Biocompatible single-chain polymer nanoparticles loaded with an antigen mimetic as potential anticancer vaccine.

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ABSTRACT: The "pancarcinoma" Tn antigen (αGalNAc-O-Ser/Thr) is a tumor-associated carbohydrate antigen (TACA) overexpressed on the surface of cancer cells and suitable target for anti-cancer vaccines. However, TACAs commonly show weak immunogenicity, low *in vivo* stability and poor bioavailability. To address these issues, the development of physiologically stable TACA synthetic mimetics and novel nanocarriers for multivalent display are object of intense research. Nanomaterials represent suitable scaffolds to multimerize antigens, but absence of toxicity, easy functionalization and capability to incorporate biomolecules are compulsory characteristics for vaccine nanocarriers. Here, we report on the conjugation of a synthetic Tn-antigen mimetic to biocompatible and water-dispersible dextran-based single-chain nanoparticles (DXT-SCPNs). *In vitro* stimulation of PBMCs and analysis of interleukins production indicated a specific innate immune modulation mediated by the multivalent presentation of the Tn mimetic at the nanoparticle surface. These preliminary results pave the way for the development of Tn-mimetic clusters on biocompatible DXT-SCPN for TACA-based vaccines.

Mucins are high molecular weight extracellular proteins, heavily glycosylated with complex oligosaccharides. Mucin-type oligosaccharides are involved in specific interactions and receptors binding; therefore, the O-glycosyl pattern is crucial for mucin structure and biological functions like cell growth, adhesion, invasion and immune surveillance.[1] Among the different types of O-glycosides, which contribute to form antigens in aberrantly over- or hypo-glycosylated mucins, the most common tumor-associated carbohydrate antigens (TACAs) are α-Tn, Thomsen-Friedenreich (TF), sialyl Tn and sialyl TF.[1,2] α-Tn antigen (Tn) is expressed at high levels, i.e. between 70% and 90%, in different cancers tumors (colon, lung, bladder, cervix, ovary, stomach, and prostate), whereas little or no expression has been observed in a broad range of healthy adult tissues.[3,4,5] Some Tn-based vaccines have produced excellent results in animal models and several clinical trials in humans have been published.[6,7,8] Nonetheless, the Tn-based vaccines currently under development have raised several criticisms: a) the enzymatic instability of the native Tn antigen *in vivo* which reduces the vaccine bioavailability, b) the presentation of the Tn sugar moiety, due to the nature of the native glycosidic linkage, is dramatically influenced by the supporting peptide/scaffold, strongly reducing the antigenic effect of the vaccine, and c) high density of Tn residues is often required for antibody recognition.[9] With this in mind, we recently synthesized a promising immunostimulant Tn-antigen mimetic, resistant to enzymatic degradation and structurally blocked in the suitable conformation for a correct recognition by the immune system.[10]

TACAs are poorly immunogenic antigens and cannot be presented to T cells for T cell responses (T cell-independent type II antigens).[11] Consequently, the class switch from IgM to IgG and the recall memory response, are not generated. A successful strategy to overcome these limiting aspects consists in coupling carbohydrates onto immunogenic protein carriers (conjugate vaccines).[11,12] A key parameter to be considered when using carbohydrates in molecular recognition events, including vaccine development, is their natural multivalent display, which can be artificially obtained by coupling carbohydrate antigens to multivalent scaffolds, such as nanomaterials.[13,14,15] For example, Tn-antigen glycopolymers were able to generate immune response *in vivo*.[16] In addition, nanomaterials-based antigen delivery systems can provide an adjuvant activity, inducing activation and maturation of antigen presenting cells.[17] Integration of multiple functions in the same nanosystem, which includes the opportunity to modulate the innate immune response and stimulate adaptive immunity, is in fact a key aspect and strength point in favor of the development of nanoparticle-based vaccines.[2, 17b] Recently, we reported that superparamagnetic iron oxide nanoparticles loaded with a mimetic of the Tn antigen were able to induce macrophage effector functions, eliciting gene expression and protein release of the TNF-α.[18] Capitalizing on these promising results but keeping in mind the major concerns affecting the use of metal nanoparticles as delivery systems *in vivo*,[19,20] we turned our interest to biocompatible/biodegradable soft-matter based nanosystems as an alternative. In this regard, single-chain polymer nanoparticles (SCPNs)[21,22] have great potential

as biomaterial nanocarriers due to their soft matter characteristics, their small and controllable size and the potential mimicking behavior towards proteins.[23] SCPNs can be prepared by controlled collapse of single polymer chains into folded nanoparticles through intra-chain cross-linking.[24] However, only few examples of bioconjugated SCPNs for potential application in intracellular delivery,[25] imaging,[26] and controlled drug delivery[27,28] have been reported. In addition, most of these SCPN-based nanosystems are based on synthetic polymers which are usually prepared in organic solvents and through experimental conditions difficult to translate to clinic. In an effort to have in hand water-dispersible and biocompatible SCPNs based on readily available and easily functionalized polymers, the synthesis and functionalization of dextran-based single-chain polymer nanoparticles (DXT-SCPNs) has been recently reported.[29] DXT-SCPNs have been designed in such a way that chemical reactive functional groups (like carboxylic acids) can be easily integrated to allow further incorporation of (bio)molecules of interest.[29] In the present study, we aimed at using DXT-SCPNs as nanocarriers to mimic the expression of Tn antigen on cancer cells (Figure 1) by multimerization of the Tn mimetic **5** (see Scheme 1), stable *in vivo* and immunogenic. The immunomodulation properties of this new glycosyl-nanosystem were studied.

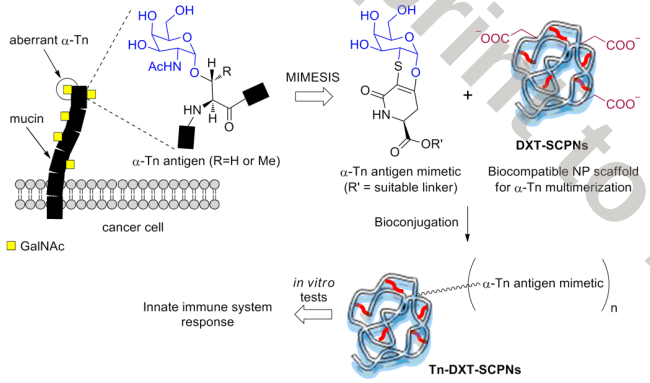
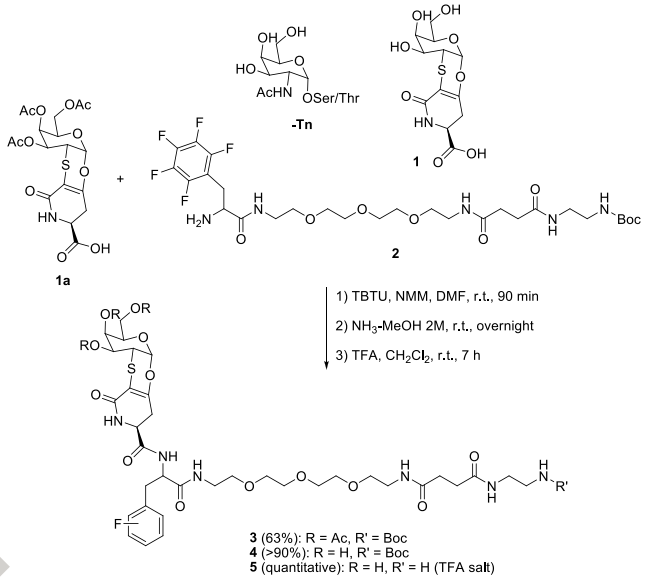


Figure 1. Outline of the "multimerization" strategy to develop single-chain polymer nanoparticle-based mimetics of aberrant expression of α-Tn antigen on cancer cells.

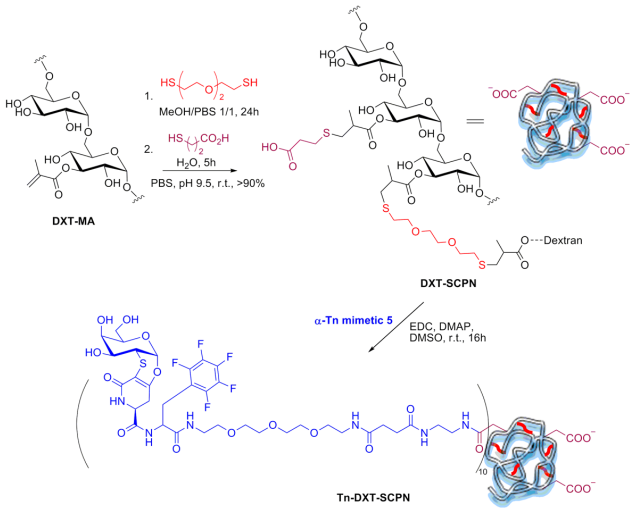
As anticipated, the host response and tolerance to nanoparticles depend on their interactions with the innate immune system, thus it is of key importance to know how nanoparticles are perceived by immune cells of the in-born immunity system.[30] To prove *in vitro* the biomimetic properties of the Tn mimetic loaded onto DXT-SCPNs, we stimulated human peripheral blood mononuclear cells (PBMC) aiming at triggering similar innate immune responses as the natural Tn clusters. It has been reported that mucins secreted from colon cancer cells are able to induce the secretion of interleukin 6 (IL-6) in peripheral blood monocytes.[31] In addition, the macrophage galactose-type C-type lectin receptor (MGL), having a specificity for terminal/linked GalNAc residues (including the Tn antigen)[32,33] has been described as a key lectin involved in the signaling cascade and toll-like receptors (TLR) cross-talk on human antigen-presenting cells.[34] We thus investigated whether DXT-SCPNs functionalized with the Tn-antigen mimetic **5** (Tn-DXT-SCPNs) are able to enhance the TLR-2-mediated IL-6 and IL-10 secretion in human PBMC triggering a specific cross-talk between Tn-receptors (such as MGL) and TLR.

The synthesis of the Tn mimetic **5** is described in Scheme 1. Briefly, compound **1a**[35] was coupled with the fluorinated spacer **2** and the acetyl derivative **3** obtained (63%) was transformed into **4** (> 90%) after removal of the acetyl protecting groups (see SI). The NBoc-protecting group of **4** was removed in acidic conditions (TFA) immediately before performing the reaction with the nanoparticles (see below). The spacer **2** has been chosen for the presence of the perfluorinated aromatic ring which allowed to take advantage of ^{19}F as internal NMR probes for the monitoring of the loading of the nanoparticles with the Tn-mimetic and for the estimation of the amount of Tn antigen loaded by UV spectroscopy (see SI).



Scheme 1. Synthesis of the Tn-antigen mimetic 5.

DXT-SCPNs functionalized with mercaptopropionic acid (MPA) were prepared as described[29] but without isolating the intermediate in a novel "one-batch" protocol (Scheme 2). Briefly, a dextran-methacrylate derivative (DXT-MA) with 52% degree of substitution (MA groups per repeating glucose unit) was reacted with the homobifunctional cross-linker 3,6-dioxa-1,8-octane-dithiol in aqueous media. After 5 hours, *in situ* addition of MPA allowed for the incorporation of carboxylic groups into the DXT-SCPN (see SI). These nanoparticles showed a number-average diameter of 12 nm as measured by transmission electron microscopy (TEM) over 100 nanoparticles and a hydrodynamic diameter of 16 nm (Z-average) as measured by dynamic light scattering (DLS) (Figures S8-S10). Tn mimetic-loaded dextran-based single-chain polymeric nanoparticles (Tn-DXT-SCPNs) were obtained by covalent coupling (Scheme 2) of the Tn mimetic amino-derivative **5** (generated by treating **4** with TFA and used without further purification) with DXT-SCPNs, whose carboxylic acid moieties were pre-activated with EDC coupling agent. After purification by dialysis against deionized water, the Tn-DXT-SCPNs were freeze-dried. Of note, compound **5** is not soluble in water, while the corresponding Tn-DXT-SCPNs could be easily redispersed in aqueous buffer (PBS, pH 7.4) or saline without flocculation.



Scheme 2. "One-batch" preparation of dextran-based single chain nanoparticles (DXT-SCPNs) and further functionalization with the Tn-antigen mimetic 5.

DLS measurements showed an increase of the hydrodynamic diameter (Z-average ~70 nm, Figure 2a) with respect to the starting DXT-SCPNs (Z-average ~16nm, Figure S8). The increase in diameter could indicate slight aggregation, but the aggregates remain in the nanometer range. TEM micrographs indicated an average diameter of 42 nm in the dry state (**Errore. L'origine riferimento non è stata trovata.**b). ^{1}H NMR of Tn-DXT-SCPNs showed a diagnostic signal at 5.75 ppm corresponding to the anomeric proton of the galactose moiety of the Tn mimetic (Figure S11), indicating a successful coupling. The ^{19}F NMR spectra confirmed the loading of the fluorinated mimetic on the surface of the DXT-SCPNs (Figure S16). The loading of Tn mimetic **5**, estimated by UV spectra (absorption at 280 nm), was 23 wt%, which corresponds to ~10 Tn units/nanoparticle.

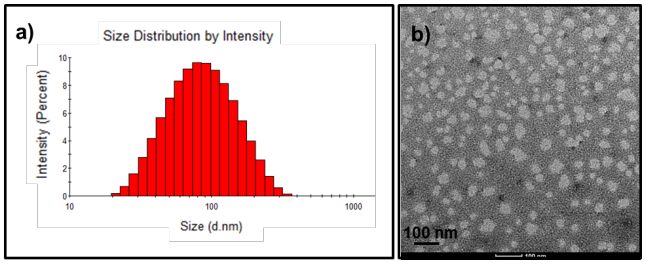


Figure 2. Characterization of Tn-DXT-SCPNs: a) DLS (NaCl 1 mM, 25 ºC); b) TEM micrograph after uranyl staining.

Dextran has been employed in several biomedical applications due to aqueous solubility, biodegradability, biocompatibility, wide availability, ease of functionalization and PEG-like non-fouling properties.[36] The absence of toxicity *in vitro* of both nanoparticles was tested with HeLa cells in a standard MTS assay. In agreement with previous results,[29] after 48h, none of the tested concentrations significantly reduced cells viability when compared with non-treated cells (Figures S17 and S18, see SI for details).

The Tn antigen is a highly specific human TACA and the Tn glycosylation of glycoproteins is able to modulate both B and T cell immunology.[37] It is also well known the enhanced antigenicity of carbohydrate-related cancer antigens when they are displayed on nanocarriers such as dendrimers, liposomes,[38] and gold nanoparticles.[16] Hence, Tn mimetic-loaded dextran-based nanoparticles prepared in this work were used to stimulate human PBMC *in vitro*.

Before performing the innate immunity experiments, the nanoparticles were preliminary assessed for possible endotoxin contamination using reporter human embryonic kidney (HEK-293) cell lines expressing TLR-2 or TLR-4 receptors. The nanoparticles did not trigger TLR-2 or TLR-4 stimulation proving the absence of detectable endotoxins (Figure S19). This is an essential pre-requisite to avoid misleading interpretation of the *in vitro* results, as endotoxins are known to promote different chemokines secretion.

Human PBMC isolated from healthy donors (n=3) were stimulated for 24 hours or 4 days with Tn-DXT-SCPNs (DXT-SCPNs were used as control. Tn-DXT-SCPNs were able to trigger the secretion of IL-6 in a similar way as the positive control LPS (Figure 3A) while non-functionalized particles induced no IL-6 secretion. It means that only Tn-DXT-SCPNs are able to stimulate the innate system eliciting the production of IL-6 as already demonstrated for mucin glycoproteins.[31] Recently, it was reported that the induction of MGL signaling (a CLR that recognizes the Tn antigen) elicits a TLR-2-mediated response and enhances the secretion of different interleukins.[34] Thus, we checked whether Tn-DXT-SCPNs could raise a similar series of innate immune responses, leading to a cross-talk between TLR-2 and CLR. As described in Figure 3B, PBMC co-stimulated with Tn-DXT-SCPNs and the synthetic triacylated lipopeptide Pam_3CSK_4 (TLR-2 ligand) triggered higher amount of IL-6 compared to PBMC treated with Pam_3CSK_4. Additionally, a similar cross-talk was observed with PBMC co-stimulated with LPS (TLR-4 ligand) and Tn-DXT-SCPNs showing an enhancement of IL-6 secretion. These data showed that the biocompatible Tn-DXT_SCPNs do mimic *in vitro* the behavior of naturally-occurring Tn clusters eliciting similar innate immune responses.[34]

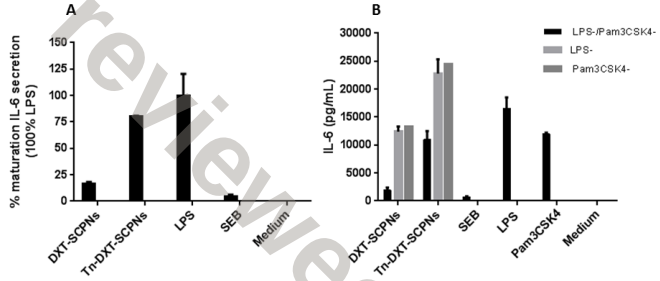


Figure 3. IL-6 analysis by ELISA on human PBMC stimulated with DXT-SCPN and Tn-DXT-SCPN. SEB (*Staphylococcus aureus Enterotoxin B* Superantigen) was used as positive control. Significant differences between DXT-SCPNs and Tn-DXT-SCPNs were calculated showing $p < 0.01$.

The cross talk between Tn-antigen receptors and TLR-2 was also studied by measuring the secretion of IL-10 (Figure 4A). Tn-DXT-SCPNs enhances the TLR-2-mediated IL-10 secretion, a phenomenon previously reported for human monocyte-derived dendritic cells.[34] DXT-SCPNs used as control, did not up-regulate the IL-10 secretion, as observed for the medium (Figure 4A). In addition, to study the autologous T-cells secretion of IFN-γ, PBMC were stimulated for longer period (4 days). As reported in Figure 4B, Tn-DXT-SCPNs were capable of stimulating the secretion of IFN-γ after 4 days of incubation. Conversely, non-functionalized particles did not induce any stimulation of IFN-γ (Figure 4B). These results

clearly demonstrated the potentials of Tn-DXT-SCPN in activating T-cell *in vitro* (Figure 4B).

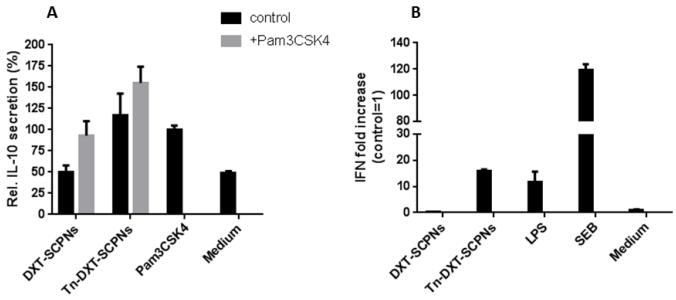


Figure 4. IL-10 (A) and IFN-γ (B) analysis by ELISA on human PBMC stimulated with DXT-based nanoparticles (n=3; combined data of three independent donors are shown). SEB was used as positive control. Significant differences between DXT-SCPNs and Tn-DXT-SCPNs were calculated showing $p < 0.01$.

In conclusion, the present work demonstrated the advantageous use of water-dispersible and biocompatible single-chain polymer nanoparticles based on dextran polysaccharide (DXT-SCPNs) as novel nanocarriers for the immune-presentation of a promising Tn-antigen mimetic. The structurally rigid Tn mimetic **1** showed stability *in vivo* (higher than that of the native antigen) and if suitably conjugated, immunogenicity against cancer.[10] Herein we showed that multimerization of the suitably functionalized Tn-antigen mimetic **5** on DXT-SCPNs affords water dispersible and relatively small nanoparticles that triggers a series of innate immune responses in human PBMC cells (*i.e.* enhancement of the TLR-2-mediated IL-6 and IL-10 secretion) like those elicited by naturally-occurring Tn clusters. DXT-SCPN nanocarriers used efficiently presented Tn mimetic **1** to the immune system. They may represent a conducive alternative to immune proteins and a unique chemical tool to better understand the role of the multivalent presentation of Tn-like antigens, including signaling and cross-talk between different innate receptors.

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website at:
Materials and Methods, *in vitro* tests, synthesis and spectroscopic characterization of compounds **2-9**, preparation and characterization of DXT-SCPNs and of Tn-DXT-SCPNs (PDF)

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Notes

PBMC from healthy volunteer donors upon their consent in accordance with the Declaration of Helsinki. The authors declare no competing financial interest.

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Biocompatible and water-dispersible dextran-based single-chain nanoparticles (DXT-SCPNs) present a structurally rigid a-Tn antigen mimetic and enhance TLR-2-mediated IL-6 and IL-10 secretion in human peripheral blood mononuclear cells.