

Peptidic nanoparticles for cancer therapy and diagnosis

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ABSTRACT

A challenging topic in cancer research is to create drug system that can deliver in a specific and non-cytotoxic manner a therapeutic compound. Usually, tumor targeting requires very specific compounds. Currently, peptide analogues like somatostatin, neurotensin, or bombesin are used to target G-coupled receptors, which are over-expressed on tumor cells.

We have recently designed a novel type of nanoparticles with regular icosahedral symmetry and a diameter of about 16 nm, which self-assemble from single polypeptide chains [1]. Here we propose an innovative drug targeting and delivery system based on such nanoparticles functionalized with somatostatin or bombesin as targeting units. Moreover, these nanoparticles can be radiolabeled with Technetium-99m or other radionuclides for diagnosis and treatment of cancer.

Keywords: peptide nanoparticles, self assembly, cancer targeting, drug delivery, drug targeting

1. INTRODUCTION

Due to the limited efficiency and high toxicity of conventional chemotherapy different strategies have been developed for non-cytotoxic cancer treatment and cancer localization [2-4]. Currently, many somatostatin synthetic analogues are developed and tested, however, many of those somatostatin analogues are rapidly degraded in the plasma and are cytotoxic [5-6]. The recent development in bio-nanotechnology offers new avenues for cancer therapy. A lot of studies have been devoted to nanoparticulate delivery systems (10-100nm) like lipid or polymer particles [7-9]. Due to the nanometer sized of such cargos, the transportation of therapeutic compounds in the blood stream is increased in terms of time circulation. But their surface functionalization to improve drug targeting properties is usually complicated and rather uneffective.

Here we describe a novel type of nanoparticle, namely peptide nanoparticles functionalized with somatostatin or bombesin peptides for drug targeting. Moreover, the nanoparticles can easily be radiolabeled with Technetium-99m or other radionuclides for treatment or visualization of cancer. These peptidic nanoparticles are formed by self-assembly of a specific peptide sequence that consists of the

pentameric coiled-coil domain of Cartilage Oligomerization Matrix Protein (COMP) [10] and a *de novo* designed minimal trimeric coiled-coil domain, which are joined by a two-glycine residue linker segment. The nanoparticle size can be varied by extending the length of the trimeric coiled-coil domain. Finally, the nanoparticles can be functionalized by insertion of a peptide sequence (somatostatin 28 or 14, bombesin, etc.) at either end of the particle-forming peptide. Such peptidic nanoparticles will be mechanically stable and have a high loading capacity. Furthermore, they are fully biocompatible as they are built up from biogenic building blocks (amino acids). For large-scale production and for efficient functionalization of the nanoparticles, the peptides are produced using a recombinant protein expression system (*E. coli*). The assembly properties of the nanoparticles are monitored by transmission electron microscopy (TEM), fluorescence correlation spectroscopy (FCS), analytical ultracentrifugation and dynamic light scattering (DLS).

2. CONCEPT

There are only five regular polyhedra, the tetrahedron, the cube, the octahedron, the dodecahedron and the icosahedron. The icosahedron is built up from 60 asymmetric units and is the largest closed shell in which every subunit is in an identical environment [11]. These asymmetric units are tri-pyramids and each of the pyramid edges corresponds to one of the rotational symmetry axes. The icosahedron has 2-fold, 3-fold and 5-fold rotational symmetry axes (see Fig. 1.a). If the symmetry axes of peptidic oligomerization domains are superpositioned onto the edges of these tri-pyramids, such three dimensional building blocks can be built up from peptidic oligomerization domains (see Fig. 1.a). As a consequence, by a superposition of the different oligomerization domains onto the symmetry axes of the icosahedron, a peptide nanoparticle can be built up from peptidic oligomerization domains (see Fig. 1.c). In our design the protein oligomerization domains where chosen from the Cartilage Oligomerization Matrix Protein (COMP) [10] which forms pentamers, and from a *de novo* designed sequence which forms a trimer. These two domains are connected via a short peptide linker to form one single peptide chain. Finally, at either end this peptide chain can be extended with peptide sequences with a specific function (epitopes, targeting peptides), or a drug or a molecular marker

(radionuclides, fluorescent probes, etc.) can be covalently attached to it and hence be presented at the surface of the particles (see Fig.2). The high density of ligands on the particle surface will increase the affinity for the specific receptor due to cooperativity of binding.

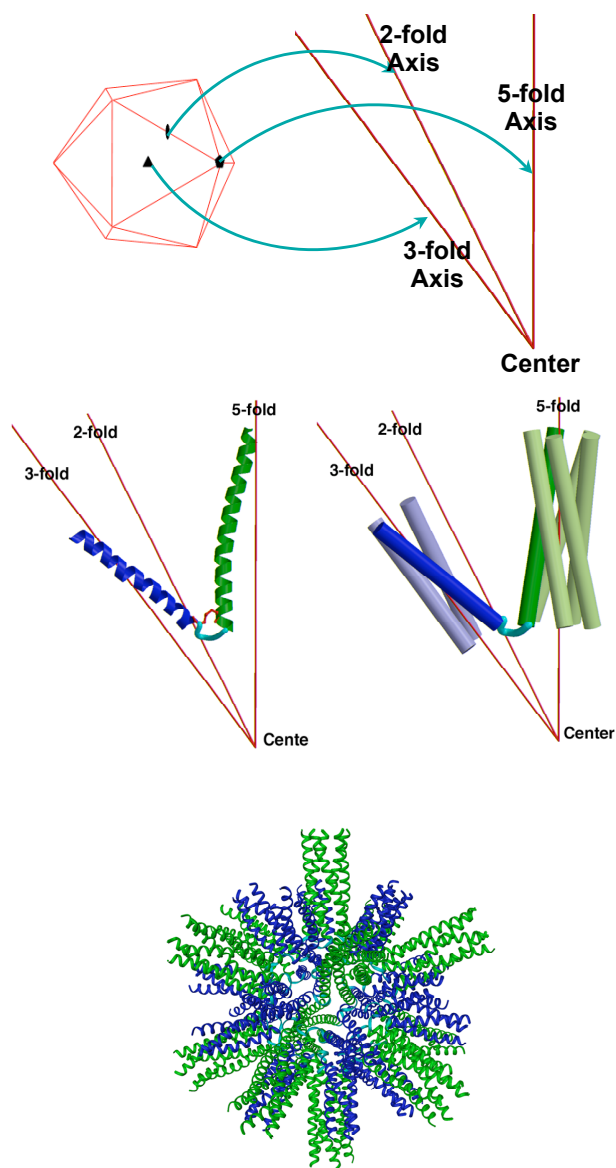


Figure 1: Basic concept of the nanoparticle design. **a:** The icosahedron is composed of 60 identical three-dimensional building blocks (asymmetric units) related by 2, 3, and 5-fold symmetry elements. **b:** A three dimensional building block composed of a coiled-coil domain with 3-fold symmetry (blue), a linker segment (cyan) and a coiled-coil domain with 5-fold symmetry (green) is aligned such that the internal symmetry elements of the two domains are superimposed onto the symmetry axes of the polyhedron. **c:** A peptidic nanoparticle assembled from 60 peptide chains.

3. RESULTS AND DISCUSSIONS

3.1 Building block design, expression and purification

According to the nanoparticle assembly principle, peptidic nanoparticles were rationally designed for drug targeting and drug delivery (see Fig. 2). For large-scale production and for efficient functionalization of the nanoparticles, the peptides are produced using a recombinant protein expression system (*E. coli*). The DNA sequences were cloned in a standard vector.

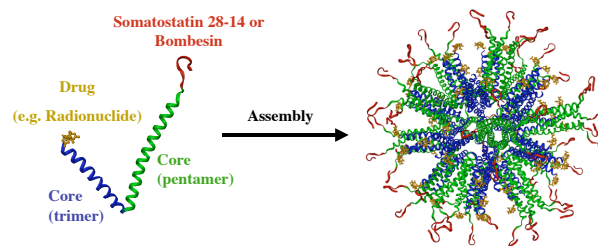


Figure 2: Functionalized nanoparticles (right) formed by self-assembly of peptides (left). The pentameric coiled-coil of the core particle is extended by a ligand and the trimeric coiled-coil of the core particle is extended by a drug.

The N-terminal domain of the core peptide (Fig. 2) corresponds to a modified pentameric coiled coil domain of COMP. The C-terminal domain corresponds to a *de novo* designed minimal trimeric coiled-coil domain [12-13]. A linker segment consisting of two glycine residues joins the two domains. Two cysteine residues are optimally placed to generate an intramolecular disulfide bridge between the two oligomerization domains. A second minimal trimeric domain, called extension I is inserted into the peptide sequence (Scheme 1). The aim of this extension is to increase the particle size and hence to improve the presentation of the different somatostatin or bombesin peptides on the surface of the nanoparticles. The peptide purification is performed under denaturing conditions, in presence of 8M urea and β -mercaptoethanol, using affinity chromatography (see Fig. 3). Due to the strong interaction of trimeric coiled-coil domain, the peptide forms trimers even under denaturing conditions as used for the preparation of the SDS page.



Scheme 1: Peptide sequences of the somatostatin 28, somatostatin 14 and bombesin nanoparticle constructs.

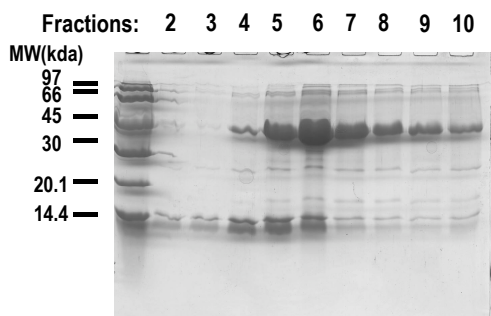


Figure 3: 15 % SDS page of the peptide purification (pH 8+ 1M Imidazole). Fraction 1, 2: monomeric form of the peptide. Fraction 3-7: monomeric and trimeric form of the peptide

3.2 Nanoparticle Self-Assembly

The refolding of the peptide into nanoparticles is performed by a stepwise dialysis of urea under reducing conditions. The assembly behavior of the nanoparticles is analyzed by analytical ultracentrifugation. Our preliminary results run at a peptide concentration of 0.1mg/mL gives a Svedberg constant of about 30S for the somatostatin 28 peptide, which corresponds to a molecular weight of the nanoparticles of roughly 900 kDa. This in turn is in agreement with nanoparticles being composed of about 60 peptide chains and hence confirming icosahedral symmetry of the nanoparticles. 30-40% of the protein fraction shows higher sedimentation constants indicating that a certain fraction of the peptide forms larger aggregates as can also be seen on electron micrographs.

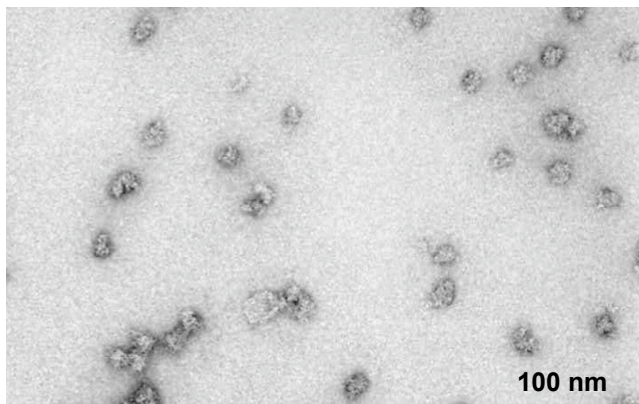


Figure 4: Electron micrograph of somatostatin 28 nanoparticles (0.5mg/mL)

The morphology of the nanoparticles with somatostatin and without somatostatin were also investigated by transmission electron microscopy (TEM, see Figs. 4 and 5). Furthermore, the polydispersity of the nanoparticles in solution was then analyzed by dynamic light scattering (DLS) and fluorescence correlation spectroscopy (FCS). Different conditions and parameters are currently tested for optimal refolding and assembly behavior of the nanoparticles. We are varying the peptide concentrations,

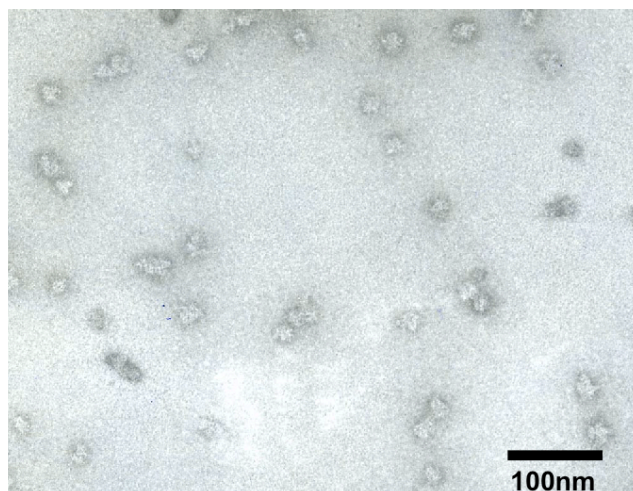


Figure 5: Electron micrographs of nanoparticle without somatostatin (0.5mg/mL)

the ionic strength and the composition and the pH of different buffers, etc. Successive dialyses allow the correct peptide refolding and the self-assembly into nanoparticles.

Nanoparticles self-assembly was further confirmed by electron microscopy of negatively stained samples. The electron micrographs nicely revealed particles of the expected size of 20-25nm that correspond to the calculated diameter of a computer model of the nanoparticle. Mostly, the nanoparticles are also single particles of discrete size and spherical shape.

Dynamic light scattering (DLS) investigation on nanoparticle solutions results in a hydrodynamic radius of 27 nm a for nanoparticles without somatostatin and a slightly larger radius of 31 nm for the somatostatin labeled nanoparticles. Somatostatin is indeed expected to increase the size of the nanoparticles a little. The diameters of the nanoparticles determined by DLS is larger as compared to electron micrographs because the electrostatic interactions due to the presence of salt in the solution contributes to an increased apparent size of the nanoparticles. Moreover, the peptidic nanoparticles cannot be considered as perfect spheres. Indeed, the particles surface is not regular due to

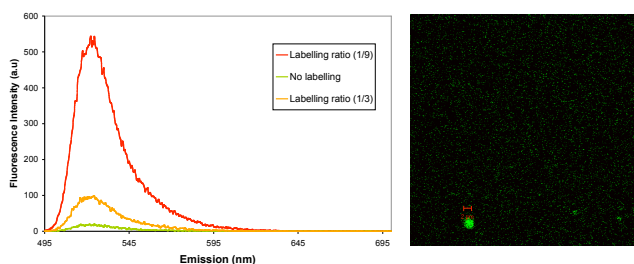


Figure 6: Emission spectra (left) and fluorescence micrograph (right) of labeled nanoparticle solutions excited with 488 nm beam line. The solutions were prepared with a particle/dye ratio of 1/3 and 1/9. The peptide concentration is 0.1 mg/mL.

the ending composition of the peptide N-and C termini (see Fig. 1 and 2). The polydispersity of the investigated solutions was determined to be about 20% from DLS that is a reasonable value in agreement with value reported for conventional lipid or polymer vesicles [14-16].

Fluorescence correlation spectroscopy (FCS) measurements were performed to obtain complementary information on the size and polydispersity of nanoparticles labeled with a fluorescence dye [17]. The nanoparticles were selectively labeled with Alexa-488 (labeling was performed according to the manufacturer's guidelines) at the N-terminus of the nanoparticle peptide. The labeling is checked by fluorescence spectroscopy and confocal microscopy (see Fig. 6).

The fluorescence correlation spectroscopy (FCS) technique gives a time diffusion of around 500 ms for 60% of the solution, which correspond to a nanoparticle diameter of 20 nm. Some larger aggregates are also observed, probably due to the electrostatic interactions between the particles. The results demonstrate that the structure of the nanoparticles and their self-assembly is not disturbed when small molecular markers are attached to it. A similar labeling procedure will therefore also be applied for radioactive compounds and it will allow us to monitor the nanoparticle in *in vivo* conditions.

4. CONCLUSIONS AND OUTLOOKS

The main objective was to develop a prototype of self-assembling functionalized peptidic nanoparticles, which can be used as a drug targeting and delivery system for the visualization and treatment of cancer. According to the computer design we obtain nanoparticles with predicted biophysical characteristics. The addition of peptide ligands, like somatostatin, or fluorescent probes does not interfere with the assembly behavior of the nanoparticles. The presence of somatostatin in many copies on the surface of the nanoparticles will considerably increase the selectivity for the target cells due to cooperativity of binding. As a consequence the concentration of radionuclide can be reduced and the side effects can be limited. The next step of the work will be the functional studies of the nanoparticles in *in vitro* and *in vivo* conditions.

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