the end of the treatment, when the mice were
336 sacrificed and the tumors were resected and processed for histological analysis.

337 **Histology.** To confirm the establishment of melanoma and to evaluate the effects of α-TOS-
338 Nps treatments at the end of the experiment, each tumor was dissected from surrounding tissues
339 and cut into several pieces (approximately 5 x 5 x 5 mm). The pieces were fixed in 10%
340 formaldehyde/phosphate-buffered saline (PBS) at pH 7, and paraffin embedded. Additionally,
341 major organs (heart, lung, kidney, liver, spleen, brain, stomach, gut and skin) of mice treated with
342 α-TOS-Nps were also dissected and prepared in the same way. Tissue sections (4 µm in thickness)
343 were stained with hematoxylin and eosin (H&E) for histopathology analysis. Images were
344 captured with an AxioCam ERc5s on a Carl Zeiss light microscope and processed using the
345 imaging software Zen 2011 SP1.

346 **Immunohistochemical.** The antigen HMB45 was evaluated on paraffin-embedded tissue
347 sections of 4 µm in thickness on the onset of study to support the confirmation of melanoma.
348 HMB45 l is an intracytoplasmic antigen in the majority of melanomas and other tumors that

349 demonstrate melanoma/melanocytic differentiation. Briefly, paraffin sections were deparaffinized
350 and rehydrated. Endogenous peroxidase activity was quenched by incubation in peroxide for 10
351 min. To unmask antigens, slides were heated at 97ºC for 5 min. Slides were incubated with
352 monoclonal antibodies HMB45 (DAKO). After incubation with the secondary antibody (DAKO),
353 immune reactive products were developed using 3´3-diaminobenzidine (DAB) as the chromogen
354 with standard development times.

355 **Nanoparticles biodistribution.** Tissue sections from the melanoma tumor and major organs
356 were stained with Prussian blue. The slides were transferred to a Coplin chamber containing
357 freshly prepared Perls´reagent (solution of 10% potassium ferrocyanide trihydrate, $K_4Fe$ [CN]
358 6.3H2O, and 20% HCl solutions mixed with a volume ratio of 1:1). The samples were left for 30
359 min for color development. Then, the slides were washed with PBS three times and counterstained
360 with nuclear fast red and incubated for 5 min. The slides were rinsed with distilled water. Blue
361 color was observed on the areas bearing iron oxide nanoparticles (Schlachter et al, 2011; Zhu et
362 al, 2012). Images were collected with an AxioCam ERc5s on Carl Zeiss light microscope and
363 processed using the imaging software Zen 2011 SP1.

364 **Statistical analysis.** Statistical analysis was conducted by Student´s t-test. Differences between
365 results were considered significant when $p<0.05$.

366

367 **Conflict of Interest:** The authors declare no competing financial interest.

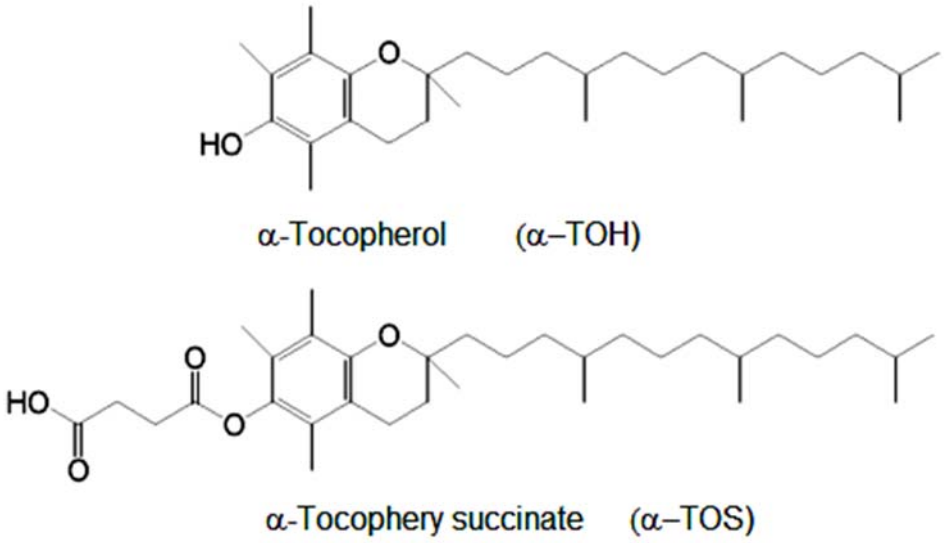
368 **Acknowledgment**. The authors are grateful for Dr. Silvia Moya (Centro de Investigación en
369 Alimentación y Desarrollo, CIAD), for comments and critical reading of the article. The work was
370 supported by SEP-CONACYT (Fondo de Investigación Científica Básica) 154602.
371

372

373

374

375




376

377 Figure 1. Molecular structure of α-Tocopherol and α-Tocopheryl succinate.

378

379

380

381

382

383

384

385

386

387

388

389

390

391

392

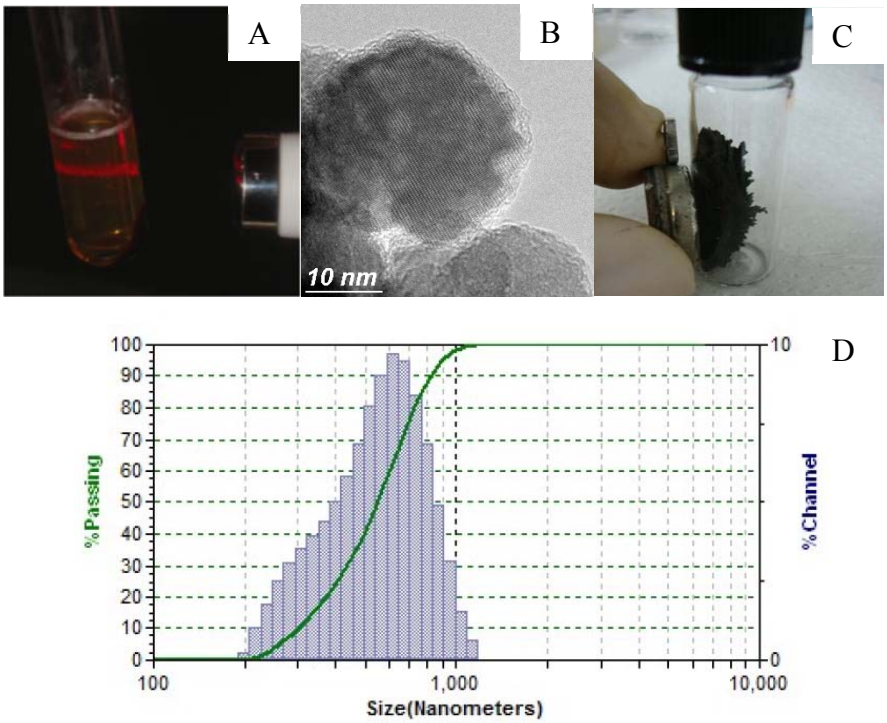
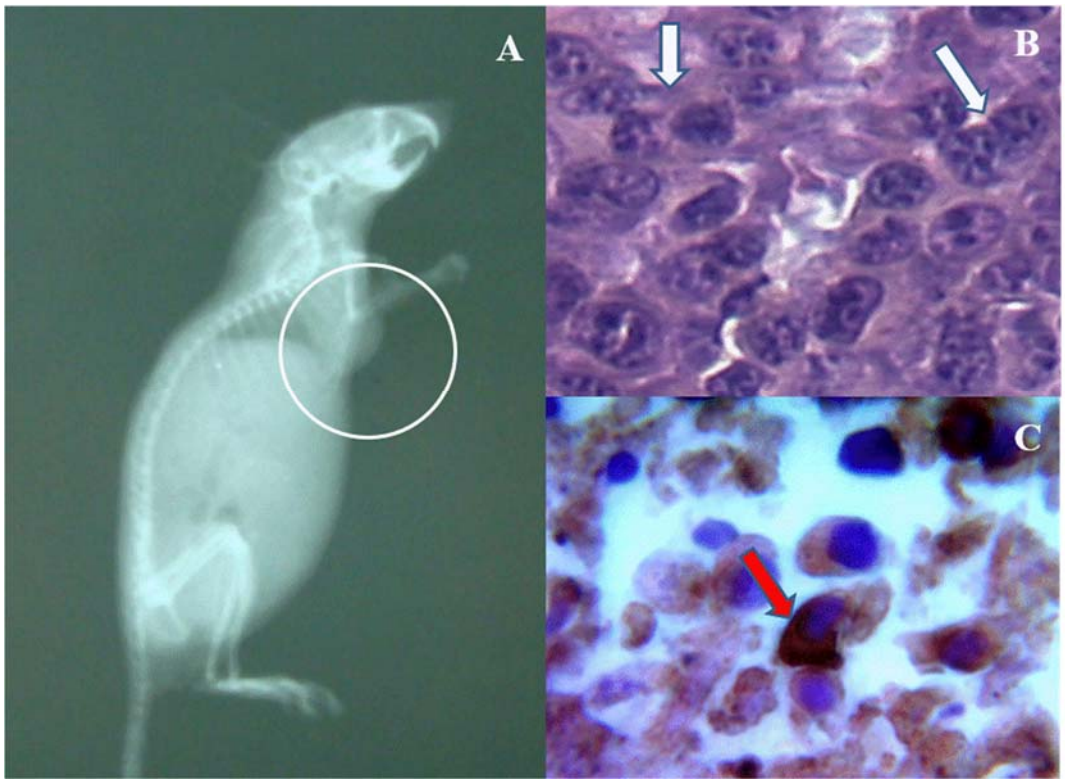



Figure 2. Characterization of α-TOS-Nps: (a) Tyndall effect was observed when the laser beam was scattered by the nanoparticles of the colloidal system; (b) Typical magnetite NP showing a spherical- like morphology of 15 nm average size observed by TEM; (c) The nanoparticles became magnetic with the application of an external magnetic field; (d) The DLS study showed a hydrodynamic size distribution in the range ≈ 190-1100 nm, with a mean value of 550 nm. The hydrodynamic size distribution was in agreement with the granules and agglomerates of nanoparticles observed inside the tumor.

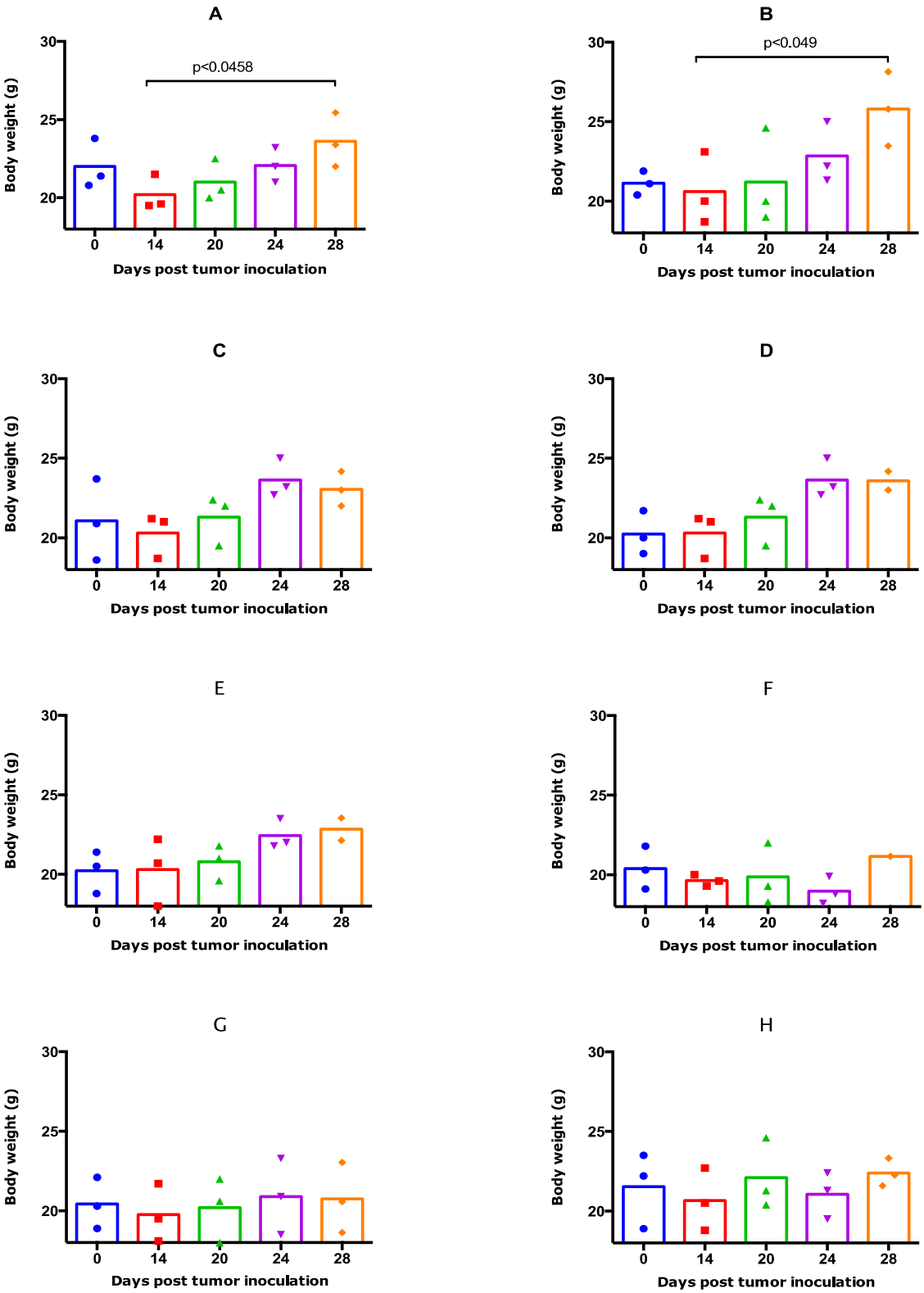
414 


415 Figure 3. The establishment of melanoma tumor was evaluated with X-Ray working at 30
416 kv and 100 mAs (a). The white circle shows a nodular mass with regular border, oval or
417 lobular morphology with heterogeneous density and nodular calcifications. The nodular
418 mass was dissected, analyzed and confirmed with histology. With H&E, a moderate
419 cellular pleomorphic form is observed (white arrow) characterized by rounded or
420 polygonal cells with oval and hyperchromatic nucleus and moderate mitotic activity (b).
421 The cytoplasmic tumor antigen HMB45, specific for melanoma, give positive brown
422 reaction (red arrow) (c). All the probes confirmed the melanoma establishment in mice
423 two weeks after inoculation of B16F0 cells. Microphotographs with 100X magnification.

424

425

426

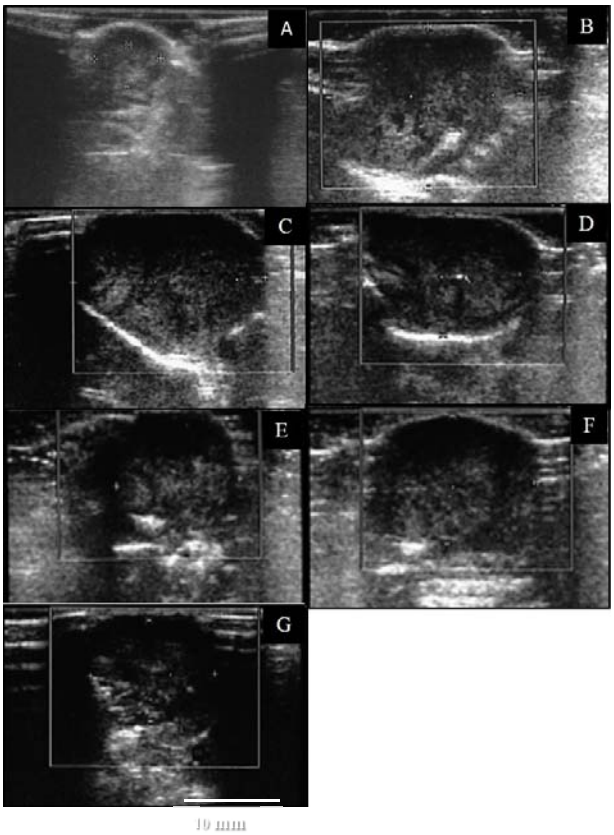

A
p<0.0458
B
p<0.049
C
D
E
F
G
H
Body weight (g)
30
25
20
0
14
20
24
28
Days post tumor inoculation

427

428 Figure 4. Effect of i.t. injection of α-TOS-Nps on body weight in a melanoma mouse
429 model. A) Control of normal mice, B) untreated control, C) Nps 2 mg, D) α -TOS 2 mg,
430 E) α-TOS-Nps 2 mg, F) α-TOS-Nps 1 mg, G) α-TOS-NPs 0.150 mg, H) α-TOS-Nps 0.75
431 mg. B16F0 cells (100,000) were implanted in the flank of nude mice (n=3 in each group).
432 The experimental treatments onset when the tumor is established (≈200 $mm^3$, day 14 after
433 cell injection). Mice were treated with α-TOS or Nps (magnetite) alone or in combination
434 as α-TOS-Nps (magnetite functionalized with α-TOS), or vehicle control. All the
435 treatments were administrated by i.t. injection in 50µL of PBS on days 14, 16, 18, 20, 22,
436 and 24 (6 times in total). Body weights were determined in 3-4 day intervals until day 28.
437 All the treatments and control groups displayed weight gain. In B, the weight gain was
438 relationated with the volume tumor

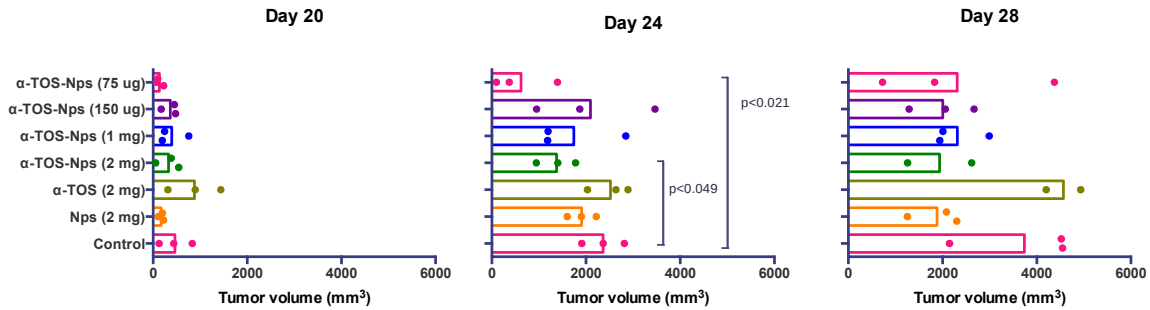
439
440
441
442
443
444

445




446 Figure 5. Evaluation by ultrasonography. (A) and (B) show representative images of an
447 untreated control mouse at the onset of the experiment and on week 3. Figures (C) and
448 (D) show a mouse treated with α-TOS (2 mg) and Nps alone (2 mg). Figures (E), (F) and
449 (G) show images of mouse treated with α-TOS-Nps at 75 μg, 150 μg and 2 mg
450 respectively. A solid tumor, non-cystic, with regular borders, and irregular and nodular
451 calcifications was observed in all the treatments. The echogenicity was heterogeneous and
452 hyperechogenic disperse semi-nodules were also observed at the onset and the end of the
453 study. The tumors showed similar ultrasonographic characteristics with all the treatments,
454 but an inhibitory effect in tumor measurements in Nps and 0.75 and 2 mg of α-TOS-Nps
455 doses was observed. Figure (C) shows the image of tumor inoculated with α-TOS alone.
456 In this case, an inhibitory effect on the growth was not observed with respect to untreated
457 control.

458




459

460 Figure 6. Effect of i.t. injection of α-TOS-Nps on tumor growth in a melanoma mouse
461 model. The experimental treatments onset when the tumor is established (≈200 $mm^3$, day
462 14 after cell injection). Mice were treated with α-TOS or Nps (magnetite) alone or in
463 combination as α-TOS-Nps (magnetite functionalized with α-TOS), or vehicle control.
464 All the treatments were administrated by i.t. injection in 50µL of PBS on days 14, 16, 18,
465 20, 22, and 24 (6 times in total).. Tumor growth was monitored by measuring the tumor
466 length (L) and width (W) in 3-4 day intervals until day 28. A significant difference in the
467 tumor growth was observed with 0.75 and 2 mg of α-TOS-Nps treatment at day 24.
468 Tumor volume was determined with the following formula: Volume= $(L \times W^2)/2$. Data
469 point represents mean tumor volume ($mm^3$).

470

471

472

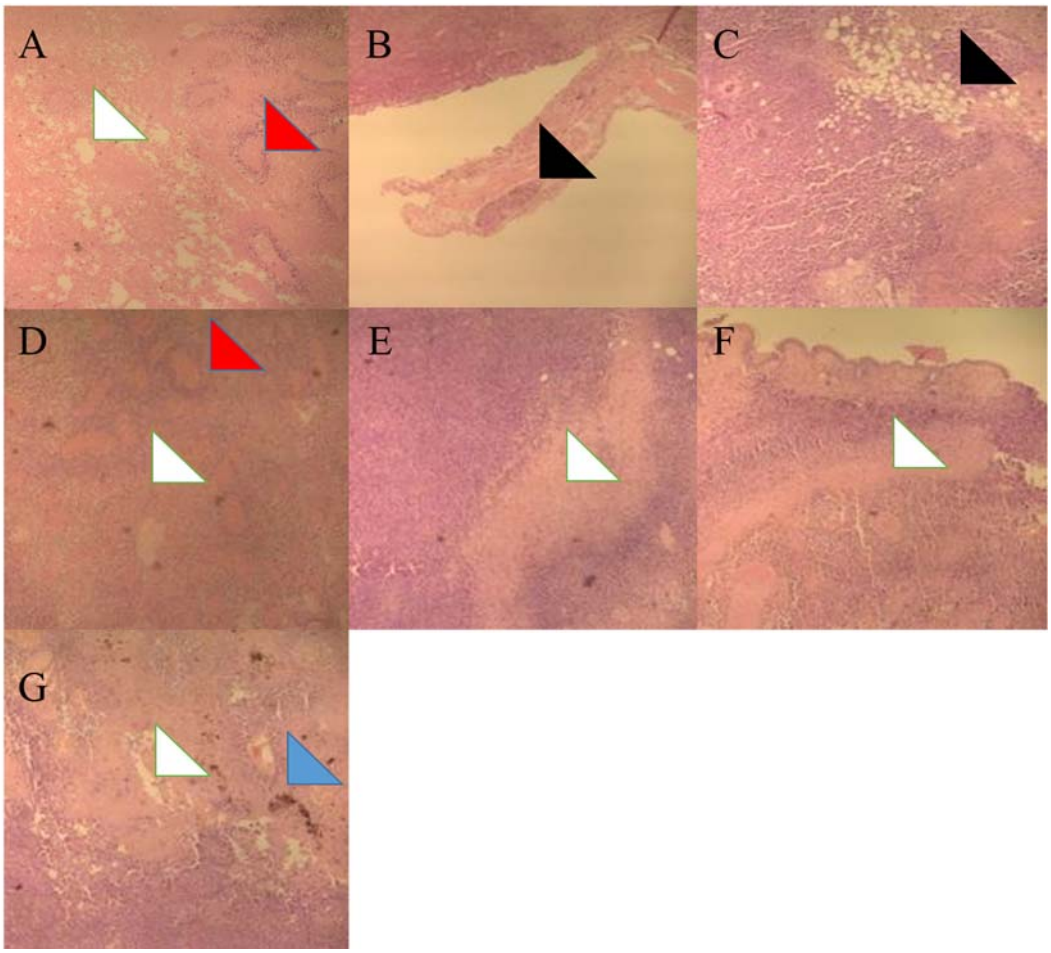
473

474

475

476

477




478

479 Figure 7. Photomicrography (H&E) illustrating at low power magnification histological
480 characteristics. A) Untreated control, showing coagulative necrosis (white arrowhead)
481 and angiotrophism (red arrowhead); B) α-TOS treatment (2 mg) where skeletal muscle
482 tissue is infiltrated with cancer cells (black arrowhead); C) Pure Nps (2 mg) where
483 adipose tissue is infiltrated with cancer cells (black arrowhead); D) α-TOS-Nps 0.075 mg,
484 E) α-TOS-Nps 0.150 mg, F) α-TOS-Nps 1 mg), G) α-TOS-Nps 2 mg; in all the treatments
485 with α-TOS-Nps there were large areas of coagulative and liquefactive necrosis (white
486 arrowshead). The blue arrowhead show melanin granules. 3x magnification.

487

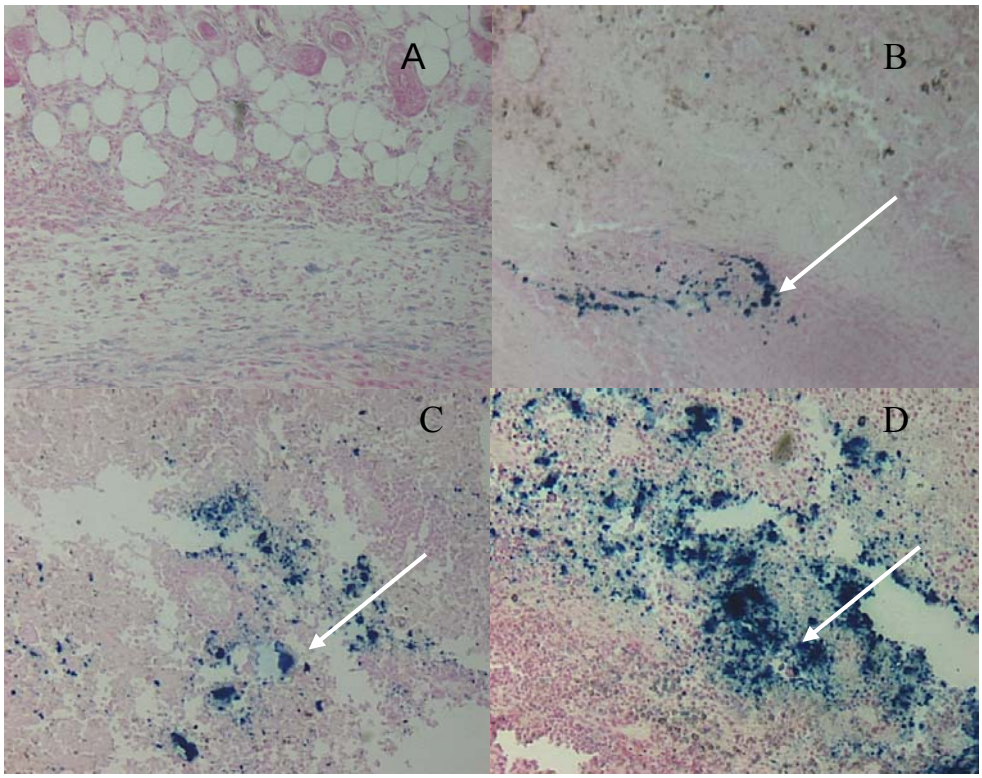



Figure 8. The Prussian blue staining detected ferric ions as blue or purple deposits (white arrows) in tumor in all the treatments doses at the end of experiment. (a) Control, untreated, (b) 0.75 mg, (c) 0.150 mg, (d) 2 mg of α-TOS-Nps applied each third day. Granules and agglomerates were detected inside the tumor in all the treatments. These observations are in agreement with hydrodinamic size distribution determined with DLS. Microphotographs (20X magnification).

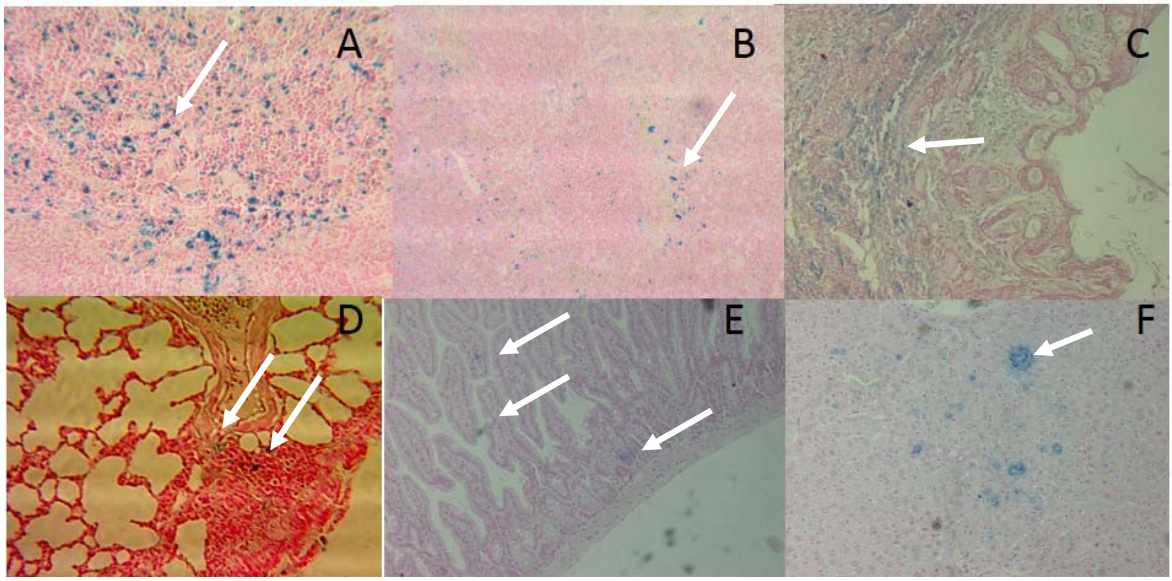



Figure 9. The white arrows show the magnetite nanoparticles functionalized with α-TOS (α-TOS-Nps) detected with the Prussian blue staining as blue or purple deposits in spleen (A), liver (B), skin (C), lung (D), cecum (E) and kidney (F) of mice treated in all the evaluated doses. (10X magnification). No abnormal histopathological gross lesions were observed in the organs of treated groups.

526 *Address correspondence to

527 jhdez@ciad.mx

528 aracely.angulo@udlap.mx

529 **REFERENCES**

530 1.-Tomasetti, M., Neuzil, J., 2007. Vitamin E analogues and immune response in cancer treatment. Vitam
531 Horm 76, 463-91; doi: 10.1016/S0083-6729(07)76018-1.

532 2.-Zhao, Y., Neuzil, J., Wu, K., 2009. Vitamin E analogues as mitochondria-targeting compounds: from
533 the bench to the bedside. Mol Nutr Food Res 53, 129-39; doi: 10.1002/mnfr.200800045.

534 3.-Kanai, K., Kikuchi, E., Mikami, S., Suzuki, E., Uchida, Y., Kodaira, K., Miyajima, A., Ohigashi, T.,
535 Nakashima, J., Oya, M., 2010. Vitamin E succinate induced apoptosis and enhanced chemosensitivity to
536 paclitaxel in human bladder cancer cells in vitro and in vivo. Cancer Sci 101, 216-23; doi: 10.1111/j.1349-
537 7006.2009.01362.x.

538 4.-Turánek, J., Wang, X.F., Knotigova, P., Koudelka, S., Dong, L.F., Vrublova, E., Mahdavian, E.,
539 Prochazka, .L, Sangsura, S., Vacek, A., Salvatore, B.A., Neuzil, J., 2009. Liposomal formulation Alpha-
540 tocopheryl maleamide: in vitro and in vivo toxicological profile and anticancer effect against spontaneous
541 breast carcinomas in mice. Toxicol Appl Pharmacol 237(3), 249-57; doi: 10.1016/j.taap.2009.01.027.

542 5.-Thanh, N.T.K., Green, L.A.W., 2010. Functionalisation of nanoparticles for biomedical applications.
543 Nano Today 5, 213-30; doi: 10.1016/j.nantod.2010.05.003.

544 6.-Cochran, D.B., Wattanwar, P.P., Wydra, R., Hilt, J.Z., Anderson, K.W., Ejter, R.J.,
545 2013. Suppressing iron oxide nanoparticle toxicity by vascular targeted antioxidant polymer nanoparticles.
546 Biomaterials.S0142-9612 (13): 00973-3. doi: 10.1016/j.biomaterials.2013.08.025.

547 7.-Baba, D., Seiko, Y., Nakanishi, T., Zhang, H., Arakaki, A., Matsunaga, T., Osaka, T., 2012. Effect of
548 magnetite nanoparticles on living rate of mcf-7 human breast cancer cells. Colloids Surf B Biointerfaces
549 95, 254-7; doi: 10.1016/j.colsurfb.2012.03.008.

550 8.-Alexiou, C., Schmid, R.J., Jurgons, R., Kremer, M., Wanner, G., Bergermann, C., Huenges, E.,
551 Nawroth, T., Arnold, W., Parak, F., 2006. Targeting cancer cells: magnetic nanoparticles as drug carriers.
552 Eur Biophys J 35, 446-50; doi: 10.1007/S00249-006-0042-1.

553 9.-Thomas, R., Park, I., Jeong, Y.Y., 2013. Magnetic Iron Oxide Nanoparticles for Multimodal Imaging
554 and Therapy of Cancer. Int J Mol Sci 14, 15910-30; doi: 10.3390/ijms140815910.

555 10.-Laurent S., Mahmoudi M., 2011. Superparamagnetic iron oxide nanoparticles: promises for diagnosis
556 and treatment of cancer. Int J Mol Epidemiol Genet 2(4), 367-90; PubMed:
557 http://www.ncbi.nlm.nih.gov/pubmed/22199999. doi

558 11.-Haglund, E., Seale-Goldsmith, M., Leary, J., 2009. Design of Multifunctional Nanomedical Systems.
559 Ann Biomed Eng 37(10), 2048-63; doi: 10.1007/s10439-009-9640-2.

560 12.-Jimbow, K., Ishii-Osai, Y., Kobayashi,T. 2013. Melanoma-Targeted Chemothermotherapy and In
561 Situ Peptide Immunotherapy through HSP Production by Using Melanogenesis Substrate, NPrCAP, and
562 Magnetite Nanoparticles. Journal of Skin Cancer, 2013, 1-12. http://dx.doi.org/10.1155/2013/742925

563 13.-Kim, J.S., Yoon, T.J., Yu, K.N., Kim, B.G., Park, S.J., Kim, H.W., Lee, K.H., Park, S.B., Lee, J.K.,
564 Cho, M.H., 2006. Toxicity and tissue distribution of magnetic nanoparticles in mice. Toxicol Sci 89(1),
565 338-47; doi: 10.1093/toxsci/kfj027.

566 14.-Zhu, X.M., Wang, Y.X., Leung, K.C., Lee, S.F., Zhao, F., Wang, D.W., 2012. Enhanced cellular
567 uptake of aminosilane-coated superparamagnetic iron oxide nanoparticles in mammkalian cell lines. Int J
568 Nanomedicine 7, 953-64; doi: 10.2147/IJN.S28316.

569 15.-Speroni, L., Bustuoabad. V.A., Gasparri, J., Chiaramoni, N.S., Taira, M.C., Ruggiero, R.A., Del
570 Valle, S. 2009. Alternative site of implantation affects tumor malignancy and metastatic potential in mice.
571 Cancer Biology & Therapy, 8:375-379. doi: 10.4161/cbt.8.4.7452.

572 16.-Feng SS, Zeng, W., Lim, Y.T., Zhao, L., Win, K.Y., Oakley, R., Teoh, S.H., Lee, R.C.H., Pan, S.
573 2007. Vitamin E TPGS-emulsified poly (lactic-co-glycolic acid) nanoparticles for cardiovascular restenosis
574 treatment. Nanomedicine 2:333-344. http://www.ncbi.nlm.nih.gov/pubmed/17716178.
575 doi:10.2217/17435889.2.3.333

576 17.-Schlachter, E.K, Wildmer, H.R., Bregy, A., Lönnfors-Weitzel, T., Vajtal, I., Corazza, N., Bernau,
577 V.J.P., Weitzel, T., Mordasini, P., Slotboom, J., Herrmann, G., Bogni, S., Hofmann, H., Frenz, M., Reinert,
578 M., 2011. Metabolic pathway and distribution of superparamagnetic iron oxide nanoparticles: in vivo study.
579 Int J Nanomedicine 6, 1793-1800; doi: 10.2147/IJN.S23638.

580 18. - Tartaj, P., Morales, M., Veintemillas-Verdaguer, S., González-Carreño, T., Serna, C. J. 2003. The
581 preparation of magnetic nanoparticles for applications in biomedicine. J Phys D: Appl Phys 36: R182-R197.
582 doi:10.1088/0022-3727/36/13/202.

583 19. - Gupta, A. K. and Gupta, M. 2005. Synthesis and surface engineering of iron oxide nanoparticles for
584 biomedical applications. Biomaterials 26, 3995-4021. doi: 10.1016/j.biomaterials.2004.10.012.

585 20.-Zhang, Y., Zhang, J., 2005. Surface modification of monodisperse magnetite nanoparticles for
586 improved intracellular uptake to breast cancer cells. J Colloid Interface Sci 283(2), 352-7; doi:
587 10.1016/j.jcis.2004.09.042.

588 21.-Amstad, E., Textor, M., Reimhult, E., 2011. Stabilization and functionalization of iron oxide
589 nanoparticles for biomedical applications. Nanoscale 3(7), 2819-43; doi: 10.1039/c1nr10173k.

590 22.-Mahmoudi, M., Simchi, A., Milani, A.S., Stroeve, P., 2009. Cell toxicity of superparamagnetic iron
591 oxide nanoparticles. J Colloid Interface Sci 336(2), 510-8; doi: 10.1016/j.cis.2009.04.046.

592 23.-Hanini, A., Schmitt A., Kacem K., Chau F., Ammar S., Gavard J.,2011 Evaluation of iron
593 oxide nanoparticle biocompatibility. Int J Nanomedicine. 6:787-94. doi: 10.2147/IJN.S17574.

594 24.-Brannon-Peppas, L., Blanchete, J.O., 2004. Nanoparticle and targeted systems for cancer therapy.
595 Adv Drug Deliv Rev 56(11), 1649-59; PubMed: http://www.ncbi.nlm.nih.gov/pubmed/15350294. doi:
596 10.1016/j.addr.2004.02.014.

597 25.-Neun, B.W., Dobrovolskaia MA. 2011, Detection and quantitative evaluation of endotoxin
598 contamination in nanoparticle formulations by LAL-based assays. Methods Mol Biol, 697:121-30. doi:
599 10.1007/978-1-60327-198-1_12.

600 26.-Quintana, E., Shackleton, M., Sabel, M.S., Fullen, D.R., Johnson, T.M., Morrison, S.J., 2008.
601 Efficient tumor formation by single human melanoma cells. Nature 456, 593-8; doi: 10.1038/nature07567.

602 27.-Lee, J., Karshafian, R., Papanicolau, N., Giles A., Kolios M.C., Czarnota, G.J., 2012. Quantitative
603 ultrasound for the monitoring of novel microbubble and ultrasound radiosensitization. Ulltrasound Med
604 Biol 38(7), 1212–21; doi: 10.1016/j.ultrasmedbio.2012.01.028.

605 28.-Malafa, M.P., Fokum, F.D., Mowlavi, A., Abusief, M., King, M., 2002. Vitamin E inhibits melanoma
606 growth in mice. Surgery 131, 85-91; doi: 10.1067/msy.2002.119191.

607 29.-Qu, S., Yang, H., Ren, D., Kan, S., Zou, G., Li, D., Li, M., 1999. Magnetite Nanoparticles Prepared
608 By Precipitation From Partially Reduced Ferric Chloride Aqueous Solutions. J Colloid Interface Sci 215
609 (1), 190-2; doi: 10.1006/jcis.1999.6185.

610 30.- Angulo-Molina, A., Méndez-Rojas, M. A., Palacios-Hernández, T.J., Contreras-López, O. E., Hirata-
611 Flores, G., Flores-Alonso, J. C., Merino-Contreras, S., Valenzuela, O., Hernández, J., Reyes-Leyva, J.
612 "Magnetite nanoparticles functionalized with α-tocopheryl succinate (α-TOS) promote selective cervical
613 cancer cell death", J Nanoparticle Res (Submitted).

614

# ANEXOS

# ANEXO I

## Riesgos Ambientales de la Nanotecnología: Evaluando la Ecotoxicidad de Nanomateriales

Méndez-Rojas MA, Sánchez-Salas JL, Angulo-Molina A and Palacios-Hernández TJ. 2014. Environmental risks of nanotechnology: evaluating the ecotoxicity of nanomaterials". In: Kharisov BI, Kharissova O, Dias RH. Nanomaterials for Environmental Protection. John Wiley & Sons, NY. In press

## RESUMEN

Las propiedades electrónicas, ópticas, magnéticas y mecánicas únicas de los materiales a nivel nanoescala abren una puerta a las nuevas alternativas tecnológicas para su aplicación en reacciones de catálisis, materiales avanzados, medicina, energía, electrónica, e incluso para su aplicación en la bio-remediación. En este sentido, la incorporación de esos nanomateriales a productos de uso común como empaques, ropa, ingredientes alimenticios, así como el incremento en los productos de desecho con componentes nanoestructurados, nos lleva a cuestionarnos sobre el impacto ambiental y el riesgo en la salud pública de su presencia. Aunque la mayoría de los nanomateriales se han estudiado de forma extensa, los efectos a largo plazo no se conocen, ya que gran parte de los estudios no han sido concluyentes. La ecotoxicología es un campo multidisciplinario que evalúa y predice el impacto de sustancias químicas tóxicas sobre los seres vivos. Una de las preguntas de mayor interés en los últimos años es el impacto que pueden tener los nanomateriales de desecho en un ecosistema. De ahí la importancia de considerar no sólo la composición química, sino también la concentración, la estabilidad, forma, tamaño, agregación y propiedades físicas de los nanomateriales y su interacción con otras sustancias. Actualmente podemos encontrar nanometales y sus derivados en diferentes productos comerciales y a la vez encontrarlos como base para la bio-remediación de agua, aire y suelo. De ahí la importancia de evaluar cuál es el impacto o riesgo de su presencia en el medio ambiente o en el ser humano. Muchos de los nanomateriales son capaces de atravesar las barreras biológicas naturales y afectar directamente a los órganos expuestos. Así mismo la exposición aguda o prolongada puede favorecer el estrés oxidativo. Actualmente, existen diferentes modelos *in vitro* e *in vivo* utilizados para evaluarlos. Sin embargo, todavía no existe estandarización en muchos de ellos, aunado a que además se desconoce las características físico-químicas de esos nanomateriales y por ende no pueden preverse los posibles efectos biológicos. Diferentes instancias internacionales, están realizando consensos para trabajar y evaluar los riesgos de los nanomateriales a los que ya estamos expuestos y con aquellos en los que existe un riesgo inminente. De gran importancia es que la información obtenida fluya y se tomen las medidas gubernamentales para su manejo y control.

**Environmental risks of nanotechnology: Evaluating the ecotoxicity of nanomaterials**

*Miguel Angel Méndez-Rojas (*), José Luis Sánchez-Salas, Aracely Angulo-Molina*

*Universidad de las Américas Puebla*

*Teresa de Jesús Palacios-Hernández*

*Universidad Popular Autónoma del Estado de Puebla*

*Corresponding /Contact Author Name: Miguel Angel Méndez-Rojas*

*Corresponding/Contact Author Phone: +52 222 2292607*

*Corresponding/Contact Author Email: miguela.mendez@udlap.mx*

*Keywords: Eco-toxicity, evaluation, environmental impact, nanomaterials*

**Abstract:**

The unique electronic, optical, magnetic and mechanical properties of materials in the nanoscale open venues to new technologically relevant alternatives for application in catalysis, advanced materials, medicine, energy and electronics and even in environmental remediation. In that sense, the incorporation of such nanomaterials in every day consumer products, food ingredients, packing materials, cosmetics, clothes, as well as the increase on the number of waste side-products containing nano-sized components has arisen many questions about their potential impact on the environment and their risks in public health. Although most of the nanomaterials have been extensively studied in research labs for decades, it is very probable than the long term effects have been overlooked or even that the test realized to evaluate their potential toxicity are not enough to reach a conclusion.

Ecotoxicology is a multidisciplinary field of study that aims to evaluate and predict the impact of toxic chemicals on biological organisms. What is the fate and effects of nanomaterials into an ecosystem is a new –and important- question for this field. To answer it, we should to consider not only the chemical composition, but also the concentration, stability, bioavailability, solubility, size, shape, aggregation and physical properties of the considered nanomaterial and their interactions with other substances. As many nanometals and their derivatives (oxides, chalcogenes and salts) as well as carbon-based nanomaterials are finding commercial uses or even being considered for polluted water, air or soil remediation, it is important to assess their potential risks and effects on the environment and human health.

In this chapter, we start by discussing the physical and chemical properties of some technologically important nanomaterials, in order to understand how such properties may have implications on human health and potential for ecological disruption if dispersed as

pollutants in the environment. After that, we present a glimpse to the actual and future markets of consumer products and environmental technologies making use of nanomaterials. In order to understand the impact on ecology and human health, we will discuss how their unique properties made them able to pass through natural barriers, their bioaccumulation in organisms and the disruption of metabolic processes by oxidative stress, enzyme inhibition, cellular interactions, inflammation or genotoxicity, creating a health risk for short and long term due their toxicological effects. At the end, we will discuss several *in vitro* and *in vivo* techniques and some of the advantages and disadvantages of exposure methods used in ecotoxicology.

## ***Introduction***

Ecotoxicology is a young, multidisciplinary, field of science concerned with the study of contaminants (chemicals or biological organisms) in the biosphere and the understanding of their effects at the population, community and ecosystem level, including humans *(1, 2)*. It is a multidisciplinary field, which uses tools and concepts from biology, chemistry, medicine, toxicology and ecology. In that sense, nano-ecotoxicology is an emergent branch of ecotoxicology specifically dedicated to engineered and natural nanomaterials. For such specialized field of interest, it is important a complete assessment along the life-cycle of the product, in order to understand the potential environmental and health hazards of such materials.

Ultrafine particles suspended in the air have been traditionally included as a topic of interest in toxicology, and their sizes ranges in the nanometer scale; when present in water or soils, they are refereed as colloids. In the normal toxicological terminology, particles with diameters less than 0.1 μm (100 nm, 0.1 μm) are called ultrafine particles (UFP), although they can be grouped in the three general categories: a) those with diameters less that 100 nm, b) those with sizes between 100 and 2,500 nm (resulting from aggregation of UFPs), and, c) coarse-mode particles larger than 2,500 nm. In the other hand, the term colloid is applied to particles with sizes in the 1 to 1000 nm range (0.001 to 1 micrometer). This kind of extremely fine and small materials may be the product of natural processes, or they may be produced as side-products of anthropogenic activities or inclusively they may be specifically manufactured for very specific applications.

Natural nanostructured materials have been around us for a long time. Humans and living beings have been exposing to naturally produced nanomaterials since the beginning of life sources (soil erosion, ocean water evaporation, forest fires, photochemical reactions, volcanic eruptions, viruses, biogenic magnetite biosynthesized by magnetotactic bacteria, mollusks, arthropods, fish, birds, or from disintegration of iron-meteorites when entering into the atmosphere) *(3, 4)*. They are also artificially produced, both intentionally for very specific applications (pigments, quantum dots, magnetic nanoparticles, catalysts, coatings, cosmetics, among several more examples) and as side-products of several manufacturing and industrial processes (fuel and charcoal combustion, mineral processing, cooking,

welding, smoking, building demolition, consumer products containing nanomaterials degradation, etc.), many of them potentially toxic.

*Figure 1. Some natural sources of nanomaterials. Clockwise: a forest fire, dust storms, sea water evaporation, volcanic eruptions.*

Engineered nanomaterials (ENMs), in the other hand, are source of concern as they have not been around us for a long time and then, living beings may have not developed appropriate biological barriers or trapping systems to avoid undesirable interactions which may harm the individual. There are several reports of toxicological studies of nanomaterials which suggest that several of them may be dangerous, although the results sometimes are not conclusive and even contradictory *(1)*.

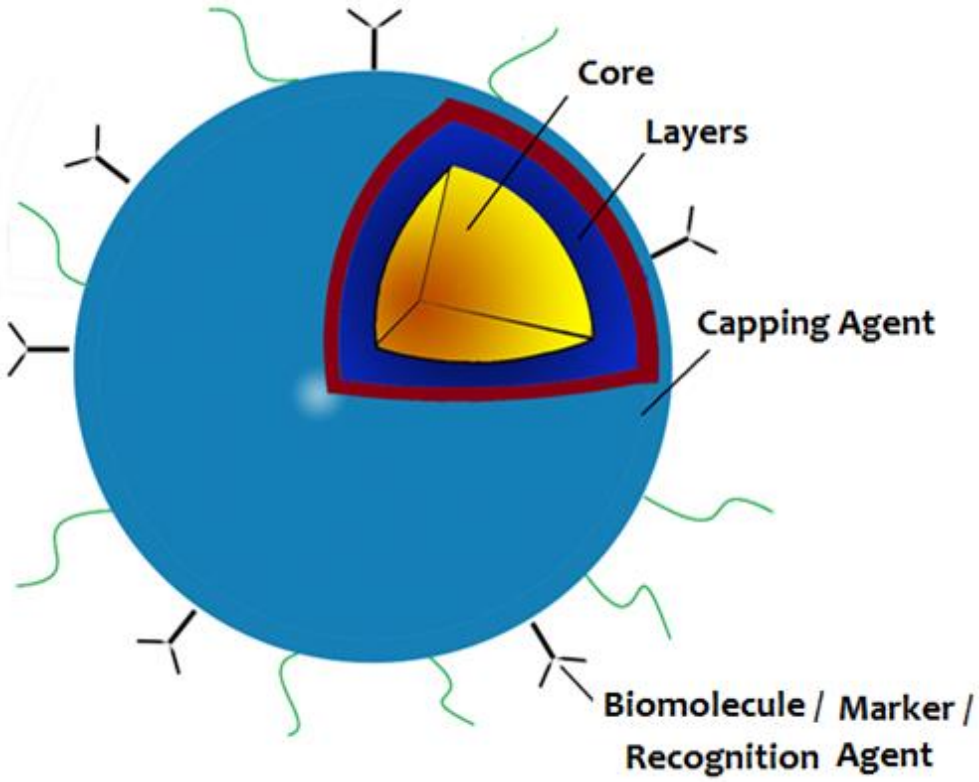



*Figure 2. Schematic representation of an Engineered Nanomaterial (ENM). The core determine some of the physical properties of the material; the Layer are molecules acting as stabilizers or modifying agents; the capping act as an agent to increase biocompatibiligy, change charge, solubility; the biomarker / fluorophere / recognition agent generates specifity for recognizing a substrate or detect the nanomaterial.*

For example, nano $TiO_2$ and nano ZnO have been reported as toxic to soil bacterial communities that may alter environmentally important soil processes *(5)*. However, other report found out that toxicity may arise not directly from the nanosized particles acting on bacteria but rather from metal ions known to be toxic for the bacteria and coming from the chemical and biological dissolution of metal oxides and sulphates in the environment *(6)*. So, it is need to establish what are the mechanism underlying the real source of toxicity is before jumping into a conclusion. It is therefore highly recommendable that the toxicological effect of nanomaterials be clarified before their commercial or practical applications or, in the other hand, to halt or modify their toxicity.

The concern on how some engineered or natural nanomaterials (NMs) may become hazardous pollutants posing a serious threat to public and environmental health is alive and growing, as careful studies to understand and modelling their complex interrelations with life systems moves slower than the rate how they are being introduced in new consumer products. Toxicological studies related to NMs started two decades ago, but most of the published papers and reports are still limited to *in vitro* studies or laboratory animal models

*in vivo* analyses, mainly concerned with human health impact. The first reports on environmental impact of nanomaterials are more recent *(7-9)*, although the terms "eco-toxicity" or "eco-nanotoxicity" were not yet used.

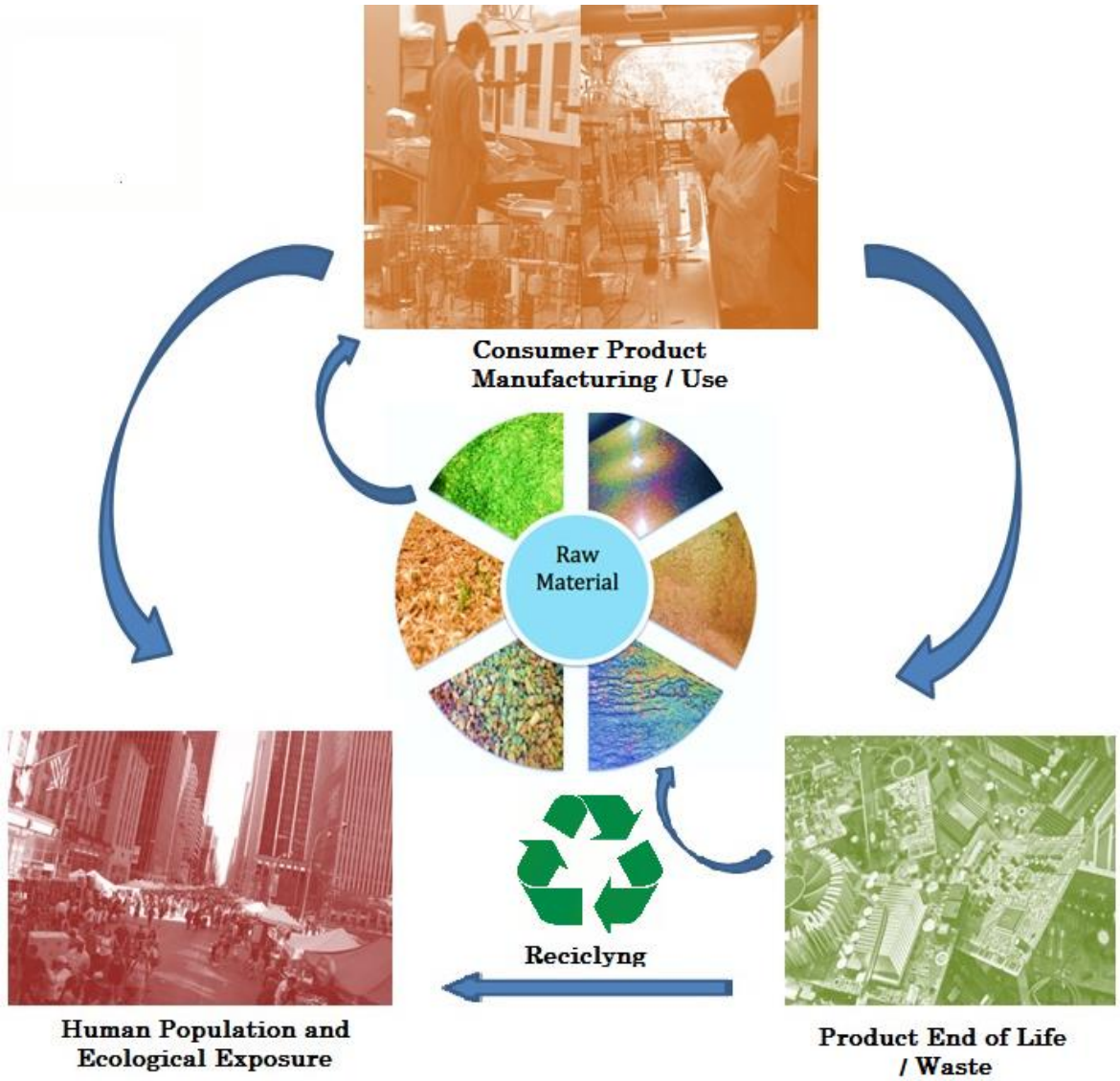



*Figure 3. Life cycle of a consumer product containing nanomaterials.*

According with Kahru and Duborguier, nanostructured $TiO_2$, ZnO, CuO, Ag, single wall carbon nanotubes (SWCNTs), muti-walled carbon nanotubes (MWCNT)s and fullerenes, $C_{60}$, are among the nanomaterials with more chances for environmental and health impact, due to their high volumes of production or extended use in consumer products *(1).* For example, the high scale manufacturing of SWCNTs was estimated to reach 1500 tons per year in 2011, while the total production of nanostructured metal oxide for cosmetic use ranged 1000 tons per year from 2005 to 2010 *(10, 11)*. The list may be extended to some

other commercially important materials such as nano Au, nano zero valent iron (Fe), quantum dots (CdS, CdSe, CdTe, ZnSe), nano iron oxides ($Fe_2O_3$, $Fe_3O_4$), nano $CeO_2$, nano $SiO_2$, graphene, as well as a huge list of nanocomposites. All the later materials are included in a list of representative manufactured NMs published by the Working Party on Manufactured Nanomaterials (WPMN), a committee formed in 2006 by the Organization for Economic Co-operation and Development (OECD) to address the safety challenges of NMs.

By 2010 in between 880 to 1000 different consumer products containing engineered nanomaterials (ENM) were identified in the market, a number that has been steadily increasing every next year *(12, 13).* Just in the span of 3 years (2007 to 2010), the number six-folded, being the largest increment for personal care and coating products, including cosmetics, textiles and anti-wetting products. If we add to that number that of those nanomaterials coming from natural, then exposition to nanostructured materials is an actual, real and complex problem that needs to be carefully analyzed to understand the potential risks and to determine the right protection measurements needed to be implemented for our own safety.

*Figure 4. Consumer products already in the market containing nanomaterials.*

In order to determine if a nanomaterial is (or not) toxic for the environment or human health, it will need a very rigorous characterization, to know their precise physical and chemical characteristics and to understand their relationships with their biological action. Some relevant properties of NMs to be considered in order to assess their potential toxicity are: how they react (chemically), the sorption of chemicals on their surfaces or their own sorption into a biological surface, the size/shape relationship, if they are soluble or not in some specific solvent, pH range or physical state, if they are susceptible to form aggregates or to agglomerate, and finally, if a coating is or not present, among some others. Among the relevant effects to monitor are: generation of reactive oxygen species (ROS), if they are able to act as carriers of toxic substances, their changes in oxidation state, their bioaccumulation, the molecular interactions that they are able to generate and other indirect effects. Solubility is important, as it affects the bioavailability of a material. Further transformation of a

nanomaterial prior to and after interacting with a biological system has to be considered, because an innocuous material may become toxic and vice versa *(14).*

### *The biosystem – nanomaterial interaction*

The chemical and physical properties of bulk materials can vary greatly with respect to their nanostructured forms. They may become toxic and harmful, in contrast of being inert in their macroscopic form. The potential toxicity of NMs has been recognized by several authors *(3, 10, 15-22).* A better understanding of the risks associated with specific NMs may reduce environmental damage or adverse health effects to the living beings in an ecosystem *(23, 24).* Interpretation of toxicity may be complex issue, as sometimes the synthetic methodology may affect the results as the processing of the material may incorporate additives, surfactants and solvents that are not completely removed from the final products, especially if their physical, chemical and biological interactions are not known in detail. For example, $C_{60}$ was initially considered to be toxic, but later studies indicated that such toxicity was related to residual tetrahydrofuran (THF) used in the processing of the material *(25).* Then, biological activity may depend on other components present in the chemical formulation of the material. Commercial sources of NMs do not often provide information regarding the synthesis or the use of stabilizing/capping agents, so a careful characterization *a priori* is highly recommended.

Interactions between nanostructured materials and biological systems may occur in several ways, being simple but or very complex. As the scale of biological relevant objects such as membrane structures, biomolecules (enzymes, proteins, DNA, RNA, antibodies), in virus, bacteria or eukaryotic cells is comparable to several kinds of NMs, then exist different unknown potential levels of complex interactions. Toxicity is a complex event *in vivo* and currently it is difficult to monitor systemic and physiological effects *in vitro*, so most assays determine effects at the cellular level. Most assays oversimplify the events they measure and are selected due to they are cheap, easily to quantify and reproducible.

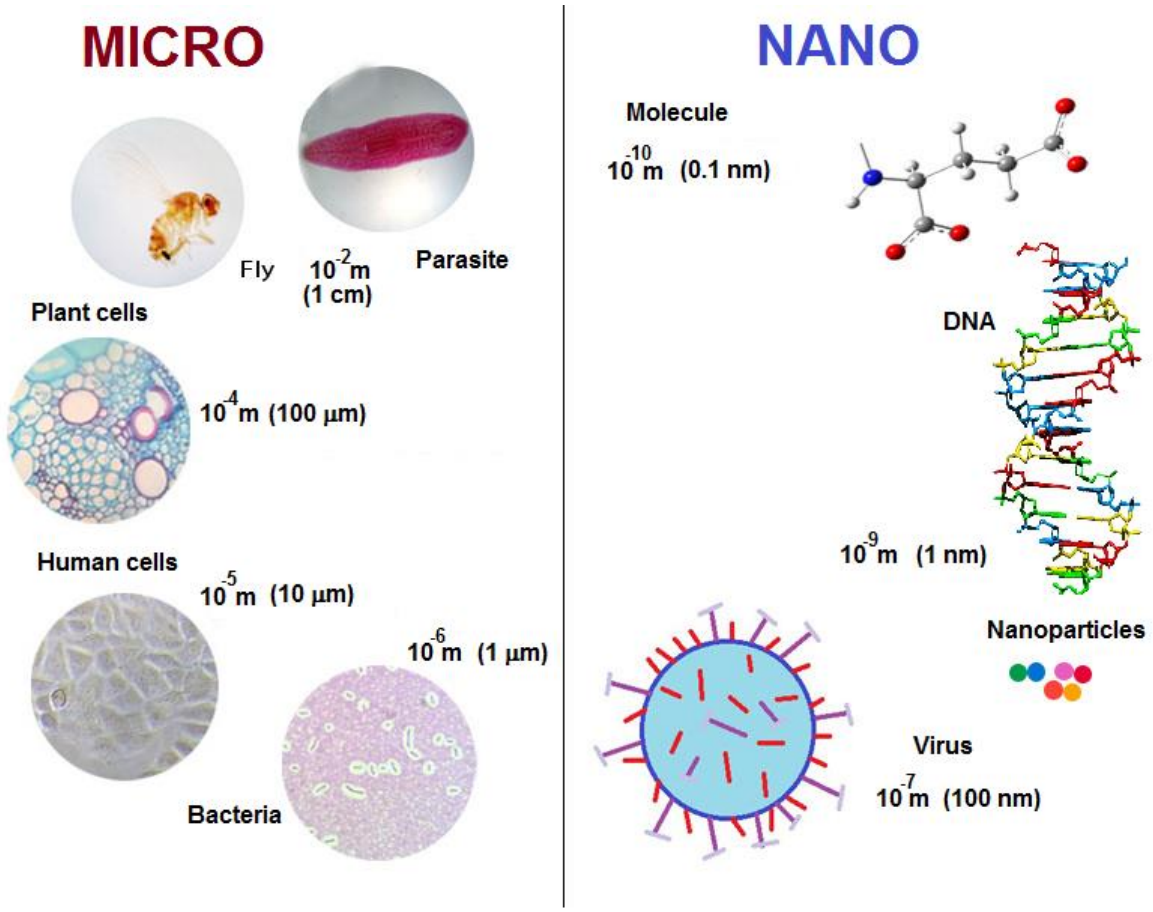



*Figure 5. Comparative size scales of nanomaterials and biological systems*

Due to their reduced size, nanomaterials may pass through several important biological barriers. An average cell membrane is able to avoid internalization of nanoparticles larger than 6 nm, although by endocytosis, materials in the range up to 100 nm may get into the intracellular space. The nuclear membrane can stop particles smaller than 40 nm. The brain blood barrier (BBB) filters particles up to 35 nm, while the alveolar-capillary barrier up to 10 to 24 nm. In the kidney, the renal systems is able to retain particles in the range of 8-12 nm, while the skin has a dermal barrier efficient in the range from 20 to 30 nm. The gastric mucosae is not very selective, allowing particles less than 500 nm in size to move across *(64)*.

Although apparently we may have a good knowledge of a nanomaterial chemical and physical characteristics, there is a lack of understanding on the intracellular activity and impact of engineered NMs on cell function. They may interact with a single cell in different ways than a tissue or whole organism, determining that not simple *in vivo* models may be suitable for complete interpretation. They also may coat their surfaces, i.e. proteins, antibodies, small biomolecules, depending on the type of biological fluid which they are in contact (blood, plasma, interstitial fluid), avoiding the immune system. They may even affect intracellular responses, inducing damage or beneficial responses. Nanostructured metal

oxides, for example, may generate reactive oxygen species such as singlet oxygen, superoxide and peroxide, as well as participate in oxidation-reduction processes on the cell surface, which may degrade cell membranes, proteins or even DNA. Interaction with biomolecules may also induce changes in their functional structures or block the active sites of enzymes, which in turn will have not always good metabolic consequences *(3).*

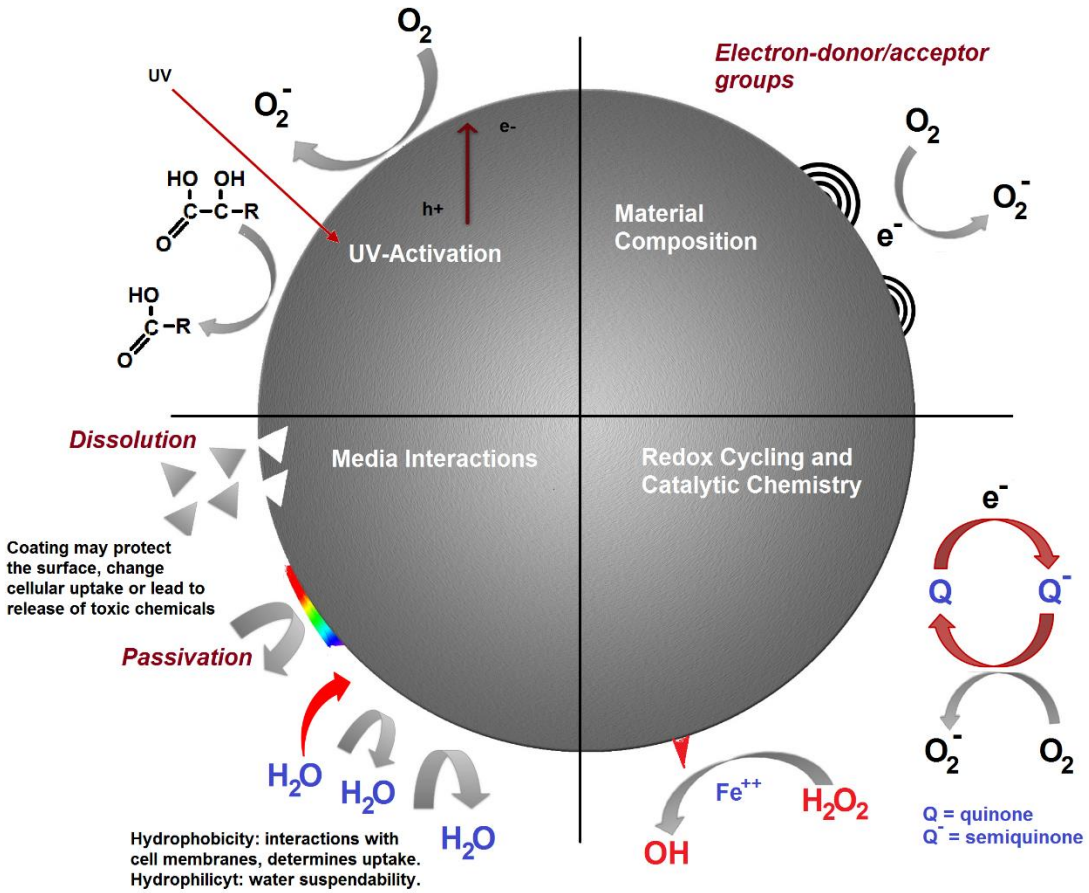



*Figure 6. Possible mechanisms by which nanoparticles interact with biological tissue (adapted from reference 3).*

In eco-nanotoxicology, it is important to understand how nanomaterials can interact with a living organism since the first moment it is exposed to them until their degradation or elimination occur, as well as whether these materials (or their by-products) are bioacummulated within cells, tissues or organs, inducing by this way intracellular changes, inflammatory responses or undesirable effects culminating with metabolic illness. Because of nanotoxicology is a new research interest topic, there are many contributions as attempts to standardize the evaluation of nanomaterials toxicity, considering that the interaction of

these materials with dying agents, DNA and cellular structures could cause some variability with the data interpretation and must be validated carefully *(26, 27).*

Ecotoxicity tests are tools used within environmental hazard assessment frameworks to answer questions about the intrinsic dangers of chemical substances which may be released into the environment *(28).* These tools can be applied to NMs and when it are evaluated the exposure scenarios should be replicate using *in vitro* and *in vivo* toxicity assays to know the potential health risk. One problem with *in vitro* assays are the results obtained in this study cannot guarantee biocompatibility *in vivo*, and therefore data from *in vitro* studies may be misleading and will require verification through animal evaluations *(29).*

In order to understand the impact of a nanomaterial into the environment and living systems, several specific methods have been developed. They can be grouped into four categories: a) chemical and physical characterization; b) microbiological assays; c) *in vitro* assays; d) *in vivo* assays.

a) Chemical and physical characterization

Very sophisticated and specialized analytical instrumentation has been developed to obtain some of the fundamental physical information about the nanomaterial we desire to study and is already available in major facilities around the globe *(30).* From several well established techniques such as scanning or transmission electron microscopy (SEM, TEM) able to obtain precise information about the size, morphology and chemical composition (when EDX detectors are available) of the nanomaterial. From Dynamic Light Scattering (DLS) instrumentation it is possible to determine the hydrodynamic radii of the nanoparticles when dispersed in a liquid, and it is possible to study the influence of pH on the surficial charge (Zeta potential), the nature of the solvent, temperature, the effect of capping agents and detergents in the stabilization of nanoparticles, stability against time, pH, temperature and to understand the kinetic of aggregation in solution. Specific area of powdered materials can be obtained by using BET analyzers, thermal stability and transformation may be determined by Thermal Gravimetric Analyzers (TGA) and for chemical composition and presence of contaminants by Atomic Absorption Spectrophotometry (AAS) or Inductively Couple Plasma Mass Spectrometry (ICP-MS). Other spectroscopies such as Ultraviolet-Visible, Infrared or Raman may be also useful to define the existence of organic or inorganic coatings, chemical modifications in the surface and chemical identity, among other characteristics.

*Figure 7. Analytical instrumentation used for nanomaterial's physical and chemical characterization (clockwise, left to right): Transmission electron microscope, scanning electron microscope, atomic absorption spectrophotometer, UV-Visible spectrometer, FTIR spectrophotometer, dynamic light scattering.*

Of course, once the chemical and physical analyses show some relevant data, *in vitro* and *in vivo* studies may give us complementary information. Bio-tests using bacterial, cell or tissue cultures, animal models (mice, rats, rabbits, dogs, fishes…), eco-toxicity models (*M. salmoides*, *C. elegans*, *D. magna*, *C. dubia*, common fly (*Drosophila melanogaster*) and some invertebrates and small vertebrates) are currently among the most used in research laboratories around the world.

b) Microbiological assays

Different approaches can be used to assess bacterial toxicity using well-characterized materials and standard bacterial assay systems. It is possible to examine the effects of nanoparticle concentration, particle size, exposure time, growth medium, and pH on the growth and viability of bacterial cells like *E. coli*, *Bacillus subtilis* or *Shewanella oneidensis*. Among other methods to assess a nanomaterial bacterial toxicity we can mention:

- *Disk diffusion tests*. Bacterial sensitivity to different-sized nanomaterials is tested by disk diffusion tests as described by Ruparelia *(31).* Small filter paper disks of uniform size (i.e., 6 mm diameter) are placed separately in each of the different nanoparticle suspensions for 5 min; then the disks are carefully removed using sterile forceps. After the bacterial suspension (100 $\mu$l of $10^4$ to $10^5$ CFU $ml^{-1}$) is uniformly plated on LB agar plates or other rich

media, a disk containing nanoparticles is placed at the center of each plate and the plate is incubated at 37°C for 18 h. The average diameter of the inhibition zone (DIZ) surrounding the disks is measured to determine inhibition. This simple method gives us an idea if some NMs have any activity; however, sometimes it is need to use minimum media in place of rich media (i.e., LB or Müeller-Hinton agar) to see if any effect is present.

- *Determination of MIC*. The MIC, defined as the lowest concentration of a compound that inhibits the growth of an organism *(32).* The MIC test can be determined for *E. coli* in LB medium at pH 7.2 and/or in M9 minimal medium at pH 6.4 (33), 7.2, and 7.8. For *B. subtilis* can be tested in LB and minimal media at pH 7.2 *(34)*, and for *S. oneidensis* is tested in LB and HBA minimal media at pH 7.2 *(35)*. Reactions are carried out in test tubes containing 5 ml of the logarithmic-phase (~0.098) bacterial cultures and different-sized nanoparticles at various concentrations (i.e. 50, 100, and 150 mg/liter). Tubes with sterile media containing no nanoparticles or nanoparticles only served as controls. Samples are incubated on a shaker (200 rpm) at 37°C (*E. coli* and *B. subtilis*) or 30°C (*S. oneidensis*), with growth monitored by obtaining measurements of the optical density at 600 nm (OD600) every 30 min for 8 h. At the end, the last tube with no-growth corresponds to the MIC of that compound.

- *CFU measurements*. Studies of *E. coli* and *B. subtilis* viability are performed in liquid cultures at a nanoparticle concentration of 100 mg/liter (or the proper concentration according the nanomaterial). Aliquots are taken at 0, 1, 5, and 24 h and serially diluted in the appropriate minimal medium, and the dilutions are seeded on LB agar plates. After overnight incubation at 37°C, the numbers of CFU are counted manually.

- *Live/dead viability assays*. *E. coli* and *B. subtilis* cultures grown to logarithmic phase in M9 medium and *B. subtilis* minimal medium, respectively, are treated with different concentrations (i.e. 50, 100, and 150 mg/liter) of nanoparticles. Following exposure, the impact on bacterial membrane integrity is assessed using a live/dead BacLight bacterial viability kit. To quantify the relative numbers of live and dead cells, the relative fluorescence intensities are measured using a fluorescence plate reader (excitation at 485 nm, emission at 525 and 625 nm).

- *Monitoring superoxide production*. Superoxide production upon exposure of bacterial suspensions to various concentrations of nanoparticles are monitored by following the absorbance at 470 nm due to the reduction of 100 μM 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) to XTT-formazan by superoxide ($O_2^-$) *(36, 37).*

- *Microarray hybridization and analysis*. For microarray experiments, an overnight *E. coli* culture is used to inoculate 250-ml flasks containing 100 ml of prewarmed M9 medium to an OD600 of ~0.1 and incubated at 37°C with shaking at 200 rpm until mid-log phase (OD600, ~0.5). Cultures are treated with either prewarmed nanoparticles (100 mg/liter), or Milli-Q water. After 1 h, cells are harvested by rapid centrifugation (5,000 X *g*, 2 min at 4°C) and snap-freezing in liquid $N_2$. Three separate controls and three experimental cultures are examined for each condition. Total cellular RNA is isolated as described by Brown and Pelletier, the cells are first resuspended in TE (Tris 10 mM-EDTA 1 mM, pH 7.6) buffer and

incubated with 1 mg/ml of lysozyme to lyse the cells *(38, 39).* Purified, fluorescently labeled cDNA is hybridized to *E. coli* K-12 gene expression 4-by-72 K arrays (or other microarrays) using a Nimblegen hybridization system. Microarrays are washed according to the array manufacturer's procedure. Briefly, microarray mixers are removed in 42°C Nimblegen wash buffer I and then washed manually in room temperature buffers: wash buffer I for 2 min, wash buffer II for 1 min, and wash buffer III for 15 s. Microarrays are dried for 80 s using a Maui wash system and then scanned and the images quantified. Microarray data are normalized using the Lowess normalization algorithm, and an analysis of variance (ANOVA) is performed to determine significant differences in gene expression levels between conditions and time points using the false discovery rate testing method ($P < 0.01$).

*Cyanobacteria and green algae models.* These microorganisms have been also used to determine the toxicity of a nanomaterial due to their ecological position at the base of the aquatic food chain and their essential role in nutrient cycling and oxygen production. Cyanobacteria constitute a phylum of bacteria that obtain their energy through plant-like photosynthesis. They are the most widespread primary producers in the marine food chain and are crucial in many other habitats including freshwater bodies, saline lakes, and biological soil crust.

For example, the toxicity of nano-$CeO_2$ suspension was determined by monitoring the growth inhibition of the green alga *'Pseudokirchneriella subcapitata* and by determining the constitutive luminescence inhibition of the recombinant bioluminescent cyanobacterium *Anabaena* CPB4337. The bioassays using the bioluminescent cyanobacterium Anabaena CPB4337 are based on the inhibition of constitutive luminescence caused by the presence of toxics *(40).*

*c) In vitro assays*

In order to evaluate the biological activity and/or toxicity of NMs there have been explored some alternatives to determine the effect of a particle upon a living organism. Conventional *in vitro* analyses and cell-based assays were performed to obtain an estimate that could mimic the *in vivo* physiologic environment of a living being, and at this way to determine their possible biological risk in case that the material could be toxic. To determine the metabolic state of a group of cells we must hold a concept known as cell viability, which indicates the potential of this group of cells to proliferate and grow. A normal cell population must be metabolically active in culture, which must indicate that all their functions are normal. In toxicology, there are many ways to determine cell viability, from simple dye exclusions to the use of sophisticated instruments. In nanotoxicology there have been explored the same techniques that have been used in toxicology to evaluate the effect of a nanomaterial when a cell population is exposed, nevertheless these studies have not resulted in the creation of standards that could be useful to most of the new NMs released to the environment *(27).* Besides, *in vitro* analyses are very popular because of their established methodologies, low costs, a broad number of replicates, small set-ups, their

safety and efficacy and few ethical issues. The important advantage about *in vitro* testing of nanomaterials is that it is a solution in the replacement or reduction of the use of laboratory animals, reducing at the same time the uncertainty caused due the variability between individuals *(41).* As with other man-manufactured materials such as cosmetics and drugs, *in vitro* evaluation of nanomaterials need to be performed, due the increase of nanostructured materials and nanoparticles that are released to the environment. Table 1 summarizes the most popular *in vitro* analyses employed in nanotoxicology, some of them validated by the Organization of Economic Co-Operation and Development (OECD), the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), and the National Institutes of Health via the Nanotechnology Characterization Laboratory (NCL-NIH). Many of them include common dye exclusions, indirect determinations of metabolic disruptions, microscopy analyses and cell viability determinations by impedance.

At this way, cytotoxicity tests can be used to predict acute toxicity of nanomaterials, and 2D and 3D are used to address specific localization of them. It is important to mention that in all colorimetric assays it is measured an specific damage and not necessarily total death, besides the cell number is very important because if there is any inhibition of growth during the experiment that is not caused directly by the application of nanoparticles this decrease in cell growth could be estimated as a false positive. Besides all colorimetric assays have demonstrated to be liable to the interaction between dye agents and nanomaterials, and it is very difficult to wash out the remaining dye *(27, 42).* At this moment, 2D and 3D systems such as light and electron microscopy, and real time analyses by impedance or platting efficiency assays appear to be the most reliable systems due the elimination of additional chemical treatments and the constant monitoring of the culture *(42-46).*

*d) In vivo assays*

Living systems are potentially exposed to NMs through ingestion, ocular, dermal or inhalation pathways. This exposition can occur when environmental pollutans are presents in air, water or soil. We do not know the effects of many of these materials on our health and if the *in vitro* assays are not satisfactory to demonstrate the potential effects, then testing in an *in vivo* model is need. In order for the selection of an appropriate and representative *in vivo* model is important to considerate the NMs exposition route. For example for oral route, although several mammalian test species may be used, the rat is the preferred species. In the case of dermal exposition the most common animal model include rat, rabbit, or guinea pig, but the albino rabbit is preferred because of its size, ease of handling, skin permeability, and extensive data base. Commonly used laboratory strains must be employed. If a species other than rats, rabbits, or guinea pigs is used, the tester must provide justification and reasoning for its selection *(47).*
Using a variety of techniques, typical *in vivo* assessments include the determination of physiological localization and the concentration of material in specific tissues, rate of excretion, macroscopic tissue analysis and organism toxicity *(48).*

ñ

Table 1. Summary of common toxicity assays for different nanomaterials

| Assay | Purposes | Applications in nanotoxicology | Cell line | References |
|---|---|---|---|---|
| Neutral red uptake | Cell viability<br>Cell death<br>Phototoxicity | Carbon nanotubes<br>$C_{60}$<br>$Fe_3O_4$ | A549 pulmonary cell line.<br>THP-I pulmonary cell line<br>Human monocyte macrophages | OECD Draft Guidance 129 *(42)*<br>ECVAM validated *(42)*<br>*(49)* |
| Trypan Blue | Proliferative ability<br>Cell viability<br>Cell growth | Colloidal Ag<br>$MoO_3$<br>$Fe_3O_4$<br>$TiO_2$<br>Carbon based nanoparticles<br>Gold nanoparticles | Bronchial epithelial cell<br>BEAS-2B.<br>Rat liver cells BRL 3A<br>Human epidermal cells A431. | *(50)*<br>*(51)*<br>*(52)*<br>*(53)* |
| Fluorescein diacetate derivatives | Reactive oxygen species. | Copper oxide<br>$TiO_2$ | Human epithelial cells HEp-2<br>SD Primary hepatocytes | *(54)*<br>*(43)* |
| Lactate dehydrogenase assay | Disruptions in cell membrane. | $Fe_3O_4$<br>Metallic Cu<br>CuO<br>$TiO_2$<br>Metallic Ag<br>Titania stoichiometric<br>Metallic cobalt<br>Copper-Zinc mixed oxide variants<br>Nickel<br>Nickel oxide<br>Zirconia<br>Alumina<br>Tungsten carbide<br>CdO<br>$MoO_3$<br>$MnO_2$ | Hepatocarcinoma cells Hep-G2<br>Kidney cells LLC-PK1<br>Rat liver cells BRL 3A | *(55)*<br>*(56)*<br>*(51)* |
| Tetrazolium salts (MTT, MTS) | Letal dose 50%<br>Mitochondrial activity. | $Fe_3O_4$<br>Metallic Cu<br>CuO<br>$TiO_2$<br>Metallic Ag<br>Titania stoichiometric<br>Metallic cobalt<br>Copper-Zinc mixed oxide variants<br>Nickel<br>Nickel oxide<br>Zirconia<br>Alumina<br>Tungsten carbide<br>CdO<br>$MoO_3$<br>$MnO_2$ | Hepatocarcinoma cells Hep-G2<br>A549 pulmonary cell line.<br>THP-I pulmonary cell line<br>Kidney cells LLC-PK1<br>Rat liver cells BRL 3A | *(57)*<br>*(55)*<br>*(56)* |
| Glutathione reduced assay | Cell signaling activity | CdO<br>$TiO_2$<br>$MoO_3$<br>Metallic Al<br>Colloidal Ag | Rat liver cells BRL 3A<br>Human epidermal cells A431. | *(51)*<br>*(52)*<br>*(58)* |
| Caspase activation kits | Cell apoptosis | $Fe_3O_4$<br>$TiO_2$ | Human fibroblasts hTERT-BJ1<br>Kidney cells LLC-PK1<br>Hepatocarcinoma cells Hep-G2 | *(59)*<br>*(60)*<br>*(61)*<br>*(62)* |
| RT-CES System | Cytotoxicity | $TiO_2$<br>Gold nanoparticles | NK 92 Cells<br>Breast cancer cells MDA-231-B and MCF-7. | *(63)*<br>*(45)* |

| | | | | |
|---|---|---|---|---|
| | | | Human alveolar epithelial cells L-132.<br>Human glioblastoma cells T98G.<br>Human primary fibroblast cells AGO-1522B | |
| Electron microscopy | Nanoparticle shape and intracellular localization | CNT<br>$TiO_2$<br>CeO<br>$Fe_3O_4$ | Human epidermal keratinocytes (HEK).<br>Neural stem cells<br>Bronchial epithelial cells. | *(64)*<br>*(65)*<br>*(66)*<br>*(59)*<br>*(67)* |

*OECD and EVCAM guidelines are summarized in reference *(42).*

Short-term (''acute'') tests are generally used first, with observations of organism survival the most common measurement of effect. Longer-term (''chronic'') tests (with observation of sublethal effects on organism growth or reproduction being the most common measurements of effect) are then used when results from short-term tests combined with large safety factors suggest that there may be risks to the environment *(28).*

Generally, the initial step for the assessment and evaluation of the toxic characteristics of a substance is to determinate the acute oral toxicity. It provides information on health hazards likely to arise from short-term exposure by the oral route *(47).* The term acute toxicity is used to describe the adverse effects of a substance that may result from a single dose of a substance or multiple doses given within a 24-hour period. The studies are carried out via oral, dermal, and inhalation routes of exposure for the purpose of estimating doses that cause lethality. Acute effects may be local and/or systemic *(47, 68).*

The acute toxicity test are made to obtain information on the biologic activity of a chemical and its mechanism of action at different levels including the cell components. The information permits us to obtain information for identification and risk management in the related context of production, handling, and use of chemical *(69).* The $LD_{50}$ (median lethal dose) value, is currently the basis for toxicologic classification of chemicals and is defined as the statistically derived dose that, when administered in an acute toxicity test, is expected to cause death in 50% of the treated animals in a given period. The $LD_{50}$ value is expressed in terms of weight of test substance per unit weight of test animal (milligrams per kilogram). In the last years, the acute systemic toxicity studies are among the most criticized of all toxicology tests on both scientific and ethical grounds. New preferences now are trying to employ like dose selection lethality limits instead of $LD_{50}$'s, applying the 3Rs principle (refinement, reduction, and replacement of animal use) *(47, 68).*

In case the need to use animal, should be reviewed and approved by the institutional animal ethics committee. The National Toxicology Program (NTP) regularly evaluates substances for a variety of health related effects. Rodent is the most common animal model used by NTP *(70).* The studies for general toxicology using rodents include single dose acute studies, repeat dose studies 2, 4, 13, 26 or 52 week´s duration, carcinogenic studies with or without genetically modified animals models, sensitization and irritation studies.

Generally, the testing laboratories adhere to the principles enunciated in the "Guide for the Care and Use of Laboratory Animals" *(71).* The regular NTP in vivo procedures are:

*Perinatal exposure*: The range finding study shall determine whether there is maternal toxicity and/or toxicity to the pups in order to provide a basis for determining the doses for the subsequent toxicity study (13-week or 2-year study). The animals shall be exposed to the substance during in utero development, through their mother's milk, and via dosed feed, dosed water, or gavage administration.

*14 Day Toxicity Protocol*: The goal of this is to provide a basis for identifying potential target organs and toxicities and to assist in setting doses for the 13-week exposure study. After a 10- to 14-day quarantine period, animals are assigned at random to treatment groups. The study includes five treatment groups each administered a different concentration of test article per sex per species plus a control group. Each group per sex per species contains five animals. The animals receive the test article through a designated route of exposure and the control animals receive vehicle alone.

*13-Week Toxicity Study:* In addition to obtaining toxicological data, the purpose of this study is to determine the treatments for each strain and species to be used in the 2-year toxicology/carcinogenesis study. Basically, after a 10- to 14-day quarantine period, animals are assigned at random to treatment groups. The study includes five treatment groups each administered a different concentration of the test problem material plus a control group. Each group contains 10 animals per sex per species. The animals receive the subject chemical by a designated route of exposure. Controls receive untreated water or feed or vehicle alone in gavage and dermal studies. For dosed-feed and dosed-water studies, animals are exposed for 90 days after which they are sacrificed with no recovery period. For inhalation, gavage and dermal studies animals are exposed five times per week, weekdays only until the day prior to necropsy.

*2-Year Study Protocol:* The purpose of this study is to determine the toxicological and/or carcinogenic effects of long-term exposure on rats and mice. Typically, after a 10- to 14-day quarantine period, animals are assigned at random to treatment groups. Rats and mice receive the test agent for 104 weeks via a defined route of exposure at 3 treatment concentrations plus controls. For inhalation, gavage and dermal studies, animals are treated five times per week, weekdays only *(70).*

*In vivo* assays, the evaluation includes to identify the treatment related lesions in target organs. In mammals the organ weights of at least liver, thymus, right kidney, right testis, heart, and lung are recorded from all animals surviving until the end of the study. A complete necropsy is performed on all treated and control animals that either die or are sacrificed. All tissues required for complete histopathology are prepared and stained with hematoxylin and eosin for histopathology evaluation.

In the NTP *in vivo* procedures all the studies animals are weighed individually on day one on test, after seven days, and at weekly periods thereafter. Animals are observed twice daily, at least six hours apart, including holidays and weekends, for morbidity and death. Animals found moribund or showing clinical signs of pain or distress are humanely euthanized.

Formal clinical observations are performed and recorded weekly. For dosed-feed or dosed-water studies, food consumption/water consumption is measured and recorded weekly. In 2-year study procedures, individual animal body weights for test and control group animals are recorded on day one on test and at 4-week intervals thereafter except for dosed-feed and dosed-water studies, which are recorded weekly for the first thirteen weeks and monthly thereafter *(70).*

Additionally, specific toxicological parameters can be evaluated and processed for hematology and clinical chemistry determinations. Blood is collected from core study mice at the end of the study for hematology determinations (Table 2). Another studies such micronuclei determinations in blood cells, genotoxicity, the sperm morphology and vaginal cytology evaluations are used too.

Table 2. Blood Clinical measurements

| **Hematology** | **Clinical chemistry** |
|---|---|
| Red blood cell count (RBC) | Sorbitol dehydrogenase (SDH) |
| Mean corpuscular volume (MCV) | Alkaline Phosphatase (ALP) |
| Hemoglobin (Hb) | Creatine Kinase (CK) |
| Hematocrit | Creatinine |
| Mean corpuscular hemoglobin (MHC) | Total Protein |
| Mean corpuscular hemoglobin concentration (MCHC) | Albumin |
| Erythrocyte morphologic assessment | Urea Nitrogen (BUN) |
| Leukocyte count (WBC) | Total Bile Acids |
| Leukocyte differential | Alanine Aminotransferase (ALT) |
| Reticulocyte count | Glucose |
| Platelet count and morphologic assessment | Cholesterol |
| | Triglycerides |

Another type of studies includes the immunotoxicology probes. Assessment of the adverse effects on the immune system is an important component for evaluating the overall health and safety of NMs. The immune system is constantly functioning to maintain homeostasis eliminating pathogens and removing cancerous cells. Small modifications to the immune system, which may occur following NMs exposure, could lead to impaired protection or an inappropriate immune response resulting in autoimmunity and damage to the host *(72).* The most common effects include an increases susceptibility to infections or cancer, autoimmune diseases, chronic inflammation or allergies. There are large spectrums of *in vitro* and *in vivo* immunological assays in comprehensive immunotoxicity studies. These include assays of immunochemistry (quantification of cytokines), immunogenicity (antibodies), immunopathology (relative weight and histopathology of lymphatic organs), immunophenotyping (analysis of cells origen), functional test (analysis of macrophages and granulocytes functions), hypersensivity testing, infections models (bacterials, viral, fungals models), and asthma models.ñ

For example, Lee evaluated the immunotoxicity of silica nanoparticles *in vivo (29).* This nanoparticles have been used in chemical mechanical polishing, varnishes, cosmetics, food, and biomedical devices. Although silica is generally considered to be non-cytotoxic, designing silica as NMs may change its biocompatibility because of changes in its physicochemical properties. In the *in vivo* assay the animals received silica NPs suspended

in distilled water for 4 weeks (5 days/week). The results indicate that *in vivo* exposure to silica nanoparticles caused damage to systemic immunity through the dysregulation of the spleen, but the *in vivo* data were inconsistent with those for *in vitro* data, which show lower cytotoxicity for silica nanoparticles. This is an example of the importance of verifying biocompatibility both *in vitro* and *in vivo* during the design of new NMs and therefore data from in vitro studies require verification through animal evaluations *(29).*

By other way, in humans, the most critical exposure route for NMs are inhalation and skin contact, although the adverse effects are mainly expected occur in the lungs *(73). In vivo* there are combinations of particle delivery techniques such as intratracheal instillation/ aspiration/ inhalation or nose-only/whole body inhalation as a means to study the pulmonary and systemic effects of nanoparticles. The evaluation of respiratory tract toxicity from airborne materials frequently involves exposure of animals via inhalation. This provides a natural route of entry into the host and, as such, is the preferred method for the introduction of toxicants into the lungs. However, for various reasons, this technique cannot always be used, and the direct instillation of a test material into the lungs via the trachea has been employed in many studies as an alternative exposure procedure.

For example, Horie and others (2012) evaluated the pulmonary toxicity of multi-wall carbon nanotubes (MWCNT) by intratracheal instillation in rat. The MWCNT dispersion was administered to rat lung by single intratracheal instillation at doses of 0.2 mg and 0.6 mg/rat. Bronchoalveolar lavage fluid (BALF) was collected at 3 days, 1 week, 1 month, 3 months,and 6 months after instillation. They found that the intratracheal instillation of MWCNT induced persistent inflammation in rat lung not only the high dose group but also in the low dose group *(74).*

More recently the efforts are focused on the development and validation of new alternative test systems (sensitive, specific, rapid) for toxicological research that will reduce, replace, or refine animal use. Model systems under development include non-mammalian species, transgenic species, genetically engineered *in vitro* cell systems, microchip array technology, and computer-based predictive toxicology models *(70).* Fish and amphibian embryo models are gaining increasing popularity in the area of toxicology, both in research and potential regulatory application. The fish and amphibian embryo models provide an ethically acceptable small scale analysis system with the complexity of a complete organism.

The *Organization for Economic Co-operation and Development* (OECD) is an intergovernmental organization, in which representatives of 34 industrialized countries in North and South America, Europe and the Asia and Pacific region, as well as the European Commission, meet to co-ordinate and harmonizes policies, discuss issues of mutual concern, and work together to respond to international problems such nanomaterials ecotoxicity. The OECD's *Working Party on Manufactured Nanomaterials* (WPMN) was established in 2006 to promote international co-operation in human health and environmental safety aspects of manufactured nanomaterials. The OECD program has focused in generating appropriate methods and strategies to ensure potential safety issues, through:

- Establishing an OECD database on manufactured nanomaterials to inform and analyze research activities and strategies on environmental, human health and safety issues;
- Testing specific nanomaterials for their human health and safety evaluation, while ensuring appropriate testing methods (*in vivo* & *in vitro*).

Some standard of OECD ecotoxicity *in vivo* tests include water flea acute (*Daphnia magna*) where the dosing method is the natural water by 48 h and the test end point is the half maximal effective concentration ($EC_{50}$) *(75). D. magna* is an organism widely used as an indicator in aquatic environmental risk assessment because *Daphnia* filter large volumes of water and water-suspended particles. It also plays an important role in freshwater food chains *(76, 77).* These features make *D. magna* a particularly useful test animal for assessing the accumulation of nanomaterials, because their uptake in this organism could result in transfer throughout the food chain. *D. magna* may be grown in artificial freshwater (Ca + Mg hardness 2.5 mM, pH 6.5 to 7.1) with a photoperiod of 16:8 light:dark at 20 ± 2°C. The population is fed three times a week with a green algae culture of *Scenedusmus sp* (dominant species), *Monoraphidium contortum* and *Selenastrum capricornutum*. Organisms used in tests must be 5 to 7 days old at the beginning of the experiments.

For fish acute (*Zebrafish*) the test medium is natural water by 96 h and the test end point is the 50% of maximum lethal concentration ($LC_{50}$). In the case for fish prolonged toxicity (*Zebrafish*) the study is monitored during 14 days following $EC/LC_{50}$ until the test end point. There are *in vivo* assays using birds, such bird dietary toxicity, where the doses is applied in the basal diet for 8 days and the test end point is $LC_{50}$. Another *in vivo* assays include others fish species, honey bee, earth worms and plants. These are some examples of *in vivo* assays approved by OECD *(68).*

Aditionally, studies to evaluate how nanomaterials may affect the different development stages of plants are also an easy alternative to assess their potential environmental effects. For example, the germination and growing of seeds of *Lactuca sativa* were tested by Yang and Watts *(78). L. sativa* is one specie which is used and recommended by EPA regularly for measuring pesticide and toxic substances in the environment. The germination average rate is usually 85%, and the seeds have to been stored in dry and dark places at room temperature. Initially, the seed are wet in a bleach solution (10% from commercial product) for 10 min to eliminate biological contaminants. The seeds are then rinsed three times and are set up for germination immediately. In a plastic try (transparent) squares of 2.25 $cm^2$ are drawn to accommodate the seeds in each intersections. The number of seeds will depend on how many substances should be tested and must be by triplicate. The system will include the nanomaterial to test in solution at different concentrations (i.e. 0.1, 0.5, 0.75 mg/mL) in sterile deionized water. All positive and negative controls must be considered.

The seeds are incubated by 168 h using a photoperiod of 12 h (light and dark) and temperature ranging 25°C ± 0.5°C. Observation and counting registration of germination is recorded each 24 h. Also the root size (mm) is registered using a Vernier scale and

comparing the exposed and non-exposed seeds. The elongation root during the exposure is calculated using the next formula

$$ER = L_{treated} – L_{non\text{-}treated}$$

$$RRG = ER_{sample} - ER_{control}$$

where $L_{treated}$ and $L_{no\text{-}treated}$ are the length of roots with or without treatment respectively. The relative root growth (RRG) is calculated according with Schildknecht and de Campos-Vidal, 2002, [cited in *(70)*].

*Conclusions*

Although no large scale spills has been reported and documented to evaluate the real ecological impacts of nanomaterials in the environment, there is a genuine concern by several groups claiming for the implementation of international standard methods and procedures for environmental, health and safety testing, in order to establish solid arguments to confirm or deny the potential and highly polemic hazards of nanomaterials in actual or future use. There are several opportunities to develop new methods for testing experimentally the potential impact of nanomaterials into the environment, in particular, simple, cheap and fast methods which may correlate specific physical properties with biological activity. Due to the lack of definitive information for most of the actual (and future) available nanomaterials, nano-ecotoxicology seems to be a field of opportunities of research for scientists in materials and environmental sciences. Gross tests of cytotoxicity are still required to screen many effects, but there is a growing need to supplement them with more subtle tests of metabolic pathway regulation and signaling and biological models, as the responses will vary with different compounds. It is important to interpret *in vitro* results in terms of the *in vivo* response on the same or similar cells. However, the *in vitro* system lacks regularly of many factors that the *in vivo* system posess, such as blood stream, blood pressure, $O_2$/$CO_2$ pressure and concentration, hormone changes, osmolality, among several others. The nature of the response has to be considered carefully. A toxic response *in vitro* may be result of changes in cell survival metabolism, whereas the major problem *in vivo* may be a tissue response (e.g. inflammatory reaction, fibrosis, organ failure) or a systemic response (e.g. pyrexia, vascular dilatation). For *in vitro* testing become effective, models of these responses must be developed and simulated *in vitro*.

Several environmental groups, non-governmental organizations and academic organizations have been involved in public discussion about the fears surrounding the production, commercial use and disposing of nanomaterials, but we have no conclusive information to definitively answer the central questions around the environmental impacts of ENMs.

In order to avoid a public rejection and misinformation around the topic, it is important to have a continuous and responsible exchange of information between society and scientists, discussing real scientific facts and not only fears feed by sci-fi books, partial interpretation

of facts or pseudo-scientific ideas. In some way, it seems like the most real –and immediately- danger for humanity, involving nanotechnology, came from the misinterpretation of their real benefits and hazards. Some civil, non-governmental groups such as ETC (among others), follow very close the development of new technologies (including genomics, biotechnology and nanotechnology), but sometimes share with their followers very limited and biased documents, exposing polemic points of view related to problems related to such technologies *(79).* A coalition of this organization and other consumer safety and environmental groups (CTA International Center for Technology Assessment, Center for Environmental Health, Food & Water Watch, Friends of the Earth and the Institute for Agriculture and Trade Policy) even filed a lawsuit against the Food and Drug Administration (FDA) over the health and environmental risks of nanotechnology and nanomaterials *(80).* Seems like nanotechnology may become a double edge sword, only in the right or wrong hands. Misconceptions have to be cleared out, to avoid public misinterpretation of their real utility –or dangers *(81).*

**Acknowledgments**

To Xiomara G. Fernandez, Violeta Fernández, Fernando Arteaga, Lizette A. Minjarez, Enrique Gonzalez and Astrid Espinoza (UDLAP) for helping on the design and making of graphic art for this manuscript.

## REFERENCES

1. Kahru, A.; Duborguier, H.–Ch. From ecotoxicology to nanoecotoxicology, *Toxicology*. **2010**, *269*, 105-119.
2. Newman, M. C.; Zhao, Y. Ecotoxicology nomenclature: LC, LD, LOC, LOEC, MAC. In *Encyclopedia of Ecology*; Jorgensen, S. E.; Fath B., Eds.; Elsevier: Amsterdam, Netherlands, 2008; pp 1187-1193.
3. Nel, A.; Xia, T.; Mädler, L.; Li, N. Toxic potential of materials at nanolevel. *Science*. **2006,** *311*, 622-627.
4. Buzea, C.; Pacheco, I. I.; Robbie, K. Nanomaterials and nanoparticles: Sources and toxicity. *Biointerphases.* **2007**, *2*, MR17-MR71.
5. Ge, Y.; Schimel, J. P.; Holden, P. A. Evidence for Negative Effects of $TiO_2$ and ZnO Nanoparticles on Soil Bacterial Communities. *Environ. Sci. Technol.* **2011**, *45*, 1659-1664.
6. Rousk, J.; Ackermann, K.; Curling, S. F.; Jones, D. L. Comparative Toxicity of Nanoparticulate CuO and ZnO to Soil Bacterial Communities. *PLOS ONE*. [Online] **2012**, *7*, e34197. http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0034197 (accessed March 19, 2013).
7. Colvin, V. L. The potential environmental impact of engineered nanomaterials. *Nat. Biotech.* **2003**, *21*, 1166-1170.
8. Oberdöster, E.; Larkin, P.; Rogers, J. Rapid Environmental Impact Screening for Engineered Nanomaterials: A case study using microarray technology. [Online] **2005**. In *Project on Emerging Nanotechnologies. Wilson Center and The Pew Charitable Trust*. http://eprints.internano.org/77/1/Environmental_Impact_Screening.pdf (accesed Mar 13, 2013).

9. Lewinski, N. Nanomaterials: What are the Environmental and Health Impacts? *Chem. Eng. Prog.* **2008**, *104*, 37-40.
10. Klaine, S. J.; Alvarez, P. J. J.; Batley, G. E.; Fernandes, T. F.; Handy, R. D.; Lyon, D. Y.; Mahendra, S.; McLaughlin, M. J.; Lead, J. R. Nano-materials in the environment: behavior, fate, bioavailability, and effects. *Environ. Toxicol. Chem.* **2008**, *27*, 1825–1851.
11. Alvarez, P. J. J.; Colvin, V. L.; Lead, J.; Stone, V. Research Priorities to Advance Eco-Responsible Nanotechnology. *ACS Nano*. **2009**, *3,* 1616- 1619.
12. Wijnhoven, S. W. P.; Dekkers, S.; Kooi, M.; Jongeneel, W. P.; de Jong, W. H. *Nanomaterials in consumer products*, RIVM Report 34370003/2010, [Online] **2011**. National Institute for Public Health and the Environment, Netherlands. http://www.rivm.nl/bibliotheek/rapporten/320029001.pdf (accessed Mar 19, 2013); Wijnhoven, S. W. P.; Dekkers, S.; Hagens, W. I.; de Jong, W. H. *Exposure to nanomaterials in consumer products*, [Online] **2009**. RIVM Letter Report 340370001/2009, National Institute for Public Health and the Environment, Netherlands. http://www.rivm.nl/bibliotheek/rapporten/340370001.pdf (accessed Mar 19, 2013).
13. Project of Emerging Nanotechnologies. *Consumer products inventory*, **2009**. http://www.nanotechproject.org/inventories/consumer/ (accessed Mar 19, 2013).
14. Som, C.; Berges, M.; Chaudhry, Q.; Dusinska, M.; Fernandes, T. F.; Olsen, S. I.; Nowack, B. The importance of live cycle concepts for the developments of safe nanoproducts. *Toxicology*, **2010**, *269*, 160-169.
15. Barnard, A. S. Nanohazards: knowledge is our first defense. *Nat. Mater.* **2006**. *5*, 245–248.
16. Brayner, R.; Ferrari-Iliou, R.; Brivois, N.; Djediat, S.; Benedetti, M. F.; Fievet, F. Toxicological impact studies based on *Escherichia coli* bacteria in ultrafine ZnO nanoparticles colloidal medium. *Nano Lett.* **2006**, *6*, 866–870.
17. Ju-Nam, Y.; and J. R. Lead. Manufactured nanoparticles: an overview of their chemistry, interactions and potential environmental implications. *Sci. Total Environ.* **2008**, *400*, 396–414.
18. Suresh, A. K.; Pelletier, D. A.; Wang, W.; Moon, J.-W.; Gu, B.; Mortensen, N. P.; Allison, D. P.; Joy, D. C.; Phelps, T. J.; Doktycz, M. J. Silver nanocrystallites: biofabrication using *Shewanella oneidensis*, and an evaluation of their comparative toxicity on Gram-negative and Gram-positive bacteria. *Environ. Sci. Technol.* **2010**, *44*, 5210–5215.
19. Thill, A.; Zeyons, O.; Spalla, O.; Chauvat, F.; Rose, J.; Auffan, M.; Flank A. M. Cytotoxicity of $CeO_2$ nanoparticles for *Escherichia coli*. Physico-chemical insight of the cytotoxicity mechanism. *Environ. Sci. Technol.* **2006**, *40*, 6151–6156.
20. Auffan, M.; Rose, J.; Bottero, J. Y.; Lowry, G. V.; Jolivet, J. P.; Wiesner, M. R. Towards a definition of inorganic nanoparticles from an environmental, health and safety perspective. *Nat. Nanotechnol.* **2009**, *4*, 634–641.
21. Handy, R. D.; Owen, R.; Valsami-Jones E. The ecotoxicology of nanoparticles and nanomaterials: current status, knowledge gaps, challenges, and future needs. *Ecotoxicology,* **2008**. *17*, 315–325.
22. Nowack, B.; Bucheli, T. D. Occurrence, behavior and effects of nanoparticles in the environment. *Environ. Pollut.* **2007**, *150*, 5–22.
23. Guzman, K. A. D.; Taylor, M. R.; Banfield, J. F. Environmental risks of nanotechnology: national nanotechnology initiative funding, 2000–2004. *Environ. Sci. Technol.* **2006**, *40*, 1401–1407.
24. Kirchner, C.; Liedl, T.; Kudera, S.; Pellegrino, T.; Javier, A. M.; Gaub, H. E.; Stolzle, S.; Fertig, N., Parak; W. J. Cytotoxicity of colloidal CdSe and CdSe/ZnS nanoparticles. *Nano Lett.* **2005**, *5*, 331–338.
25. Henry, T. B.; Menn, F. M.; Fleming, J. T.; Wilgus, J.; Compton, R. N.; Sayler, G. S. Attributing effects of aqueous $C_{60}$ nano-aggregates to tetrahydrofuran decomposition products in larval zebrafish by assessment of gene expression. *Environ. Health Perspect.* **2007**, *115*, 1059–1065.

26. Singh, N.; Manshian, B.; Jenkins, G. J. S.; Griffiths, S. M.; Williams, P. M.; Maffeis, T. G. G.; Wright, C. J.; Doak, S. H. Nanogenotoxicology: the DNA damaging potential of engineered nanomaterials. *Biomaterials,* **2009**, *30*, 3891-3914.
27. Laurent, S.; Burtea, C.; Thirifays, C.; Häfeli, U. O.; Mahmoudi, M. Crucial ignored parameters on nanotoxicology: The importance of toxicity assay modifications and "cel vision". *Plos One*, [Online] **2012,** *7*, e29997. http://www.plosone.org/article/info:doi/10.1371/journal.pone.0029997 (accessed Mar 19, 2013).
28. Crane, M.; Handy, R. D.; Garrod, J.; Owen, R. Ecotoxicity test methods and environmental hazard assessment for engineered nanoparticles. *Ecotoxicol.* **2008**, *17*, 421–437.
29. Lee, S.; Kim, M. S.; Lee, D.; Kwon, T. K.; Khang, D.; Yun, H. S.; Kim, S. H. The comparative immunotoxicity of mesoporous silica nanoparticles and colloidal silica nanoparticles in mice. *Int. J. Nanomed.* **2013**, *8*, 147–158
30. Sayre, P.; Klaper, R. Eds. *Nanomaterials and the Environment & Instrumentation, Metrology, and Analytical Methods*. Project Report 2009-2010, National Nanotechnology Initiative Series of EHS Workshops and Reports, **2011**. http://www.nano.gov/sites/default/files/pub_resource/nanoandenvironmentandinstrumentation.pdf (accessed Mar 19, 2013).
31. Ruparelia, J.P.; Chatterjee, A. K.; Duttagupta, S. P. Strain specificity in a antimicrobial activity of silver and copper nanoparticles, *Acta Biomater.* **2008**, *4*, 707-716.
32. Qi, L.; Xu, Z.; Jiang, X.; Hu C.; Zou X. Preparation and antibacterial activity of chitosan nanoparticles. *Carbohydr. Res.* **2004**, *339*, 2693–2700.
33. Kim, Y. S.; Seo, J. H.; Cha, H. J. Enhancement of heterologous protein expression in *Escherichia coli* by co-expression of nonspecific DNA binding stress protein, Dps. *Enzyme Microb. Technol.* **2003**, *33*, 460–465.
34. Stein, D. C.; Kopec, L. K.; Yasbin, R. E.; Young, F. E. Characterization of *Bacillus subtilis* DSM704 and its production of 1-deoxynojirimycin. *Appl. Environ. Microbiol.* **1984**, *48*, 280–284.
35. Gorby, Y. A.; Yanina, S.; McLean, J. S.; Rosso, K. M.; Moyles, D.; Dohnalkova, A.; Beveridge, T. J.; Chang, I. S.; Kim, B. H.; Kim, K. S.; Culley, D. E.; Reed, S. B.; Romine, M. F.; Saffarini, D. A.; Hill, E. A.; Shi, L.; Elias, D. A.; Kennedy, D. W.; Pinchuk, G.; Watanabe, K.; Ishii, S. I.; Logan, B.; Nealson, K. H.; Fredrickson, J. K. Electrically conductive bacterial nanowires produced by *Shewanella oneidensis* strain MR-1 and other microorganisms. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 11358–11363.
36. Dumas, E. M.; Ozenne, V.; Mielke, R. E.; Nadeau, J. L. Toxicity of CdTe quantum dots in bacterial strains. *IEEE Trans. Nanobiosci.* **2009**, *8*, 58–64.
37. Lyon, D. Y.; Brunet, L.; Hinkal, G. W.; Wiesner, M. R.; Alvarez, P. J. J. Antibacterial activity of fullerene water suspensions (nC(60)) is not due to ROS-mediated damage. *Nano Lett.* **2008**, *8*, 1539–1543.
38. Brown, S. D.; Thompson, M. R.; VerBerkmoes, N. C.; Chourey, K.; Shah, M.; Zhou, J.; Hettich, R. L.; Thompson. D. K. Molecular dynamics of the *Shewanella oneidensis* response to chromate stress. *Mol. Cell. Proteomics,* **2006**, *5*, 1054–1071.
39. Pelletier D.A.; Suresh A.K.; Holton G.A.; McKeown C.K.; Wang W.; Gu B.; Mortensen N.P.; Allison D.P.; Joy D.C.; Allison M.R.; Brown S.D.; Phelps T.J.; Doktycz M.J. Effects of engineered cerium oxide nanoparticles on bacterial growth and viability. *Appl Environ Microbiol.* **2010**, *6*, 7981–7989
40. Rodea-Palomares, I.; Gonzalez-Garcia, C.; Leganes, F.; Fernandez-Piñas, F. Effect of pH, EDTA, and anions on heavy metal toxicity toward a bioluminescent cyanobacterial bioreporter. *Arch. Environ. Contam. Toxicol.* **2009**, *57*, 477–487.
41. Valant, J.; Iavicoli, I.; Drovne, D. The importance of a validated standard methodology to define in vitro toxicity of nano-$TiO_2$. *Protoplasma,* **2012**, *249*, 493-502.

42. Dusinka, M.; Runden-Pran, E.; Carreira, S. C.; Saunders, M. Critical evaluation of toxicity tests. In *Adverse Effects of Engineered Nanomaterials. Exposure, Toxicology, and Impact on Human Health;* Fadeel, B.; Pietroiusti, A.; Shvedova, A. A., Eds.; Academic Press: New York, NY, **2012**; pp. 63-83.
43. Stern, S. E.; Zolnik, B. S. NCL Method GTA-7. Hepatocyte Primary ROS Assay. *Nanotechnology Characterization Laboratory*; [Online], **2010**. http://ncl.cancer.gov/NCL_Method_GTA-7.pdf (accessed Mar 13, 2013).
44. Ma, Y. *In vitro* models for nanotoxicity testing. In *Nanotoxicity: From In vivo and In vitro Models to Health Risks;* Sahu, S.; Casciano, D., Eds.; John Wiley and Sons Ltd.: Hoboken, NJ; **2009**; pp. 349-377.
45. Butterworth, K. T.; Coulter, J. A.; Jain, S.; Forker, J.; McMahon, S. J.; Schettino, G.; Prise, K. M.; Currell, F. J.; Hirst, D. G. Evaluation of cytotoxicity and radiation enhancement using 1.9 nm gold particles: potential application for cancer therapy. *Nanotechnology*, **2010**, *21*, 295101.
46. Arora, S.; Rajwade, J. M.; Paknikar, K. M. Nanotoxicology and *in vitro* studies: The need of the hour. *Tox. App. Pharm*. **2012**, *258*, 151-165.
47. Code of Federal Regulation. 799.9110 - TSCA acute oral toxicity. Protection of Environment. 2010; http://cfr.vlex.cm/vid/799-tsca-acute-oral-toxicity-19834216 (accessed Feb 17, 2013).
48. Maurer-Jones, M. A.; Hayne, C. L. *In Vivo* and *In Vitro* Nanotoxicology Studies. SYMPOSIUM 1: Nanodiagnostics and Nanotherapeutics: Building Research Ethics and Oversight. *J. Law, Med. & Ethics*, **2012**, *40*, 795–801.
49. Lewinski, N.; Colvin, N.; Drezer, V. Cytotoxicity of nanoparticles. *Small,* **2008**, *4*, 26-49.
50. Goodman, C. M.; McCusker, C. D.; Yilmaz, T.; Rotello, V. Toxicity of gold nanoparticles functionalized with cationic and anionic side chains. *Bioconj. Chem.* **2004**, *15*, 897-900.
51. Hussain, S. M.; Hess, K. L.; Gearhart, J. M.; Geiss, K. T.; Schlager, J. J. *In vitro* toxicity of nanoparticles in BRL 3A rat liver cells. *Toxicol. In Vitro,* **2005**, *19*, 975-983.
52. Shukla, R. K.; Sharma, V.; Pandey, A. K.; Singh, S.; Sultana, S.; Dhawan, A. ROS-mediated genotoxicity induced by titanium dioxide nanoparticles in human epidermal cells. *Toxicol. In Vitro,* **2011**, *25*, 231-241.
53. Herzog, E.; Casey, A.; Lyng, F. M.; Chambers, G.; Byrne, H. J.; Davoren, M. A new approach to the toxicity testing of carbon-based nanomaterials – the clonogenic assay. *Toxicol. Lett.* **2007**, *174*, 49-60.
54. Fahmy, B.; Cormier, S. A. Copper oxide nanoparticles induce oxidative stress and cytotoxicity in airway epithelial cells. *Toxicol. In Vitro,* **2009**, *23*, 1365-1371.
55. Stern, S. E.; Potter, T. NCL Method GTA-2. Hep-G2 Hepatocarcinoma Cytotoxicity Assay. *Nanotechnology Characterization Laboratory*. [Online], **2010**. http://ncl.cancer.gov/NCL_method_GTA-2.pdf (accessed Mar 13, 2013).
56. Stern, S. E.; Potter, T. NCL Method GTA-1. LLC-PK1 Kidney Cytotoxicity Assay. *Nanotechnology Characterization Laboratory*. [Online], **2010**. http://ncl.cancer.gov/NCL_Method_GTA-1.pdf (accessed Mar 13, 2013).
57. Lanone, S.; Rogerieux, F.; Geys, J.; Dupont, A.; Maillot-Marechal, E.; Boczkowski, J.; Lacroix, G.; Hoet, P. Comparative toxicity of 24 manufactured nanoparticles in human alveolar epithelial and macrophage cell lines. *Part. Fibre. Tox.* [Online]; **2009**, *6*, 4. http://www.particleandfibretoxicology.com/content/6/1/14 (accessed Mar 19, 2013).
58. Stern, S. E.; Potter, T.; Neun, B. W. NCL Method GTA-3. Hep-G2 Hepatocyte Glutathione Assay. *Nanotechnology Characterization Laboratory*. [Online], **2010**. http://ncl.cancer.gov/NCL_Method_GTA-3.pdf (accessed Mar 13, 2013).
59. Berry, C. C.; Wells, S.; Charles, S.; Curtis, A. S. G. Cell response to dextran-derivatized iron oxide nanoparticles post-internalization. *Biomaterials,* **2003**, *24*, 4551-5413.

60. Stern, S. E.; Potter, T. NCL Method GTA-5. LLC-PK1 Kidney Cell Apoptosis Assay. *Nanotechnology Characterization Laboratory*. [Online], **2010**. http://ncl.cancer.gov/ncl_method_gta-5.pdf (accessed Mar 13, 2013).
61. Stern, S. E.; Potter, T. NCL Method GTA-6. HepG2 Hepatocarcinoma Apoptosis Assay. *Nanotechnology Characterization Laboratory*. [Online], **2010**. http://ncl.cancer.gov/NCL_Method_GTA-6.pdf (accessed Mar 13, 2013).
62. Stern, S. E.; Neun, B. W. NCL Method GTA-14. Hep G2 Hepatocarcinoma Homogeneous Apoptosis Assay. *Nanotechnology Characterization Laboratory*. [Online], **2010**. http://ncl.cancer.gov/NCL_Method_GTA-14.pdf (accessed Mar 13, 2013).
63. Dobrovolskaia, M.; Potter, T. NCL Method ITA-11. Measurement of Nanoparticle Effects on Cytotoxic Activity of NK Cells by Label-Free RT-CES System. *Nanotechnology Characterization Laboratory*. [Online], **2009**. http://ncl.cancer.gov/NCL_Method_ITA-11.pdf (accessed Mar 13, 2013).
64. Oberdörster, G.; Oberdörster, E.; Oberdörster, J. Nanotoxicology: an emergent discipline evolving from studies of ultrafine particles. *Environ. Health Perspect.* **2005**, *113*, 823-839.
65. Monteiro-Riviere, N. A.; Nemanich, R. J.; Inman, A. O.; Wang, Y. Y.; Riviere, J. E. Multi-walled carbon nanotube interactions with human epidermal keratinocytes. *Toxicol. Lett.* **2010**, *2*, 2004-2010.
66. Wang, Y.; Wang, J.; Wu, M.; Deng, X.; Wen, T.; Chen, C.; Zhang, H.; Liu, Y.; Jiao, Z. Internalization, translocation and biotransformation of silica-coated titanium dioxide nanoparticles in neural stem cells. *J. Nanosci. Nanotechnol.* **2010**, *10*, 7121-7125.
67. Raemy, D. O.; Limbach, L. K.; Rothen-Rutishauser, B.; Grass R. N.; Gehr, P.; Birbaum, K.; Brandenberger, C.; Günther, D.; Stark, W. J. *Eur. J. Pharm. Biopharm*. **2011**, *77*, 368-375.
68. Seidle, T.; Robinson, S.; Holmes, T.; Creton, S.; Prieto, P.; Scheel, J.; Chlebus, M. Cross-sector review of drivers and available 3Rs approaches for acute systemic toxicity testing *Toxicol. Sci.* **2010**, *116*, 382–396.
69. Walum, E. Acute Oral Toxicity. *Environ. Health Perspect.* **1998**, *106* (Supplement 2), 497-503.
70. National Toxicological Program, Department of Health and Human Services, *National Institute of Environmental Health Sciences*. [Online], **2010**. http://ntp.niehs.nih.gov/ (accessed Feb 23, 2013).
71. National Research Council (NRC) Guide for the care and use of Laboratory Animals, 8th Edition, [Online], **2011**. http://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-Use-of-Laboratory-Animals.pdf (accessed Apr 12, 2013).
72. Wang, X.; Reece, S. P.; Brown, J. M. Immunotoxicological impact of engineered nanomaterial exposure: mechanisms of immune cell modulation. *Tox. Mech. Meth.* **2013**, *23*, 168-177.
73. Groso, A.; Fink, A. P.; Magrez, A.; Riediker, M.; Meyer, T. Management of nanomaterials safety in research environment. *Part. Fibre Toxicol.* [Online]; **2010**, *7*, 40. http://www.particleandfibretoxicology.com/content/7/1/40 (accessed Mar 13, 2013).
74. Horie, M.; Stowe, M.; Kambara, T.; Lee, B. W.; Endoh, S.; Maru, J.; Oyabu, T.; Myojo, T.; Ogami, A.; Uchida, K.; Yamamoto, K.; Kobayashi, N.; Kuroda, E.; Nakazato, T.; Morimoto, Y. Pulmonary Inflammation of Well-Dispersed Multi-Wall Carbon Nanotubes Following Intratracheal Instillation: Toxicity by Fiber of 1–5 μm in Length. *Materials*, **2012**, *5*, 2833-2849
75. Tervonen K.; Waissi G.; Petersen E.J.; Akkanen J.; Kukkonen V.K. Analysis of fullerene-$C_{60}$ and kinetic measurements for its accumulation and depuration in Daphnia magna. *Environ. Toxicol. Chem*. **2010**, *29*, 1072-1078.
76. Geller, W.; Müller H. The filtration apparatus of cladocera; Filter mesh-sizes and their implications on food selectivity. *Oecologia,* **1981**, *49*, 316–321.
77. Ebert, D. Ecology, Epidemiology and Evolution of Parasitism in *Daphnia*. *National Library of Medicine, National Center for Biotechnology Information,* 2005; Bethesda, MD, USA.

78. Yang L.; Watts D.J. Particle surface characteristics may play an important role in phytotoxicity of alumina nanoparticles. *Toxicol. Lett.* **2005**, *158*, 122-132
79. ETC Group. Nanotech and the Precautionary Prince. *Genotype*, [Online]; 2003. May 2. http://www.etcgroup.org/sites/www.etcgroup.org/files/publication/158/01/precautionprince.pdf (accessed Dec 21, 2012).
80. International Center for Technology Assessment, Citizen Petition to FDA. *Petition Requesting FDA Amend Its Regulations for Products Composed of Engineered Nanoparticles Generally and Sunscreen Drug Products Composed of Engineered Nanoparticles Specifically*, Docket 2006P-0210. [Online]; 2006. http://www.icta.org/doc/Nano%20FDA%20petition%20final.pdf (accessed Mar 19, 2013).
81. Warheit, B. Debunking Some Misconceptions about Nanotoxicology. *Nano Lett.* **2010**, *10*, 4777-4782.

# Anexo II

## Nutrición y Biotecnología Alimentaria. Bases para la Sustentabilidad Social.

Ortega Regules AE, Angulo Molina A, Lozada Ramirez JD. Nutrición y biotecnología alimentaria, bases para la sustentabilidad social. En: Asili, N. Vida sustentable, la experiencia de un sueño compartido. Universidad de las Américas Puebla. 2012, 1ra edic. Pág. 408-426, ISBN 978-607 7690-12-2.

# RESUMEN

A lo largo de la historia diversas civilizaciones han basado su progreso o fracasos en función de su capacidad para mantener saludables y eficientemente alimentadas a sus poblaciones. La nutrición humana está basada no sólo en los nutrimentos que se ingieren sino también en cómo el organismo es capaz de metabolizar dichos nutrimentos. Para asegurarnos que los nutrimentos son correctamente metabolizados es necesario conocer los aspectos genéticos de cada individuo en particular; con este fin, la rama de la nutrición que evalúa la interacción de aquellos componentes de la dieta de un individuo con sus genes, provocando efectos adversos es conocida como nutrigenética. Esta rama se apoya en el conocimiento de algunos componentes de la dieta que en ciertas condiciones condicionan al genoma humano, alterando la expresión de genes y al metabolismo del individuo. Los productos de expresión de los genes son generalmente proteínas.

Una vez sintetizadas las proteínas, éstas maduran para poder llevar a cabo funciones específicas en el organismo. Desde el punto de vista nutricional juegan un papel muy importante ya que forman parte de todos los tejidos del organismo. Los sistemas biológicos requieren de cuatro tipos de biomoléculas: carbohidratos, lípidos, ácidos nucleicos y proteínas; además de otros átomos y moléculas para llevar a cabo los procesos esenciales de la vida. La obtención de las biomoléculas necesarias se da a través de los alimentos y deben ser suficientes en cantidad y calidad para un buen funcionamiento del metabolismo. Cualquier desequilibrio o mal funcionamiento del metabolismo se ve reflejado como padecimientos o enfermedades.

La nutrición es el conjunto de procesos biológicos, psicológicos y sociológicos involucrados en la obtención, asimilación y metabolismo de los nutrimentos. El

estado de nutrición es el balance entre consumo y gasto de esas fuentes de energía, al ocurrir desviaciones patológicas se denomina mala nutrición y acarrea otros problemas importantes de salud. Se han diseñado herramientas de orientación alimentaria asesorando en cuanto a la combinación y cantidades de los alimentos, un ejemplo de esto es el plato del buen comer. Por otra parte, en los últimos años se han incorporado a la alimentos tradicionales alimentos transgénicos, nuevos alimentos, alimentos funcionales, nutracéuticos, alimentos bioactivos entre otros cómo alternativas o complementos de la dieta regular. La inseguridad alimentaria es un tema de vital importancia, ya que trae consigo hambre, desnutrición y una serie de graves problemas en diversos ámbitos. El modelo de traspatios es una alternativa para combatir la inseguridad alimentaria y para favorecer la sustentabilidad, ya que propone la autoproducción. El desarrollo alimentario debe ser sustentable evitando comprometer las necesidades futuras y dando lugar al uso de nuevas tecnologías y alternativas a los procesos tradicionales.

# Nutrición y biotecnología alimentaria. Bases para la sustentabilidad social

Ana Eugenia Ortega Regules;
Aracely Angulo Molina;
José Daniel Lozada Ramírez

«Somos lo que comemos», sin duda que esta frase contiene mucha «sustancia». La nutrición se ha convertido en uno de los aspectos más estudiados y valorados en nuestros tiempos. Las civilizaciones a lo largo de la historia de la humanidad han progresado o fracasado en función de su capacidad para alimentar eficientemente y mantener saludables a sus poblaciones. La escasez de alimentos, las enfermedades y las guerras han provocado la desaparición de civilizaciones enteras, mientras que el poderío de algunas se ha basado en su capacidad para comercializar con productos alimenticios con sus vecinos. Sin embargo, la nutrición humana no sólo se basa en lo que comemos, es decir, en los nutrientes que podemos ingerir para asegurar una buena salud, sino en cómo es que el organismo es capaz de metabolizar dichos nutrientes. El ejemplo más simple lo podemos observar en bacterias capaces de degradar compuestos «tóxicos» para los seres humanos. Dichos microorganismos utilizan como alimento estos compuestos, por lo que pueden llegar a formar parte de su «carta». La pregunta entonces es ¿cómo asegurarnos de que los nutrientes que ingerimos son correctamente metabolizados en nuestro organismo? Esta pregunta sólo puede contestarse si conocemos los aspectos genéticos de cada individuo en particular. Los genes determinarán por lo tanto si una persona es capaz de ingerir leche o productos lácteos (metabolismo de la lactosa), si una persona es celíaca (metabolismo del gluten), si una persona es fenilcetonúrica (metabolismo del aminoácido fenilalanina), si una persona es deficiente en ácidos grasos esenciales (metabolismo de lípidos), entre otros. De esta manera podemos hablar de una nueva rama de la nutrición conocida como nutrigenética, la cual evalúa la interacción de aquéllos componentes de la dieta de un individuo con sus genes, provocando efectos adversos. Conociendo estas interacciones podemos eliminar ciertos componentes de la dieta que pueden contribuir al desarrollo de enfermedades como el cáncer o la diabetes. La nutrigenética se apoya, por tanto, en el conocimiento de que algunos componentes de la dieta en condiciones particulares condicionan al genoma humano, alterando la expresión de genes y, por lo tanto, al metabolismo del individuo. Este conocimiento forma parte de un área conocida como Nutrigenómica. Partiendo de este principio, podemos asegurar que la «nutrición con conciencia» puede contribuir a la prevención o cura de enfermedades crónicas y genéticas. Con lo anterior estamos en la antesala del desarrollo de investigación centrada en una alimentación basada en el conocimiento de aquellos nutrientes saludables ingeridos en cantidades adecuadas para el aseguramiento de la salud. Por tanto podemos enfatizar que... «Somos lo que comemos y de qué forma lo metabolizamos».

## ••• Del ADN al metabolismo, las biomoléculas

La información contenida en los genes está almacenada en una molécula descrita hace poco más de 55 años por James Watson y Francis Crick, el ADN. El ADN (Ácido Desoxirribonucleico) es la molécula que posee toda la información que determinará todas las características particulares de un organismo vivo. Desde esta perspectiva, el ADN determina si un organismo es capaz de adaptarse a las condiciones medioambientales a las cuales está siendo sometido, además de establecer los patrones de salud y enfermedad en cualquier individuo. El ADN de un organismo es el genoma de dicho organismo. Lo anterior implica que todos y cada uno de los genes que forman parte de un organismo conforman el genoma. Un gen se define como un fragmento de ADN que contribuye a una función; la unidad de la herencia. La información contenida en el ADN determinará nuestras características y funciones como individuos, nos hará funcionar de una forma particular en presencia de ciertos factores ambientales (nutrientes, luz, temperatura, estrés, etc.)

Los productos de la expresión de los genes son, la gran mayoría de las veces, proteínas. El paso de ADN a proteínas requiere de una molécula que funciona como intermediario, el ARN. Lo anterior ocurre para preservar la integridad del ADN, es decir, el ARN es una copia de trabajo que puede ser manipulada y sufrir degradación sin afectar la información genética, es decir, sin que la información de «lo que somos» se vea alterada. Una vez sintetizadas las proteínas a través de mecanismos complejos que requieren de una gran cantidad de energía en la célula, éstas maduran para poder llevar a cabo funciones específicas en el organismo, proporcionándole sus características específicas. Desde el punto de vista nutricional, las proteínas juegan un papel muy importante que va desde el desdoblamiento de los nutrientes de la dieta, hasta la formación y fortalecimiento de estructuras ya que forman parte de cartílago, uñas, pelo, piel y músculo, aunque están distribuidas en todos los tejidos del organismo.

Los sistemas biológicos requerimos de cuatro tipos de biomoléculas y otros átomos y moléculas para poder llevar a cabo los procesos esenciales de la vida. Estas cuatro biomoléculas son carbohidratos o hidratos de carbono, lípidos o grasas, ácidos nucleicos y proteínas. Anteriormente se ha mencionado la importancia de ácidos nucleicos (ADN y ARN) y de las proteínas, siendo ahora el turno de los carbohidratos. Los azúcares o carbohidratos, satanizados en los últimos tiempos, están constituidos por moléculas con átomos de carbono, hidrógeno y oxígeno. Los carbohidratos forman a las dextrinas, almidones, glucógeno, celulosas, hemicelulosas, pectinas y gomas en los alimentos. Estas moléculas proporcionan unidades de glucosa, la cual es necesaria para la obtención de energía inmediata para que la célula sea capaz de llevar a cabo sus funciones básicas. Los azúcares también son utilizados para almacenar la energía dentro de tejidos específicos. Sin embargo, un organismo que presenta un exceso en los niveles de glucosa suele estar enfermo.

Para el caso de las grasas, se trata del grupo más heterogéneo de biomoléculas ya que, a diferencia de carbohidratos, ácidos nucleicos y proteínas, no hay una unidad básica que se repita en su estructura. Generalmente se trata de sustancias suaves e insolubles en agua. Son muy importantes desde el punto

de vista funcional, estructural y nutricional para los sistemas vivos. Por ejemplo, todas las membranas biológicas están formadas de lípidos, es decir, todas nuestras células requieren de este tipo de biomoléculas para funcionar.

Las biomoléculas interaccionan entre sí para asegurar el funcionamiento adecuado del metabolismo. El metabolismo se define como el grupo de reacciones químicas que ocurren en los sistemas biológicos y es el encargado de dirigir todos los procesos que ocurren a nivel celular, por ejemplo, la absorción de nutrientes y su transporte hacia el interior de la célula, la obtención de energía a partir de esos nutrientes, la síntesis de moléculas complejas a partir de esa energía, la formación de tejidos a partir de las moléculas complejas formadas, etc. El correcto funcionamiento del metabolismo es, por lo tanto, un requisito para que un organismo pueda funcionar adecuadamente. Cualquier desequilibrio o mal funcionamiento del metabolismo se ve reflejado como padecimientos o enfermedades que pueden ser leves, graves o mortales. De esta manera, las biomoléculas son las encargadas de que los procesos metabólicos funcionen, química pura. Pero, ¿de dónde podemos obtener estas biomoléculas para garantizar un correcto desempeño metabólico? La respuesta es simple, de los alimentos.

Cabe señalar que los componentes de los alimentos no son utilizados de forma íntegra en los organismos que las consumen, primero deben ser degradados en sus componentes más simples los cuales son usados en los innumerables procesos celulares. Esos componentes deben ser suficientes en cantidad y en calidad para garantizar el buen funcionamiento del metabolismo. Por ejemplo, la gelatina es un alimento rico en proteínas pero de baja calidad (es proteína formada por un grupo reducido de aminoácidos), por lo que un individuo sería incapaz de sobrevivir con una dieta rica en este tipo de alimentos.

## ••• ***Nutrición***

En épocas pasadas, se consideraba a los alimentos como simples proveedores de energía y de nutrimentos que de alguna forma se relacionaban a enfermedades. Sin embargo, ahora se sabe que existen moléculas bioactivas en los alimentos que pueden interaccionar con los genes, proteínas y otras biomoléculas implicadas en la regulación metabólica y la expresión genética, y por ello asociarse a la aparición, progresión y/o cura de ciertas enfermedades donde el alimento es un factor ambiental clave.

Los seres vivos nos alimentamos para obtener energía y poder realizar desde las funciones más básicas para el mantenimiento de la vida como el respirar y el comer, hasta las funciones más complejas que nos permiten hablar, pensar e interactuar unos con otros. Hasta hace algunos años, el término alimentación se utilizaba como sinónimo de nutrición. Sin embargo, actualmente la nutrición se define como el conjunto de procesos biológicos, psicológicos y sociológicos involucrados en la obtención, asimilación y metabolismo de los nutrimentos por el organismo y es fundamentalmente un proceso celular que ocurre en forma continua (Bourges, 2001). En este sentido, la alimen-

tación queda inmersa en el concepto de nutrición, aunque independientemente de hablar de nutrición o de alimentación, ambos términos convergen en un factor común, el alimento.

Los alimentos varían según su origen y pueden incluir órganos, tejidos o secreciones que contienen cantidades apreciables de nutrimentos, que son inocuos si son consumidos en cantidades habituales, que son de fácil y suficiente disponibilidad, que son atractivos a los sentidos y que son seleccionados según la cultura a la que pertenece el consumidor (Bourges, 2001).

Por otro lado, la nutrición se refiere a la serie de fenómenos que determinan que algunas sustancias del medioambiente (los alimentos) sean utilizadas como fuente de energía e incorporadas como materia útil por el organismo. Este proceso determina el estado de nutrición, el cual resulta del balance entre el consumo y el gasto de esas fuentes de energía. Un individuo puede tener una nutrición adecuada, pero cuando ocurren desviaciones patológicas de la misma, se denomina mala nutrición. Este término puede referirse tanto a alteraciones nutricionales por defecto (desnutrición) o por exceso (sobrenutrición), así como por desequilibrio (disnutrición) (Verdú, 2009).

La desnutrición afecta el desarrollo intelectual de los individuos, particularmente cuando se presenta durante el crecimiento, desarrollo y maduración del sistema nervioso durante los primeros años de vida. Está asociada a situaciones de pobreza, la cual impide una adecuada educación y formación del individuo. Además, la desnutrición favorece la aparición de algunas enfermedades, tanto infecciosas como físicas, las cuales generan un retraso en la adquisición de capacidades y habilidades motoras e incluso una disminución de las mismas (Verdú, 2009; Casanueva, 2008). Con un incremento estimado de 105 millones de hambrientos en 2009, existen actualmente un aproximado de 1 020 millones de individuos desnutridos en el mundo, lo cual significa que casi una sexta parte de la humanidad padece hambre (OMS, 2009). Lo anterior representa una grave amenaza difícil de resolver, dado que la desnutrición influye en la tasa de mortalidad y morbilidad por infecciones, además de incidir en la tasa de mortalidad y en la esperanza de vida materna, infantil y perinatal (Casanueva, 2008).

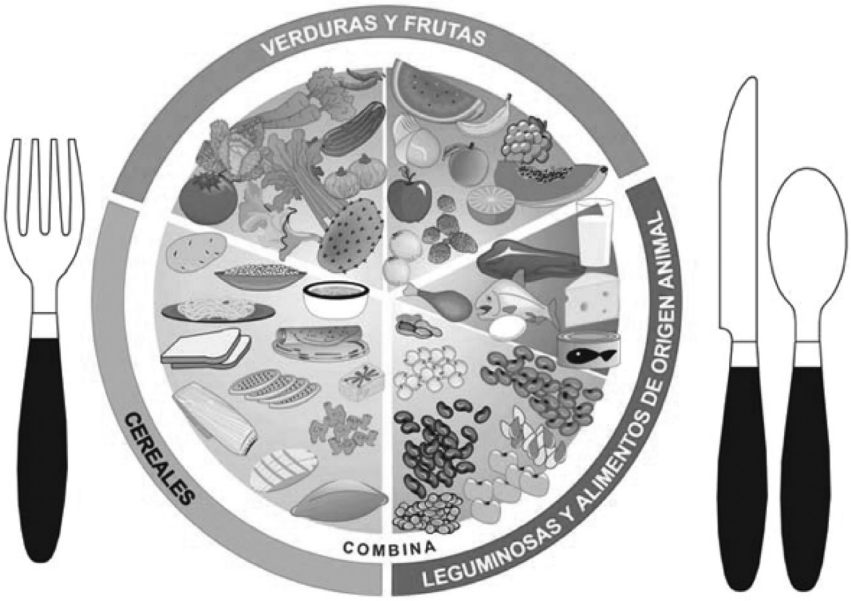
En México, más de 30 millones de mexicanos padecen desnutrición. Aunado a lo anterior, muchos de los precios de los alimentos básicos se han incrementado entre 15 % al 60 % durante los últimos dos años, llevando a una disminución en el consumo de algunos alimentos o a la adquisición de otros de baja calidad. Los aumentos de la gasolina y el gas LP provocaron a principios del 2010 que el precio de granos básicos como el arroz, frijol, lentejas y maíz, así como el kilogramo de huevo y azúcar repercutieran de inmediato su precio en el consumidor final. Además estos aumentos se suman a los del 2009, cuando el costo de la canasta básica, elaborada con 27 productos alimenticios, aumentó a $650, por lo que ésta quedó fuera del alcance de muchas familias mexicanas, para quienes el consumo de carnes rojas, ciertas frutas y verduras, es considerada un lujo.

Además, la pobreza afecta a más de la mitad de la población mexicana (50 a 55 millones de personas), debido a la crisis mundial, a la falta de poder adqui-

sitivo, a la falta de ingresos y al desempleo que se presenta en nuestro país (*La Jornada*, 2009). Al igual que en otros países latinoamericanos, la desnutrición en México, cuando ocurre durante la infancia, debe ser considerada como un problema de salud pública. Además, la desnutrición no se distribuye de manera homogénea a lo largo del territorio mexicano, ni entre las zonas rurales y las urbanas, ni entre las distintas áreas rurales, donde se sabe que su prevalencia es mayor (Casanueva, 2008). El sureste del país es la región más afectada por la desnutrición, debido en gran parte a que una gran proporción de la población vive en zonas rurales pobres. Según la última Encuesta Nacional de Salud (ENSANUT), realizada, el desmedro o baja estatura en escolares es de 10.4 % en niños y de 9.5 % en niñas, siendo éste uno de los indicadores más importantes de desnutrición crónica. Por ello, la desnutrición debe considerarse un problema de salud pública importante que está directamente relacionado a una mayor morbilidad y mortalidad por enfermedades infecciosas, así como alteraciones en el desempeño físico y mental de los niños (Casanueva, 2008; Verdú, 2009).

Otro problema de salud pública importante, el cual está relacionado a una deficiente alimentación, es la anemia por deficiencia de hierro. En Puebla existe una prevalencia del 28 % en niños menores de cinco años a este mal, y del 16.9 % en niños de entre 5 a 11 años. Para el caso de adultos mayores de cincuenta años, la prevalencia es del 24.4 %, así como una prevalencia muy alta de anemia en mujeres adolescentes. Es importante mencionar que la prevalencia de anemia en niños menores de cinco años en nuestro estado es mayor que el promedio nacional, lo que ubica a Puebla dentro de los diez estados con mayor prevalencia de anemia y desnutrición infantil (ENSANUT, 2006).

Otro caso es el de la mala nutrición por exceso que es la responsable de una de las grandes pandemias de la actualidad, el sobrepeso asociado a la obesidad infantil. Nuestro país es, desafortunadamente, uno de los países con mayor prevalencia de obesidad en niños. Para el Estado de Puebla, aun cuando la prevalencia de esta enfermedad en escolares es menor a la media nacional, cerca de uno de cada cinco niños padece sobrepeso y obesidad. Desgraciadamente, esta problemática no sólo se presenta en la población infantil sino también en la adulta (ENSANUT, 2006).




Plato del bien comer

En México, un grupo de expertos diseñó una herramienta de orientación alimentaria a la cual se le llamó el Plato del Bien Comer. Esta herramienta se le utiliza para promover buenos hábitos de alimentación y orientar a la población sobre la elección adecuada de los alimentos disponibles para su consumo. Como regla general se debe elegir un alimento de cada sección (diferenciadas por los colores verde, rojo y amarillo) en cada una de nuestras comidas. Los alimentos asociados al color verde comprenden verduras y frutas, los asociados al rojo comprenden leguminosas y alimentos de origen animal, y los asociados al amarillo los cereales (NOM-043-SSA2-2005 ) (Casanueva, 2008). Todo lo anterior debe cumplir con los seis requisitos esenciales de una buena alimentación, es decir, la dieta debe ser:

- Completa; con todos los nutrimentos requeridos por el individuo.
- Suficiente; que cubra sus necesidades.
- Equilibrada; que contenga proporciones apropiadas entre los nutrimentos.
- Variada; que incluya diferentes alimentos.
- Adecuada; debe ser acorde con los gustos, la cultura y accesibles económicamente.
- Inocua; que no implique riesgos para la salud.

La dieta debe de cumplir con el mayor número de las características antes descritas, por lo que la elección de los alimentos debe ser cuidadosa. De aquí que, recientemente, se ha incrementado el interés por la obtención de salud a

través de la alimentación, por lo que la correcta elección de aquellos alimentos ricos en sustancias bioactivas y que cumplan con los requisitos para formar parte de una buena dieta, ha ganado mucho interés (Casanueva, 2008).

Lo anterior no implica que existan «buenos» o «malos» alimentos, sino que se debe ser muy cuidadoso con la combinación y las cantidades que se eligen como parte de una dieta. Anteriormente, se decía que los «malos alimentos» podían provocar enfermedades crónicas como el cáncer si los genes «buenos» no intervenían para evitarlo. Ahora se sabe que la interacción alimento-gen es un proceso continuo altamente regulado y complejo donde ciertos constituyentes de los alimentos pueden proteger o dañar, según sea el caso, a los genes implicados en el desarrollo de la enfermedad. Hoy en día se considera que las recomendaciones alimentarias para la población deberán diversificarse más en el futuro. Es muy probable que en pocos años los especialistas en nutrición tendrán los expedientes genéticos de su pacientes, podrán identificar las enfermedades específicas a las cuales son más propensos y, con base en ello, serán capaces de diseñar los mejores planes de alimentación tomando en cuenta la nueva tendencia de los consumidores; saciar su apetito y encontrar beneficios adicionales en su salud, en su longevidad y en una mejor calidad de vida.

## ••• Los nuevos alimentos y la nueva alimentación: Biotecnología y alimentación

En los últimos años han surgido nuevos términos relacionados a la alimentación y a la nutrición; alimentos transgénicos, nuevos alimentos, alimentos funcionales, nutracéuticos, alimentos bioactivos, por mencionar algunos. Estos conceptos se han visto envueltos en una serie de controversias y debates internacionales, que han afectado su distribución y la posibilidad de utilizarse en la lucha contra el hambre. Esta problemática sobre todo ha afectado a los alimentos transgénicos. Un alimento transgénico se define como aquel que ha sido sometido a una modificación en la información genética original del organismo que lo originó, a través de ingeniería genética y usando herramientas de biología molecular. Por lo anterior, se puede deducir que los alimentos transgénicos son generados a partir de un organismo sometido a la incorporación de genes provenientes de otro organismo para poder producir una característica específica deseada o eliminar alguna característica indeseable. Hoy en día la mayor cantidad de alimentos transgénicos provienen de plantas transgénicas, los cuales en su mayoría son cereales como el maíz, el arroz, la cebada, entre muchos otros.

Las primeras plantas transgénicas comercializadas fueron plantas resistentes a ciertos herbicidas y plantas que producían su propio insecticida dirigido hacia ciertas especies de insectos, pero inocuos para el humano y los animales (Balbás, 2002). Ejemplos específicos de plantas transgénicas que se cultivan en nuestro país son el algodón, soya y arroz, a las cuales se les ha conferido la resistencia a plagas, virus y enfermedades; tolerancia a herbicidas; adaptación

a ambientes extremos y mejoras alimenticias (SEMARNAT, 2009). Existe un gran debate sobre el uso de maíz transgénico, el cual se ha estudiado de forma intensa para la obtención de información sobre los beneficios y riesgos en el uso de éste cereal, por ello, el gobierno federal de nuestro país prohíbe el cultivo de maíz transgénico, además que nuestro país es el centro de origen del maíz (SEMARNAT, 2009).

Se han realizado diferentes proyectos encaminados a mejorar las propiedades fisicoquímicas, sensoriales y nutrimentales de algunos productos agrícolas. En los últimos años se está trabajando sobre la generación de alimentos funcionales a través de la ingeniería genética de alimentos. Las herramientas proporcionadas por la biología molecular involucra la modificación deliberada del material genético de microorganismos, de plantas o de animales para consumo animal o humano a través de la biotecnología.

El Convenio sobre la Diversidad Biológica (CDB) define la biotecnología como: «Toda aplicación tecnológica que utilice sistemas biológicos y organismos vivos o sus derivados para la creación o modificación de productos o procesos para usos específicos».

Según la Organización de las Naciones Unidas para la Agricultura y la Alimentación (FAO por sus siglas en inglés), la biotecnología ofrece instrumentos poderosos para el desarrollo sostenible de la agricultura, la pesca y la actividad forestal, así como de las industrias alimentarias. Cuando se integra debidamente con otras tecnologías para la producción de alimentos, productos agrícolas y servicios, la biotecnología puede contribuir en gran medida a satisfacer las necesidades de la población mundial en continuo crecimiento. Por lo anterior, se debe considerar que la biotecnología puede ser una alternativa si es usada responsablemente como herramienta para la generación de un desarrollo sostenible en la alimentación. Sin embargo, la biotecnología moderna también es la responsable de la creación de los denominados organismos modificados genéticamente, los cuales han llegado a ser objeto de un debate muy intenso.

Existen una gran cantidad de grupos que se encuentran en una postura en contra de la aplicación de alimentos transgénicos. Los detractores apuntan que los alimentos transgénicos no han sido probados respecto a su total inocuidad. Algunos de los problemas asociados al uso de cultivos transgénicos son la transferencia del material genético nuevo hacia otros organismos, el crecimiento de organismos transgénicos en lugares no deseados, posible daño tóxico a organismos benéficos, coexistencia con la agricultura convencional y orgánica. Sin embargo, los especialistas consideran que estos riesgos pueden ser evaluados y controlados a través de medidas de bioseguridad (SEMARNAT, 2009).

En México, el Instituto Nacional de Ecología (INE) está encargado de evaluar los riesgos de la liberación de transgénicos en el medio ambiente a través de la Coordinación de Bioseguridad, propone medidas de control y mitigación de riesgos y emite una opinión técnica para la toma de decisiones. También colabora con información científica y técnica con la Procuraduría Federal de Protección al Ambiente, la Secretaría de Salud, la Comisión Nacional para

la Biodiversidad, y la Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (SAGARPA). Además, apoya el monitoreo en campo para determinar si ocurre siembra accidental o no intencional de cultivos transgénicos, ya que cuenta con un laboratorio para la detección de material transgénico en cultivos e informa a través de su página web sobre la bioseguridad de transgénicos (SEMARNAT, 2009).

La SAGARPA y la SEMARNAT son las encargadas de expedir permisos para siembra experimental de maíz genéticamente modificado en nuestro país, respetando la Ley de Bioseguridad Sobre Organismos Genéticamente Modificados. Los cultivos autorizados se mantienen en la fase de experimento y se harán en terrenos controlados y totalmente aislados de otro tipo de cultivos (por lo menos 500 metros respecto de otros cultivos), además de estar rodeados de mallas ciclónicas para evitar el fácil acceso a estas pequeñas parcelas y de marcar un aislamiento temporal después de un mes para evitar flujo génico a un posible maíz convencional. Otros aspectos de seguridad precisados para asegurar que no exista flujo de los cultivos transgénicos a los convencionales son la precisión de las coordenadas geográficas del cultivo, establecer una bitácora, instalar plantas de polen para que no pueda haber flujo por esta vía e incinerar el producto, puesto que está prohibido su acceso al mercado alimentario (SEMARNAT, 2009). El gobierno federal, a través de la SAGARPA se ha pronunciado sobre la inminente liberación de maíz transgénico en nuestro país; «No se permitirá la experimentación ni la liberación al ambiente de maíz genéticamente modificado que contenga características que impidan o limiten su uso o consumo humano o animal, o bien su uso en procesamiento de alimentos para consumo humano». Además, tanto SAGARPA como SEMARNAT han establecido que la liberación de maíz transgénico se hará de forma paulatina para determinar los posibles aspectos negativos asociados a este proceso de liberación. Por lo anterior, ha sido creado el Régimen Especial de Protección del Maíz, el cual, junto con la reglamentación sobre el uso de transgénicos creada en 2005 y mejorada en 2008, se cree que existen los elementos para transitar adecuadamente en el uso sustentable y responsable de la biotecnología de este producto tan importante desde el punto de vista alimentario y cultural (Diario Oficial de la Federación, 2009).

A pesar de los esfuerzos por regular la liberación de transgénicos en nuestro país, la asociación no gubernamental Greenpeace asegura contar con pruebas de casos de contaminación de maíz transgénico consumido por la población. Greenpeace ha detectado maíz transgénico en cultivares de Oaxaca, Chihuahua, Veracruz, Morelos, Durango, Estado de México, San Luis Potosí, Tlaxcala y Puebla (Greenpeace, 2009). Con lo anterior surge la necesidad de establecer controles estrictos que permitan que el uso de la biotecnología sea implementada de forma responsable para asegurar la sustentabilidad del campo y de la producción de alimentos.

Por otro lado, en 1984 surge en Japón (Ministerio de Salud Japonés) el término «alimento funcional», el cual se refiere a cualquier alimento en forma natural o procesada que además de poseer nutrimentos, contiene componentes adicionales que favorecen la salud, la capacidad física y el estado mental de

una persona. De esta manera se creó FOSHU (Foods for Specified Health Use) para regular el desarrollo de dichos alimentos. Actualmente, esta definición se ha extendido y se considera que un alimento es funcional cuando además de proveer nutrimentos para los procesos de mantenimiento y desarrollo del organismo, brinda un beneficio adicional que puede ser reducir el riesgo de enfermedades y/o promover un estado de salud óptimo. Dada la controversia sobre si cualquier alimento podría considerarse como funcional, el Centro de Información Internacional de Alimentos (IFIC) define a los alimentos funcionales como «aquellos productos a los cuales se adiciona un compuesto específico para incrementar sus propiedades saludables» y alimentos saludables como «aquéllos que en su estado natural o con un procesamiento mínimo que tienen compuestos con propiedades beneficiosas para la salud». De esta manera, se puede agrupar por un lado a los alimentos naturales como saludables y por otro a los alimentos funcionales como aquéllos que han sido modificados para aumentar sus propiedades saludables.

Hace poco menos de 10 años se acuña en los Estados Unidos el término «nutracéutico», el cual se refiere a aquellos componentes de los alimentos con propiedades bioactivas y que pueden extraerse de los mismos. En ocasiones se confunde el término nutracéutico y alimento funcional, una forma sencilla de diferenciarlos es a través de su presentación. El funcional es un alimento como tal (fruta, verdura, etc.) y el nutracéutico tiene una presentación farmacéutica como una tableta, cápsula o polvo.

Actualmente, existe un mayor interés respecto a la relación que existe entre la alimentación y el estado de salud ya que va más allá de una preocupación por prevenir problemas de desnutrición. Temas como la prevención del cáncer o las enfermedades cardiovasculares están íntimamente relacionados con el consumo de alimentos de origen vegetal; tal es el caso de los granos integrales, las frutas y las verduras. Este hecho se debe a que diversas investigaciones se han enfocado en el estudio de la relación entre el consumo de algunos alimentos y prevención de enfermedades. Estos trabajos de investigación demuestran que la presencia de fitoquímicos como los antioxidantes podría participar en la prevención de enfermedades. Aunque este tipo de compuestos no son indispensables para el organismo, su consumo aporta al organismo efectos quimiopreventivos sobre los procesos dañinos como el cáncer. Entre estos compuestos se encuentran los polifenoles, carotenoides, vitaminas antioxidantes (A, C y E), así como algunos otros fitoquímicos. Una gran cantidad de trabajos de investigación están relacionados al estudio de nuevos alimentos funcionales que permitirán la revalorización de una gran cantidad de productos, principalmente de cultivos agrícolas que habían sido abandonados o descuidados y que se sabe que proporcionan moléculas bioactivas capaces de tener efectos benéficos en la salud humana. Tal es el caso de algunos compuestos fenólicos presentes en frutos rojos como la granada, fresa, zarzamora, mora azul, jamaica, etc., a los que se les asocia una elevada capacidad antioxidante y efectos benéficos considerables en el ser humano. Por lo anterior, este tipo de trabajos de investigación apoya a la sustentabilidad de la cadena alimentaria, favoreciendo la diversificación de muchos productos.

Un nuevo término con incipiente impacto es el de «nuevos alimentos», el cual, según el Reglamento CE 258/97 del Parlamento Europeo, se refiere a todos los alimentos e ingredientes de los mismos que sean de reciente desarrollo, que no hayan sido utilizados para consumo humano de una forma significativa por parte de los ciudadanos de la Unión Europea antes del 15 de mayo de 1997. Todas estas tendencias permitirán que los productores encuentren más opciones y diversifiquen las aplicaciones en las que los alimentos sean usados de la forma más adecuada para buscar el beneficio social.

## ••• Seguridad alimentaria

Independientemente de las nuevas tendencias en el desarrollo de alimentos, de su origen y demás, es indiscutible que los seres vivos tenemos que alimentarnos para poder nutrirnos. Además, tanto el exceso como la falta de éstos podrían llevarnos a problemas de mala nutrición. La desnutrición por ejemplo, es un problema mundial multifactorial que incide negativamente sobre las seis características de la una dieta adecuada. Tales son los casos de los problemas en la calidad y de la inocuidad de los alimentos. Factores como el desabasto, la falta de producción, plagas, cambios climáticos, entre otros, influyen directamente en el tipo y la calidad del alimento que llegará a los hogares y por ende a la nutrición de un individuo, una comunidad o un país, por lo que la seguridad alimentaria puede verse afectada. Por ello, en el sentido más amplio, no sólo debemos preocuparnos por las necesidades que deben ser cubiertas para una nutrición individual, sino para la nutrición comunitaria e incluso para la nutrición internacional.

La preocupación por asegurar a los habitantes de una nación los alimentos necesarios cobró importancia cuando Malthus escribe en 1798 un ensayo donde se establece un crecimiento de la población dispar (más acelerado) respecto a la producción de alimentos y que, por lo tanto, la humanidad estaba condenada a sufrir sobrepoblación y escasez alimentaria. Es en esta publicación que se plantea por vez primera vez el paradigma de actualidad: La seguridad alimentaria (Camberos, 2000).

En la actualidad, el tema de seguridad alimentaria y de la producción sustentable de alimentos debe ser considerado como prioritario para México y para el resto de los gobiernos debido a la crisis mundial alimentaria. Esta crisis se ha dado como consecuencia del aumento en el precio de los alimentos, acaparamiento de productos y reducción de reservas alimentarias, así como del aumento de la población en condiciones de pobreza. En México la seguridad alimentaria y la nutrición se han visto afectadas por una constante serie de crisis económicas, políticas y sociales que afectan a las poblaciones más vulnerables de zonas rurales y urbanas (Barquera, 2001). Es por ello que, en momentos en que la crisis económica mundial domina, es imprescindible proteger a las explotaciones agrícolas vulnerables acechadas por los impactos negativos de esta crisis, explotaciones en las que habitan y trabajan el 70% de los hambrientos del mundo (OMS, 2009).

La inseguridad alimentaria trae consigo hambre y desnutrición, por lo que es nuestra obligación hacer hincapié en las trágicas consecuencias de la desnutrición, las cuales incluyen la muerte, discapacidades, retraso del desarrollo mental y físico y, como resultado, retraso del desarrollo socioeconómico nacional (Organización de las Naciones Unidas, 2004). Por ello, el acceso normal a cantidades suficientes de alimentos de buena calidad e inocuos, es esencial para una nutrición apropiada (OMS, 2009). Desafortunadamente, en nuestro país existe un gran número de familias en condiciones de inseguridad alimentaria, por lo que es necesario urgir a nuestro gobierno a combatir este problema, para lo cual es necesario promover y fortalecer una cultura de autoproducción de alimentos, que favorezca la buena nutrición de la población.

## ••• *El modelo de traspatios*

Una alternativa interesante para combatir la inseguridad alimentaria y favorecer su sustentabilidad, es el uso de sistemas de autoproducción de alimentos. Dentro de este marco se encuentra en modelo de traspatios, el cual consiste en la producción familiar de alimentos en el mismo sitio en el que dicha familia habita. Los patios de las casas son utilizados como huertos en los que las familias son capaces de cultivar sus productos de elección, además de que aquellos que no sean autoconsumidos, pueden ser comercializados a pequeña escala. Este sistema requiere de una adecuada extensión de tierra, por lo que su aplicabilidad se reduce a áreas rurales. El gobierno del Estado de Puebla, y los gobiernos de otros estados de nuestro país, apoyan este modelo que ha resultado ser positivo en otros lugares en donde se ha implementado. Este modelo favorece la sustentabilidad alimentaria, además asegura la preservación de una serie de aspectos sociales y culturales. Lo anterior combate una amenaza creciente en nuestro país, los cambios en la alimentación que implican cambios culturales, asociados a la falta de poder adquisitivo y pobreza. Podemos mencionar que existe un número creciente de comunidades rurales en la que las actividades agrícolas son abandonadas y el suministro de alimentos proviene de productos industrializados de dudosa calidad alimentaria, los cuales son incluidos como base de la dieta. Cada vez es más frecuente encontrar que poblaciones enteras usan como base de su dieta alimentos procesados como comida chatarra y comida instantánea porque «son más baratas», abandonando de su dieta aquellos alimentos que forman parte de sus tradiciones culturales y que son base de una buena alimentación. Por lo anterior, el modelo de traspatios asegura una continuidad en la producción de alimentos locales, propios de la región, los cuales han formado parte de la cultura de las personas que los producen y que pueden ser adecuados a dietas saludables.

## ••• Sustentabilidad alimentaria

Todos los aspectos asociados al desarrollo tecnológico implicados en la mejora de la productividad y rentabilidad en la sustentabilidad alimentaria deben ser, al menos, considerados como posibilidades. Dentro de las alternativas para evitar la inseguridad alimentaria está el incremento de la productividad a través de tecnologías alternativas como la biotecnología moderna, cuya aportación más reconocida son los alimentos transgénicos. Desafortunadamente, muchos todavía ignoran los beneficios asociados a la biotecnología moderna. Los modernos avances ofrecen una oportunidad considerable para dirigirse al mejoramiento de la sustentabilidad en la producción de alimentos. Sin embargo, es importante considerar que la sustentabilidad será inalcanzable si la biotecnología se considera como una solución milagrosa y remplaza los pilares clave, como un control integral de plagas o variedades adaptadas. La biotecnología debe ser considerada como parte de los sistemas tecnológicos interdependientes y convergentes, es decir, una herramienta poderosa. El 2005 marcó el décimo aniversario de la comercialización de las cosechas biotecnólogicas. La Conferencia sobre Medio Ambiente y Desarrollo de las Naciones Unidas en 1992 afirmó que la biotecnología

> promete hacer una contribución significativa al permitir el desarrollo de, por ejemplo, mejor cuidado de la salud, promover la seguridad alimentaria a través de prácticas agrícolas sustentables, mejores suministros de agua potable, procesos de desarrollo industrial para la transformación de materias primas de un modo más eficiente, apoyo de métodos sustentables de forestación y la detoxificación de desechos peligrosos.

Como ejemplo del impacto de la biotecnología en el desarrollo alimentario se puede mencionar que el número de países productores que aprobó las cosechas biotecnológicas de algodón, maíz, soya y canola, llegó a 21 en 2005. Catorce de estos países tienen áreas de cultivo con cosechas biotecnológicas mayores de las 50 000 hectáreas y 90 % de los agricultores que cultivan estas cosechas de los países desarrollados, eran de escasos de recursos (Clive, 2005).

La FAO ha reconocido que la ingeniería genética puede contribuir a elevar la producción y productividad en la agricultura, silvicultura y pesca, y que puede dar lugar a mayores rendimientos en tierras marginales de países donde actualmente no se pueden cultivar alimentos suficientes para alimentar a sus poblaciones. También se ha manifestado a favor de su aplicación para el mejoramiento nutrimental de alimentos, lo que mejora la salud de muchas comunidades de bajos ingresos.

En países como el nuestro, otras tecnologías de menor costo y accesibles como la tecnología de trasplantes deben estar disponibles a los pequeños productores sobre todo en regiones marginales y sobrepobladas, que a su vez son las que requieren más alimentos (Camberos, 2000). Otros sistemas que avalan la sustentabilidad alimentaria están asociados a la producción orgánica de

comestibles de consumo humano y animal, la cual permitirá asegurar la calidad de la alimentación y con ello la seguridad alimentaria.

La seguridad alimentaria debe considerar la soberanía alimentaria y la mejora en el acceso a los alimentos, en base a una política de desarrollo agropecuario que combine eficientemente la productividad y el bienestar, particularmente para las poblaciones rurales, encargadas del suministro de alimentos. Desafortunadamente, el problema de la seguridad alimentaria rebasa aspectos técnicos y tecnológicos y está al margen del desarrollo económico, debido a que las políticas agrícolas apuntan en una dirección y las de seguridad alimentaria en otra (Camberos, 2000).

Por lo anterior, los gobiernos deben considerar que para que el desarrollo sea sustentable, se deben satisfacer las necesidades actuales sin comprometer las necesidades futuras, esto es, las de las generaciones venideras. Dentro de los indicadores sociales para medir la sustentabilidad de los alimentos están el estado nutricional de la población infantil, peso suficiente al nacer, tasa de mortalidad infantil bajo los cinco años, esperanza de vida al nacer y la tasa de mortalidad derivada de la maternidad (Organización de las Naciones Unidas, 2004). Estos indicadores se asocian directa o indirectamente a la seguridad alimentaria, la cual a su vez se relaciona con la alimentación sustentable.

Otros aspectos relacionados son la manutención de la calidad y el estado de salud de los suelos, su fertilidad. Los sistemas de sustentabilidad deben velar por preservar las condiciones de salud de las tierras para no comprometer la productividad de las mismas. Otros factores igualmente importantes para garantizar la sustentabilidad alimentaria son la calidad del agua, la calidad de vida de los productores y el equilibrio social, cultural y económico entre los productores y los consumidores.

La seguridad alimentaria se podrá alcanzar cuando toda la gente, en todo momento, tenga acceso físico y económico a alimentos seguros, nutritivos y suficientes para alcanzar sus necesidades dietarias y preferencias alimentarias para desarrollar una vida activa y saludable (Reyes y Atalha, 2006).

## ••• Conclusiones y perspectivas

Los nuevos y más recientes hallazgos y tecnologías emergentes pueden constituir una alternativa válida para apoyar la sustentabilidad alimentaria, aunque es necesario que las políticas y acciones relacionadas a su implementación se haga de forma responsable y escuchando a los especialistas en las correspondientes áreas. No debe permitirse que las presiones generadas por consorcios poderosos influyan en la toma de decisiones debido a los grandes intereses económicos en los que el tema está envuelto. Alternativamente, existen mecanismos que pueden garantizar el suministro alimenticio usando tecnologías modernas, no convencionales, aunque se requieren apoyos gubernamentales destinados a la investigación en éstas áreas. Sin embargo, la parte medular radica en los apoyos al campo y a las personas que lo trabajan. Es indispensable modificar las conductas y prejuicios respecto a la concepción que se

tiene sobre las personas dedicadas al trabajo en el campo. Las personas que habitan zonas rurales deben ser dotadas de tecnología y herramientas educativas necesarias para garantizar el desarrollo sustentable del campo. También deben equilibrarse los aspectos socioculturales entre productores y consumidores, disminuyendo la influencia de intermediarios, quienes son realmente los beneficiados desde el punto de vista económico. La implementación de cultivos de traspatio puede ser una alternativa para mantener la integridad genética de los productos cultivados, además de garantizar el uso del suelo con fines alimentarios y preservar la carga cultural asociada al cultivo de ciertos productos. Este sistema, también contribuye a la diversificación de los cultivos en función de las propias necesidades y su comercialización sin la amenaza de sobreexplotación del suelo y de la intermediación. Los gobiernos de todo el mundo están obligados a asegurar el suministro de alimento y las políticas establecidas deben de estar en consonancia con el abasto y el no desperdicio. Para todo ello se necesita invertir en ciencia y tecnología.

# Referencias


American Heart Association Science Advisory: Phytochemicals and cardiovascular disease (1997). Circulation, 95, 2591-2593.

Bourges, H. (2001). Orientación alimentaria: Glosario de términos. Cuadernos de Nutrición, 24(1), 7-32.

Balbás, P. (2002). De la biología molecular a la biotecnología. México: Trillas.

Barquera, S., Rivera, J. A. y Gasca, A. (2001). Políticas y programas de alimentación y nutrición en México. Sal Púb Mex, *43*, 1-14.

Barquera, S., Rivera, J. y Gasca, A. (2001). Políticas y programas de nutrición en México. Sal Púb Mex, 43, 5.

Barón, M.A., Liseti, R., Páez, M.C. y Pabón, M. (2007). Estado nutricional de hierro y parasitosis intestinal en niños de Valencia, Estado Carabobo, Venezuela. An Venez Nutr, 20(1), 5-11.

Camberos, M. (2000). La seguridad alimentaria de México en el año 2030. Ciencia Ergo Sum, 7(1), 49-55.

Cantrell, R.C. (2006). El papel de la biotecnología para mejorar la sustentabilidad del algodón. Consultado el 18 de marzo de 2009 en http://es.cottoninc.com/Sustainabilityes/BiotechnologyRoleImprovingCottonSustainability- es/BiotechnologyRoleImprovingCottonSustainability-es.pdf

Casanueva, E., Kaufer, M., Pérez, A.B. y Arrollo, P. (2008). Nutriología Médica. México: Fundación Mexicana para la Salud: Editorial Médica Panamericana.

Clive, J. (2005). Estado Global de las Cosechas Transgénicas Comercializadas: 2005 ISAAA Breviario 34.

Diario Oficial de la Federación (2009). Convenio de Coordinación para el Desarrollo Rural Sustentable, que celebran la Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación y el Gobierno del Distrito Federal. Consultado el martes 10 de febrero de 2009 en http://www.finanzas.df.gob.mx/transparencia/docs/4DOF_SEDEREC.pdf

Gibson, R.S. (2005). Principles of nutritional assessment. Nueva York: Oxford University Press.

Greenpeace (2009). Guía roja y verde de alimentos transgénicos. Consultado el 23 de marzo de 2009 en http://www.greenpeace.org/raw/content/espana/reports/gu-a-roja-y-verde.pdf

Halverson, B.L., Holte, K., Myhrstad, M.C.W., Barikmo, I., Hvattum, E., et al. (2002). A systematic screening of total antioxidants in dietary plants. J Nutr, 132, 461-471.

Hasler, C.M. (2002). Functional foods: benefits, concerns and challenges-A position paper from the American Council on Science and health. J Nutr, *132*, 3772-3781.

National Center for Health Statistics (1994). Consensus workshop on dietary assessment: nutrition monitoring and tracking the year 2000 objetives. Hyattsville, Maryland: Departament of Health and Human Services.

Milner, J.A. (2000). Functional foods: the US perspective. Am J Clin Nutr, *71*, 1654S- 1659S.

Murakami, A., Koshimizu, K. y Ohigashi, H. (1998). Chemoprevention with food phytochemicals: screening, rodent studies, and action mechanisms. J Med Food, *1*, 29-38.

Olaiz, G., Rivera, J., Shamah, T., Rojas, R., Villalpando, S., et al. (Eds.). (2006). Encuesta Nacional de Salud y Nutrición. Cuernavaca, México: Instituto Nacional de Salud Pública.

Organización Mundial de la Salud (2009). Día Mundial de la alimentación. Consultado el 4 de marzo del 2009 en http://www.fao.org/getinvolved/worldfoodday/worldfoodday-history/worldfoodday-09/es/

Organización Mundial de la Salud (2003). Dieta, nutrición y prevención de enfermedades crónicas, Informe de una consulta Mixta de Expertos OMS/FAO (916). Ginebra, Suiza. Consultado el 6 de marzo de 2009 en http://www.nutrinfo.com.ar/pagina/e-books/dnptsp.pdf

Organización de las Naciones Unidas (2004). Indicadores de los aspectos sociales del desarrollo sostenible. Consultado el 10 de febrero de 2009 en http://www.un.org/esa/sustdev/natlinfo/indicators/indisd/spanish/social.htm

Perez, A.B., Palacios, B. y Castro, A.L. (2008). Sistema Mexicano de Alimentos Equivalentes. México: Fomento de Nutrición y Salud.

Reyes, J.M. y Atalah, S.E. (2006). Intervención nutricional en prevención de enfermedades cardiovasculares: el caso de Noruega. Rev. chil. Nutr, *33*(3), 464-472 2006.

Secretaría de Medio Ambiente y Recursos Naturales (2009). Boletín SEMARNAT 13109. Consultado el 15 de febrero de 2009 en http://www.semarnat.gob.mx/informacionambiental/noticias/boletindeprensa/Pages/BOLETIN SEMARNAT 13109.aspx

Vásquez, E.M., Romero, E., Nápoles, F., Nuño, M.E., Trujillo, F., et .al. (2002). Prevalencia de deficiencia de hierro y yodo, y parasitosis en niños de Arandas, Jalisco, México. Sal Púb Mex 44, 195-2002.

Verdú, J.M. (2009). Tratado de Nutrición y Alimentación, situaciones fisiológicas y patológicas. Barcelona: Editorial Océano/ Ergon.

# Anexo III

## Presentaciones en Congresos y Estancias

## PRESENTACIONES EN CONGRESOS Y ESTANCIAS

Estancias Académicas

-Centro de Investigación Biomédica de Oriente CIBIOR, Metepec, Puebla, 2010-2013.

- Centro de Nanociencias y Nanotecnología de la UNAM (CNYN), Ensenada, BCN, Julio del 2012.

Posters y Presentaciones Orales

De este trabajo se derivaron 10 posters y 5 presentaciones orales, las cuales se enlistan a continuación.

**Poster 1**. Nanoalimentos funcionales con análogos de vitamina E: actividad anticancerígena in vivo.

Primer lugar del concurso de poster del 5to Congreso Nacional de Nutrición LINUAP. 22 de Nov del 2013, Puebla, Puebla, Méx.

X. Fernández Garibay[1], L. Minjarez Espinoza[1], S. Valerino Perea[1], L. Ramírez[1], F. Vázquez Luna[1], A. Espinoza Sanchez[1], F. Arteaga Cardona[1], M.A. Méndez Rojas[1], T. Palacios Hernández[1,2], G.A. Hirata Flores[3], O.E. Contreras[3], S. Hernández Gutiérrez[4], J. Reyes Leyva[5], J. Hernandez[6], A. Angulo Molina[1,5,6]

[1]Universidad de las Américas Puebla, UDLAP, Puebla, México; [2]Universidad Popular Autónoma del Estado de Puebla, UPAEP, Puebla, México; [3]Centro de Nanociencias y Nanotecnología, CNYN, Ensenada, México, [4]Universidad Panamericana; [5]Centro de Investigaciones Biomédicas de Oriente CIBIOR, IMSS, Metepec, Puebla, México; [6]Investigación en Alimentación y Desarrollo, CIAD, AC, Hermosillo, México.

**Poster 2**: Cytotoxic and Antitumor Effect of Vitamin E Analogues Functionalized to Magnetite Nanoparticles.

A. Angulo Molina [1, 2, 3], J. Reyes Leyva[3], J. Hernández[1], T. Palacios Hernández [2, 4], M.A. Méndez Rojas[2], M. Cerro López[2], J. Flores[3], F. Ruiz[5], O.E. Contreras[5] and G. A. Hirata Flores[5].

[1] Centro de Investigación en Alimentación y Desarrollo, CIAD, AC, Hermosillo, México; [2] Universidad de las Américas Puebla, UDLAP, Puebla, México; [3]Centro de Investigaciones Biomédicas de Oriente CIBIOR, IMSS, Metepec, Puebla, México; [4]Universidad Popular Autónoma del Estado de Puebla, UPAEP, Puebla, México; [5]Centro de Nanociencias y Nanotecnología, CNYN, Ensenada, México.

Presentado en: The SOT (Society of Toxicology) 52nd Annual Meeting and ToxExpo, San Antonio, Texas. Marzo 10-14, 2013

**Poster 3**: Synthesis, Characterization and Evaluation of Biological In Vitro Activity of Eu3+ Doped Hydroxyapatite nanoparticles.

J. Delgado-Jimenez[1], T. D. Palacios-Hernandez[2], A. Angulo Molina[1], J. L. Varela[3], R. Agustin[3] and E. Rubio-Rosas[3].

[1]Ciencias Químico-Biológicas, UDLAP, Puebla, México; [2]Ciencias Biológicas, UPAEP, Puebla, México; [3]Centro de Vinculación Universitaria y Transferencia de Tecnología, BUAP, Puebla, México.

Presentado en: The SOT (Society of Toxicology) 52nd Annual Meeting and ToxExpo, San Antonio, Texas. Marzo 10-14, 2013

**Poster 4:** Effect of Surface Modification of Metal Oxide Nanoparticles upon Cell Viability and Genotoxicity of Epithelial Breast Cells.

T. D. Palacios-Hernandez[1], E. Gonzalez[2], M. A. Mendez[3], G. A. Hirata[4], D. Momot[5], E. E. Hernandez[5], A. Marogi[5], M. Poirier[5], A. Angulo Molina [3], O. Olivero[5] and R. E. Cachau[6].

[1]Biological Sciences, UPAEP, Puebla, Mexico; [2]Chemistry Center, ICUAP, Puebla, Mexico; [3]Chemical and Biological Sciences, UDLAP, Puebla, Mexico; [4]Nanosciences and Nanotechnology Center, UNAM, Ensenada, Mexico; [5]National Cancer Institute, NIH, Bethesda, MD; [6]Frederick National Laboratory of Cancer Research, Frederick, MD.

Presentado en: The SOT (Society of Toxicology) 52nd Annual Meeting and ToxExpo, San Antonio, Texas. Marzo 10-14, 2013

**Poster 5:** Efecto anticancerígeno de nanopartículas de magnetita funcionalizadas con $\alpha$-tocoferil succinato.

A. Angulo Molina[1,2,3], J. Reyes Leyva[3], J. Hernández[3], T. Palacios Hernández[2,4], M.A. Méndez Rojas[2], M. Cerro López[2], Y. Brito Barrera[2], G.A. Hirata Flores[5], O.E. Contreras López[5], F. Ruiz Medina[5], J.C. Flores Alonso[3], L. Flores Mendoza[3].

[1]Centro de Investigación en Alimentación y Desarrollo, CIAD, AC, Hermosillo, Mexico; [2]Universidad de las Américas Puebla, UDLAP, Puebla, Mexico; [3]Centro

de Investigaciones Biomédicas de Oriente CIBIOR, IMSS, Metepec, Puebla, Mexico; [4]Universidad Popular Autónoma del Estado de Puebla, UPAEP, Puebla, Mexico; [5]Centro de Nanociencias y Nanotecnología, CNYN, Ensenada, México.

Presentado en: XXII Jornadas interinstitucionales de investigación en salud del Estado de Puebla. Febrero 6-8, 2013.

**Poster 6:** Cáncer, un acercamiento teragnóstico con nanopartículas luminiscentes.

A. Angulo Molina[1,2,3], T. Palacios Hernández[2,4], G.A. Hirata Flores[5], S. Hernández Gutiérrez[6], J.A. Flores Alonso[3], X. Fernández Garibay[2], L. MinjarezEspinoza[2] y F. Arteaga Cardona[2].

[1]Centro de Investigación en Alimentación y Desarrollo, CIAD, AC, Hermosillo, México; [2]Universidad de las Américas Puebla, UDLAP, Puebla, México; [3]Centro de Investigaciones Biomédicas de Oriente CIBIOR, IMSS, Metepec, Puebla, México; [4]Universidad Popular Autónoma del Estado de Puebla, UPAEP, Puebla, México; [5]Centro de Nanociencias y Nanotecnología, CNYN, Ensenada, México. Universidad Panamericana UP, México, DF.

Presentado en: XXII Jornadas Interinstitucionales de investigación en salud del estado de Puebla. Febrero 6-8, 2013.

**Poster 7.** Magnetite nanoparticles functionalized with alpha tocopheryl succinate: cytotoxicity and antitumor effect in breast cancer cells.

A. Angulo Molina [1, 2, 3], J. Reyes Leyva[2], J. Hernández[3], T. Palacios[1], M. A. Méndez Rojas[1], M. Cerro López[1] and O. Olivero[4].

[1]Químico Biológicas-Nutrición, Universidad de las Américas Puebla, Puebla, Puebla, México, [2]Centro de Investigaciones Biomédicas de oriente CIBIOR,

IMSS, Metepec, Puebla, México, [3]Nutrición, CIAD, Hermosillo, Sonora, México and [4]Laboratory of Cancer Biology and Genetics, National Cancer Institute, Bethesda, MD.

Presentado en: The SOT (Society of Toxicology) 51st Annual Meeting & ToxExpo. March 11-15, 2012. San Francisco, California.

**Poster 8.** Magnetite nanoparticles functionalized with alpha tocopheryl succinate: citotoxicity and antitumor effect in breast cancer cells.

A. Angulo Molina[1,2,3], J. Reyes Leyva[2], J. Hernández[3], T. Palacios[1], M. Méndez Rojas[1], M. Cerro López[1], and O. Olivero[4].

[1]Universidad de las Américas Puebla, Puebla, México; [2]Centro de Investigaciones Biomédicas de oriente CIBIOR, IMSS, Metepec, Puebla, México; [3]Nutrición, CIAD, Hermosillo, Sonora, México; and [4]Laboratory of Cáncer Biology and Genetics, National Cáncer Institute, Bethesda, MD.

Presentado en: NanoMex. Junio 13-15, 2012. Puebla, Puebla.

**Poster 9:** Efecto anticancerígeno de nanopartículas de magnetita funcionalizadas con $\alpha$-tocoferil succinato.

A. Angulo Molina[1,2,3], J.R Reyes Leyva[3], J. Hernández[2], T. Palacios Hernández[1,4], M.A. Méndez Rojas[1], M. Cerro López[1], Y. Brito Barrera[1], G.A. Hirata Flores[5], O.E. Contreras López[5], F. Ruiz Medina[5], J.C. Flores Alonso[3], L. Flores Mendoza[3]

[1]Centro de Investigación en Alimentación y Desarrollo, CIAD, AC, Hermosillo, México; [2]Universidad de las Américas Puebla, UDLAP, Puebla, México; [3]Centro

de Investigaciones Biomédicas de Oriente CIBIOR, IMSS, Metepec, Puebla, México; [4]Universidad Popular Autónoma del Estado de Puebla, UPAEP, Puebla, México; [5]Centro de Nanociencias y Nanotecnología, CNYN, Ensenada, México.

Presentado en: XXI foro nacional de investigación en salud. IMSS, 2012. Oaxtepec, Morelos.

**Poster 10:** Establecimiento de un modelo de cáncer murino por xenoinjerto para evaluación anticancerígena de factores nutrimentales.

R. Carrasco Macías [1] y A. Angulo Molina [1,2]
[1]Universidad de las Américas Puebla, [2] Centro de Investigación en Alimentación y Desarrollo, AC.

Primer lugar en el consurso de carteles del 2do Congreso de Nutrición de la Asociación LINUAP (Licenciados en Nutrición Asociados en Puebla, AC) 2010.

## Presentaciones Orales

1: Nanoalimentos funcionales en cáncer: citotoxicidad in vitro e in vivo. Presentado en IX Congreso Nacional de Toxicología, Nuevo Vallarta Nayarit. 8 de Nov del 2013

2: Nanomedicamentos: implicaciones y riesgos.
Presentado en: 7mo Congreso internacional de farmacovigilancia. Puebla, Puebla. 27 de Junio del 2013.

3: Functional nanofood: synthesis, characterization and nanotoxic effects in cancer cells.

Presentado en: Simposium "Nanoparticles and oxidative strees". IV congreso de especies reactivas del oxígeno en biología y medicina, de la sociedad mexicana de bioquímica. Querétaro, marzo 19 del 2013.

4: Nanobiotecnología y su aplicación en el desarrollo de agentes teragnósticos. Presentado en: Congreso de Biotecnología de la Universidad Popular del Estado de Puebla (UPAEP); Marzo 13, 2013

5: Nanobiotecnología y combate al hambre.

Presentado en: Encuentro Interinstitucional por el día mundial de la alimentación. Octubre 15-16, 2012. Puebla, Puebla. Tecnológico de Monterrey.

6. Magnetite nanoparticles used as nanocarriers of vitamin E analogues.

Presentado en: 5th international Congress Food science and food biotechnology in developing countries, Noviembre 2012. Nvo. Vallarta, Nayarit.