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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISIÓN DE NUEVAS CREACIONES
E. _____ S. _____ D. _____

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 09-035922- -00006-0000

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Re: Expediente No. 09-035922
Solicitud de Patente de Invención Titulada: "PARTÍCULAS DE NÚCLEO DIVIDIDO PARA LA PRESENTACIÓN DE MOLÉCULAS EXTRAÑAS, ESPECIALMENTE PARA LA APLICACIONES DE VACUNAS, Y MÉTODOS PARA PRODUCIRLAS".
Solicitante: UNIVERSITÄTSKLINIKUM.
Nuestra Ref.: P2009/000043

RESPUESTA AL OFICIO No. 14001 NOTIFICADO POR FIJACIÓN EN LISTA
(RESPUESTA ARTÍCULO 45)

J. IAN RAISBECK, mayor de edad y vecino de Bogotá, D.C., identificado tal como aparece al pie de mi firma, en mi carácter de apoderado de la sociedad solicitante, me permito manifestar lo siguiente ante el auto emanado por ese Despacho, dentro del periodo de prórroga previsto en el artículo 45 de la Decisión 486, en los siguientes términos:

1. Con la finalidad de dar mayor claridad a la materia reivindicada me permito adjuntar un nuevo **Capítulo Reivindicatorio Modificado** que remplaza en su totalidad al Capítulo Reivindicatorio actualmente vigente.

Nueva Reivindicación	Antigua Reivindicación	Modificación
6	6	Se elimina la característica en la que el eucariota patogénico es el patógeno causante de la malaria, <i>Plasmodium falciparum</i> .
7	N/A	Se agrega una nueva Reivindicación 7 en la cual se incluye la característica en la que el patógeno eucariota patogénico es el patógeno causante de la malaria, <i>Plasmodium falciparum</i> , que se encontraba en la antigua Reivindicación 6.
9	8	Se elimina la característica de la proteína particular, mencionada en la antigua

		Reivindicación 8.
10	N/A	Se agrega una nueva Reivindicación 10, donde se incluye la característica de la proteína particular, mencionada en la antigua Reivindicación 8.
1, 5, 6, 8, 9, 11, 12, 13, 16	1, 5, 6, 7, 8, 9, 10, 11, 15	El término "molécula" fue remplazado por "secuencia peptídica o proteica" y se modificaron las dependencias.
14, 15, 17, 18, 19	12, 13, 17, 16, 17	Las antiguas reivindicaciones fueron reenumeradas y se modificaron las dependencias.

Vale la pena anotar que las modificaciones efectuadas en el Capítulo Reivindicatorio cumplen a cabalidad con lo estipulado en el Artículo 34 de la Decisión 486 de la Comunidad Andina de Naciones, ya que estas no implican de ninguna manera una ampliación de la protección correspondiente a la divulgación contenida en la solicitud inicial. Se adjunta recibo de pago por 9 Reivindicaciones adicionales.

2. a- El Examinador establece que el Capítulo Reivindicatorio carece de claridad.

b- Con la finalidad de superar las objeciones del Examinador, se adjunta un nuevo Capítulo Reivindicatorio Modificado.

3. a- El Examinador establece que la denominación "proteína extraña" carece de claridad.

b- Respetuosamente difiero de la objeción del Examinador, ya que la palabra proteína es totalmente clara para una persona medianamente versada en el arte.

4. a- El Examinador cita el documento "Hepatitis B virus capsid-like particles can display the complete dimeric outer surface protein C and stimulate production of protective antibody responses against *Borrelia burgdorferi* infection" (D1) como el documento del arte previo más cercano. El Examinador establece que D1 divulga el uso de una proteína nuclear del virus de la hepatitis B para ser usado como el transportador en una vacuna contra la bacteria *Borrelia sp*, aprovechando la formación de partículas como

cápside que hace esta proteína. Adicionalmente, el Examinador establece que para la construcción de la proteína de fusión se utiliza el dímero de la proteína nuclear del virus de la hepatitis B y se inserta entre éste, la proteína de la superficie externa OspC de la bacteria. Por otra parte, el Examinador menciona que D1 evidencia la actividad de la proteína transportadora de antígenos de la proteína nuclear del virus de la hepatitis B, la producción de anticuerpos contra la proteína OspC que era el antígeno transportado y el potencial de la molécula para ser usado en vacunas. De esta forma, el Examinador concluye que por medio de D1 se puede desarrollar un sistema portador de núcleo dividido como el de la presente invención, usándolo con diferentes antígenos, por lo tanto, la presente invención carece de novedad.

b- Respetuosamente difiero del análisis del Examinador, y señalo que D1 fue citado previamente en la descripción de la presente invención. El Examinador en su análisis no ha considerado la principal diferencia de la técnica anterior respecto de la presente invención. De hecho, D1 revela una construcción en la que el polipéptido extraño se inserta en la proteína del núcleo viral (véase, por ejemplo, figura 2, página 17477). La proteína extraña (OspC) está unida en ambos extremos a la proteína portadora. Contrariamente a esto, el objeto de la presente solicitud es un sistema portador de núcleo dividido en el que la secuencia peptídica o proteica extraña se une o bien al C-terminal del dominio N del núcleo o al extremo N-terminal del dominio C del núcleo. Este principio se ilustra, por ejemplo, en la figura 2 de la presente solicitud. Lo anterior no es anticipado de ninguna manera por D1. Por lo tanto, la presente invención cumple con el requisito de novedad.

c- Con la finalidad de dar mayor sustento en favor de la presente invención, se adjuntan dos documentos: una declaración del Dr. Michael Nassal, uno de los inventores de la presente invención, explicando los efectos superiores del sistema de núcleo dividido reivindicado y el documento no-patente (artículo) al cual hace referencia el Dr. Nassal, el cual fue publicado en la Revista Nature, donde también se demuestran los efectos superiores obtenidos mediante el sistema reivindicado. De hecho, el documento demuestra que la presente solicitud provee por primera vez proteínas divididas como portadoras en la construcción de VLPs y/o CLPs quiméricos. Lo anterior no es anticipado ni sugerido por el arte previo citado, con lo cual una persona versada en la materia, disponiendo de las enseñanzas del estado de la técnica, no podría derivar de manera evidente la presente invención.

5. Teniendo en cuenta lo anterior, solicito se proceda a conceder la patente de invención. En caso de subsistir cualquier objeción, solicito se emita un nuevo examen con el ánimo de corregirla.

Atentamente,



J. IAN RAISBECK
C.C. 79'376.996 de Bogotá D.C.
T.P. 135.799 del C. S. de la J.

Anexos:

- Capítulo Reivindicatorio Modificado.
- Declaración del Dr. Michael Nassal.
- Artículo titulado: "SliptCore: An exceptionally versatile viral nanoparticle for native whole protein display regardless of 3D structure."
- Recibo de pago No. 12-141014 por concepto de 9 reivindicaciones adicionales.

REIVINDICACIONES

1. Un sistema portador de núcleo dividido que, como polipéptidos separados, tiene el dominio nuclear N y el dominio nuclear C de la proteína nuclear de un virus de la hepatitis B, y al menos una secuencia peptídica o proteica extraña contra la cual ha de provocarse una respuesta inmune, donde dicha secuencia peptídica o proteica extraña está fusionada al extremo C del dominio nuclear N o el extremo N del dominio nuclear C, donde la proteína nuclear es capaz de unirse para formar partículas similares a cápsides, y donde la secuencia peptídica o proteica extraña está fusionada a un aminoácido de la proteína nuclear que está localizado entre las posiciones 73 y 94 de la proteína nuclear.

2. El sistema portador de núcleo dividido reivindicado en la reivindicación 1, **caracterizado** porque el virus de la hepatitis B es específico para mamíferos.

3. El sistema portador de núcleo dividido reivindicado en la reivindicación 2, **caracterizado** porque la proteína nuclear tiene la secuencia de un virus de la hepatitis B humano.

4. El sistema portador de núcleo dividido reivindicado en la reivindicación 2, **caracterizado** porque la proteína nuclear tiene la secuencia de aminoácidos del WHV.

5. El sistema portador de núcleo dividido de acuerdo con cualquiera de las reivindicaciones 1 a 4, **caracterizado**

porque la secuencia peptídica o proteica extraña es la secuencia de aminoácidos de una proteína de una bacteria patogénica.

6. El sistema portador de núcleo dividido de acuerdo con cualquiera de las reivindicaciones 1 a 4, **caracterizado** porque la secuencia peptídica o proteica extraña es la secuencia de aminoácidos de una proteína de un eucariota patogénico.

7. El sistema portador de núcleo dividido de acuerdo con la reivindicación 6, **caracterizado** porque el eucariota patogénico es el patógeno causante de la malaria, *Plasmodium falciparum*.

8. El sistema portador de núcleo dividido de acuerdo con cualquiera de las reivindicaciones 1 a 4, **caracterizado** porque la secuencia peptídica o proteica extraña es la secuencia de aminoácidos de una proteína de un virus.

9. El sistema portador de núcleo dividido de acuerdo con cualquiera de las reivindicaciones 1 a 4, **caracterizado** porque la secuencia peptídica o proteica extraña es la secuencia de aminoácidos de una proteína que tiene su propio potencial basado en patogenicidad.

10. El sistema portador de núcleo dividido de acuerdo con la reivindicación 9, **caracterizado** porque la proteína es una proteína marcadora tumoral.

11. El sistema portador de núcleo dividido de acuerdo

con cualquiera de las reivindicaciones 1 a 4, **caracterizado** porque la secuencia peptídica o proteica extraña se selecciona del grupo que consiste en

- a) la secuencia del péptido aceptor de biotina
- b) la secuencia del péptido ACID
- c) la secuencia del dominio Z33 derivado de la proteína A
- d) la secuencia del dominio GB1 de la proteína G.

12. Un método para preparar un portador para péptidos o proteínas extrañas, contra las cuales ha de inducirse una respuesta inmune, donde el portador, primero, tiene la secuencia proteica del antígeno nuclear de un virus de la hepatitis B, y segundo, tiene una secuencia peptídica o proteica heteróloga,

donde la secuencia peptídica o proteica heteróloga está fusionada con el dominio nuclear N o el dominio nuclear C, después de la posición 73 o antes de la posición 94 de la región nuclear de la hepatitis B, y

donde la cadena polipeptídica del portador está interrumpida después del dominio nuclear N o antes del dominio nuclear C, **caracterizado** porque comprende

expresar el dominio nuclear N y la secuencia peptídica o proteica heteróloga, o el dominio nuclear C y la secuencia peptídica o proteica heteróloga, como proteínas de fusión por separado,

o insertar una secuencia de reconocimiento para una proteasa

entre el núcleo N y la secuencia peptídica o proteica heteróloga, o entre la secuencia peptídica o proteica heteróloga, y el núcleo C, y cortar la vacuna de núcleo dividido con una proteasa, para obtener dos polipéptidos, después de la expresión pero antes de su uso, y formar partículas similares a cápsides.

13. El método reivindicado en la reivindicación 12, **caracterizado** porque la secuencia peptídica o proteica heteróloga se une por fusión después del aminoácido 74 o antes del aminoácido 85 del antígeno nuclear de la hepatitis B.

14. El método de acuerdo con cualquiera de las reivindicaciones 12-13, **caracterizado** porque la secuencia de la proteína extraña heteróloga tiene al menos 40 aminoácidos de longitud.

15. El método reivindicado en la reivindicación 12, **caracterizado** porque la secuencia de la proteína extraña heteróloga tiene al menos 120 aminoácidos de longitud.

16. El método de acuerdo con cualquiera de las reivindicaciones 12-15, **caracterizado** porque se expresan dos polipéptidos separados con la ayuda de un vector bicistrónico, donde el primer polipéptido comprende el dominio nuclear N y el segundo polipéptido comprende el dominio nuclear C, y donde la secuencia peptídica o proteica heteróloga está fusionada al extremo C del dominio nuclear N

o el extremo N del dominio nuclear C.

17. Un medicamento que tiene partículas similares a cápsides, **caracterizado** porque dichas partículas tienen un sistema portador de núcleo dividido de acuerdo con cualquiera de las reivindicaciones 1 a 11.

18. El medicamento reivindicado en la reivindicación 17, **caracterizado** porque es una vacuna.

19. Un agente de diagnóstico que tiene partículas similares a cápsides, **caracterizado** porque dichas partículas tienen un sistema portador de núcleo dividido de acuerdo con cualquiera de las reivindicaciones 1 a 10.

PATENT/OFFICIAL

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Michael NASSAL et al. : **Confirmation No.:** 2936
: :
Serial No.: 12/440,454 : **Group Art Unit:** 1648
: :
Filing Date: May 5, 2009 : **Examiner:** A. BOESEN

For: SPLIT-CORE-PARTICLES FOR THE PRESENTATION OF FOREIGN
MOLECULES, ESPECIALLY FOR VACCINE APPLICATIONS AND METHOD
FOR THEIR PRODUCTION

International Application No.: PCT/EP2007/006190 filed July 12, 2007

Attorney Docket Number: LNK-048

MAIL STOP AMENDMENT
Honorable Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. 1.132

I, Michael NASSAL, do hereby declare and state that:

1. I am a co-inventor of the subject matter described and claimed in the above-referenced patent application. As such, I am familiar with the above-referenced patent application, including the pending claims and the office action of June 1, 2011.
2. I am also a co-author of the article "SplitCore: An Exceptionally Versatile Viral Nanoparticle for Native Whole Protein Display Regardless of 3D Structure" published by Scientific Reports in June, 2011 (see Walker, Andreas et al., *Scientific Reports*, 2011 June; 1(5) Supplement: 1-8).

3. The afore-mentioned 2011 "Split-Core" article illustrates the advantages obtainable by the present invention. In my informed opinion, the fact that the invention has been published in such a well-renowned journal speaks to the patentability of the split-core carrier system as presently claimed. Further to that position, I submit the following quotes from reviewers of the original manuscript that were included in an e-mail sent to me on Monday, March 7, 2011 from Marianne Manchester, member of the Editorial Board for Scientific Reports.

4. From Reviewer #1 (under the heading "Remarks to the Author"):

"This manuscript describes a **remarkable advance in the ability to decorate hepatitis B capsid-like-particles** (CLPs). The authors genetically split the capsid protein at the outer surface junction between helices that form the 4-helix bundle at the dimer symmetry axes of the CLPs. Co-expression of the two fragments in E.coli spontaneously assembles to form particles that appear to be CLPs comparable to those made when the intact subunits are expressed. The authors then proceed to utilize this newfound "split-core" assembly flexibility to produce a variety of vaccine candidates for lyme disease and malaria. Insertion of moderate to large protein antigens at the termini of either core lead to efficient assembly with the proteins displayed on the surface. The effectiveness of the antigenic epitopes depended on their orientation relative to the cores in some cases. This was particular evident when the Osp A antigenic protein for a lyme disease vaccine was expressed. When expressed at the C-terminus of the N-terminal core there was excellent production of neutralizing antibodies in the mouse model, but when expressed as the N-terminal addition for the C-terminal core there was almost no neutralization. Some very clever additional experiments were performed in which it was demonstrated that different fragments of green fluorescent protein could be attached to each core and they would spontaneously assemble in the context of the quaternary structure to form active GFP. Additional proteins could be added to the GFP fragment termini and they still assembled to provide a simultaneous labeled protein with further decoration. The paper is well written and the data well documented. **The results are of truly exceptional interest. This is an important breakthrough in the technology of CLP decoration and will have an important impact on the field.**" (emphasis added).

5. From Reviewer #2 (under the heading "Remarks to the Author"):

"The paper presents an outstanding contribution to the development of virus-like particle (VLP) technology, which is one of the most promising fields of biomedical nanotechnology including not only vaccinology, but also gene therapy. This is the first successful attempt of application of splitted proteins as carriers in the construction of chimeric VLPs and/or CLPs (core-like particles). The paper is technically sound at the utmost level. Research methodology is highly appropriate. Authors provided sufficient methodological detail, which allows reproduction of the experiments. The manuscript is written clearly and concisely. The claims of the paper are convincing. The authors are fair by their treatment of previous literature. The claims are appropriately discussed in the context of earlier findings. It is important that the study adopts not only human HBc, but also its non-human homologues and starts novel line of putative highly-specific biomedical products. In general, the presented approach paves a way for efficient commercial production of CLP-based vaccines in bacteria, overcoming drawbacks of eukaryotic cell-based vaccine production systems. There are no ethical concerns in the study. Scientific and applied content of the paper is highly relevant to the major ideology of the Journal. Concluding, the paper deserves publication in the Scientific Reports in its present form." (emphasis added)

6. From Reviewer #3 (under the heading "Remarks to the Author"):

"This is an exciting paper in which authors describe a novel and potentially very useful method for preparing HBV capsid-like antigen display platforms. By expressing the sequences from before and after the 'immuno-dominant loop' separately but from the same plasmid they can assemble capsids on which it is possible to display whole-protein antigens whose termini are not close and therefore cannot be displayed by mere insertion into the c/e1 loop. They demonstrate that it is possible to display sizable (>300 aa) proteins, particularly ones where the N- and C-termini are far apart, to display them in either orientation depending on which fragment they are attached to, and even to display more than one protein at the same time. They show that the displayed antigens are properly folded and can elicit high-titer neutralizing antibodies...The experimental methods are described in detail, the results bear out the claims and the manuscript is well organized, sufficiently referenced, and for the most part, succinct."

U.S. Serial No.: 12/440,454

Atty Docket No.: LNK-048

Declaration Under Rule 132: Michael NASSAL

7. Given the enthusiastic reception from these art-accepted experts, it is my informed opinion that one of ordinary skill in the art would find the presently claimed invention to be both adequately described and enabled and novel and non-obvious over the teachings of the prior art of record.
8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true. I further declare that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: Sep 20th, 2011


Dr. Michael NASSAL

EN LA OFICINA DE MARCAS Y PATENTES DE DE LOS ESTADOS UNIDOS

A solicitud de: Michael NASSAL et al.

: Confirmación No.: 2936

Serial No.: 12/440,454

: Unidad Grupo Técnico: 1648

Fecha de presentación: Mayo 5, 2009

: Examinador: A. BOESEN

Para: PARTÍCULAS DE NÚCLEO DIVIDIDO PARA LA VISUALIZACIÓN DE MOLECULAS EXTRAÑAS, ESPECIALMENTE PARA APLICACIÓN EN VACUNAS Y MÉTODO PARA SU PRODUCCIÓN.

Solicitud Internacional No.: PCT/EP2007/006190 presentada el 12 de julio de 2007

Número de Expediente del Apoderado: LNK-048

DESTINO DE CORREO MODIFICACIÓN

Honorable Comisionado de Patentes

P.O. Box 1450

Alexandria, VA 22313-1450

DECLARACIÓN BAJO EL 37 C.F.R. 1.132

Yo, Michael NASSAL, por la presente declaro y afirmo que:

1. Yo soy co-inventor de la materia objeto descrita y reivindicada en la solicitud de patente arriba referenciada. Como tal, estoy familiarizado con la solicitud de patente anteriormente referenciada, incluyendo las reivindicaciones pendientes y la acción oficial de junio 01 de 2011.
2. También soy co-autor del artículo "Núcleo dividido: Una Excepcionalmente Versátil Nano-partícula Viral para la Visualización de la Proteína Nativa Completa Independientemente de la estructura 3D" publicado por Scientific Reports en junio de 2011 (ver Walker, Andreas et al., *Scientific Reports*, 2011 June: 1(5) Supplement: 1-8).

3. El artículo antes mencionado "Núcleo dividido" de 2011, ilustra las ventajas obtenidas por la presente invención. En mi opinión informada, el hecho de que la invención haya sido publicada en una revista bien conocida habla de la patentabilidad del sistema portador de núcleo dividido como se reivindica actualmente. Como consecuencia de esta posición, presenté las siguientes citas de los críticos del manuscrito original que fueron incluidas en un e-mail enviado a mí el lunes, 07 de marzo de 2011, por Marianne Manchester, miembro del Consejo editorial de Scientific Reports.

4. Del Crítico #1 (bajo el título "Comentarios al Autor"):

"Este manuscrito describe un **notable avance en la capacidad para ornamentar partículas tipo cápside de hepatitis B** (CLPs). Los autores genéticamente dividen la proteína cápside en la unión de la superficie exterior entre hélices que forma el haz de 4 hélices a los ejes de simetría del dímero de la CLPs. La co-expresión de los dos fragmentos en *E. coli* ensambla espontáneamente para formar partículas que parecen ser CLPs comparables a aquellas hechas cuando las subunidades intactas se expresan. Los autores entonces proceden a utilizar esta recién descubierta flexibilidad de ensamblaje de "núcleo dividido" para producir una variedad de candidatos a vacuna para la enfermedad de Lyme y la malaria. La inserción de antígenos de proteína desde moderados a grandes en los extremos de cualquier núcleo lleva a un ensamble eficiente con las proteínas que aparecen en la superficie. La efectividad de los epítomos antigénicos depende de su orientación relativa a los núcleos en algunos casos. Esto fue particularmente evidente cuando la proteína antigénica Osp A para una vacuna para la enfermedad de Lyme fue expresada. Cuando se expresó en el extremo C-terminal del núcleo N-terminal hubo una excelente producción de anticuerpos neutralizantes en el modelo de ratón, pero cuando se expresó como la adición N-terminal para el

núcleo C-terminal casi no hubo neutralización. Algunos experimentos adicionales muy inteligentes se realizaron en los que se demostró que diferentes fragmentos de la proteína verde fluorescente se podrían unir a cada núcleo y que ellos harían espontáneamente el ensamble en el contexto de la estructura cuaternaria para formar GFP activa. Adicionalmente, podrían ser añadidas proteínas al fragmento terminal de la GFP y aún serían ensambladas para proporcionar una proteína etiquetada simultánea con una ornamentación adicional. El artículo está bien escrito y los datos bien documentados. **Los resultados son de interés verdaderamente excepcional. Este es un avance importante en la tecnología de ornamentación de CLP y tendrá un impacto importante en el campo.** (Resaltado adicionado).

5. Del Crítico #2 (bajo el título "Comentarios al Autor"):

"Este artículo presenta **una excepcional contribución al desarrollo de la tecnología de partículas tipo virus (VLP)**, que es uno de los más promisorios campos de la nanotecnología biomédica incluyendo no solo la vacunología, sino también la terapia génica. **Este es el primer intento exitoso de aplicación de proteínas divididas como portadores en la construcción de VLPs quiméricas y/o CLPs (partículas tipo núcleo)**. El artículo es técnicamente sólido al máximo nivel. La metodología de investigación es altamente apropiada. Los autores proporcionan suficientes detalles metodológicos, que permiten la reproducción de los experimentos. El manuscrito está escrito de forma clara y concisa. **Las pretensiones del artículo son convincentes**. Los autores son justos por su tratamiento de la literatura anterior. Las reivindicaciones son apropiadamente discutidas en el contexto de conclusiones anteriores. **Es importante que el estudio adopte no solo HBc humano, sino también sus homólogos no humanos e iniciar una nueva línea de productos biomédicos putativos altamente específicos. En general, el enfoque**

presentado allana el camino para la producción comercial eficiente de vacunas en bacterias basadas en CLP, superando los inconvenientes de los sistemas de producción de vacunas basadas en células eucariotas. No hay preocupaciones éticas en este estudio. El contenido científico y aplicado del artículo es altamente relevante para la ideología principal de la revista. Conclusión, el artículo merece su publicación en el Scientific Reports en su forma actual.” (Resaltado adicionado).

6. Del Crítico #3 (bajo el título “Comentarios al Autor”):

“Este es un artículo muy emocionante en el cual los autores describen un nuevo y potencialmente muy útil método para preparar plataformas que visualizan antígenos tipo cápside de HBV. Por medio de la expresión de secuencias de antes y después del ‘bucle inmuno dominante’ en forma separada pero desde el mismo plásmido ellos pueden ensamblar cápsides en los cuales es posible visualizar antígenos de proteína completa cuyos extremos no están cerrados y por lo tanto, no pueden ser visualizados por la mera inserción en el bucle c/e1. **Ellos demuestran que es posible visualizar proteínas considerables (>300 aa), particularmente unas donde el N- y C- terminales están muy separados, para visualizarlos en cualquier orientación dependiendo de a qué fragmento están unidos, e incluso para mostrar más de una proteína al mismo tiempo.** Ellos muestran que los antígenos visualizados están adecuadamente plegados y pueden obtener titulaciones elevadas de anticuerpos neutralizantes... Los métodos experimentales son descritos en detalle, los resultados confirman las afirmaciones y el manuscrito está bien organizado, suficientemente referenciado, y en su mayor parte sucinto.

SERIAL U.S. No.: 12/440,454

No. de Expediente del Apoderado: LNK-048

Declaración Bajo la Regla 132: Michael NASSAL

7. Dada la gran acogida de estos expertos aceptados en la técnica, es mi opinión informada que una persona con conocimientos medios en la materia encontraría la invención reivindicada en la actualidad tanto adecuadamente descrita y realizable, como novedosa y no obvia a partir de las enseñanzas de la técnica anterior al registro.

8. Por medio de la presente declaro que todas las afirmaciones hechas en este documento de mis propios conocimientos son verdaderas y que todas las declaraciones hechas con base en la información y creencias se tienen por verdaderas. Además, declaro que estas afirmaciones fueron hechas con el conocimiento de que las declaraciones intencionales falsas y similares son castigables con multa o encarcelamiento, o ambos, bajo la sección 1001 del Título 18 del Código de los Estados Unidos, y que dichas declaraciones intencionalmente falsas pueden poner en peligro la validez de la solicitud o de cualquier patente emitida al respecto.

Fecha: Septiembre 20 de 2011

Firma Ilegible

Dr. Michael NASSAL



SUBJECT AREAS:
NANOBIOTECHNOLOGY
PROTEIN DESIGN
STRUCTURAL BIOLOGY
VACCINES

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SplitCore: An exceptionally versatile viral nanoparticle for native whole protein display regardless of 3D structure

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Nanoparticles displaying native proteins are attractive for many applications, including vaccinology. Virus-based nanoparticles are easily tailored by genetic means, commonly by inserting heterologous sequences into surface-exposed loops. The strategy works well with short peptides but is incompatible with the structures of most native proteins, except those with closely juxtaposed termini. Here we overcome this constraint by splitting the capsid protein of hepatitis B virus, one of the most advanced and most immunogenic display platforms, inside the insertion loop (SplitCore). The split parts, coreN and coreC, efficiently form capsid-like particles (CLPs) in *E. coli* and so do numerous fusions to coreN and/or coreC of differently structured proteins, including human disease related antigens of >300 amino acids in length. These CLPs induced high-titer antibodies, including neutralizing ones, in mice. The concept was easily expanded to triple-layer CLPs carrying reporter plus targeting domains, and should be applicable to protein-based nanoparticle design in general.

Viral capsids are natural self-assembling nanoparticles amenable to genetic modification, with diverse applications in material science^{1,2} and biomedicine³ including vaccinology⁴. The icosahedral nucleocapsid of hepatitis B virus (HBV) is particularly well characterized in the latter aspect⁵. Authentic nucleocapsids are formed by 120 (triangulation number $T=4$; diameter ~34 nm) or to a lesser extent 90 dimers ($T=3$; diameter ~30 nm)⁶ of a single 183 amino acid (aa) core protein (HBc; reviewed in⁷); its N terminal ~140 aa are required for assembly^{8,9}, the capsid-internal C terminal domain (CTD) binds nucleic acids. In the largely α -helical fold of the assembly domain^{10–12}, a hairpin formed by helices $\alpha 3$ and $\alpha 4$ serves as dimerization interface (Fig. 1a); the resulting four-helix bundles protrude from the capsid surface as prominent spikes. The exposed loop connecting $\alpha 3$ and $\alpha 4$ is part of the immunodominant c/e1 B-cell epitope between aa 74–84 which evokes >90% of the anti-HBc response during HBV infection¹³. HBc particles are exceptionally immunogenic, likely due to the repetitive surface presentation of B cell epitopes¹⁴, the presence of potent T cell epitopes, plus their ability to act as T cell independent antigen¹⁵. These properties contribute to the potent immunogenicity enhancement experienced by foreign sequences displayed on HBc CLPs, especially if inserted into the most surface-exposed, yet sequence-internal, c/e1 loop; however, such insertions must not compromise assembly competence.

Display of small peptides in the c/e1 loop is well established¹⁶, and HBc CLPs presenting a peptide from the circumsporozoite protein (CSP) of the malaria agent *Plasmodium falciparum* have proven safe in a Phase I study¹⁷; results with CLPs presenting an influenza A virus peptide are soon to be expected (ClinicalTrials.gov Identifier: NCT00819013). However, one or a few epitopes account neither for the genetic diversity and adaptability of pathogens nor for immunological polymorphisms in vaccinees; furthermore, peptides wedged into the carrier may adopt new, antigenically irrelevant structures¹⁸. Displaying native whole proteins would overcome these limitations¹⁹ and provide new opportunities also for non-vaccine applications. Successful whole-protein display has first been reported for terminal fusions to small bacteriophage λ and T4 accessory proteins that bind to preformed capsids^{20,21}. However, due to their complexity these systems have not found wide-spread application. A much simpler access to protein-displaying particles was provided by our demonstration that HBc can present the 238 aa green fluorescent protein (GFP)²², as well as the outer surface protein C (OspC) of the Lyme disease agent *Borrelia burgdorferi* (Bb)²³, with those CLPs inducing neutralizing antibodies²⁴. Both proteins are exceptional in that their closely juxtaposed termini^{25,26} fit naturally into the c/e1 acceptor sites. In contrast, proteins with farther apart termini interfere with assembly, as exemplified by Bb OspA²⁷. Bridging the distance with long connecting linkers was only partially effective, and although the mostly irregular multimers evoked protective

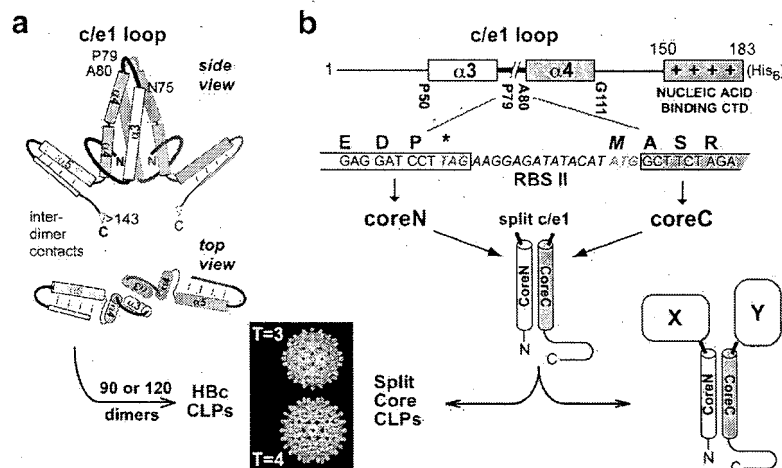


Figure 1 | Structural rationale of the SplitCore approach. (a) Scheme of the HBC assembly domain (aa 1–143), based on x-ray data (pdb:1QGT¹²). In the dimer (the second monomer is shown in grey), the $\alpha 3$ (white) - $\alpha 4$ (skyblue) hairpins associate into four-helix-bundles; the c/e1 epitope locates to the loop connecting $\alpha 3$ and $\alpha 4$. Helices $\alpha 1$ and $\alpha 2$ are omitted for clarity; $\alpha 5$ and the sequence to position 140 (orange) mediate multimerization of 90 or 120 dimers into T=3 and T=4 CLPs; cryo EM reconstructions are from ³⁴. The CTD (not shown) locates to the interior. (b) SplitCore concept. The HBC sequence was split inside c/e1 between P79 and A80 via artificial stop and start codons. Efficient co-expression in *E. coli* of coreN (aa 1–79) and coreC (aa 80–149 or 183) was achieved by bicistronic vectors with the 3' cistron controlled by a second ribosome binding site (RBS II), or via overlapping stop and start codons (e.g. Supplementary Fig. 2c). If the separate coreN and coreC fragments maintained the ability to assemble into HBC-like particles (left arrow), the resulting SplitCore CLPs would expose new termini on their surface, either of which could serve as one-sided, sterically unrestrained attachment site (right arrow) for heterologous molecules (X, Y).

antibodies ²⁸, structural heterogeneity and the unknown immunogenic potential of the linkers are undesirable for a vaccine.

Hence the conformational stress imposed on carrier and insert by the two-sided fixation emerged as key problem. Opening one of the two linkages should relieve this stress, as was corroborated by enhanced CLP formation by an HBC-OspA fusion upon site-directed cleavage via an engineered protease site ²⁹. However, the extra sequences and processing steps prompted us to seek a more general solution, leading to the “SplitCore” concept (Fig. 1b).

As in some non-multimerizing proteins ³⁰, fragment complementation of the separately expressed N and C terminal parts (coreN and coreC) of HBC split inside the c/e1 loop might yield CLP-forming split subunits. Foreign sequences fused to coreN and/or coreC would be attached to such CLPs via one end only and thus without imposing conformational stress, regardless of their structure (Fig. 1b, bottom right). The data reported below demonstrate the validity of this concept and suggest its applicability to protein-based nanoparticle scaffolds in general.

Results

Separately expressed coreN and coreC fragments assemble efficiently into HBC-like CLPs. Efficient complementation requires that the two fragments be expressed in near equimolar amounts, with minimal non-specific aggregation. This was best achieved by using bicistronic mRNAs (Fig. 1b). The original constructs encoded coreN (aa 1–79) and coreC (aa 80–149 or 80–183) in that order, each preceded by its own ribosome binding site (RBS). Alternative functional designs included overlapping stop and start codons (see Supplementary Fig. S2 online). For some fusion proteins (e.g. Supplementary Fig. S7 online), reversing the cistron order provided superior expression, likely due to differential start codon accessibility ³¹. Their functionality further excluded that read-through products comprising both fragments, as were occasionally formed (e.g. Supplementary Fig. S2), were required as association scaffolds.

Sucrose gradient sedimentation of cleared lysates from bacteria expressing coreN plus coreC149 (SplitCore149) or coreC183 (SplitCore183) revealed cosedimentation (Fig. 2a) of two peptides

of the expected sizes into the particle-typical center fractions ³², in yields of 10–15 mg/L of culture. Complementation was corroborated by native agarose gel electrophoresis (NAGE) where particles

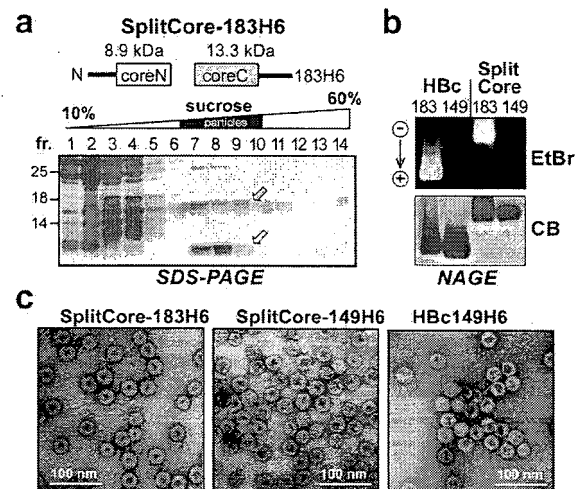


Figure 2 | Efficient assembly of split wild-type HBC. (a) Co-sedimentation in sucrose gradients of separately expressed coreN and coreC fragments (indicated by the downward pointing arrows) into particle-typical fractions. Cleared lysates from bacteria co-expressing coreN and coreC183H6 were sedimented through sucrose gradients, and aliquots of each fraction were analyzed by SDS-PAGE and Coomassie-Blue (CB) staining; coreN plus coreC149H6 and constructs lacking the His-tag gave comparable results. The somewhat lower than expected mobility of the coreC183H6 fragment observed in these and all subsequent experiments likely results from the high arginine-content of the CTD. (b) Native agarose gel electrophoresis (NAGE). Aliquots from the center gradient fractions were subjected to NAGE; particle-borne RNA was visualized by ethidium bromide (EtBr), protein by CB. (c) Negative staining EM of SplitCore and conventional HBC149_H6 CLPs. Additional structural and antigenic properties of SplitCore are shown in Supplementary Figure S1 online.



migrate as distinct bands⁸. Like contiguous HbC, the split 183 and 149 derivatives each produced a single band upon Coomassie Blue staining; their slower mobility (Fig. 2b, bottom) likely relates to changes in surface charge mediated by the new exposed termini. HbC CLPs containing the CTD package bacterial RNA^{8, 33} which can be stained with ethidium bromide. The same was observed for SplitCore183, proving integrity of the CLPs (Fig. 2b, top). Abundant intact particles were also detected by negative staining EM (Fig. 2c). These data demonstrate efficient self-complementation of coreN and coreC with and without CTD into assembly-competent structures.

Splitting HbC in c/e1 preserves the global particle structure but destroys the immunodominant epitope and subtly affects others. By high-percentage NAGE and direct evaluation of electron micrographs we observed an increased fraction of about 30–40% vs. ~5% T=3 particles for the SplitCore CLPs (see Supplementary Fig. S1). As insertions into c/e1 can exert a similar effect³⁴, these data confirm independently that there is structural cross-talk between the spike tip and the C proximal multimerization sites³⁴. Immunoblotting of NAGE gels (see Supplementary Fig. S1 online) with monoclonal antibodies (mAb's) revealed a complete loss of c/e1 reactivity whereas reactivity with the particle-specific mAb's 275¹³ and 3120³⁵ was only slightly reduced. Hence SplitCore CLPs mimic the structure of HbC though with subtle alterations in regions away from the spike tip. The absence of c/e1 reactivity was also reflected in a 15- to 80-fold reduced reactivity of SplitCore and SplitCore-GFP CLPs (see below) with anti-HbC antibodies from human serum in three widely used diagnostic anti-HbC assays (see Supplementary Fig. S1 and Supplementary Protocols online).

SplitCore-CLPs can natively display GFP fused to either coreN or coreC. Next we tested whether the SplitCore system is compatible with native surface display of a heterologous protein. GFP is an ideal model²² because chromophore formation depends on native folding. Constructs with GFP fused to either coreN or coreC were well expressed, including from a stop/start vector (see Supplementary Fig. S2 online), cosedimented in sucrose gradients, and ran as

distinct, green fluorescent bands in NAGE (see Supplementary Fig. S2); similar results were obtained with the coreC149 constructs and with GFP replaced by the red fluorescent mCherry (T.T.A. Nguyen and M. Nassal, unpublished results). Also Bb OspC fused to coreN formed CLPs with comparable efficiency (see Supplementary Fig. S3 online) as the previously described contiguous chain construct²⁴; hence for such favorable structures the SplitCore platform is as suited as conventional HbC.

SplitCore allows CLP presentation of an unfavorably structured protein that prevents CLP formation in the conventional HbC system. The extended structure of OspA (Fig. 3, and Supplementary Fig. S4) is incompatible with efficient CLP formation upon c/e1 insertion²⁸ (Fig. 3a, coreOspA183H6). In contrast, OspA fused to either coreN or coreC, and thereby exposing different parts of OspA (see below), efficiently formed regular CLPs, as indicated by sedimentation (see Supplementary Fig. S4 online) and EM (Fig. 2a, coreN-OspA183H6, OspA-coreC183H6). Similar results were obtained with coreC149 constructs (data not shown).

For immunogenicity evaluation, the further purified (see Supplementary Fig. S4 online) and endotoxin-depleted³⁶ CLP preparations were used to immunize mice as described²⁸ and Supplementary Information, lipidated (Pam3-Cys) OspA (LipOspA), the active ingredient of the commercial Lymerix vaccine in which the lipid acts as TLR agonist, served as reference. While details will be reported elsewhere (A. Walker, T. Stehle, R. Wallich, M. Simon and M. Nassal, manuscript in preparation) the most pertinent results were: (i) both CLP preparations, and OspA-coreC in particular, induced higher levels of OspA-specific antibodies than LipOspA (Fig. 3b); (ii) in an established mouse model of Lyme disease³⁷, passive transfer of the antisera to coreN-OspA and Lymerix protected 6 of 6 and 5 of 6 animals, respectively, from chronic Bb infection whereas only 1 of 6 mice was protected by the OspA-coreC induced antibodies (Fig. 3c). This result is rationalized by the location of the major neutralizing epitope, LA2, in the C terminal part of OspA³⁸ which is solvent-exposed in coreN-OspA but not OspA-coreC CLPs (Fig. 3a); indeed, the

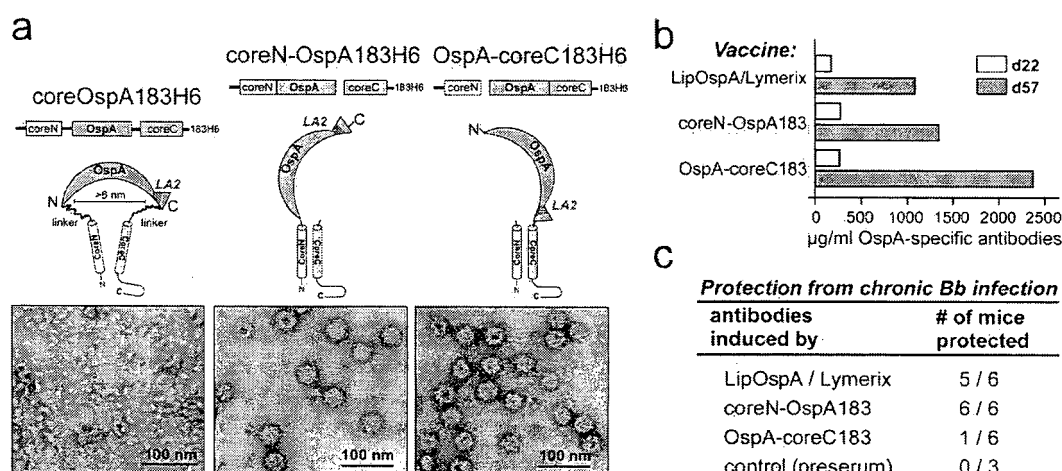


Figure 3 | Drastically enhanced CLP formation by SplitCore OspA fusions, and distinct immunological properties of SplitCore-OspA CLPs. (a) The ectodomain (aa 18–273) of Bb OspA adopts an extended β -sheet structure, with N and C terminus >6 nm apart (see Supplementary Fig. S4a for more details). Conventional insertion into c/e1 required very long linkers to generate soluble, though rarely regular particle forming, aggregates (left). In contrast, SplitCore with OspA fused to either coreN or coreC efficiently formed regular CLPs. Depending on orientation, the neutralizing LA2 epitope³⁸ is solvent-exposed, or buried. (b) SplitCore-OspA CLPs induce comparable if not higher anti-OspA antibody titers than the LipOspA Lymerix vaccine. Groups of 5 Balb/c mice each were immunized with 10 μ g each of the indicated CLPs (endotoxin content <50 EU/mg protein) or LipOspA on day 0, 14, 18 and 49. anti-OspA titers (in μ g/ml serum) in sera collected at day 22 and 57 are shown. (c) Protectivity against chronic Bb infection. The indicated immune sera were transferred into SCID mice, and the animals were challenged with 10^4 borreliae (strain ZS7) each. Development of arthritic disease was monitored over 52 days²⁶; animals remaining symptom-free were considered protected from chronic infection. The low protective potential despite high overall anti-OspA titer of the OspA-coreC183 induced immune sera correlated with a 4- to 5-times lower content in LA2-equivalent antibodies (A. Walker, T. Stehle, R. Wallich, M. M. Simon, and M. Nassal; manuscript in preparation).

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fraction of LA2-equivalent antibodies was drastically lower (not shown). Thus the SplitCore system allows to deliberately direct antibodies against a specific antigen region, with potentially relevant consequences for protectivity.

SplitCore-CLPs can display near full-length *P. falciparum* CSP and induce very high titer anti-CSP yet lower titer anti-HBc antibodies. Next we tested the SplitCore system's ability to display an even larger protein, CSP, from *Plasmodium falciparum*. CSP consists of ~400 aa (Fig. 4a), including an immunodominant central repeat region with a variable number (~40) of repetitions of NANP and fewer NVDP motifs³⁹. Two of the most advanced, yet only modestly protective, malaria vaccine formulations are RTS,S³⁹ and ICC-1132¹⁷. RTS,S comprises the C terminal half of CSP without the GPI anchor signal fused to the small HBV surface protein (HBs); ICC-1132 features several of the central repeat motifs inserted into HBc c/e1 plus the CSP universal T cell epitope fused to the HBc149 C terminus. Beyond the much lower intrinsic immunogenicity of HBs vs. HBc antigen⁴⁰, the lack in either vaccine of potentially important epitopes in the N terminal CSP sequence⁴¹ may limit their efficacy. Here we fused the 319 aa CSP sequence from the well characterized derivative CS27IVC⁴² to coreN. A conventional c/e1 insertion construct was completely insoluble, but the SplitCore versions (with coreC149H6 and coreC183H6) efficiently formed CLPs (see Supplementary Fig. S5 online); EM revealed abundant particles with a regular inner and a more elaborate, often ninja-star-like outer shell (Fig. 4c), consistent with the recently proposed extended rod-like structure of CSP⁴¹.

For immunogenicity testing further purified SplitCoreN-CSP149 CLPs (see Supplementary Fig. S5 online) were used to immunize mice⁴³ and Supplementary Information⁴⁴, a conventional repeat peptide construct (HBc149-NANP) analogous to ICC-1132 (Fig. 4b) served as reference (A. Walker, J. Billaud, D. Milich, M. Nassal; manuscript in preparation). Either CLP induced very high titers of NANP peptide-specific antibodies. When tested against recombinant CSP, the antisera induced by the CSP-presenting CLPs gave even stronger signals, likely owing to the presence of additional antibodies recognizing

non-NANP epitopes; in contrast, anti-HBc titers were 5- to 20-fold lower. Hence SplitCore-CSP CLPs may overcome shortcomings of both RTS,S and ICC-1132, warranting further investigation of their protective potential.

Triple-layer fluorescent SplitCore CLPs exposing a targeting domain. Next we evaluated the ability of SplitCore CLPs to simultaneously present two different sequences per split subunit. As a model for a heterodimeric insert, we chose split GFP because of its known self-complementation ability when separated at various sites³⁰. Furthermore, the GFP-internal new termini, if exposed, might allow to add yet another protein shell around the HBc scaffold (Fig. 5a); finally, GFP provides a convenient reporter to monitor how such additional domains affect the properties of the CLPs. A solubility-enhanced GFP variant was split in the loop between β -strands 10 and 11 of the barrel structure⁴⁴ so as to expose the splitting site to the solvent; GFP β 1-10 was fused to coreN, and GFP β 11 to coreC. The resulting SplitCore-SplitGFP (SC-SGFP) protein was well expressed in largely soluble, particulate and fluorescent form (see Supplementary Fig. 6 online).

As a third domain, we fused the B1 immunoglobulin-binding domain from protein G (GB1) to the GFP β 11-coreC fragment (Fig. 5). GB1 is relatively small (56 aa) but retains a high affinity for immunoglobulins. The bipartite triple fusion termed SC-SGFP-GB1 was highly expressed (~40 mg soluble protein/L culture), and efficiently formed fluorescent CLPs according to sedimentation, NAGE analysis and EM (Fig. 5b–d). Immunoglobulin binding was demonstrated by indirect immunofluorescence microscopy (Fig. 5e), where the CLPs generated a very similar, primary antibody-dependent staining pattern as an Alexa-488 conjugated secondary antibody. SC-SGFP-GB1 CLPs could also substitute for dye-labeled secondary antibody in fluorescent Western blots with comparable sensitivity (Fig. 5f), and they detected antibody-decorated cells in flow cytometry (A. Walker, PhD thesis, University of Freiburg, 2008).

The small GB1 domain does not push the system to its limits. We have successfully generated fluorescent SplitCore CLPs with OspC fused to coreN-GFP β 1-10, or to GFP β 11-coreC; and with OspA

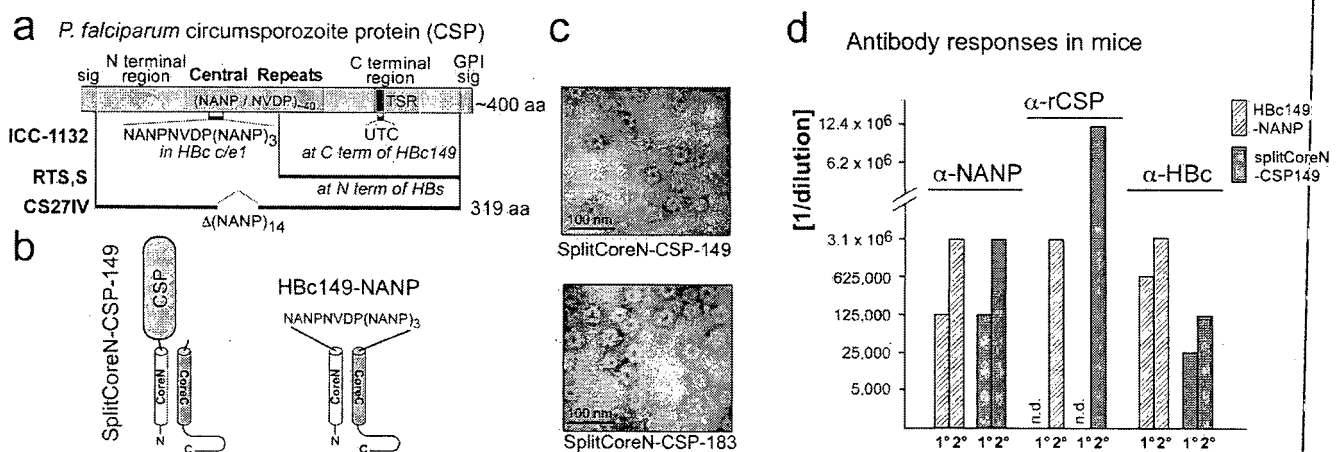


Figure 4 | SplitCore-CLPs can display the entire *P. falciparum* circumsporozoite protein (CSP) ectodomain. (a) General organization of CSP. CSPs are ~400 aa in length, with an N terminal signal sequence and a C terminal glycosylphosphatidyl-inositol (GPI) anchor signal. The central region contains a variable number of NANP and fewer NVDP repeats (~40 total), the C proximal region a thrombospondin type-1 repeat (TSR) including a universal T cell epitope (UTC). Experimental vaccine ICC-1132 carries a few of the repeats inserted into c/e1 of HBc, plus the UTC fused to the C terminus¹⁷; vaccine RTS,S comprises the C terminal half of CSP except the GPI signal, fused to the small surface antigen (HBs) of HBV³⁹. CS27IV⁴², based on the T4 isolate/Thailand (Genbank accession no. M19752), lacks the terminal signals and 14 NANP repeats. (b) Schematic structures of SplitCoreN-CSP-149, carrying the entire CS27IV sequence, and HBc149-NANP. (c) CLP formation by SplitCoreN-CSP-149 and -183 revealed by negative staining EM. (d) Antibody responses in mice against (NANP)₅ peptide (α -NANP), recombinant CS27IV protein (α -rCSP), and HBc. (B10.xB10.S) F1 mice were vaccinated with 20 μ g each of low endotoxin (<40 EU/mg protein) preparations of SplitCoreN-CSP149 (Supplementary Fig. S5) or HBc149-NANP CLPs in incomplete Freund's adjuvant (IFA) and boosted once 56 days later with 10 μ g antigen in IFA. Antibody responses after the first (1°) and second immunization (2°) were assessed by ELISA via antiserum dilution series⁴³; the highest dilution giving a signal at least three-fold above background (pre-immune sera) is indicated.

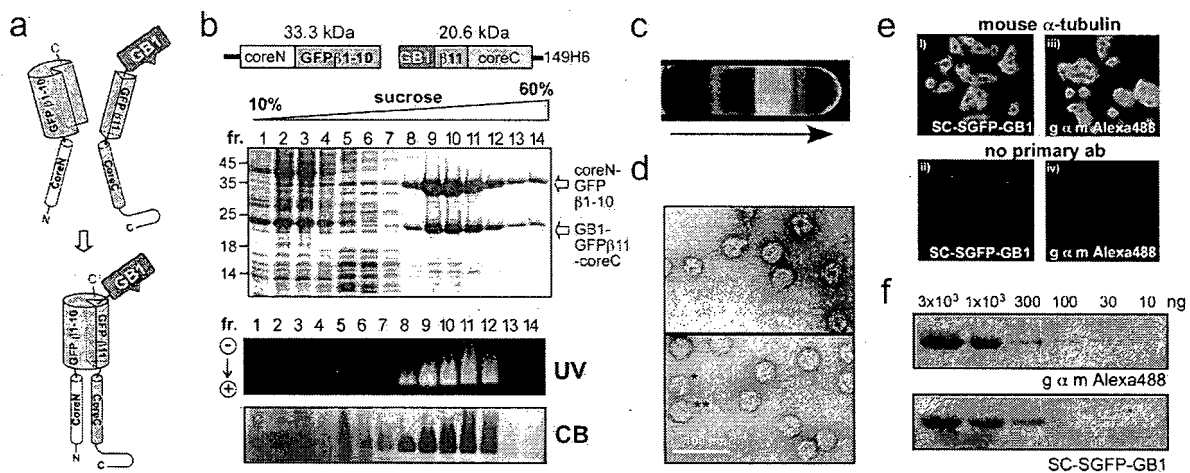


Figure 5 | SplitCore-SplitGFP-GB1: Triple-layer fluorescent particles with exposed targeting domains. (a) Design of SplitCore-splitGFP-GB1 fusions (SCSGFP-GB1). GB1 fused to one of the new ends provided by the split GFP insert (see Supplementary Fig. S3 online) should yield fluorescent CLPs with exposed immunoglobulin binding GB1 domains. (b) Expression and CLP formation. Bacterial lysates were subjected to sedimentation as in Fig. 1c. SDS-PAGE (top) demonstrated cosedimentation of two proteins of the expected sizes into fractions 8 to 12 which produced distinct green fluorescent bands in NAGE (UV) that also stained with CB. Similar results were obtained with GB1 fused to GFPβ11-10 on coreN (not shown). (c) Visualization of folded GFP containing material in the gradient tube from which the samples in (b) were derived. (d) Negative staining EM. The two micrographs are from independent grids onto which material from fraction 8, diluted 10-fold in PBS, was applied. Smaller (*; diameter ~38 nm) and larger (**; diameter ~42 nm) particles were observed. (e,f) SCSGFP-GB1 binds antibodies. (e) Indirect immunofluorescence. Permeabilized HeLa cells were incubated with mouse anti-tubulin antibody (i, iii) or not (ii, iv), then with SCSGFP-GB1 (i, ii) or an Alexa488-conjugated goat anti-mouse IgG (γm-Alexa488) antibody (iii, iv). Both generated similar, primary antibody-dependent staining patterns. (f) Fluorescent Western blot. Decreasing amounts of GFP were separated by SDS-PAGE, blotted on nitrocellulose and incubated with a mouse anti-GFP mAb mixture (Roche), then with either γm-Alexa488 or SC-SGFP-GB1. Fluorescent signals were detected using a Typhoon 7000 imager (GE Healthcare).

fused to the N terminus of GFPβ11-coreC and even to both core fragments (see Supplementary Fig. S7 online). That construct encompasses a total mass of ~100 kDa of heterologous sequence per 15 kDa carrier subunit. We also obtained CLPs by fusing an attenuated variant of *S. aureus* enterotoxin B (SEB⁴⁵) to GFPβ11-coreC (Supplementary Fig. S7). SEB comprises ~240 aa, and in the contiguous HBC system had not produced any soluble fusion protein (J. Vorreiter, B. Stiles, M. Nassal; unpublished data).

SplitCore is adaptable to the core proteins of other hepadnaviruses. Preexisting immunity to a carrier might limit repeated applications via “carrier induced epitopic suppression”⁴⁶ although for some HBC-presented antigens there is even evidence for the opposite^{47, 48}. Perhaps more important for human vaccines, tolerance against HBC in chronic HBV carriers could limit immune enhancement by carrier dependent T cell responses. This may be circumvented by using core proteins from rodent (woodchuck, ground squirrel) hepadnaviruses⁴³ which diverge by ~40% in their sequences from HBC (see Supplementary Fig. S8a online).

Split woodchuck core protein (SplitWHC) efficiently formed CLPs, as based on sedimentation, NAGE and EM (see Supplementary Fig. S8 online), and was able to display full-length OspA and CSP (not shown). Immunogenicity in mice of the SplitWHC CSP-CLPs was comparable to that of the respective HBC CLPs (A. Walker, J. Billaud, M. Nassal, D. Milich; manuscript in preparation), with generation of very high levels of NANP-specific and relatively low levels of anti-carrier antibodies (see Supplementary Fig. 8 online). Furthermore, CLPs formed when coreN was derived from one and coreC from the other virus (A. Walker, PhD thesis, University of Freiburg, 2008), consistent with a substantial sequence tolerance of coreN - coreC association.

Discussion

Genetic insertion of heterologous sequences into surface-exposed loops of viral capsid proteins provides convenient access to tailored

nanoparticles⁴. However, insertion of native proteins nearly always suffers from structural incompatibility. The SplitCore approach overcomes this fundamental restriction for HBC, one of the most attractive carrier scaffolds to date⁵, and provides access to particles with sophisticated surface structures as illustrated in Fig. 6a. The same concept should be applicable to other protein-based nanoparticles including for, but not limited to, vaccine applications.

To our knowledge, SplitCore is the first truly multimeric split protein (for a compilation of previously established split protein systems, see reference⁴⁹). Efficient association of the coreN and coreC fragments may be promoted by the extensive contacts provided by the α3 - α4 hairpin interface (Fig. 1); this should allow for flexibility regarding choice of the exact splitting site. The insolubility of either fragment expressed alone (not shown) suggests that the bicistronic mRNA design also promotes fragment association by providing spatial proximity during translation. Efficient CLP formation with various differently structured heterologous proteins (Figs. 3–5; Supplementary Figs. S2–S7) indicates that many more will be displayable on SplitCore CLPs, regardless of tertiary structure. Though some restrictions remain, including very large size (exceeding the geometrically restricted space available on the CLP surface), quaternary structure (e.g. antiparallel dimers, higher order multimers; see³²), or inability of the heterologous protein as such to be solubly expressed in *E. coli* (e.g. membrane proteins, glycoproteins), a multitude of protein antigens remain for vaccine applications. Particularly useful is the SplitCore-intrinsic ability to generate opposite surface orientations, allowing to control specificity of the anti-insert immune response (Fig. 3). If neutralization is not as confined to a specific spatial location as in OspA, mixing CLPs presenting the antigen in either orientation provides an easy route to broaden the anti-insert antibody repertoire.

A point to consider for using HBC as vaccine carrier in humans is that anti-HBC testing is used diagnostically to distinguish HBV infection (routinely resulting in a long-lasting anti-HBC response) from anti-HBV vaccination (with HBs, and thus without inducing

anti-HBc). Our data show that disruption of the immunodominant c/e1 epitope and, on top, sterical shielding by the displayed whole proteins strongly reduce the anti-HBc response (Fig. 4d and Supplementary Fig. S8 online); this was also reflected by the much reduced reactivity of SplitCore CLPs with patient serum anti-HBc (Supplementary Fig. S1 online). Furthermore, SplitCore CLP-induced vs. infection-induced anti-HBc antibodies are easily distinguished by the absence of any anti-c/e1 response. If considered necessary, animal HBV based SplitCore CLPs may be used instead (Supplementary Fig. S8 online). Finally, cross-complementation between HBc- and WHc-derived coreN and coreC implies that undesired epitopes may be eliminated via engineered mutations.

Because of its exceptional immunological properties, most previous efforts towards manipulating HBc were devoted to its use as a vaccine carrier. However, the SplitCore system also opens new avenues to nanoparticle design in general. The surface-exposed termini of SplitCore-displayed heterologous proteins, either on a genuinely extended structure (X_u in Fig. 6b), or on a split insert (split X_f in Fig. 6b), can be used to build sophisticated multilayer architectures. Just one example are the SC-SGFP-GB1 CLPs, in which the HBc parts provide the particle scaffold, GFP an easily followable reporter, and GB1 a specific targeting device. Successful CLP formation with different proteins much larger than GB1 (Supplementary Fig. S7) indicates few limitations for the targeting domain. Moreover, the inner CLP lumen can serve as a cargo container. One example is CTD mediated packaging of RNA⁸ which, in vaccine applications, can act as a TLR7 agonist⁵⁰; hence coreC149 vs. coreC183 derivatives may be exploited to modulate the type of immune response induced. The CTD can be replaced by heterologous sequences (Z in Fig. 5) of up to ~17 kDa⁵¹, which might themselves have specific functionalities, including binding of non-nucleic acid and non-peptidic ligands that could serve as cargo. With appropriate targeting structures on their surface, such internally modified CLPs could be envisaged as specific delivery vehicles.

The SplitCore-intrinsic surface exposure of two accessible new termini also provides distinct advantages for enzymatic and chemical modification. An example is the display of a biotin-acceptor peptide

which can be biotinylated by the *E. coli* BirA ligase; such CLPs do indeed specifically interact with streptavidin (see Supplementary Fig. S9). Many other enzymatic modifications require accessible termini, amongst them sortase-mediated tagging⁵² and N terminal fatty acylation. According to our preliminary data (T.T.A. Nguyen and MN) myristoylation via N-myristoyl-transferases can efficiently be achieved when a suitable substrate sequence is exposed on the SplitCore CLPs. Chemical conjugation has greatly been advanced by bioorthogonal “Click” chemistries⁵³. Site-specific incorporation of Click-compatible non-natural amino acids into conventional HBc has been reported but the Click adducts were incompatible with CLP integrity⁵⁴, most likely because of sterical strain. Evidently, this would be overcome by adding the non-natural amino acids to coreN or coreC.

Various other viral capsid proteins are explored as display scaffolds⁴ yet up to now the ~400 aa Flock House virus coat protein is the only one for which native surface display of a complete protein, the anthrax receptor 2 (ANTRX2), has been achieved by genetic insertion⁵⁵; that protein was chosen because, as in GFP, its termini fit naturally into surface-exposed loops. Given that the sterical constraints for loop insertions are fundamentally similar in any protein, the advantages of the split capsid protein concept demonstrated here for HBc should as well be applicable to numerous other protein-based nanoparticle carriers.

Methods

Plasmid constructs. Parental plasmids pET28a2-HBc183H6 and pET28a2-HBc149H6⁵² feature a T7 promoter controlled synthetic HBc gene⁵⁶ encoding full-length (183) or truncated (149) HBV core protein (HBV genotype D, serotype ayw; accession no.: CAA 24706) with a C terminal His₆ tag. SplitCore derivatives were generated by introducing between HBc codons P79 and A80 a stop codon, a second RBS, and an initiator codon (Fig. 1b); in stop-start vectors the stop codon of coreN overlapped with the start codon of coreC (Supplementary Fig. S2c); in some cases, the cistron order was reversed (Supplementary Fig. S7e). All constructs were generated using standard PCR methods and verified by DNA sequencing.

Insert protein coding sequences. Plasmids used as sources for the genes of GFP,²² OspA²³ and OspC₂₄ have previously been described. For CSP, plasmid pDS56-32/RBSII-CS27iVC_6XHis⁴² was obtained from the Malaria Research and Reference Reagent resource (MR4; code MRA-272). A plasmid encoding genetically attenuated

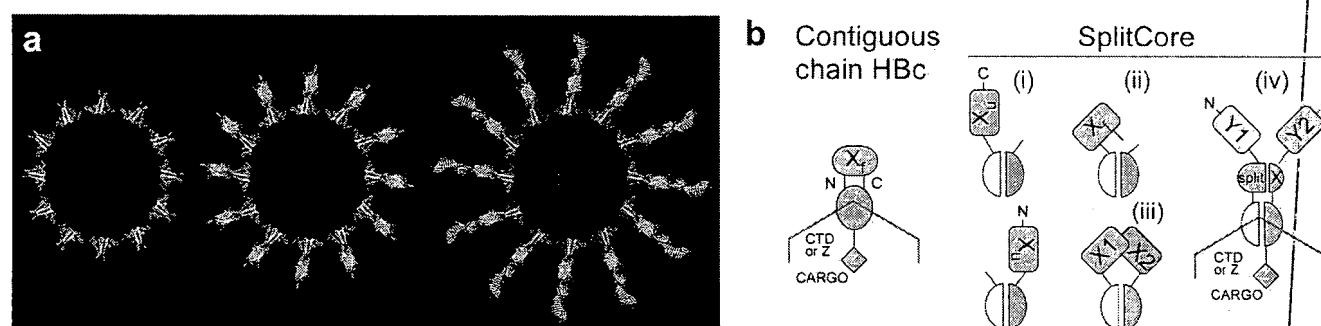


Figure 6 | Summary of unique SplitCore features. (a) Graphical illustration of representative, increasingly complex SplitCore CLPs generated in this study. The structural models of each protein are drawn approximately to scale; heterologous molecules fused to the second subunit per HBc dimer (grey) have been omitted for clarity. The cross-sections shown are hypothetical and only serve to illustrative pertinent SplitCore features. Splitting the insertion loop in HBc (left) leaves two surface-accessible termini (in red) for fusion of heterologous proteins regardless of their 3D structure. A protein with a favorable structure for insertion, such as GFP (middle), may itself be split to provide new exposed termini, to which an additional protein, such as OspA (right), can be added. The accessible termini on SplitCore, or the second or third layer proteins, are as well available for other types of modification. (b) Schematic comparison of the conventional contiguous chain HBc vs. SplitCore platforms. The half-hexagon symbolizes the icosahedral particle shell. In conventional HBc (left) surface presentation by c/e1 insertion of foreign protein ligands X is restricted to the few having a naturally favorable structure (X_f). The CLP lumen may be modulated by C terminal modifications; the authentic CTD packages RNA as cargo but may be replaced, within limits (<17 kDa⁵¹), by heterologous moieties (Z) that could act as cargo or provide specific functions (ligand binding, reporter probes). In SplitCore the new exposed termini allow (i) presentation of unfavorably structured inserts (X_u) in either orientation; of X_f ligands with comparable efficiency as in the contiguous system (ii); and of two ligands per carrier subunit (iii). Solvent-exposed termini of X_u ligands may directly be used as further attachment sites (not tested); however, as demonstrated, this is definitely possible for a split X_f ligand like GFP. Particle-internal features can be manipulated as in contiguous chain HBc. One out of various applications are CLPs carrying a targeting ligand (Y1, Y2), a monitoring reporter (split X), plus a cargo specified by Z.

S. aureus enterotoxin B (SEB; mutations: L45F, Y89A, Y94A⁴⁵) was kindly provided by Dr. B. Stiles.

Function sequences in individual constructs. The linkers connecting coreN or coreC with the inserts were typically G₄S or G₄T. In the SplitCore OspA fusions the original linkers ((G₄S)₂; (G₄S)₂RG₄DLG₄) from the contiguous construct²⁸ were maintained for comparability. SplitGFP was based on a solubility enhanced version⁴⁴; the N terminal part consisted of GFP aa 1–214 (GFPβ1–10), followed by the dipeptide DP; GFPβ11 contained the sequence 214–238, preceded by an artificial initiator codon.

Protein Expression and Purification. *E. coli* BL21(DE3) Codonplus cells (Stratagene) were used throughout as described^{24,29,32}. Conditions for sedimentation through 10–60% sucrose step gradients at 20°C were as follows, depending on sample volume: SW28 rotor, 3:45 h at 28,000 rpm²⁴; TST41.14 rotor 2 h at 41,000 rpm; TLS-55 rotor, 45 min at 55,000 rpm. Gradients were harvested in 14 fractions (2.6 ml each for SW28; 860 µl each for TST41.14; 100 µl each for TLS-55) from the top. For further purification, the pooled center gradient fractions were dialysed against TN150 buffer (25 mM Tris-Cl [pH7.5], 150 mM NaCl) and subjected to a second gradient sedimentation or size exclusion chromatography on Superose 6 (GE Healthcare).

Endotoxin depletion. Endotoxin was depleted by phase separation with Triton X-114 essentially as described³⁶. Residual detergent was depleted by repeated dialysis against excess PBS at 4°C. Endotoxin contents dropped from 10⁵–10⁶ EU/mg protein in the crude lysates to below 50 EU/mg, as determined by the Pyrochrome Assay (Associates of Cape Cod, Inc., MA).

Native agarose gel electrophoresis (NAGE) and immunoblotting. NAGE was performed in 1% agarose gels (2.6% for T=3 vs. T=4 particle distinction; Supplementary Fig. S1a) as described⁸. For immunological detection, NAGE or SDS-PAGE gels were blotted to PVDF membranes which were probed with specific primary antibodies and peroxidase-conjugated secondary antibodies plus chemiluminescent substrates²⁹. Monoclonal anti-HBc antibodies used were 10E11 and 10F10 (anti-coreN and anti-coreC, respectively⁵⁷); 312 and 3105 (anti-c/e1^{13,58,59}); 275¹³ and 3120³⁵, both recognizing particle-specific epitopes.

Electron Microscopy. Negative staining EM using 2% uranyl acetate was performed as described³⁴.

Immunoglobulin binding by SC-SGFP-GB1 CLPs. For indirect immunofluorescence, 4% paraformaldehyde fixed HeLa cells were permeabilized using 0.2 % Triton X-100 in PBS, blocked with 5% BSA in PBS and incubated with 0.05 µg/ml of mouse-anti-tubulin mAb, or not; for detection, either 0.13 µM goat-anti-mouse IgG antibody conjugated to Alexa488 (gox-Alexa488; Invitrogen) or 0.05 µM SC-SGFP-GB1 CLPs were used. Images were taken with a Zeiss AxioVert 35 microscope. For fluorescent Western blot detection, a dilution series of recombinant GFP mixed with constant amounts (1 µg/lane) each of BSA and HBc149H6 was separated by SDS-PAGE and electroblotted to nitrocellulose membranes. After blocking, the blots were incubated with mouse-anti-GFP mAb (Roche), then with either 2.0 µg/ml gox-Alexa488 or 2.5 µg/ml SC-SGFP-GB1 CLPs. Fluorescent signals were recorded using a Typhoon 7000 imager (GE Healthcare).

Details of the evaluation of the reactivity of SplitCore CLPs with human anti-HBc in commercial ELISAs and the immunizations with OspA- and CSP-presenting SplitCore CLPs are given in the Supplementary Protocols.

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Author contributions

CS performed and designed experiments and analyzed data. JW performed and designed experiments, analyzed data and assisted in writing and editing the manuscript. MN designed and performed the initial experiments, designed, analyzed and interpreted others and wrote the manuscript.

Additional information

Supplementary Information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: The authors declare competing financial interests. Authors MN and CS are, and author AW has been, employed by the Universitätsklinikum Freiburg which has filed a patent application for the SplitCore system described in this report [Ref: WO/2008/028535] with the authors listed as inventors.

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