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Making Minds Matter

Boosted transport of Theranostic Angiopep-2 engineered crosslinked Hyaluronic Acid NanoParticles (Thera-ANG-cHANPs) in glioblastoma cells

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***Abstract*—Angiopep-2 is a 19 amino acid synthetic peptide that demonstrated the ability to shuttle active agents across the BBB in clinics by the interaction with LRP-1 receptors. The combination of such peptide with properly designed drug delivery systems can strongly improve the outcome of patients with brain tumours, a pathology in which the standard of care is scarcely effective because of poor transport and resistance. In this work, we engineered theranostic crosslinked hyaluronic acid nanoparticles (Thera-cHANPs), co-encapsulating Gd-DTPA with boosted relaxivity and the chemotherapeutic drug Irinotecan (CPT-11), with Angiopep-2, to produce Thera-ANG-cHANPs. Thera-ANG-cHANPs demonstrated stability in the biological environment, boosted uptake by patient derived Glioblastoma cells and, as consequence of improved transport, a boosted therapeutic effect exerted with a lower dose of drug in a reduced time window.**

***Keywords*—Angiopep-2; Glioblastoma (GBM); Nanoparticles; Hydrodenticity.**

I. INTRODUCTION

Angiopeps (ANGs) are a family of synthetic peptides that selectively target Low-density Lipoprotein Receptor Related Protein 1 (LRP-1). [1, 2]. Those receptors are relevant for the targeted transport across the Blood Brain Barrier (BBB) since they are highly overexpressed at the endothelial layer of the Neurovascular Unit, being also present in a greater extent with respect to other receptors (e.g. Transferrin and Insulin receptors) [3]. Demeule et al., demonstrated that one specific ANG peptide, Angiopep-2 (TFFYGGSRGKRNNFKTEEY) bears 10-folds higher transcytosis capacities at the BBB in vivo with respect to the endogenous substrates of LRP1 receptors, such as aprotinin [4]. Moreover, Angiopep-2 confirmed its shuttling ability into the Central Nervous System (CNS) in clinical settings, determining successful brain tumour accumulation of the drug paclitaxel, when formulated as a drug-peptide conjugate (ANG1005) in patients with recurrent gliomas [5]. Indeed, further evidence proved that LRP1 receptors are also overexpressed at glioma cell membrane, and thus that Angiopep-2 bears dual targeting ability, probably because of the marked proliferating phenotype characterizing cells of higher grade tumours, such as Glioblastomas (GBMs) [6] [7, 8].

GBM is the most aggressive and incurable CNS malignancy, with very poor patient prognosis[9]. Clinical protocols for diagnosis and therapy are constantly revised and updated based on the developing knowledge around tumour genotyping and stratification. The latest guidelines have been released in 2017 by the European Association of Neuroncology (EANO) [10]. They identify the tumour burden by T1-enhanced regions in MRI and confirm the Stupp protocol, based on chemo-radio-combinatorial therapy, as standard of care. However, Gadolinium (Gd)-based contrast agents are not able to cross the BBB and can accumulate only in the regions of the tumour where it is disrupted. Moreover, the only approved chemotherapeutic drug, Temozolomide (TMZ) has been proved to be ineffective in more than 50% of patients, in details those affected by the methylation of the O [6]-methylguanine-DNA methyltransferase (MGMT) promoter [11]. For all the aforementioned reasons, the use of Angioepep-2 engineered formulations constitutes a huge opportunity to improve GBM standard of care and expand it both for diagnosis and therapy, repurposing strongly effective active agents that are currently excluded because of poor CNS accumulation.

Hyaluronic Acid (HA) demonstrated the ability of boosting the relaxivity of Gd-based CAs through the properties of Hydrodenticity[12, 13]. In particular, when encapsulated in properly crosslinked HA nanoparticles, it is packed in gado-meshes, domains of packed water that is determining the boosting Gd-DTPA relaxivity up to 12-fold as a consequence of the improved hydration degree of the metal chelate [12-14]. Secondly, the camptothecin prodrug Irinotecan (CPT-11), approved for the treatment of colorectal cancer since 1996, has been recently proposed for the treatment of Glioblastoma as valuable alternative to the poorly effective TMZ. Despite some encouraging results, its poor serum stability and CNS penetration are still posing limitations to its approval for GBM treatment. Based on these observations, we designed Angiopep-2 engineered theranostic crosslinked HA NPs (Thera-ANG-cHANPs) encapsulating both Gd-DTPA with boosted relaxometric properties and the camptothecin drug Irinotecan for an earlier diagnosis and more effective therapy of GBM [14]. In this regard, the present study aims at highlighting the improved interaction of Thera-ANG-cHANPs with a relevant model of patient derived glioblastoma cells and its potential implication on GBM theranostics.

II. RESULTS

Table 1 reports the main methodologies applied for the production and in-vitro testing of Thera-ANG-cHANPs. The use of patient tumour material for cell culture establishment was approved by the 600 institutional review board of the ErasmusMC and obtained with patient's informed consent. Ethical Commette: MEC-2013-090.

Table 1. Main methodologies applied for Thera-ANG-cHANPs production and testing

	TECHNIQUE	*METHODOLOGY*
NP Production	Microfluidic Hydrodynamic Flow Focusing (HFF)	Nanoprecipitation in a quartz X-junction chip. Solvent phase: HA 0.05% w/V; Gd-DTPA 0.1% w/V: CPT-11: 0.025% w/V + EtOH 10% V/V. Non-solvent phase: Acetone; Divinyl sulfone 4% V/V. Process parameters: Middle Flow rate 30 µL/min Side Flow rate 110 µL/min
Angiopep-2 Conjugation	NH_2-COOH chemical crosslinking	EDC (0.02M) and NHS (0.006M) activation and 50 µg Angiopep-2 per mL of NPs. BCA assay kit for peptide quantification
NP physicochemical properties	Morphology: TEM Average Size: DLS	TEM observation of 5 µL of sample on copper grids; DLS measurement of 1 mL sample by Zetasizer Nano (Malvern)
NP relaxivity	1T MRI	Spin-echo sequence (repetition times 550, 880, 1400, 2000 ms, echo time 80 ms) and T1 signals calculated using MLE method to fit saturation recovery curves (a*exp(-TR/T1)+c). FOV 80-mm, a 96 × 96 matrix, and 2 mm slice thickness
Active Agents Encapsulation	ICP-MS Uv-VIS	Gd-DTPA: ICP-MS CPT-11: Abs 368 nm
Cell uptake	Flow cytometry (FACS)	Cell culture: U87 MG: Dulbecco's modified Eagle's medium (DMEM), 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine; PDX: (DMEM)–537 F12 with 1% penicillin/streptomycin, B27, human epidermal growth factor (EGF; 5 538 µg/mL), human basic fibroblast growth factor (FGF; 5 µg/mL), and heparin (5 539 mg/mL). Seeding density: 50000 cells/well .

The theranostic design of Thera-ANG-cHANPs is schematically presented in Figure 1a.

The following sections present the rational for particle testing and the achieved results.

A. Impact of Angiopep-2 conjugation on LRP1 targeting ability

The targeting ability of Thera-ANG-cHANPs entirely depends on Angiopep-2 interaction with the receptor LRP1. For this reason, as a first step, a molecular docking simulation between the peptide and the LRP-1 portion responsible for the ligand binding (the double complement type repeats CR56) is performed. It allows to characterize the peptide/receptor binding in terms of energy, binding modes and, most importantly, know about both peptide and receptor amino acids involved, to assess the impact of peptide conjugation to NPs on its interaction with the LRP1, if any. As presented in Figure 1b, the binding is characterized by a quite high binding energy, -5.67 kcal/mol. Moreover, it appears that a specific region of the CR56 is involved. The analysis of the amino acids reveals that this LRP1 region comprises the Asp959, reported as the residue involved in the Receptor Associated Protein (RAP) binding, directly connected to the recycling of the receptors from the endosomes to the cell surface, thus to its transcytosis capacity [15, 16]. Moreover, all the residues of Angiopep-2 result to be involved in the binding with equal probability, allowing to conclude that a chemical crosslinking of the peptide on NP surface, preventing some amino acids from the binding with LRP1, should not negatively impact on its overall targeting ability.

B. ANG-cHANPs: production and characterization

Microfluidic production of crosslinked Hyaluronic Acid Nanoparticles (cHANPs) encapsulating multiple agents is a consolidated process[14, 17-19]. Briefly, by implementing a nanoprecipitation process in an X-junction chip through a hydrodynamic flow focusing regime, a highly uniform population of nanoparticles with tightly controlled features is produced. For the theranostic design, a co-solvation strategy is implemented allowing to maximize encapsulation of the poorly soluble CPT-11 without perturbing HA precipitation and crosslinking, and Gd-DTPA encapsulation. In optimal process conditions, a uniform population of spherical particles with mean size of 106.5 ± 46 nm is achieved. Encapsulation efficiencies (EE%) of Thera-cHANPs are 30% (80µM) and 18% (151µM) for Gd-DTPA and CPT-11, respectively.

After production, Angiopep-2 is conjugated to -COOH groups of HA exposed onto NP surface to produce engineered Thera-ANG-cHANPs. Briefly, after -COOH activation by EDC/NHS, Angiopep-2 is put in contact with Thera-cHANPs (50 µg per mL of NPs). After 4h, excess peptide is purified and particle size, morphology, and relaxometric properties are characterized. Dynamic Light Scattering (DLS) reveals a particle population of increased mean size (368 ± 61 nm). Neverthless, as showed by Transmission Electron Microscopy (TEM) observation in Figure 1c, particle morphology is preserved, and Angiopep-2 is successfully and uniformly conjugated to the polymer, as further confirmed by the BCA assay, measuring 32 µg/mL of Angiopep-2 in the purified sample. The increase in NP size might be due to polymer chain sliding consequent to interaction with the peptide causing water imbibition and hydrogel swelling.

Relaxometric properties of NPs are evaluated under a preclinical 1T MRI. Figure 1d present the T1 maps as measured for increasing concentration of Gd-DTPA encapsulated in NPs showing, as expected, T1 shortening as Gd-DTPA concentration increases. Figure 1e reports fitting of mean T1 values for longitudinal relaxivity evaluation. This analysis reveals a $r_1 = 13.08\, mM^{-1}s^{-1}$ for Thera-ANG-cHANPs that is 3.45 times higher than free Gd-DTPA ($r_1 = 4\, mM^{-1}s^{-1}$). This result suggests that the Hydrodenticity

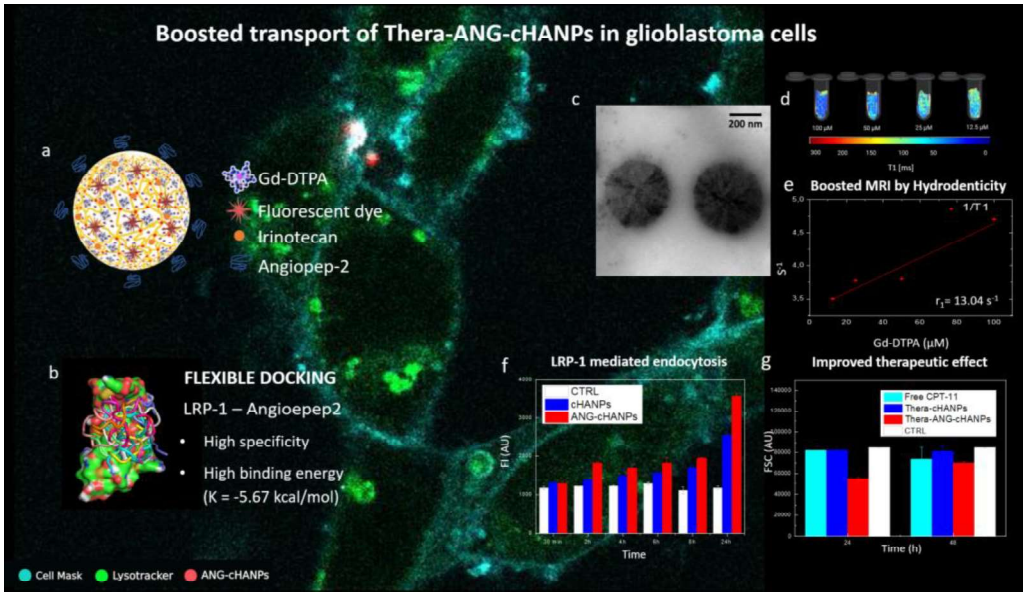


Fig. 1: Thera-ANG-cHANPs for boosted theranostics of Glioblastoma cells; a) Schematic representation of Thera-ANG-cHANPs architecture; b) Flexible Molecular Docking between Angiopep-2 and LRP-1 receptor; c) Transmission Electron Microscopy (TEM) of ANG-cHANPs; d) T1-map of NPs at 1T MRI; e) Longitudinal relaxivity estimation by T1 measurement; f) Uptake of ANG-cHANPs vs cHANPs by Flow Cytometry (FACS); g) Cytotoxicity of Thera-ANG-cHANPs vs free-CPT-11 and bare Thera-cHANPs by Forward Scattering Measurement in FACS.

boosting is preserved, as consequence of preserved hydrogel network.

C. 2.3 Boosted ANG-cHANPs transport into glioma cells

The interaction of Thera-ANG-cHANPs with glioma cells is evaluated by Flow Cytometry measurements, a single cell analysis technique that additionally allows to interrogate a statistically relevant cell population. NIR emitting cHANPs and ANG-cHANPs are produced by encapsulating the standard dye ATTO 633 ($\lambda_{ex/em}$ = 633/652 nm) to reduce the signal overlapping with cell autofluorescence, mostly present in the Vis spectrum. NP uptake is evaluated in a patient derived (PDX) cell population, as relevant model of human glioblastoma.

To quantify NP uptake, cells are seeded in 48-well plates at a density of 50000 cells/well and let adhere for 24h. Afterwards, cells are exposed to bare or engineered NPs (50 μg/mL). At selected time points, cells are washed three times with PBS for removal of non-internalized particles, detached by trypsinization and uptake is measured by flow cytometry. The analysis of the fluorescence at 650 nm is presented in Figure 1f comparing the uptake of bare cHANPs with engineered ANG-cHANPs over time. At the analysis of cHANPs internalization profile a linear increase in fluorescence over time can be observed. Differently, ANG-cHANPs show higher value of fluorescence, as indication of higher amount of internalized particles and also a sharper, exponential increase of signal after 24h of contact. As widely reported in literature, this behaviour is proper of energy dependent mechanisms which are not saturated over time as the gradient of concentration of the moving specie is vanishing across the cell membrane[20-22]. For this reason, it is possible to conclude that Angiopep-2 is mediating the boosted uptake of NPs by human derived glioma cells. Similar results are achieved in the standard U87-MG cell line (data not shown).

To investigate the possible implications of such improved transport properties, Thera-ANG-cHANPs are tested on U87-MG cells for their therapeutic potential. The cytotoxicity induced by encapsulated CPT-11 is compared to cell death caused by free CPT-11 at the same concentration (10 μM), both evaluated through the Forward Scattering Signal (FSC) measured by Flow cytometry that is widely recognized as indication of cell viability[22]. Results are presented in figure 1g. Cytotoxicity is measured at 24h and 48h since CPT-11 is a topoisomerase II inhibitor, and for this reason, is exerting its therapeutic effect at cell replication. At the analysis of the results, it is clear that free CPT-11 is producing slight or no effect on U87 cells, even after 48h of contact, as bare cHANPs. This result is not surprising if considering that the tested dose of 10 μM is 10-folds lower than the measured ED50 for CPT-11 in many cell lines. Differently, Thera-ANG-cHANPs are producing a strong reduction in cell viability already after 24h of contact, consolidating this result at 48h. In light of these results, it is possible to conclude that as a consequence of the boosted particle accumulation in U87 cells, a lower dose of CPT-11 resulted highly effective in a reduced time window.

III. Conclusion

GBM is characterized by several biological barriers that are very different in nature (physical, chemical, biological and mechanical) and highly dynamic among tumoral stages. They strongly limit the effectiveness of the standard of care and

vanish most of the efforts that literature is posing to design alternative but complex diagnostic and therapeutic approaches. To significantly impact on GBM diagnosis and therapy a deeper understanding of the cross-talk of active agents with disease-specific biological barrier is needed. In this sense, Thera-ANG-cHANPs are designed starting from approved materials and active agents, that already proved their effectiveness in clinics. The effort in this case is devoted to the understanding of the impact of their combination and the evaluation of nano-bio-interactions that arise at the cellular level between Thera-ANG-cHANPs and a relevant human model of Glioma.

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