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Ana María
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intracellular delivery of
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Ana María López Estévez

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TESIS DE DOCTORADO

NANOENSAMBLAJES PARA LA LIBERACIÓN INTRACELULAR DE ANTICUERPOS MONOCLONALES EN LA TERAPIA DEL CÁNCER

Ana María López Estévez

ESCUELA DE DOCTORADO INTERNACIONAL DE LA UNIVERSIDAD DE SANTIAGO DE COMPOSTELA

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TESE DE DOUTORAMENTO

NANOENSAMBLAXES PARA A LIBERACIÓN INTRACELULAR DE ANTICORPOS MONOCLONAIS NA TERAPIA DO CANCRO

Ana María López Estévez

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SUPERVISOR / TUTOR AUTHORISATION

Nanoassemblies for the intracellular delivery of monoclonal antibodies in cancer therapy

Prof. María Josefa Alonso Fernández, Full Professor in the Department of Pharmacology, Pharmacy, and Pharmaceutical Technology at the University of Santiago de Compostela.

Prof. María Dolores Ramona Torres López, Full Professor in the Department of Pharmacology, Pharmacy, and Pharmaceutical Technology at the University of Santiago de Compostela.

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**Nanoensamblajes para la liberación intracelular de anticuerpos
monoclonales en la terapia del cáncer**

Prof. María Josefa Alonso Fernández, Catedrática del Departamento de Farmacología, Farmacia y Tecnología Farmacéutica de la Universidad de Santiago de Compostela.

Prof. María Dolores Ramona Torres López, Catedrática del Departamento de Farmacología, Farmacia y Tecnología Farmacéutica de la Universidad de Santiago de Compostela.

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Nanoensamblaxes para a liberación intracelular de anticorpos monoclonais na terapia do cancro

Prof. María Josefa Alonso Fernández, Catedrática do Departamento de Farmacoloxía, Farmacia e Tecnoloxía Farmacéutica da Universidade de Santiago de Compostela.

Prof. María Dolores Ramona Torres López, Catedrática do Departamento de Farmacoloxía, Farmacia e Tecnoloxía Farmacéutica da Universidade de Santiago de Compostela.

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A mi familia
A los míos

“No dejes apagar el entusiasmo, virtud tan valiosa como necesaria;
trabaja, aspira, tiende siempre hacia la altura”
Rubén Darío.

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ABSTRACT

RESUMEN

ABSTRACT

The poor prognosis of cancer disease, partially caused by the severe off-target toxicities of chemotherapy, has pointed out the necessity to develop personalized strategies aimed to exploit the uniqueness of tumor biology. Mutations in the Kirsten Rat Sarcoma Virus (KRAS) oncoprotein are particularly relevant therapeutic targets in lung cancer, among others. Indeed, RAS mutations have been subscribed to 9-30% of human cancers. Although one KRAS mutation has been addressed, KRAS is still considered an *undruggable* target. In this scenario, monoclonal antibodies (mAbs) have emerged as the main alternative to achieve a positive efficacy/toxicity balance while blocking intracellular oncoproteins.

Within this context, the main objective of this thesis has been the development of nanocarriers specially adapted for the intracellular delivery of mAbs. Hence, we have developed novel polymer-based nanoassemblies (HANAs) functionalized or not with the tumor penetrating peptide tLyP1 and evaluate them according to i) their capacity to load mAbs and sustain their release, ii) their ability to modify the biodistribution profile of the mAb, iii) their performance in terms of activity and target engagement towards the KRAS^{G12V} oncoprotein.

In Chapter 1 the aim was to develop novel a new nanotechnology name HANAs for the intracellular delivery of mAbs. Bevacizumab was chosen as a model mAb. We have investigated the structural organization of the HANAs and deeply characterized their physicochemical properties. Furthermore, the tumor penetrating peptide, tLyP1, was anchored to the HANAs surface, optimized, and characterized accordingly. The HANAs were tested in terms of *in vitro* viability and cell uptake in lung cancer cells and macrophages.

In Chapter 2 we investigated the potential of the HANAs developed in Chapter 1 to modulate the biodistribution profile of the mAb after its labeling with the radionuclide ⁸⁹Zr. Furthermore, the extent of the tLyP1 peptide in the tumor accumulation was investigated by PET imaging in a human lung cancer model.

In Chapter 3, the HANAs technology was validated for the intracellular delivery of the clinically relevant antiKRAS^{G12V} mAb. The capacity of HANAs to impair the proliferation and colony formation was studied in KRAS-mutant lung cancer cell lines. Afterward, the ability to engage the target after reaching intracellular domains was also explored.

Overall, this work highlights the translational potential of HANAs' technology. The knowledge generated by understanding the key parameters that allow an efficient entrapment of the bevacizumab will serve as feedback for the HANAs validation with other mAbs. Moreover, the study of the biodistribution and the capacity to access novel biological compartments will open new avenues for the development of the next generation of cancer therapeutics.

RESUMEN

El mal pronóstico de las enfermedades oncológicas, causado en parte por los graves efectos adversos de la quimioterapia, ha señalado la necesidad de desarrollar terapias personalizadas que exploten la singularidad de la biología tumoral. Las mutaciones en la oncoproteína del virus Kirsten de sarcoma de rata (KRAS, siglas en inglés) son dianas terapéuticas especialmente relevantes en cáncer de pulmón, entre otros. De hecho, las mutaciones RAS están relacionadas con entre el 9-30% de los cánceres humanos. Aunque ya existe un fármaco dirigido a una de las mutaciones KRAS, esta diana se sigue considerando difícil de tratar. En este contexto, los anticuerpos monoclonales (mAbs, siglas en inglés) han surgido como la principal alternativa para lograr un balance positivo de eficacia/toxicidad y, al mismo tiempo, bloquear las oncoproteínas intracelulares.

En este contexto, el principal objetivo de esta tesis ha sido el desarrollo de nanotransportadores especialmente adaptados para la liberación intracelular de mAbs. Por lo tanto, hemos desarrollado nuevos nanoensamblajes basados en polímeros (HANAs) funcionalizados o no con el péptido de penetración tumoral tLyP1, y los hemos evaluado en función de i) su capacidad para cargar mAbs y mantener su liberación, ii) su capacidad para modificar el perfil de biodistribución del mAb, iii) su actividad funcional y capacidad para interactuar con la oncoproteína KRAS^{G12V}.

En el Capítulo 1, el objetivo era desarrollar una nueva nanotecnología denominada HANAs para la administración intracelular de mAbs. Para ello, se eligió el Bevacizumab como mAb modelo. Tras ello, hemos investigado la organización estructural de las HANAs y caracterizado en profundidad sus propiedades fisicoquímicas. Además, la superficie de las HANAs han sido funcionalizadas con el péptido de penetración tumoral tLyP1, optimizado y caracterizado en consecuencia. Finalmente, las HANAs se testaron en términos de viabilidad *in vitro* y captación celular en células de cáncer de pulmón y macrófagos.

En el Capítulo 2 investigamos el potencial de las HANAs desarrolladas en el Capítulo 1 para modular el perfil de biodistribución del mAb tras su marcaje con el radionucleido ⁸⁹Zr. Además, se investigó la función del péptido tLyP1 en la acumulación tumoral en un modelo de cáncer de pulmón humano mediante imágenes PET.

En el Capítulo 3, se validó la tecnología HANAs para la administración intracelular del mAb clínicamente relevante, antiKRAS^{G12V}. Se estudió la capacidad de las HANAs para impedir la proliferación y la formación de colonias en líneas celulares de cáncer de pulmón con la mutación KRAS. Posteriormente, también se exploró la capacidad de reconocer la diana KRAS^{G12V} tras alcanzar dominios intracelulares.

En conclusión, este trabajo pone de relieve el potencial traslacional de la tecnología HANAs. Los conocimientos generados mediante la comprensión de los parámetros clave que permiten un atrapamiento eficaz del bevacizumab servirán de retroalimentación para la validación de las HANAs con otros mAbs. Además, el estudio de la biodistribución y la capacidad de acceder a nuevos compartimentos biológicos abrirá nuevas vías para el desarrollo de la próxima generación de terapias contra el cáncer.

RESUMO *IN EXTENSO*

INTRODUCCIÓN

Sen dúbida, o éxito das terapias actuais contra o cancro [1] foi cuestionado durante os últimos anos debido a aparición de efectos adversos e ineficacia contra cancros severos. Isto forzou o desenvolvemento de terapias máis eficaces e selectivas como é o caso das proteínas. Dentro desta clasificación, os anticorpos monoclonais (mAbs) supuxeron o inicio dunha nova era no que ao tratamento contra o cancro se refire. A especificidade e selectividade dos mAbs situáronos á cabeza dos tratamentos oncolóxicos, reducindo así os efectos adversos dalgúns tratamentos actuais [2,3] como a quimioterapia. Tanto é o seu potencial que actualmente ocupan o 43% do mercado de biolóxicos aprobados pola FDA contra o cancro [4].

A pesar do seu claro beneficio, o certo é que a inadecuada biodistribución dos mAbs e a incapacidade para atravesar membranas celulares limitan a súa área de aplicación. Neste contexto, a nanotecnoloxía eríxese como unha alternativa plausible para a resolver estas limitacións, o cal abre novas vías para a creación e mellora da medicina personalizada [5].

O deseño de nanopartículas (NPs) para a liberación de biolóxicos non é unha tarefa doada. Entre os principais problemas aos que se teñen que enfrontar atópase a inestabilidade que experimentan durante o seu paso polo torrente circulatorio. Este problema foi abordado durante décadas mediante o uso de materiais que aumenten a estabilidade das NPs e, ademais, evadan o sistema inmune [6]. Outro dos principais problemas é a escasa acumulación no tecido diana que estes experimentan. Parte da solución a este problema pasa polo uso de ligandos que permitan unha acumulación selectiva das NPs e o deseño racional das mesmas [7]. Outros moitos factores relacionados coa bioloxía e o dinámico progreso da progresión tumoral supoñen un reto para o acceso das NPs ao tumor [8].

O coñecemento xerado durante anos sobre a nanotecnoloxía e as barreiras biolóxicas que ésta enfronta impulsaron o seu uso para solucionar as limitacións que os biolóxicos presentan. En xeral, éstes poderían resumirse en inadecuada biodistribución, toxicidade inespecífica e incapacidade para atravesar membranas celulares. Neste contexto, diferentes nanotransportadores foron explorados para a liberación dos biolóxicos con maior relevancia clínica no tratamento do cancro, entre os que se atopan as enzimas [9,10], proteínas apoptóticas [11–13], inmunomoduladores [14,15] e, con maior relevancia, os mAbs [16–20]. En concreto, os mAbs pódense clasificar en base ao seu lugar de acción en i) mAbs no microambiente tumoral (TME, siglas do inglés) [20–25] ii) inhibidores de puntos de control inmunitario [18,19,26] e iii) mAbs para alcanzar unha diana intracelular [17,27].

En canto á liberación de mAbs en relación ao TME, varios traballos procuraron alcanzar diferentes moléculas relevantes no TME implicadas no progreso tumoral. Un claro exemplo diso é a modificación da organización do TME tras a administración de liposomas funcionalizados con mAbs antiCD44 e cargados con mAb antiIL6R. Este tratamento provocou unha diminución da expresión de diferentes rutas de sinalización como MMP-9 e Sox2, ademais da conversión fenotípica dos macrófagos de M1 a M2 [25]. Outros traballos desenvolveron complexos micelares para a liberación do trastuzumab, demostrando a capacidade para modificar o perfil de biodistribución e reducir o volume tumoral nun modelo humano de cancro de mama [21].

A pesar do éxito dos inhibidores de puntos de control inmunitario, éstos están asociados a efectos adversos relacionados coa súa inadecuada biodistribución [28,29]. Un exemplo relevante é o punto de control inmunitario CTLA-4, cuxa administración en liposomas, antes da administración de Doxil®, deu lugar a unha redución tumoral nun modelo de melanoma [30].

Por último, pero de gran relevancia, é a liberación de mAbs para alcanzar dianas intracelulares. Neste sentido, o noso grupo foi pioneiro en demostrar a posibilidade de alcanzar a diana intracelular gasdermina mediante a administración de nanocápsulas de ácido hialurónico (HA, siglas do inglés) cargadas co mAb anti-gasdermina. A relevancia desta terapia confirmouse tras observar unha redución do cociente de migración das células canceríxenas e unha diminución do volume do tumor nun modelo de cancro de mama HER2-positivo [31].

CAPÍTULO 1: NANOENSAMBLAXES DE POLÍMERO PARA A LIBERACIÓN DE ANTICORPOS MONOCLONAIS (MABS): DESENVOLVEMENTO E FUNCIONALIZACIÓN

O desenvolvemento de novas nanotecnoloxías contra o cancro non é un asunto trivial. As numerosas barreiras biolóxicas que as NPs teñen que encarar antes de alcanzar o tumor, esixen un deseño racional que abarque dende as características fisicoquímicas das NPs ata os requirimentos estruturais das mesmas. A importancia dun deseño personalizado é tal que as NPs que se atopan actualmente en ensaios preclínicos e clínicos xa consideran estas especificacións durante o seu desenvolvemento [32,33]. A grandes liñas, estas especificacións buscan aumentar a cantidade de NPs que se acumulan no tumor tras evadir unha captura inespecífica durante o seu paso pola circulación sanguínea. Con este obxectivo, varias estratexias consideráronse efectivas como é o caso do tamaño de partícula (≤ 100 nm) [34–36] ou o recubrimento das NPs con materiais que permitan evadir unha absorción inespecífica de proteínas, aumentando o tempo de vida media en sangue [37,38]. Entre os materiais máis explorados para este fin atópase o polietilenglicol (PEG). En canto as aproximacións destinadas a aumentar a acumulación no tumor destacan os péptidos de penetración tumoral, como é o caso do tLyP1, cuxa afinidade polos receptores de neuropilin sobreexpresados no tumor [39,40] e vasos linfáticos [41] colócanlo como un candidato excelente para aumentar a penetración en tecido tumoral [42,43]. [Apartado afectado pola protección de dereitos]

A pesar de toda esta información, o desenvolvemento de NPs para a liberación intracelular de mAbs demostrou ser un gran desafío. Neste ámbito, o noso grupo de investigación foi o primeiro en demostrar o beneficio da nanotecnoloxía para alcanzar dianas intracelulares mediante a liberación de mAbs [31].

En base a esta información, o obxectivo principal deste traballo foi deseñar e desenvolver novas nanoensamblaxes constituídas por polímeros (HANAs) para a liberación de mAbs. [Apartado afectado pola protección de dereitos]. Deste xeito, elaboráronse HANAs con i) o menor número de compoñentes posible ii) elevada eficacia de asociación (EA), capacidade de carga (CC), e liberación controlada do anticorpo iii) elevada estabilidade durante o almacenamento, iv) orientación selectiva por células tumorais e v) capacidade para liberar o mAb intracelularmente. O cumprimento destas características por parte da nosa tecnoloxía HANAs foi avaliado

mediante diferentes técnicas. Así mesmo, o comportamento biolóxico avalíase en diferentes liñas celulares.

1.1 DESENVOLVEMENTO E CARACTERIZACIÓN DOS HANAs NON FUNCIONALIZADAS

As NPs que foron exploradas para liberación intracelular de mAbs presentan características moi variadas entre as que destacan elevadas eficacias de asociación [17,51,52] e a mínima liberación en condicións fisiolóxicas [51,53]. É destacable que o uso de péptidos que melloren a penetración en tumor é unha práctica que foi só testada por un autor [51].

[Apartado afectado pola protección de dereitos]

[Apartado afectado pola protección de dereitos]. Como resultado, obtivéronse un total de 6 prototipos con tamaños de partícula desde 79 ata 196 nm, PDI<0.3 e carga superficial negativa (Táboa 1). Ademais, a eficacia de asociación (EA) oscilou entre 32 e 99% e as correspondentes capacidades de carga (CC) entre 13 e 46%. [Apartado afectado pola protección de dereitos].

Táboa 1. Propiedades fisicoquímicas, eficacia de asociación (EA, %) e capacidade de carga (CC, %) de prototipos de A a C1 despois de concentrar a unha concentración de BVZ final de 3.2 mg/mL. [Apartado afectado pola protección de dereitos]. O dato é expresado como media $\pm$ SD, $n \geq 3$, excepto prototipo B2 ($n=2$). A análise estatística feita foi unha proba Mann-Whitney (A vs B**; B vs B1**) e una proba t non apareada (C vs C1).

Prototipo	Propiedades fisicoquímicas			EA (%)	CC (%)
	Tamaño de partícula (nm)	PDI	Potencial Zeta (mV)		
A ²	196 $\pm$ 13	0,24	-14 $\pm$ 1	99 $\pm$ 0,3**	46,6
B ¹	162 $\pm$ 17	0,23	-13 $\pm$ 2	86 $\pm$ 11	34,4
B1 ¹	156 $\pm$ 11	0,26	-13 $\pm$ 3	33 $\pm$ 18**	13,0
B2 ¹	150 $\pm$ 15	0,27	-14 $\pm$ 1	52 $\pm$ 6	19,6
C ¹	107 $\pm$ 14	0,23	-13 $\pm$ 3	74 $\pm$ 11	29,7
C1 ¹	79 $\pm$ 8	0,25	-13 $\pm$ 2	67 $\pm$ 21	22,2

O perfil de liberación de BVZ foi estudado en medios que imitan o sangue (pH 7.4) e as condicións endosomais (pH 5.0). En termos xerais, a cinética de liberación foi similar entre todos os prototipos, excepto para o C, o cal presentou unha liberación do 52% tras unha semana de incubación a pH 5.0. [Apartado afectado pola protección de dereitos].

Finalmente, todos eles foron amplamente caracterizados co fin de ser validados como potenciais produtos farmacéuticos. Como resultado, as HANAs presentaron unha elevada estabilidade en medios biolóxicos simulados de polo menos 8 horas e en suspensión a 4°C de entre 6 meses a 1 ano e 3 meses. Ademais, mantiveron as súas propiedades fisicoquímicas e a cantidade de BVZ asociado tras liofilizar.

Debido ás prometedoras características destes prototipos, as HANAs denominadas B e C1 foron seleccionadas para a funcionalización da súa superficie co tLyP1.

1.1.1 Desenvolvemento e caracterización das HANAs funcionalizadas con tLyP1 (tLyP1-HANAs)

Para o desenvolvemento dos prototipos funcionalizados co tLyP1, foi necesario desenvolver dous protocolos para a conxugación deste ao polímero e ao surfactante-PEG. [Apartado afectado pola protección de dereitos]. Tras caracterizar por resonancia magnética nuclear os conxugados polímero-tLyP1 e surfactante-PEG-tLyP1, usáronse para formular os HANAs.

A introdución do polímero-tLyP1 nas HANAs realizouse seguindo o mesmo protocolo de formulación xa descrito, pero substituíndo o polímero polo polímero-tLyP1. Como se mostra na Táboa 2, a substitución do polímero non induciu cambios fisicoquímicos, mantendo o tamaño de partícula en 160 nm. O prototipo resultante foi denominado BtLyP1. A determinación da EA si revelou cambios significativos (de 86 a 26%), [apartado afectado pola protección de dereitos].

Para a incorporación do surfactante-PEGtLyP1 ao prototipo C1 foi necesario realizar un novo cribado tras observar que a substitución do surfactante-PEG polo surfactante-PEGtLyP1 daba lugar a un aumento do tamaño de partícula. Tras reducir a cantidade de surfactante-PEGtLyP1, o tamaño de partícula seguiu sendo superior ao da HANA non funcionalizada. Con todo, para manter un correcto balance tamaño de partícula/grao de funcionalización, este prototipo foi incluído ao panel de candidatos finais e denominado C1tLyP1. Neste caso, a EA mantívose tras a funcionalización.

Táboa 2. Composición e propiedades fisicoquímicas das HANAs non funcionalizadas e funcionalizadas con tLyP1. Os datos exprésanse como media $\pm$ SD, $n \geq 3$. #HANAs funcionalizadas con tLyP1. A análise estadística do tamaño de partícula evaluouse mediante unha proba t non apareada (B fronte BtLyP1; C1 fronte C1tLyP1). N.A. Non aplicable.

Prototype	Concentración final [mg/mL]				Propiedades fisicoquímicas		
	Polymer	Surfactant	Surfactant.PEG	BVZ	Tamaño de partícula (nm)	PDI	Potencial Zeta (mV)
B	3,2	1,6	N.A.	3,2	162 $\pm$ 17	0,23	-13 $\pm$ 2
BtLyP1	3,2 [#]	1,6	N.A.	3,2	166 $\pm$ 9	0,18	-11 $\pm$ 6
C1	1,6	3,2	1,6	3,2	79 $\pm$ 8	0,25	-13 $\pm$ 2
C1tLyP1	1,6	3,2	0,064 [#]	3,2	121 $\pm$ 14****	0,25	-13 $\pm$ 2

Dende o punto de vista das propiedades como produtos farmacéuticos, os tLyP1-HANAs demostraron ter características comparables ás HANAs non funcionalizados en termos de estabilidade en medios biolóxicos simulados, estabilidade en suspensión a 4°C e capacidade para ser liofilizado.

1.1.2 Avaliación do comportamento biolóxico *in vitro*

Co obxectivo de estudar o comportamento *in vitro* dos HANAs, seleccionáronse HANAs non funcionalizados designados como prototipos B e C1. Deste xeito, a influencia do tamaño de partícula e da presenza dunha coroa de PEG avaliaríase simultaneamente en liñas celulares de cancro de pulmón (CMT 167) e macrófagos (RAW 264.7).

Nun primeiro estudo, avaliouuse a viabilidade celular das dúas liñas celulares citadas, tras o contacto cos HANAs. Os nanosistemas mostraron un perfil de toxicidade baixo na liña de cancro de pulmón. Ao contrario que en macrófagos, onde se observou unha toxicidade tamaño-dependente. Aínda que este feito xa foi previamente atribuído ao tamaño de partícula [59], o certo é que outros traballos mostran resultados contrarios [60,61].

Nun segundo estudo avaliouuse a capacidade dos HANAs de internalizar o mAb tras a marcaxe dos HANAs co fluoróforo DiD a 37°C. Para dilucidar o mecanismo de internalización das NPs, realizouse o mesmo estudo a 4°C, inhibindo así a actividade da membrana celular [62]. Mentres que o prototipo B na liña CMT 167 demostrou ter unha captura similar independentemente da concentración e temperatura, o comportamento foi o contrario en macrófagos. A natureza fagocítica dos macrófagos podería ser responsable destas observacións. Ademais, estes resultados foron confirmados mediante microscopía confocal xa que se observou un depósito difuso da fluorescencia na superficie ou interior celular.

Os resultados de captura para o prototipo C1 foron contrarios ao observado para o prototipo B (Figura 1). Deste xeito, a captura a 37°C en ambas liñas celulares pareceu ser dependente da temperatura e da concentración. Estes resultados foron confirmados por microscopía confocal.

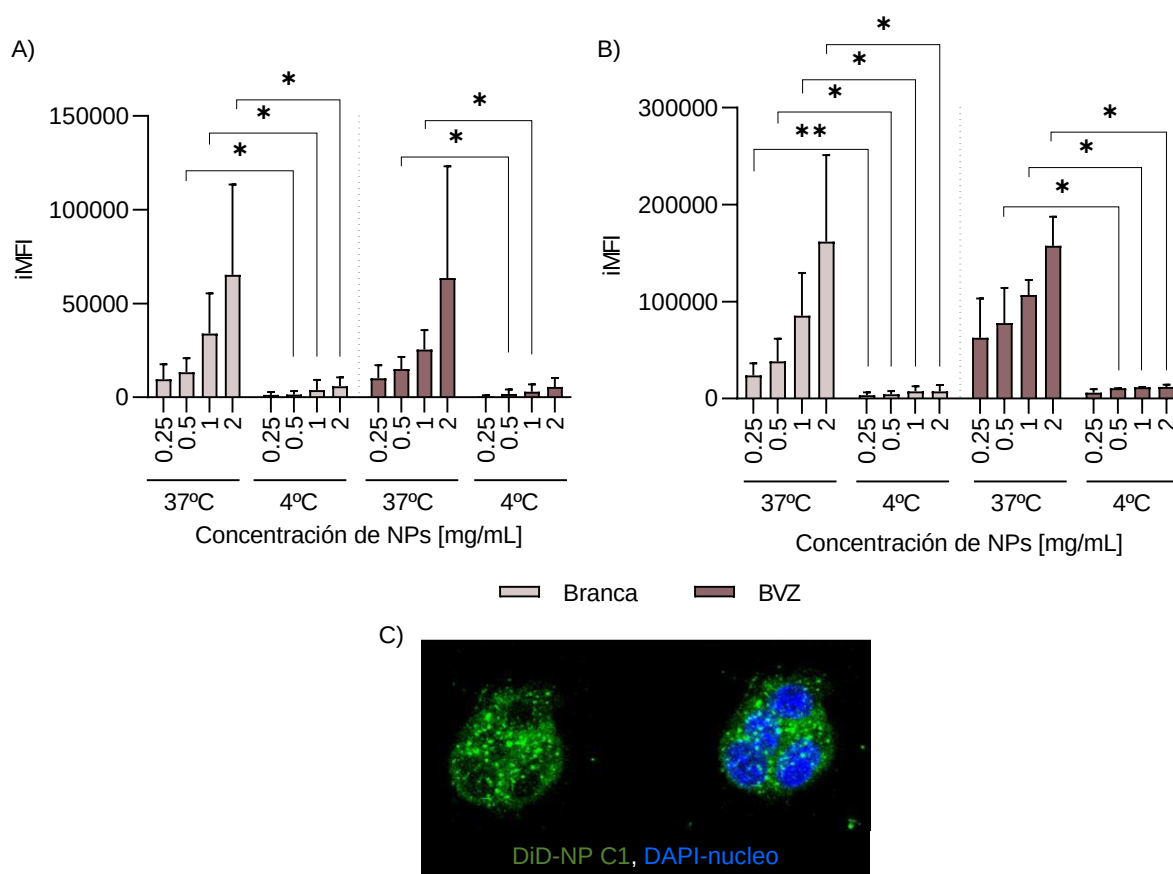


Figura 1. Captura celular do prototipo C1 branco marcado con DiD e cargado con BVZ. Análise cuantitativa de DiD iMFI en CMT167 (A) e RAW264.7 (B) tras 4 horas de incubación a concentracións crecentes de HANAs. C) Imaxes representativas de microscopía confocal do prototipo C1 branco a 2mg/mL tras 24 horas de incubación na liña celular de cancro de pulmón. As comparacións estatísticas realizáronse seguindo unha proba t paramétrica non apareada entre temperaturas. Os datos exprésanse como media $\pm$ SD, $n \geq 3$. iMFI (intensidade media de fluorescencia integrada).

A tecnoloxía HANAs foi deseñada para a liberación intracelular de mAbs. [Apartado afectado pola protección de dereitos]. Ademais, a inserción dun péptido de penetración tumoral non deu lugar a cambios nas devanditas propiedades. Finalmente, a internalización e baixa toxicidade foi demostrada nunha liña de cancro de pulmón e macrófagos.

CAPÍTULO 2: NANOENSAMBLAXES MULTIFUNCIONAIS COMO ESTRATEXIA PARA A MELLOR CAPTURA TUMORAL DE MABS: AVALIACIÓN *IN VITRO* E IMAXE PET *IN VIVO*.

O deseño e desenvolvemento de novos sistemas de liberación de mAbs en cancro implica, en última instancia, presentar unha acumulación favorable no tumor. Isto foi ata agora só conseguido empregando PEG, unha estratexia que habilita a acumulación pasiva das NPs. Desafortunadamente, a eficacia de liberación en tumores sólidos é baixa.

Entre os ligandos de orientación á tecido diana máis usados na funcionalización de NPs, destacan os anticorpos, polisacáridos ou péptidos de penetración celular. Con todo, a súa inmunoxenicidade [66] ou a dubidosa sobreexpresión do receptor en células cancerixenas de

forma exclusiva [67,68] limitaron a súa explotación. Por iso, neste traballo propoñemos o uso dun péptido de penetración tumoral, denominado LyP1 truncado (tLyP1, polas súas siglas en inglés). Este pertence a unha familia de péptidos que comparten unha mesma secuencia denominada CendR, caracterizada por recoñecer os receptores neuropilin-1 e -2 [43,69–71], sobreexpresados en células tumorais [39,40] e vasos linfáticos [41]. Este demostrou mellorar a eficacia antitumoral de diferentes NPs [72–75]. De feito, o noso laboratorio demostrou como a funcionalización con tLyP1 de nanocápsulas de HA cargadas con docetaxel provocou unha acumulación tumoral de fármaco 36,6 veces superior ao fármaco libre [76].

En base a estes prometedores resultados, o obxectivo deste traballo foi coñecer o comportamento *in vitro* e *in vivo* dos HANAs tras a súa funcionalización co tLyP1. Para iso, desenvolveuse un protocolo para a marcaxe cun isótopo de vida media longa, o zirconio-89 (^{89}Zr). O ^{89}Zr é un radioisótopo ideal para estudar a biodistribución de mAbs debido á súa longa vida media. De feito, este foi amplamente explorado en ensaios clínicos como probas diagnósticas [77,78]. Mediante o uso da técnica de imaxe nuclear non invasiva, tomografía por emisión de positrones (PET), conséguese unha adecuada sensibilidade e resolución espaciotemporal [44]. Así, os HANAs foron marcados con ^{89}Zr e avalíouse a súa biocompatibilidade e captura *in vitro* en liñas celulares de cancro de pulmón. Finalmente, estudouse o seu perfil de biodistribución en animais sans e con cancro de pulmón mediante PET/tomografía computarizada (TC).

2.1 RADIOMARCAJE CON ^{89}Zr DOS HANAs CARGADOS CON DFO-BVZ

Os prototipos denominados con anterioridade como C1 e C1tLyP1 foron seleccionados para estudar o seu comportamento *in vitro* e *in vivo*, evaluando así a influencia do tLyP1. Para a adecuada marcaxe dos HANAs, desenvolveuse un protocolo para a conxugación do quelante deferoxamina (DFO) ao BVZ [79] e posterior coordinación co ^{89}Zr .

Co fin de obter un método que dese lugar ás mellores propiedades radioquímicas mantendo as características fisicoquímicas dos HANAs, exploráronse dous métodos de marcaxe. Os resultados reflectidos na Figura 2, indican a obtención dun rendemento radioquímico moderado, sendo dun $66 \pm 22 \%$ (^{89}Zr -DFO-BVZ), $68 \pm 7 \%$ (^{89}Zr -C1) e $61 \pm 19 \%$ (^{89}Zr -C1tLyP1). Ademais, o rendemento de produción foi baixo mentres que a pureza radioquímica foi superior ao 90%. [Apartado afectado pola protección de dereitos].

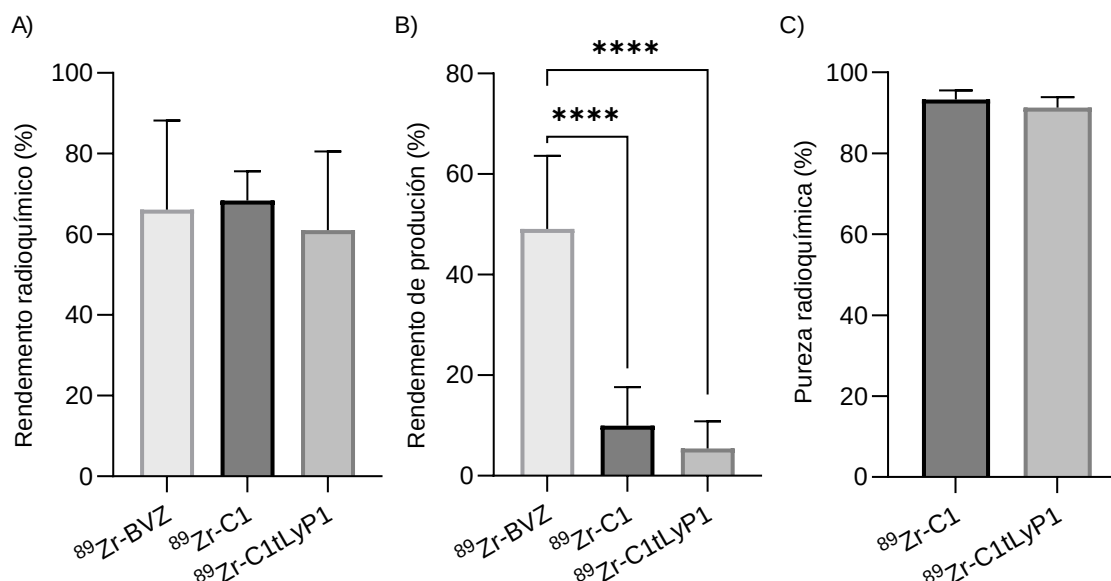


Figura 2. Caracterización radioquímica de C1 cargadas con BVZ e marcadas con ^{89}Zr (^{89}Zr -C1), C1tLyP1 cargado con BVZ marcado con ^{89}Zr (^{89}Zr -C1tLyP1) preparado polo método 1, e ^{89}Zr -BVZ. A) Rendemento radioquímico (RCY, %), B) rendemento de produción (%) e C) pureza radioquímica (RCP, %). Os datos exprésanse como media $\pm$ SD, $n \geq 3$. A análise estatística realizouse mediante unha proba de Mann-Whitney (A) e unha proba t non apareada (B-C).

Co obxectivo de aumentar o rendemento de produción, desenvolveuse outro procedemento, denominado método 2. Nestas condicións, aínda que o rendemento radioquímico foi inferior ao método 1, os rendementos de produción foron superiores, alcanzando valores de ata o 16% para o ^{89}Zr -C1tLyP1, en contraposición co método 1, un 5%.

Finalmente, ambos métodos resultaron ser reproducibles e deron purezas radioquímicas aceptables. Por tanto, decidiuse validar a marcaxe para estudos *in vivo*, confirmando a estabilidade da marcaxe en plasma humano durante polo menos 72 horas.

2.1.1. Avaliación do comportamento biolóxico *in vitro*

Co fin de coñecer a influencia do tLyP1 na captura celular, seleccionóuse a liña de cancro de pulmón humano A549. Previamente, confirmóuse a expresión do NRP-1 e o baixo perfil de toxicidade dos HANAs non marcados e marcados con ^{89}Zr .

Para maximizar o efecto terapéutico dos HANAs, éstos deben alcanzar o tecido diana e penetrar na masa tumoral. Por iso, a presenza do tLyP1 na superficie dos HANAs debe dar lugar a unha maior captura celular tras a súa interacción co NRP-1. En consonancia co esta hipótese, a Figura 3 reflectíu a ausencia de captura por parte do control (^{89}Zr -BVZ) e dos HANAs non funcionalizados (^{89}Zr -C1), mentres que as ^{89}Zr -C1tLyP1 deron lugar a unha captura 9.2 e 34.1 veces superior, respectivamente. Ademais, a saturación do NRP-1 cun exceso de tLyP1, reduciu a captura, confirmando así unha interacción superior mediada polo tLyP1 [72,80,81].

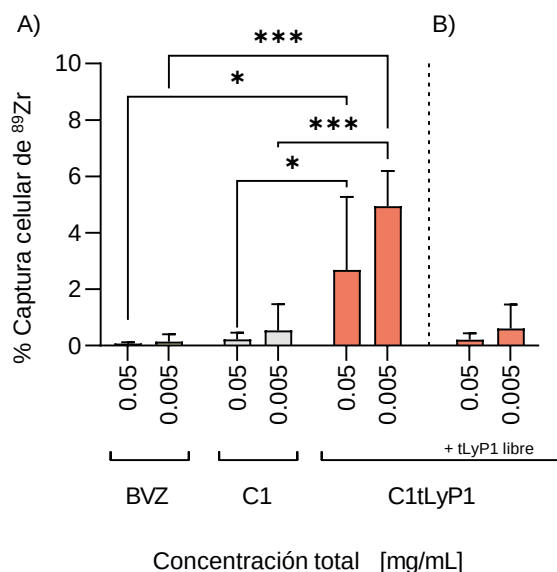


Figura 3. Captura celular *in vitro* de ^{89}Zr (A) e ensaio competitivo (B) na liña celular A549. As células tratáronse durante 4 horas despois da incubación con ^{89}Zr -BVZ (BVZ) e co prototipo C1 cargado con BVZ marcado con ^{89}Zr (^{89}Zr -C1) e C1tLyP1 cargado con BVZ marcado con ^{89}Zr (^{89}Zr -C1tLyP1) no medio suplementado con soro a 37°C. Para o ensaio competitivo, as células tratáronse cun exceso de tLyP1 durante 1 hora antes do tratamento. A comparación estatística realizouse mediante un ANOVA de 2 vías seguido dunha proba LSD de Fisher. Os datos exprésanse como media $\pm$ SD ($n \geq 3$, excepto para o ensaio competitivo, $n=2$).

2.1.2. Avaliación do perfil de biodistribución *in vivo*

En base aos prometedores datos *in vitro*, avalíouse o perfil de biodistribución de ^{89}Zr -BVZ e ^{89}Zr -C1 en animais sans. Tras a súa administración intravenosa, as imaxes PET/CT revelaron a capacidade de ^{89}Zr -C1 para modificar a biodistribución do ^{89}Zr -BVZ libre. Mentres que o ^{89}Zr -BVZ permaneceu en circulación sanguínea polo menos 72 horas, o ^{89}Zr -C1 acumulouse rapidamente no fígado e bazo. Estes resultados foron confirmados mediante estudos de farmacocinética en sangue e *ex vivo* tras a extracción dos órganos.

Tras corroborar o potencial da nosa tecnoloxía para modificar a biodistribución dos mAbs en animais sans, a biodistribución dos ^{89}Zr -C1 e ^{89}Zr -C1tLyP1 foi estudada nun modelo relevante de cancro de pulmón no microcítico (A549) (Figura 4).

A farmacocinética en circulación sanguínea revelou a ausencia de diferenzas entre ^{89}Zr -C1 e ^{89}Zr -C1tLyP1 durante 24 horas (Figura 4.A), revelando que a maior presenza de PEG en ^{89}Zr -C1 non produciu un aumento da vida media do sistema ao comparalo co ^{89}Zr -C1tLyP1, ao contrario do descrito por outros autores [82,83].

Doutra banda, as imaxes de PET/CT na Figura 4. B revelaron unha acumulación no fígado e bazo, resultados que coinciden co estudo en animais sans. Debido a que a acumulación no tumor foi difícil de observar, realizáronse estudos *ex vivo*. Tras 24 horas da administración intravenosa, os resultados *ex vivo* (Figura 4. C) demostraron unha maior acumulación de ^{89}Zr -C1 en médula ósea e pulmóns. Este resultado non está necesariamente asociado ao perfil de biodistribución do ^{89}Zr -C1, senón que pode deberse á acumulación do ^{89}Zr -BVZ nos devanditos órganos tras a súa liberación do ^{89}Zr -C1 en circulación sanguínea. En canto aos tumores, non

se atoparon diferenzas significativas na acumulación sendo do 2 ± 1 %ID/g para $^{89}\text{Zr-C1}$ e 3 ± 1 para $^{89}\text{Zr-C1tLyP1}$. Con todo, diferentes relacións tumor/tecido revelaron diferenzas entre prototipos (Figura 4. D). Aínda que o cociente tumor/músculo non foi significamente diferente entre HANAs, unha acumulación preferente no caso dos HANAs con tLyP1 foi obvia (12 ± 2 $^{89}\text{Zr-C1tLyP1}$ vs $^{89}\text{Zr-C1}$ 8 ± 8). Ademais, as relacións tumor/sangue e tumor/fígado indicaron unha acumulación significativamente superior para o $^{89}\text{Zr-C1tLyP1}$ 24 horas post-administración.

Este traballo confirmou que as HANAs son capaces de modificar o perfil de biodistribución do anticorpo e aumentar a súa acumulación en tumor.

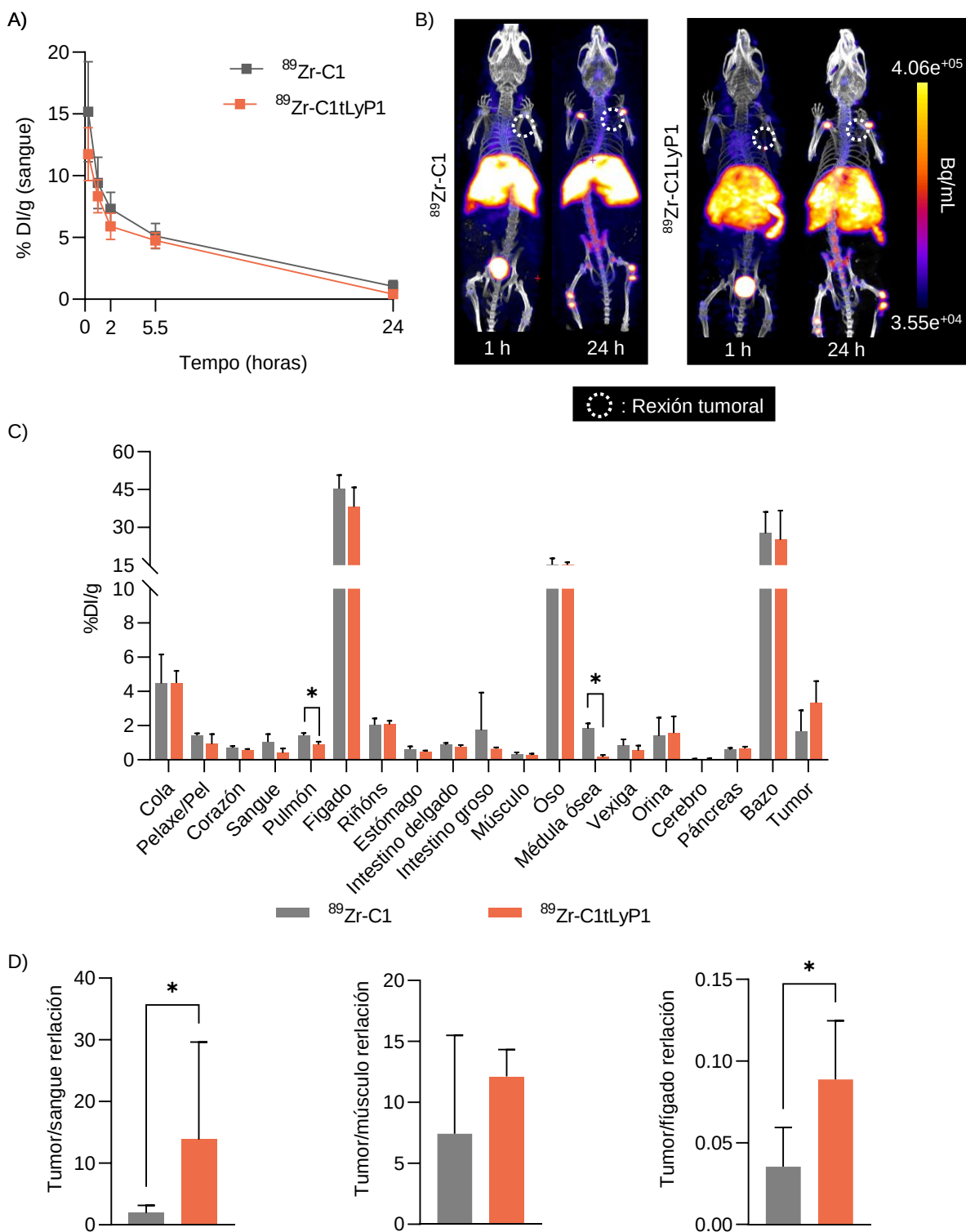


Figura 4. Perfiles sanguíneos, biodistribución en órganos e imaxes PET de C1 cargado con BVZ marcado con ^{89}Zr (^{89}Zr -C1) e C1tLyP1 cargado con BVZ marcado con ^{89}Zr (^{89}Zr -C1tLyP1) nun modelo de rato Balb/c portador do tumor A549. A) Porcentaxe de dose inxectada de ^{89}Zr por gramo (%DI/g) de sangue tras a inxección i.v. B) Proxeccións PET/CT de intensidade máxima de 2.5-3 (^{89}Zr -C1) e 1.5-2.5 (^{89}Zr -C1tLyP1) tras a administración i.v. C) Captura en órganos representada como %DI/g de tecido ás 24 h da inxección i.v. D) Cociente de captación tumor/sangre, tumor/músculo e tumor/figado. Os datos represéntanse como media $\pm$ SD, $n=4$. A comparación estatística realizouse mediante unha proba t non apareada (A, D relación tumor/músculo e tumor/figado) e unha proba de Mann-Whitney (B, D relación tumor/sangue).

CAPÍTULO 3: DESPREGANDO O PODER DA NANOMEDICINA PERSONALIZADA: LIBERACIÓN INTRACELULAR DE ANTIKRAS^{G12V} MABS PARA COMBATER O CANCRO DE PULMÓN

Malia os grandes avances na terapia do cancro e da aparición da medicina personalizada, a maioría das oncoproteínas intracelulares seguen sen poder ser tratadas. Cando se produce unha mutación en RAS, o ciclo de intercambio GDP a GTP queda inhibido, producíndose unha activación continua do estado GTP, responsable de promover a proliferación celular, a anxioxénese e a apoptosis [84–86]. En concreto, a isoforma KRAS gañou unha maior atención debido a que está expresada no 9-30% de cancros humanos [87,88]. Neste contexto, parece razoable inhibir o estado KRAS-GTP mediante o desenvolvemento de fármacos que teñan punto de unión específicos para iso.

Actualmente, só existen fármacos para bloquear a mutación KRAS^{G12C} no tratamento do cancro de pulmón non microcítico [89,90]. Con todo, a aparición de resistencias puxo en cuestión o seu uso [91]. Doutra banda, desenvóléronse terapias alternativas como son o uso de ARN interferente [92–96] e de proteínas de repetición de anquirina (DARPin) para inhibir o intercambio de nucleótidos RAS [97,98].

A pesar destas estratexias, o certo é que o uso de mAbs con longas áreas de contacto que permitan a formación de interaccións proteína-proteína emerxe como unha alternativa prometedora [53]. No noso laboratorio, demostramos como mediante o uso da nanotecnoloxía é posible alcanzar dianas intracelulares. Neste caso, desenvóléronse nanocápsulas de HA cargadas co mAb antigasdermina B [31]. Outros autores exploraron esta posibilidade a través do desenvolvemento de diferentes NPs para a liberación de mAb antiBCR/ABL [51] e antiMYC [53].

Así, propoñemos o uso da nosa tecnoloxía HANAs para a encapsulación dun mAb dirixido contra a mutación KRAS^{G12V} (antiKRAS^{G12V} mAb). Ademais, estas serán funcionalizadas co tLyP1 para maximizar o seu acceso tumoral [72,75,76,99]. Os prototipos foron deseñados e optimizados en base ás súas características fisicoquímicas, caracterizados por diferentes técnicas ortogonais. Ademais, a biocompatibilidade, funcionalidade e capacidade para recoñecer a súa diana intracelular foron ensaiadas en diferentes liñas celulares que expresaban a mutación de interese, KRAS^{G12V}.

3.1 DESENVOLVEMENTO E CARACTERIZACIÓN DAS HANAS CARGADAS CON ANTIKRAS^{G12V} MAB (ANTIKRAS^{G12V} MAB-HANAS)

Seguindo o protocolo de formulación descrito con anterioridade, realizouse un cribado considerando diferentes concentracións de polímero-tLyP1, surfactante e antiKRAS^{G12V} mAb para a obtención de HANAs. Dos prototipos resultantes, o candidato composto por concentracións finais de 0.25 polímero-tLyP1, 0.5 surfactante e 0.5 antiKRAS^{G12V} mAb (mg/mL) foi seleccionado debido a súa reproducibilidade e pequeno tamaño, pasando a denominarse mAb antiKRAS^{G12V} tLyP1-HANAs. Nun segundo estudo, introduciuse o surfactante-PEGtLyP1 e os prototipos resultantes caracterizáronse igualmente. Entre eles, seleccionouse as HANAs con 0.01 mg/mL de surfactante-PEGtLyP1 debido aos resultados de biodistribución obtidos no anterior capítulo e as súas aceptables características fisicoquímicas, pasando a ser chamado mAb antiKRAS^{G12V} PEGtLyP1-HANAs. Os prototipos homólogos

brancos presentaron características fisicoquímicas similares aos HANAs cargados con mAb antiKRAS^{G12V}.

Co obxectivo de estudar en maior profundidade as propiedades fisicoquímicas e a estrutura das nosas antiKRAS^{G12V} mAb-HANAs, decidimos realizar a caracterización mediante NTA e cryoTEM. Esta caracterización permitiu confirmar o tamaño de partícula observado por DLS. [Apartado afectado pola protección de dereitos].

Finalmente, o estudo da EA e CC demostraron como a nosa tecnoloxía presenta uns valores aceptables de EA (65%) pero, sobre todo, destaca a CC 18.7%. Ademais, tal e como se afirmou para os HANAs cargadas con BVZ, o potencial destas HANAs como produtos farmacéuticos foi validado, debido a que presentaron os requisitos mínimos en termos de estabilidade en medios biolóxicos simulados (8 horas), en suspensión a 4°C (≥ 6 meses) e como produto liofilizado.

Os estudos de liberación do mAb en condicións fisiolóxicas revelaron unha cinética de liberación similar entre HANAs, é dicir, ausencia de liberación do mAb rápida tras a incubación, seguida dunha liberación controlada de ata un 26% durante 1 semana. En termos xerais isto indicaría a ausencia mAb debilmente asociado á superficie das HANAs. [Apartado afectado pola protección de dereitos]

Táboa 1. Propiedades fisicoquímicas mediante DLS e NTA, eficacia de asociación (EA, %) e capacidade de carga (CC, %) dos mAb antiKRAS^{G12V} tLyP1-HANAs e PEGtLyP1-HANAs tras concentrado 6.4 veces. #Material conjugado con tLyP1. Os datos exprésanse como media $\pm$ SD, $n \geq 3$. As diferenzas estatísticas relativas ao tamaño de partícula por DLS e EA dos mAb antiKRAS^{G12V} tLyP1-HANAs e os mAb antiKRAS^{G12V} PEGtLyP1-HANAs analizáronse mediante unha proba t non apareada, mentres que o tamaño de partícula por DLS e NTA fíxose seguindo unha proba t non apareada (mAb antiKRAS^{G12V} tLyP1-HANAs) e Mann-Whitney (mAb antiKRAS^{G12V} PEGtLyP1-HANAs).

Prototipo	Tamaño de partícula (nm) (DLS)	PDI	Tamaño de partícula (nm) (NTA)	Concentración ($\times 10^{10}$ partículas /mL)	Potencial Zeta (mV)	EA (%)	CC (%)
tLyP1-HANAs	141 $\pm$ 7	0.20	136 $\pm$ 20	459 $\pm$ 50	-11 $\pm$ 2	66 $\pm$ 9	18,7
PEGtLyP1-HANAs	142 $\pm$ 11	0.23	122 $\pm$ 8**	396 $\pm$ 131	-11 $\pm$ 2	64 $\pm$ 6	18,2

3.1.1 Avaliación funcional das antiKRAS^{G12V} – HANAs

Previo á avaliación da funcionalidade da nosa tecnoloxía, comprobouse que os HANAs brancas e cargadas con antiKRAS^{G12V} tiñan un perfil de toxicidade baixo en NCI-H1568 (salvaxe), A549 (KRAS^{G12S}-mutante), CMT167 e H441 (KRAS^{G12V}-mutante).

Para avaliar a capacidade de inhibir a proliferación celular e formación de colonias da nosa nanotecnoloxía tras a liberación intracelular do mAb antiKRAS^{G12V} realizáronse ambos ensaios en diferentes liñas celulares que expresaban a mutación G12V (H441 and CMT167). Así, os tLyP1-HANAs demostraron inhibir a proliferación celular nun 9.4% (H441) e 35.8% (CMT167) tras 7 días. Así mesmo, a inhibición do PEGtLyP1-HANAs foi dun 27.3% en CMT167 e 18.9% en H441, con actividade xa evidente tras 5 días de tratamento. Doutra banda, os controis e a liña salvaxe non diminuíron a viabilidade celular, confirmando así a actividade

específica do mAb grazas á liberación das HANAs. Estes resultados están en liña co reportado por outros autores con terapias de siRNA [104,105].

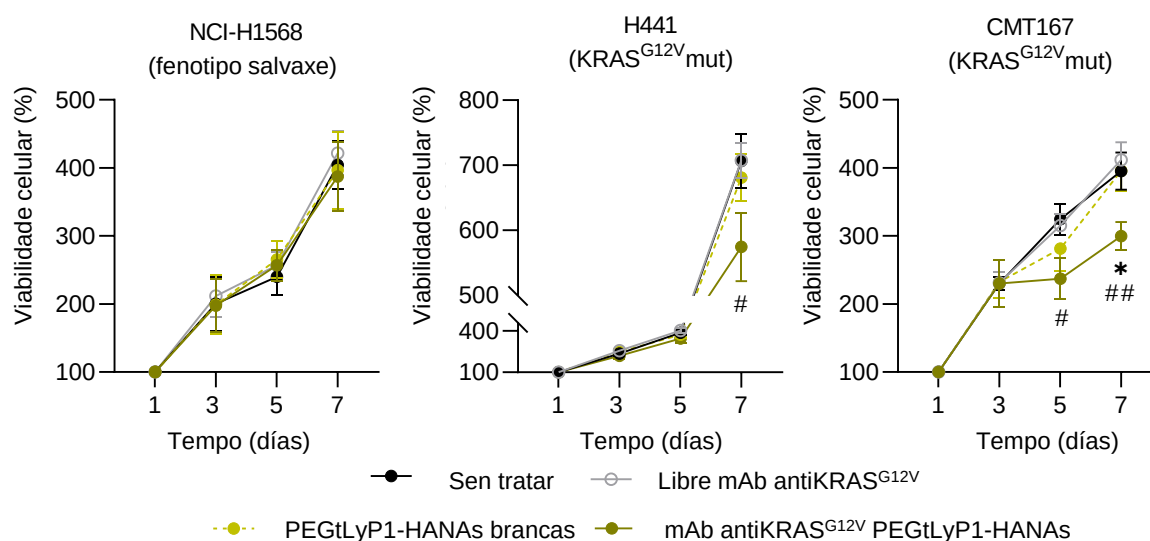


Figura 5. As PEGtLyP1-HANAs cargadas con mAb antiKRAS^{G12V} reduciron a proliferación de células de cancro de pulmón con mutación G12V. A liña celular de cancro de pulmón incubouse durante 7 días con medio de cultivo (sen tratar), mAb antiKRAS^{G12V} libre, HANAs brancas e mAb antiKRAS^{G12V} PEGtLyP1-HANAs. Os datos exprésanse como media $\pm$ SEM, $n \geq 3$. As diferenzas estadísticas analizáronse mediante a proba t múltiple en cada tempo entre grupos. # e * refírensen ás diferenzas entre o mAb antiKRAS^{G12V} libre ou as PEGtLyP1-HANAs brancas, e os mAb antiKRAS^{G12V} PEGtLyP1-HANAs, respectivamente. Non se representaron as diferenzas entre os PEGtLyP1-HANAs con mAb antiKRAS^{G12V} e o grupo non tratado.

A capacidade para inhibir a formación de colonias foi confirmada mediante ensaios clonoxénicos. De feito, observóuse unha redución dun 36%. Aínda que estes resultados indican unha menor eficacia por parte da nosa tecnoloxía cando son comparados con outros sistemas de liberación de siRNA [91,106], o certo é que debido ao seu mecanismo de acción e a súa pauta de administración [107,108] a comparación é difícil.

3.1.2. Capacidade dos antiKRAS^{G12V} – HANAs para alcanzar a oncoproteína intracelular KRAS^{G12V}

Tras comprobar que o anticorpo na nosa tecnoloxía manteniase funcional, comprobouse que os HANAs foron capaces de penetrar nas células. Tras iso, realizáronse estudos para comprobar a capacidade dos HANAs para recoñecer a diana intracelular, KRAS^{G12V}. Os resultados na Figura 6 reflicten unha dinámica de interacción tempo-dependente, alcanzando o seu máximo ás 48 horas. Isto pode ser debido a unha perda de actividade do mAb liberado polos HANAs [109], diminución da afinidade polo target ou unha cinética de liberación diferente no medio intracelular. O certo é que este ensaio é crucial para entender o mecanismo de acción destes candidatos e a súa capacidade para bloquear a oncoproteína.

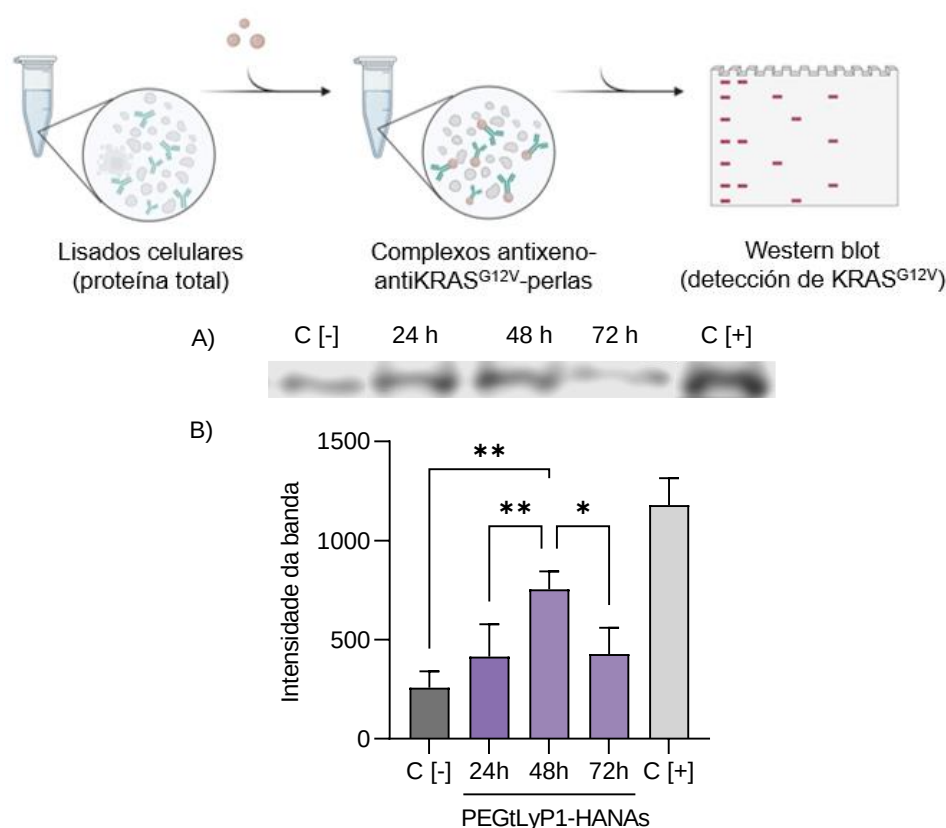


Figura 6. Resultados do estudo de compromiso de diana consistente na análise por inmunoprecipitación (IP) da interacción da proteína KRAS^{G12V} co mAb antiKRAS^{G12V} liberado das PEGtLyP1-HANAs. A) Representación esquemática do procedemento xeral respecto ao ensaio de compromiso de diana, que implica a IP do complexo de oncoproteína antiKRAS^{G12V} mAb-KRAS^{G12V} e, posterior análise por western blotting. Imaxes en bruto (A) e intensidades de banda da membrana western blot (B). A cantidade de produto IP determinouse ás 24, 48 e 72 horas na liña celular CMT167. O control negativo [Ct (-)] realizouse inmunoprecipitando 1 mg do lisado celular CMT167 coas perlas anti-mAb. Para o control positivo [Ct (+)], engadiuse 1 mg do lisado celular CMT167 a 5 µg do mAb antiKRAS^{G12V}. As diferenzas estatísticas determináronse mediante un ANOVA unidireccional entre grupos exceptuando o Ct (+). Os datos represéntanse como media ± SD, n=3.

Como conclusión deste traballo, a tecnoloxía HANAs demostrou a súa versatilidade para atrapar o mAb antiKRAS^{G12V}. Como resultado, inhibiuse a proliferación celular e capacidade para a formación de colonias. Ademais, confirmouse a capacidade dos HANAs para recoñecer a proteínas KRAS^{G12V} e a súa dinámica de interacción.

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INTRODUCTION

1 INTRODUCTION

This chapter reproduces a published review: Matilde Durán-Lobato^a, Ana María López-Estévez^{b,c}, Ana Sara Cordeiro^d, Tamara G. Dacoba^{b,c}, José Crecente-Campo^{b,c}, Dolores Torres^c, María José Alonso^{b,c}. “Nanotechnologies for the delivery of biologicals: Historical perspective and current landscape” [1].

^a Department of Pharmacy and Pharmaceutical Technology, School of Pharmacy, Universidad de Sevilla, Seville, Spain

^b Center for Research in Molecular Medicine & Chronic Diseases (CIMUS), Health Research Institute of Santiago de Compostela (IDIS), Universidade de Santiago de Compostela, Campus Vida, Santiago de Compostela, Spain

^c Department of Pharmacology, Pharmaceutics and Pharmaceutical Technology, School of Pharmacy, University of Santiago de Compostela, Santiago de Compostela, Spain

^d Leicester School of Pharmacy, De Montfort University, Leicester, United Kingdom

1.1 CANCER DISEASE. WHERE ARE WE?

Just one cell mutation is enough to trigger uncontrolled cell proliferation that inevitably leads to cancer. After replicating, these aberrant cells invade their cellular environment and replace the vital function of the local tissue. In the worst scenario, the malignant tumor disseminates throughout the body [2], developing metastasis. Unfortunately, 28.9 million people will be suffering this situation in 2040 [3]. Cancer is a cross-cutting problem that not only affects the quality of life of patients and their ecosystem, but also generates a large annual cost to the health-care system. In fact, in 2018, the total cancer cost in Europe was €199 billion, of which €32 billion were invested in cancer treatments, specifically drugs [4].

1.2 BIOLOGICALS FOR THE INDICATION OF CANCER

Although classical cancer treatments combining chemotherapy, radiotherapy and other small molecules, have led with some positive outcomes [5], there is a clear need to develop advanced oncological therapies with a higher efficacy/toxicity ratio and able to cure severe cancers. In this context, protein therapeutics has emerged as a new promising alternative. Of the 113 biologics approved by the FDA in the last decade, 15 of them were approved this last year, of which 40% were indicated for cancer treatment [6]. Accordingly, it has been estimated that the global protein therapeutics market will reach \$155.06 billion by 2025 [7]. Within this frame, mAbs represent the leading class of proteins investigated for cancer treatment. As shown in Fig. 1, the number of oncological mAbs approved in the last ten years has grown exponentially. Currently, only in the US, 49 therapeutic antibodies have been authorized for cancer treatment (43% of the total Abs approvals), and 10 are under regulatory review. The category of mAbs covers naked mAbs, antibodies-drug conjugates and other mAbs derivatives. The naked mAbs represent 69% of those currently under regulatory review [8]. Only a few of them have been withdrawn from the market for commercial reasons or due to the impossibility to ensure a significant clinical benefit as is the case of tositumomab [9] and olaratumab [10].

MAbs operate with high specificity according to different mechanisms of action: i) induction of apoptosis by directly targeting tumor cells, either as receptor agonists or blockers, and ii) induction of vascular and stromal cell disruption or immune-related cell death activation at the level of the tumor microenvironment (TME). This last mechanism, typical of immune

checkpoint inhibitors antiCTLA-4 or antiPD-1/PD-L1 (discussed in section 4.5) has set up the basis of a new concept of immunotherapy. Although their interaction with the target oncoproteins is very specific, the activation of alternative signalling mechanisms may occur, thus diminishing the efficacy of the treatment [11]. These, among others, are the reasons why mAbs are normally co-administered with other therapeutic options, often involving small molecules.

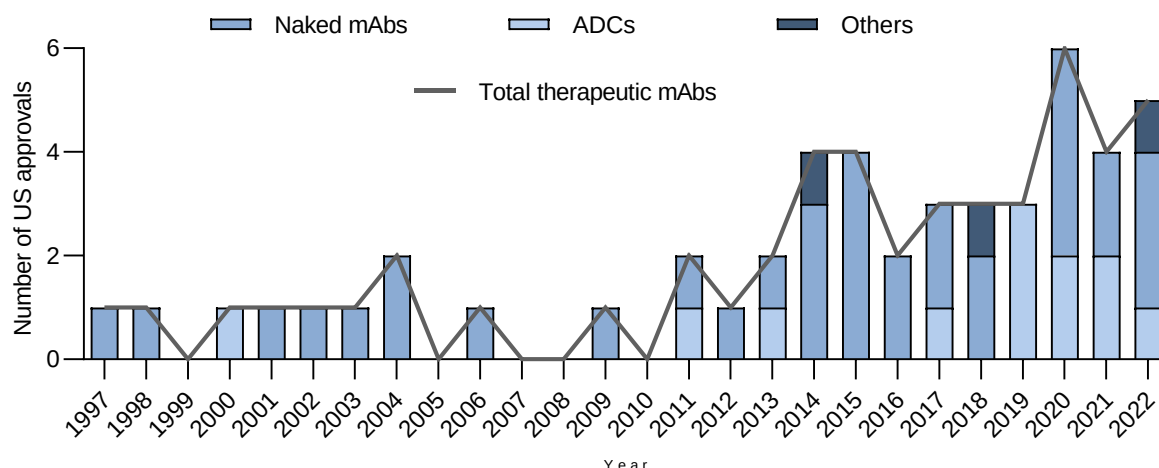


Figure 1. Monoclonal antibodies approved in US for the indication of cancer from 1997 to 2022. MAbs were classified based on their format as naked mAbs (IgG format), ADCs and others. Biosimilar products were omitted. Products withdrawn or marketing discontinued for the first approved indication were covered [8].

Despite the great potential of biological drugs in general and mAbs, in particular, their full exploitation in cancer is being significantly constrained by a number of biopharmaceutical problems, including their susceptibility to degradation, their incapacity to cross biological barriers and their inadequate biodistribution. In this context, nanotechnology [12], with particle sizes below 200 nm, preferably 100 nm, has emerged as a potential strategy to deal with the above mentioned problems, further discussed in section 4.2. Nevertheless, the design of the appropriate nanocarrier is not a simple and straight-forward approach. For example, a classical problem of nanodelivery carriers is related to their susceptibility to alteration upon contact with the blood stream, a process that impairs their targeting capacity and may exacerbate immune reactions against biological drugs. Although the use of materials such as PEG and other molecules that provide nanocarriers with stealth attributes [13] has been part of the solution, significant variability in the outcomes has been highlighted as a major concern. The use of targeting ligands together with the rational design of the nanocarriers based on the specific physiopathological characteristics of the tumor environment are expected to enhance the chances of the drug-loaded carriers to reach their targets [14].

In this sense it is important to keep in mind that cancer is a dynamic process highly conditioned by the tumor progression and the changes in the surrounding environment. For example, high vascular permeability together with unperfused tumor regions may result in heterogenous blood distribution in tumors. This situation leads to a variable access of the nanoformulations to the different tumor regions [15]. In addition, the stroma composition and the presence of tumor associated macrophages and other immune cells may influence the intratumoral distribution of the formulations. Finally, as the tumor grows, the chances of developing metastatic lesions increase and the access of the nanoformulations to the targeted cells becomes more difficult. The pathogenesis of metastasis involves several events such as angiogenesis progression and

the secretion of proangiogenic factors, among others [16]. Consequently, each stage of cancer progression may need different formulation approaches for the effective cure of the disease.

1.2.1 The unmet need: *undruggable* oncoproteins

The biology of the tumor is highly conditioned by the intracellular expression of mutant oncoproteins. Despite their relevance, some of these oncoproteins have been considered *undruggable* due to the lack of defined binding pockets of interaction with drug candidates. Examples of them are Rat sarcoma virus (RAS), nuclear factor-kB (NF-kB), myelocytomatosis proto-oncogene (c-MYC), myeloblastosis proto-oncogene (MYB) and androgen receptor variant 7 (AR-V7) [17]. However, the emergence of therapies that address oncoproteins has changed the concept of *undruggable*. In this context, we can highlight the Omomyc, the only direct MYC inhibitor that have reached Phase I/II (NCT04808362) for the treatment of advanced solid tumors. This “miniprotein” is designed to hamper the MYC protein-protein interactions [18].

Regarding the RAS family, although the KRAS isoform is the most frequent alteration in cancer [19], it was not until 2021 when the first small molecule antiKRAS^{G12C} received the FDA approval for NSCLC indication [20]. Unfortunately, the efficacy of these new drugs in the clinics is currently under question [21].

The efforts undertaken by the scientific community in recent years to design nano-oncologicals for the delivery of therapeutic proteins, with special focus on mAbs, are reviewed in the following sections.

1.3 HISTORY PERSPECTIVE OF PROTEIN DELIVERY

The first strategy to improve the biopharmaceutical properties of proteins was probably the formulation introduced by Prof. Frank Davis at the end of **1960** [22]. It involved the chemical modification of a model protein, the bovine liver catalase, with the hydrophilic polymer PEG. The resulting complex exhibited an increased circulation time and a reduction of its immunogenicity [23]. Despite this early achievement, it was not until **1993** when the first protein-polymer conjugate consisting of neocarzinostatin and a styrene-maleic acid copolymer (SMANCS), known as **Zinostatin stimalamer®**, was marketed for the indication of cancer [24]. Subsequently, three PEGylated proteins have reached the market for cancer indications [25]. In 1994, **Oncaspar®** (PEG-L-asparaginase) became the first PEGylated protein approved for the treatment of acute lymphoblastic leukemia [26,27], soon followed by **Asparlas™**, a PEGylated asparagine enzyme marketed for the same indication but with a lower frequency of administration [28]. In **2011**, a PEGylated interferon-anti 2b (**Sylatron™**) was approved as an adjuvant for the treatment of melanoma [29].

Other strategies explored for the formulation of proteins as oncological therapies have involved the use of NPs. In this frame, our lab pioneered in **1997** the encapsulation of model proteins in PLGA nanospheres [30] and CS NPs [31]. The same kind of technologies were later applied to the formulation of the therapeutic enzyme, L-asparaginase [32] and **interferon-alfa** [33].

In the area of mAbs, it should be highlighted that their production started only in 1975 [34], using the hybridoma technique. The immune related problems encountered with these original mAbs, was soon solved with their humanization. In 1997, rituximab was the first mAb approved for non-Hodgkin lymphoma treatment, and just one year later, trastuzumab was approved for breast cancer therapy [35]. In the same decade mAbs started being produced using phage display libraries [36] and, in 2014, ramucirumab, the first mAb produced by this technique for cancer indications, reached the market [37]. In parallel, in 2011, the first cancer immunotherapy against the cytotoxic T- lymphocyte antigen-4 (CTLA-4) reached the market, ipilimumab (Yervoy®) [38]. More recently, the first immune checkpoint inhibitor against LAG-3, relatlimab, was approved and used in combination with an antiPD-1 for treatment of melanoma [39].

The application of nanotechnology to the formulation of these complex started with the antibody-drug conjugates (ADCs) which were first explored in clinical trials in **1983** [40]. After a number of attempts and failures, finally, the first ADC, **Mylotarg™** (Gemtuzumab ozogamicin), was approved in **2000** for the treatment of relapse CD33-acute myeloid leukemia (CD33-AML). However, ten years later, it was withdrawn from the market due to safety issues, and, then, approved again in **2017** for the treatment of relapse or refractory CD33-AML with a different dosing protocol [41]. Currently, there are 12 ADCs commercialized for cancer indications (**Figure 1**). These technologies are not the focus of this review however because detailed reviews have recently been published on this topic [42–44].

Overall, the studies on protein delivery using nanotechnology in the context of cancer are all quite recent and the use of NPs still remain at the preclinical level. Seminal works in this field will be commented in subsequent sections.

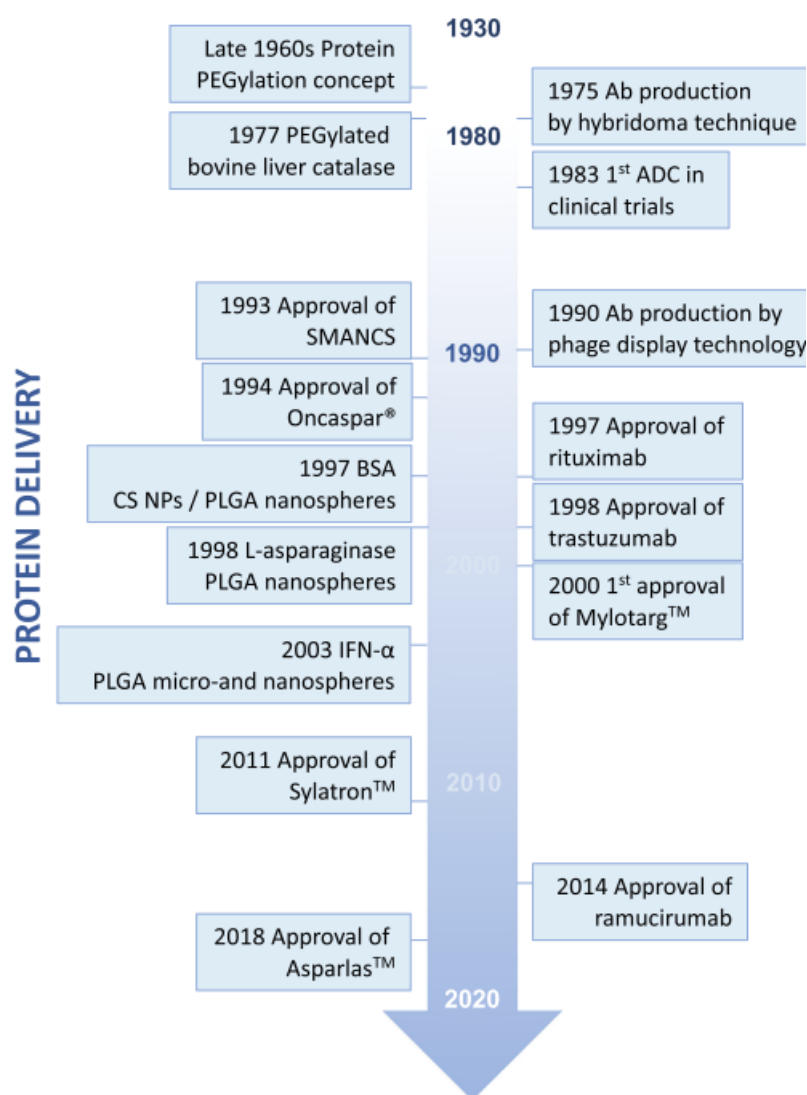


Figure 2. Timeline of the introduction of seminal advances in drug delivery technologies in the protein parenteral delivery field. PLGA poly(lactic-glycolic acid); PLG poly (lactide-co-glycolide); BSA bovine serum albumin; CS chitosan; IFN-α interferon-α; NPs nanoparticles

1.4 RECENT SEMINAL WORK IN PROTEIN DELIVERY IN CANCER THERAPY

In this section we cover the most impactful protein delivery nanotechnologies reported in the last years for the treatment of cancer. Due to the different nature and mechanistic aspects of the proteins delivered so far, we have chosen to classify the delivery nanotechnologies in the following groups: enzymes, apoptotic proteins, immunomodulators and, finally, mAbs.

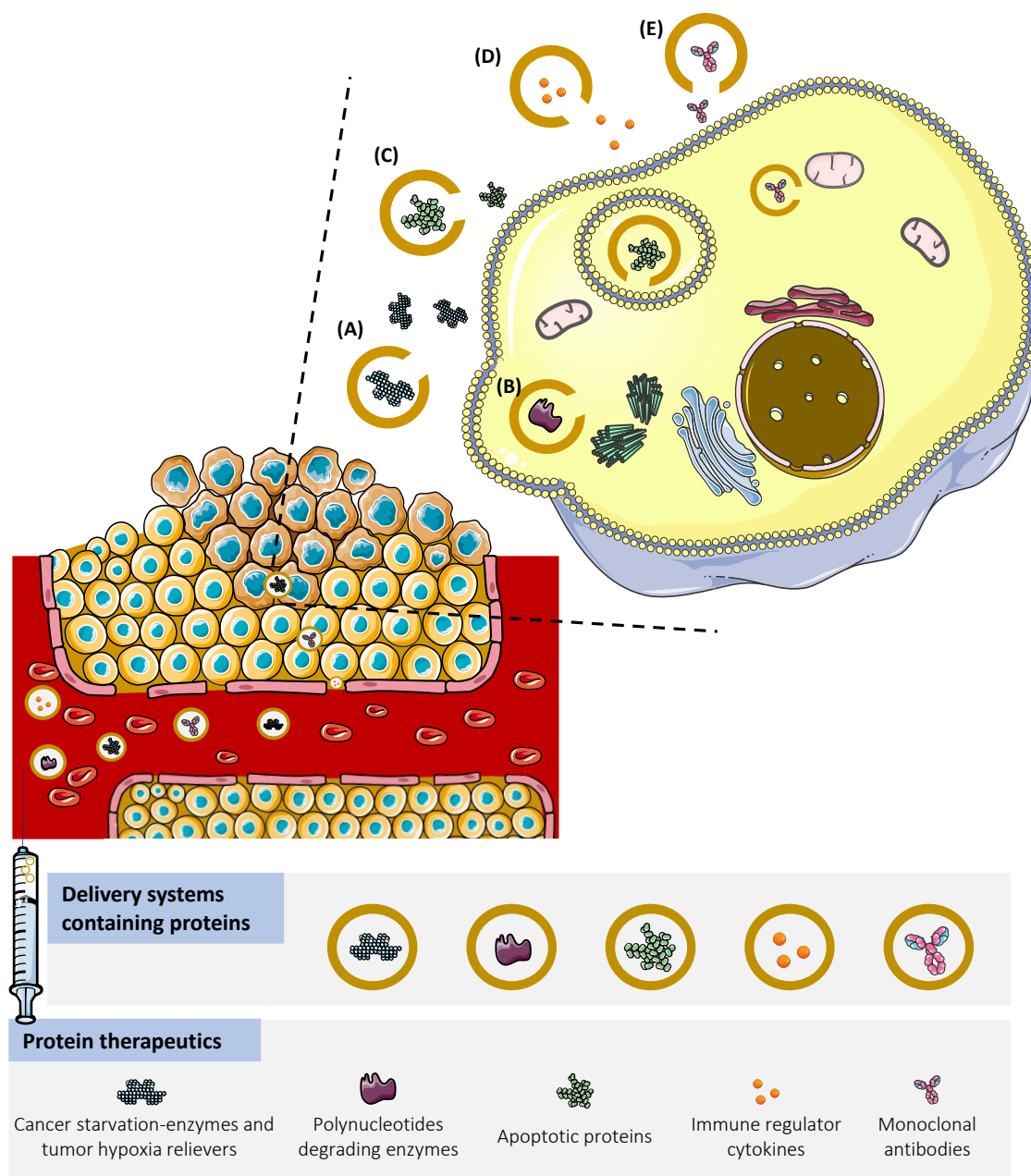


Figure 3. Summary of current protein delivery strategies for cancer treatment. Delivery of therapeutic proteins into the target tumor is highly challenging. The proper design of nanoplatfroms containing proteins, in addition to protecting biologicals, can modify their biodistribution, allowing them to reach the tumor. Once they are in their functional area, nanomedicines will release their cargo extra- and/or intracellularly: (A) Nanotherapies containing glucose oxidase or catalase can induce or relieve hypoxia, enhancing the efficacy of tumor therapies. (B) Nanomedicines containing DNase or RNase can be applied to catalyse the degradation of DNA or RNA, respectively. (C) Delivery of proteins such as Cytochrome C, Granzyme B or TRAIL may allow to exploit their apoptotic activities. (D) Several nanostrategies can be used to deliver cytokines, exploiting their pro-apoptotic or a proliferative activity, or induce the immune response against tumor cells. (E) Nanoplatfroms encapsulating monoclonal antibodies can induce apoptosis by direct targeting to tumor cells, or induce vascular and stromal cell disruption, or immune-related cell death activation at the TME.

1.4.1 Delivery of enzymes using nanotechnology

1.4.1.1 Tumoral delivery of cancer starvation-enzymes and tumor hypoxia relievers

One of the major physiological characteristics of cancer is the extremely rapid growth of cancer cells and their abnormal consumption of nutrients (e.g., glucose). Besides, the distance from the core of the tumor to the blood vessels makes the diffusion of oxygen difficult, which leads to tumor hypoxia. Different antagonistic strategies have been described to manage the tumor hypoxia: (1) to activate hypoxia by enzymes which will provoke the decrease of tumor oxygen (cancer starvation enzymes), thus preventing the development of the tumor and, on the other hand, (2) to relieve the hypoxia (tumor hypoxia relievers), thus favoring the diffusion of antitumor drugs and, hence, the efficacy of tumor therapies. The use of cancer starvation-enzymes, such as **glucose oxidase**, has attracted interest as it converts glucose and oxygen into gluconic acid and H_2O_2 , which results in an increase in cell apoptosis. For example, PEG-liposomes containing glucose oxidase in combination with a liposomal hypoxia-activated pro-drug resulted in a positive outcome in terms of tumor growth in a breast cancer mice model [45]. However, the production of H_2O_2 is a double-edged sword since it may cause DNA damage [46,47]. To confront this problem, Ma and co-workers, developed nanoclustered enzymes consisting of covalently crosslinked **glucose oxidase and catalase**, a catalytic enzyme able to decompose H_2O_2 into $H_2O + O_2$, via a pH-responsive linker. These nanostructures were coated with a conjugate of BSA and the hypoxia-activated chemotherapeutic, tirapazamine. Using this strategy, it was expected that once the enzymes were released in the acid conditions of the TME, the H_2O_2 would be decomposed by catalase to cause lower toxicity. Then, the hypoxia would activate the effect of tirapazamine. Indeed, the result of this treatment was the almost total eradication of the tumor growth in mice bearing EMT-6 mammary cancer cells [48].

A different strategy to mitigate tumor hypoxia has been trying to enhance the efficacy of the treatments. To this aim, catalase was entrapped in NCs or liposomes, in association with therapies, such as photodynamic [49] or radio-immunotherapy [50], respectively. In both cases, the therapeutic effects were remarkably improved. A similar approach to overcome hypoxia involved the encapsulation of catalase in antiPDL1 surface-modified liposomes. The result of this treatment was an enhanced tumor accumulation, a decrease in tumor growth and an increase in survival time, all of which was attributed to the tumor infiltration of $CD8^+$ T cells and hypoxia relief [51].

So far, the use of nanoplatfroms for the efficient delivery of these therapeutic enzymes in the TME has demonstrated the ability to exploit the tumor hypoxia condition, following co-delivery strategies. It remains to be seen whether ongoing research efforts will lead to next-generation strategies based on protein delivery.

1.4.1.2 Tumoral delivery of polynucleotide degrading enzymes

Several approaches have also been explored to achieve the intracellular accumulation of polynucleotide degrading enzymes. In an example, HA nanogels were developed for the co-encapsulation of **DNase I** and modified-acidic-activatable hyaluronidase (aHAase). The success of this system lies in turning tumor conditions into therapeutic opportunities. Thus, under slightly acidic conditions, aHAase is partially activated, causing the degradation of the

major constituent of the HA present in the extracellular matrix, thus allowing a deeper tumor penetration of the system. Once at the intracellular level, the nanogels escape from the endosomal compartment releasing DNase I intracellularly. This formulation led to a significant inhibition of tumor growth in human lung adenocarcinoma epithelial tumor bearing mice [52]. A similar strategy was described for ribonuclease A (RNase A). In this case, nanoassemblies including RNase A-loaded NCs and an antibiotic against cancer stem-like cells, doxycycline, were developed. A marked tumor growth inhibitory activity was observed when these nanoassemblies were intravenously administered in a breast cancer xenograft tumor model [53]. More recently, the encapsulation of RNase into poly (L-glutamic acid)-graft-PEG methyl ether-based nanogels was proven to trigger the protein release under hypoxic conditions thanks to the destruction of the cross-linking point between β -cyclodextrin and azobenzene. The application of this system in a breast cancer model achieved a significant tumor suppression rate (68.7%). Interestingly, the efficacy of this approach was improved when it was combined with a nanosystem containing a vascular disrupting agent, i.e. combretastatin A4 [54]. Recent advances in biotechnology and the identification of case-by-case needs will contribute to the success of polynucleotides degrading enzymes-based therapies.

1.4.2 Delivery of apoptotic proteins

Some strategies have been applied to the intracellular delivery of apoptotic proteins, i.e., Cytochrome C (Cyt C). Cyt C is a key component of the electron transport chain in mitochondria that binds to the protease activating factor-1 and triggers cell apoptosis. In a report, Cyt C was encapsulated into HA nanogels [55] and, also co-encapsulated with a plasmid DNA encoding the p53 protein [56]. The results of both approaches were similar in a SC and orthotopic breast tumor bearing mice model, respectively.

In a different work, the pro-apoptotic protein Granzyme B (GrB) loaded into HA nanogels could be delivered at the intracellular level, thus causing the reduction of tumor growth in subcutaneous human breast and orthotopic human lung tumor-xenografts mice models [57]. The outcome of the study could be improved by adding to the delivery carrier an epidermal growth factor receptor (EGFR) ligand, the GE11 peptide. The result of this treatment was the almost total suppression of the tumor growth in human ovarian carcinoma and human breast tumor-xenograft in mice [58]. A different approach using again GrB consisted on its conjugation with the cell-penetrating peptide, TAT, and further encapsulation into porous p-2-methacryloyloxy ethyl phosphorylcholine (PMPC)/HA NCs. This strategy led to a significant tumor accumulation which was correlated with a great reduction in tumor growth in a breast cancer model [59]. Similar strategies have been adopted for multiple myeloma (MM) treatment. In this context, polymersomes containing GrB were functionalized with HA for targeting CD44 in MM cells. This delivery approach led to a high accumulation of GrB in subcutaneous LP1 MM tumor as well as in the bone marrow of the orthotopic LP1 MM model. This accumulation was translated into a significant suppression of subcutaneous LP1 tumor and an increase survival in the orthotopic LP1 model [60]. In a recent contribution, the authors have applied their technology to cross the blood brain barrier (BBB) through the encapsulation of GrB or CpG oligonucleotide immunoadjuvant into polymersomes with 20% of ApoE density. The co-administration of GrB-loaded polymersomes and CpG-loaded polymersomes led to a remarkable apoptosis and immune response in an orthotopic glioma tumor-bearing mice model when compared with the control and the single treatments, respectively [61]

The Apo2 ligand, also called tumor necrosis factor (TNF)-related apoptosis-inducing ligand (Apo2L/TRAIL or TRAIL), has attracted attention due to its ability to induce apoptosis upon binding to the surface death receptors DR4 and DR5 on cancer cells. Unfortunately, so far TRAIL approaches could not be translated to the clinic due the unstable nature of the soluble form of the protein and to its short half-life (60 min in humans) among other reasons [62–64]. To overcome these limitations, TRAIL was associated to the surface of liposomes in combination with the adhesion receptor E-selectin. This receptor facilitates adhesion to selectin ligands leukocytes in blood and on tumor cells. Minimal administration resulted in a significant reduction of the metastatic burden following tumor resection in a 4T1 breast carcinoma mice model [65]. In this regard, the surface liposomal display of Apo2 ligand/TRAIL protein led to marked tumor growth inhibition in HT-29 colorectal carcinoma, following intraperitoneal administration [66]. In an alternative approach, TRAIL was associated to the surface of paclitaxel-loaded nanodisks functionalized with a fibronectin-targeting peptide. This strategy led to a significant reduction of the lung weight and metastatic nodules when compared with the single treatments or a liposomal counterpart in a lung metastatic melanoma mice model. The authors argued that nanodisks might have had a superior lung accumulation in comparison with the liposomes [67]. Likewise, the combination of RGD peptide and TRAIL with stimuli responsive elastin-like polypeptide-based NPs was reported to increase the half-life and to achieve an almost complete tumor regression in a colon carcinoma tumor xenograft model after single intraperitoneal administration [68].

Recently, an alternative approach to interfere with apoptosis has relied on the use of designed ankyrin repeat proteins (DARPs) to inhibiting RAS nucleotide exchange [69,70]. Indeed, DARPs have been recently included into lipid nanoparticles for RAS inhibition. Unfortunately, in this case the efficacy was comparable to the free protein [70].

Overall, the study of the delivery of apoptotic proteins is in its early-stages with only a few examples showing the efficacy of strategies. Subsequent development of this kind of treatments will shed light on its translational potential.

1.4.3 Immune regulator cytokines

Cytokines are immune regulatory proteins that have shown promising results in cancer therapy. However, their poor pharmacokinetic profile and their associated systemic toxicity have limited their therapeutic exploitation. Nanotechnology has been presented as a tool to overcome these drawbacks as illustrated by a number of examples described below.

As previously mentioned, the PEGylation of cytokines has been useful for increasing their half-life, however, other approaches have been later explored to improve their biodistribution and reduce their toxicity. For example, the intravenous administration of IL-2 Fc and antiCD137 anchored on the surface of PEGylated liposomes resulted efficacious in terms of reducing tumor growth in the absence of systemic toxicity, while the free drugs caused a lethal immunotoxicity. In this sense, the rapid accumulation of the liposomes in tumors could be responsible for their success [71]. Other combination therapies investigated have involved the co-administration of two cytokines, IL-2 and IFN- γ , and doxorubicin (DOX) into nanovesicles (NV). This combination of drugs was administered intravenously to triple negative breast cancer bearing mice leading to a significant inhibition of the primary tumor growth and lung

metastasis. These results were explained by the induction of DCs maturation that was accompanied by the infiltration and activation of natural killer cells and CD8⁺ T lymphocytes, as well as the recruitment of CD45⁺ immune cells and Ly6G⁺ neutrophils. In addition, the absence of systemic toxicity in terms of body weight was noticed in a murine melanoma model when IL-2-NV-DOX was compared to the free drugs [72].

In brief, the clinical use of IL-2 and IFN-alpha, as the only FDA-approved cytokines, were initially suggested as milestones in cancer treatment [73]. However, early findings of their short half-life and dose-related toxicities prompted the search for novel approaches. As noted above, several technologies have been shown to overcome these drawbacks and could potentially find their way to the clinic. Meanwhile, the development of specific immunotherapies, such as immune inhibitors checkpoints and others described in the following section, have attracted the attention of researchers and clinicians and, as a consequence, this still inefficiently exploited therapeutic approach was abandoned.

1.4.4 Monoclonal antibodies

The discovery of mAbs represented a critical milestone in cancer therapy. However, the therapeutic potential of these complex molecules has not yet been fully exploited due to their biopharmaceutical limitations. In this context, nanotechnology has been shown to be a useful tool for improving the accumulation of mAbs in tumors, thereby enhancing their efficacy and reducing their systemic exposure. In addition, our laboratory [74,75] and others [76,77] have shown it is possible to design nanocarriers intended to target intracellular oncoproteins that, historically, have been considered "undruggable targets". This section will be devoted to highlighting the most disruptive technologies developed in the last few years for improving the delivery and, hence, the clinical benefit of mAbs. Table 4 shows the delivery carriers developed for either tissue or intracellular targeting, and the results of their proof-of-concept studies in different animal models, following their administration as a mono- or a combination therapy.

Table 1. Summary of the most relevant nanocarriers for the delivery of mAbs N.A.: not applicable, HSA: human serum albumin, Treg cells: regulatory T cells; MPC: 2- methacryloyloxyethyl phosphorylcholine, GDMA: glycerol dimethacrylate, PLA-PEG-PLA: poly(d,l-lactide)-b-poly(ethylene glycol)- b-poly(d,l-lactide)-diacrylate triblock copolymers, HER2: human epidermal growth factor receptor 2 , VitE vitamin E; hTERT human telomerase reverse transcriptase; EM erythrocyte membrane; FITC: fluorescein 5(6)-isothiocyanate; VEGF-A: vascular endothelial growth factor-A, PEG-b-poly{N-[N'-(2-amino-ethyl)-2-aminoethyl]aspartamide} PEG-pAsp(DET), PLL -poly(L-lysine).

mAb	Cellular target (location)	Delivery system	Therapy	Tumor model	Ref.
Delivery of mAbs to the TME					
antiIL6R	IL-6 receptor (extracellular)	AntiCD44 Ab-liposomes	Monotherapy	Breast cancer (orthotopic)	[78]
Trastuzumab	HER2 (extracellular)	Micellar nanocomplexes	Monotherapy	Breast cancer	[79]
Trastuzumab	HER2 (extracellular)	PEG-VitE based hydrogels	Monotherapy	Breast cancer	[80]
Bevacizumab	VEGF-A (extracellular)	PEG-HSA NPs	Monotherapy	Colorectal cancer	[81]
Bevacizumab	VEGF-A (extracellular)	Lipid-polymer hybrid NPs	Erlotinib/ bevacizumab	Non-small cell lung cancer	[82]
Bevacizumab	VEGF-A (extracellular)	Liposomes	Doxorubicin-loaded HER2 Ab decorated liposomes/bevacizumab co-administration	Breast cancer	[83]
Bevacizumab	VEGF-A (extracellular)	HSA-bound paclitaxel (Abraxane®)	Abraxane®/bevacizumab	Melanoma cancer	[84]
Nimotuzumab / trastuzumab	EGFR / HER2 (extracellular)	MPC-peptide crosslinker based NCs	Monotherapy	Glioma (orthotopic)	[85]
Cetuximab	EGFR	MPC-peptide crosslinker based NCs	Cetuximab / AKT-inhibitor co-administration	Glioma	[86]
Rituximab	CD20 protein (extracellular)	MPC/PLA-PEG-PLA/GDMA-based NCs	Monotherapy	B-cell lymphoma (orthotopic)	[87,88]
Delivery of immune checkpoint inhibitors to the TME					
antiPD-1	PD-1 ligands (extracellular)	PEG-PLGA NPs	Monotherapy	Melanoma cancer	[89]
antiPD-1	PD-1 ligands (extracellular)	GRGDS peptide - PEG-PLGA NPs	Iron oxide/perfluoropentane/antiPD-1	Melanoma cancer	[90]
antiPDL-1	PDL-1 ligands (extracellular)	HA-polylysine NPs	Chlorin e6/dextro-1-methyl tryptophan/antiPDL-1	Melanoma cancer	[91]
antiPDL-1	PDL-1 ligands (extracellular)	Liposomes conjugated to Treg cells	Imiquimod/ IL-2/antiPDL-1	Melanoma cancer	[92]
antiCTLA-4	CTLA-4 ligands (extracellular)	Liposomes	Doxil®/antiCTLA-4 co-administration or monotherapy	Melanoma and colorectal cancer	[93,94]
antiCD47	CD47 ligands	MPC-NCs	antiCD47 / STING agonist	Glioblastoma (orthotopic)	[95]

Table 2. Summary of the most relevant nanocarriers for the delivery of mAbs (continuation)

Intracellular delivery of mAbs					
antigasdermin B	Gasdermin B protein (intracellular)	HA-NCs	Monotherapy	Breast cancer (orthotopic)	[74]
antiKRAS	Mutated KRAS (intracellular)	HA-NCs	Monotherapy	Pancreatic and colorectal cancer (orthotopic)	[75]
Bevacizumab	VEGF-A (extra- and intracellular)	CD44v6 Fab-PEG-PLGA NPs	Monotherapy	N.A.	[96]
Bevacizumab	VEGF-A (extra- and intracellular)	Tween 80/Tween 20/Brij 97 NPs	Monotherapy	N.A.	[97]
Bevacizumab	VEGF-A (extra- and intracellular)	Liposomes	Benzoporphyrin derivative-loaded PLGA-PEG NPs/bevacizumab	Pancreatic cancer (orthotopic)	[98]
Bevacizumab	VEGF-A (extra- and intracellular)	Liposomes	Benzoporphyrin derivative/bevacizumab	Pancreatic cancer	[76]
Cetuximab	EGFR (extracellular)	GSH-responsive silica NPs	Cetuximab/siRNA	Cervical carcinoma	[99]
antiKi-67	Ki-67 protein (intracellular)	Liposomes	FITC-antiKi-67-Ab conjugate	N.A.	[100]
antiTERT	hTERT (intracellular)	EM-coated self-assembling NPs	Monotherapy	N.A.	[101]
antiS100A4	S100A4 protein (intracellular)	Fusogenic liposomes	Doxorubicin/antiS100A4	Breast cancer	[77]
antiBCR/ABL	BCR/ABL protein (intracellular)	Transferrin targeted-PLGA NPs	Monotherapy	Chronic myeloid leukemia	[102]
antic-MYC	MYC protein (intracellular)	PEG-pAsp(DET)-PLL Polymeric micelles	Monotherapy	Colon cancer	[103]

1.4.4.1 Delivery of mAbs targeted to the TME

The diversity of the TME composition is responsible for its therapeutic complexity since it supports tumor growth and participates in the development of resistances [104,105]. In this sense, fibrosis and high interstitial pressure have been reported as two of the main causes for the limited tumor accumulation [104]. Therefore, several efforts in the nanotechnology field have been directed towards target different molecules that are relevant in the TME. For example, the modification of the TME organization was possible with the administration of antiIL6R Ab loaded liposomes decorated with an antiCD44 Ab. This treatment blocked IL6R-Stat3 signaling and led to the reduction of the expression of several downstream molecules, such as MMP-9 and Sox2, which was accompanied by a reduction of angiogenesis and the macrophages phenotype conversion from M1 to M2. Ultimately, these effects translated into the inhibition of tumor metastasis in different breast cancer models [78].

Similarly, different nano-delivery systems have been proposed for the intratumoral delivery of trastuzumab, which targets the HER2 [79,80] or bevacizumab (BVZ), a VEGF-A blocker [81–84]. For instance, it is worth mentioning that the association of trastuzumab to micellar nanocomplexes resulted in a change in the pharmacokinetic and biodistribution profile of the mAb with a final output in terms of reduction of the tumor volume in a human breast

cancer xenograft tumor model, following intravenous administration [79]. In another example, trastuzumab-loaded PEG- vitamin E hydrogels were also investigated for the treatment of breast cancer. Single SC administration near the tumor site led to a remarkable reduction in the tumor growth compared to both, the SC and intravenous administration of the free mAb [80]. Unfortunately, in the specific case of BVZ, the benefit achieved by the encapsulation in terms of improving the biodistribution or efficacy of the encapsulated monoclonal is still under debate. For example, some authors have found that the administration of BVZ-loaded PEG-HSA-NPs led to significantly higher intratumoral levels of BVZ, when compared to the free BVZ. However, this accumulation did not translate to a higher antitumor efficacy when the mAb was administered as a monotherapy [81]. More positive outcomes were reported for treatments involving the co-encapsulation of BVZ and other drugs in the same NPs [82–84]. This should not be surprising if we take into account that, at the clinical level, BVZ is always administered as a combination therapy.

Nanotechnology has also been investigated as a way to facilitate the delivery of drugs to tumor areas of difficult access, i.e., the central nervous system (CNS). For example, NCs made of choline and acetylcholine analogues (2-methacryloyloxyethyl phosphorylcholine: MPC polymer) were loaded with nimotuzumab or trastuzumab and the resulting formulations were found to facilitate the accumulation of the mAbs in the cerebrospinal fluid and brain and showed remarkable antitumor efficacy in a glioma mice model [85]. The same delivery vehicle was recently expanded for the delivery of cetuximab in combination with an AKT inhibitor. The early administration of the NCs prior to the small molecule yielded a prolonged survival time and reduced the expression of phosphorylated EGFR and AKT, and Ki67 protein [86]. In a different study NCs composed by PLA-PEG-PLA and MPC and loaded with rituximab (RTX), were found to facilitate the delivery of the mAb to the CNS following intravenous administration. The final output of this formulation was a decrease in the brain lymphoma metastases in a non-Hodgkin lymphoma xenograft murine model [87]. The relevance of this approach in immunocompetent animals, rats and non-human primates (NHPs), was also reported. The results showed higher RTX levels in CNS (10-fold) and in LN (2-3 fold) in both animal models when compared with the free molecule. Interestingly, NCs revealed lack of blood, liver or brain toxicity in NHPs [88].

1.4.4.2 Delivery of immune checkpoint inhibitors to the TME

The discovery of ICIs, such as antiCTLA-4, antiPD-1 or antiPDL-1 represented a major milestone in oncological therapy. However, these mAbs are far from ideal as significant immune-related side effects associated with their indiscriminate biodistribution have been reported [106,107]. Once more, researchers have used nanotechnology to overcome this limitation. For example, Ordikhani et al. proposed the incorporation of anti-PD-1 in NPs composed of mPEG-PLGA for the treatment of melanoma. Results demonstrated the uptake of NPs by CD11c+ dendritic cells (DCs) in the spleen, which triggered DCs activation and maturation. As a result, effector T cells were activated, resulting in a strong immune response. The administration of a low dose of antiPD-1 demonstrated a marked antitumor efficacy in a murine melanoma model, which was mainly attributed to the selective uptake of NPs by DCs in secondary lymphoid tissues [89]. A different technological approach consisted in encapsulating antiPD1, in PLGA NPs, functionalized with PEG and Gly-Arg-Gly-Asp-Ser peptides in combination with a photothermal therapy. This synergistic treatment allowed a controlled release of the antiPD1 by thermal treatment and increased the infiltration of CD8+

T cells to the TME, reaching an almost complete tumor regression in a melanoma mouse model [90]. Another approach combined the use of NPs of HA, polylysine derivatives and antiPDL1, with photodynamic therapy. This immunotherapy was found to be efficacious against tumor metastasis, relapse, and postsurgical regrowth [91]. Similarly, a combination therapy consisting of imiquimod, antiPDL1 and IL-2 encapsulated in liposomes and, then, conjugated on the surface of regulatory T cells, triggered the maturation of DCs, inhibition of the PD-1/PD-L1 immune-checkpoint, and infiltration of CD8⁺ T cells, resulting in a strong suppression of primary and metastatic tumors [92].

Similar strategies have been adopted for the delivery of the immune-checkpoint inhibitor, antiCTLA-4. For instance, antiCTLA-4-loaded PEG-liposomes led to a significant tumor growth reduction and increased CD8⁺ population and CD8⁺/Treg ratio in tumor-infiltrated lymphocytes, when compared to the free CTLA-4 administration. Moreover, the administration of antiCTLA-4 liposomes prior to Doxil® led to an improvement of the antitumor efficacy in a melanoma mouse model [93]. These results correlate with previous studies, where the antiCTLA-4 encapsulation into PEGylated liposomes significantly increased the tumor accumulation via the EPR effect [94].

The recent entry to phase II clinical trials of an antiCD47 (AK117) [108] has led to the development of strategies that improve its efficacy. On this basis, NCs made of a choline and acetylcholine analogue polymer (i.e., MPC) were used for the delivery of antiCD47, and the stimulator of interferon genes cyclic diguanylate monophosphate (CDG) to cross the BBB. The main outcome of this strategy was a remarkable reduction of the tumor growth accompanied by the polarization of the M1-phenotype in an orthotopic glioblastoma tumor model, compared with the free mAb [95].

1.4.4.3 Intracellular delivery of mAbs

The majority of the oncoprotein targets remain elusive because they are localized at the intracellular level and do not exhibit the adequate pockets for interaction with small molecules. The term “*undruggable*” has been coined to describe this situation. Although the most frequent oncogene mutation is the Kirsten Rat Sarcoma Viral Oncogene Homologue (KRAS), others such as MYC, MYB, and nuclear factor- κ B (NF- κ B) have been described as undruggable oncogenes [17]. MABs could be ideal candidates to interact with these targets, however, so far, their use has been restricted by their inability to enter the cancer cells. Although several approaches such as cell-penetrating peptides and physical methods (e.g., electroporation or microinjection) have been used to this end, alternative strategies must be explored in order to protect the mAb and reach a translation in humans, clearly discarded in the case of physical methods [109].

A few years ago, our group, was pioneer in demonstrating the feasibility of targeting the intracellular oncoprotein gasdermin B (GSDMB). Indeed, HA NCs were developed for the delivery of antigasdermin B (AbGB). Among the results that support the preclinical relevance of this prototype, it is important to mention the ability of AbGB-loaded NCs in reducing the migration rate of tumor cells and the reduction of the tumor volume in a HER-2⁺ breast cancer model. These outcomes were a consequence of the accumulation of the AbGB inside the tumor cells and its ability to interact with the target GSDMB [74].

These results encouraged us to move forward and develop a proprietary nanotechnology platform (Multi-functional Polymeric Nanocapsules, MPN Technology®) for the intracellular delivery of antiKRAS mAb [75]. The KRAS target is one of the most relevant and challenging oncological targets. One specific mutation, KRAS^{G12C} could be finally targeted after decades of efforts [110,111] however, other mutations of the same target still remain “undrugged”. Therefore, our laboratory’s efforts have focused on finding ways to reach other KRAS mutations. The results obtained so far have shown promising *in vivo* responses in several murine models harboring G12D and G12V KRAS mutations. To our knowledge, this is the first nanotechnology that has been proven to facilitate the intracellular delivery of the antiKRAS mAb, thereby eliciting antitumoral responses following intravenous injection in different mice cancer models [75].

Beside KRAS, other relevant intracellular targets that have been confronted with the use of nanotechnology include BCR/ABL and MYC. For example, transferrin decorated PLGA NPs loaded with an antiBCR/ABL mAb, which were found to elicit a remarkable anti-leukemia efficacy in mice [102]. In another approach, antiMYC mAb entrapped into PEGylated-lysine and -aspartamide micelles was found to achieve a significant reduction of the tumor volume in a murine colon adenocarcinoma model [103].

Other authors have explored the effect of nanoformulations of mAbs whose target is localized either inside or outside the cells. This is the case of the VEGF-A, whose inhibition at the intracellular level has been proposed as a therapeutic strategy. Based on this premise, BVZ was encapsulated into PLGA-PEG NPs decorated with an antibody fragment (F_{ab}) specific for CD44v6-expressing human cancer cells and the resulting formulation was found to be effective in terms of reducing VEGF intracellular levels in a human stomach adenocarcinoma epithelial cell line [96]. Additionally, self-associated NPs further demonstrated its effective internalization in a non-small cell lung cancer cell line [97]. In other examples, liposomal strategies for the co-delivery of BVZ and the photodynamic therapy agent benzoporphyrin derivative monoacid (BPD), have been found to significantly reduce tumor growth in a SC pancreatic ductal adenocarcinoma mouse model [76] and orthotopic murine pancreatic model [98]. With both strategies, a BDP synergistic effect and VEGF-A intracellular blocking could be obtained. A similar rationale has been applied for cetuximab. Although the EGF receptor has been clearly identified as an extracellular receptor, a recent work has aimed to intracellularly deliver PEGylated stimuli-responsive silica NPs co-encapsulating siAKT and cetuximab. The reason behind this approach resides in the expected comparable efficacy after the extra- or intracellular delivery of the mAb. As a result, a significant reduction of the tumor growth was achieved in a HeLa tumor-bearing mice model, in contrast to the NPs monotherapy. These results were partially attributed to the passive accumulation within the tumor of the NPs [99].

Other intracellular delivery approaches explored so far include a liposomal formulation containing a photoactivatable antiKi-67 Ab conjugate that aimed to reach the nucleus [100] and erythrocyte membrane-coated antiTERT mAb Tween 80 NPs [101], although no *in vivo* data have been reported. Furthermore, fusogenic liposomes loaded with antiS100A4 mAb and doxorubicin have been developed to achieve a cytoplasmatic delivery. This last formulation was also tested in a breast cancer xenograft model, and the results showed not only the reduction of the tumor growth but also the suppression of liver metastasis [77]. Despite the great achievements regarding the tumor accumulation by passive mechanisms,

actively targeted nanocarriers are expected to trigger the generation of novel delivery systems for the entrapment of mAbs.

1.5 CONCLUSIONS AND PROSPECT VIEW

In the context of oncological therapies, the development of protein delivery strategies is still in early stages. Despite the number of nanomedicines that have made their way to the market, its clinical and preclinical failures observed lies in numerous factors yet to be resolved such as NPs accumulation in liver and spleen, the limited diffusion across the tumor environment, the difficult access to metastatic niches and, ultimately, the hurdles associated to the intracellular delivery. The true potential of targeted delivery systems is yet to be realized specially for the protein/peptide drugs.

Aiming at confronting these challenges from early stages of formulation design and characterization, including *in vivo* anticancer efficacy studies, we must keep in mind the difficulties for mimicking the human tumor complexity, heterogenicity and immune status. Particular considerations such as the EPR effect, tumor size and mouse strains must be prudently considered in order to provide conclusive data. For example, high tumor volumes at the onset of treatment normally reduce the efficacy of the therapy, which might be due to low penetration and increased hypoxia [112]. Moreover, NPs clearance may be governed by the mouse immune status, thus, Th2 strains such as BALB/c elicit faster clearance when compared with Th1 strains (e.g., C57BL/6) [113]. As a whole, the generation of advanced animal models such as patient-derived xenografts, humanized or genetically engineered mouse models with aggressive metastasis are fundamental for the clinical translatability of the nanomedicine products [114]. In this sense, the use of *in silico* predictive models that could merge tumor variability over time, resistance as well as transport barriers faced by NPs, which will allow to improve predictive equations. However, to properly implement this and similar strategies, further knowledge of the physiological scenario, tumor type, NP-cell interaction, and transport mechanisms, must be generated.

It should also be mentioned that the development of nanocarriers for the intracellular delivery of mAbs has been an important achievement within this field. We are confident that the design of new mAbs and nanobodies against undruggable targets is a promising next step for nanoncologicals therapeutics. In parallel, the emergence of immunotherapies has changed the concept of tumor therapy, providing the possibility to target immune cells located both in the tumor area and in relevant distant organs such as the spleen. Thus, the application of nanotechnology offers the opportunity to manage the antitumor immune response, either by blocking or by boosting its response.

As a whole, the field is moving towards introducing special chemical modifications in the nanosystems that can exploit tumor pathophysiological conditions. Ultimately, the design of personalized strategies based on case-by-case needs and a better understanding of oncological demands will be crucial to open up new avenues of treatment.

Finally, in our view, the main common challenge in the development of new nanomedicines, regardless of its intended application, relies on the fact that small changes in the composition may drastically change the pharmacokinetic and pharmacodynamic profile. This together with

the limited standardized regulatory guidelines by the FDA and EMA for nanomedicines makes harder reaching a clinical approval. Significant efforts are being taken in this direction, as illustrated in the REFINE NANOMED project [115], which aims to provide a regulatory science framework for the risk-benefit assessment of nanomedicine. Nevertheless, beyond these recognized challenges, the case of COVID mRNA vaccines making use of nanoparticles together with recent increase of nanomedicines in the market, we could anticipate that, overall, the clinical exploitation of biological drugs will significantly benefit from the advances in the nanomedicine field.

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BACKGROUND, HYPOTHESES AND OBJECTIVES

1 BACKGROUND

It has been estimated that the number of people that will suffering cancer by 2040 will be 28.9 million [1]. This discouraging situation is a consequence of the poor therapeutic outcome of chemotherapy, associated with a questionable safety profile and the still limited amount of advanced and targeted therapies. Among the different causes of cancer mutations in the Kirsten rat sarcoma virus (KRAS) oncoprotein are particularly relevant as they have been subscribed to 9-30% of human cancers [2,3]. However, only one KRAS mutation have been addressed [4,5], considering the other as *undruggable*.

Within this context, monoclonal antibodies (mAbs) have settled a new era in the treatment cancer as their efficacy and selectivity stands out. However, the drawbacks associated to their inadequate biodistribution and incapacity to cross cell membranes have limited their full exploitation [6].

Nanotechnology has been pointed out as a promising strategy to overcome the biopharmaceutical problems of biological drugs. Nevertheless, the exploitation of the nanotechnology for the delivery of mAbs is still in early stage. The work done so far regarding the design of nanoparticles (NPs) for the delivery of mAbs [7–10] have been mainly oriented to targets localized on the cell membranes or at the extracellular level, whereas the attention to intracellular targets has been very low. In this regard, our group pioneered the development of nanotechnologies for the intracellular delivery of mAbs. More specifically, we could show *in vivo* that hyaluronic acid-based nanocapsules could efficiently deliver *in vivo* the mAb anti-gasdermine [11]. We believe this was a clear milestone in the field. While other researchers have follow similar approaches, this specific area of research is in an early phase [12–14].

The clinical outcome of marketed nanoncologics in terms of efficacy and toxicity [15] is unquestionable, however, their accumulation and delivery efficiencies remains low [16]. Likewise, the development of nanomedicines able to address *undruggable* oncogenes is poorly explored, pointing out the necessity to shed light on those of particular concern, such as KRAS-mutated cancers. Further understanding of how to develop disease-oriented NPs able to overcome the biological barriers faced after administration, are crucial to ensure the industry transability of the new generation of oncological treatments [17,18]. Therefore, the background information allows us to propose the below hypotheses.

2 HYPOTHESES

1. Polymer-based nanoassemblies (HANAs) are potential delivery systems for mAbs. The control of the critical parameters that governs their properties are crucial for their clinical translation.
2. The entrapment of mAbs into the HANAs can modulate the biodistribution of the biological. By functionalizing their surface with an active targeting ligand, an enhanced tumor accumulation of the HANAs should be achieved.
3. The validation of the HANAs for the entrapment of the clinically relevant antiKRAS^{G12V} mAb is a strategy to impair the cell proliferation and colony formation

of KRAS-mutant cell lines. Indeed, an efficient intracellular delivery will allow the antiKRAS^{G12V} mAb recognition and interaction to the KRAS^{G12V} oncoprotein.

3 OBJECTIVES

Taking into account the background and hypotheses stated above, the main objective of this thesis was to develop polymer-based nanoassemblies (HANAs) for the entrapment of mAbs, aiming to achieve an intracellular delivery in the context of cancer. To this end, the first experimental goal was to design, develop and functionalize the HANAs, with a special focus on the critical parameters that determines the resulting physicochemical properties of the NPs. Secondly, to study if the biodistribution of a mAb can be modulated by its incorporation into the HANAs, and more in detail, whether the presence of a tumor penetrating peptide on the HANAs improve the tumor accumulation. Lastly, to prove the HANAs technology versatility for the intracellular delivery of the antiKRAS^{G12V} mAb as a therapeutic agent and their capacity to facilitate the engagement of the KRAS^{G12V} oncoprotein. These objectives were achieved following the next steps:

1. Design, development, functionalization, and *in vitro* evaluation of the HANAs entrapping a model mAb.

The results corresponding to this objective are presented in Chapter 1 “Polymer nanoassemblies for the delivery of monoclonal antibodies: development and functionalization”

2. Evaluate the role played by the tumor penetrating peptide on the functionalized ⁸⁹Zr-radiolabeled HANAs *in vitro* and *in vivo* and the capacity to modulate the biodistribution profile of the mAb.

The results corresponding to this objective are presented in Chapter 2 “Multifunctional nanoassemblies as a strategy to improve the tumor uptake of monoclonal antibodies: *in vitro* evaluation and PET *in vivo* imaging”

3. Design, development, and *in vitro* evaluation of the HANAs for the intracellular delivery of the antiKRAS^{G12V} mAb.

The results corresponding to this objective are presented in Chapter 3 “Unleashing the power of personalized nanomedicine: intracellular delivery of anti-KRAS^{G12V} mAb to combat lung cancer”

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CHAPTER 1

Polymer Nanoassemblies for the delivery of monoclonal antibodies: development and functionalization

CHAPTER 1:

Polymer Nanoassemblies for the delivery of monoclonal antibodies: development and functionalization

This work has been done in collaboration with the CIC bioGUNE research institute and with the University of Macau.

ABSTRACT

Monoclonal antibodies (mAbs) have entered the field of cancer therapeutics with unquestionable success. However, their inability to cross cell membranes has limited their exploitation for targeting only extracellular targets. In order to overcome this critical limitation, our laboratory has pioneered the development of nanotechnologies that enables the transport and release of mAbs into the intracellular compartments. The objective of this work was to design and develop a nanotechnology characterized by its simplicity in the manufacturing process and composition, both being critical for the translational potential. [Section with copyright protection]. After a screening of over 124 NAs, we were able to identify the experimental conditions that led to HANAs with a size smaller than 200 nm, a neutral Zeta potential and association efficiency values of up to 99%. These prototypes presented a final mAb loading of up to 46.6%, never reported before for biological drugs, and this amount was actively and slowly released *in vitro*. In addition, HANAs were found to be stable in simulated biological media, and also during long-term storage in suspension. Moreover, the HANAs could be freeze-dried and resuspended without altering the initial properties. Finally, in order to further validate our technology for targeting cancer cells to the tumor, the surface of HANAs was functionalized with the peptide tLyP1. Cellular studies in CMT 167 and RAW 264.7 showed the very low cytotoxicity of HANAs and their capacity to enter into the target cell. In short, this is a new approach for the intracellular delivery of mAbs and, as such, opens new avenues for the intracellular targeting of so far known as undruggable targets.

GRAPHICAL ABSTRACT

[Section with copyright protection]

1 INTRODUCTION

MAbs have settled the new era of oncologicals. Their selectivity and specificity have resulted in an increase of the efficacy and a reduction of side effects of oncological treatments [1,2]. The best example of their success is the exponential number of Abs that have reached the market, nowadays representing the 69.4 % of the biologicals approved in the context of cancer [3]. Despite this success, these complex molecules suffer of important limitations such as inadequate biodistribution and inability to cross cell membranes, which have hampered their full exploitation. Our laboratory was the first to show that nanotechnology may be a very useful tool to overcome these limitations, thus opening a new area of research that has room for significant improvements in cancer therapy and, more explicitly, in personalized medicine [4].

The use of nanotechnology for the targeted delivery of oncological drugs is still challenging. Although marketed nano-oncologicals have shown unquestionable improvements in terms of efficacy and toxicity [5], their targeting capacity remains low. In this sense, the disease-oriented design of nanocarriers taking into consideration the multiple biological barriers, is critical for the emerging generation of oncological treatments [6,7]. In this regard, the particle size has driven the efforts in the field over the years as it influences the pharmacokinetics and biodistribution of the nanocarrier. It has been widely accepted that particle sizes close to or below 100 nm are desirable [8–10] to enhance the tumor accumulation since it may reduce the opsonization and enhance the blood circulation time [11]. Besides, the chances to improve the tumor accumulation of the NPs are also closely related to their stealth properties, which could be achieved by various strategies. For instance, neutral and hydrophilic coating surfaces are expected to diminish the unspecific uptake [12–14] and extend the blood circulation time of the nanocarriers [15,16]. [Section with copyright protection].

[Section with copyright protection].

Among the active targeting strategies, we and others have observed that tumor-homing peptides offer opportunities for enhancing tumor accumulation [17–19]. These peptides are defined by a common denominator, the Cend-R motif. CendR peptides are defined by a vascular recognition motif R/KXXR/K in which X can be a random amino acid, and the exposure of the C-terminal motif for receptor recognition and neuropilin (NRP) affinity for tissue penetration [20,21]. Within this family of peptides, we have found that truncated LyP-1 (tLyP1), which interacts with the NRP-1/2 [22] overexpressed in tumor cells [23,24] and lymphatic vessels [25], offers interesting targeting opportunities.

Interestingly, very few nanotechnology approaches have been explored for the intracellular delivery of mAbs [26–31]. With regard to the design of nanocarriers for this aim, it is remarkable the fact that only the potential of liposomes, polymeric nanoparticles (NPs,) and nanocapsules have been explored. In this line, these delivery systems have shown a favorable biodistribution profile for their accumulation into the tumor [28,29] and high intracellular delivery efficiencies of the mAb. These have led to a remarkable suppression of the tumor growth after addressing the intracellular located oncoprotein [30,31]. Among them, it is important to highlight that our laboratory has pioneered this field by developing a multifunctional polymeric nanocapsules technology which enables the intracellular delivery of anti-gasdermin [28] and also anti-KRAS mAbs.

1.1 OBJECTIVES

Based on this background, the objective of this work was to develop a new nanotechnology for the intracellular delivery of mAbs with high possibilities for clinical translation. Accordingly, we designed polymer-based nanoassemblies (HANAs) endowed with the following features: (i) [section with copyright protection]. In addition, BVZ was selected as a model mAb; (ii) exhibiting a high association efficiency of the selected mAb and a high loading capacity; (iii) providing a controlled release of the selected mAb; (iv) having an adequate biological and physical stability during storage, and (v) having a cell internalization and targeting capacity. To achieve this last point and taking into account our previous experience with the tLyP1 peptide as a targeting ligand, we linked this peptide to one of the components of the HANAs, [section with copyright protection]. Resulting bevacizumab-loaded HANAs were characterized based on the physicochemical properties, architecture, and capacity to entrap Abs. Ultimately, the biocompatibility and capacity to internalize Abs was evaluated *in vitro* in lung and macrophages cancer cell lines.

2 MATERIALS AND METHODS

2.1 MATERIALS

The humanized mAb bevacizumab was kindly donated by mAbxience (Spain). [Section with copyright protection]. Fetal bovine serum (FBS) was obtained from Gibco (Thermo Fisher Scientific, United States). Polysorbate 80 (Tween® 80) and methanol-D4 (CD₃OD), deuteration degree 99.8% were provided by Merck (Darmstadt, Germany). 1,1'-Diocetyl-3,3,3',3'-Tetramethylindodicarbocyanine (DiD) was obtained from Invitrogen, Thermo Fisher Scientific (United States). The truncated LyP-1 peptide (aminoacidic sequence: CGNKRTR) was synthesized by ChinaPeptides Co., Ltd. (Shanghai, China). Resazurin sodium salt, Triton X-100, Accutase®, [section with copyright protection], HEPES buffer, deuterium oxide (D₂O) and dimethyl sulfoxide-d6 (DMSO-d6) were acquired from Sigma-Aldrich (Darmstadt, Germany). [Section with copyright protection]. The 96 multiwells plate, recombinant Human VEGF165, SnakeSkin™ Dialysis Tubing (regenerated cellulose, SnakeSkin 7K molecular weight cutoff MWCO, 22 mm) were provided by Thermo Fisher Scientific (United States). Goat anti-human IgG HRP conjugated was obtained from Jackson Immuno Research Laboratories, Inc. (United States). 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid (ABTS) solution was acquired from Roche (Switzerland).

2.2 SYNTHESIS OF POLYMER-tLYP1 CONJUGATE

2.2.1 Carbodiimide chemistry – polymer-maleimide (polymer-Mal) conjugation

[Section with copyright protection]. The product was purified by dialysis (MWCO 7.0 kDa) against EtOH:NaCl 150 mM (50%, v/v) for 4-6 h and then, then three cycles against ultrapure water overnight and 2 more cycles of 4-6 h. The intermediate polymer-Mal was frozen at -80°C for at least 1 h, freeze-dried (Genesis™ 25 EL, S.P Industries, PA, USA) and stored at -20°C. The scale up process was conducted by increasing 5-times the volume of reagents. For the synthesis of other molar ratios, the condition reactions were properly adjusted.

[Section with copyright protection].

2.2.2 Maleimide chemistry – polymer-tLyP1 conjugation

[Section with copyright protection]. The resulting conjugate was purified by dialysis (MWCO 7.0K) against EtOH:NaCl 150 mM (60%, v/v) for 4-6 h and then, three cycles against ultrapure water for 4-6 h, overnight and 4-6 h. The polymer-tLyP1 conjugate was frozen at -80°C for at least 1 h, freeze-dried (Genesis™ 25 EL, S.P Industries, PA, USA), and stored at -20°C.

[Section with copyright protection].

2.2.3 Synthesis of surfactant-PEG-tLyP1 conjugate

2.2.3.1 Maleimide chemistry – surfactant-PEG-Mal-tLyP1 conjugation

Surfactant-PEG-Mal was synthesized following a maleimide-thiol coupling reaction between the maleimide group of the PEG chains and the thiol group of the peptide. Surfactant-PEG-Mal and tLyP1 were dissolved at an initial concentration of 1 mg/mL and 40 mg/mL, respectively, in HEPES buffer 10mM (60% (v/v) ethanol in ultrapure water, pH 7.2). Then, the peptide was placed into the PEG-lipid solution. The molar ratio for tLyP1: surfactant-PEG-Mal was set at 2:1. After stirring at RT overnight, the unreacted peptide was removed by dialyzing against NaCl 150 mM (2 h and then, two more cycles against ultrapure water. Finally, the functionalized lipid was kept at -80°C for at least 1 h, freeze-dried, and stored at -20°C. The final product was analyzed by proton nuclear magnetic resonance (¹H-NMR) spectroscopy.

2.2.4 ¹H-NMR spectroscopy

¹H-NMR spectra were recorded using a Bruker DRX-500 or NEO-750 spectrometer. [Section with copyright protection]. When required, trimethylsilylpropanoic acid (TSP) was used as an internal standard. ¹H-NMR spectra were recorded at 300 K, 64 to 2048 accumulated scans and a relaxation delay of 1.6-10s.

Surfactant-PEG-tLyP1 was dissolved in DMSO-d₆ at a concentration of 2 mg/mL, and transferred into 5-mm NMR tubes. Surfactant-PEG-Mal and tLyP1 standard solutions were used as controls at the same conditions. The spectra were acquired at a temperature of 300K, with 64-128 accumulated scans and a relaxation delay of 10-12s.

MestreNova Software (Mestrelab Research S.L.) was used for spectral processing. [Section with copyright protection].

2.3 PREPARATION OF POLYMER-BASED NANOASSEMBLIES (HANAs)

2.3.1 HANAs

[Section with copyright protection].

The NPs were submitted to a Tangential Flow Filtration (TFF) or concentration under nitrogen procedure until reach a BVZ concentration of 3.2 mg/mL following the method described in section 2.7.2.

2.3.2 PEGylated HANAs

[Section with copyright protection].

The NPs were submitted to a TFF or concentration under nitrogen procedure until reach a BVZ concentration of 3.2 mg/mL following the method described in section 2.7.2.

2.3.3 Scaling-up of HANAs

[Section with copyright protection]. NPs were finally diafiltrated and concentrated up to 3.2 mg/mL BVZ by TFF as described in section 2.7.1. Likewise, the main physicochemical properties of the NPs were characterized by DLS (see section 2.8.)

2.4 PREPARATION OF tLyP1 TARGETED HANAs

2.4.1 tLyP1-targeted HANAs

[Section with copyright protection].

tLyP1 NPs were submitted to a nitrogen concentration until reach a BVZ concentration of 3.2 mg/mL, following the method described in section 2.7.2.

2.4.2 tLyP1-targeted PEG-HANAs

[Section with copyright protection].

tLyP1 NPs were submitted to a nitrogen concentration until reach a BVZ concentration of 3.2 mg/mL, following the method described in section 2.7.2.

2.5 FLUORESCENCE LABELING OF HANAs

Fluorescent labelled nanocomplexes were prepared adding 25 μ L or 10 μ L of DiD into the ethanolic phase. [Section with copyright protection]. Samples were concentrated by TFF (prototype B) or under nitrogen evaporation (prototype C1).

2.6 MICROFLUIDICS

A NanoAssemblr® Benchtop device (Precision Nanosystems, Vancouver, Canada) was used for the preparation of the NPs. [Section with copyright protection].

2.7 CONCENTRATION OF HANAs

2.7.1 Diafiltration and concentration by TFF

In order to diafiltrate and concentrate the formulation, it was used a KrosFlo® KR2i Tangential Flow Filtration System (Repligen, Waltham, MA) with a membrane MWCO 100 kDa (modified polyethersulfone, surface: 20cm²). Thus, the formulation is added to the feed reservoir and diafiltrated 5 times (previously equilibrated with PBS at 12 mL/min) using PBS as eluent at 12 mL/min. Then, NPs are concentrated by following the above-described process, but no eluent is used. Following this method, the formulation medium of the NPs was shifted from ethanol/ultrapure water to PBS and concentrated 1.6 times.

2.7.2 Nitrogen evaporation

NPs were concentrated, removing the ethanol under a nitrogen stream until the samples reached a BVZ final concentration of 3.2 mg/mL.

2.8 PHYSICOCHEMICAL AND MORPHOLOGICAL CHARACTERIZATION

The physicochemical characterization of the NPs was performed using a Malvern Zeta-Sizer (NanoZS, ZEN 3600, Malvern Instruments, Worcestershire, United Kingdom). Particle size, polydispersity index (PDI) and derived count rate (DCR) were determined using Dynamic Light Scattering (DLS) after diluting accordingly the samples in PBS. The zeta potential was calculated measuring the mean electrophoretic mobility after dilution in PBS by Laser Doppler Anemometry (LDA), using the same device. Further NPs characterization was performed by NPs Tracking Analysis (NTA) after a dilution 1:1000 or 1:10000 in PBS (NanoSight NS3000, Amesbury, United Kingdom).

Morphological analysis was performed by field emission scanning electron microscopy (FESEM) (Zeiss Gemini Ultra Plus, Oberkochen, Germany) and cryo-Transmission Electron Microscopy (cryo-TEM).

For FESEM analysis, NPs were accordingly diluted in PBS, and then mixed with phosphotungstic acid (2% in ultrapure water) in a volume ratio 1:1. A volume of 1 μ L of the previous mixture was placed on a copper grid with carbon films and left it dry in the fume hood. Finally, grids were washed with 1 mL of ultrapure water. After drying, samples were analyzed using a scanning electron microscopy (STEM) and immersion lens (InLens) detectors.

Cryo-TEM evaluation was conducted in the prototypes designated as B and C1. To do so, prototype B was prepared in on the Automatic Plunge Freezer EM GP2 (Leica). Vitrification was done in >90% humidity at $\sim 8^{\circ}\text{C}$. Regarding the preparation of prototype C1, it was done in the Vitrobot Mark IV (FEI – Thermo Fisher Scientific) and vitrified in 70-80% humidity at RT. Data acquisition was carried out on a JEM-2200FS/CR (JEOL, Ltd.) 200 kV TEM equipped with a K2 Summit direct detection camera (GATAN) at x2,000 magnification for ‘aerial views’ and at x25,000 or x30,000 for high magnification micrographs. The size histogram was calculated by manually measuring the NPs.

2.9 IN SILICO MODELLING OF HANAS

2.9.1 Binding mechanism of the antibody with the components of the system by molecular dynamic (MD) simulation

[Section with copyright protection]

2.9.2 System settings of MD simulation

[Section with copyright protection]

2.10 ASSOCIATION EFFICIENCY (AE) AND LOADING CAPACITY (LC)

BVZ association efficiency (AE %) to NPs was quantified using an enzyme-linked immunosorbent assay (ELISA). NPs were isolated by ultracentrifugation (35000 rpm, 1.5 hour, 15°C) by using a Beckman Coulter (optime L90K) ultracentrifuge equipped with a Beckman type 70.1 Ti rotor. The amount of free BVZ in the supernatant was recovered and quantified. As control, a solution of BVZ at 3.2 mg/mL was treated in the same conditions and subsequently quantified.

A 96-multiwell plate was coated with 0.005 µg antigen/well (i.e., recombinant human VEGF₁₆₅) at a concentration of 0.05 µg/mL (100 µL/well) and incubated overnight at 4°C. The antigen was prepared in coating buffer (1.59 mg/mL, Na₂CO₃, 2.94 mg/mL, NaHCO₃, pH 9.6). After the antigen attachment, the plate was washed four times with washing buffer (Tween 20 0.05% (v/v) in PBS, pH 7.4). The process was followed with a blocking step that was performed with 300 µL/well of blocking buffer (2% v/w of dry milk powder prepared in washing buffer) for 2h at 37°C under orbital shaking at 300rpm. By the end, another washing step is done, and the antigen-coated plate is ready to use. Calibration curves for untreated BVZ and for the BVZ recovered from the supernatant were done by diluting accordingly and loading the samples on the previously prepared antigen-coated plate (incubation for 1h at 37°C). Then, the plate was submitted to a washing step with the addition of the secondary goat anti-human HRP antibody at a concentration of 0.08 µg/mL and incubated for 1h at 37°C. Finally, after this last washing step, the detection substrate (ABTS) was added (50 µL/well). After 25 min of incubation at RT, samples absorbances were measured at 405 nm by using a microplate reader (Synergy H4, BioTek, VT, USA). The percentage of entrapped antibody and the loading capacity of the NPs were calculated as follows:

$$AE (\%) = [(\text{theoretical amount of BVZ} - \text{free BVZ}) / \text{theoretical amount of BVZ}] \times 100$$

$$LC (\%) = [\text{associated BVZ} / \text{total theoretical concentration of the system}] \times 100$$

2.11 STABILITY IN SIMULATED BIOLOGICAL FLUID

The short-term colloidal stability of the NPs in simulated biological medium was performed in PBS supplemented with 10% FBS. For this purpose, NPs were diluted 10-times in the medium and incubated at 37°C under orbital shaking at 300 rpm. At different time points (0, 2, 4, 8 and 24 h), 100 µL of sample was withdrawn and particle size and PDI were evaluated by DLS. As control, NPs were diluted in PBS under the same conditions.

2.12 STABILITY DURING STORAGE AND FREEZE-DRYING STUDIES

Both storage stability in suspension (4°C) and freeze-drying studies have been performed. For the stability in suspension, the selected formulations were stored in its liquid form at 4°C and characterized based on particle size, PDI and Zeta potential. For the freeze-drying studies, 2.5 and 5% (w/v) of trehalose were added, then frozen at -80°C for at least 1 h and freeze-dried. Then, formulations were resuspended to its original volume in ultrapure water and physicochemical properties and AE were measures as described in sections 2.8 (DLS) and 2.10, respectively. As control, NPs were freeze-drying in the absence of trehalose.

As control of the possible interference of the trehalose in the ELISA measurement, a calibration curve in the presence of 10% (w/v) of trehalose was performed.

2.13 RELEASE PROFILE

The amount of released BVZ was quantified by ELISA. Samples were diluted 10-times in PBS at pH 7.4 and 5.0 (adjusted with NaOH) and incubated under orbital agitation at 37°C. Each time point was prepared in an independent low binding Eppendorf and at different time points (0, 2, 4, 8, 24, 168 h), 500µL of the samples were ultracentrifuged (35000 rpm, 1.5 h, 15°C). After isolation, the free antibody in the supernatant was recovered and quantified by ELISA. As control, a standard of free BVZ was treated in the same conditions and quantified. The release pattern was expressed as the % of cumulative BVZ released calculated as follows:

$$\text{Cumulative BVZ released (\%)} = \text{free BVZ at } t_1 - \text{free BVZ at } t_0$$

t_0 indicates the condition before incubation, t_1 corresponds to the condition at the time point under study.

Release studies performed in the presence of 10mM of glutathione (GSH) pH 5.5 were conducted following the above-described method.

2.14 CYTOTOXICITY STUDIES

The murine-derived lung cancer and macrophages cell lines CMT 167 and RAW 264.7 were seeded in a 96-well plate at densities of 2×10^3 and 5×10^3 , respectively, in DMEM supplemented with 10% FBS, penicillin and streptomycin for 24 h at 37°C. Cells were incubated with increasing concentrations of BVZ-loaded and blank NPs. As controls, cells were incubated with DMEM-5%FBS and Triton X-100 0.5% (v/v) in culture medium. After 24 h of treatment, the supernatant was removed, and cells were treated with 100µL/well of Resazurin 1X for 40 min at 37°C. The levels of produced resorufin were determined by measuring the absorbance at 544 and 590nm using a microplate reader (Synergy H4, BioTek, VT, USA). Cells treated with DMEM 5%FBS were used as positive control and considered 100% of cell viability and cells treated with Triton X-100 were considered as negative control. Cell viability was calculated as follows:

$$\% \text{ Cell viability} = \frac{[(\text{sample fluorescence} - \text{negative control fluorescence}) / \text{positive control fluorescence}] \times 100}{1}$$

2.15 UPTAKE STUDIES

Cells were seeded in a 24-well plate at densities of 50×10^3 CMT 167 and 10×10^4 RAW 264.7 cells per well. Total concentrations of BVZ-loaded NPs at 0.38, 0.75, 1.5, 3 mg/mL and the correspondent dose of blank NPs were added in DMEM-5% FBS supplemented with penicillin and streptomycin and incubated with the cells either at 37°C and 4°C. Cells were treated in the same conditions and considered as the negative control. After 4 h of incubation, the cell

supernatant was discarded, and cells were washed with cold PBS three times. Then, cells were detached from the wells with accutase for 15 min at 37°C and collected in FACS tubes. Thereafter, cells suspensions were washed with 3mL of cold FACS washing buffer (PBS 2% FBS) and centrifugated at 1000 rpm for 5 min. Finally, cells were fixed with 100μL of paraformaldehyde (PFA 4%) for 30 min. Treated cells were analyzed by flow cytometry in a FACScalibur instrument (10000 events) and resulting data were analyzed by FlowJo software BDLifescience. Data were represented based on integrated mean fluorescence intensity (iMFI), calculated by using the below equation.

$$\text{iMFI} = \text{mean fluorescence intensity} \times \% \text{ of DiD positive cells}$$

To evaluate the internalization capacity of the nanocarriers, the CMT 167 and RAW 264.7 cells were incubated for 24 h at densities of 2×10^3 and 5×10^3 , respectively. After the incubation period, cells were exposed to empty prototypes B and F for 24 h. Then, cells were rinsed with cold PBS 3-times and fixed in PFA 4%. The slides were imaged in the Confocal TCS SP5 X instrument and analyzed with the LASX software (Leica).

2.16 STATISTICAL ANALYSIS

Data analysis was performed by using the GraphPad Prims version 9.3.0. Gaussian distribution was determined following a Shapiro-Wilk test. Differences were considered significant for * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ with a confidence level of 0.05. When analyzing the particle size, statistical comparisons were only done for those prototypes with a PDI < 0.3.

Statistical comparisons between the different parameters of non-targeted NPs were done as follows. The stability of NPs particle size in simulated biological media was statistically analyzed by using a parametric unpaired t-test comparing NPs particle size at each time point in PBS-10%FBS or PBS with the NPs particle size *prior* incubation. The statistical analysis of NPs size upon storage at 4°C was done using an unpaired t-test comparing NPs particle size at each time point and the NPs size *prior* storage (0h). Particle size and Zeta potential of prototype B and particle size of prototype C were compared by a Mann-Whitney test. Regarding the cumulative BVZ released, a Mann-Whitney (A vs B pH 5.0) and unpaired t-test was done among time points (C vs C1 pH 7.4; C vs C1 pH 5.0).

For the tLyP1 targeted NPs, statistical analysis was done following the below tests. The stability in simulated biological media of tLyP1 NPs size was analyzed by using unpaired t-test comparing the size in PBS-10%FBS or PBS at each time point with the NPs size *prior* incubation. Particle size, PDI and Zeta potential of tLyP1 NPs in suspension at 4°C were statistically analyzed by using an unpaired t-test to compare the parameters at each time point at 4°C and the values *prior* storage (0 weeks). Regarding the cumulative BVZ released, a unpaired (BtLyP1 vs C1tLyP1 pH 7.4) and Mann-Whitney (BtLyP1 vs C1tLyP1 pH 5.0) t-test were done among time points.

3 RESULTS AND DISCUSSION

The interest of the scientific community in developing intracellular delivery systems for the encapsulation of mAbs has slowly increased over the last decades. The main delivery strategies explored so far includes liposomes, nanocapsules and polymeric NPs. Overall, these nanocarriers have reported high association efficiencies [27,29,30] and minor release of the mAb in physiological conditions [30,31]. Other pharmaceutical features such as the biological and physical stability during storage, and the loading capacity have been scarcely reported. The same applies to the use of targeting ligands (e.g., peptides) to ensure their tumor accumulation, as far as we are aware only one work has explored this possibility [30].

[Section with copyright protection].

3.1 DEVELOPMENT AND CHARACTERIZATION OF POLYMER-BASED NANOASSEMBLIES (HANAs).

[Section with copyright protection].

3.1.1 Development and optimization of non-targeted HANAs

[Section with copyright protection].

Microfluidics offers the opportunity to produce NPs in a reproducible way, with the additional advantage of scalability and particle size tuneability [44]. Consequently, prototype A was also manufactured using microfluidics and the results showed that the properties of these prototypes did not differ from those of prototypes formed by simple mixing.

To sum up, leading candidates were concentrated by TFF or nitrogen evaporation up to a final BVZ concentration of 3.2 mg/mL. The initial and final composition are reported in Table 1 and the physicochemical properties of the final product in Table 2.

Table 1. Theoretical composition of resulting prototypes before (initial composition) and after concentrating by TFF (A-B2) or nitrogen (C, C1) up to 3.2 mg/mL of BVZ (final composition). [Section with copyright protection].

Prototype	Initial composition [mg/mL]				Final composition [mg/mL]			
	Polymer	Surfactant	Surfactant-PEG	BVZ	Polymer	Surfactant	Surfactant-PEG	BVZ
A	2 ²	0.25	-	2	3.2 ²	0.4	-	3.2
B	2 ¹	1	-	2	3.2 ¹	1.6	-	3.2
B1	2 ¹	1	0.025	2	3.2 ¹	1.6	0.04	3.2
B2	2 ¹	1	0.25	2	3.2 ¹	1.6	0.4	3.2
C	0.25 ¹	0.5	-	0.5	1.6 ¹	3.2	-	3.2
C1	0.25 ¹	0.5	0.25	0.5	1.6 ¹	3.2	1.6	3.2

Table 2. Composition and physicochemical properties of resulting prototypes after concentrating by TFF (A-B2) or nitrogen (C, C1) up to 3.2 mg/mL of BVZ. [Section with copyright protection]. Data are expressed as mean $\pm$ SD, n $\geq$ 3. Statistical comparison of the particle size was done following an unpaired t-test (B vs A, B2 and C vs C1) and Mann-Whitney test (B vs B1).

Prototype	Final composition [mg/mL]			Physicochemical properties		
	Polymer	Surfactant	Surfactant-PEG	Particle size (nm)	PDI	Zeta potential (mV)
A	3.2 ²	0.4	-	196 $\pm$ 13***	0.24	-14 $\pm$ 1
B	3.2 ¹	1.6	-	162 $\pm$ 17	0.23	-13 $\pm$ 2
B1	3.2 ¹	1.6	0.04	156 $\pm$ 11	0.26	-13 $\pm$ 3
B2	3.2 ¹	1.6	0.4	150 $\pm$ 15	0.27	-14 $\pm$ 1
C	1.6 ¹	3.2	-	107 $\pm$ 14	0.23	-13 $\pm$ 3
C1	1.6 ¹	3.2	1.6	79 $\pm$ 8****	0.25	-13 $\pm$ 2

3.1.1.1 Characterization of the size distribution and morphology

The screening presented above was done based on the analysis of particle size and zeta potential using DLS and LDA, respectively. Although DLS is a fast and easy tool, the assumption of spherical particles or the fact that large particles scatter the light more intensely could limit the accuracy of the measure [45]. To overcome these limitations and for a deeper characterization on selected prototypes, their particle size and morphology were determined by NTA and FESEM.

As shown in Figure 1, NTA analysis of HANAs revealed that the particle size of prototype A was smaller to the one observed by DLS, a fact that could be attributed to the NTA goodness for the characterization of heterogeneous populations [46]. Moreover, mean particle size characterization of prototypes C and C1 revealed the poor stability of the samples upon high dilutions, which led to particle sizes medians >105 nm. NTA distribution parameters were described in detail in Supplementary Table 1.

The particle size observed by FESEM (Figure 1) was larger than the one observed by NTA/DLS analysis. While microscopy has been described as the standard method for structural and

morphological analysis, the drying and staining of samples can affect their characterization. Therefore, more sophisticated techniques such as cryo-TEM were required.

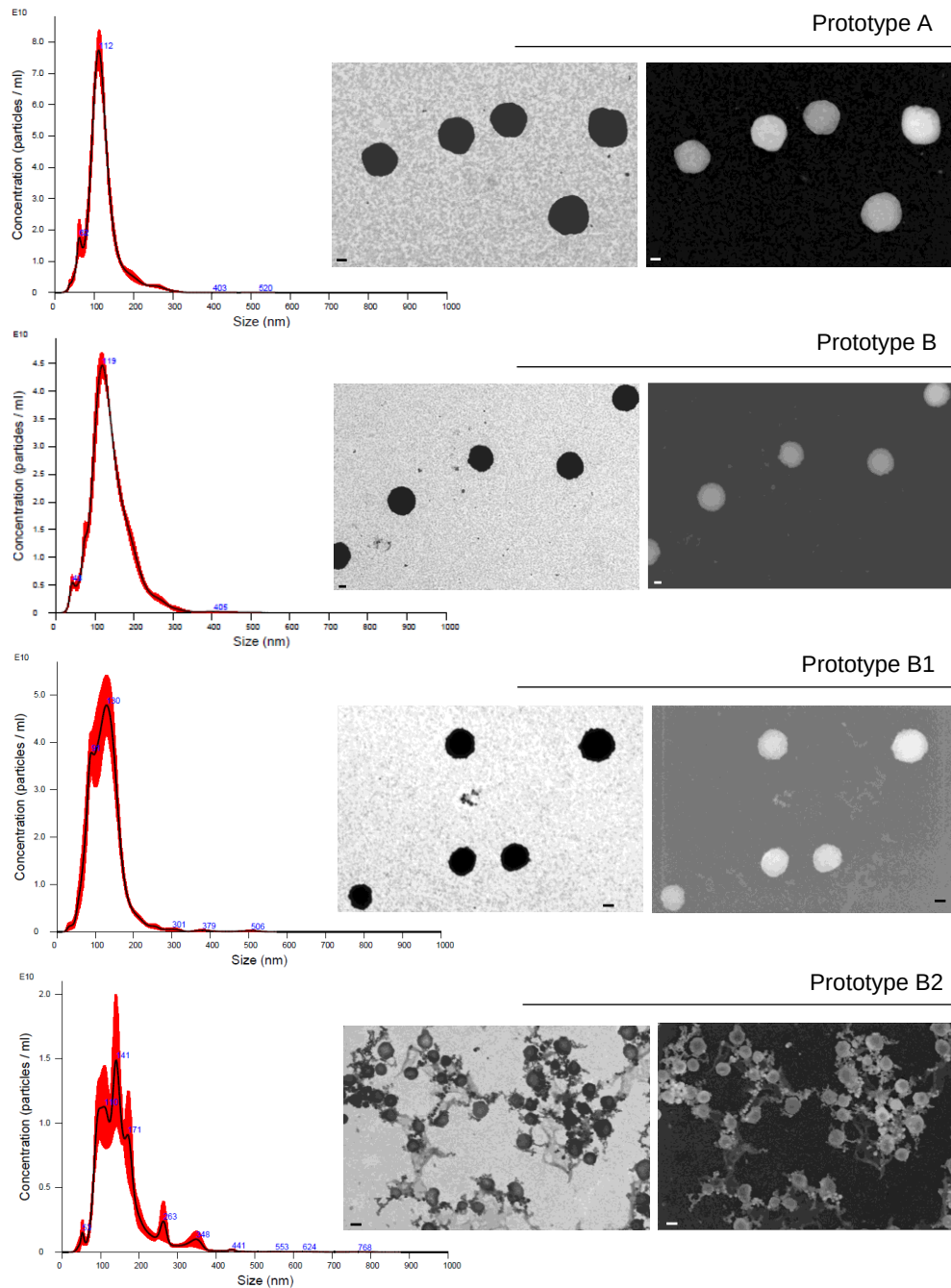
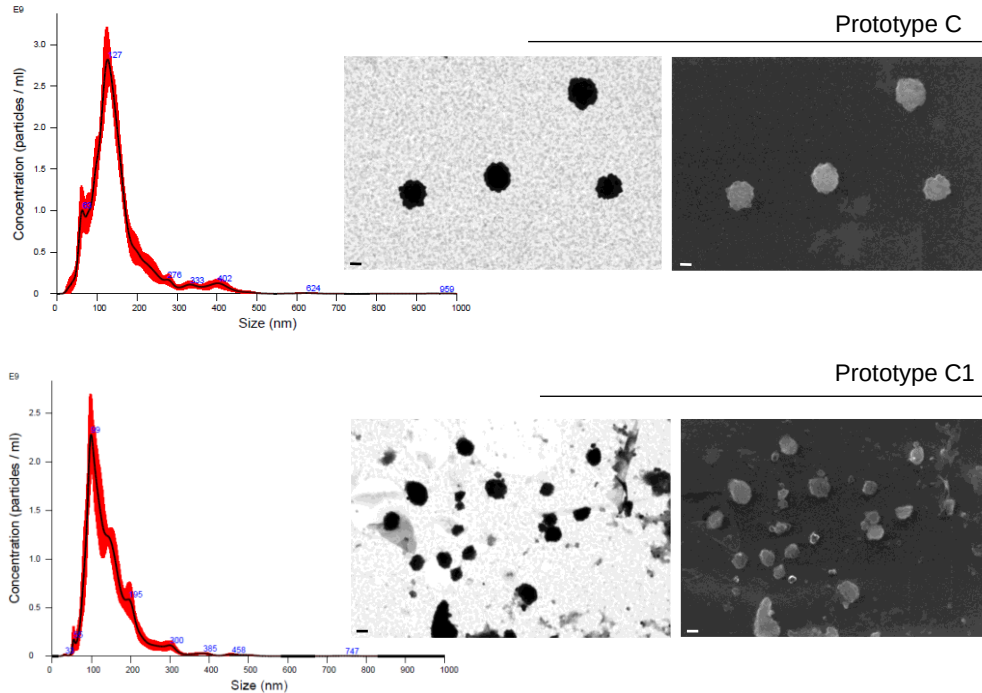


Figure 1. Graph distribution of BVZ-loaded final prototype A-D by Nanoparticle Tracking Analysis (NTA) and FESEM images using STEM (second column) and InLens (third column) detectors. Scale bar 100nm.



...continuation from Figure 6. Graph distribution of BVZ-loaded final prototype C-C1 by Nanoparticle Tracking Analysis (NTA) and FESEM images using STEM (second column) and InLens (third column) detectors. Scale bar 100nm.

[Section with copyright protection].

To allow reasonable conclusions to be drawn, the prototypes will be compared on the basis of the initial concentration of materials used to obtain the HANAs since the manufacturing procedure defines their features (see Table 1). Accordingly, prototypes from A to B2 and C-C1 will be analyzed separately.

3.1.1.2 Computational modelling

[Section with copyright protection].

3.1.1.3 Stability in simulated biological fluids

A critical feature of nanoparticulate drug delivery systems is their stability in plasma. Thus, the stability of selected prototypes was evaluated in PBS-10%FBS and PBS at 37°C. The results in Figure 2 indicate that the apparent polydispersity of the HANAs after incubation in PBS-10%FBS was attributed to the interferences coming from the FBS, since overlapped signals were observed between the control and the NPs in the media but not in PBS. This observation is in agreement with earlier reports [47,48]. Consequently, the analysis of the particle size in PBS-10%FBS was done based on the mean peak by intensity.

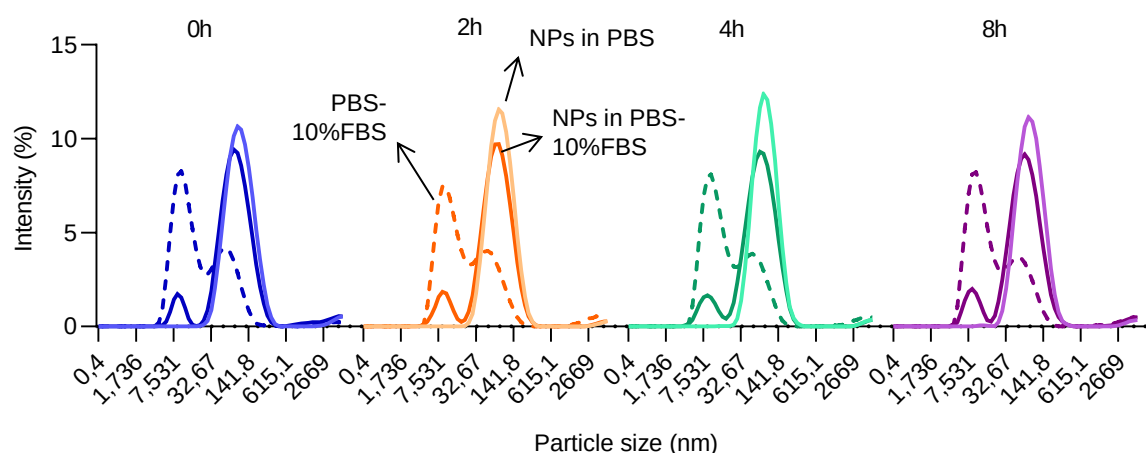


Figure 2. Stability in simulated biological fluids of prototype C1 at 37°C. Stability intensity histogram of the NP upon incubation in PBS-10%FBS, PBS and the control media (PBS-10%FBS). Data are represented as mean, n=3.

A general pattern upon incubation in PBS-10%FBS was observed, that is an increment of the particle size followed by a size stabilization comparable to the NPs prior incubation (Figure 3). This behavior could be attributed to an initial bulk association of the FBS proteins to the NPs, the extent of which depends on their affinity for the NPs [47]. Despite this fact, overall, it could be assumed that the size distribution is apparently the same for at least 8 hours in PBS-10%FBS and 24 hours in PBS at 37°C.

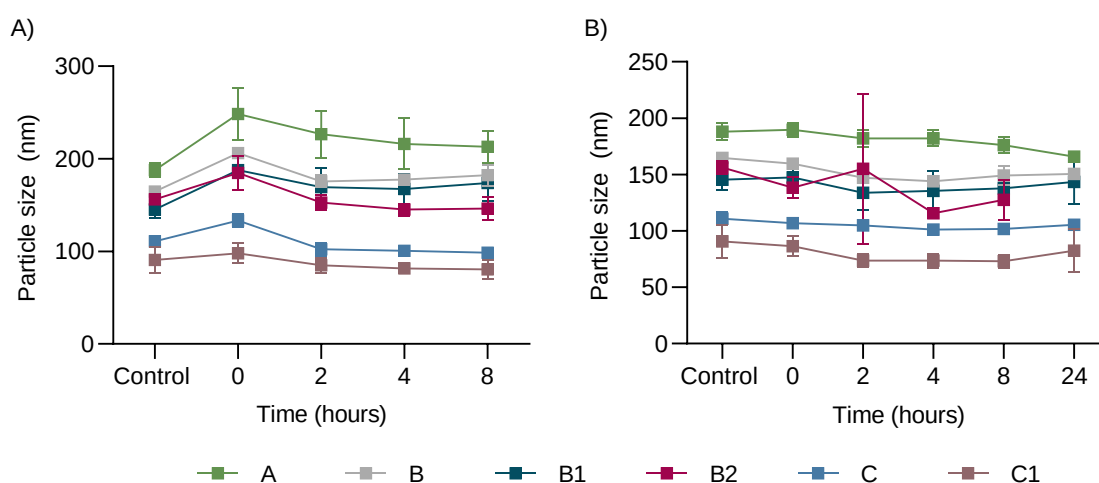


Figure 3. Stability in simulated biological fluids of prototypes A-C1 at 37°C. NPs were incubated in PBS supplemented with 10% FBS (A) and PBS (B), and the distributive and cumulative particle size were evaluated overtime, respectively. Control indicates the mean particle size of the NPs before incubation. Data are represented as mean $\pm$ SD, n=3. PBS phosphate buffer saline, FBS fetal bovine serum.

3.1.1.4 Association efficiency (AE) and loading capacity (LC)

As shown in Table 3, the AE of BVZ to the different prototypes ranged from 32 to 99% and the corresponding LCs varied between 13 and 47%. As expected, the amount of each critical component has an influence on the loading capacity. For example, by comparing prototypes B and B1, we conclude that the introduction of PEG had a negative impact on the loading. [Section with copyright protection].

Surprisingly, the conclusions derived from the comparison of prototype C and C1 was contrary to the above statement. That is, the addition of PEG did not reduce the AE. The explanation behind this pattern could also be found in the formulation method. It should be noted that prototypes C and C1 were formulated with initial concentrations 4 times lower than the other HANAs (see Table 1). [Section with copyright protection].

[Section with copyright protection].

Table 3. Physicochemical properties, association efficiency (AE, %) and loading capacity (LC, %) of prototypes A-C1 after concentrate to a final BVZ concentration of 3.2 mg/mL. [Section with copyright protection]. Data are expressed as mean $\pm$ SD, $n \geq 3$, except for prototype D ($n=2$). Statistical analysis was evaluated by using a Mann-Whitney (A vs B, B vs B1**) and unpaired (C vs C1) t-test.**

Prototype	Physicochemical properties			AE (%)	LC (%)
	Particle size (nm)	PDI	Zeta potential (mV)		
A ²	196 $\pm$ 13	0.24	-14 $\pm$ 1	99 $\pm$ 0.3**	46.6
B ¹	162 $\pm$ 17	0.23	-13 $\pm$ 2	86 $\pm$ 11	34.4
B1 ¹	156 $\pm$ 11	0.26	-13 $\pm$ 3	33 $\pm$ 18**	13.0
B2 ¹	150 $\pm$ 15	0.27	-14 $\pm$ 1	52 $\pm$ 6	19.6
C ¹	107 $\pm$ 14	0.23	-13 $\pm$ 3	74 $\pm$ 11	29.7
C1 ¹	79 $\pm$ 8	0.25	-13 $\pm$ 2	67 $\pm$ 21	22.2

Although prototypes B1 and B2 showed acceptable stability in simulated biological fluids, the AE was low. Consequently, they were discarded.

3.1.1.5 Long-term stability in suspension

A key characteristic of NPs' robustness and manufacturability is the long-term stability in suspension. Therefore, the stability of the NPs upon storage in suspension at 4°C was monitored overtime. HANAs seemed to maintain the particle size and PDI upon incubation for at least 60 (prototype A) and 24 weeks (prototypes B, C, C1), that is 1 year and 3 months, and 6 months, respectively. Similar results were obtained for the PDI. When examined the Zeta potential, prototypes A, B and C1 experimented a drop in the surface charge at several time points. Given the large variability of the Zeta potential, an increment in the number of replicates would be expected to reduce such differences.

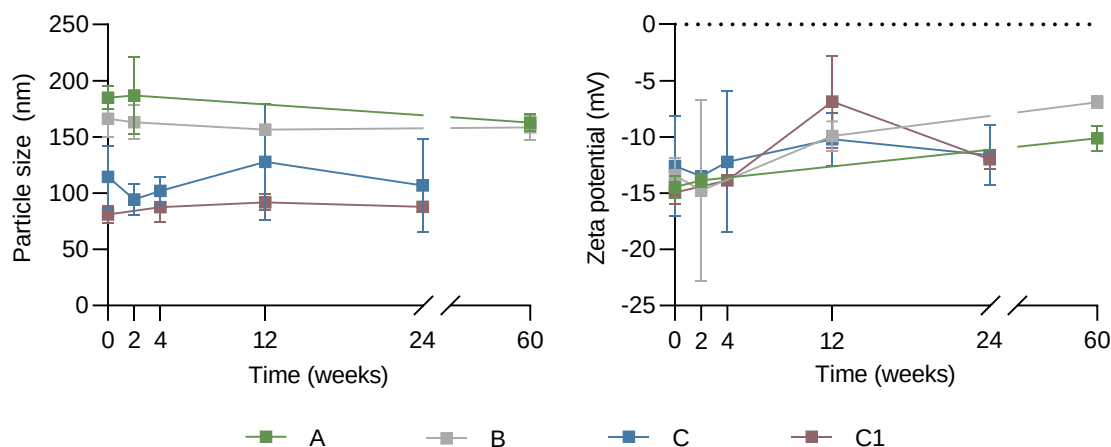


Figure 4. Stability of prototypes A, B, C and C1 upon storage in suspension at 4°C. (A) Particle size and Zeta potential (B) were measured at the indicated time points. Data are represented as mean $\pm$ SD, $n \geq 3$, except for prototype A at 60 weeks and prototypes C-C1 at 24 weeks ($n=2$).

Overall, it could be concluded that NPs were stable within 6 months, and 1 year and 3 months.

3.1.1.6 Freeze-drying studies

In order to complete the characterization as potential pharmaceutical products, selected prototypes were freeze-dried and resuspended to their original volume (Table 4). In a first attempt, a low percentage of trehalose was tested (i.e., 2.5% w/v) in order to avoid the obtention of a hypertonic formulation [49]. Results in Table 4 revealed that the physicochemical properties for the NPs after resuspension were preserved except for prototype C1, $PDI > 0.3$. Although the AE after freeze-drying was reduced in prototype B, their extent was minor. Thus, the amount of trehalose was doubled (i.e., 5%) for prototype C1 without improvement.

Table 4. Physicochemical characterization and association efficiency (AE %) before and after freeze-drying studies in the presence of 2.5 or #5% (w/v) trehalose. Data are represented as mean $\pm$ SD, $n \geq 3$. Statistical comparison of the particle size and AE of each prototype before and after freeze-drying was done using an unpaired t-test but Mann-Whitney test for the AE of prototype B. N.D. not determined

Proto-type	Before freeze-drying				After freeze-drying			
	Particle size (nm)	PDI	Zeta potential (mV)	AE (%)	Particle size (nm)	PDI	Zeta potential (mV)	AE (%)
A	196 $\pm$ 13	0.24	-14 $\pm$ 1	99 $\pm$ 0.3	192 $\pm$ 5	0.23	-14 $\pm$ 1	99 $\pm$ 0.5
B	162 $\pm$ 17	0.23	-13 $\pm$ 2	86 $\pm$ 11	167 $\pm$ 4	0.22	-13 $\pm$ 2	85 $\pm$ 3
C	107 $\pm$ 14	0.23	-13 $\pm$ 3	74 $\pm$ 11	122 $\pm$ 9	0.26	-14 $\pm$ 1	53 $\pm$ 21
C1	79 $\pm$ 8	0.25	-13 $\pm$ 2	67 $\pm$ 21	115 $\pm$ 15	0.36	N.D.	N.D.
#C1	79 $\pm$ 8	0.25	-13 $\pm$ 2	67 $\pm$ 21	103 $\pm$ 4	0.32	N.D.	N.D.

3.1.1.7 Release profile

The *in vivo* fate of nanotherapeutics is highly restricted by their controlled release behavior during circulation in the blood stream and thereafter, in the TME. Particularly, it is expected that the antibody remains entrapped to the HANAs during circulation while being gradually released in the target tumor and intracellularly. This gradient is accompanied by a decrease in pH [50]. Thus, prototypes B, C and C1 were selected in order to study whether the superior amount of polymer in the formulation process, or presence of PEG leads to a different release kinetics. Therefore, in the present work, we study the behavior of our systems at pH 7.4 and 5.0, mimicking the blood conditions and the endo-lysosomal compartments, respectively.

As shown in Figure 5, the burst release was found to be lower than 20% with minor differences overtime, except for prototype C, which showed a cumulative BVZ released of 52% after 1 week of incubation at pH 5.0. These results suggest that prototype C may have a different architectural organization, resulting in a weaker interaction between the HANA's components and the BVZ.

In order to mimic the *in vivo* circumstances, other conditions were tested. For instance, PBS supplemented with 10 mM GSH pH 5.5. Nevertheless, the condition was discarded since the BVZ was degraded after 1 week of incubation (Supplementary Figure 1).

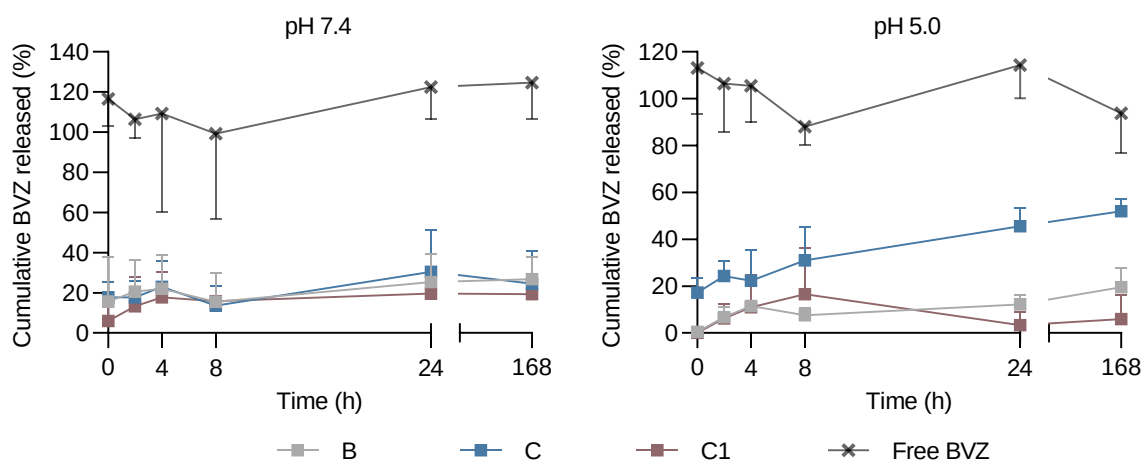


Figure 5. Release profile of HANAs upon incubation in PBS pH 7.4 and 5.0 at 37°C. The cumulative amount of released BVZ of prototype A, B, C and C1 was quantified by ELISA. As control, free BVZ was treated in the same conditions than the NPs. Data are represented as mean $\pm$ SD, $n \geq 3$.

Overall, these results underline the important interaction between BVZ and other components of the formulations. [Section with copyright protection].

3.1.2 Development and characterization of tLyP1 functionalized polymer-NPs (tLyP1-HANAs)

To validate the versatility of above HANAs for their incorporation of the tumor penetrating peptide, tLyP1, the functionalization of two components of the system was carried out. Hence, the polymer and the surfactant.PEG were selected for the synthesis to ensure the peptide exposure to the TME. To ensure the obtention of a pure conjugate while preserving the original physicochemical properties of the NPs, several optimizations of the synthesis and formulation conditions were conducted.

3.1.2.1 tLyP1 functionalization of PEG-lipid and polymer

For the functionalization of the nanoassemblies, we have adopted two different strategies: the functionalization of polymer and the functionalization of PEG-lipid. [Section with copyright protection]. In the case of surfactant-PEG, we adopted the reactions conditions described in the literature [51,52], which simply involves the thiol-maleimide reaction.

The described reaction allows the functionalization of polymers and surfactants via a simple and reproducible method. The use of mild conditions ensures the preservation of the biological functionality.

3.1.2.1.1 Polymer

[Section with copyright protection].

3.1.2.1.2 Surfactant.PEG-Maleimide

tLyP1 was conjugated to the maleimide group of the PEGylated lipid through a thiol-maleimide reaction. The formation of a covalent adduct was achieved after carried out a coupling reaction among reagents in a molar ratio 1:2, which was confirmed by ¹H-NMR analysis. [Section with copyright protection].

3.1.2.2 Development of tLyP1 targeted NPs

Based on the screening performed on section 3.1, prototypes B and C1 were selected for their surface functionalization with the tLyP1 peptide, aiming to compare the particle size and presence of PEG simultaneously. In the case of prototype B, native polymer was shifted by the tLyP1-polymer conjugate with a DS of $2.1 (\%) \pm 0.3$ (condition 2) while the amount of each component was maintained (Table 5). The composition and physicochemical properties of the tLyP1 targeted HANA's were similar to those of the non-targeted counterpart and named, from now on, BtLyP1.

Table 5. Composition and physicochemical properties before and after the incorporation of the tLyP1 targeted conjugate. Data are expressed as mean $\pm$ SD, $n \geq 3$. #tLyP1 targeted material, tLyP1 DS 2.1 (%) $\pm$ 0.3. Statistical analysis of the particle size was done by using an unpaired parametric t-test.

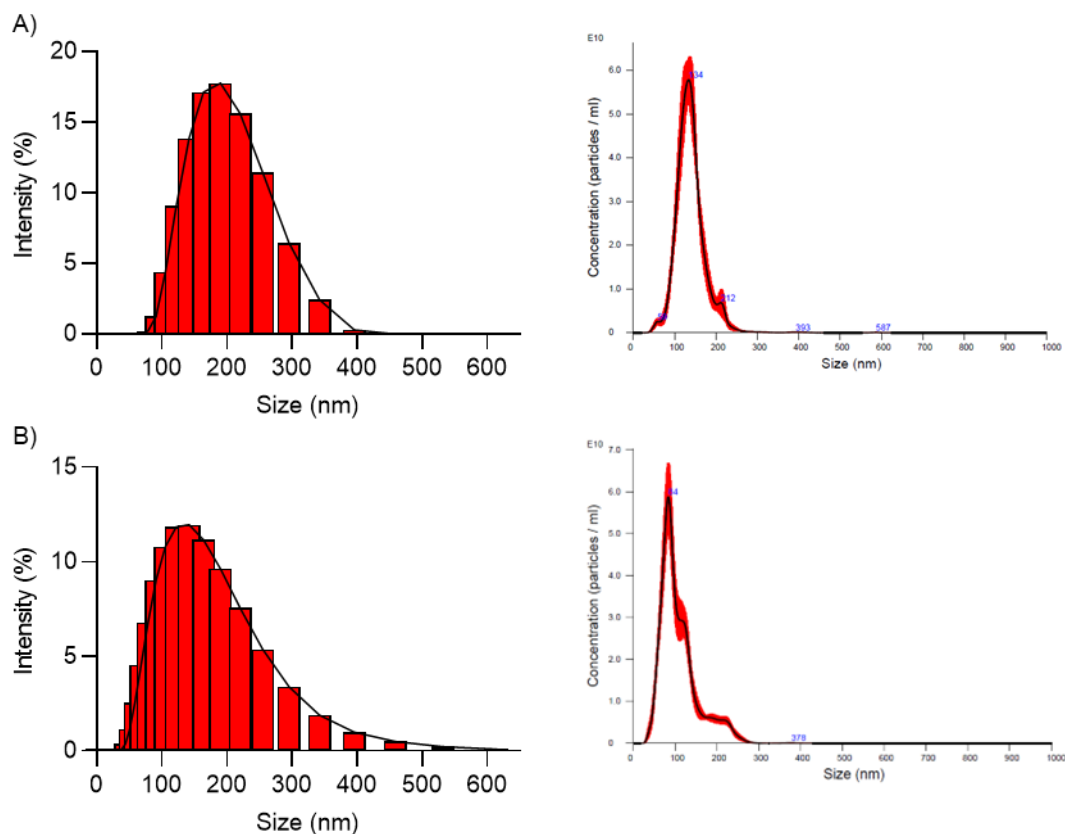
Prototype	Final concentration [mg/mL]			Physicochemical properties		
	Polymer	Surfactant	BVZ	Particle size (nm)	PDI	Zeta potential (mV)
B	3.2	1.6	3.2	162 $\pm$ 17	0.23	-12 $\pm$ 1
BtLyP1	3.2 [#]	1.6	3.2	166 $\pm$ 9	0.18	-11 $\pm$ 6

In the case of prototype C1, the decided option was the functionalization of the surfactant.PEG. However, an optimization of the formulation was required since large particles were obtained when we introduced functionalized surfactant.PEG in the formulation ([Section with copyright protection]). As shown, the results indicate a direct correlation between the concentration of surfactant.PEG-tLyP1 and the particle size. In other words, the particle size was reduced by decreasing the amount of tLyP1 conjugate. Although statistically different, only the lowest concentration tested, i.e., 0.01 mg/mL final concentration in the system, resulted in NPs with a particle size closest to the non-targeted equivalent. This low functionalization degree was, then, selected for further studies and the resulting prototype named C1tLyP1. We hypothesized that the different particle size among targeted and non-targeted surfactant.PEG may be attributed to the reaction conditions carried out to synthesize the conjugate, which modify their solubility properties and thereafter, the assembling mechanism into the NP. This is a behavior that has been previously described. For instance, the chemical modification of the polystyrene maleic acid polymer with neocarzinostatin have changed the original solubility properties of the drug [53].

To sum up, final tLyP1-HANAs presented a particle size ranging from 120 to 170 nm, PDI<0.3 and neutral surface charge (see Table 6).

Table 6. Composition and physicochemical properties of the non-targeted and tLyP1 targeted HANAs. Data are expressed as mean $\pm$ SD, $n \geq 3$. #tLyP1 targeted material. Statistical analysis of the particle size was evaluated by using an unpaired t-test (B vs BtLyP1; C1 vs C1tLyP1). N.A. not applicable.

Prototype	Final concentration [mg/mL]				Physicochemical properties		
	Polymer	Surfactant	Surfactant.PEG	BVZ	Particle size (nm)	PDI	Zeta potential (mV)
B	3.2	1.6	N.A.	3.2	162 $\pm$ 17	0.23	-13 $\pm$ 2
BtLyP1	3.2 [#]	1.6	N.A.	3.2	166 $\pm$ 9	0.18	-11 $\pm$ 6
C1	1.6	3.2	1.6	3.2	79 $\pm$ 8	0.25	-13 $\pm$ 2
C1tLyP1	1.6	3.2	0.064 [#]	3.2	121 $\pm$ 14****	0.25	-13 $\pm$ 2



Prototype	DLS	Nanoparticle tracking analysis (NTA)					
	Physicochemical properties						Concentration (particles/mL)
	Mean	Mean	Mode	D10	D50	D90	
BtLyP1	166 ± 9	137 ± 4***	132 ± 8	90 ± 13	133 ± 6	184 ± 5	$3.9 \times 10^{12} \pm 1.0 \times 10^{12}$
C1tLyP1	121 ± 14	107 ± 16	91 ± 11	66 ± 16	99 ± 15	155 ± 27	$3.6 \times 10^{12} \pm 7.3 \times 10^{12}$

Figure 6. Physicochemical characterization of BtLyP1(A) and C1tLyP1 (B). Intensity histograms based on particle size distribution by DLS (left column) and size distribution by NTA (left column). The table represents the NTA distribution parameters (mean, mode and D10/50/90) and concentration of BVZ-loaded NPs. Statistical analysis was performed by using an unpaired t-test between mean particle size of DLS and NTA. Data are expressed as mean ± SD, n≥3.

To deeply characterize the tLyP1-HANAs, the orthogonal technique NTA was used (Figure 6). The results derived from the NTA analysis revealed smaller particles sizes for BtLyP1 than the ones yielded by DLS. As previously discussed, these differences may be attributed to the DLS limitations attributed to its principle [45,46]. Broadly speaking, NTA analysis confirmed the particle size revealed by DLS.

3.1.2.3 AE and LC

The capacity of the tLyP1-HANAs to entrap the antibody was evaluated by ELISA. As shown in Table 7, the tLyP1 incorporation to prototype B led to a remarkable reduction of the AE (from 86 to 26 %). In contrast, minor modifications in the AE were exhibited for prototype C1 upon functionalization with tLyP1.

We speculate that since the addition of the hydrophilic tLyP1 induces changes in the physical properties (i.e., solubility) of the tLyP1-polymer and tLyP1-surfactant.PEG conjugates, it is expected to observe structural changes in the NPs and thus a reduction in the AE. Furthermore, the percentage that each tLyP1-conjugate represents within the system (40% in BtLyP1 vs 0.8% in C1tLyP1) may be responsible for observing such variations in BtLyP1.

Table 7. Physicochemical properties, association efficiency (AE, %) and loading capacity (LC, %) of BtLyP1 and C1tLyP1. Data are expressed as mean $\pm$ SD, $n \geq 3$. Statistical analysis of the AE was done by using a Mann-Whitney (B vs BtLyP1) and unpaired (C1 vs C1tLyP1) t-test.

Prototype	Physicochemical properties			AE (%)	LC (%)
	Particle size (nm)	PDI	Zeta potential (mV)		
B	162 $\pm$ 17	0.23	-13 $\pm$ 2	86 $\pm$ 11	34.4
BtLyP1	166 $\pm$ 9	0.18	-11 $\pm$ 6	26 $\pm$ 11****	10.3
C1	79 $\pm$ 8	0.25	-13 $\pm$ 2	67 $\pm$ 21	22.2
C1tLyP1	121 $\pm$ 14	0.25	-13 $\pm$ 2	65 $\pm$ 12	26.0

3.1.2.4 Stability in simulated biological media

tLyP1-HANAs were incubated in PBS supplemented with 10%FBS at 37°C and the mean peak by intensity was evaluated overtime. To discriminate interference coming from the FBS, the media was treated in the same conditions and the NPs were incubated in PBS. Hence, the peak observed at 20 nm during the NPs incubation in PBS-10%FBS was attributed to the media and not the NP destabilization as has been shown in Figure 7.A. Consequently, HANAs exhibited an increment in the particle size upon incubation in the rich protein media, followed by a particle size stabilization comparable to the NPs prior to incubation. NPs were incubated in PBS confirming the above statement since no differences were observed overtime (Figure 7. B-C).

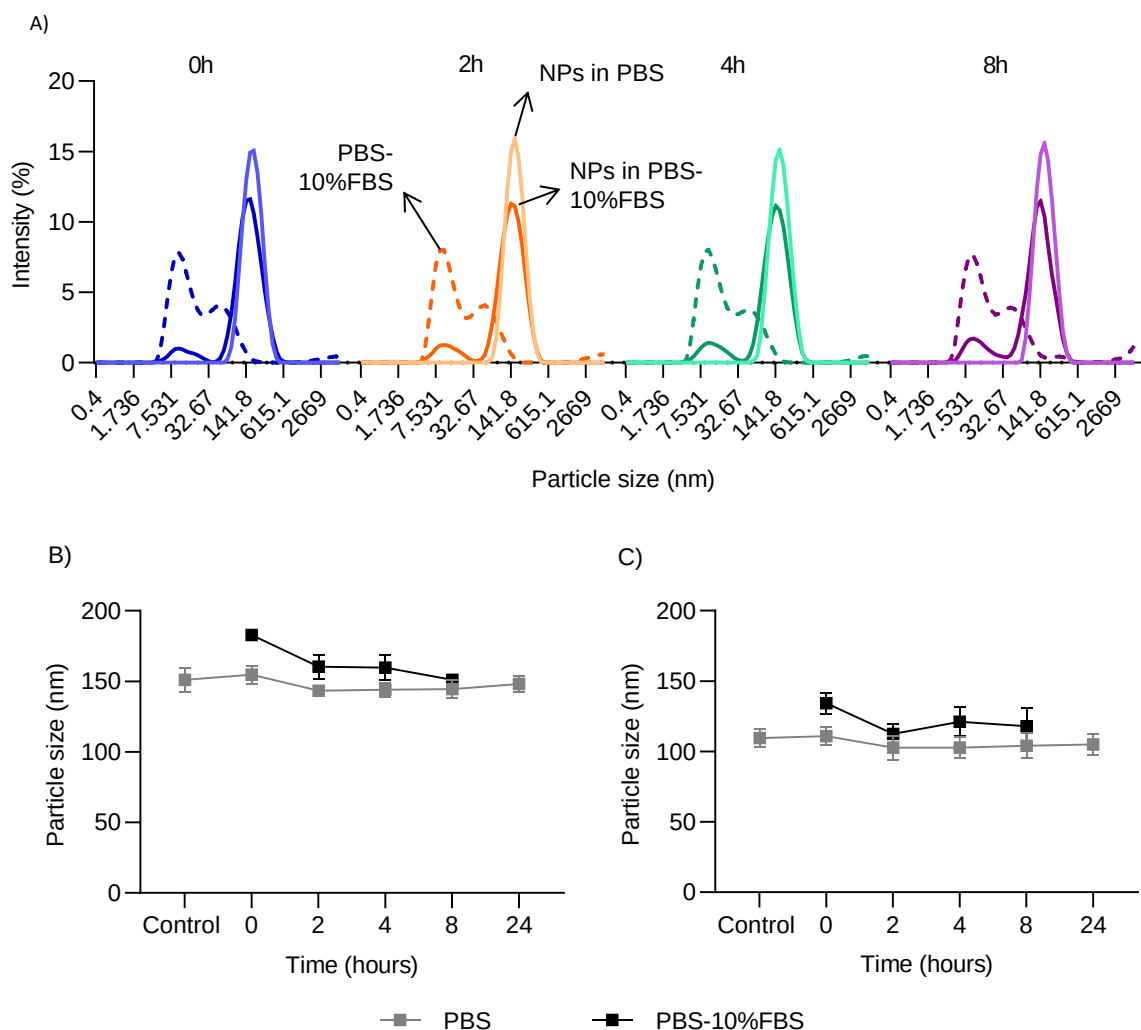


Figure 7. Stability in simulated biological fluids of BtLyP1 and C1tLyP1 upon incubation at 37°C. **A)** Representative stability intensity histogram of BtLyP1 upon incubation in PBS-10%FBS, PBS and the control media (PBS-10%FBS) at 37°C. Particle size evolution of (A) BtLyP1 and (B) C1tLyP1 in PBS supplemented with 10% FBS and PBS (control). Data are represented as mean $\pm$ SD, $n=3$.

Overall, these results confirmed the favorable behavior of the NPs *in vivo* conditions as they demonstrated high stability in simulated conditions for at least 8 hours.

3.1.2.5 Long-term stability in suspension

NPs were stored at 4°C and at different time points the particle size, PDI and surface charge were evaluated. Results in Figure 8 demonstrated the long-term stability of BtLyP1 and C1tLyP1 for at least 24 weeks (i.e., 6 months) in terms of particle size and surface charge, despite having undergone a significant deviation from the media after 2 days (*) of incubation. Moreover, the PDI was maintained over time.

The obtained storage profiles guarantee the potential of these candidates as pharmaceutical products.

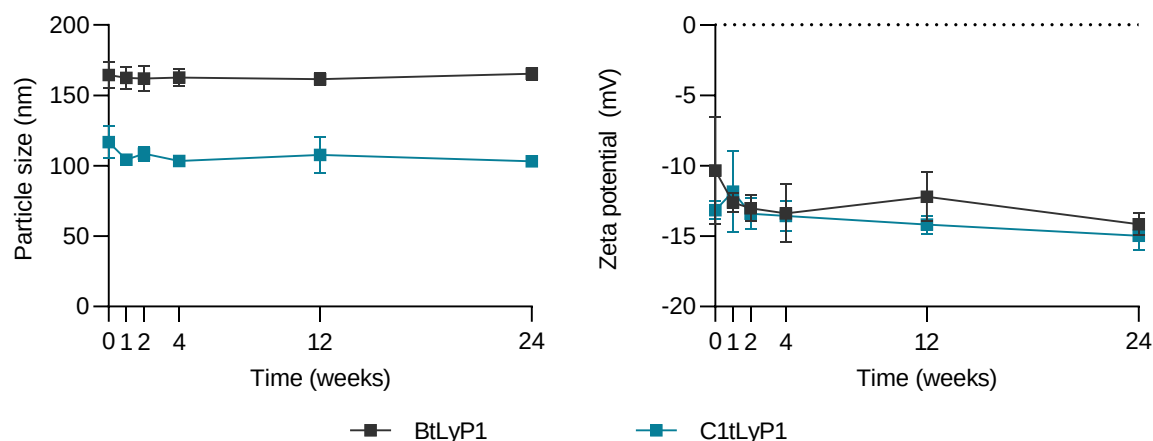


Figure 8. Long-term stability in suspension of BtLyP1 and C1tLyP1 at 4°C. Particle size and Zeta potential evolution upon storage for up to 24 weeks. Data are represented as mean $\pm$ SD, $n \geq 3$.

3.1.2.6 Freeze-drying studies

To validate the NPs as a biopharmaceutical product, BtLyP1 and C1tLyP1 were freeze-dried and resuspended to its original volume. Thus, increasing concentrations of trehalose were tested (i.e., 2.5 and 5%, w/v) and the physicochemical properties and the capacity to retain the Ab were evaluated before and after the freeze-drying process (Table 8). At the lowest concentration of trehalose, the PDI of BtLyP1 was above 0.3 and the particle size of C1tLyP1 suffered an increment, ruling out this condition. Consequently, an increment of trehalose to 5% preserved the original physicochemical properties and the amount of entrapped BVZ to the HANAs after resuspension.

Table 8. Physicochemical characterization and association efficiency (AE %) before and after freeze-drying studies in the presence of trehalose (% w/v). Data are expressed as mean $\pm$ SD, $n \geq 3$. Statistical comparison of the particle size and AE between the NP before and after freeze-drying was done following unpaired t-test.

Physicochemical properties	Before freeze-drying	After freeze-drying
BtLyP1(2.5% trehalose)		
Particle size (nm)	166 $\pm$ 9	206 $\pm$ 9
PDI	0.18	0.31
Zeta potential (mV)	-11 $\pm$ 6	-13 $\pm$ 1
AE (%)	26 $\pm$ 11	N.D.
BtLyP1(5% trehalose)		
Particle size (nm)	166 $\pm$ 9	164 $\pm$ 10
PDI	0.18	0.22
Zeta potential (mV)	-11 $\pm$ 6	-12 $\pm$ 1
AE (%)	26 $\pm$ 11	20 $\pm$ 21

... continuation from Table 9. Physicochemical characterization and association efficiency (AE %) before and after freeze-drying studies in the presence of trehalose (% w/v). Data are expressed as mean $\pm$ SD, $n \geq 3$. Statistical comparison of the particle size and AE between the NP before and after freeze-drying was done following unpaired t-test.

Physicochemical properties	Before freeze-drying	After freeze-drying
C1tLyP1 (2.5% trehalose)		
Particle size (nm)	121 $\pm$ 14	142 $\pm$ 16*
PDI	0.25	0.28
Zeta potential (mV)	-13 $\pm$ 2	-14 $\pm$ 1
AE (%)	65 $\pm$ 12	60 $\pm$ 15
C1tLyP1 (5% trehalose)		
Particle size (nm)	121 $\pm$ 14	120 $\pm$ 1
PDI	0.25	0.25
Zeta potential (mV)	-13 $\pm$ 2	-14 $\pm$ 0.3
AE (%)	65 $\pm$ 12	50 $\pm$ 14

3.1.2.7 Release profile

The mAb release from tLyP1 targeted HANAs was studied at pH 7.4 and 5.0 in order to emulate the blood circulation and the extra- and intracellular tumor conditions. Figure 9 indicates that irrespective of the functionalization approach, the release profiles were similar and only differ in the initial burst effect. [Section with copyright protection].

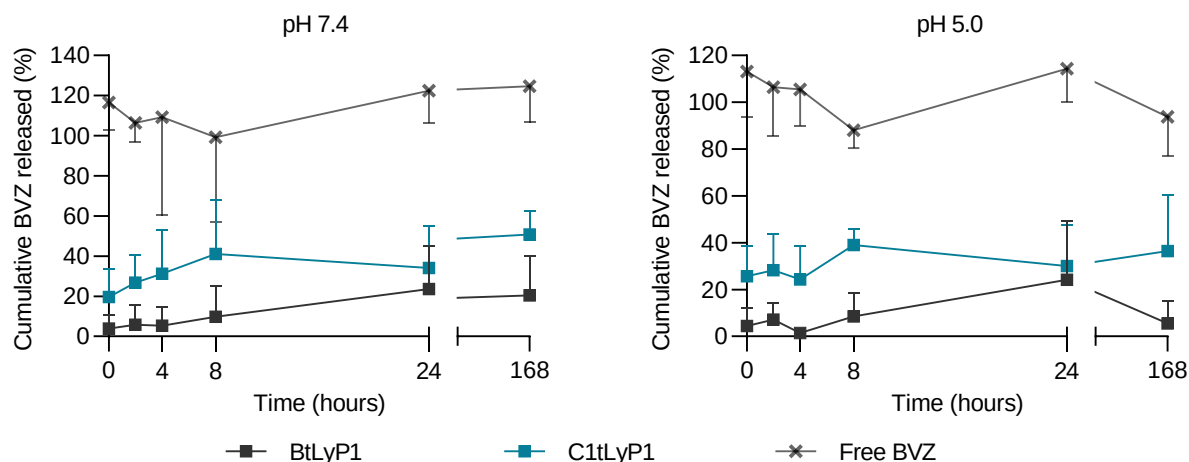


Figure 9. Release profile of BtLyP1 and C1tLyP1 upon incubation in PBS pH 7.4 and 5.0 at 37°C. The cumulative amount of released BVZ was quantified by ELISA. As control, free BVZ was treated in the same conditions than the NPs. Data are represented as mean $\pm$ SD, $n \geq 3$.

It should be noted that the *in vivo* conditions were not mimicked in this study. [Section with copyright protection].

3.1.3 Preliminary scaling-up of the HANAs technology

The obtained insights encourage the idea of an industrial translatability of our technology. Consequently, prototype B was scaled-up from 1 mL to a 50 mL batch by using a blade mechanical agitator. We find that by simply proportionally increasing the volume of each phase, the main physicochemical properties were presumable maintained. Hence, the suitability of our NPs for an industrial production was confirmed.

Table 9. Physicochemical properties of a 1mL batch of prototype B (n≥3), in comparison with the 50mL scale-up (n=1). N.D. not determined

Prototype (batch)	Physicochemical properties			AE (%)	LC (%)
	Particle size (nm)	PDI	Zeta potential (mV)		
B (1 mL)	162 ± 17	0.23	-13 ± 2	86 ± 11	34.4
B (50 mL)	177	0.22	-13	N.D.	N.D.

Overall, results presented above describing the development and physicochemical properties of HANAs and tLyP1-HANAs outline the encouraging features of these novel system for the delivery of mAbs. The remarkable AE and loading capacity, together with the control of release that the NPs exhibited, stand out from other systems available for the same application. So far, the fact that they can be functionalized with a tumor-penetrating peptide gives them unique characteristics.

Hence, to study the *in vitro* performance of the NPs, in a first attempt non-targeted HANAs were selected since their characterization was the main goal of this chapter. Therefore, HANAs designated in section 3.1.1 as prototypes B and C1 were chosen. Hence, the influence of the particle size and the presence of a PEG corona would be evaluated simultaneously in a lung (CMT 167) and macrophages (RAW 264.7) cancer cell lines.

3.1.4 Preparation of fluorescent (DiD) labelled of HANAs

Prior to evaluate the interaction of the NPs with relevant cancer cell lines, NPs were labelled with the fluorescent dye DiD. Thus, Table 10 exhibited how the DiD incorporation to the systems maintained the physicochemical properties of the NPs. In this case, blank NPs were also labelled to allow comparison to be drawn.

Table 10. Physicochemical properties of DiD-labelled blank and BVZ-loaded prototypes B and C1. DiD final concentration is 10 and 0.01 ppm, respectively. Data are expressed as mean ± SD, n=3.

Prototype	Physicochemical properties		
	Particle size (nm)	PDI	Zeta potential (mV)
B (blank)	174 ± 9	0.20	-15 ± 2
B (BVZ)	158 ± 16	0.17	-13 ± 2
C1 (blank)	80 ± 5	0.23	-13 ± 1
C1 (BVZ)	75 ± 10	0.14	-13.3

3.2 CELL VIABILITY STUDIES

To assess the toxicity profile of the NPs, the percentage of metabolic activity was determined by using the resazurin reduction assay after 24 hours of treatment. Thus, cell viability was studied in lung cancer (CMT 167) and macrophages (RAW 264.7) cell lines exposed to increasing concentrations (up to 1.8 or 2 mg/mL) of NPs, either blank or BVZ-loaded. As shown in Figure 10.A, lack of toxicity was found after 24 hours of exposure in the lung cancer cell line (CMT 167) in both of the prototypes since cell viability values were >70%, in accordance with ISO 10993-5 standards [54].

On the other hand, a size-dependent toxicity profile was observed in RAW 264.7 cells (Figure 10.B). Regardless of the NPs composition, we speculate that the compromised viability may be attributed to the smallest particle size yielded for prototype C1. Although this outcome have been previously described [55], the size-dependent uptake by macrophages is controversial since early reports suggest a direct correlation between the particle size and the magnitude of the uptake [13,56].

Overall, a low toxicity profile was observed in the different cell lines.

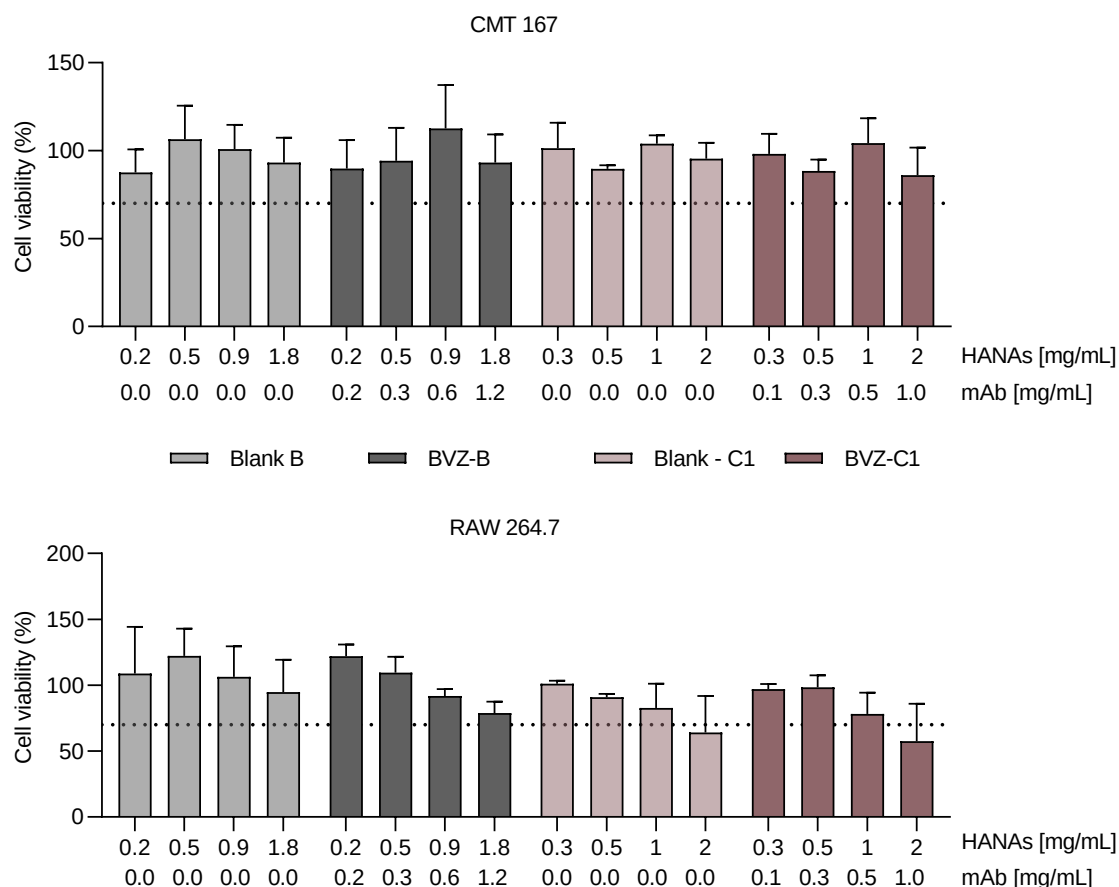


Figure 10. Metabolic activity of prototypes B and C1 in CMT 167 (A) and RAW 264.7 (B) cell lines after 24 of exposure. Cells were exposed to increasing concentrations of DiD-labelled empty and BVZ-loaded NPs. Statistical analysis was done by using a parametric unpaired t-test for comparison between blank and BVZ-loaded prototype C1 in RAW 234.7. Cell viability values below 70% were considered toxic (dashed lines). Data are expressed as mean $\pm$ SD, n=3.

3.3 UPTAKE STUDIES

To evaluate the capacity of non-targeted NPs to internalize their cargo, cellular uptake studies were carried out in lung cancer (CMT 167) and macrophages (RAW 264.7) cell lines at 37°C. Moreover, cellular energy depletion at 4°C was conducted in parallel to roughly elucidate the internalization mechanism of the NPs [57].

Using DiD-labelled NPs, similar uptake patterns were observed for prototype B irrespective of the temperature and the NPs concentration (Figure 11. A) in the CMT 167 cell line, which indicates the leakage of the dye or the deposition of the NPs onto the cell surface [57]. In contrast, the uptake behavior of the NPs in macrophages exhibited significant differences between temperatures (Figure 11. B). The higher phagocytic rate of macrophages could be the responsible for the discrepant uptake, preventing the observation of dye leakage. Fluorescence confocal images in the CMT 167 cell line revealed a diffuse fluorescence distribution in the inner part of the membrane or intracellularly, confirming the above statement.

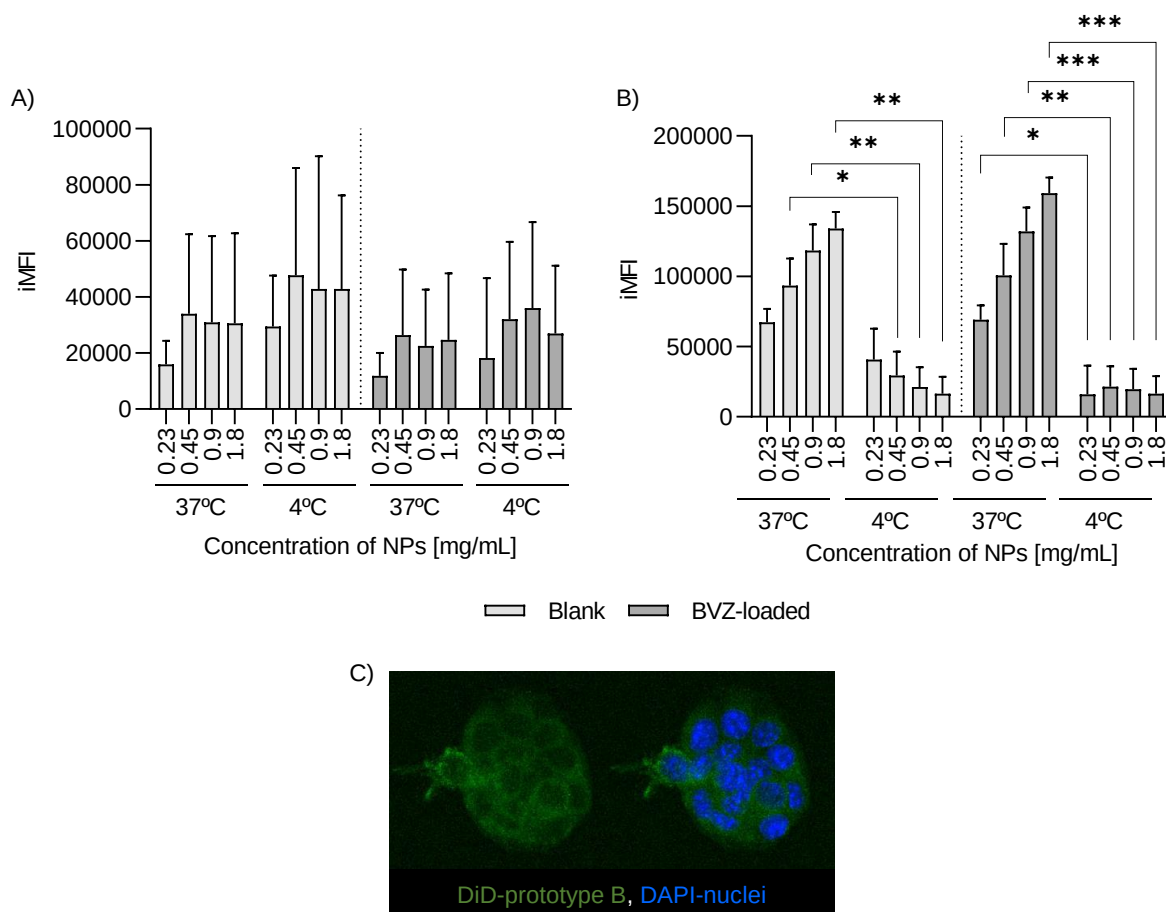


Figure 11. Cellular uptake of DiD-labelled blank and BVZ-loaded prototype B. Quantitative analysis of DiD iMFI in CMT167 (A) and RAW264.7 (B) following 4 hours of incubation at increasing concentrations of NPs. C) Representative confocal microscopy images of blank prototype B at 2mg/mL after 24 hours of incubation in the lung cancer cell line. Statistical comparisons were done following a parametric unpaired t-test between temperatures. Data are expressed as mean $\pm$ SD, $n \geq 3$. iMFI integrated mean fluorescence intensity.

In the case of the prototype C1, the absence of fluorescence at 4°C suggest the dye retention to the NP and therefore, validates the observations at 37°C. Thus, temperature-dependency and an apparent dose dependency was observed in both cell lines, this being more remarkable in macrophages, a result that is consistent with their phagocytic nature (Figure 12).

To confirm the intracellular location, confocal microscopy was performed showing a defined intracellular fluorescence in the lung cancer cell line. [Section with copyright protection].

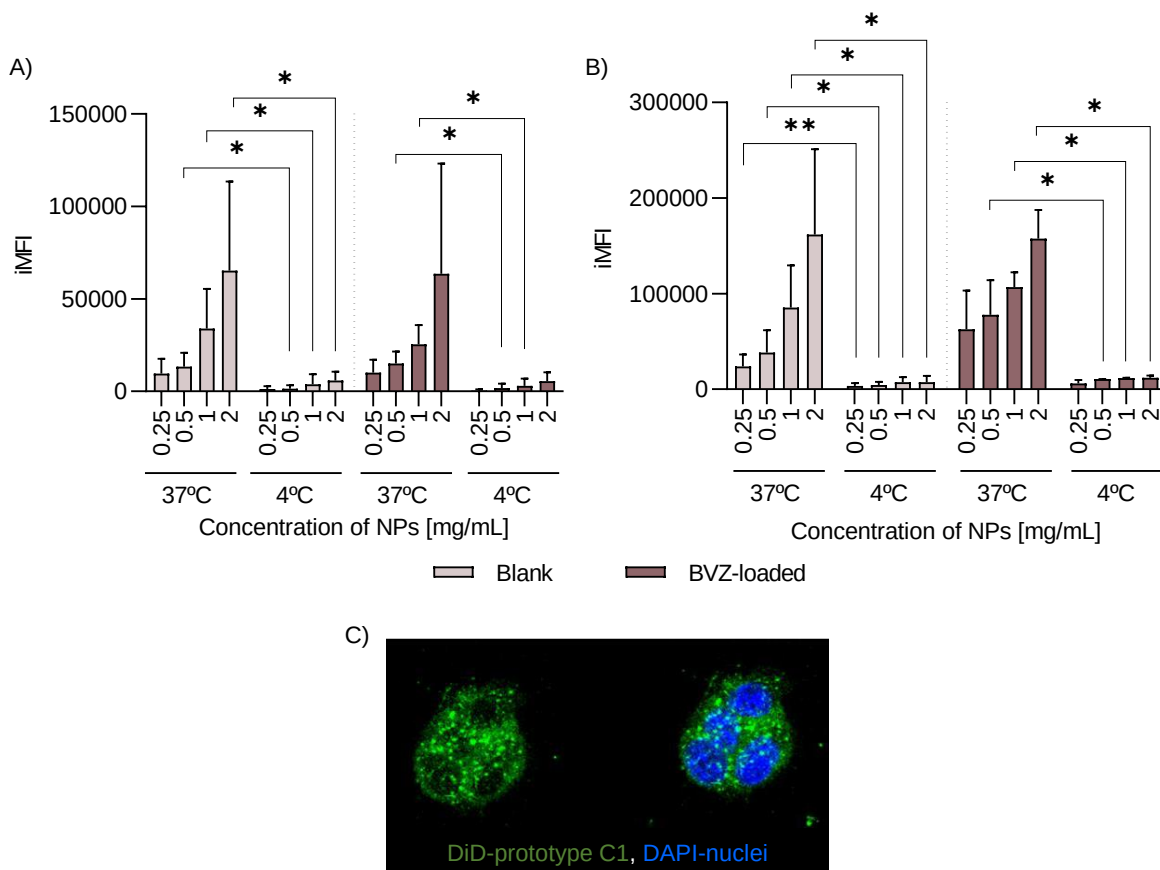


Figure 12. Cellular uptake of DiD-labelled blank and BVZ-loaded prototype C1. Quantitative analysis of DiD iMFI in CMT167 (A) and RAW264.7 (B) following 4 hours of incubation at increasing concentrations of NPs.

C) Representative confocal microscopy images of blank prototype C1 at 2mg/mL after 24 hours of incubation in the lung cancer cell line. Statistical comparisons were done following a parametric unpaired t-test between temperatures. Data are expressed as mean $\pm$ SD, $n \geq 3$. iMFI integrated mean fluorescence intensity.

4 CONCLUSIONS

Overall, we engineered a new composition of polymer-based nanoassemblies (HANAs), functionalized with a tumor-penetrating peptide, specially adapted for the association of mAbs through a simple assembling technique. [Section with copyright protection]. The developed HANAs showed a favorable uptake in relevant cancer cell lines, and, hence they could be proposed as nanocarriers for intracellular delivery of mAbs.

Further *in vivo* studies would be performed to evaluate the biodistribution profile of the NPs and the role played by the tumor-penetrating peptide tLyP1. In addition, assessing the efficacy of the NPs in clinically relevant tumor models would be mandatory to elucidate the relevance of these NPs for the intracellular delivery of mAbs.

5 ACKNOWLEDGMENTS

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6 REFERENCES

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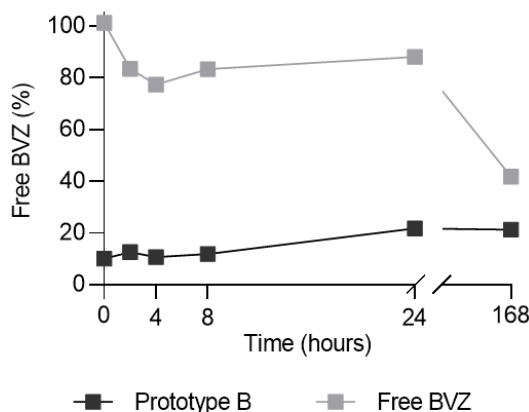
7 SUPPLEMENTARY

[Section with copyright protection]

Supplementary Table 1. DLS mean particle size and NTA distribution parameters (mean, mode and D10/50/90) of BVZ-loaded HANAs. Data are expressed as mean $\pm$ SD, $n \geq 3$. Statistical analysis was done following an unpaired t-test but prototype C (Mann-Whitney test) between mean particle sizes.

Proto- type	DLS	Nanoparticle tracking analysis (NTA)					
	Physicochemical properties						Concentration (particles/mL)
	Mean	Mean	Mode	D10	D50	D90	
A	195.4 $\pm$ 14	132.9 $\pm$ 18**	86.7 $\pm$ 66	86.5 $\pm$ 11	124.3 $\pm$ 14	187.9 $\pm$ 36	3.9 $\times 10^{12}$ $\pm$ 7.2 $\times 10^{11}$
B	161.8 $\pm$ 17	144.3 $\pm$ 13	130.0 $\pm$ 20	83.5 $\pm$ 7	137.3 $\pm$ 14	214.7 $\pm$ 20	3.3 $\times 10^{12}$ $\pm$ 1.1 $\times 10^{12}$
B1	155.6 $\pm$ 11	136.6 $\pm$ 14	122.9 $\pm$ 3	84.7 $\pm$ 5	130.2 $\pm$ 11	191.8 $\pm$ 35	3.9 $\times 10^{12}$ $\pm$ 6.9 $\times 10^{11}$
B2	150.2 $\pm$ 16	149.9 $\pm$ 14	130.6 $\pm$ 15	92 $\pm$ 5	137.9 $\pm$ 11	222.2 $\pm$ 29	1.7 $\times 10^{12}$ $\pm$ 6.4 $\times 10^{11}$
C	107.5 $\pm$ 14	150.9 $\pm$ 55*	119.1 $\pm$ 38	80 $\pm$ 23	134.4 $\pm$ 44	240 $\pm$ 105	2.4 $\times 10^{11}$ $\pm$ 1.2 $\times 10^{11}$
C1	78.9 $\pm$ 8	138.7 $\pm$ 9	98.2 $\pm$ 7	74 $\pm$ 18	123.6 $\pm$ 5	220 $\pm$ 24	2.4 $\times 10^{11}$ $\pm$ 6.6 $\times 10^{10}$

[Section with copyright protection]



Supplementary Figure 1. % of free BVZ after the incubation of prototype B in GSH 10mM pH 5.5 at 37°C. As control, BVZ was incubated in the same conditions, $n=1$.

[Section with copyright protection]

CHAPTER 2

Multifunctional nanoassemblies as a strategy to improve the tumor uptake of monoclonal antibodies: *in vitro* evaluation and PET *in vivo* imaging

CHAPTER 2:

Multifunctional nanoassemblies as a strategy to improve the tumor uptake of monoclonal antibodies: *in vitro* evaluation and PET *in vivo* imaging

This work has been done in collaboration with the group of Rafael T. M. de Rosales, School of Biomedical Engineering & Imaging Sciences from King's College London in London, United Kingdom.

ABSTRACT

The clinical potential of current nanotherapeutics for monoclonal antibodies (mAbs) delivery relies on the ability to modify their biodistribution profile while increasing the penetration into the tumor tissue. Although great success has been achieved over the years, most nanoparticles (NPs) still face short circulation times, resulting in poor tumor accumulation. Hence, in this work, we explored the possibility of functionalizing the surface of polymer-based nanoassemblies (HANAs) with the tumor-penetrating peptide, tLyP1, through a poly(ethylene glycol)-PEG-motif, aiming to preserve the stealth properties while enhancing the tumor penetration of the HANAs. Thus, PEGylated HANAs entrapping a model mAb (bevacizumab) were functionalized with the tLyP1 peptide and evaluated *in vitro* and *in vivo*. For tracking purposes, the mAb was radiolabeled with the positron emitter radionuclide zirconium-89 (^{89}Zr). The resulting mAb-loaded HANAs showed good radiochemical properties and particle sizes ranging from 70 to 170 nm. The results of the *in vitro* cell culture studies revealed selective accumulation of tLyP1 targeted HANAs, this being associated with the overexpression of the neuropilin-1 receptor (NRP-1) on the A549 cell line. To investigate whether the PEGylated HANAs could change the distribution profile of the Ab, biodistribution studies in healthy animals were carried out using positron emission tomography (PET) imaging for 72 hours. The results confirmed the above hypothesis while showing a higher uptake for the entrapped mAb as compared to the free version in the liver and spleen. Finally, mice bearing human subcutaneous (A549) tumors were used to investigate the tumor accumulation of the HANAs by PET-computed tomography (CT) studies and *ex vivo* biodistribution. When intravenously administered, tLyP1-targeted HANAs showed a tendency towards an improved accumulation in the tumor tissue compared to their non-targeted counterpart. Overall, these data suggest the potential of tLyP1-targeted HANAs for the delivery of mAbs into the tumor.

GRAPHICAL ABSTRACT

[Section with copyright protection].

1 INTRODUCTION

Despite the outstanding outcomes in cancer treatment over the past decades, cancer continues to leave catastrophic mortality rates. In fact, a worldwide increment of 62.5% is expected by 2040 [1]. This dramatic scenario is not only due to the inadequate biodistribution and questionable safety profile of classical chemotherapy (e.g., docetaxel [2]) but also to the difficulties for drug candidates to engage their specific intracellular targets. Current efforts towards personalized nanomedicine are expected to confront these critical challenges [3]. In this context, mAbs have gained ground to traditional treatments due to their specificity and potency. Proof of this is the exponential number of mAbs that have entered the market over the past ten years for cancer treatment (i.e., 43% of total mAbs and derivatives approved in the US [4]). This success has been tightly related to the increasing understanding of tumor environment complexity, immune signaling pathways [5], and their controversial role in cancer progression, responsible for the creation of unique tumor features such as anomalous tumor microenvironment phenotypes or oncogene-driver tumors [6–8].

In spite of the extraordinary outcome of mAbs in the clinic, their suboptimal biodistribution, together with their incapacity to cross cell membranes [9,10] has limited their full exploitation. Being conscious of this relevant problem, our lab pioneered the idea of using nanotechnology to overcome the above-indicated biological barriers [10]. To achieve this goal, nanocarriers must exhibit specific critical properties. For instance, the particle size (<100 nm) [11–13] has been found to play a crucial role in the biodistribution of the NPs. On the other hand, the nanocarrier must have a minimal protein corona so that it can have extended blood circulation [14,15] and, hence, efficient tumor extravasation. This has been so far achieved using PEG, a strategy that enables the passive accumulation of the NPs. Unfortunately, the passive targeting strategy has led to low delivery efficiencies to solid tumors [16]. Within this context, active strategies where the specific biology of the tumor is exploited, have shown room for improvement [17,18].

Functionalization of nanosystems can be exploited to impart unique and purposeful properties to the system. Although multiple targeting ligands have been proposed over the years, such as Abs, polysaccharides, or cell-penetrating peptides, immunogenicity [19], poorly defined cancer receptor expression [20,21] or receptor-independent uptake [22,23] have limited their clinical translation. Hence, tumor-penetrating peptides (TPP) able to recognize tumor endothelium, extravasate, and enhance the penetration into the extravascular tissue, are expected to overcome previous drawbacks. TPPs are defined by a common denominator, the Cend-R motif (R/KXXR/K). CendR peptides are characterized by the recognition of NRP-1 or NRP-2 receptors [24–27], overexpressed in tumor cells [28,29], and lymphatic vessels [30]. In general, this mechanism is preceded by the recognition of a primary receptor on the tumor endothelium. Although little is known about the exact uptake mechanism, it has been suggested an endocytosis or cell-to-cell transport is upregulated during nutrient deprivation [31]. Within TPPs, we can highlight the truncated LyP-1 (tLyP1) peptide which specifically binds to NRP-1/2 receptors and exerts deeper tumor penetration than the parent LyP-1 [27]. Indeed, the blockade of NRP-1 has been suggested as an adjuvant alternative for angiogenesis inhibition [32]. tLyP1 peptide has gained considerable attention as a directed strategy to improve the antitumor efficacy of NPs [33–36]. Besides, previous results in our lab with tLyP1 functionalized HA-nanocapsules loading docetaxel have demonstrated a 36.6-fold increase in

tumor accumulation compared with the free drug, Taxotere® [37]. Despite these achievements, nanomedicine still suffers from poor tumor accumulation and penetration into the target tissue.

Our laboratory showed for the first time the capacity to deliver mAbs intracellularly. In fact, we have pioneered the development and *in vivo* evaluation of multifunctional polymeric nanocapsules as carriers for the intracellular delivery of mAbs such as anti-gasdermin [10] and anti-KRAS mAbs. Afterward, other authors have proposed the use of liposomes or polymeric micelles, among others, for the same purpose [9,10,38–41]. Of particular note, these technologies have shown the ability to modify the biodistribution profile of mAbs, leading to their tumor accumulation [10,40]. Furthermore, a remarkable suppression of the tumor growth after the engagement of the intracellular-located oncoprotein [9,41] has been also proved.

1.1 OBJECTIVES

Based on the background information, this work is intended to shed light on an unsolved problem, the poor tumor accumulation and subsequent deficient delivery efficiencies of NPs. To do so, we have selected the HANAs technology described in Chapter 1 and hypothesized that the functionalization of the PEG with the tLyP1 could expand the properties of the active targeting, whereas the stealth properties are retained. To perform a rigorous characterization of the *in vitro* and *in vivo* behavior of the targeted and non-targeted nanosystems, HANAs were radiolabeled with the long half-life radioisotope ^{89}Zr . The positron-emitting radionuclide ^{89}Zr has been extensively used in clinical trials for imaging probes of mAbs [42,43]. Using this radionuclide a high-quality spatiotemporal resolution can be achieved [44]. HANAs loaded with ^{89}Zr were tested for their cytotoxicity and capacity to enter cells in a human lung cancer cell line (A549). Then, *in vivo* PET/CT and *ex vivo* biodistribution studies were performed in the relevant human lung tumor-bearing mice model, A549.

2 MATERIALS AND METHODS

2.1 MATERIALS

The humanized monoclonal antibody bevacizumab (BVZ) was kindly donated by mAbxience (Spain). [Section with copyright protection]. The truncated Lyp-1 (tLyP1) peptide (aminoacidic sequence: CGNKRTR) was synthesized by ChinaPeptides Co., Ltd. (Shanghai, China). GMP-grade zirconium-89 as [89Zr]Zr-oxalate in 1 M oxalic acid was purchased from PerkinElmer (BV Cyclotron, VU Amsterdam, NL). N1-hydroxy-N1-(5-(4-(hydroxy(5-(3-(4-isothiocyanatophenyl)thioureido)pentyl)amino)-4-oxobutanamido)pentyl)-N4-(5-(N-hydroxyacetamido)pentyl)succinimide (p-NCs-Bz-deferoxamine (DFO)) was obtained from Chematech (Dijon, France). Human serum was acquired from Sigma-Aldrich (Darmstadt, Germany). Amicon centrifuge filters (30 and 100 kDa MWCO), resazurin sodium salt, and Triton X-100 were provided by Merck (Darmstadt, Germany). PD-10 column filled with Sephadex G25 gel and the Superose 6 10/300 GL column were purchased from Cytiva (Marlborough, USA). Radioactivity was measured with a CRC-25R dose calibrator (Capintec, USA) or a 1282 CompuGamma γ -counter (LKB Wallac, Finland). The 96 multiwells plate and the recombinant human VEGF₁₆₅ were provided by Thermo Fisher Scientific (United States). Goat anti-human IgG HRP conjugated was purchased from Jackson Immuno Research Laboratories, Inc. (United States). 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid (ABTS) solution was obtained from Roche (Switzerland). The mouse monoclonal IgG1 κ neuropilin-1 antibody was acquired from Santa Cruz Biotechnology (Dallas, Texas, USA).

2.2 SYNTHESIS OF THE SURFACTANT.PEG-TLYP1 CONJUGATE

Surfactant.PEG-Mal was synthesized following a maleimide-thiol coupling reaction between the PEG chains and the peptide. Briefly, surfactant.PEG-Mal and tLyP1 were dissolved in HEPES buffer 10 mM (60% (v/v) ethanol in water, pH 7.2) at 1 mg/mL and 40 mg/mL, respectively. Then, the tLyP1 solution was added over the surfactant.PEG-Mal at a molar ratio tLyP1: surfactant.PEG-Mal of 2:1. The mixture was incubated overnight at room temperature under magnetic agitation. After it, the conjugate was purified by dialysis against 150 mM NaCl and then, 3-times against water. Once purified, the product was lyophilized. The resulting surfactant.PEG-tLyP1 conjugate was characterized by proton nuclear magnetic resonance (¹H-NMR) spectroscopy.

2.2.1 ¹H-NMR spectroscopy

¹H-NMR spectra were recorded using a Bruker DRX-500 spectrometer. surfactant.PEG₂₀₀₀-tLyP1 was dissolved at 2 mg/mL in DMSO-d₆ and transferred into 5-mm NMR tubes. surfactant.PEG₂₀₀₀-Mal and tLyP1 standard solutions at the same conditions were used as controls. The extent of the conjugation was calculated based on the integral peak corresponding to the methyl group of the threonine of the peptide at 1.2 ppm and the methyl groups of the surfactant at 0.8 ppm.

2.3 SYNTHESIS OF THE DEFEROXAMINE (DFO)-BVZ CONJUGATE

For labeling the Ab, a first conjugation reaction was done between the BVZ and the DFO. The as-obtained 1 mL of PBS containing 5 mg of Ab was mixed with 110 μL 0.1 M Na_2CO_3 for an acceptable pH range of 8.9 – 9.1 and homogenized by vortex. Over this solution, 20 μL of 5 mM DFO dissolved in DMSO were added in 5 μL steps. The mixture was incubated at 37°C. After 30 minutes, the final product was purified by centrifugal filters and washed 3 times with ultrapure water. Finally, the purified DFO-BVZ conjugate was made up to 1mL with ultrapure water.

2.4 PREPARATION AND CHARACTERIZATION OF BVZ-DFO-LOADED HANAS

Previously synthesized BVZ-DFO was entrapped into the HANAs. [Section with copyright protection].

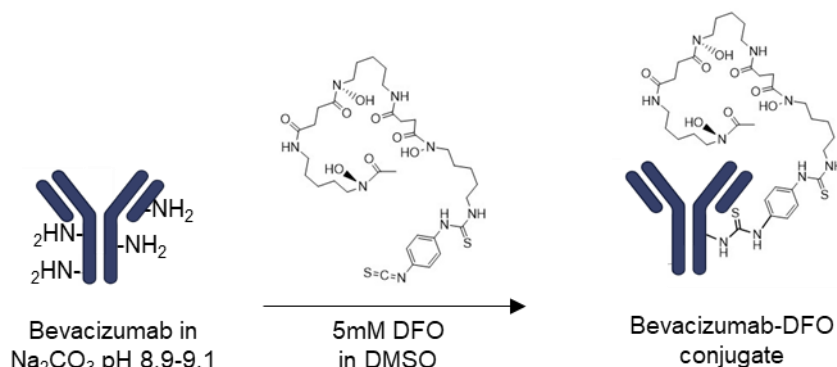


Figure 1. Synthetic scheme of the conjugation of deferoxamine (DFO) to the Ab, leading to the formation of a thiourea linkage; DMSO: dimethyl sulfoxide.

[Section with copyright protection].

2.5 RADIOLABELING OF HANAS WITH ^{89}Zr

2.5.1 ^{89}Zr -labeling of BVZ-DFO-loaded HANAs and post-purification procedure (method 1)

The chelation of ^{89}Zr to the HANAs was performed as follows; 200 μL of 1M oxalic acid containing ^{89}Zr were neutralized with 90 μL of 2 M sodium carbonate pH 7-8 and incubated for 3 minutes at room temperature. Then, 300 μL of 0.5 M HEPES buffer pH 7.2, 700 μL of DFO-BVZ-HANAs, and 700 μL of 0.5 M HEPES buffer pH 7.2 were consecutively added over the previous solution and mixed at room temperature under horizontal agitation. After 1 hour of incubation, the resulting mixture was loaded onto a size exclusion P-10 column (previously equilibrated with 20mL of PBS). ^{89}Zr -labeled BVZ-loaded HANAs (^{89}Zr -HANAs) were eluted with 2 mL of PBS while free ^{89}Zr was trapped in the column. Then, isolated ^{89}Zr -HANAs were concentrated at least 4 times by using centrifugal filters at 13000 rpm. To remove the ^{89}Zr -HANAs from free ^{89}Zr -BVZ, concentrated ^{89}Zr -HANAS were added into a size-exclusion chromatography column in an ÄKTA FPLC (GE Healthcare, Amersham, UK) using PBS as eluent at 0.5 mL/min. Eluted fractions containing the ^{89}Zr -HANAS, which are fractions

between 7 to 12 mL, were collected and concentrated by centrifugal filters. As a control, ^{89}Zr -labeling of the Ab was carried out following the above-described method but ÄKTA purification. For instance, isolation was carried out by eluting the ^{89}Zr -BVZ in a pre-equilibrated P-10 column with 2.5 mL of PBS. All the fractions were gamma counted and compared with the UV/VIS chromatogram to confirm the fractions of interest.

2.5.2 HANAs purification and post- ^{89}Zr -labeling procedure (method 2)

For the second radiolabeling method, the above-described method was slightly changed. Thus, 500 μL of concentrated BVZ-DFO-loaded HANAs were purified by injecting the mixture into a size-exclusion column using an ÄKTA FPLC (GE Healthcare, Amersham, UK) at 0.5 mL PBS buffer/min. Eluted HANAs between fractions 7 and 12 mL were collected and concentrated by using centrifugal filters at 13000 rpm. Then, purified HANAs were radiolabeled by mixing 200 μL of 1M oxalic acid containing ^{89}Zr (1-10 MBq) with 90 μL of 2M sodium carbonate pH 7-8 for neutralization and incubated for 3 minutes at room temperature. The previous solution was subsequently mixed with 300 μL of 0.5 M HEPES buffer pH 7.2, 700 μL of purified HANAs, and 700 μL of 0.5 M HEPES pH 7.2 and incubated for 1 hour at room temperature under continuous agitation. Then, free ^{89}Zr was removed by loading the ^{89}Zr -HANAS into a pre-equilibrated size exclusion P-10 column. Removal of unreacted ^{89}Zr was ensured by adding 50 μL of DFO 5 mM to the column or the reaction. Finally, ^{89}Zr -HANAS were concentrated by using centrifugal filters at 13000 rpm up to 500 μL .

2.6 PHYSICOCHEMICAL CHARACTERIZATION OF THE ^{89}Zr -HANAS

The particle size and polydispersity index (PDI) of the HANAS were determined by Dynamic Light Scattering (DLS) after diluting the samples in PBS accordingly. Both parameters were analyzed using a Zetasizer® Nano ZS instrument (Malvern Instruments, UK).

2.7 RADIOCHEMICAL CHARACTERIZATION OF THE ^{89}Zr -HANAS

The radiochemical yield (RCY) was calculated as the percentage of activity HANAs in the eluted fraction post-removal of the free ^{89}Zr compared to the initial activity. The radiochemical purity (RCP) was estimated after correlating the activity bounded to the HANAs fraction with the unbounded activity after purification. For this purpose, purified ^{89}Zr -HANAs were applied into the size-exclusion column of the ÄKTA equipment using PBS as eluent at 0.5 mL/min and the activity in the eluted fractions were gamma counted. For the radiochemical stability in human serum, ^{89}Zr - HANAs were incubated in human serum at 37°C for 24, 48, and 72 hours and the retained activity in the ^{89}Zr -HANAs was determined following the same procedure as the RCP. The production yield was determined based on the measured activity in the final product compared to the initial activity expressed as a percentage.

Radiochemical parameters were determined by gamma counter or ionization chamber. MBq and counts per minute were correlated with an ^{89}Zr calibration curve (Supplementary Figure 1).

2.8 ASSOCIATION EFFICIENCY (AE) AND LOADING CAPACITY (LC) OF THE NON-RADIOLABELED HANAS

The AE and LC of BVZ were determined in the non-radiolabeled HANAS by using an enzyme-linked immunosorbent assay (ELISA). A Beckman Coulter (optime L90K) ultracentrifuge equipped with a Beckman type 70.1 Ti rotor was used to isolate the HANAS (35000 rpm, 1.5 hours, 15°C). The amount of free BVZ in the supernatant was recovered and quantified. As a control, a solution of BVZ at 3.2 mg/mL was treated in the same conditions and subsequently quantified.

A 96-multiwell plate was coated with 0.005 µg antigen/well (i.e., recombinant human VEGF₁₆₅) at a concentration of 0.05 µg/mL (100 µL/well). After incubation overnight at 4°C, the plate was washed with Tween 20 0.05% (v/v) in PBS, pH 7.4. Then, a blocking step was performed to avoid unspecific bindings with 300 µL/well of blocking buffer (2% v/w of dry milk powder prepared in washing buffer) for 2h at 37°C under orbital shaking at 300 rpm. It was followed by another washing step. Once the plate was antigen-coated, the treatment of the samples was done. A BVZ calibration curve and the BVZ retrieved from the supernatant were diluted in PBS accordingly and loaded onto the previous plate. After incubation at 37°C for 1h, the plate was submitted to a washing step. Thereafter, the secondary goat anti-human HRP antibody was added at a concentration of 0.08 µg/mL and incubated for 1h at 37°C. After washing, the detection substrate (ABTS) was added (50 µL/well). The plate was incubated at room temperature for 25 minutes and samples absorbances were measured at 405 nm by using a microplate reader (Synergy H4, BioTek, VT, USA). The AE and LC were calculated as follows:

$$AE (\%) = [(\text{theoretical amount of BVZ} - \text{free BVZ}) / \text{theoretical amount of BVZ}] \times 100$$

$$LC (\%) = [\text{associated BVZ} / \text{total theoretical concentration of the system}] \times 100$$

2.9 NRP-1 QUANTIFICATION IN THE A549 CELL LINE

The surface expression of NRP-1 on the A549 cell line was quantified by immunofluorescence staining. Cells were harvested with TrypLE™, centrifugated, and washed with cold PBS. Then, 1×10^6 cells/mL were incubated with the anti-NRP1 primary Ab (0.2 µg) in cold PBS and stored at room temperature protected from the light. After 30 minutes of incubation, cells were washed 3 times with cold PBS and fixed with 4% paraformaldehyde in PBS. Surface expression of NRP-1 was confirmed by fluorescence-activated cell sorting (FACS) analysis. Results were analyzed using FlowJo software.

2.10 CELL VIABILITY ASSAYS

To evaluate the toxicity profile of the ⁸⁹Zr-HANAs, an Alamar Blue assay was performed in the A549 human lung cancer cell line. Thus, 7500 cells/well were seeded in a 96-well plate and incubated overnight at 37°C and 5% CO₂. Dulbecco's Modified Eagle Medium (DMEM) (high glucose) containing 10% fetal bovine serum, penicillin, and streptomycin was used as cell culture media. When the confluence was 70-80%, the cell culture media was discarded and

replaced with 100 μL /well of increasing concentrations of empty and BVZ-loaded HANAs. After 24 hours of exposure, the media was removed and 100 μL /well of the 1X resazurin reagent diluted in cell culture media was added and incubated for 40 minutes. Fluorescence intensity as an indicator of the number of live cells is proportional to the reduction of resazurin to resorufin. Thus, fluorescence values were recorded in a microplate reader (Promega, Madison, WI, USA), after setting up the excitation/emission intensity at 525/580-640 nm. Cells treated with cell culture media were used as positive controls. Triton X-100 0.5% (v/v) in cell culture media was added to cells and used as the negative control. The percentage of cell viability was calculated, after subtracting the values from the negative control, as the fluorescence of the samples divided by the positive control.

The cytotoxicity profile of ^{89}Zr -HANAs was determined following the above-described method. In this case, cells were exposed to increasing concentrations of the ^{89}Zr -HANAs (i.e., 0.0005, 0.005, 0.05, 0.5 mg/mL) for 4 hours. ^{89}Zr -BVZ was used as control. The percentage of cell viability was calculated as above described. The radiolabeling process of the HANAs requires several concentration and purification procedures. To calculate the mass of components to be used for the study, the synthesis yield of the HANAs was determined. Thus, 40 μL of purified ^{89}Zr -HANAs and ^{89}Zr -BVZ were added to a glass vial and dried by evaporation at 100°C . After it, the vial was weighted, and the final concentration was calculated after subtracting the weight of the empty vial.

Cell viability (%) = fluorescence of the sample/ fluorescence of the positive control

2.11 *IN VITRO* UPTAKE OF ^{89}Zr -C1 AND ^{89}Zr -C1tLyP1

The uptake capacity of the HANAs and the influence of the tLyP1 peptide were studied on the A549 cell line. An initial density of 5×10^4 cells/well was seeded in a 24-well plate and incubated overnight in a 5% CO_2 atmosphere at 37°C . DMEM supplemented with 10% FBS penicillin and streptomycin was the culture media of selection. When confluence 70-80%, ^{89}Zr -C1, ^{89}Zr -C1tLyP1, and ^{89}Zr -BVZ were dispersed in culture media at final concentrations of 0.05 and 0.005 mg/mL. To do so, the final concentration of HANAs used was calculated after considering the loss of components during the ^{89}Zr labeling as above-described. Afterward, 500 μL /well were added to the cultured plate and incubated for 4 hours at 37°C . Then, the supernatant was collected, and cells were washed 3 times with cold PBS 1X. Cells were detached by incubation with TrypLETM for 15 minutes at 37°C and resuspended in cell culture medium. The cell culture medium was used as the negative control. The radioactivity in the supernatant, wash, and cells suspension was gamma counted, and the % ^{89}Zr cellular uptake was calculated after subtracting the activity from the negative control:

Cellular uptake of ^{89}Zr (%) = activity in the cells suspension/ total activity (supernatant + wash + cells)

The competitive receptor assay was done as described in another report [45]. Briefly, prior to HANAs treatment, cells were treated with an excess of free tLyP1 (i.e., 1 mM) in serum-supplemented medium and incubated for 1 h at 37°C . Then, cells were exposed to increasing concentrations of ^{89}Zr -C1tLyP1 following the procedure above-described.

2.12 *IN VIVO* PET/CT IMAGING OF ^{89}Zr -HANAs IN HEALTHY MICE

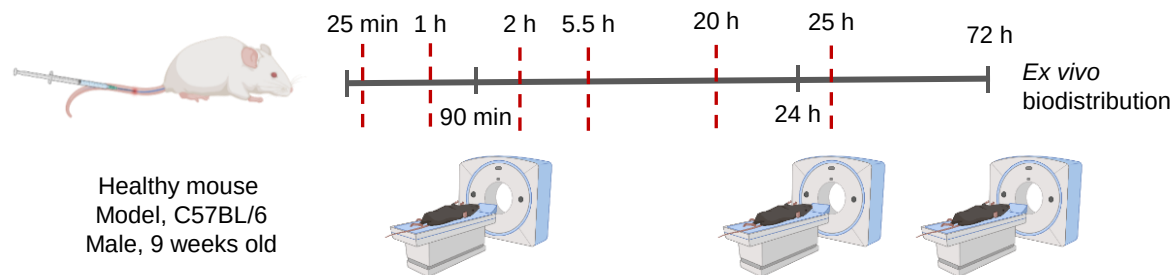


Figure 2. Scheme of *in vivo* PET experiments in C57BL/6 healthy mice. Blood samples were withdrawn at 25 minutes and 1, 2, 5.5, 20, 25 and 72 hours. PET/CT images were recorded at 90 minutes, 24 and 72 hours. Dashed lines indicates the mean blood withdrawal time points.

Animal imaging studies were ethically reviewed and carried out in accordance with the Animals (Scientific Procedures) Act 1986 (ASPA) UK Home Office regulations governing animal experimentation. *In vivo* imaging was conducted in healthy 8-week-old C57BL/6 mice. Animals were anesthetized with isoflurane (2–3% in oxygen) and the radiolabeled NPs (1.5 - 3 MBq) intravenously administrated 1 h before the PET acquisition (1:5 coincidence mode; 5 ns coincidence time window). PET was recorded for 30 min and then a semicircular CT scan was performed. Animal body temperature and respiratory rate were monitored during the whole process. PET/CT images were reconstructed using Tera-Tomo 3D reconstruction (400–600 keV energy window, 1:3 coincidence mode, 4 iterations, and subsets) at a voxel size of $(0.4 \times 0.4 \times 0.4) \text{ mm}^3$ and corrected for attenuation, scatter, and decay.

2.13 *IN VIVO* PET/CT IMAGING IN A LUNG TUMOR MODEL

Tumors were induced 1 week prior to the imaging studies by subcutaneous injection of 2×10^6 A549 lung cancer cells in Balb/c nu/nu mice. Tumor growth and animal welfare were regularly monitored after that.

2.14 STATISTICAL ANALYSIS

Data analysis was performed by using GraphPad Prim version 9.3.0. Gaussian distribution was determined following a Shapiro-Wilk test. The differences were considered significant for * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ with a confidence level of 0.05.

The statistical analysis of the uptake profile among concentrations was done following a paired t-test.

The statistical analysis within the *in vivo* studies in healthy mice comparing ^{89}Zr -BVZ and ^{89}Zr -C1 was done following an unpaired t-test among time points (pharmacokinetics study in blood) and Mann-Whitney test for %ID/g of tissue among groups (*ex vivo* biodistribution).

3 RESULTS AND DISCUSSION

The design of NPs with the capacity to target the tumor microenvironment has been extremely challenging. In particular, the poor tumor accumulation and penetration of the NPs, together with the scarce understanding of the NPs' *in vivo* biodistribution are still unsolved constraints. Our strategy towards this aim has been the functionalization of the HANAs, with demonstrated capacity for the efficient loading of mAbs, with the tumor penetrating peptide tLyP1. The HANAs developed in Chapter 1 (Table 1) so-called prototype C1 and C1tLyP1 were selected to study the pharmacokinetic pattern and the role played by the peptide.

Table 1. Composition, physicochemical properties, and BVZ association efficiency (AE) and loading capacity (LC) of the after concentration of the selected HANAs prototypes for the radiolabeling. Data are expressed as mean $\pm$ SD, $n \geq 3$. *tLyP1 targeted material.

Proto- type	Final composition [mg/mL]				Physicochemical properties			AE (%)	LC (%)
	Polymer	Surfactant	Surfactant.EG	BVZ	Particle size (nm)	PDI	Zeta potential (mV)		
C1	1.6	3.2	1.6	3.2	79 $\pm$ 8	0.25	-13 $\pm$ 2	67 $\pm$ 21	22.2
C1tLyP1	1.6	3.2	0.064*	3.2	121 $\pm$ 14	0.25	-13 $\pm$ 2	65 $\pm$ 12	26.0

3.1 RADIOLABELING OF DFO-BVZ-LOADED HANAS WITH ^{89}Zr

The suitability of the radiolabeling method of the HANAs for *in vivo* studies is determined by the capacity to radiolabel the HANAs without causing appreciable modifications of the physicochemical properties while achieving proper radiochemical characteristics. Thus, in order to perform nuclear imaging, the Ab was radiolabeled with the radionuclide ^{89}Zr [46], a positron emitter radioisotope with a half-life of 78.4 h. To accomplish it, the naturally occurring bifunctional chelate DFO was selected due to its well-established stability with Abs [47] and by-products [48], and safety profile [49,50].

[Section with copyright protection].

To define the most suitable radiolabeling strategy in terms of reproducibility, radiochemical properties, and production yield for PET imaging, two labeling strategies were explored as summarized in [Section with copyright protection].

Prior to the radiolabeling, the tLyP1 peptide was conjugated to the surfactant.PEG following a maleimide-thiol chemistry, described in detail in Chapter 1. ^1H -NMR analysis was carried out to determine the degree of substitution (DS) through the aliphatic chains of the PEG-lipid and the methyl group of the threonine of the peptide. Thus, the DS was 94.8 ± 12 ($n=4$). A schematic representation of the surfactant.PEG-tLyP1 conjugate, and the chemical characterization carried out is shown in Chapter 1.

[Section with copyright protection].

3.1.1 ^{89}Zr -labeling of BVZ-DFO-loaded HANAs and post-purification procedure

In a first labeling strategy (Method 1, [Section with copyright protection]), the BVZ-DFO conjugate was loaded into the HANAs and the ^{89}Zr labeling process was initiated. As shown in Figure 3.A, moderate radiochemical yield (RCY) of 66 ± 22 % (^{89}Zr -DFO-BVZ, denoted as ^{89}Zr -BVZ from now), 68 ± 7 % (^{89}Zr -C1), and 61 ± 19 % (^{89}Zr -C1tLyP1) were obtained, revealing minor differences among the radiochemical yield of the mAb and those of HANAs. These values are lower than what has been published for other radiolabeled mAbs [51]. However, a comparison with other mAb-loaded nanocarriers cannot be drawn as there is no previous work in this regard.

The production yield, radiochemical purity, and the physicochemical properties of the ^{89}Zr -HANAs were determined. Regarding the production yield, low efficiencies were obtained for the HANAs, independently of their composition (Figure 3.B). When compared with the Ab, the differences were noticeable, being 49 ± 15 % (^{89}Zr -BVZ), 10 ± 8 % (^{89}Zr -C1), and 5 ± 5 % (^{89}Zr -C1tLyP1). In parallel, the radiochemical purity was determined (Figure 3.C), revealing values $>90\%$, which demonstrate the successful removal of free ^{89}Zr and ^{89}Zr -BVZ. Once ^{89}Zr -HANAs were purified and concentrated, the obtained ^{89}Zr -HANAs were applied for further experiments.

[Section with copyright protection]. After the removal of non-entrapped ^{89}Zr -BVZ (see Supplementary Figure 2 for the UV/VIS spectra and radioactivity profile) and the concentration of the HANAs, a process required to produce the adequate dose of HANAs, a clear loss of activity is also observed.

The physicochemical properties of the HANAs after and before the process underwent minor changes (Supplementary Table 1).

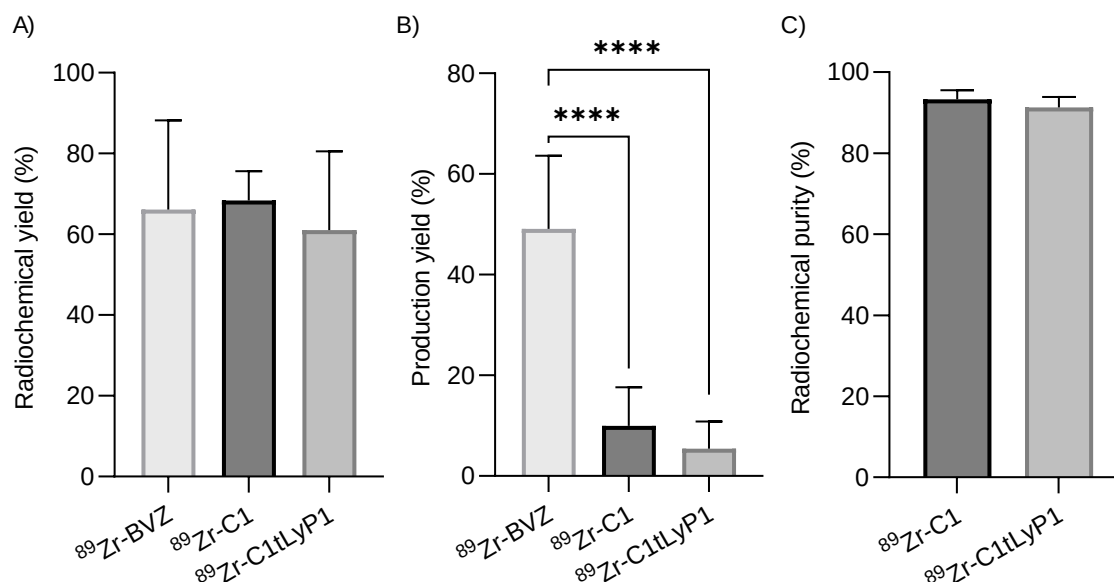


Figure 3. Radiochemical characterization of ^{89}Zr -labeled BVZ-loaded HANAs-PEG (^{89}Zr -C1), ^{89}Zr -labeled BVZ-loaded C1tLyP1 (^{89}Zr -C1tLyP1) prepared by method 1, and ^{89}Zr -BVZ. A) Radiochemical yield (RCY, %), B) production yield (%), and C) radiochemical purity (RCP, %). Data are expressed as mean $\pm$ SD, $n \geq 3$. Statistical analysis was done using a Mann-Whitney (A) and unpaired t-test (B-C).

3.1.2 HANAs purification and post- ^{89}Zr -labeling procedure

In a second stage, we aimed to improve the production yield by purifying the HANAs prior to the ^{89}Zr -labeling (method 2, [Section with copyright protection]). We hypothesized that the removal of the non-entrapped DFO-BVZ complex prior to the radiolabeling would lead to a high radiochemical yield. Contrary to what was observed with method 1, obvious differences in terms of radiochemical yield were detected upon labeling the Ab already associated to the HANAs. Indeed, the radiochemical yield was 2.7- and 1.5-times lower for ^{89}Zr -C1 and ^{89}Zr -C1tLyP1, respectively (Figure 4.A) as compared to ^{89}Zr -BVZ. [Section with copyright protection]. In addition, the removal of the non-entrapped BVZ-DFO may led to a reduction of the available DFO, being also responsible for the low radiochemical yield values since the isotope has less opportunities to form coordination complexes. Although this information helps to understand the nature of these novel HANAs, further studies must be performed to fully understand this behavior. Noteworthy, ^{89}Zr -HANAs were concentrated achieving production yields of $6 \pm 2\%$ (^{89}Zr -C1) and $16 \pm 0.1\%$ (^{89}Zr -C1tLyP1) and RCP values similar to method 1. Further evaluation of the UV/VIS and radioactivity profiles (Supplementary Figure 3) revealed the absence of free ^{89}Zr or ^{89}Zr -BVZ confirming the high RCP of both samples.

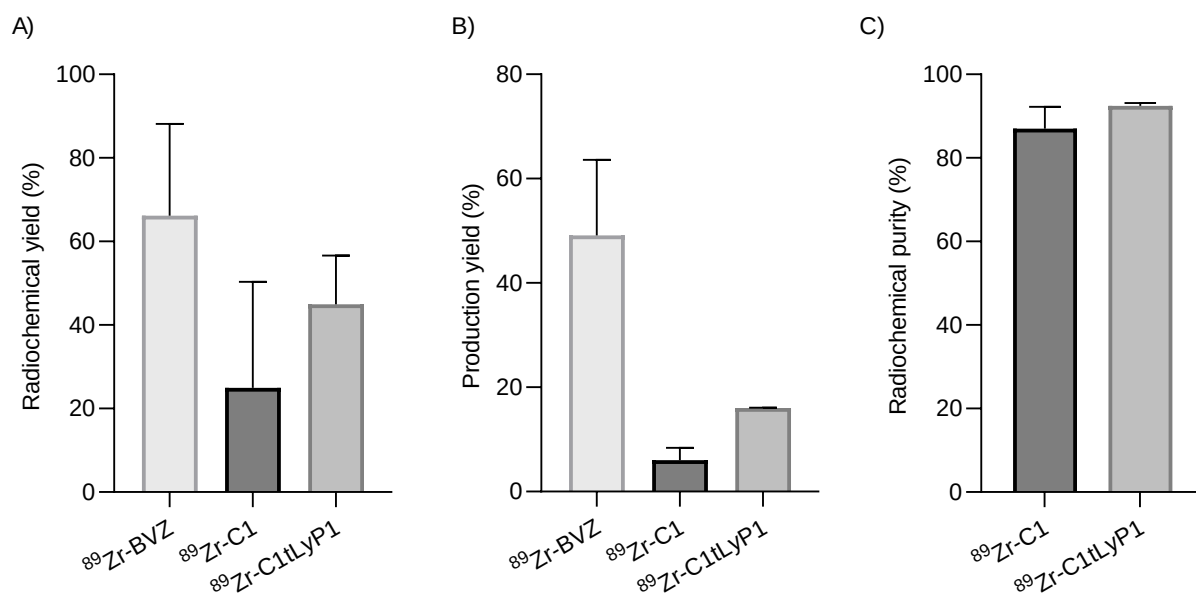


Figure 4. Radiochemical characterization of ^{89}Zr -labeled BVZ-loaded C1 (^{89}Zr -C1) and BVZ-loaded C1tLyP1 (^{89}Zr -C1tLyP1). A) Radiochemical yield, B) production yield, and C) radiochemical purity (RCP, %) of C1, C1tLyP1, and BVZ. Data are represented as mean $\pm$ SD, $n \geq 2$.

Finally, the physicochemical properties were monitored during the process with an apparent increase in particle size regarding ^{89}Zr -C1tLyP1 (Supplementary Table 2).

Overall, acceptable radiochemical purities and reproducible procedures were associated with both methods. Low to moderate but still reasonable production yields resulted from the processes, with apparent improvements in method 2 for ^{89}Zr -C1tLyP1 (16 % compared with 5% from method 1).

3.2 STABILITY OF THE ^{89}Zr -HANAS IN HUMAN SERUM.

To assess whether the radiolabeling was stable for carrying out *in vivo* studies, the ^{89}Zr -HANAs were incubated in human serum. Therefore, the ^{89}Zr -retention in the HANAs at 37°C was assessed at different times. As shown in Figure 5, radiochemical stabilities of $69 \pm 6\%$ and $70 \pm 4\%$ for ^{89}Zr -C1 and ^{89}Zr -C1tLyP1, respectively, were obtained after 72 hours with no variations overtime or between samples. Overall, the radiochemical properties of both ^{89}Zr -HANAs are suitable for further *in vivo* studies.

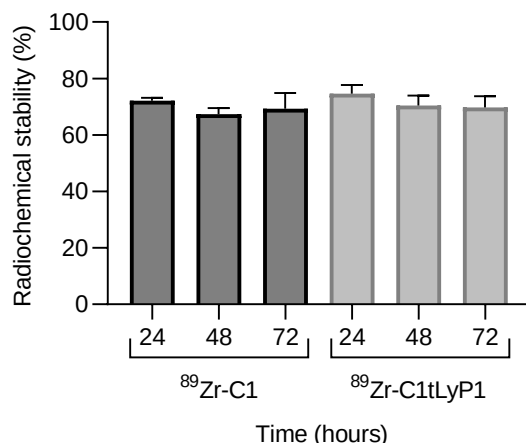


Figure 5. Radiochemical stability of ^{89}Zr -labeled BVZ-loaded C1 (^{89}Zr -C1) and BVZ-loaded C1tLyP1 (^{89}Zr -C1tLyP1) in human plasma at 37°C. ^{89}Zr -HANAs were incubated in human plasma and the amount of ^{89}Zr retained to the HANAs was quantified at 24, 48, and 72 hours. Data are expressed as mean $\pm$ SD (^{89}Zr -C1; n=2 and ^{89}Zr -C1tLyP1; n=3). Statistical analysis of ^{89}Zr -C1tLyP1 was done using an unpaired t-test. Study performed following radiolabeling method 1.

3.3 IN VITRO CELL CULTURE STUDIES

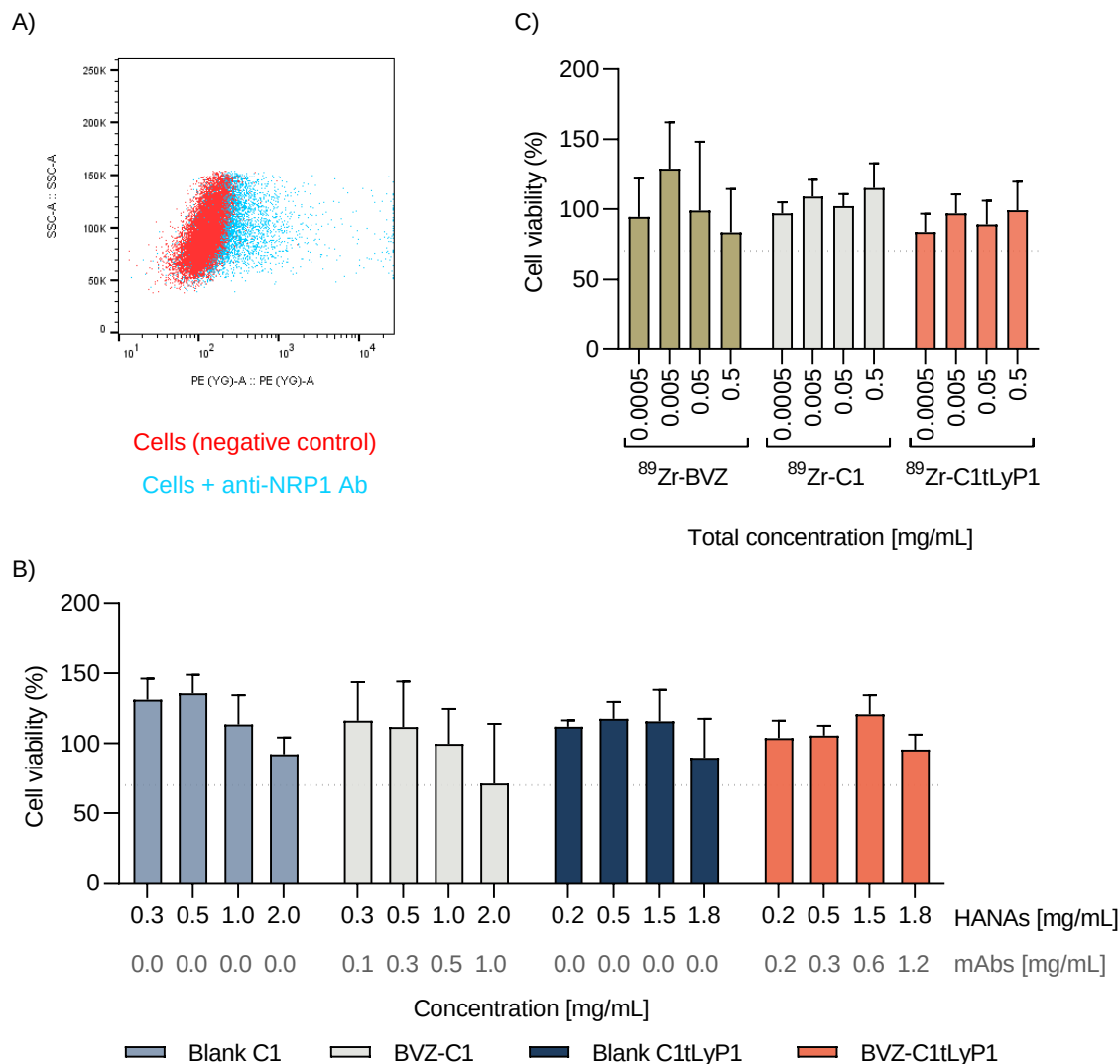
3.3.1 NRP-1 expression

Surface disposal of NRP-1 receptors triggers the tLyP1-targeted HANAs internalization via the CendR pathway, enhancing tissue penetration. Previous reports have demonstrated the NRP-1 overexpression on the surface of the human lung cancer cell line, A549. As shown in Figure 6. A, the expression of NRP-1 was detected by flow cytometry. Accordingly, the A549 cell line was selected for further *in vitro* and *in vivo* studies.

3.3.2 Cell viability studies

Novel systems for the delivery of Abs must maintain the biocompatible nature of the protein while enhancing its efficacy. Hence, the cytotoxicity profile of increasing concentrations of empty and BVZ-loaded HANAs was studied in the A549 cell line. In accordance with the ISO 10993-5 standards, cell viability $<70\%$ was considered toxic [52]. After 24 hours of exposure, HANAs showed a low-toxicity profile of up to 1.8-2 mg/mL, independently of the tLyP1 presence on their surface. Since no statistical differences between blank and BVZ-loaded HANAs were found (Figure 6. B), the biocompatible profile of the

prototypes was confirmed, establishing a novel candidate for the association of clinically relevant proteins.



Radiolabeling of HANAs may modify their *in vitro* behavior and reduce their chances of *in vivo* success. In fact, radiotoxicities caused by ionizing radiation can be directly caused by their physical characteristics such as radiochemical impurities or side products, or indirectly due to the water radiolysis [53]. The decomposition of water due to ionizing radiation led to the production of reactive species such as $\text{OH}^\cdot$ or H_2O_2 which ultimately give rise to cell death or mutations [54]. Therefore, aiming to evaluate the potential radiotoxicity of both, the ^{89}Zr -HANAs and the ^{89}Zr -BVZ, a metabolic activity assay was carried out (Figure 6. C). Thus, cells were exposed to ^{89}Zr -labeled non-targeted and tLyP1-targeted HANAs for 4 hours. ^{89}Zr -BVZ was treated in the same conditions and used as control. In accordance with the above results, increasing concentrations of ^{89}Zr -HANAs or ^{89}Zr -BVZ did not modify the cell viability within the range of tested activity per cell.

In conclusion, the results of these experiments indicate that the cytotoxicity of the HANAs formulations is acceptably low since concentrations of up to 1.8-2 mg/ml of HANAs did not exhibit any cytotoxicity.

3.3.3 Uptake studies

To maximize a therapeutic outcome, targeted HANAs must reach the target tissue and penetrate the tumor mass. Thus, the presence of the tLyP1 peptide on the HANAs surface should enhance cell uptake through its interaction with the NRP-1 receptor. To support this hypothesis, the uptake profile of ^{89}Zr -labeled non-targeted and tLyP1 targeted HANAs was studied in the A549 cell line (Figure 7.A). For that, increasing concentrations of HANAs were incubated with the cells for 4 hours. As expected, ^{89}Zr -BVZ (control) showed negligible cell uptake. When the Ab was entrapped into the HANAs, two different uptake patterns were observed. Regarding non-targeted HANAs, minor uptake was found for both concentrations, which was comparable with the control. This result could be attributed to the high PEG concentration of the non-targeted HANAs which reduces the uptake [55,56] by the formation of a hydrophilic stealth corona, reducing the cell membrane interaction. In contrast, C1tLyP1 demonstrated superior cellular uptake. In fact, for the lowest concentration tested, the extent of the uptake was 9.2- and 34.1 times higher than the non-targeted counterpart and the Ab, respectively. Therefore, since we hypothesize that the presence of tLyP1 on the surface of HANAs is a condition *sine qua non* for HANAs internalization, the uptake extent would be expected to be independent of the HANAs concentration and not greater than the percentage of NRP-1 expressed. In agreement, no differences were observed among concentrations of HANAs.

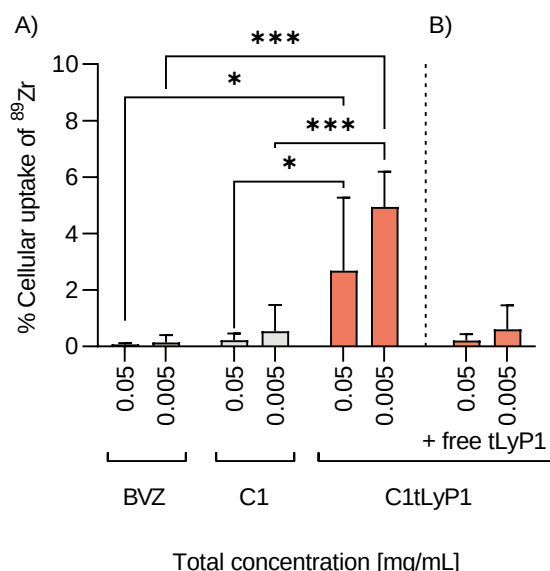


Figure 7. *In vitro* cell uptake of ⁸⁹Zr (A) and competitive assay (B) in the A549 cell line after 4 hours. Cells were with total concentration (mg/mL) of ⁸⁹Zr-BVZ (BVZ) and ⁸⁹Zr-labeled BVZ-loaded C1 (C1) and ⁸⁹Zr-labeled BVZ-loaded C1tLyP1 (C1tLyP1) in serum-supplemented media at 37°C. For the competitive assay, cells were treated with an excess of tLyP1 for 1 hour prior treatment. Total concentration (mg/mL) was calculated considering the dry mass of the final product after the radiolabeling. Statistical comparison was performed by a 2-way ANOVA followed by a Fisher's LSD test. Data are expressed as mean ± SD (n≥3, except for the competitive assay, n=2).

In order to confirm whether the uptake is mediated by the overexpression of the NRP-1 on the cell surface, a competitive study in the same cell line was carried out (Figure 7.B). After saturation of the NRP-1 receptor with an excess of peptide, this hypothesis was confirmed. As shown, the cellular uptake was reduced. These results evidenced an improved cellular uptake related to the presence of the tLyP1, as reported before by other authors in several cancer cell lines [33,45,57].

Although the absence of *in vitro* uptake has been shown with C1, the further addition of tLyP1 undeniably improved the outcome of the HANAs. Therefore, in order to study the correlation of the *in vitro* studies with an *in vivo* model, the biodistribution of C1 and C1tLyP1 was conducted in healthy and tumor-bearing mice.

3.4 *IN VIVO* PET/CT IMAGING OF ⁸⁹ZR-C1 IN HEALTHY MICE

Encouraged by the former results and aiming to validate the *in vivo* performance of the HANAs, PET/CT imaging and biodistribution studies were conducted in healthy mice (C57BL/6). *In vivo* studies in healthy mice will allow us to settle the bases for a disease model as the immune system remains intact. Based on the results disclosed by the stability studies in human serum, biodistribution studies were performed for up to 72 hours. To compare the capacity of ⁸⁹Zr-C1 to modify the biodistribution profile of the mAb, two groups were evaluated: the free mAb (⁸⁹Zr-BVZ) and the BVZ-loaded HANAs (⁸⁹Zr-C1). Thus, PET/CT studies revealed a very different biodistribution profile of the PEGylated HANAs compared to the mAb. While, as expected, the free mAb remains in blood for long periods, the ⁸⁹Zr-C1 rapidly accumulated in the liver and spleen, where it remained for more than 72 h (Figure 8). The intravenous

administration of neutralized ^{89}Zr (Supplementary Figure 4) confirmed the uptake pattern of the HANAs as no overlapped signals were detected.

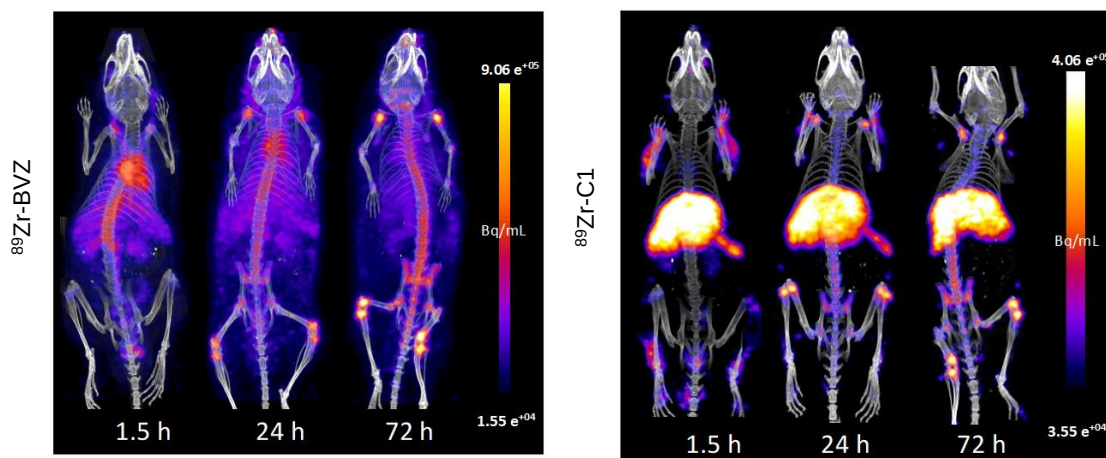


Figure 8. Maximum intensity projection PET/CT imaging of ^{89}Zr -BVZ and ^{89}Zr -labeled BVZ-loaded C1 (^{89}Zr -C1) in 9 weeks old healthy C57BL/6J mice. PET/CT maximum intensity projections of 1-2 MBq of ^{89}Zr -BVZ and ^{89}Zr -C1 after i.v. administration (n=4). Study performed following radiolabeling method 1.

PET/CT images were consistent with the pharmacokinetics study in blood. At 2 hours postinjection, a rapid clearance of the HANAs from the blood stream of 7 ± 3 % of the injected dose (Figure 9.A) and significantly prolonged circulation times for the mAb of 47 ± 4 % were found, accordingly to the half-life of BVZ (approx. 3.9 days) [58].

Ex vivo gamma counter biodistribution after 72 hours revealed the uptake profile of the mAb and the HANAs in different organs (Figure 9.B). All tissues but muscle and bone marrow led to significant differences in the biodistribution of free mAb (^{89}Zr -BVZ) and HANA's-loaded Ab (^{89}Zr -C1). ^{89}Zr -C1 remains in liver and spleen after 72 hours, indicating the ^{89}Zr -C1 accumulation without minor modifications over time, as suggested by the PET/CT images. As the ^{89}Zr -BVZ is highly exposed to the disruptive environment of the blood, ^{89}Zr is more prone to be released. Consequently, bone superior accumulation was found, which is in line with the bone affinity of ^{89}Zr .

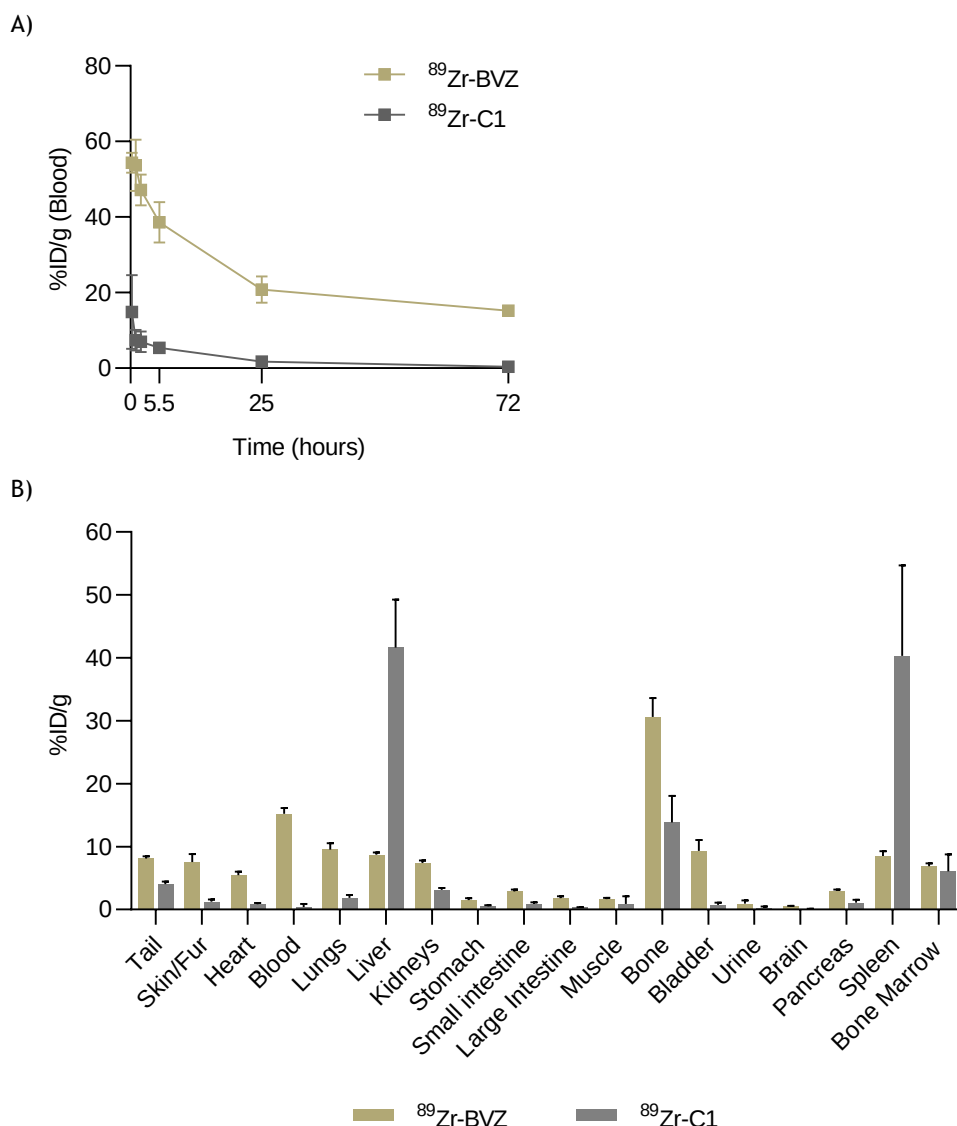


Figure 9. Blood profiles and organ biodistribution of ^{89}Zr -BVZ and ^{89}Zr -labeled BVZ-loaded C1 (^{89}Zr -C1) in 9 weeks old healthy C57BL/6J mice. A) Percentage of injected dose of per gram (%ID/g) in blood after i.v injection of 1-2 MBq of ^{89}Zr -BVZ and ^{89}Zr -C1 (n=4 per group). B) Organ uptake represented as %ID/g of tissue at 72 h after i.v injection of ^{89}Zr -BVZ and ^{89}Zr -C1 (n=4). Study performed following radiolabeling method 1.

Overall, we consider that above evidence supports the idea of using the prototype C1 for the delivery of mAbs to the tumor tissue, given the corroborated capacity of our prototype C1 to modify the biodistribution profile of the BVZ. Hence, the usefulness of prototype C1 as a potential candidate for the delivery of Abs was further explored in a relevant non-small cell lung (NSCL) cancer tumor model.

3.5 IN VIVO PET/CT IMAGING IN A LUNG CANCER TUMOR MODEL

Based on our previous results in healthy mice the HANAs prototype ^{89}Zr -C1, PET/CT studies were conducted in a subcutaneous A549 NSCL carcinoma mouse model. To understand the role of the tumor penetrating peptide on the HANAs' surface, together with the presence of PEG, the biodistribution profile of ^{89}Zr -C1tLyP1 and ^{89}Zr -C1 were compared in this second study.

The pharmacokinetic profile of the HANAs in blood circulation was studied overtime, revealing no-significant differences up to 24 hours between both prototypes (Figure 10.A). Contrary to what was expected [59,60], the higher density of PEG in ^{89}Zr -C1 (11.9 %) in comparison to ^{89}Zr -C1tLyP1 (0.4 %) did not change the pharmacokinetic profile of ^{89}Zr -HANAS. The higher PEG density and the presence of tLyP-1 could give rise to a different protein corona on the HANAs [68], making it difficult to predict their behavior in terms of permanence in blood. Despite this, ^{89}Zr -C1 and ^{89}Zr -C1tLyP1 were monitored by PET/CT imaging for 1 and 24 hours after i.v. injection (Figure 10.B). Results were consistent with the biodistribution pattern observed in healthy mice, this meaning that HANAs were primarily accumulated in liver and spleen at both, 1 and 24 hours postinjection. It is worth noting that a poor accumulation of the HANAs was found in the tumor, leading us to a further evaluation by *ex vivo* studies.

Ex vivo biodistribution studies at 24 hours following HANAs injection were consistent with the PET/CT images, showing high uptake in liver and spleen and minor differences between targeted and non-targeted HANAs (Figure 10.C). Moreover, the signal found in lungs and bone marrow was significantly higher for ^{89}Zr -C1. However, when compared with the results obtained in the healthy mouse model (section 3.4.), the accumulation may be attributed to the released ^{89}Zr -BVZ and not the ^{89}Zr -HANAs, although it should be confirmed in this tumor model. Within tumors, no significant differences in uptake were found with 2 ± 1 %ID/g for ^{89}Zr -C1 and 3 ± 1 %ID/g for ^{89}Zr -C1tLyP1. Further tumor to tissue uptake ratios were determined (Figure 10.D). As shown, no-significant tumor to muscle ratios but a preferential tumor accumulation of ^{89}Zr -C1tLyP1 of 12 ± 2 in contrast to 8 ± 8 for ^{89}Zr -C1 was observed. Moreover, the values of tumor to blood ratios showed a significantly greater accumulation of tLyP-1 HANAs in the tumor of 14 ± 16 compared to 2 ± 1 (^{89}Zr -C1) after 24 hours. In accordance, higher tumor/liver ratios were found for the functionalized HANA's (^{89}Zr -C1tLyP1).

In brief, the results of the tumor accumulation of tLyP1-HANAs indicated the functionalization with tLyP1 had a significant effect on tumor uptake [33,37,63–65]. When our results are compared with other tumor-homing peptides such as iRGD or LyP1 decorating porous silicon NPs [66] and dendrimers [67], respectively, consistent results are observed. For instance, iRGD-NPs demonstrated a 4.4 tumor-to-muscle ratio (%ID/g) after its intravenous administration in a prostate cancer mouse model, which was 1.6 times higher than the non-targeted counterpart. Similarly, LyP1- ^{131}I -PEG-NPs revealed a tumor-to-muscle ratio of 5.9 in a breast cancer model, which was found to be sufficient to reduce the tumor volume. Furthermore, in other approaches carried out in the same A549 tumor model, tumor-to-muscle ratios for up to 21% have been described 6 hours after injection [68] as well as doxorubicin %ID/g of 8.60 determined by fluorescence spectroscopy was found after anchoring the cNGQ peptide to the surface of polymersomes, which was 2.4 fold higher than the non-targeted counterpart [69].

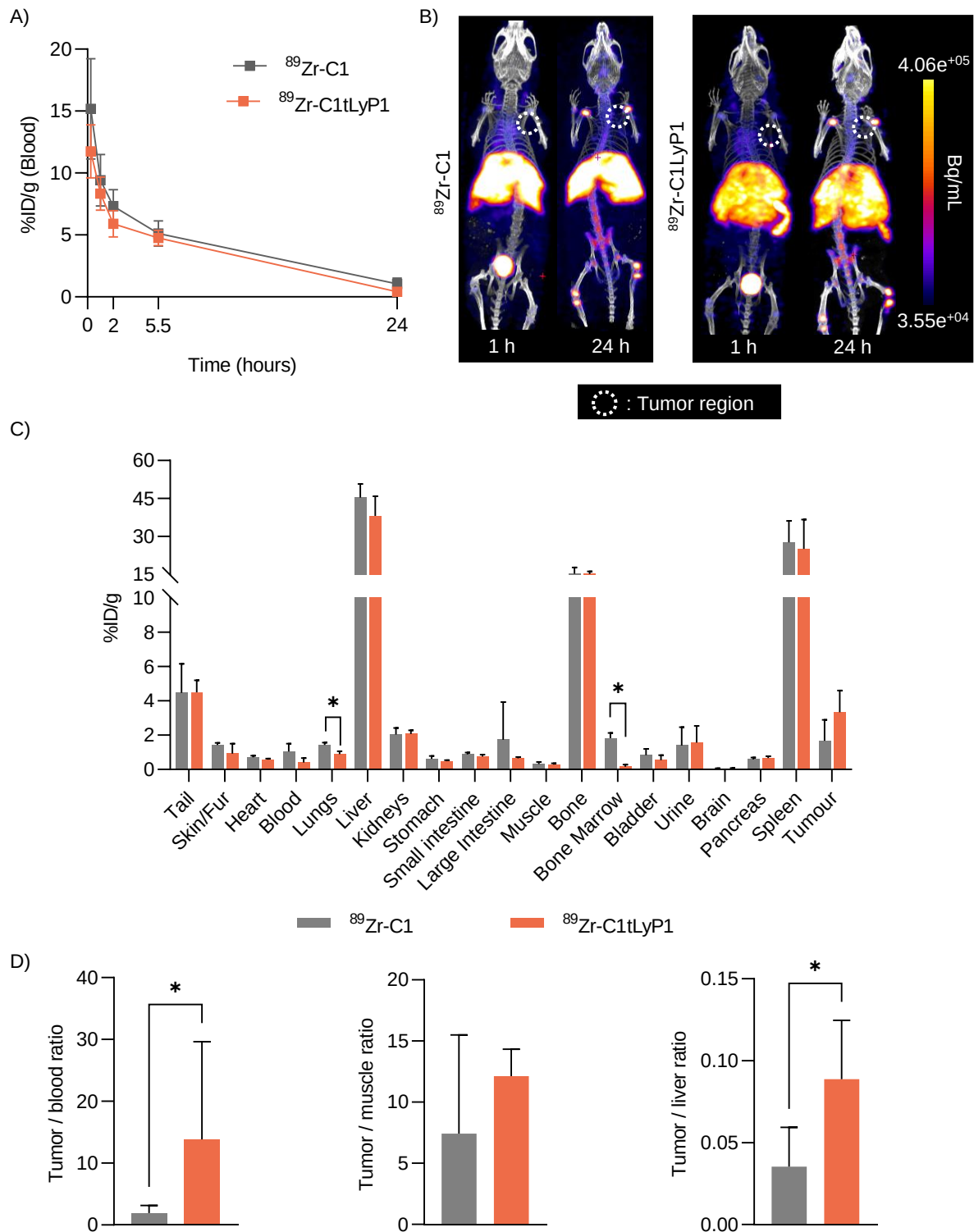


Figure 10. Blood profiles, organ biodistribution, and PET imaging of ^{89}Zr -labeled BVZ-loaded C1 (^{89}Zr -C1) and ^{89}Zr -labeled BVZ-loaded C1tLyP1 (^{89}Zr -C1tLyP1) in an A549 tumor-bearing Balb/c mouse model. **A)** Percentage of the injected dose of ^{89}Zr per gram (%ID/g) of blood after i.v injection. **B)** PET/CT maximum intensity projections of 2.5-3 (^{89}Zr -C1) and 1.5-2.5 (^{89}Zr -C1tLyP1) after i.v. administration. **C)** Organ uptake represented as %ID/g of tissue at 24 h after i.v injection. **D)** Ratio of tumor/blood, tumor/muscle, and tumor/liver uptake. Data are represented as mean $\pm$ SD, $n=4$. Statistical comparison was done following an unpaired t-test (A, D tumor/muscle and tumor/liver ratio) and Mann-Whitney test (B, D tumor/blood ratio).

It is our opinion that the efficacy studies will be highly conditioned by the potency of the loaded drug since moderate tumor accumulations of alternative HANAs have led to significant therapeutic outcomes. Nevertheless, to maximize the *in vivo* fate, a more relevant tumor model should be explored.

Overall, tLyP1-functionalized HANAs were accumulated in the tumor in a greater extent than the non-targeted counterpart, confirming the feasibility of these HANAs as active targeting delivery systems.

4 CONCLUSIONS

A key parameter in the translational assessment of nanomedicine is to identify the biodistribution profile of NPs following the prescribed administration route. More in detail, the design of delivery strategies aiming to modify the biodistribution profile of naked mAbs while enhancing their tumor accumulation are key for their clinical translation. Here, we report two different radiolabeling strategies of BVZ-loaded HANAs with minor implications in the physicochemical properties. Moreover, ⁸⁹Zr-radiolabeled HANAs have revealed acceptable production yields and high radiochemical purities, which have validated their use for *in vitro* and *in vivo* studies. By functionalizing the HANAs' surface with a tumor penetrating peptide, we enhanced the *in vitro* uptake of the HANAs. Indeed, the higher uptake was not accompanied by a superior toxicity, confirming the low toxicity profile of the HANAs, even after the labeling. Our work further demonstrated the potential of our technology to modify the biodistribution of mAbs, a limiting factor for the NPs' accumulation. Furthermore, the NPs' functionalization with the tLyP1 enhanced the accumulation of the NPs in tumors and presumably, the mAb delivery efficiency.

In summary, these novel nanotherapeutics have demonstrated their ability to modify the biodistribution profile of the mAb, suggesting their translatability to other clinically relevant mAbs.

5 ACKNOWLEDGMENTS

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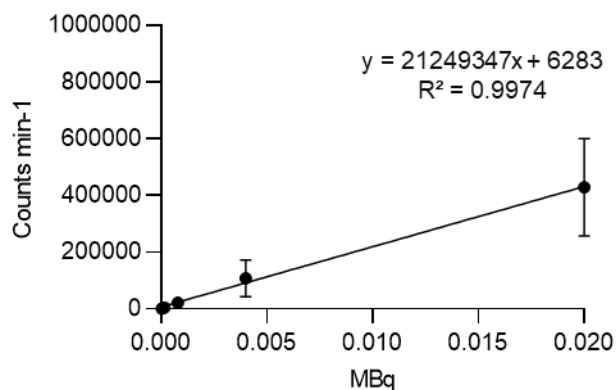
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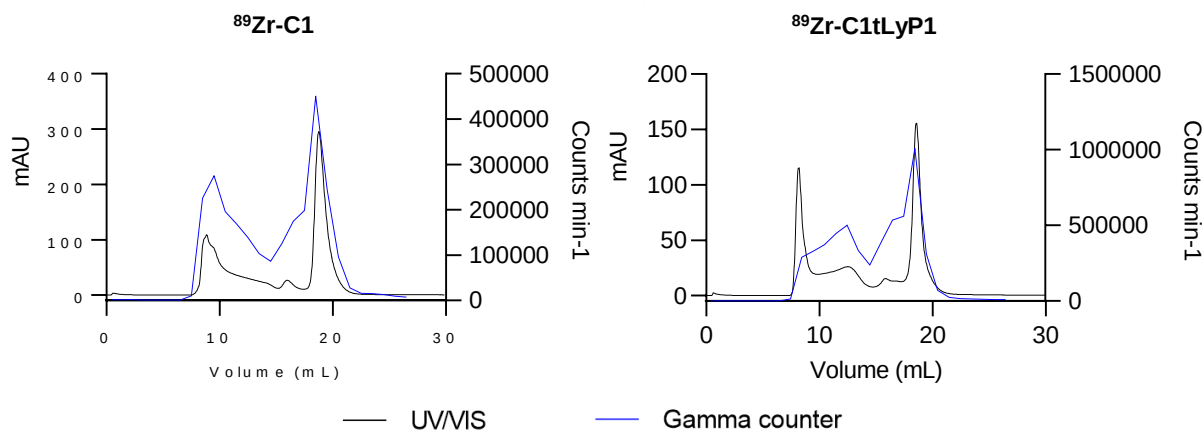
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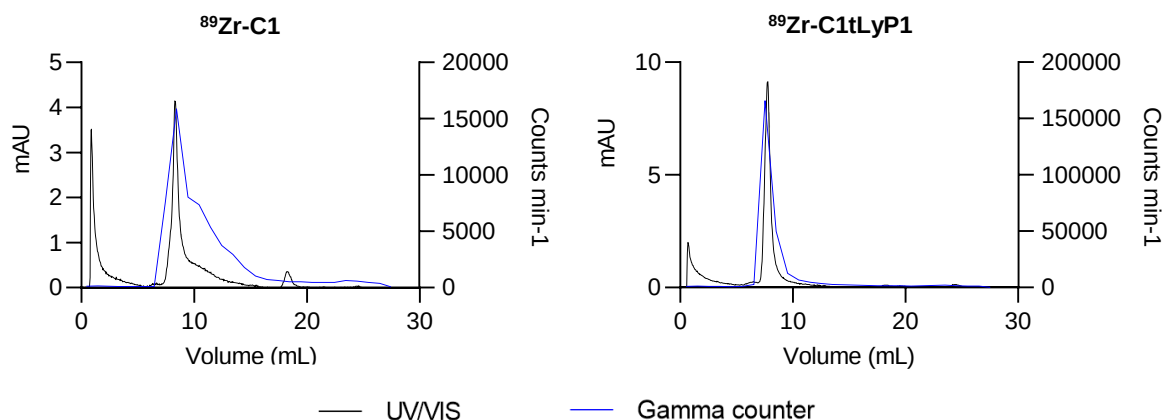
7 SUPPLEMENTARY



Supplementary Figure 1. Calibration curve of ^{89}Zr for the correlation of MBq and counts per minute. The linear regression was calculated only with the mean value of each point. Data are represented as mean $\pm$ SD (n=3).



Supplementary Figure 2. UV and radioactivity profile of C1 and C1tLyP1 during the ^{89}Zr -labeling and post-purification procedure (method 1). Graphs indicate the process required for BVZ removal. UV absorbance at 280nm was obtained after using the ÄKTA system and counts min⁻¹ after gamma-counted the eluted fractions. mAU milli-absorption units.



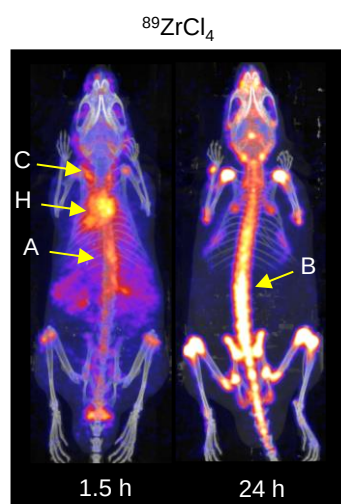
Supplementary Figure 3. UV and radioactivity profile of C1 and C1tLyP1 after the purification and post- ^{89}Zr -labeling method (method 2). Graphs indicate the validation of the radiolabeling process through the radiochemical purity. UV absorbance at 280nm was obtained after using the ÄKTA system and counts min⁻¹ after gamma-counted the eluted fractions. mAU milli-absorption units.

Supplementary Table 1. Physicochemical properties of ^{89}Zr -labeled BVZ-loaded C1 (^{89}Zr -C1) and BVZ-loaded C1tLyP1 (^{89}Zr -C1tLyP1) after the radiolabeling and purification process by following method 1. Data are expressed as mean $\pm$ SD, $n \geq 3$

Prototype	Physicochemical properties	
	Particle size (nm)	PDI
C1	70 ± 7	0.23
C1tLyP1	127 ± 5	0.18

Supplementary Table 2. Physicochemical properties of ^{89}Zr -labeled BVZ-loaded C1 (^{89}Zr -C1) and BVZ-loaded C1tLyP1 (^{89}Zr -C1tLyP1) after the purification and radiolabeling process by following method 2. Data are expressed as mean $\pm$ SD, ^{89}Zr -C1 $n=1$, ^{89}Zr -C1tLyP1 $n=2$.

Prototype	Physicochemical properties	
	Particle size (nm)	PDI
C1	85	0.25
C1tLyP1	168 ± 6	0.21



Supplementary Figure 4. PET/CT images of $^{89}\text{Zr-Cl}_4$ ($^{89}\text{Zr-Cl}_4$) in 9 weeks old healthy C57BL/6J mice. PET/CT maximum intensity projections of 1-2 MBq of $^{89}\text{Zr-Cl}_4$ at 1.5 and 24 after i.v. administration ($n=4$). A aorta, B bone, C carotids, H heart

CHAPTER 3:
**Unleashing the power of personalized
nanomedicine: intracellular delivery of
anti-KRAS^{G12V} mAb to combat lung cancer**

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ABSTRACT

Despite significant research efforts, rat sarcoma virus (RAS) has remained elusive as a therapeutic target. In fact, so far there are not drugs approved for the mutated allele Kirsten-RAS^{G12V} (KRAS^{G12V}), which is the second most prevalent mutation in lung cancer. The failure to find an effective therapy relies on the lack of well-defined binding pockets and the intracellular location of these oncoproteins, making them *undruggable*. In this frame, the use of monoclonal antibodies (mAbs) with large surface area paratopes might be an efficient therapeutic alternative to address intracellular targets. However, a major limitation to achieve this goal relies on the mAb's incapacity to cross cell membranes. The main objective of this work has been to confront this limitation through the use of nanotechnology. Here, we report the design and development of tLyP1-targeted polymer-based nanoassemblies (HANAs) adapted for the association and intracellular delivery of the antiKRAS^{G12V} mAb. We developed a library of functionalized nanocarriers and selected those having adequate physicochemical properties (less than 200 nm), drug loading capacity (greater than 10%), and biological stability. The antiKRAS^{G12V} mAb activity on the selected HANAs prototypes was assessed in terms of inhibition of cell proliferation and colony formation in a panel of KRAS^{G12V}-mutated lung cancer cell lines. Furthermore, we performed target engagement studies, which showed that antiKRAS^{G12V} mAb-loaded HANAs were able to recognize the KRAS^{G12V} oncoprotein. Overall, our studies suggest that our technology could potentially be an effective therapeutic approach for KRAS^{G12V}-mutant tumors

GRAPHICAL ABSTRACT

[Section with copyright protection].

1 INTRODUCTION

Despite the great advances in cancer therapy and the emergence of personalized medicine, the majority of intracellular oncoproteins remain undrugged. A particularly relevant example of the so-called undruggable targets is the RAS family, whose mutated forms are present in 9-30% of human cancers [1,2]. Mutations in RAS are responsible for the accumulation of RAS proteins in the GTP-bound state leading to a permanent “on” conformation of the protein. In consequence, the GTP to GDP exchange cycle is inhibited, triggering the activation of RAS-dependent downstream signaling pathways, which ultimately promote cellular proliferation and angiogenesis while interfering with apoptosis [3–5]. Among the RAS isoforms, KRAS mutations present in ~93% of pancreatic ductal adenocarcinoma [6], ~43% of colorectal adenocarcinoma [7], and ~32% of lung adenocarcinomas [8] have been recognized as highly challenging. In this context, the possibility to switch off RAS through the development of drugs with adequate pockets of interaction with the target is the key for novel targeted therapies.

Numerous attempts have been undertaken to address the main RAS effector pathways, mitogen activated kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signaling cascades, by using small-molecule inhibitors [2,9]. While these inhibitors remain desirable for cancer therapy, they are related to feedback loops and off-target toxicities, which has pointed the need to address the specific KRAS oncoprotein. After decades of efforts, two antiKRAS^{G12C} small molecules have been finally approved for the treatment of non-small cell lung cancer (NSCLC) [10,11], although they have been recently related with the development of acquired resistances [12].

On the other hand, small interfering RNA (siRNA) has also been explored as a way to knockdown a specific or PAN-KRAS mutations [13–17]. Although the reported data are promising, not apparent efforts to the translation to the clinic of these therapies have been disclosed. A different alternative has been the design of ankyrin repeat proteins (DARPs) to inhibit RAS nucleotide exchange [18,19]. For instance, DARPs loaded into lipid nanoparticles (NPs) and administered in a hepatocellular carcinoma model. Unfortunately, only a moderate and similar effect was observed for the free and the encapsulated protein [19]. Finally, it should be highlighted that KRAS^{G12V} T-cell-based therapies have found their way into phase I-II clinical trials (NCT04146298, NCT03190941), however, scarce information is known about their outcome.

Beyond the described strategies, the use of mAbs with large surface area paratopes enabling adequate protein-protein interactions (PPIs) has been identified as a promising therapeutic option [20]. To facilitate the intracellular penetration of the mAbs some researchers have engineered mAb covalently modified with a cytosol penetrating motif. The results reported so far have been positive in murine models of colorectal, pancreatic, and fibrosarcoma carcinomas [21,22]. Unfortunately, this mAb engineering approach does not enable the control of the biodistribution, nor the potential immunotoxicity associated to the limited access of the mAb to the target.

In our lab, we have gone a step forward and chosen nanotechnology as an enabling technology for helping mAbs overcoming biological obstacles and facilitating their access to intracellular

targets [20,23,24]. More precisely, we have developed a platform particularly adapted for the intracellular delivery of mAbs [24–26]. Among the results that support the preclinical relevance of this concept, it is important to stand out the ability to reduce the tumor volume in a HER-2+ breast cancer model and overcome the breast-tumor resistance to trastuzumab after the administration of antigasdermin B mAb-loaded nanocapsules [24]. Other efforts carried out in this context have led to some promising results as is the case of transferrin targeted-PLGA NPs for delivering antiBCR/ABL mAb [23] or antiMYC mAb-loaded polymeric micelles [20].

In the current work we have attempted to go beyond by designing a technology intended to enhance the accumulation of the NPs in the tumor tissue. In this regard, several ligands have been addressed to ensure the selective accumulation of NPs into tumors, of which a new generation of peptides so-called tumor-penetrating peptides (TPPs) stands out. Among the TPPs that have been disclosed, our lab and others have found out that the truncated LyP-1 (tLyP1) peptide is an excellent candidate to enhance the uptake and intratumoral diffusion of NPs [25,27–29]. Indeed, we have demonstrated that the surface functionalization of docetaxel-loaded HA-nanocapsules with tLyP1 leads to a 36.6-fold increase in tumor accumulation over the free cytotoxic agent [25].

1.1 OBJECTIVES

The foremost objective of this work was to validate the HANAs technology described in Chapter 1 for the intracellular delivery of the clinically relevant antiKRAS^{G12V} mAb. Specifically, we aimed at optimizing this technology for the entrapment of the antiKRAS^{G12V} mAb and evaluate their potential for the selected target. We developed and characterized a number of prototypes for their physicochemical properties, loading capacity and biological stability. Furthermore, we assayed the efficacy and the capacity to directly target the KRAS^{G12V} oncoprotein after antiKRAS^{G12V} mAb internalization. To this end, the HANAs were evaluated for their ability to impair the proliferation and formation of colonies. Finally, intracellular staining and target engagement studies were carried out to assess the mAb intracellular location and KRAS^{G12V} oncoprotein recognition in KRAS^{G12V} mutant lung cancer cell lines.

2 MATERIALS AND METHODS

2.1 MATERIALS

AntiKRAS^{G12V} mAb was acquired from bioGenes GmbH (Germany). [Section with copyright protection]. Fetal bovine serum (FBS) was provided by Gibco (Thermo Fisher Scientific, United States). Methanol-D4 (CD₃OD), deuteration degree 99.8%, polyvinylidene difluoride (PVDF) protein blotting membrane and antiKRAS mAb produced in mice acquired from clone 3B10-2F2 were provided by Merck (Darmstadt, Germany). Resazurin sodium salt, Triton X-100, Accutase®, [Section with copyright protection], HEPES buffer, deuterium oxide (D₂O) and dimethyl sulfoxide-d6 (DMSO-d6) were obtained from Sigma-Aldrich (Darmstadt, Germany). [Section with copyright protection]. The 96 multiwells plate, SnakeSkin™ Dialysis Tubing (regenerated cellulose, SnakeSkin 7K MWCO, 22 mm), anti-mouse IgG DyLight™ 680 antibody produced in goat, pierce Ip lysis buffer were provided by Thermo Fisher Scientific (United States). The truncated LyP1 peptide (aminoacidic sequence: CGNKRTR) was synthesized by ChinaPeptides Co., Ltd. (Shanghai, China). SYPRO® Ruby was purchased from Lonza (Basel, Switzerland). Protein G PLUS-agarose beads was acquired from Santa Cruz Biotechnologies (United States).

2.2 SYNTHESIS OF tLYP1-TARGETED CONJUGATES

2.2.1 Polymer-tLyP1

[Section with copyright protection]. The product was purified by dialysis against EtOH:NaCl 150 mM (50 %, v/v EtOH/ultrapure water). The resulting polymer-Mal conjugate was frozen at -80°C and freeze-dried (Genesis™ 25 EL, S.P Industries, PA, USA).

[Section with copyright protection]. The resulting conjugate was purified by dialysis against EtOH:NaCl 150 mM (60%, v/v) and ultrapure water. Afterward, the polymer-tLyP1 conjugate was frozen at -80°C, freeze-dried, and stored at -20°C.

2.2.2 Surfactant.PEG-tLyP1

Stock solutions of surfactant-PEG-Mal (40 mg/mL) and tLyP1 (1 mg/mL) in HEPES buffer 10 mM (60% (v/v) EtOH in ultrapure water, pH 7.2) were prepared. The peptide was added to the surfactant.PEG-Mal at a molar ratio of 2:1 tLyP1: surfactant.PEG-Mal and let it stir at RT overnight. Afterward, the free tLyP1 was removed by dialyzing against 150 mM NaCl (2h) and then, two more cycles against ultrapure water. Finally, the functionalized lipid was freeze-dried, and stored at -20°C.

2.2.3 ¹H-NMR spectroscopy

¹H-NMR spectra were recorded using a Bruker DRX-500 or NEO-750 spectrometer. [Section with copyright protection]. When required, trimethylsilylpropanoic acid was used as an internal reference. ¹H-NMR spectra were recorded at 300 K, 64 to 2048 accumulated scans and a relaxation delay of 8-12 s Spectral analysis was done by using the MestreNova Software (Mestrelab Research S.L.), and the extent of the conjugation was calculated as the number of

double bond of the maleimide or tLYP1 molecules per 100 carboxylic acid groups of polymer or methyl groups from the surfactant. PEG₂₀₀₀.

2.3 PREPARATION OF tLYP-1 TARGETED POLYMER-BASED NANOASSEMBLIES (tLYP1-HANAs)

[Section with copyright protection].

2.4 PREPARATION OF tLYP-1 TARGETED PEGYLATED POLYMER-BASED NANOASSEMBLIES (PEGtLYP1-HANAs)

[Section with copyright protection].

2.5 PHYSICOCHEMICAL AND MORPHOLOGICAL CHARACTERIZATION

The physicochemical characteristics of the HANAs were determined by using a Malvern Zeta-Sizer (NanoZS, ZEN 3600, Malvern Instruments, Worcestershire, United Kingdom). For the analysis, HANAs were accordingly diluted in PBS and particle size, PDI and derived count rate (DCR) were determined using Dynamic Light Scattering (DLS). By using the same device, the zeta potential was recorded by Laser Doppler Anemometry (LDA) after measuring the mean electrophoretic mobility. For a further physicochemical characterization based on particle size and HANAs concentration, samples were analyzed by Nanoparticle Tracking Analysis (NTA) after diluting the samples 10000 times in PBS (NanoSight NS3000, Amesbury, United Kingdom).

2D-cryogenic transmission electron microscopy (cryo-TEM) micrographs were collected on a 120 kV TEM equipped with an UltraScan 4000 SP cooled slow-scan CCD camera (GATAN) at x30,000 magnification. High quality data were collected on a JEM-2200FS/CR (JEOL, Ltd.) 200 kV TEM equipped with a K2 Summit direct detection camera (GATAN) at x30,000 magnification. To do so, sample preparation was performed using the Vitrobot Mark IV, applying 4 µL of HANAs (at 1:1 concentration) on a copper mesh. Vitrification was done in 70-80% humidity at RT. For quality control data was acquired on a JEM-1230 (JEOL, Ltd.)

2.6 COLLOIDAL STABILITY

The colloidal stability of the HANAs was determined based on their physicochemical properties in simulated biological fluids and in suspension at 4°C.

The stability in simulated biological fluids was evaluated upon incubation in PBS supplemented with 10% FBS after diluting the samples 10 times in the media. AntiKRAS^{G12V} mAb-loaded HANAs were incubated at 37°C under orbital shaking at 300 rpm and, at different time points (0, 2, 4, 8, 24 hours), 100 µL of the samples were withdrawn and both, particle size based on highest intensity peak and PDI were measured by DLS. As control, the HANAs were incubated in PBS and, the mean particle size and PDI were evaluated.

The stability of antiKRAS^{G12V} mAb -loaded HANAs in suspension at 4°C was also evaluated. At different time points (0, 7, 14, 12, and 24 weeks), the particle size, PDI and zeta potential were determined as described in section 2.5.

2.7 QUANTIFICATION OF ANTIKRAS^{G12V} MAb ASSOCIATION EFFICIENCY (AE) AND LOADING CAPACITY (LC) BY USING SODIUM DODECYL-SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

The amount of associated antiKRAS^{G12V} mAb was determined by SDS-PAGE for separation under reducing conditions. HANAs were isolated by ultracentrifugation following the previously described conditions (i.e., 35000 rpm – 1.5 hour – 15 °C). Then, the amount of non-associated antiKRAS^{G12V} mAb present in the supernatant was quantified. Briefly, the supernatant was diluted accordingly to fit in the calibration curve. AntiKRAS^{G12V} mAb standard solutions of known concentrations (from 2 to 0.2 µg/lane) were prepared in PBS. Treated samples were diluted in a volume ratio 1:1 with 2X Laemmli buffer and vortexed for several seconds. Then, samples were boiled at 99.5 °C for 5 minutes. 20 µL of the denatured samples were resolved using stacking and resolving polyacrylamide gels of 4 and 12 %, respectively, in the presence of 0.1 % SDS in the running buffer. After 1 hour with variable milliamps and a voltage ranging from 100 to 120 V, the gel was fixed in 10% methanol and 7% acetic acid for 45 min. Finally, samples were stained using a SYPRO® Ruby protein gel stain solution overnight at RT in head-to-tail rotation. Prior to analysis, the gel was washed twice with ultrapure water.

GelAnalyzer 19.1 software (www.gelanalyzer.com) by Istvan Lazar Jr., PhD and Istvan Lazar Sr., PhD, was used to process and analyze the samples. Total bands areas of the calibration curve were referred to the theoretical value and those ranging between 80 and 120% were considered. Samples' bands intensities referred to the calibration curve. The AE and LC were determined as follows:

$$AE (\%) = [1 - (\text{mass of free antiKRAS}^{G12V} \text{ mAb}) / \text{theoretical mass of antiKRAS}^{G12V} \text{ mAb}] \times 100$$

$$LC (\%) = [\text{mass of associated antiKRAS}^{G12V} \text{ mAb} / \text{total theoretical mass of the HANAs}] \times 100$$

2.8 ANTIKRAS^{G12V} MAb RELEASE PROFILE

AntiKRAS^{G12V} mAb released from the HANAs was quantified using SDS-PAGE. Thus, 60 µL of the samples were diluted in 540 µL PBS at pH 7.4 and incubated at 37°C under orbital agitation (300 rpm). At different time points (0, 2, 4, 8, 24 and 168 hours), the formulation was isolated by ultracentrifugation (35000 rpm – 1.5 hour – 15°C). The free antiKRAS^{G12V} mAb in the supernatant was recovered and quantified by using the below equation where t_0 indicates the condition prior incubation, and t_1 corresponds to the condition at the time point of study.

$$\text{Cumulative antiKRAS}^{\text{G12V}} \text{ mAb released (\%)} = \frac{\text{free antiKRAS}^{\text{G12V}} \text{ mAb at } t_1 - \text{free antiKRAS}^{\text{G12V}} \text{ mAb at } t_0}{\text{antiKRAS}^{\text{G12V}} \text{ mAb at } t_0}$$

2.9 CYTOTOXICITY STUDIES

The lung cancer human H441, NCI-H1568 and murine cell lines A549 and CMT167 were harvested in a 96-well plate at densities of 25×10^2 cell/well for the CMT167 cell line and 5×10^3 cell/well for the rest. Cells were incubated in 100 μL /well DMEM supplemented with a penicillin/streptomycin solution and 10% of FBS at 37 °C. After 20 hours of incubation, increasing concentrations of HANAs were resuspended in DMEM (5% FBS final concentration). Then, cells were cultured for 24 hours at 37 °C and 100 μL /well of the reactive resazurin, previously diluted in DMEM-10%FBS, were added. To evaluate the reduction of resazurin to resorufin as indicator of the mitochondrial respiratory chain in live cells, the resazurin was allowed to react during 40 min at 37°C. Thus, resorufin levels were recorded at 544-590 nm by using a microplate reader (Synergy H4, BioTek, VT, USA). As positive control, cells were incubated in supplemented cell culture medium and considered as 100% of viability. Treated cells with Triton X-100 (0.5%, v/v in culture medium) were used as negative control, 0% of viability. Cell viability was calculated after subtracting negative control values to crude fluorescence values as follows:

$$\text{Cell viability (\%)} = \frac{[(\text{sample fluorescence} - \text{negative control fluorescence}) / \text{positive control fluorescence}] \times 100}{1}$$

2.10 ANTIKRAS^{G12V} MAb ANTIPROLIFERATIVE ACTIVITY

Cell proliferation studies were performed in the human cell lines H441 and NCI-H1568, and the murine cell line CMT167. Thus, an initial cell density of 5×10^3 cells/well was seeded on a 24-well plate by using DMEM supplemented with a penicillin/streptomycin solution and 5% of FBS as medium. Cells were treated with free antiKRAS^{G12V} mAb and antiKRAS^{G12V} mAb-loaded HANAs at a 50 nM antiKRAS^{G12V} mAb concentration on days 1, 3 and 5 at 37°C. Cells were exposed to blank HANAs at doses equivalent to the antiKRAS^{G12V} mAb. At days 1, 3, 5 and 7 an alamarBlue staining assay was performed. Thus, the medium was discarded and 400 μL /well of resazurin diluted in medium was added at the indicated time points. Resazurin was allowed to react for 40 minutes at 37°C. Afterward, the medium was transferred to a 96-well plate and fluorescent levels were determined at 544-590 nm by using a microplate reader (Synergy H4, BioTek, VT, USA). Cells incubated in culture medium supplemented with 5% FBS was considered 100% viability. Aiming to compare cells growth over time, reads were referred to the cell viability at day 1 and considered 100%. When needed, reads were scaled up to a negative control well, Triton X-100 (0.5%, v/v in culture medium). The reduction of the cell proliferation at day 7 (%) was calculated by dividing the viability after the treatment with antiKRAS^{G12V} mAb-loaded HANAs by the viability after the treatment with the free antiKRAS^{G12V} mAb.

Regarding the dose-response assay, the above method was adapted. Thus, the CMT167 cell line was exposed to the antiKRAS^{G12V} mAb-loaded HANAs at antiKRAS^{G12V} mAb concentrations

of 50, 100, and 200 nM on days 1 and 3 at 37°C. Equivalent doses of blank NAs were used as control. Cell viability was determined at 1, 3, and 5 days.

2.11 COLONY FORMATION

The colony formation assay was performed by treating the CMT167 cells with the antiKRAS^{G12V} mAb-loaded PEGtLyP1-HANAs at a 100 nM antiKRAS^{G12V} mAb concentration. After 48 hours, the cells were harvested and reseeded at 2×10^2 cells/well in a 6-well plate. Cells were treated with equivalent doses of blank PEGtLyP1-HANAs and used as control. The number of colonies was determined after 7 days of incubation. Prior to it, cells were washed with PBS, fixed, and stained with 0.05% crystal violet. Colonies were counted and analyzed with ImageJ software [30].

2.12 ANTIKRAS^{G12V} MAb INTRACELLULAR STAINING

The lung murine cell line CMT167 was seeded at densities of 25×10^3 cells/well in a 24-well plate. The selected cell culture medium was DMEM supplemented with a penicillin/streptomycin solution and 10% FBS. When confluent, antiKRAS^{G12V} mAb-loaded HANAs were diluted in DMEM (5% FBS final concentration) at a final antiKRAS^{G12V} mAb concentration of 1 μ M. Equivalent antiKRAS^{G12V} mAb and blank NAs doses were added. After 4 h of incubation at 37°C and 4°C, cells were rinsed 3 times with cold PBS and let it incubate with 100 μ L/well of Accutase® at 37°C for maximum 40 min. Once detached, cell suspension was collected in FACS tubes and washed with cold 3 mL of FACS washing buffer. Cells were centrifuged (1500 rpm, 5 min, 4 °C) and after discarding the supernatant, fixed with 100 μ L of paraformaldehyde (4%). After incubation for at least 30 min, tubes were washed and centrifuged following the above conditions. Finally, cells were incubated with 100 μ L/tube of blocking and permeabilization buffer at 4°C for 1 h.

For antiKRAS^{G12V} mAb quantification, a PE-Ab was diluted in blocking and permeabilization buffer. Then, 1 μ L per FACS tube (1:100 dilution) was added and incubated at 4°C for at least 45 min. Treated cells were washed with 3 mL of FACS washing buffer and centrifuged (1200 rpm for 5 min). Supernatant was discarded and cells pellet were resuspended in 200 μ L PBS. Finally, cells were analyzed by flow cytometry in a FACSCalibur instrument (BD Biosciences, Franklin Lakes, NJ, USA). Percentage of intracellular antiKRAS^{G12V} mAb was determined by the integrated mean fluorescence intensity (iMFI) calculated based on the % of positive cells with the MFI of that population.

2.13 TARGET ENGAGEMENT

Target engagement studies were performed in the lung murine CMT167 cell line at densities of 15×10^4 cells/well in a 6-well plate. The culture media of selection was DMEM supplemented with a penicillin/streptomycin solution and 10% FBS. Cells were treated with HANAs at 50 nM of mAb. After 24, 48, or 72 h, cells were washed twice with PBS, and centrifuged at 1500 rpm 5min. Then, the cell pellet was incubated with lysis buffer on ice for 2 h. Cell lysates were

centrifuged at 140000 rpm for 15 min at 4 °C to remove nuclei and cell debris. Afterward, the supernatant was collected, and protein content was quantified by the bicinchoninic acid assay. To pull down the mAb, 1 mg of total protein (carrying a theoretical amount of 20 µg of mAb) was immunoprecipitated for 1 h with 40 µL of Protein G PLUS-agarose beads at 4 °C in head-to-tail rotation. For positive target engagement control, 1 mg of CMT 167 cell lysate was incubated for 4 h at 4 °C (head-to-tail rotation) with 5 µg of free antiKRAS^{G12V} mAb and then immunoprecipitated as previously mentioned.

Samples were loaded in 12 %-acrylamide PAGE gels and transferred to a PVDF protein blotting membrane. Membranes were incubated with antiKRAS mAb produced in mice followed by an anti-mouse IgG DyLight™ 680 antibody produced in goat. Finally, membranes were scanned using the LI-COR Odyssey Scanner system, and band intensities were quantified with LI-COR Image Studio™ Lite Quantification Software (LI-COR Biosciences, Lincoln, NE, USA).

2.14 STATISTICAL ANALYSIS

The statistical analysis was performed by fixing the confidence level at 0.05 and significant differences were considered for *[#]p<0.05, **^{##}p<0.01, ***p<0.001, and ****p<0.0001. GraphPad Prism version 9.3.0 was the software of selection for the data analysis. Gaussian distribution was defined by following a Shapiro-Wilk test.

Statistical analysis of the evolution of the particle size in PBS or in PBS-10%FBS compared with the control (particle size prior incubation) was done following an unpaired t-test. In contrast, the evolution of the particle size, PDI and zeta potential upon storage at 4°C was evaluated by using a Mann-Whitney t-test, except for the PDI of the PEGtLyP1-HANAs, which was analyzed following an unpaired t-test.

To analyze the differences between the release profiles at each time point, the Mann-Whitney t-test was applied.

3 RESULTS AND DISCUSSION

The number of reported approaches exploiting the use of nanotechnology for the intracellular delivery of mAbs is scarce [20,23,24,31–33], being liposomes the most explored strategy so far [31,33]. The features of these delivery systems are diverse, however, a limitation to all of them is their unknown stability in biologically relevant media or access and penetration across the tumor tissue. Therefore, our work was aimed to overcome the several extracellular and intracellular barriers faced by the NPs in order to block the KRAS^{G12V} oncoprotein. To do so, we have adapted the HANAs technology ([Section with copyright protection]) described in Chapter 1 for the intracellular delivery of the antiKRAS^{G12V} mAb. Moreover, we functionalized the HANAs with the tLyP1 peptide and studied *in vitro* the capacity to deliver the mAb intracellularly.

[Section with copyright protection].

3.1 DEVELOPMENT AND CHARACTERIZATION OF ANTIKRAS^{G12V} mAb-LOADED tLYP1-TARGETED HANAS

3.1.1 Development and characterization of antiKRAS^{G12V} mAb-loaded tLyP1-polymer HANAs (tLyP1-HANAs)

[Section with copyright protection]. By adjusting the proportion of components, we could achieve a polymer-tLyP1 degree of substitution (DS) of $2.1 \% \pm 0.9$ ($n \geq 3$) (¹H-NMR spectra in Chapter 1). The DS was selected based on our previous work on the functionalization of HA-based nanocapsules carrying docetaxel [34].

[Section with copyright protection]. Finally, the prototype initially composed by polymer-tLyP1 0.25, surfactant 1, antiKRAS^{G12V} mAb 0.5 (mg/mL) was selected for further studies, from now on designated as tLyP1-HANAs, due to the small size (~130 nm) and acceptable AE (~67 %).

3.1.2 Development and characterization of antiKRAS^{G12V} mAb-loaded tLyP1-surfactant.PEG₂₀₀₀-tLyP1 HANAs (tLyP1PEG-HANAs)

In order to confer stealth properties to the NPs [35,36] while potentiating their tumor penetration and intratumoral diffusion [25,29], surfactant.PEG was chosen and further functionalized with the tLyP1 peptide. Thus, the maleimide group of the PEGylated lipid was anchored to the peptide through the thiol group. [Section with copyright protection].

[Section with copyright protection]. Finally, the acceptable physicochemical properties and AEs, and aiming to at least reproduce the conditions studied in the biodistribution of Chapter 2, led us to the selection of the HANA with the lowest amount of surfactant.PEG₂₀₀₀-tLyP1 (i.e., 0.01 mg/mL) and so-called antiKRAS^{G12V} mAb PEGtLyP1-HANAs.

Overall, the developed formulations were concentrated 6.4 times (i.e., final antiKRAS^{G12V} mAb concentration of 3.2 mg/mL, Table 1) to accomplish the *in vivo* dose requirements of the

antiKRAS^{G12V} mAb (data not published) and further characterized in terms of physicochemical properties, AE, and LC. As control, blank HANAs were produced (see Supplementary Table 1).

Table 1. Composition of the antiKRAS^{G12V} mAb tLyP1-HANAs and PEGtLyP1-HANAs before (initial) and after (final) 6.4 fold concentration. #tLyP1-targeted material

Prototype	Initial composition [mg/mL]				Final composition [mg/mL]			
	Polymer	Surfactant	Surfactant. PEG	antiKRAS ^{G12V} mAb	Polymer	Surfactant	Surfactant. PEG	antiKRAS ^{G12V} mAb
tLyP1-HANAs	0.25 [#]	1	N.A.	0.5	1.6 [#]	6.4	N.A.	3.2
PEGtLyP1-HANAs	0.25	1	0.01 [#]	0.5	1.6	6.4	0.064 [#]	3.2

As depicted in Table 2, antiKRAS^{G12V} mAb tLyP1-HANAs and antiKRAS^{G12V} mAb PEGtLyP1-HANAs presented a particle size close to 140 nm, PDI<0.25 and neutral to negative surface charge by DLS/LDA analysis. In good agreement, mean values acquired by NTA confirmed the particle size of antiKRAS^{G12V} mAb tLyP1-HANAs. In contrast, NTA analysis of antiKRAS^{G12V} mAb PEGtLyP1-HANAs indicated a smaller particle size than DLS data, observation that is in consonance with the NTA accuracy for the characterization of heterogeneous populations [37]. This finding was further observed in the NTA histogram by intensity (Supplementary Figure 1). Despite this, final candidates fit a particle size close to 130 nm, PDI<0.3, and neutral to negative surface charge.

The AE values of the antiKRAS^{G12V} mAb, for both selected prototypes were greater than 60%. More importantly, LC values were as high as ~18.7%. Contrary to a previous report [38], where increasing molar % of PEG induces the reduction of the AE, the use of surfactant-PEG did not influence the physicochemical properties or LC of the HANAs. This may be related to the low proportion that the surfactant.PEG-tLyP1 represents over the total system (mass ratio surfactant.PEG-tLyP1:total components of 0.01:1). [Section with copyright protection].

Table 2. Physicochemical properties by DLS and NTA, association efficiency (AE, %) and loading capacity (LC, %) of antiKRAS^{G12V} mAb tLyP1-HANAs and PEGtLyP1-HANAs after concentrate 6.4 times. #tLyP1 targeted material. Data are expressed as mean ± SD, n≥3. Statistical differences regarding the particle size by DLS and AE of antiKRAS^{G12V} mAb tLyP1-HANAs and antiKRAS^{G12V} mAb PEGtLyP1-HANAs were analyzed by using an unpaired t-test, whereas particle size by DLS and NTA was done following an unpaired t-test (antiKRAS^{G12V} mAb tLyP1-HANAs) and Mann-Whitney (antiKRAS^{G12V} mAb PEGtLyP1-HANAs).

Prototype	Particle size (nm) (DLS)	PDI	Particle size (nm) (NTA)	Concentration (×10 ¹⁰ particles/mL)	Zeta potential (mV)	AE (%)	LC (%)
tLyP1-HANAs	141 ± 7	0.20	136 ± 20	459 ± 50	-11 ± 2	66 ± 9	18.7
PEGtLyP1-HANAs	142 ± 11	0.23	122 ± 8**	396 ± 131	-11 ± 2	64 ± 6	18.2

3.2 MORPHOLOGICAL CHARACTERIZATION

[Section with copyright protection].

3.3 COLLOIDAL STABILITY

3.3.1 Stability examination in simulated biological media

We studied the colloidal stability of selected HANA prototypes upon incubation at 37°C in a relevant biological media (i.e., PBS supplemented with 10% of FBS). The analysis of the particle size evolution in supplemented media was done based on the mean peak by intensity since some interferences coming from the FBS were observed, in accordance with other reports [39,40]. As shown in Figure 1 and despite the significant variations suffered, the particle size of tLyP1-HANAs was found to be stable upon incubation in biorelevant media for up to 8 hours. [Section with copyright protection].

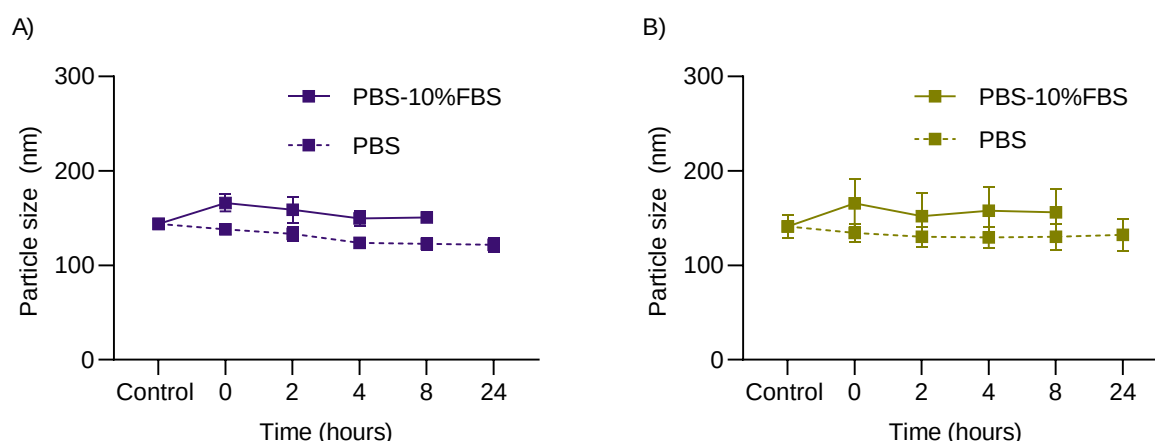


Figure 1. Stability of antiKRAS^{G12V} mAb tLyP1-HANAs (A) and antiKRAS^{G12V} mAb PEGtLyP1-HANAs (B) upon incubation in relevant biological media at 37°C. Mean particle size in PBS and particle size by intensity in PBS-10%FBS were recorded overtime. The control indicates the particle size prior incubation. Values represent mean $\pm$ SD, n=3.

3.3.2 Stability in storage conditions

Performing preliminary storage stability studies is critical to ensure the adequate use of the prototypes and to anticipate stability issues in the potential preclinical development of the prototypes. Therefore, the stability in suspension at 4°C of the HANAs in terms of particle size, PDI and Zeta potential was explored overtime. Results presented in Figure 2 show the great long-term stability in terms of particle size, which was found to be of at least 24 weeks. Regarding the surface charge, although significant variations were observed after 4 and 12 weeks upon storage for tLyP1-HANAs, minor modifications were exhibited after 24 weeks. Besides, the PDI did not experience any modification overtime. Consequently, it could be confirmed the HANAs stability for at least 6 months.

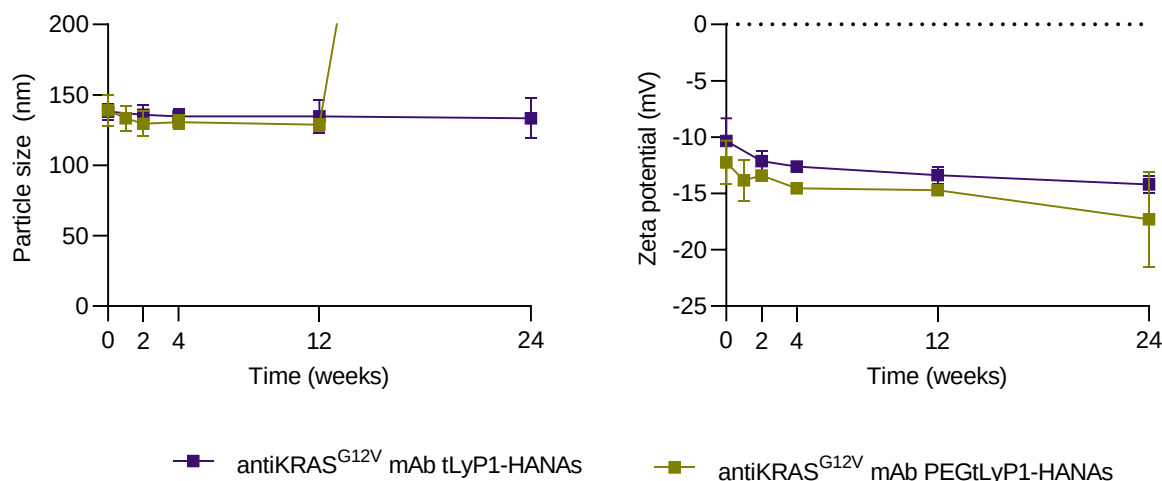


Figure 2. Stability in suspension of antiKRAS^{G12V} mAb tLyP1-HANAs and antiKRAS^{G12V} mAb PEGtLyP1-HANAs at 4°C. Particle size and zeta potential were monitored overtime. Data are expressed as mean $\pm$ SD ($n \geq 3$), except for antiKRAS^{G12V} mAb PEGtLyP1-HANAs at 12 and 24 weeks, $n=2$.

3.4 RELEASE PROFILE

The quantification of the antiKRAS^{G12V} mAb released from the HANAs was studied after incubation in PBS pH 7.4 at 37°C. The results in Figure 3 indicate that both prototypes, independently of the functionalization approach show the same release profile, characterized by a very slow release, reaching approximately 26% after 1 week of incubation. The lack of burst release is a consequence of the important interaction of antiKRAS^{G12V} mAb with the components of the HANAs.

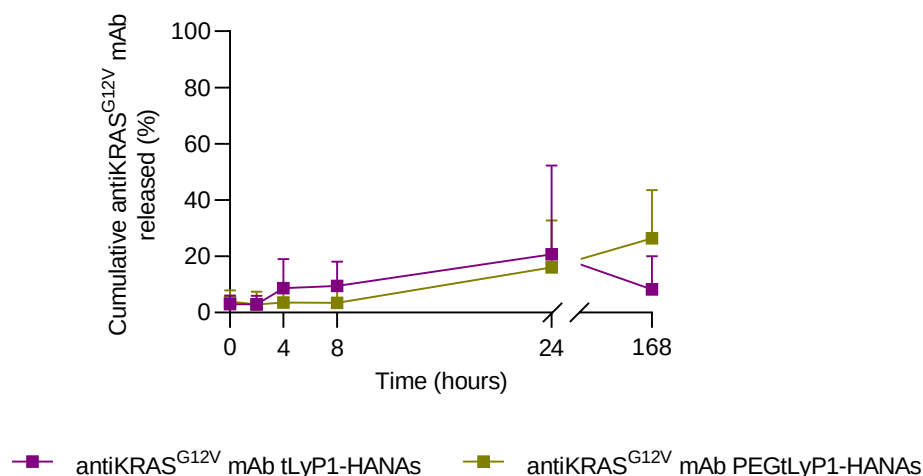


Figure 3. Cumulative % of released antiKRAS^{G12V} mAb from antiKRAS^{G12V} mAb tLyP1-HANAs and antiKRAS^{G12V} mAb PEGtLyP1-HANAs quantified by SDS-PAGE. HANAs were incubated in PBS pH 7.4 at 37°C for up to 1 week. Data are expressed as mean $\pm$ SD, $n=3$.

Based on these data, it could be expected that the antiKRAS^{G12V} mAb molecules would not be prematurely released during their circulation in plasma and subsequent biodistribution. [Section with copyright protection].

3.5 IN VITRO CYTOTOXICITY OF HANAS

We evaluated the toxicity of blank and antiKRAS^{G12V}-loaded tLyP1-HANAs and PEGtLyP1-HANAs, across a panel of four 2D cell lines: NCI-H1568 (wild-type), A549 (KRAS^{G12S}-mutant), CMT167 and H441 (KRAS^{G12V}-mutants). To allow conclusion to be drawn, a cell viability reduction of 30% was considered toxic, in accordance with the ISO 10993-5 standards [41]. Results presented in Figure 4 revealed a low toxicity profile for blank HANAs after 24 hours of treatment, irrespective of the cell line.

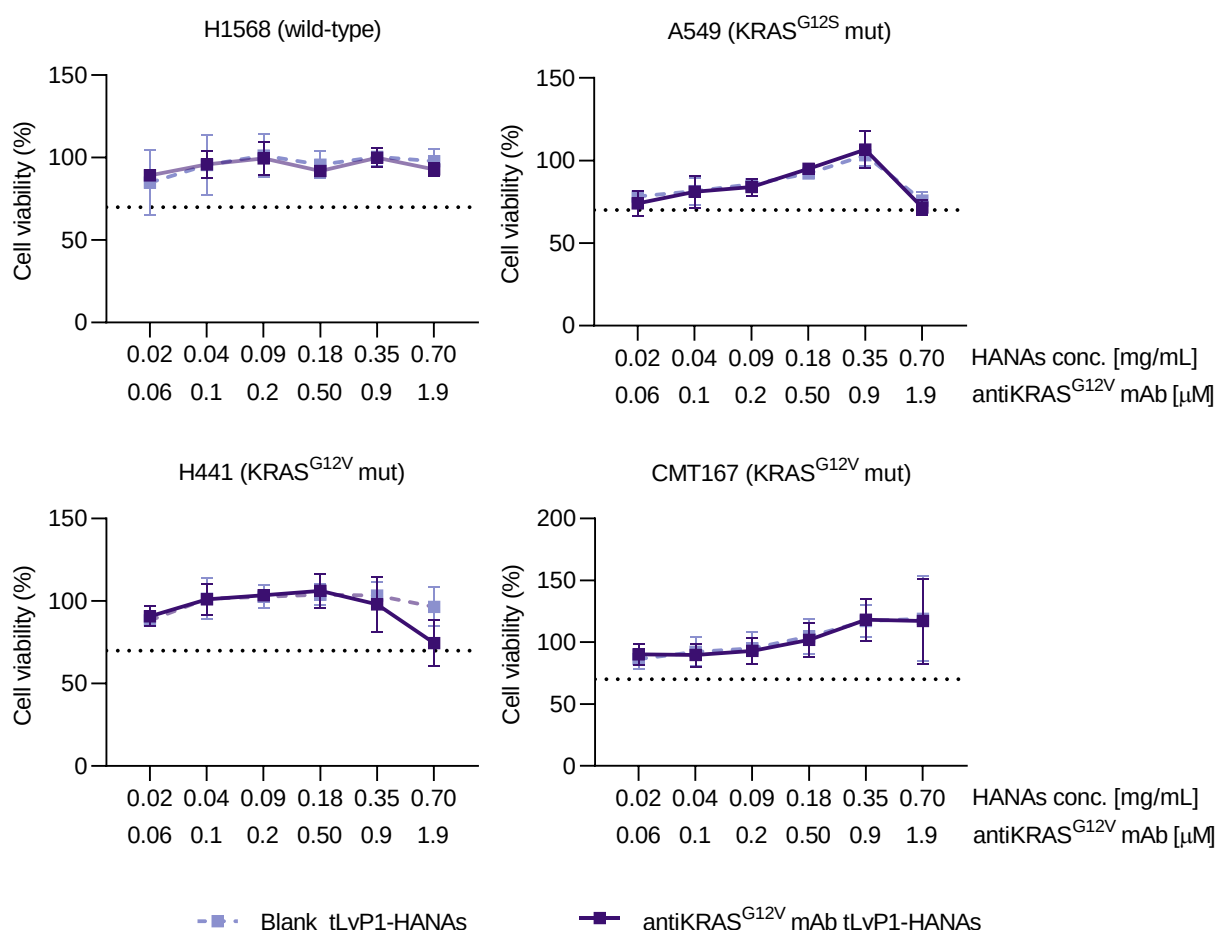


Figure 4. Metabolic activity of tLyP1-HANAs in several lung cancer cell lines after 24 of exposure. Cells were exposed to increasing concentrations of blank and antiKRAS^{G12V} mAb tLyP1-HANAs. Cell viability values below 70% were considered toxic (dashed lines). Data are expressed as mean ± SD, n ≥ 3. Conc. concentration

A different tendency was noted for antiKRAS^{G12V}-loaded tLyP1-HANAs and PEGtLyP1-HANAs (Figure 5). While a low toxicity profile was observed in the non KRAS^{G12V}-mutant cell lines, even at the highest concentrations, an obvious fall of the viability was detected in the H441 cell line (KRAS^{G12V}-mutant). In this case, the mismatched viability was greater for antiKRAS^{G12V} mAb PEGtLyP1-HANAs. Contrary to what was expected, no effect was observed in the other KRAS^{G12V}-mutant, CMT167. Overall, these results suggest a specific cell line-dependent activity triggered by the intracellular delivery of the mAb, in accordance with previous reports [42,43].

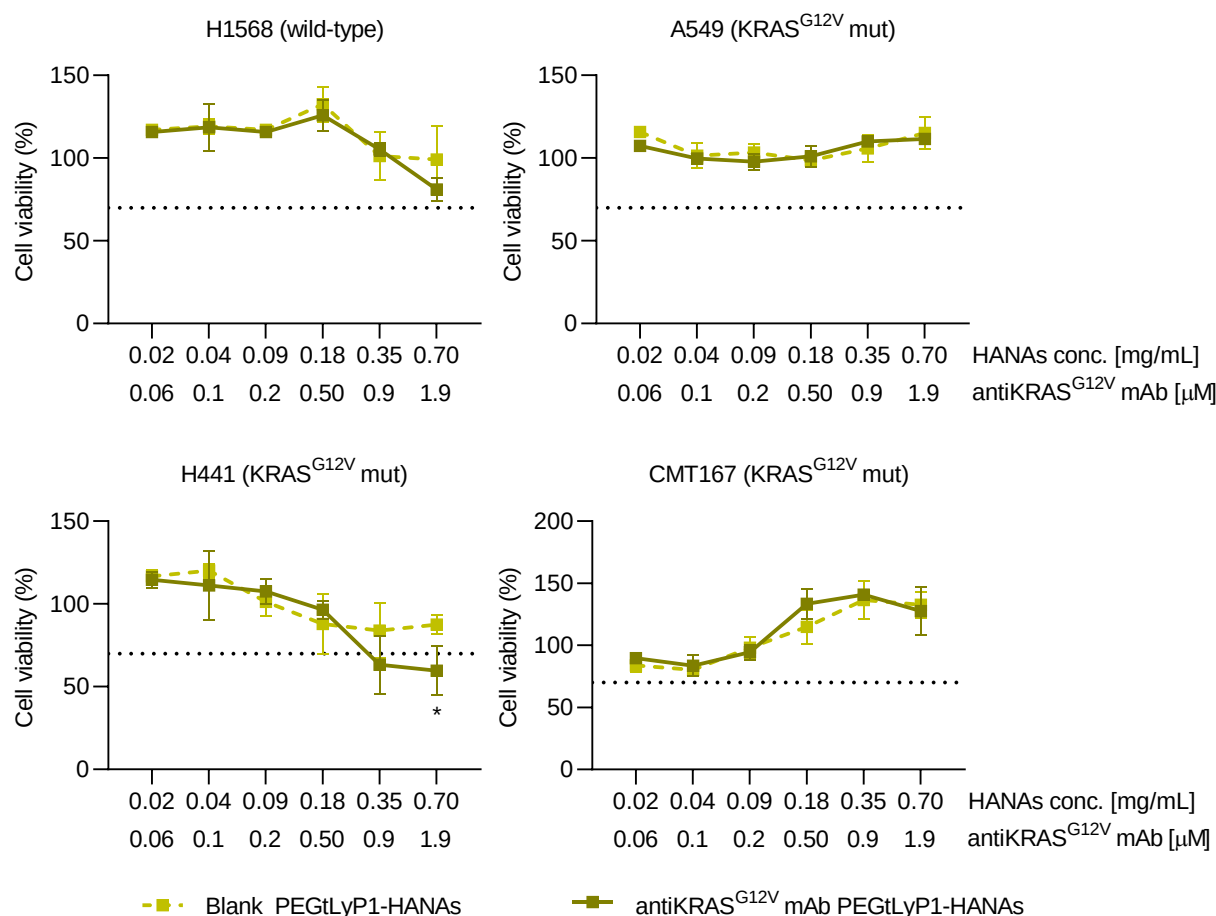


Figure 5. Metabolic activity of PEGtLyP1-HANAs in several lung cancer cell lines after 24 of exposure. Cells were exposed to increasing concentrations of blank and antiKRAS^{G12V} mAb PEGtLyP1-HANAs. Statistical analysis was done by using an unpaired t-test for comparison between blank and antiKRAS^{G12V} mAb PEGtLyP1-HANAs in the H411 cell line. Cell viability values below 70% were considered toxic (dashed lines). Data are expressed as mean $\pm$ SD, $n \geq 3$. Conc. Concentration.

These results confirmed the low cytotoxicity of the HANAs and allowed the selection of the most suitable HANAs concentration for further studies. Therefore, concentrations below 0.7 mg/mL of HANAs, which corresponds to 1.9 μ M of antiKRAS^{G12V} mAb, were established.

3.6 FUNCTIONAL EVALUATION OF ANTIKRAS^{G12V} mAb-LOADED HANAS

3.6.1 Proliferation studies

It is well known that RAS mutations are implicated on cell proliferation [3,5], outcome advocated by the persistent activation of the RAS protein. As far as we are aware, there is no available work aiming to switch off the GTP-bound active state through the intracellular delivery of mAbs in the context of nanotechnology. Consequently, to initially estimate the breadth of inhibition of the antiKRAS^{G12V} mAb, a dose-response proliferation study was performed for up to 5 days in the KRAS^{G12V}-mutant cell line, H441. Thus, increasing concentrations of blank and antiKRAS^{G12V} mAb tLyP1-HANAs were tested. Since no dose-response was observed (Supplementary Figure 2), an antiKRAS^{G12V} mAb dose of 50 nM was settled for further studies.

To study the potential of antiKRAS^{G12V} mAb-loaded HANAs to interfere on cell proliferation, the cell viability of tLyP1-HANAs and PEGtLyP1-HANAs was evaluated for longer periods. Consequently, when the activity of the antiKRAS^{G12V} mAb tLyP1-HANAs was tested in the KRAS^{G12V}-mutant cell lines (H441 and CMT167), a suppression of the proliferation of 9.4% and 35.8% was observed, respectively (Figure 6).

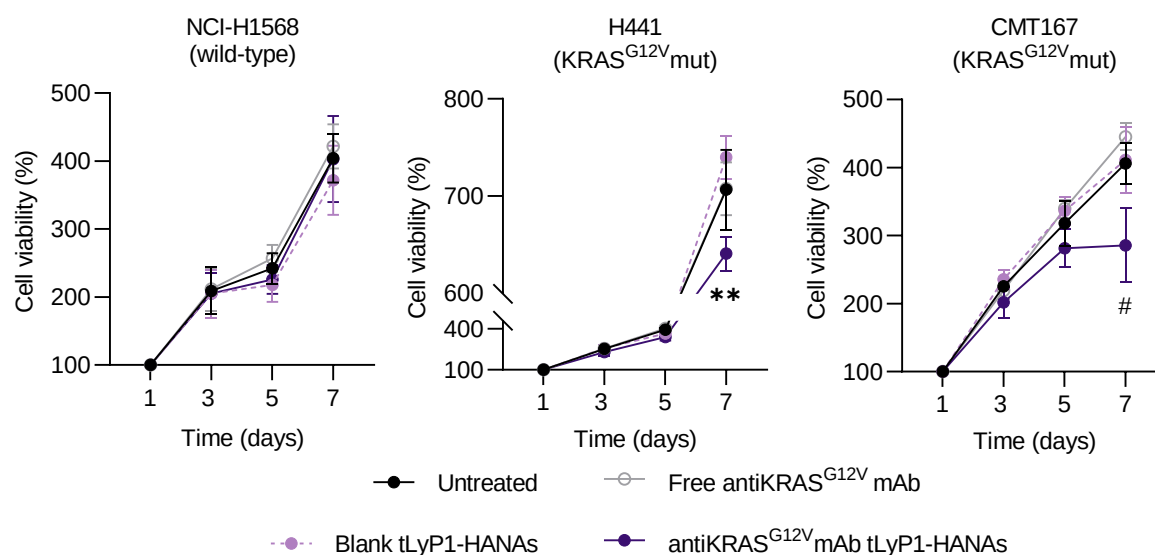


Figure 6. AntiKRAS^{G12V} mAb tLyP1-HANAs impaired G12V mutated lung cancer cell proliferation. Lung cancer cell line were incubated for 7 days with culture medium (untreated), free mAb, blank, and antiKRAS^{G12V} mAb tLyP1-HANAs. Data are expressed as mean $\pm$ SEM, $n \geq 3$. Statistical differences were analyzed by multiple t-test at each time point between groups. Significant differences between the antiKRAS^{G12V} mAb tLyP1-HANAs and the untreated group were not plotted. Differences among the free antiKRAS^{G12V} mAb or blank tLyP1-HANAs and antiKRAS^{G12V} mAb tLyP1-HANAs were indicated as # and *, respectively.

In agreement with the above results, PEGtLyP1-HANAs demonstrated a similar delay on cell proliferation (Figure 7). In this case, the extent of the inhibition was also greater on the CMT167 cell line (27.3% vs. 18.9% in H441), with the differences being significant even on day 5 of treatment.

The lack of activity displayed with controls (untreated cells, free mAb, or blank HANAs) or in the wild-type cell line confirmed the specific activity ascribe to the antiKRAS^{G12V} mAb triggered by the HANAs.

Although the extent of the inhibition varies among prototypes and cell lines, the above results are in line with other publications on the *in vitro* evaluation of KRAS siRNA-based therapeutics [44,45]. The superior rate of inhibition observed in the CMT167 cell line could be related to a higher uptake of the HANAs as a consequence of a greater expression of the tLyP1 receptor, NRP-1 [25,29] in this cell line. Likewise, the development of intrinsic and acquired resistance mechanisms [12,46] in the H441 cell line may also be responsible for the greater efficacy observed in the CMT167 cell line. This observation has been previously described in other KRAS-mutant cell lines, in which KRAS knockdown led to the activation of the downstream pathways, ERK and AKT [43,47].

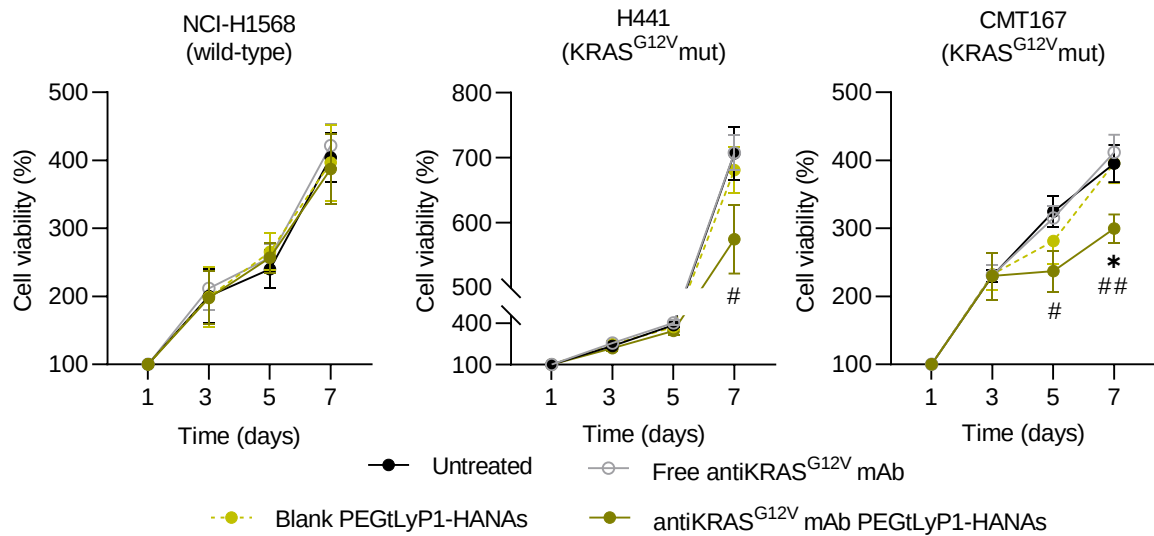


Figure 7. AntiKRAS^{G12V} mAb-loaded PEGtLyP1-HANAs impaired G12V mutated lung cancer cell proliferation. Lung cancer cell line were incubated for 7 days with culture medium (untreated), free antiKRAS^{G12V} mAb, blank, and antiKRAS^{G12V} mAb PEGtLyP1-HANAs. Data are expressed as mean $\pm$ SEM, $n \geq 3$. Statistical differences were analyzed by multiple t-test at each time point between groups. # and * refers to differences among the free antiKRAS^{G12V} mAb or blank PEGtLyP1-HANAs, and antiKRAS^{G12V} mAb PEGtLyP1-HANAs, respectively. Differences among the antiKRAS^{G12V} mAb PEGtLyP1-HANAs and the untreated group were not plotted.

In brief, the results of this study showed the inhibition of the KRAS-GTP bound state, suggesting the degree of involvement of the mAb in reducing the proliferation after reaching intracellular compartments. Moreover, the results indicate that PEGtLyP1-HANAs displayed greater activity in terms of cell proliferation.

3.6.2 Colony formation

To further prove the long-term oncogenic effects of antiKRAS^{G12V} mAb PEGtLyP1-HANAs, a clonogenic assay was performed. As displayed in Figure 8, the relative degree of inhibition in the oncogenic KRAS^{G12V} mutant cell line CMT167 was 36 (%) $\pm$ 11.

The HANAs technology appears to have a limited capacity to inhibit the formation of colonies, as compared with previously reported siRNA-loaded carriers [13,48]. Nonetheless, the comparison of these two types of therapies is challenging, not only because of their different mechanism of action but also because of their dosage and administration schedules [49,50]. It is our hypothesis that an increment of the dose or frequency of the administrations would allow to obtain a different outcome.

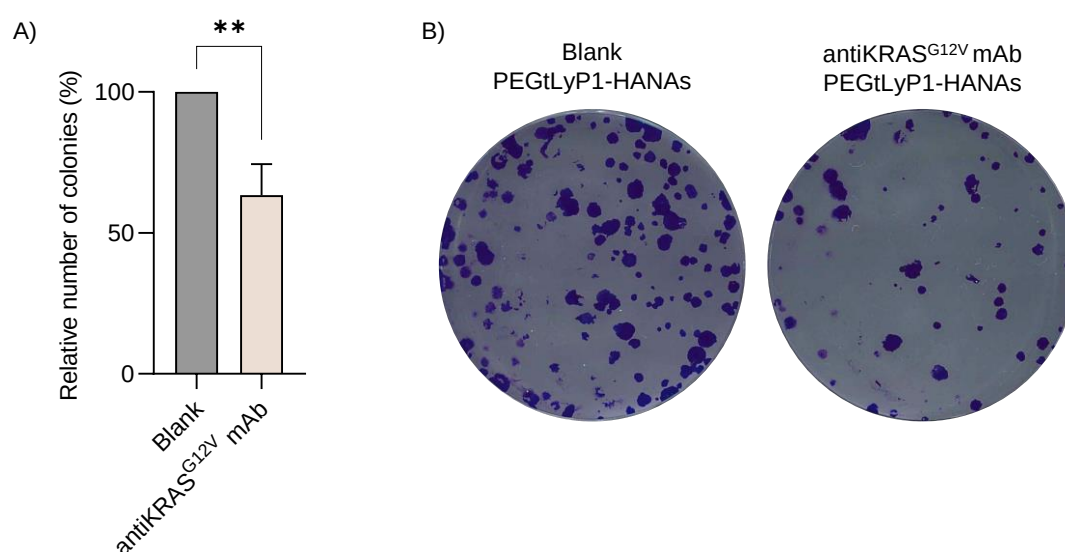


Figure 8. Inhibition of tumor colony formation by antiKRAS^{G12V} mAb PEGtLyP1-HANAs in the G12V mutated CMT167 lung cancer cell line. A) Results are expressed as the percentage of colonies after antiKRAS^{G12V} mAb PEGtLyP1-HANAs treatment (antiKRAS^{G12V} mAb) compared to blank PEGtLyP1-HANAs control group (blank). B) Representative plates of blank and antiKRAS^{G12V} mAb PEGtLyP1-HANAs from colony formation. Statistical differences were determined by Kolmogorov-Smirnov test. Values are expressed as mean $\pm$ SD (n $\geq$ 3).

To sum up, the functional evaluation of the mAb triggered by the HANAs was confirmed. However, experiments related to the HANAs' capacity to reach intracellular domains and interact with its epitope would be required to fully understand their mechanism.

3.7 EVALUATION OF ANTIKRAS^{G12V} mAb tLyP1-HANAS TO TARGET INTRACELLULAR KRAS^{G12V} ONCOPROTEIN

3.7.1 Intracellular detection

Taking into account the main goal of this thesis, which is to achieve the intracellular delivery of functional mAbs, it becomes crucial to investigate the capacity of developed HANA technology to release antiKRAS^{G12V} mAb at the intracellular level.

Therefore, intracellular levels of antiKRAS^{G12V} mAb upon tLyP1-HANAs and PEGtLyP1-HANAs treatment were quantified after 4 h of incubation at 37°C. As controls, blank HANAs, and free mAb were added. To further probe an active intracellular transport mechanism, the uptake was studied at 4°C, aiming to reduce the cell membrane activity [51,52]. As shown in Figure 9, the antiKRAS^{G12V} mAb fluorescence signal detected for PEGtLyP1-HANAs was 1.5 times higher than for tLyP1-HANAs, being this intensity significantly greater than the one observed for the free mAb. As expected, minimal signal was observed at 4°C for blank and antiKRAS^{G12V} mAb-loaded HANAs, suggesting energy-dependent uptake pathways as the main mechanisms to access the interior of the cells. Interestingly, the ability to reach intracellular domains was superior for PEGtLyP1-HANAs. It is our hypothesis that the presentation of tLyP1 to the tumor microenvironment through the PEG spacer [53] might be

more appropriate than the one achieved for tLyP1-polymer in terms of its recognition by the NRP-1 receptors.

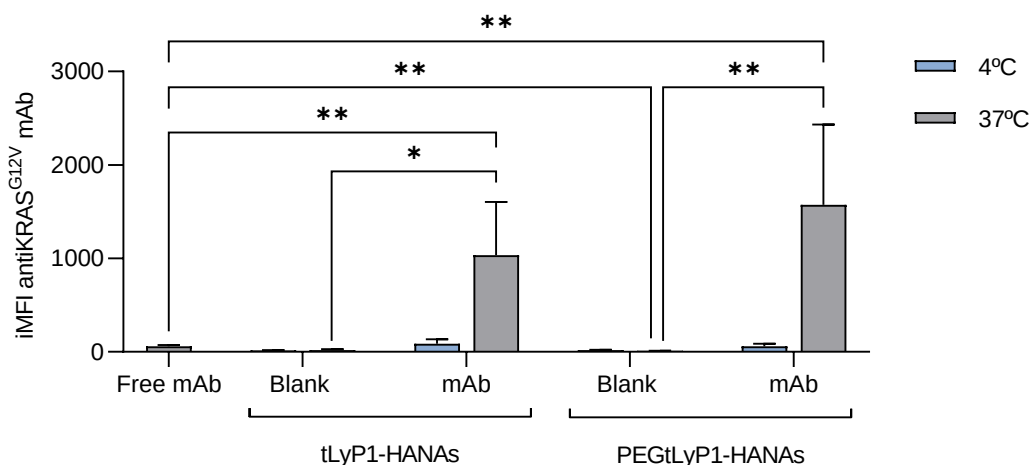


Figure 9. Intracellular detection of antiKRAS^{G12V} mAb tLyP1-HANAs and antiKRAS^{G12V} mAb PEGtLyP1-HANAs in the CMT167 cell line after 4h of exposure at 37°C and 4°C. Cells were treated with 1 μ M of antiKRAS^{G12V} mAb which corresponds to 0.075 mg/mL of tLyP1-HANAs. As controls, free antiKRAS^{G12V} mAb and blank tLyP1-HANAs were used. Statistical differences were determined by using a Mann-Whitney t-test between antiKRAS^{G12V} mAb tLyP1-HANAs or antiKRAS^{G12V} mAb PEGtLyP1-HANAs vs free antiKRAS^{G12V} mAb or blank tLyP1-HANAs at 37°C. Unpaired t-test between 37°C and 4°C for each tLyP1-HANAs. Data are expressed as mean $\pm$ SEM (n $\geq$ 3). iMFI integrated mean fluorescence intensity.

3.7.2 Target engagement

Following the confirmation of the intracellular delivery of the antiKRAS^{G12V} mAb, we found important to study its interaction with the KRAS^{G12V} oncoprotein. To do so, the CMT167 cell line was treated with the antiKRAS^{G12V} mAb PEGtLyP1-HANAs for up to 72 hours. The results in Figure 11 show time-dependent dynamics of target engagement, which increased for up to 48 hours. Surprisingly, the intensity of the engagement was apparently reduced at 72 hours. Although the level of significance of this reduction was low, we could speculate that the released mAb was gradually damaged [54] and loosed affinity for its target. On the other hand, it may also be possible that the kinetics of release at the intracellular level was affected by the intracellular disruptive conditions.

In our opinion, this assay has been crucial to assess the mechanism of action of these novel candidates for KRAS targeting and inhibition. Furthermore, this method contributes significantly to the knowledge of the mAbs intracellular delivery field as it has only been explored before to confirm the BCR/ABL and cMYC oncoprotein degradation through antiBCR/ABL mAb-loaded PLGA NPs [23] and antiMYC mAb-loaded polymeric micelles [20].

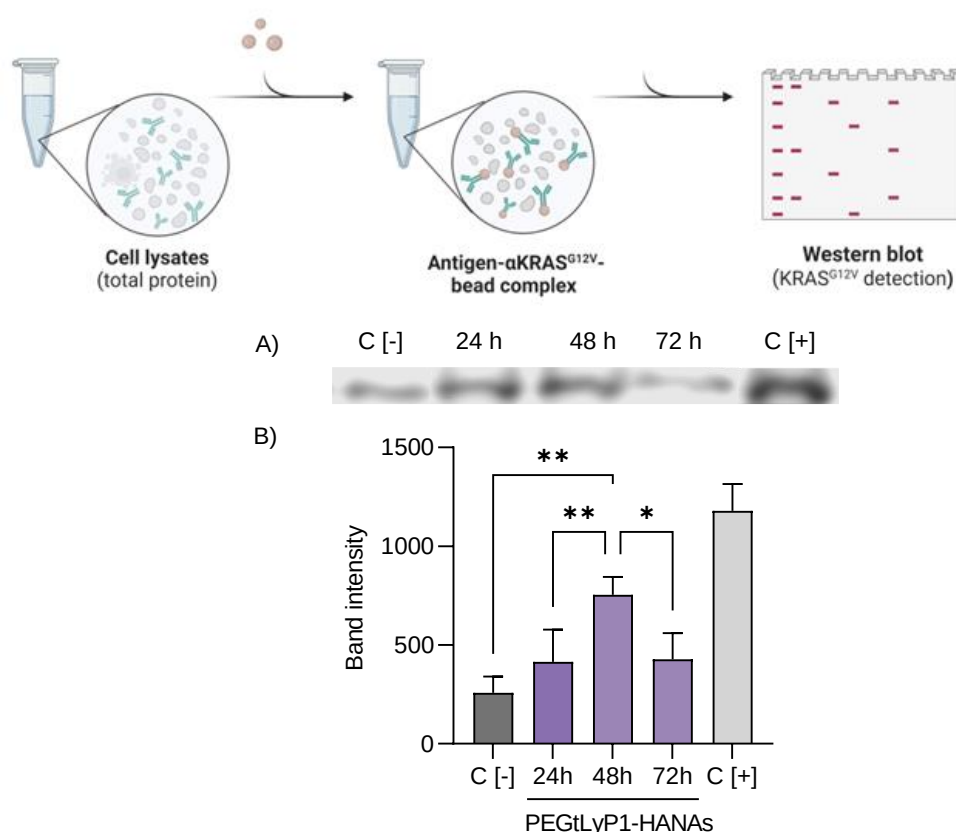


Figure 10. Results of the target engagement study consisting on the analysis by immunoprecipitation (IP) of the interaction of KRAS^{G12V} protein with the antiKRAS^{G12V} mAb delivered from the PEGtLyP1-HANAs. **A)** Schematic representation of the general procedure regarding the target engagement assay, which involves the IP of the antiKRAS^{G12V} mAb-KRAS^{G12V} oncoprotein complex and, subsequent analysis by western blotting. Raw images (A) and band intensities of the western blot membrane (B). The amount of IP product was determined at 24, 48, and 72 hours in the CMT167 cell line. The negative control [Ct (-)] was done by immunoprecipitating 1 mg of the CMT167 cell lysate with the anti-mAb beads. For the positive control [Ct (+)], 1 mg of the CMT167 cell lysate was added to 5 µg of the antiKRAS^{G12V} mAb. Statistical differences were determined by using a one-way ANOVA between groups but Ct (+). Data are represented as mean ± SD, n=3.

Overall, the greatest capacity of PEGtLyP1-HANAs to inhibit cell proliferation and colony formation was correlated with an efficient intracellular delivery of the antiKRAS^{G12V} mAb. Moreover, the capacity of the delivered mAb molecules to recognize their intracellular target represents a clear assessment of the potential of the HANA's nanotechnology.

4 CONCLUSIONS

The HANAs nanotechnology was validated for the entrapment and intracellular delivery of the antiKRAS^{G12V} mAb. By entrapping the antiKRAS^{G12V} mAb into the HANAs, a mismatched proliferation and colony formation across a panel of KRAS^{G12V} lung mutant cell lines was achieved. Intracellular staining and target engagement assays shed light to the underlying mechanism of the antiKRAS^{G12V} mAb, confirming its specific binding to the KRAS^{G12V} oncoprotein. The evaluation of the therapeutic outcomes will be tested in clinically relevant tumor models in further studies. Hence, we believe that the versatility of our technology may be used to shed light to, so far, untreatable KRAS^{G12V}-driven cancers.

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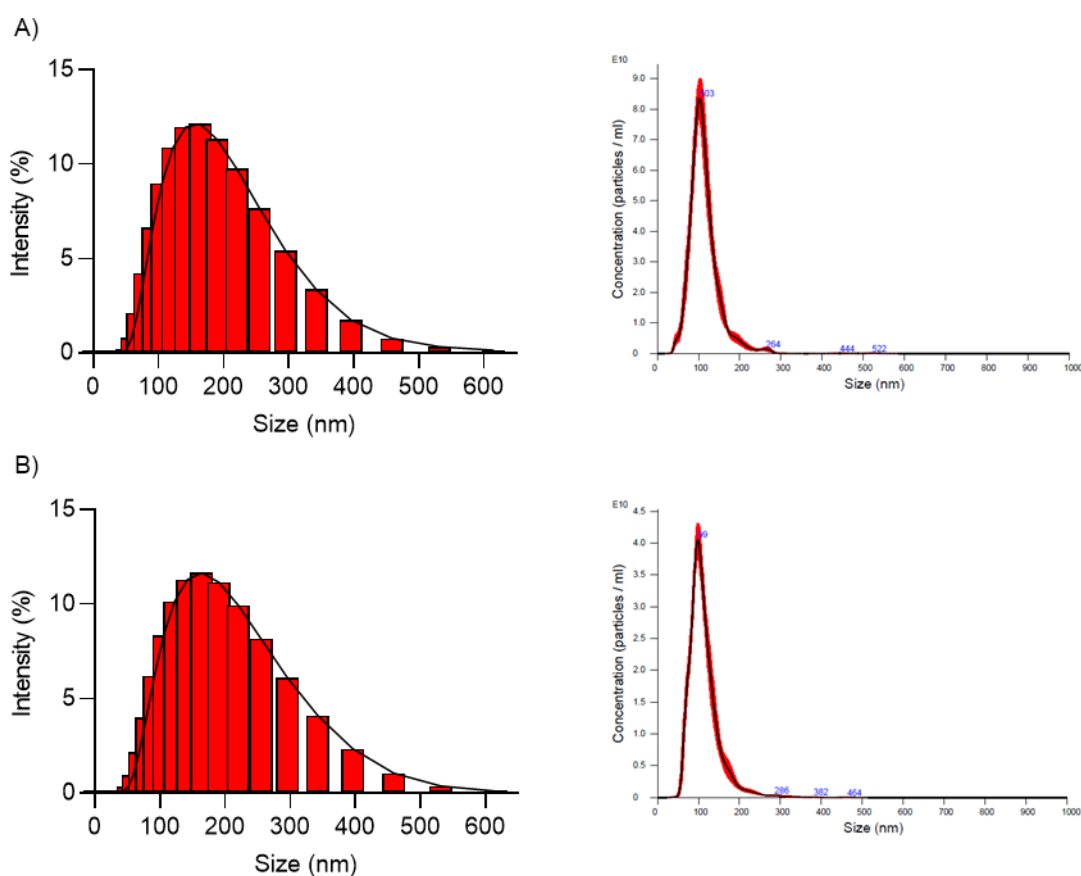
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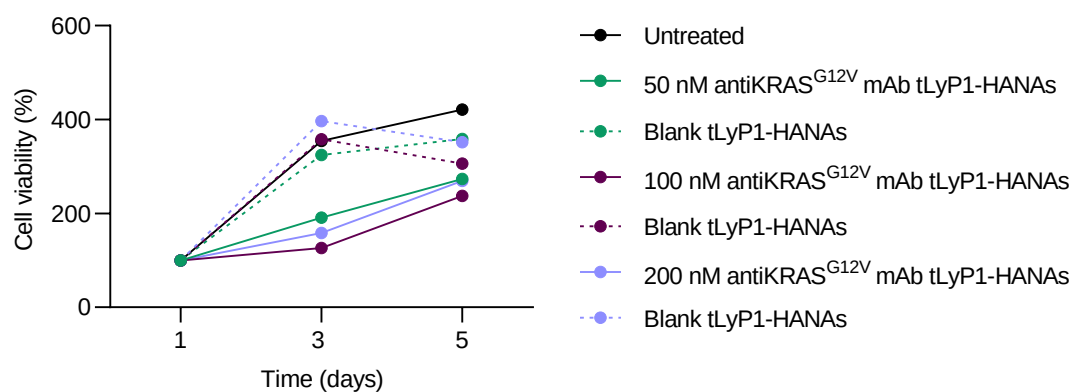
7 SUPPLEMENTARY INFORMATION

Supplementary Table 1. Physicochemical properties by DLS of blank and antiKRAS^{G12V} mAb tLyP1-HANAs and PEGtLyP1-HANAs after concentrate 6.4 times. #tLyP1 targeted material. Data are expressed as mean $\pm$ SD, n $\geq$ 3.

Prototype	Particle size (nm)	PDI	Zeta potential (mV)
tLyP1-HANAs	129 $\pm$ 10	0.15	-13 $\pm$ 3
PEGtLyP1-HANAs	132 $\pm$ 14	0.14	-12 $\pm$ 3



Supplementary Figure 1. Physicochemical characterization of tLyP1-HANAs (A) and PEGtLyP1-HANAs (B) by DLS and NTA. Left column: histogram of the particle size distribution by intensity; data are expressed as mean, n=3. Right column: size distribution based on particles per mL.



Supplementary Figure 2. Cell viability performed in a KRAS^{G12V}-mutant cell line (H441) after exposure to antiKRAS^{G12V} mAb tLyP1-HANAs at different antiKRAS^{G12V} mAb doses. Equivalent doses of blank tLyP1-HANAs were used as control. Data are expressed as mean, n=1.

OVERALL DISCUSSION

1 OVERALL DISCUSSION

Nanotechnology has been pointed as a promising strategy to enhance the efficacy of mAbs by improving their accumulation into the target tissue and their access to so far known as *undruggable* targets [1]. Our group has experience in the design of targeted nanocarriers for improving the tumor accumulation of anti-cancer drugs. For instance, the functionalization of docetaxel-loaded HA-nanocapsules (NCs) with the tumor penetrating peptide tLyP1 has resulted in a 36.6-fold increase in tumor accumulation, when compared with the free drug [2]. On the other hand, our group have pioneered the development of nanotechnologies for the intracellular delivery of mAbs. We reported for the first time the efficient intracellular delivery of the anti-gasdermin mAb upon its encapsulation into HA NCs. The encapsulated mAb led to a remarkable reduction of the tumor size and lung metastasis in a breast-cancer mice model [3]. Despite this unquestionable achievement [4,5], there is still room for additional improvement of nanotechnologies adapted for the intracellular delivery of mAbs. Certainly, a deeper characterization of the pharmaceutical features [5–7] of the reported delivery strategies is needed in order to settled the basis for the next generation of targeted nanocarriers. Likewise, the enhancement of their access and penetration across the tumor tissue is still a challenge.

Based on this background information, we have addressed these concerns by dividing this PhD thesis in three chapters:

1. Design, development, functionalization, and in vitro evaluation of the HANAs entrapping a model mAb.
2. Evaluate the role played by the tumor penetrating peptide on the functionalized ^{89}Zr -radiolabeled HANAs in vitro and in vivo and the capacity to modulate the biodistribution profile of the mAb.
3. Design, development, and in vitro evaluation of the HANAs for the intracellular delivery of the antiKRAS^{G12V} mAb.

The main achievements of the said chapters will be summarized below.

1.1 CHAPTER 1: POLYMER NANOASSEMBLIES FOR THE DELIVERY OF MONOCLONAL ANTIBODIES: DEVELOPMENT AND FUNCTIONALIZATION

Aiming to develop new nanotechnologies for the intracellular delivery of mAbs with high possibilities for the clinical translation, we have designed polymer-based nanoassemblies (HANAs). [Section with copyright protection] .In a subsequent step, the HANAs were provided with a PEG shell.

[Section with copyright protection].

A first screening was done aiming to find the most suitable condition for the obtention of HANAs with a particle size <200 nm, PDI <0.3, negative surface charge and loading capacity >15%. [Section with copyright protection].

Aiming to incorporate a PEG shell to the above HANAs, a second screening was performed. Through this strategy, it was expected to grant stealth properties to the NPs while facilitate their

functionalization with a tumor-penetrating peptide, tLyP1, when required. [Section with copyright protection].

Overall, the developed HANAs displayed the desired physicochemical properties (i.e., particle size <200 nm and negative to neutral surface charge) as have been described in Table 1. For a deeper characterization of the size and morphology, several orthogonal techniques were used. Indeed, it was found that NTA and FESEM analysis were not suitable techniques for all the prototypes due to the limitations of the methods. Thus, cryo-TEM was employed for the characterization of prototypes B and C1. [Section with copyright protection].

Table 1. Composition and physicochemical properties of resulting prototypes after concentrating by TFF (A-B2) or nitrogen (C, C1) up to 3.2 mg/mL of BVZ. [Section with copyright protection]. Data are expressed as mean $\pm$ SD, $n \geq 3$. Statistical comparison of the particle size was done following an unpaired t-test (B vs A, B2 and C vs C1) and Mann-Whitney test (B vs B1).

Prototype	Final composition [mg/mL]			Physicochemical properties		
	Polymer	Surfactant	Surfactant.PEG	Particle size (nm)	PDI	Zeta potential (mV)
A	3.2 ²	0.4	-	196 $\pm$ 13***	0.24	-14 $\pm$ 1
B	3.2 ¹	1.6	-	162 $\pm$ 17	0.23	-13 $\pm$ 2
B1	3.2 ¹	1.6	0.04	156 $\pm$ 11	0.26	-13 $\pm$ 3
B2	3.2 ¹	1.6	0.4	150 $\pm$ 15	0.27	-14 $\pm$ 1
C	1.6 ¹	3.2	-	107 $\pm$ 14	0.23	-13 $\pm$ 3
C1	1.6 ¹	3.2	1.6	79 $\pm$ 8****	0.25	-13 $\pm$ 2

Given the novelty of these delivery systems, the molecular behavior of the HANAs was studied at the atomic level by molecular dynamics (MD) simulation.

[Section with copyright protection].

Once the assembling mechanisms of the HANAs were investigated, we proceeded to the characterization in terms of colloidal stability, AE, LC and release profile. Thus, the stability of the final prototypes (Table 1) was studied in simulated biological fluids (PBS-10%FBS), exhibiting high stability of at least 8 hours.

Then, the amount of BVZ entrapped into the HANAs was determined, yielding values from 32 to 99%. More importantly, the LC ranged from 13 to 46%. The array of values displayed were attributed to the composition. For instance, the presence of a PEG-lipid reduced the AE, which may be related to the competition between the components for their interaction with the BVZ [8,9]. However, this observation was not extrapolated to prototypes C and C1. It is our opinion that when the formulations were developed with a low initial concentration of materials, the competitions were dismissed.

[Section with copyright protection].

Table 2. Physicochemical properties, association efficiency (AE, %) and loading capacity (LC, %) of prototypes A-C1 after concentrate to a final BVZ concentration of 3.2 mg/mL. [Section with copyright protection]. Data are expressed as mean $\pm$ SD, $n \geq 3$, except for prototype D ($n=2$). Statistical analysis was evaluated by using a Mann-Whitney (A vs B**; B vs B1**) and unpaired (C vs C1) t-test.

Prototype	Physicochemical properties			AE (%)	LC (%)
	Particle size (nm)	PDI	Zeta potential (mV)		
A ²	196 $\pm$ 13	0.24	-14 $\pm$ 1	99 $\pm$ 0.3**	46.6
B ¹	162 $\pm$ 17	0.23	-13 $\pm$ 2	86 $\pm$ 11	34.4
B1 ¹	156 $\pm$ 11	0.26	-13 $\pm$ 3	33 $\pm$ 18**	13.0
B2 ¹	150 $\pm$ 15	0.27	-14 $\pm$ 1	52 $\pm$ 6	19.6
C ¹	107 $\pm$ 14	0.23	-13 $\pm$ 3	74 $\pm$ 11	29.7
C1 ¹	79 $\pm$ 8	0.25	-13 $\pm$ 2	67 $\pm$ 21	22.2

The potential of the HANAs as pharmaceutical product in terms of long-term stability and freeze-drying was confirmed. Indeed, a stability in suspension at 4°C ranging from 6 months to 1 year and 3 months was yielded. Moreover, a low percentage of trehalose (i.e., 2.5 -5% w/v) was needed to maintain the physiochemical properties and amount of entrapped BVZ after its resuspension to their original volume.

Finally, the release kinetics of the prototypes B, C and C1 in PBS at pH 7.4 and 5.0 was studied to understand whether the superior amount of polymer, or presence of PEG leads to a different release profile. In general terms, a burst release <20% was found, with minor modifications overtime. [Section with copyright protection].

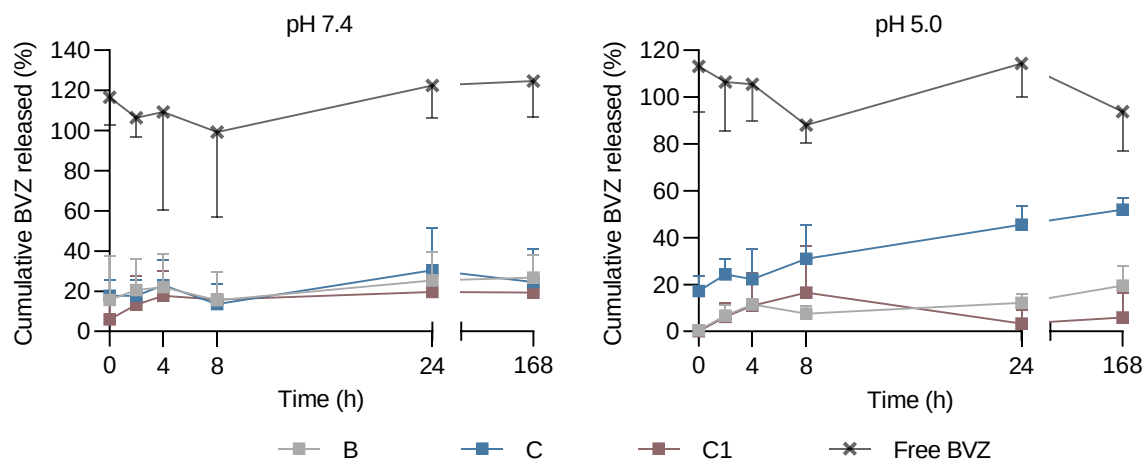


Figure 1. Release profile of HANAs upon incubation in PBS pH 7.4 and 5.0 at 37°C. The cumulative amount of released BVZ of prototype A, B, C and C1 was quantified by ELISA. As control, free BVZ was treated in the same conditions than the NPs. Data are represented as mean $\pm$ SD, $n \geq 3$.

The attractive features of the HANAs encouraged us to go further and introduce the tumor penetrating peptide, tLyP1, to the HANAs' surface in order to enhance the chances for the tumor accumulation. To do so, prototypes B and C1 were selected and functionalized through the polymer and PEG motif, respectively. Thus, the tLyP1 exposure to the TME was presumed. [Section with copyright protection]. In the case of the surfactant.PEG-maleimide, only a thiol conjugation was needed. After purification and characterization, the resulting tLyP1-conjugates were used for the formation of prototypes B and C1. While the physicochemical properties and AE were maintained when used the polymer-tLyP1 polymer, so-called BtLyP1, an increment

of the particle size was observed for C1. It was stated that the superior particle size was attributed to the modified solubility of the surfactant.PEG after the insertion of a hydrophilic molecule (i.e., tLyP1), as have been described for other drugs [10]. Thus, a screening was performed to find the most suitable condition for the incorporation of the surfactant.PEGtLyP1 to prototype C. Although the lowest concentration of surfactant.PEGtLyP1 led to higher particle sizes than C1, it was selected for further studies and named C1tLyP1. Resulting prototypes were characterized based on the main physicochemical properties, AE and LC. As shown in Table 3, the AE of BtLyP1 was dramatically dropped upon functionalization with tLyP1, while C1 preserved the amount of entrapped BVZ. These results suggested a different structural organization of the BVZ within the HANAs due to the presence of the tLyP1, being evident in prototype BtLyP1 due to its superior percentage of tLyP-1 conjugate (40% in BtLyP1 vs 0.8% in C1tLyP1).

Finally, the release profile of tLyP1-HANAs was assayed at pH 7.4 and 5.0 to mimic the blood and the endo-lysosomal conditions, respectively. As shown in Figure 2, a higher burst and release overtime was observed for C1tLyP1. [Section with copyright protection].

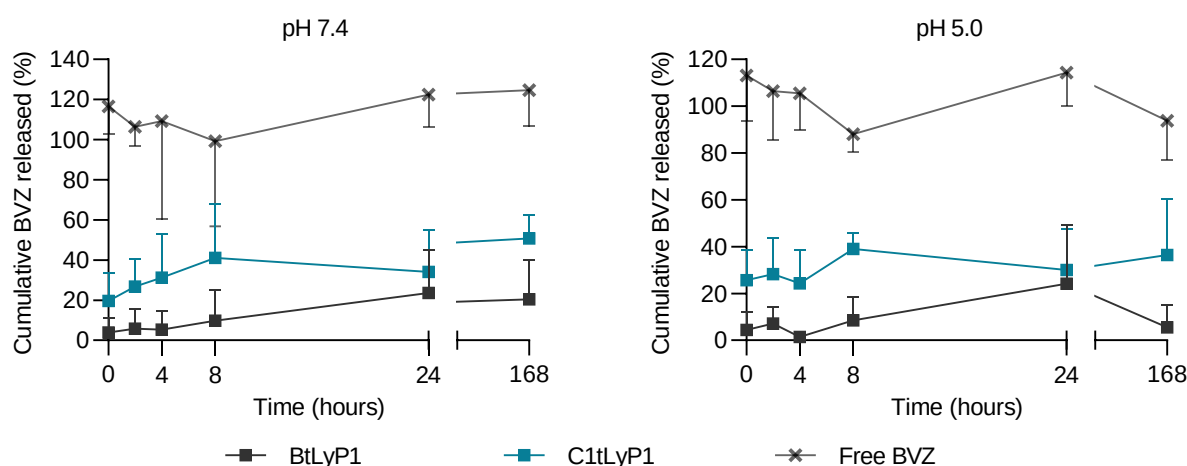


Figure 2. Release profile of BtLyP1 and C1tLyP1 upon incubation in PBS pH 7.4 and 5.0 at 37°C. The cumulative amount of released BVZ was quantified by ELISA. As control, free BVZ was treated in the same conditions than the NPs. Data are represented as mean $\pm$ SD, $n \geq 3$.

The stability in biological simulated media (PBS-10%FBS), long-term stability and freeze-drying were studied to validate the tLyP1-HANAs as potential pharmaceutical products. In general, the NPs showed a good stability in PBS-10%FBS of at least 8 hours and in suspension at 4°C of 6 months. Moreover, a 5% (w/v) of trehalose was sufficient to maintain the particle size and AE after resuspension to its original volume. These results pointed out comparable profile of tLyP1-targeted and non-targeted HANAs in terms of colloidal stability and storage conditions.

Table 3. Physicochemical properties, association efficiency (AE, %) and loading capacity (LC, %) of BtLyP1 and FtLyP1. Data are expressed as mean $\pm$ SD, $n \geq 3$. Statistical analysis of the AE was done by using a Mann-Whitney (B vs BtLyP1) and unpaired (C1 vs C1tLyP1) t-test.

Prototype	Physicochemical properties			AE (%)	LC (%)
	Particle size (nm)	PDI	Zeta potential (mV)		
B	162 $\pm$ 17	0.23	-13 $\pm$ 2	86 $\pm$ 11	34.4
BtLyP1	166 $\pm$ 9	0.18	-11 $\pm$ 6	26 $\pm$ 11****	10.3
C1	79 $\pm$ 8	0.25	-13 $\pm$ 2	67 $\pm$ 21	22.2
C1tLyP1	121 $\pm$ 14	0.25	-13 $\pm$ 2	65 $\pm$ 12	26.0

A preliminary scaling-up of the HANAs technology from 1 mL to 50 mL batch was done, confirming that the physicochemical properties were maintained. Hence, the suitability of the HANAs technology for an industrial production was suggested.

After discarding a possible toxicity caused by the HANAs, the cellular uptake of the DiD-labelled prototype B and C1 was studied in a lung cell line (CMT167) and macrophages (RAW 264.7) at 37°C. Likewise, cellular energy depletion at 4°C was conducted to confirm the an uptake mediated by active mechanisms [11]. In the case of prototype B, no dose- and temperature-response was observed in CMT167, revealing the dye leakage or deposition of the HANAs on the cell surface [11]. Instead, the uptake in macrophages led to significant differences in at 37°C and 4°C, which may indicate a superior rate of uptake in this cell line.

Regarding the uptake profile of prototype C1 dose- and temperature- dependent uptake was displayed, which was confirmed by confocal microscopy. [Section with copyright protection].

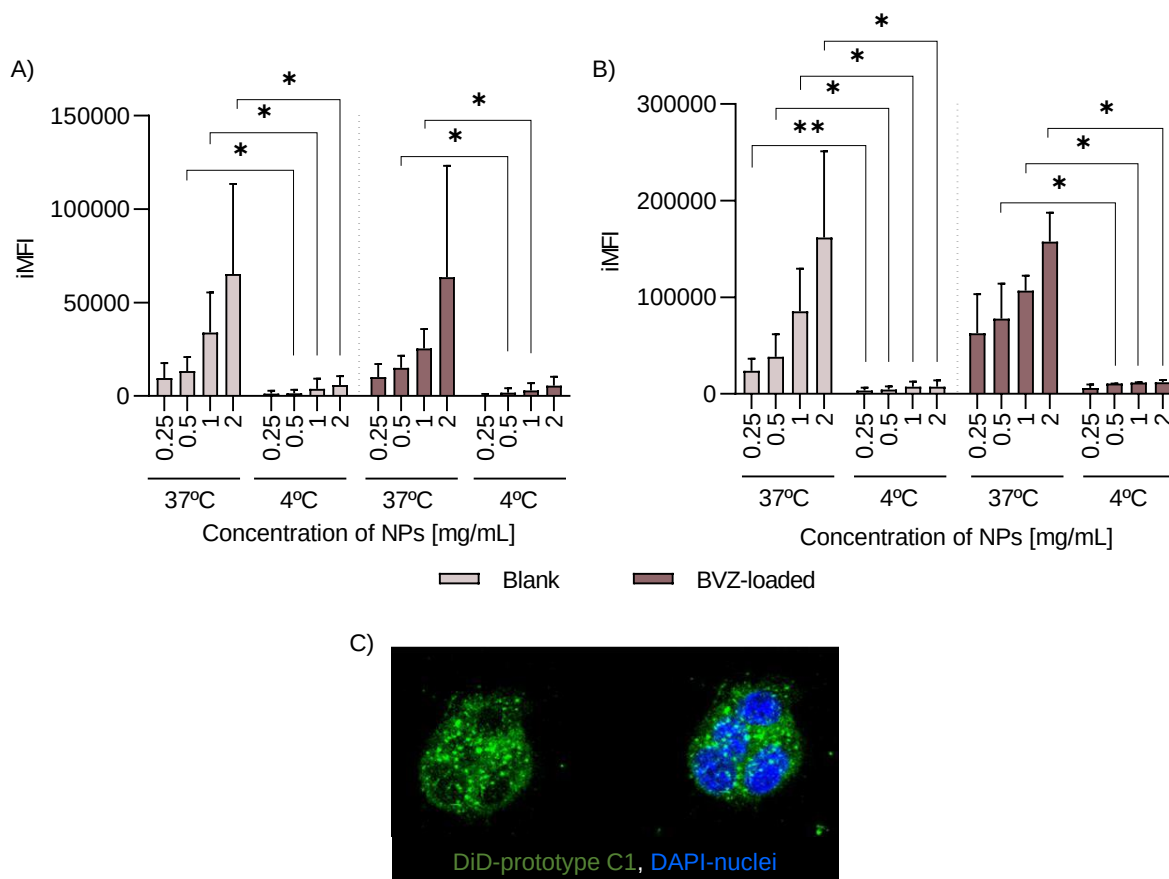


Figure 3. Cellular uptake of DiD-labelled blank and BVZ-loaded prototype C1. Quantitative analysis of DiD iMFI in CMT167 (A) and RAW264.7 (B) following 4 hours of incubation at increasing concentrations of HANAs. C) Representative confocal microscopy images of blank prototype C1 at 2mg/mL after 24 hours of incubation in the lung cancer cell line. Statistical comparisons were done following a parametric unpaired t-test between temperatures. Data are expressed as mean $\pm$ SD, $n \geq 3$. iMFI integrated mean fluorescence intensity.

1.2 CHAPTER 2: MULTIFUNCTIONAL NANOASSEMBLIES AS A STRATEGY TO IMPROVE THE TUMOR UPTAKE OF MONOCLONAL ANTIBODIES: *IN VITRO* EVALUATION AND PET *IN VIVO* IMAGING

In this section, we aimed to investigate whether prototypes C1 and C1tLyP1 developed in Chapter 1 could change the biodistribution profile of the mAb and enhance its tumor accumulation by PET imaging. To this end, a radiolabeling strategy with the radionuclide ^{89}Zr , [12], a positron emitter radioisotope with a half-life of 78.4 h, was developed. More in detail, the chelate deferoxamine (DFO) [13] was conjugated to the BVZ and entrapped into the HANAs for a posterior coordination with the ^{89}Zr .

In order to obtain a radiolabeling strategy reproducible and with radiochemical features acceptable for PET imaging, two alternative labeling methods were developed for prototypes C1 and C1tLyP1. [Section with copyright protection]. Nevertheless, there is no available information regarding the labeling of mAbs after its entrapment into NPs.

For the first method (Method 1), the BVZ-DFO was entrapped into the HANAs and afterwards, coordinated with the ^{89}Zr . After removal of free ^{89}Zr but not free ^{89}Zr -BVZ, a moderate

radiochemical yield, comparable to the control ^{89}Zr -BVZ was achieved (Figure 4.A). Moreover, low production yield were obtained for the ^{89}Zr -HANAs, independently of their composition (Figure 4.B). Furthermore, the radiochemical purity values revealed values $>90\%$, which demonstrate the successful removal of free ^{89}Zr and ^{89}Zr -BVZ.

The low production yields but high radiochemical purities may be attributed to several reasons. [Section with copyright protection].

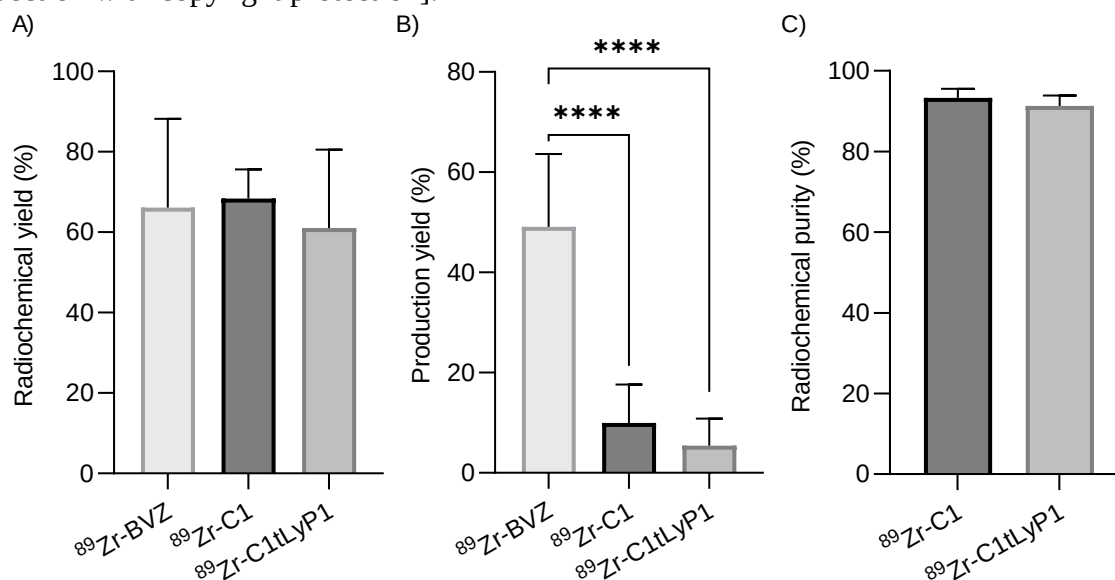


Figure 4. Radiochemical characterization of ^{89}Zr -labeled BVZ-loaded HANAS-PEG (^{89}Zr -C1), ^{89}Zr -labeled BVZ-loaded FtLyP1 (^{89}Zr -C1tLyP1) prepared by method 1, and ^{89}Zr -BVZ. A) Radiochemical yield (RCY, %), B) production yield (%) and C) radiochemical purity (RCP, %). Data are expressed as mean $\pm$ SD, $n \geq 3$. Statistical analysis was done using a Mann-Whitney (A) and unpaired t-test (B-C).

Aiming to improve above data, a second radiolabeling method (method 2) was developed. In this case, the non-entrapped BVZ-DFO was removed prior to the coordination with ^{89}Zr , achieving a production yield for prototype C1tLyP1 3 times higher than when Method 2 was followed. Finally, the radiochemical purities and reproducible procedures associated to both methods were acceptable.

To validate the ^{89}Zr -HANAs for *in vivo* studies, the stability of the ^{89}Zr -HANAs in human serum was studied for up to 72 hours. The results indicate that the amount of ^{89}Zr coordinated to the HANAs was maintained overtime.

As stated above, the tLyP1 functionalization of the HANAs is expected to enhance the uptake profile after recognition by the tLyP1 receptor, the NRP-1. In consequence, the uptake profile of ^{89}Zr -C1tLyP1 was 9.2 and 34.1 times higher than ^{89}Zr -BVZ and ^{89}Zr -C1, respectively. Indeed, after saturation of the NRP-1, the uptake was dropped, confirming the implication of the tLyP1 in the superior uptake [14–16].

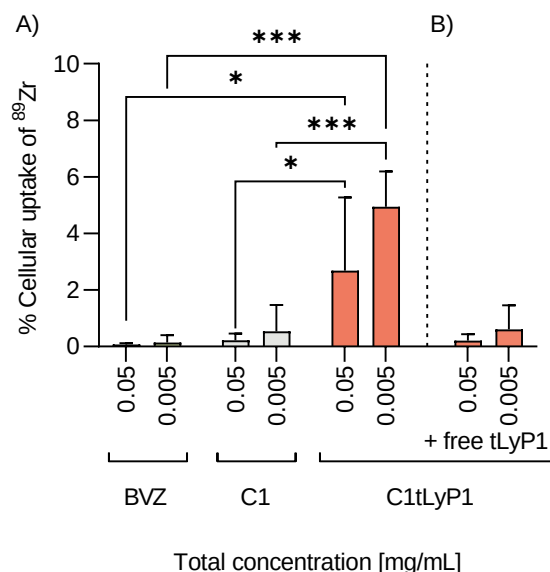


Figure 5. *In vitro* cell uptake of ^{89}Zr (A) and competitive assay (B) in the A549 cell line. Cells were treated for 4 hours after incubation with ^{89}Zr -BVZ (BVZ) and ^{89}Zr -labeled BVZ-loaded C1 (^{89}Zr -C1) and ^{89}Zr -labeled BVZ-loaded C1tLyP1 (^{89}Zr -C1tLyP1) in serum-supplemented media at 37°C . For the competitive assay, cells were treated with an excess of tLyP1 for 1-hour prior treatment. Statistical comparison was performed by a 2-way ANOVA followed by a Fisher's LSD test. Data are expressed as mean $\pm$ SD ($n \geq 3$, except for the competitive assay, $n=2$).

Aiming to establish a correlation of the *in vitro* results and *in vivo* fate of the HANAs, the biodistribution of ^{89}Zr -C1 and ^{89}Zr -C1tLyP1 was carried out in a healthy mice model, and thereafter, in a tumor-bearing mice.

PET/CT imaging (Figure 6) and biodistribution studies of ^{89}Zr -BVZ and ^{89}Zr -C1 in healthy mice (C57BL/6) revealed how the entrapped of the BVZ into the HANAs modified the biodistribution profile of the BVZ. A rapid accumulation of ^{89}Zr -C1 in liver and spleen was observed, while the ^{89}Zr -BVZ remains in blood circulation for long periods, in consonance with the BVZ half-life (approx. 3.9 days) [17]. *Ex vivo* biodistribution studies confirmed the above statements.

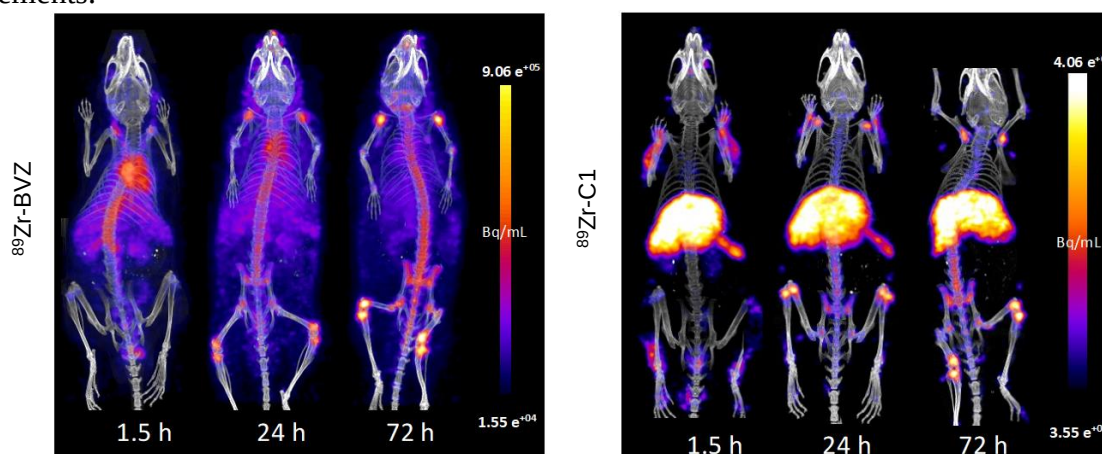


Figure 6. Maximum intensity projection PET/CT imaging of ^{89}Zr -BVZ and ^{89}Zr -labeled BVZ-loaded C1 (^{89}Zr -C1) in 9 weeks old healthy C57BL/6J mice. PET/CT maximum intensity projections of 1-2 MBq of ^{89}Zr -BVZ and ^{89}Zr -C1 after i.v. administration ($n=4$). Study performed following radiolabeling method 1.

After validating the performance of ^{89}Zr -C1 in healthy mice, PET/CT studies with ^{89}Zr -C1 and ^{89}Zr -C1tLyP1 were performed in a subcutaneous A549 NSCL carcinoma mouse model (Figure 7). Thus, the implication of the tLyP1 on the tumor accumulation will be elucidated. In accordance with the previous PET/CT images in healthy mice, both ^{89}Zr -HANAs were accumulated in liver and spleen. Since non-obvious accumulation was found in the tumor, *ex vivo* studies were conducted.

As shown, *ex vivo* biodistribution studies at 24 hours within tumors displayed, although no-significant, a superior accumulation in those tLyP1-targeted HANAs (2 ± 1 %ID/g for ^{89}Zr -C1 and 3 ± 1 for ^{89}Zr -C1tLyP1). Furthermore, the values of tumor to blood and tumor to liver ratios showed significant greater accumulation of the tLyP1-HANAs in the tumor.

Overall, the tLyP1 implication in terms of tumor accumulation was suggested, which is in consonance with previous reports [2,16,18–20]. Moreover, when compared with other tumor penetrating peptides such as iRGD decorating porous silicon NPs [21] and LyP1-dendrimers [22], superior tumor-to-muscle ratios have been found for our technology. Certainly, other delivery systems have been ascribed to tumor-to-muscle ratios for up to 21% after 6 hours of injection [23] in the same A549 tumor model. It should be emphasized that the comparison among peptides and NPs is challenging as the disposition of the peptide on the surface, components, physicochemical properties, tumor model and irrigation are factors that determines the *in vivo* outcome of the NPs.

In brief, we have developed two strategies for the radiolabeling of a new nanotechnology intended for the delivery of mAbs. More in detail, we have labeled BVZ-loaded HANAs with ^{89}Zr and validated their performance *in vitro* and *in vivo*, demonstrating that our technology was able to modify the biodistribution profile of the mAb while enhance their tumor accumulation after anchoring the tLyP1 peptide to their surface.

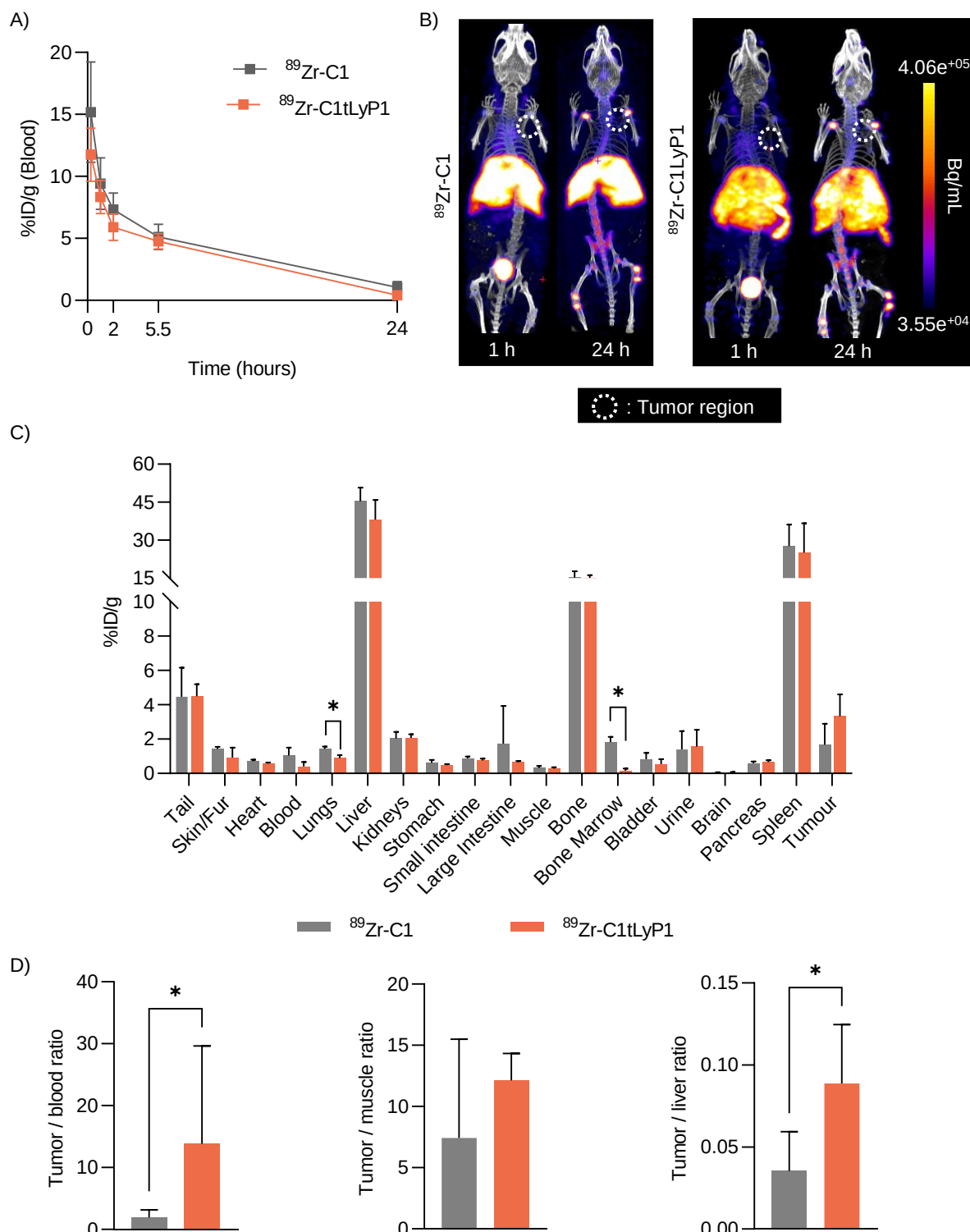


Figure 7. Blood profiles, organ biodistribution and PET imaging of ^{89}Zr -labeled BVZ-loaded C1 ($^{89}\text{Zr-C1}$) and ^{89}Zr -labeled BVZ-loaded C1tLyP1 ($^{89}\text{Zr-C1tLyP1}$) in a A549 tumor bearing Balb/c mouse model. **A)** Percentage of injected dose of ^{89}Zr per gram (%ID/g) of blood after i.v. injection. **B)** PET/CT maximum intensity projections of 2.5-3 ($^{89}\text{Zr-C1}$) and 1.5-2.5 ($^{89}\text{Zr-C1tLyP1}$) after i.v. administration. **C)** Organ uptake represented as %ID/g of tissue at 24 h after i.v. injection. **D)** Ratio of tumor/blood, tumor/muscle and tumor/liver uptake. Data are represented as mean $\pm$ SD, $n=4$. Statistical comparison was done following an unpaired t-test (A, D tumor/muscle and tumor/liver ratio) and Mann-Whitney test (B, D tumor/blood ratio).

1.3 CHAPTER 3: UNLEASHING THE POWER OF PERSONALIZED NANOMEDICINE: INTRACELLULAR DELIVERY OF ANTI-KRAS^{G12V} mAb TO COMBAT LUNG CANCER

In this chapter, we have adapted the HANAs technology for the intracellular delivery of the antiKRAS^{G12V} mAb for the treatment of lung cancer.

As a first step, and based on the results derived from Chapters 1 and 2, tLyP1-targeted HANAs were optimized for the entrapment of the antiKRAS^{G12V} mAb. For this, we investigated the influence of an array of concentrations of polymer-tLyP1 and surfactant of the physicochemical properties and drug loading of the developed prototypes. The results derived from this screening led to a number of promising prototypes with particle sizes ≥ 150 nm and AE values of $\sim 60\%$, [Section with copyright protection]. Finally, the prototype initially composed by polymer-tLyP1 0.25, surfactant 1, antiKRAS^{G12V} mAb 0.5 (mg/mL) was selected for further studies, from now on designated as tLyP1-HANAs, due to the small size (~ 130 nm) and acceptable AE ($\sim 67\%$).

Aiming to incorporate a PEG shell while facilitate the functionalization through the PEG motif to the above tLyP1-HANAs, a second screening was performed involving the incorporation of increasing concentrations of surfactant.PEG-tLyP1. The results showed that the use of concentrations of up to 0.025 mg/mL displayed a particle size ~ 150 nm and AE $\sim 55\%$. The lack of differences in the main properties were subscribed to the mass percentage that the surfactant.PEG-tLyP1 represents within the system (0.8% and 2%, respectively), avoiding the mAb competition with the PEG motif. Finally, the prototype with a surfactant.PEG-tLyP1 concentration of 0.01 mg/mL, so-called antiKRAS^{G12V} mAb PEGtLyP1-HANAs, was selected for further experiments.

Final prototypes were characterized after concentrating up to 3.2 mg/mL of antiKRAS^{G12V} mAb in order to fulfill the requirements for the intravenous administration. Although NTA analysis indicated a smaller particle size than DLS, results in Table 4 showed a final particle size of 130 nm, PDI <0.3 and neutral to negative surface charge. The amount of entrapped antiKRAS^{G12V} mAb was greater than 60%. In fact, LC values were as high as $\sim 18.7\%$.

Table 4. Physicochemical properties by DLS and NTA, association efficiency (AE, %) and loading capacity (LC, %) of antiKRAS^{G12V} mAb tLyP1-HANAs and PEGtLyP1-HANAs after concentrate 6.4 times. #tLyP1 targeted material. Data are expressed as mean $\pm$ SD, $n \geq 3$. Statistical differences regarding the particle size by DLS and AE of antiKRAS^{G12V} mAb tLyP1-HANAs and antiKRAS^{G12V} mAb PEGtLyP1-HANAs were analyzed by using an unpaired t-test, whereas particle size by DLS and NTA was done following an unpaired t-test (antiKRAS^{G12V} mAb tLyP1-HANAs) and Mann-Whitney (antiKRAS^{G12V} mAb PEGtLyP1-HANAs).

Prototype	Particle size (nm) (DLS)	PDI	Particle size (nm) (NTA)	Concentration ($\times 10^{10}$ particles /mL)	Zeta potential (mV)	AE (%)	LC (%)
tLyP1-HANAs	141 $\pm$ 7	0.20	136 $\pm$ 20	459 $\pm$ 50	-11 $\pm$ 2	66 $\pm$ 9	18.7
PEGtLyP1-HANAs	142 $\pm$ 11	0.23	122 $\pm$ 8**	396 $\pm$ 131	-11 $\pm$ 2	64 $\pm$ 6	18.2

A deep morphological characterization was conducted by cryo-TEM. [Section with copyright protection].

[Section with copyright protection].

The colloidal stability of the antiKRAS^{G12V} mAb tLyP1-HANAs and PEGtLyP1-HANAs was studied in simulated biological fluids (PBS-10%FBS) and in suspension at 4°C. HANAs displayed a high stability of at least 8 hours in PBS-10%FBS and 6 months in storage conditions.

To discard a premature release of the mAb, the release profile of the HANAs was studied in PBS at pH 7.4, displaying a maximum release of 26% after 1 week of incubation. [Section with copyright protection].

In a subsequent step, the functional evaluation of antiKRAS^{G12V} mAb tLyP1-HANAs and PEGtLyP1-HANAs was carried out by proliferation and colony formation studies in KRAS^{G12V}-mutant cell lines (CMT167 and H441). Hence, we will shed light over the mAb capacity to switch off the GTP-bound active state through the intracellular delivery of mAbs. Therefore, tLyP1-HANAs yielded a suppression of the proliferation of 9.4% and 35.8% in the H441 and CMT167-mutant cell lines, respectively. In fact, PEGtLyP1-HANAs showed a similar delay on the cell proliferation, this being greater in the CMT167 cell line (27.3% vs. 18.9% in H441). As expected, no activity was observed for the controls (free mAb or blank HANAs) or the wild-type cell line.

When the above results were compared with KRAS siRNA-based therapeutics [24,25], similarities were found. The higher response observed in the CMT167 cell line was attributed to several factors such as the NRP-1 overexpression [2,26] in this cell line or the development of intrinsic and acquired resistance mechanisms [27,28], which hinder the identification of clear responses in this cell line.

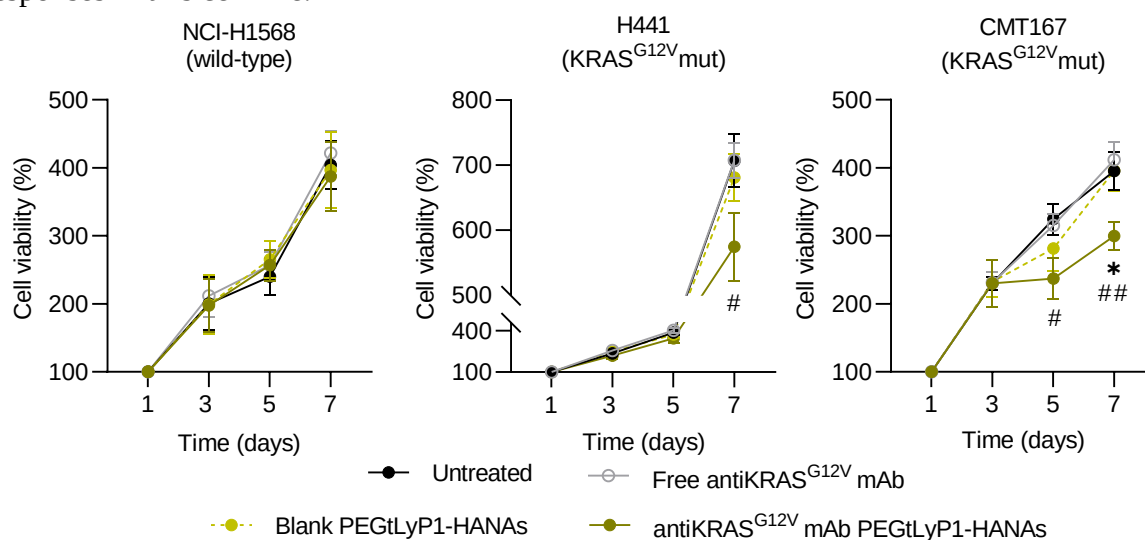


Figure 8. antiKRAS^{G12V} mAb-loaded PEGtLyP1-HANAs impaired G12V mutated lung cancer cell proliferation. Lung cancer cell line were incubated for 7 days with culture medium (untreated), free antiKRAS^{G12V} mAb, blank and antiKRAS^{G12V} mAb PEGtLyP1-HANAs. Data are expressed as mean $\pm$ SEM, $n \geq 3$. Statistical differences were analyzed by multiple t-test at each time point between groups. # and * refers to differences among the free antiKRAS^{G12V} mAb or blank PEGtLyP1-HANAs, and antiKRAS^{G12V} mAb PEGtLyP1-HANAs, respectively. Differences among the antiKRAS^{G12V} mAb PEGtLyP1-HANAs and the untreated group were not plotted.

As mentioned before, the capacity of the anti-KRAS-loaded prototype to inhibit the formation of colonies was confirmed. Indeed, the relative number of colonies in the KRAS^{G12V} mutant cell line CMT167 was of 64 (%). The results of the efficacy of the HANAs was somehow

comparable to those obtained following other therapeutic strategies such as siRNA-loaded carriers [29,30].

To understand the mechanism by which HANAs could impair cell viability by the specific inhibition of the KRAS oncoprotein, we investigated their capacity to enter the cells and delivery the mAb at the intracellular level. Thus, the cellular uptake was evaluated in the CMT167-mutated lung cancer cell line. By intracellular staining, a 1.5 fold uptake was observed for PEGtLyP1-HANAs when compared with tLyP1-HANAs, which may be attributed easiness for the recognition when the tLyP1 was anchored to the PEG spacer [31]. In contrast, no uptake was found at 4°C, thus suggesting a temperature-dependent uptake.

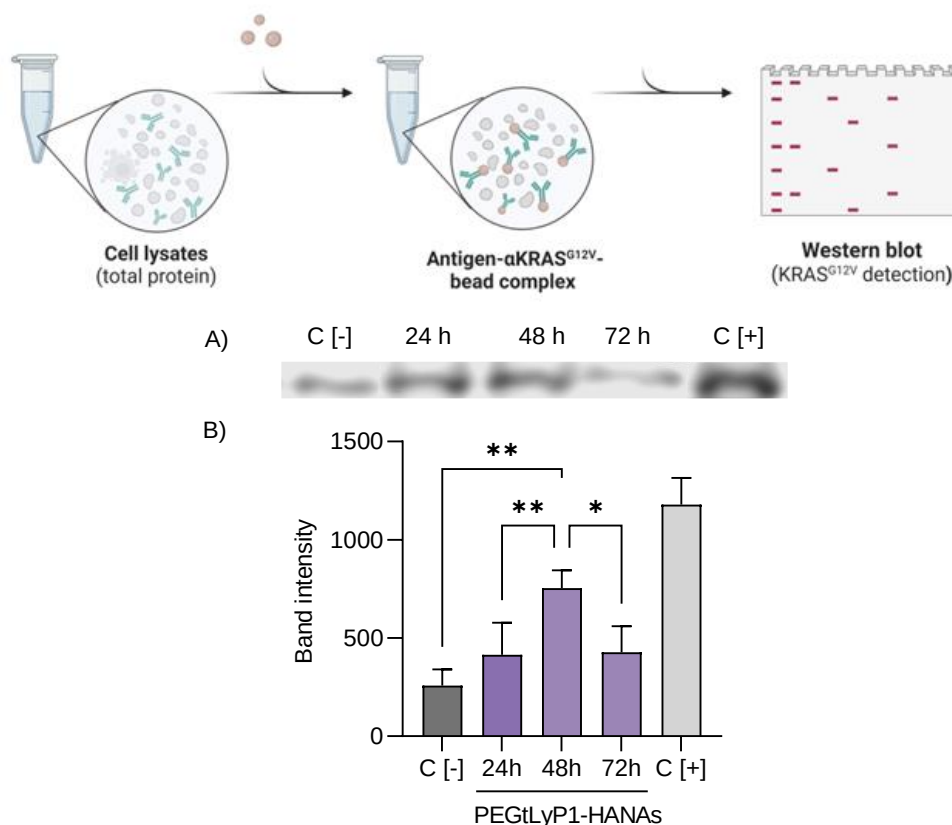


Figure 9. Results of the target engagement study consisting on the analysis by immunoprecipitation (IP) of the interaction of KRAS^{G12V} protein with the antiKRAS^{G12V} mAb delivered from the PEGtLyP1-HANAs. A) Schematic representation of the general procedure regarding the target engagement assay, which involves the IP of the antiKRAS^{G12V} mAb-KRAS^{G12V} oncoprotein complex and, subsequent analysis by western blotting. Raw images (A) and band intensities of the western blot membrane (B). The amount of IP product was determined at 24, 48 and 72 hours in the CMT167 cell line. The negative control [Ct (-)] was done by immunoprecipitating 1 mg of the CMT167 cell lysate with the anti-mAb beads. For the positive control [Ct (+)], 1 mg of the CMT167 cell lysate were added to 5 µg of the antiKRAS^{G12V} mAb. Statistical differences were determined by using a one-way ANOVA between groups but Ct (+). Data are represented as mean ± SD, n=3.

To further prove that the antiKRAS^{G12V} mAb was able to recognize the KRAS^{G12V} oncoprotein, target engagement studies were conducted. As shown in Figure 9, a time-dependent dynamics of interaction was observed. The intensity of the interaction was maximum after 48 hours, followed by a significant reduction at 72 hours. It is our hypothesis that the dynamics of interactions was conditioned by the possible intracellular damage of the mAb [32], causing a loss of activity or due to a controlled release kinetics of the mAb from the HANAs in intracellular compartments.

In brief, the HANAs have demonstrated to be a versatile technology for the intracellular delivery of mAbs, particularly, the antiKRAS^{G12V} mAb. With this platform, the activity of the mAb was preserved and the capacity to recognize the oncoprotein intracellularly allowed.

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CONCLUSIONS

The experimental work carried out in this thesis allowed us to conclude:

1. Polymer-based nanoassemblies (HANAs) were designed for the intracellular delivery of the model mAb, bevacizumab. [Section with copyright protection]. The resulting HANAs presented adequate physicochemical properties (particle size from 80 to 200 nm; zeta potential ~ -10 mV) with remarkable loading capacities (up to 46.6 %). These properties were maintained after the HANAs' surface functionalization with the tumor penetrating peptide, tLyP1. HANAs prototypes exhibited a high cell internalization and a very low toxicity profile in lung cancer cells and macrophages.
2. The bevacizumab-loaded HANAs showed a very different biodistribution profile as compared to the free mAb. Importantly, an obvious increment of the tumor accumulation was achieved after the functionalization of the HANAs' surface with the tLyP1 peptide.
3. The HANAs technology was found to be a promising platform for the intracellular delivery of relevant mAbs such as antiKRAS^{G12V} mAb. Selected anti-KRAS-loaded HANAs displayed a remarkable capacity to impair the cell proliferation and colony formation as a result of the intracellular delivery of the mAb. Of note, a time-dependent target engagement was confirmed.

As a whole, this work highlights the potential for translation of the HANA's technology. We have shown that, by understanding the parameters that determine the entrapment of the mAb and the *in vitro* behavior, it is possible to rationally design novel nanocarriers for the intracellular delivery of mAbs. Moreover, in this thesis we disclose the potential of the HANAs technology for modifying the biodistribution of mAbs and enhancing the tumor accumulation, and for reaching *undruggable* targets, i.e., the KRAS^{G12V}.

LIST OF ABBREVIATIONS

% w/v percentage of weight/volume	% δ
δ chemical shift	
AE association efficiency	A
BVZ bevacizumab	B
CD ₃ OD deuterated methanol	C
Cryo-TEM cryogenic transmission electron microscopy	
CT computed tomography	
D ₂ O deuterium oxide	D
DCR derived count rate	
DiD 1,1'-Diocetadecyl-3,3,3',3'-Tetramethylindodicarbocyanine	
DLS dynamic light scattering	
DMEM Dulbecco's Modified Eagle Medium (	
DMSO-d ₆ deuterated dimethyl sulfoxide	
DS degree of substitution	
[Section with copyright protection]	
[Section with copyright protection]	E
ELISA enzyme-linked immunosorbent assay	
EtOH ethanol	
Fab fragment antigen-binding region	F
FACS Fluorescent Activated Cell Sorter	
FBS fetal bovine serum	
Fc fragment crystallizable region	
FDA United States Food and Drug Administration	
[Section with copyright protection]	H
HEPES N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid	
¹ H NMR proton nuclear magnetic resonance	
iMFI integrated mean fluorescence intensity	I
KRAS Kirsten Rat Sarcoma Virus	K

L

LC loading capacity
LDA Laser Doppler Anemometry
[Section with copyright protection]

M

MAbs monoclonal antibodies
Mal maleimide
MD molecular dynamics
[Section with copyright protection]
MW molecular weight
MWCO molecular weight cutoff

N

N.A. not applicable
NaCl sodium chloride
NaOH sodium hydroxide
N.D. not determined
[Section with copyright protection]
NPs nanoparticles
NRP neuropilin
NSCL non-small cell lung
NTA nanoparticle tracking analysis

P

PBS phosphate buffered saline
PDI polydispersity index
PET positron emission tomography

R

RCP radiochemical purity
RCY radiochemical yield
 R_g radius of gyration
RT room temperature

S

SD standard deviation

T

TFF tangential flow filtration
tLyP1 truncated LyP-1
TME tumour microenvironment

V

VEGF vascular endothelial growth factor

ETHICAL CONSIDERATIONS

The animal experiments described in Chapter 2 were ethically reviewed and carried out in accordance with the Animals (Scientific Procedures) Act 1986 (ASPA) UK Home Office regulations governing animal experimentation.

1.1 IN VIVO STUDIES IN HEALTHY ANIMAL MODELS

Subject: New protocol - Juan Pellico - Sunassee PPL Normal Animals

Dear Julie

Please find attached an approved protocol from Juan Pellico. He is adding a new cohort to his in vivo data (new complexes – sample 2).

Many thanks

Best wishes

Kavitha

Dr Kavitha Sunassee

Pre-Clinical Operations Manager
King's College London
School of Biomedical Engineering & Imaging Sciences
4th Floor, Lambeth Wing
St Thomas Hospital
London, SE1 7EH

Tel No: +44(0)20 7188 7188 ext. 56327



information on data protection and the
General Data Protection Regulation

1.2 IN VIVO STUDIES IN TUMOR ANIMAL MODELS

From: Laniyan, Floyd <floyd.laniyan@kcl.ac.uk>

Date: Tuesday, 26 July 2022 at 11:55

To: Pellico Saez, Juan <juan.pellico@kcl.ac.uk>

Cc: kcl - raynebsu <raynebsu@kcl.ac.uk>, Gower, Kate <kate.hance@kcl.ac.uk>, Kim, Jana <jana.kim@kcl.ac.uk>, Sunassee, Kavitha <kavitha.sunassee@kcl.ac.uk>

Subject: FW: [MCMS] Facility Sign-off required for Study Plan Pellico-89ZrNanocomplexes in tumour model (SP113076)

Hi Juan,

The study plan **Pellico-89ZrNanocomplexes in tumour model (SP113076)** has been approved. I have altered changed the cohort name for the study plan to AMONSTJP03 . When placing orders or booking procedure rooms could you please include the unique code.

Checked your competencies and the 2 listed below have not been updated as competent so could this please be checked.

081601 Subcutaneous injection in mice

124501 Tumour measurement by calipers for subcutaneous tumours in mice

Also, you may find adding the schedule for the procedures on the study plan will save you time later.

Could you please add the data capture for the weighing of the animals to the schedule so that the baseline weight of the animals can be recorded at the start of the experiment.

Best wishes,
Floyd

**PUBLICATION DERIVED
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Nanotechnologies for the delivery of biologicals: Historical perspective and current landscape

Author:

Matilde Durán-Lobato, Ana María López-Estévez, Ana Sara Cordeiro, Tamara G. Dacoba, José Crecente-Campo, Dolores Torres, María José Alonso

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Mutations in the Kirsten Rat Sarcoma Virus (KRAS) oncoprotein are particularly relevant therapeutic targets in lung cancer, among others. Although one KRAS mutation has been addressed, KRAS is still considered an undruggable target. In this scenario, monoclonal antibodies (mAbs) have emerged as the main alternative to achieve a positive efficacy/toxicity balance while blocking intracellular oncoproteins. Hence, we have developed novel nanoassemblies (HANAs) for the intracellular delivery of mAbs and functionalized or not with the tumor penetrating peptide tLyP1. HANAs were evaluated according to i) the capacity to load mAbs, ii) the ability to modify the biodistribution profile of the mAb, and iii) the performance in terms of activity and target engagement towards the KRASG12V oncoprotein.