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Design and Solid Phase Synthesis of New DOTA Conjugated (+)-Biotin Dimers Planned to Develop Molecular Weight-Tuned Avidin Oligomers†

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Chemical modifications of the biotin carrier in pretargeted avidin-biotin radionuclide therapy may be of a paramount importance for tuning the amount of the radioactivity delivered to the cancer cells by the labelled biotins. We report here the synthesis of a collection of new synthetic DOTA-constructs bearing two (+)-biotin molecules (bis-biotins), designed for the creation of multimeric Av units (tetramers) bonded to the antibody. All the syntheses were carried out following the solid phase strategy, and growing up the molecules on a Rink Amide resin. The biotin heads are connected through spacers containing PEG or non-PEG residues. Molecular modelling calculations suggested that the Av cross-linking ability of the bis-biotins depends mainly on the spacers' length, the best results being expected for arms affording distances in the range of 10-25 Å between the biotin carboxylate atoms, in the fully extended conformation. SEC-HPLC MALLS analysis of the products of our Av/bis-biotins reaction mixtures confirmed this hypothesis. The bis-biotin **16**, where the non-PEG linker ensured a distance between the biotin moieties of 26.7 Å, gave about 50% of Av oligomers while the shorter analogue **18** (19.5 Å) afforded 100% of a Av polymer containing about 21 protein units. Remarkably, both the bis-biotins i.e. **16** and **18** were well soluble in aqueous solutions and showed an excellent stability against the action of peptidases.

Introduction

Cancer radioimmunotherapy (RIT), employing targeted monoclonal antibody–avidin (or streptavidin) conjugates (MoAbs-Av), and biotin derivatives carrying macrocyclic chelators, i.e., the tetraazamacrocycle DOTA, disclosed new horizons in the treatment of malignant tissues.[1,2,3] Pretargeting protocols, based on the avidin/biotin system, are currently used in clinical oncology. In particular, it has been extensively reported, either in preclinical and clinical studies, that two-step or three-step pretargeting approaches based on the strong binding of (+)-biotin head with avidin pockets (see Fig. 1) result in improved tumor-to-non tumor ratios, compared to the more conventional one-step with MoAbs.[4,5]

We previously reported the synthesis of a mono-biotin derivative (r-BHD), conjugated to the DOTA chelator and designed to be resistant to serum-biotinidases. r-BHD turned out to be a good candidate for tumor diagnosis and therapy

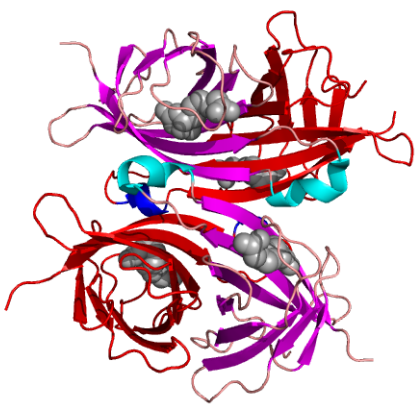


Fig. 1. The tetrameric protein contains four identical subunits (homotetramer – PDB entry 2avi), each of which can bind to (+)-biotin (depicted in gray) with a $K_D \approx 10^{-15}$ M. In its tetrameric form, avidin is estimated to have a molecular weight of about 66 kDa.

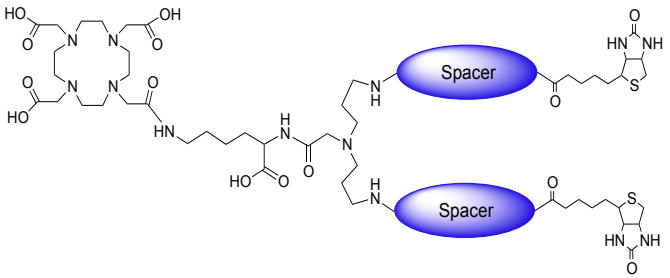
when used in the context of the pretargeting strategy.[6,7] However, in the three step pretargeting technique only a small percentage of radiolabeled biotin really localizes on the tumor region.[8] Recently, Paganelli's group pioneered a novel application of ^{90}Y-DOTA-biotin targeted radionuclide therapy in early breast cancer patients named IART.[9,10] In the Intraoperative Avidination for Radionuclide Therapy (IART®), the biotinylated MoAbs are not involved and native avidin is directly injected by the surgeon, in and around the tumor bed immediately after tumor removal, followed by ^{90}Y-DOTA-biotin targeting molecule. Inflammation due to surgery results into the residual breast tissue becoming a cation exchange material, so that avidin is held in that tissue for several days providing a target for one-day-later radiolabeled biotin *i.v.* injection.[11]

Notably, when surgery is not performed, particularly in the case of bladder[12] and prostate cancer,[13] intravesically-administered avidin followed by radiolabeled biotin was selectively accumulated in superficial bladder tumors compared to normal mucosa. Despite this fact, the rapid washout of the complex from the tumor limited the therapeutic outcomes[14] of the direct injection of avidin and confirmed the need of a longer in-situ binding between avidin and the radioactive biotin. An approach directed to increase the radiation/dose ratio was attempted in our laboratory where we synthesized a series of biotin derivatives carrying two DOTA moieties per molecule.[8] Unfortunately, subsequent measurements regarding the cellular uptake in different tissues did not show any improvement compared to r-BHD (personal communications), because these molecules were not able to overcome the major problem of the rapid washout. Alternatively, multivalent binding of the radioisotope carrier to avidin could be used for generating oligomeric architectures of higher molecular weight, capable to extend the residence time on the tumor surface and to increase the amount of the radiating constructs on the cells. Wilbur *et al.* attempted this method and prepared rigid di- or tri-functionalised benzene ring scaffolds bearing two or three biotin moieties *via* PEG spacers.[15] However, only a low extent of r-SAv oligomers, not useful for pre-targeting protocols, was formed by these biotin dimers.[15] On the contrary the molecules containing three biotin residues afforded only a polymer of r-SAv of not defined molecular weight, which the authors claimed to be of very difficult clearance after the labeling.

However, the ion exchange mechanisms of bladder tumor uptake strongly suggested that right sized, oligomeric architectures of avidin tetramers cross-linked by DOTA-bis-biotins, could well interact with the negatively charged tumor tissues and lectine molecules present on tumor cell membrane,[11] increasing the residence time of the labeled avidin.

With this in mind, we decided to re-evaluate this issue and we designed and synthesized a novel class of biotin dimers containing flexible spacer arms between the biotin heads, easy to be sized, in order to evaluate their in vitro effectiveness on the avidin polymerization. *In silico* calculations, performed starting from the crystallographic structure of the Av tetramers,

Chart 1. General structure for the bis-biotin compounds.



gave us suggestions on the nature and length of the spacer arms connecting the two biotin molecules.

The new bis-biotins prepared in our laboratories were engineered around a tri-functional flexible junction (spacer-C_3) conjugated to the DOTA chelating agent by a Lys residue and to the biotin heads through different arms of variable length and different chemical nature, ensuring the optimal solubility in aqueous solution, and an excellent resistance to the biotinidases action. The general formula of the bis-biotins is highlighted in Chart 1. The selected spacers were PEG or non-PEG residues, symmetrically arranged around the tri-amine junction linked to DOTA via the Lys residue.

Moreover, in our research the cross-linking efficiency of all the synthetic bis-biotins were investigated measuring the amount and the nature of avidin oligomers by multi-angle laser light scattering (MALLS) coupled to size-exclusion chromatography equipped with a Uv-Vis detector.

Results and discussion

Molecular Modelling

Fifteen different conceptual models of bis-biotins were taken into consideration where the biotins units are connected through PEG or non-PEG spacers (Figure 1S in Supporting Information). The spacers length between the two amide C=O of the biotin residues, considered for the calculations, were in the range from 4 to 80 Å, on the basis of the fully extended, planar, conformation. For *in silico* calculations, a simplified interaction between the bis-biotin and avidin tetramers was took into account, i.e. we considered that the biotin compounds bound only two avidin subunits and not the entire tetramers, as it is schematized in Fig. 2. Therefore, in type 1 complexes two biotin heads are linked to two adjacent avidin subunits, belonging to the same tetramer, while in type 2 complexes, the bis-biotin cross-link two subunits on different tetramers.

The spatial coordinates of the avidin tetramer for the study of type 1 and 2 complexes were obtained from the Protein Data Bank (3VHH).

In order to assess their stability, the Av/bis-biotin complexes were submitted to molecular dynamics simulation at three different temperatures (150, 300 and 450 K), in implicit solvent. For each of the fifteen structures the average potential energy (E_p) was obtained from 50 conformations recorded at a constant temperature.

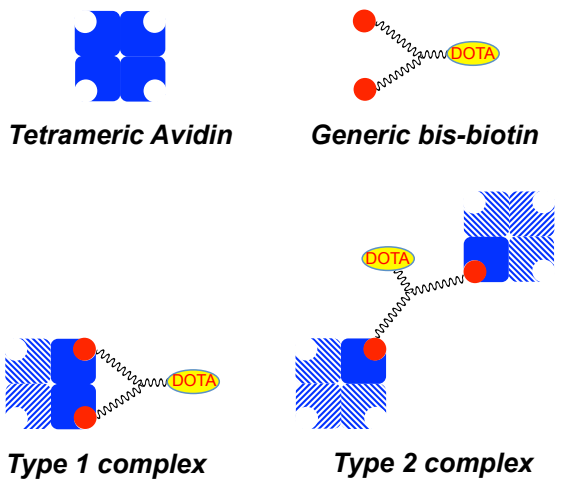


Fig. 2. Schematic representation of avidin/bis-biotin complexes. The solid-coloured portions represent the monomers of the tetrameric avidin considered in the molecular modelling calculations.

To distinguish the E_p changes due only to the molecular size of the biotinylated molecules and not to the temperature, for each temperature was calculated the potential energy difference (EpD – See Table 1S and 2S in Supporting Information) between the E_p of a single complex and the E_p of all the complexes at that temperature.

The trend of the E_pD values of the two types of avidin dimers in function to the length of the spacer arms, as above defined, is shown in the graphs in Fig. 3.

The graphics show that the Av dimers formed by the bis-biotins with the shortest spacer arms are the most stable in both complex types.

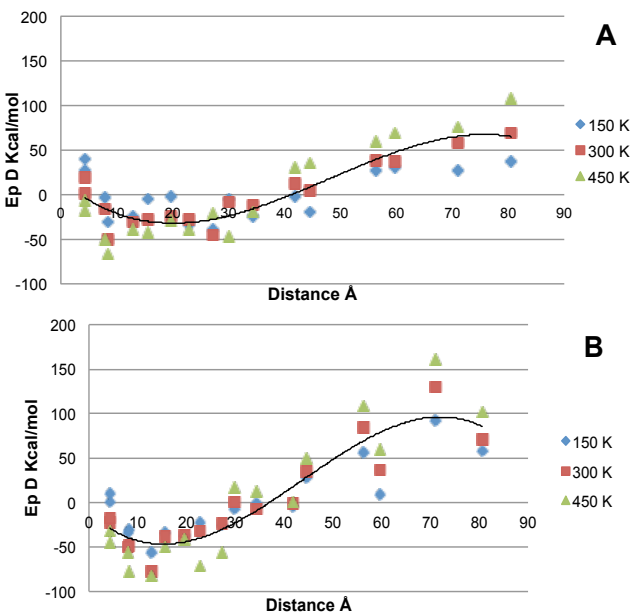


Fig. 3. Potential energy (average) of the complexes of Fig. **2** *vs* the distance between the two amide C=O of biotin residues. **A**: type 1 complexes; **B**: type 2 complexes. The value of the potential energy E_pD is the difference of potential energy of a complex, compared to the average of the potential energy of all complexes at the same temperature.

Moreover, it appears that in both complexes, with spacers characterized by very short distances (< 10 Å), the repulsive effects begin to have influence on the E_pD of the Av/bis-biotin structures. At the same time, the graph of Fig. 3 shows that the range of the variation of potential energy is greater in type 2 complexes than in type 1 complexes. Therefore, the potential energy difference (ΔE_p) between the type 2 complex and the corresponding type 1 is not constant, but depends on the length of the spacers. The trend of ΔE_p ($\Delta E_p = E_{pType2Compl.} - E_{pType1Compl.}$) is evident in the following graph (Fig. 4), highlighting that for spacer arms longer than about 25 Å, the type 1 complexes are favoured ($\Delta E_p > 0$) while for shorter distances the type 2 complexes are more stable ($\Delta E_p < 0$).

In summary, the molecular modeling calculations evidenced that while the bis-biotins containing the longest spacers favoured the formation of avidin complexes of type 1 respect to

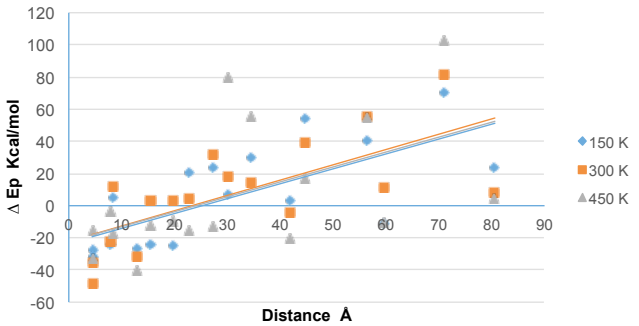


Fig. 4. Potential energy difference (ΔE_p) *vs* the distance between the biotin residues. The difference is defined as: $\Delta E_p = E_{pType2Compl.} - E_{pType1Compl.}$ Each straight line represents the trend of the potential energy difference at three temperatures. In the calculation of potential energy difference between type 2 and type 1 complexes, the energy gain due to the non-covalent bonds of the two protein chains was considered.

those of type 2, the probability of forming these latter strongly increased by shortening the chains connecting the biotin heads, and this probability was not apparently affected by the chemical nature of the spacer (*i.e.* PEG or non-PEG). Moreover, these results suggested that the optimal distances between the two biotin amide groups for obtaining a significant amount of avidin cross-linking, should be in the range of 10-25 Å.

Chemistry

An efficient synthetic pathway to the new bis-biotins was performed following the solid phase strategies. The key step in this synthetic strategy was represented by the use of suitable orthogonal protecting groups like Fmoc, *t*-Bu and Mtt. Moreover, this technique rapidly afforded the products in the range from μM to mM scale.

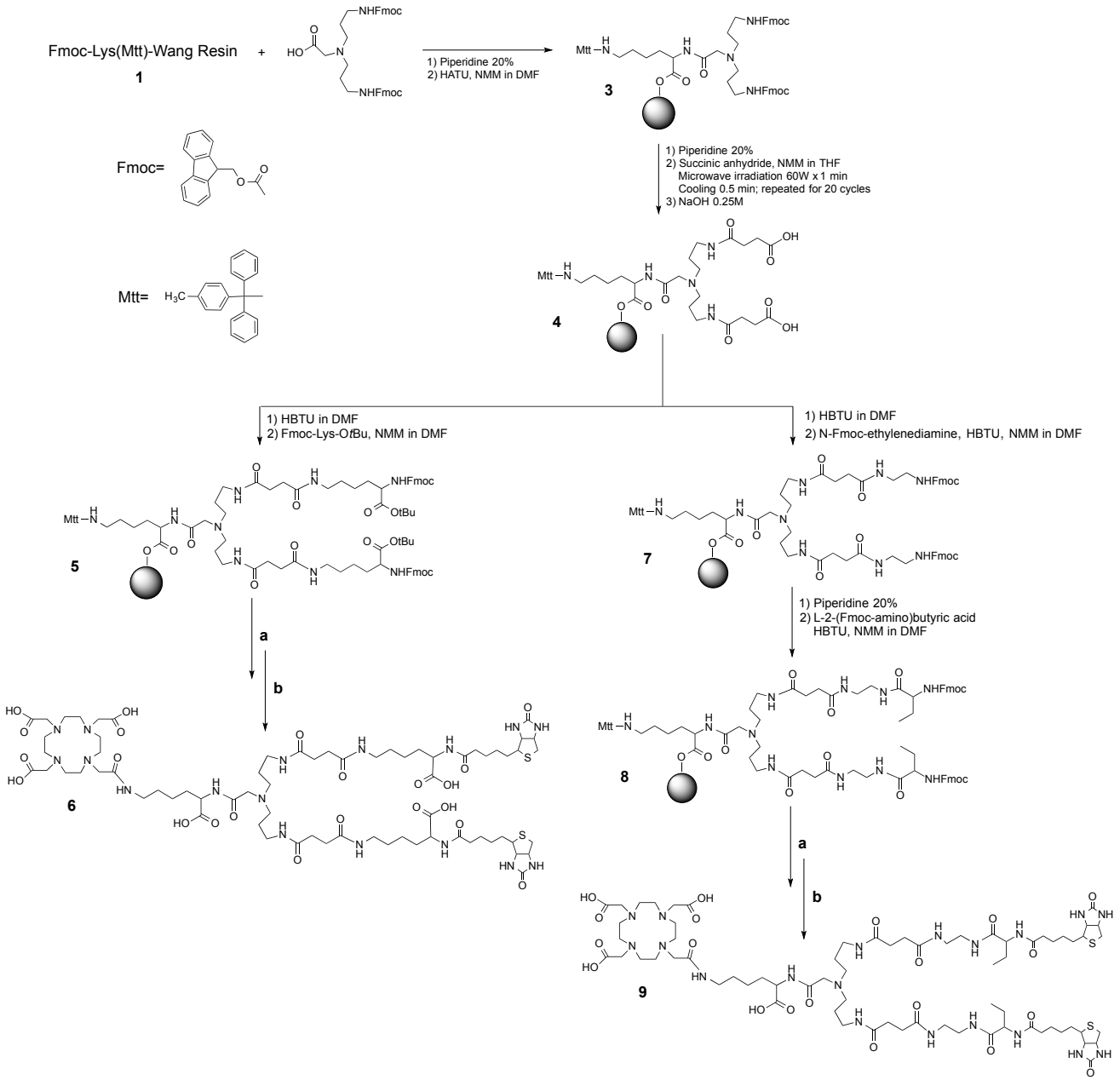
The syntheses of the new bis-biotins were carried out by anchoring the growing molecules on the Fmoc-Lys(Mtt)-Wang resin (**1**). All the coupling reactions were checked by the Kaiser test.[16] The first step, the same for all the bis-biotins, was the coupling between the bi-functional spacer *N,N*-Bis [3-(Fmoc-amino) propyl] glycine (hereafter called spacer-C_3), (**2**) (Scheme 1) with the α-amino group of Lys. Before the

coupling, the free carboxyl group of **2** was activated using HATU/NMM in DMF. The subsequent spacer elongation was carried out following the Scheme 1.

The next step was the microwave-driven coupling of the intermediate with twenty eq. of succinic anhydride which gave rise to an amide bond on a side, leaving a free carboxyl group on the other side (**4**). The literature data reported severe heating and long reaction time for this coupling.[17,18] For avoiding these experimental conditions, we decided to perform the reaction in a microwave reactor, without adding any activating agent and using anhydrous THF. The choice of this solvent was due to the fact that THF has a relatively low dispersion factor, that prevents a high growth of temperature after irradiation. In fact, during the reaction, the temperature remained between 40 and 65 °C. Under these mild conditions the reaction took place in a very short time (approx. 30 min.). As stated by the study of Stieber and Waldmann,[19] the intermediate **4** must be treated in 0.25 M NaOH in solution of THF to slight alkaline pH value, for the obtainment of the corresponding open bicarboxylate.

The presence of the carboxylic group in α position respect to the biotinamide bond, useful for blocking biotinidases activity,[20] was achieved by the coupling of Fmoc-Lys-O*t*Bu

Scheme 1. Synthetic routes to compounds **6** and **9**.



(**a**) 1) Piperidine 20% in DMF, 2 x 10 min; 2) (+)-biotin, HATU, NMM in DMF, rt, 50 min. (**b**) 1) TFA 2%, TIS 2.5% in DCM, 35 washes for 1.5 min each; 2) DOTA-3*t*Bu ester, HBTU, NMM in DMF; 3) TFA 95%, TIS 2.5%, H_2O 2.5%, rt, 3h.

(Scheme 1S) with the on-resin intermediate **4**. In the case of construct **9**, a residue of L-2-(Fmoc-amino)butyric acid was included in the growing molecule. The choice of this reagent was dictated by the evidences reported in the literature. In fact, the amino acid side chain was able to protect the amide bond with biotin, making the molecule resistant to biotinidases.[20,21] Moreover, the introduction of the hydrophobic ethyl residue was used to check a potential change in the avidin-biotin binding affinity, in comparison with the hydrophilic carboxyl group of Lys of compound **6**.

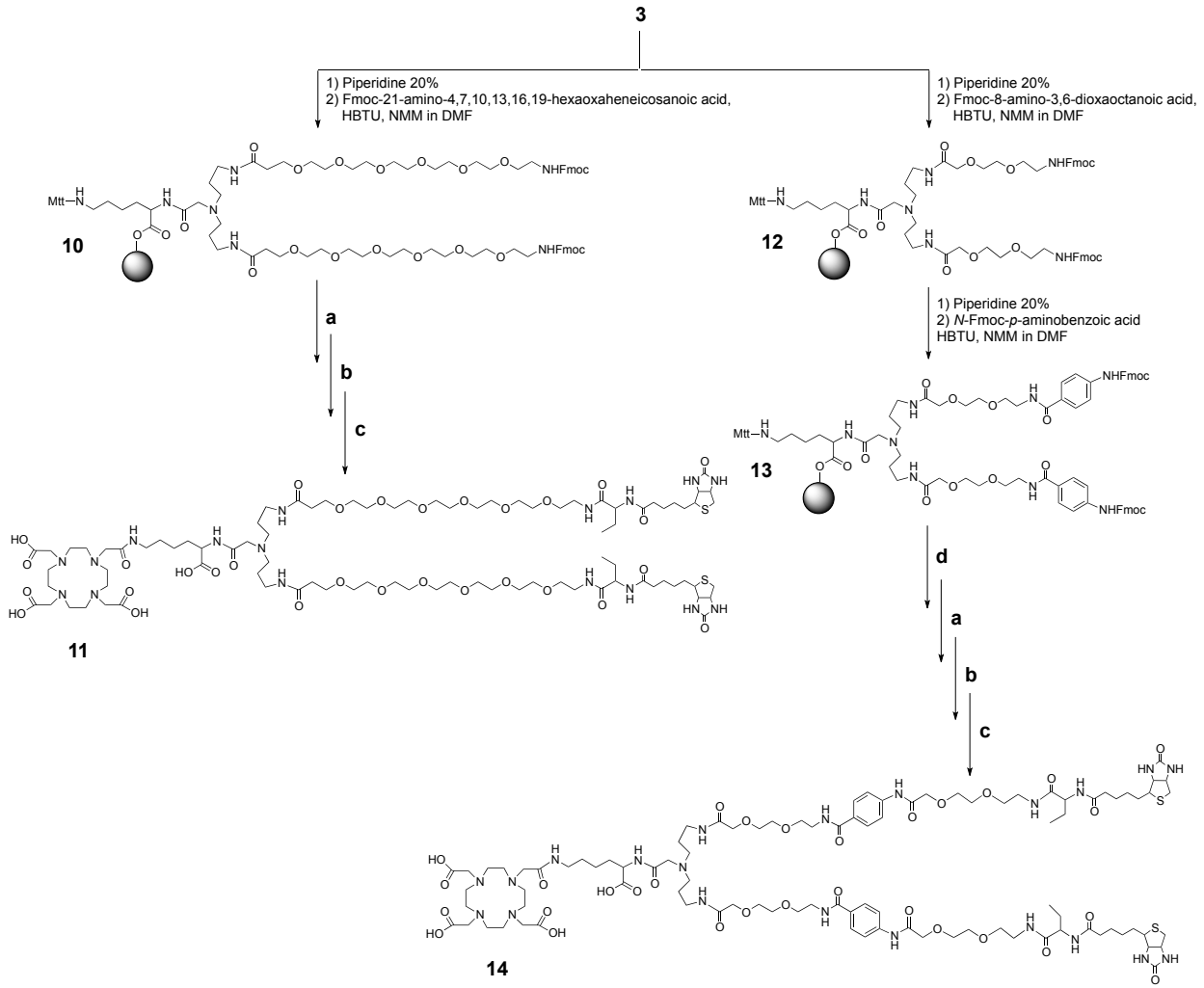
From this point, the synthetic pathway of all the bis-biotins became the same, and the elongation of the spacer arms was completed by the insertion of two residues per molecule of (+)-biotin, activated by HATU/NMM in DMF. The removal of the Mtt protecting group from the growing molecule was done in weakly acidic conditions (TFA/TIS/DCM 1:2.5:96.5). DOTA-tris-*t*Bu ester, suitably activated with HBTU/NMM was then coupled to the on-resin compound. After the reaction, the free molecules **6** and **9** were cleaved from the resin by hydrolysis with a strong acid solution (95% TFA), which also allowed the complete removal of all the *tert*-butyl protecting groups.

Compounds **11** and **14** were synthesized, as in the previous cases, starting from the intermediate **3**. The synthetic pathway of these two derivatives, however, provides some significant differences from constructs **6** and **9** (Scheme 2).

Compound **11** was obtained by the coupling of **3** with Fmoc-21-amino-4,7,10,13,16,19-hexaoxaheneicosanoic acid, suitably activated with HBTU and NMM in DMF.

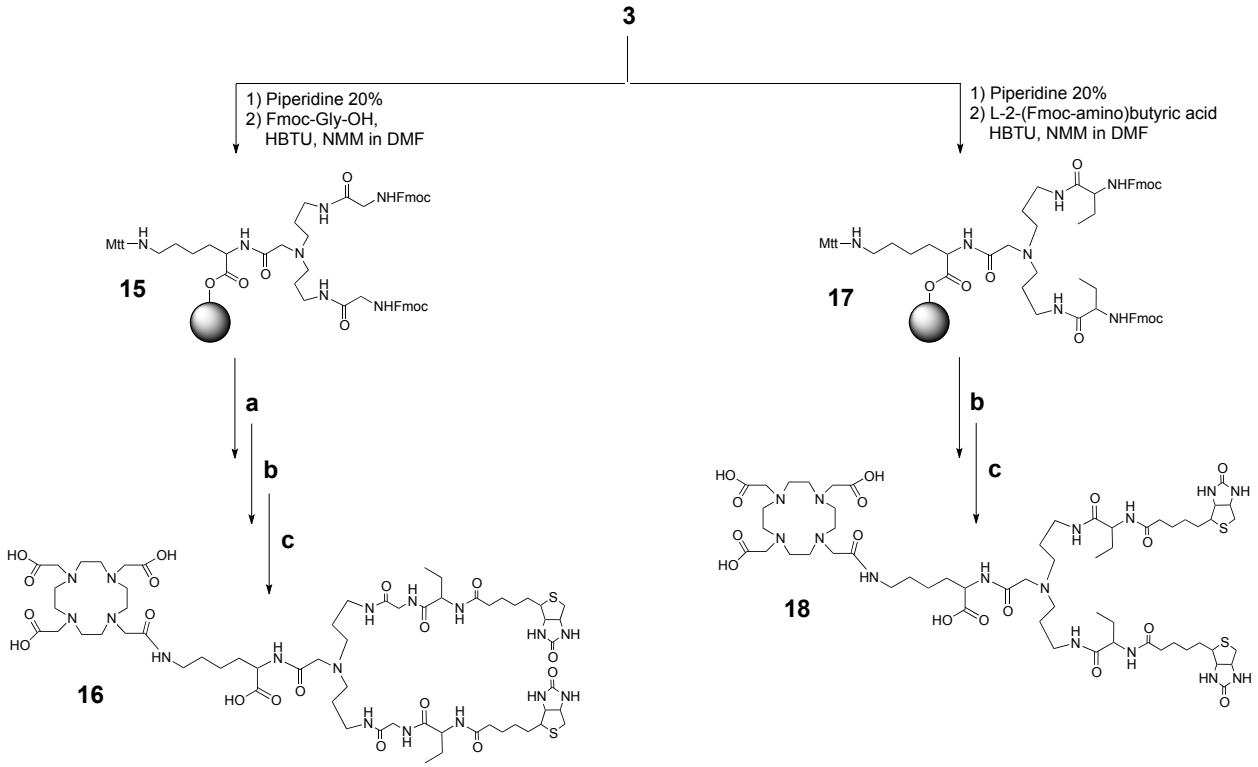
The latter reagent had the function to significantly extend, by a single synthetic step, the arms that hold the biotins and, most importantly, to reduce the fold of the aliphatic chains in a very hydrophilic medium as the physiological one.

Scheme 2. Synthetic routes to compounds **11** and **14**.



(**a**) 1) Piperidine 20% in DMF, 2 x 10 min; 2) L-2-(Fmoc-amino)butyric acid, HBTU, NMM in DMF, rt, 45 min. (**b**) 1) Piperidine 20% in DMF, 2 x 10 min; 2) (+)-biotin, HATU, NMM in DMF, rt, 50 min. (**c**) 1) TFA 2%, TIS 2.5% in DCM, 35 washes for 1.5 min each; 2) DOTA-3*t*Bu ester, HBTU, NMM in DMF; 3) TFA 95%, TIS 2.5%, H_2O 2.5%, rt, 3h. (**d**) 1) Piperidine 20% in DMF, 2 x 10 min; 2) Fmoc-8-amino-3,6-dioxaoctanoic acid, HBTU, NMM in DMF, rt, 45 min.

Scheme 3. Synthetic routes to compounds **16** and **18**.



(**a**) 1) Piperidine 20% in DMF, 2 x 10 min; 2) L-2-(Fmoc-amino)butyric acid, HBTU, NMM in DMF, rt, 45 min. (**b**) 1) Piperidine 20% in DMF, 2 x 10 min; 2) (+)-biotin, HATU, NMM in DMF, rt, 50 min. (**c**) 1) TFA 2%, TIS 2.5% in DCM, 35 washes for 1.5 min each; 2) DOTA-3*t*Bu ester, HBTU, NMM in DMF; 3) TFA 95%, TIS 2.5%, H_2O 2.5%, rt, 3h.

On the contrary, the construct **14** was designed with the aim of merging the characteristics of hydrophilicity and flexibility of PEG with the structural rigidity carried by the *p*-amino benzoic acid residue. This purpose was achieved in few synthetic passages. Initially, the compound **3** reacted with Fmoc-8-amino-3,6-dioxaoctanoic acid, activated with HBTU and NMM in DMF. Therefore, the Fmoc protected *p*-amino benzoic acid was smoothly coupled by activation with HBTU/NMM in DMF and only one coupling was necessary to obtain a negative Kaiser test. The second molecule of Fmoc-8-amino-3,6-dioxaoctanoic acid was then coupled as already described. The intermediates **10** and **13**, gave rise to the final molecules **11** and **14**, simply following the last synthetic passages common for all the bis-biotins and described in Scheme 2.

The synthesis of the last two molecules, **16** and **18**, featured by the short non-PEG spacer arms was of fundamental importance for the completeness of the set of bis-biotins to be tested. Also in this case, the starting point was the intermediate **3** that, for the compound **16**, was coupled to a residue of Fmoc-Gly-OH, as showed in Scheme 3. In case of compound **18**, which has the shortest arms length, the Fmoc-L-α-aminobutyric acid was coupled directly to the intermediate **3**. From this point, the final molecules **16** and **18** were obtained following the same synthetic steps elucidated in Scheme 3. All the new compounds were purified and completely characterized using RP-HPLC, mass spectrometry and elemental analysis.

Evaluation of the cross-linking abilities of the new bis-biotins

In accordance to the method reported by Wilbur,[15] the synthetic bis-biotins (**6**, **9**, **11**, **14**, **16** and **18**), were evaluated for their ability to induce polymerisation of Av in aqueous solution. In order to assess the proper Avidin/bis-biotin proportion, 1:0.8; 1:1.25; 1:2; 1:4; 1:8 molar ratios were tested (data not showed). However, in all cases, the Avidin/bis-biotin ratio that gave the best results was 1:1. Moreover, the polymerizations were tested also at different temperatures, but the best yields in avidin oligomers were obtained when the reaction was carried out at room temperature (about 25 °C). All the tests were performed by slowly adding 1.0 molar equiv. of the selected bis-biotin into a solution of avidin in PBS at pH 6.8, incubated at r.t. for 45 min and then analysed by SEC-HPLC. All the chromatograms were edited using Xcalibur software in order to obtain the area percentage of the various peaks. With the same method was recorded also a reference chromatogram of Av and a chromatogram of Av/(+)-biotin in 1:4 molar ratio as reference for the per-biotinylated Av. The results are showed in Fig. 5.

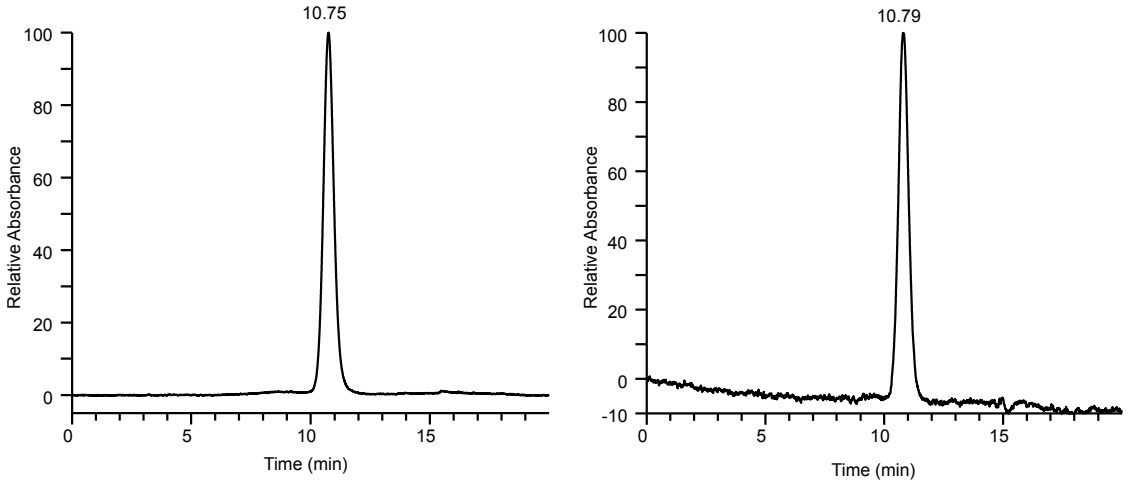


Fig. 5. Size exclusion chromatograms of avidin (left panel) and reaction mixtures of avidin with (+)-biotin in 1:4 molecular ratio (right panel).

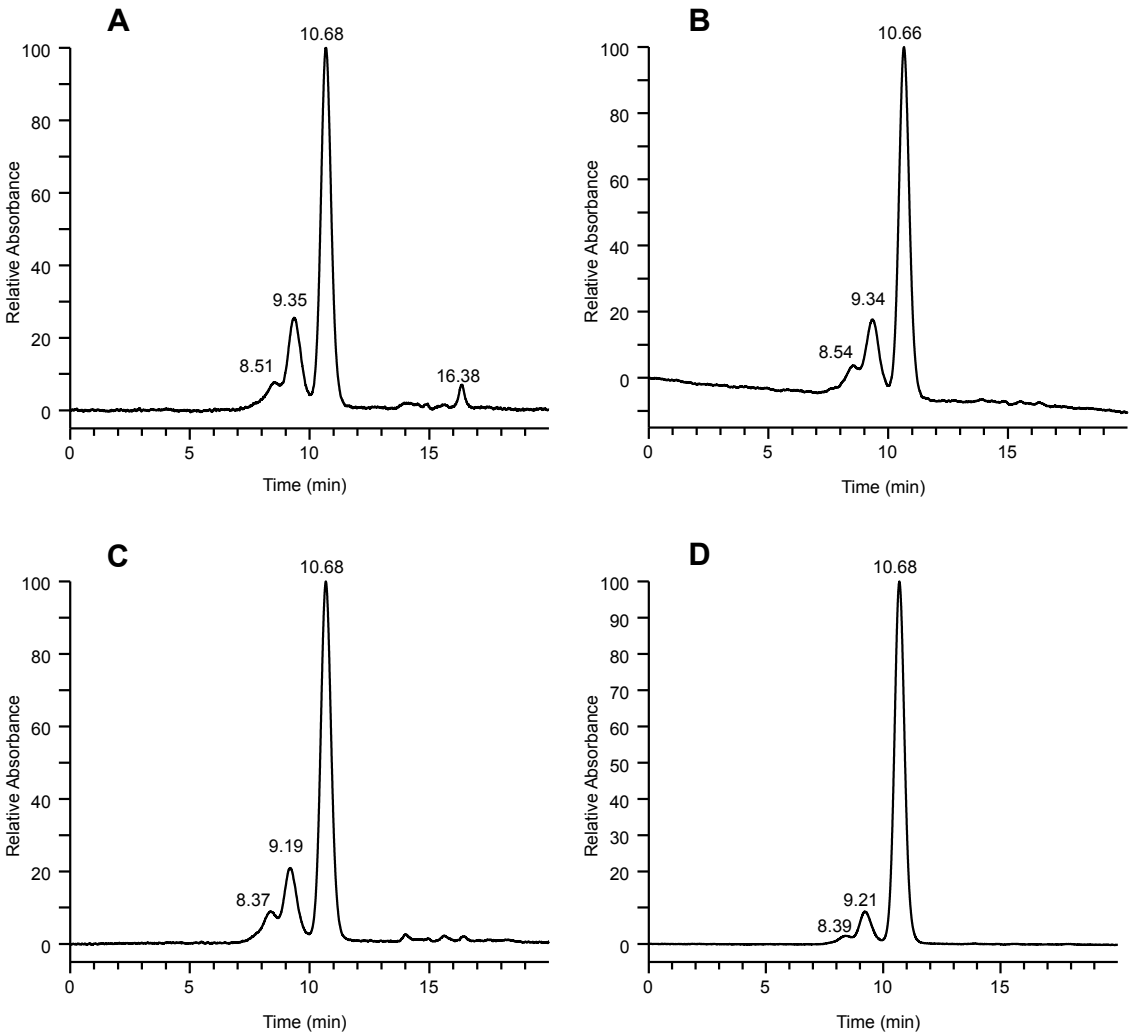


Fig. 6. Size exclusion chromatograms of reaction mixtures of Av with bis-biotin **6**, **9**, **11**, **14**. Panel A - Av/**6** (^{t}R 8.51 min, 6%; ^{t}R 9.35 min, 23%); Panel B - Av/**9** (^{t}R 8.54 min, 6%; ^{t}R 9.34 min, 20%); Panel C - Av/**11** (^{t}R 8.37 min, 6%; ^{t}R 9.19 min, 17%); Panel D - Av/**14** (^{t}R 8.39 min, 2%; ^{t}R 9.21 min, 10%). All the mixtures are in 1:1 molecular ratio and reacted for 45 min at r.t. The chromatograms are grouped taking into account the length and the nature of the spacer arms (Panel A and B – non-PEG spacers; Panel C and D – PEG spacers).

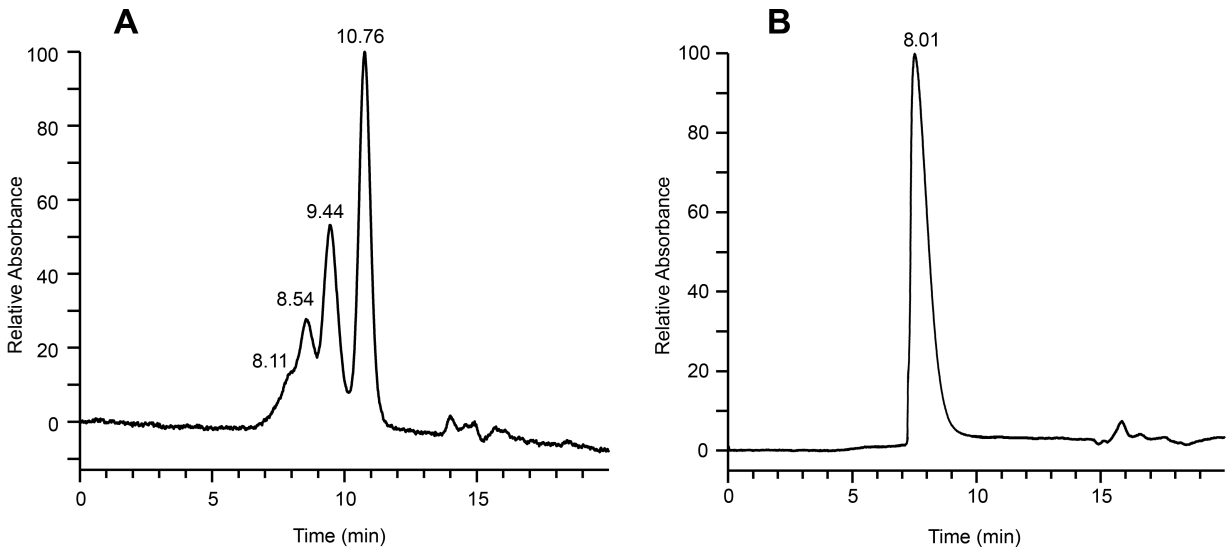


Fig. 7. Size exclusion chromatograms of reaction mixtures of Av with bis-biotin **16** and **18**. Panel A - Av/**16** (^{t}R 8.54 min,18%; ^{t}R 9.44 min, 33%); panel B - Av/**18** only one peak (^{t}R 8.01 min), due to oligomer with high molecular weight.

In both cases, the same ^{t}R was obtained, indicating that the SEC-HPLC cannot distinguish the Av alone from the per-biotinylated form that differs only by ~1000 Da, corresponding to weight of four biotin molecules.

The chromatograms obtained by the mixtures of compounds **6**, **9**, **11**, and **14** with avidin, in 1:1 molecular ratio, are depicted in Fig. 6.

The chromatograms reported in Fig. 6 clearly shows the Av oligomers peaks, formed by the cross-linking action of the tested bis-biotins. All compounds showed the presumed Av oligomers in an amount ranging from 10 to 23%. At the same time, smaller peaks (2 to 6%) at lower ^{t}R, probably due to oligomers with higher molecular weight, were detected in all the chromatograms. Among the six PEG-tethered biotin derivatives reported by Wilbur, only one gave significant evidence of r-Sav cross-linking (about 40% of Av dimers and trimers).[15] Interestingly, this compound had the longest distance between the two biotin heads calculated on the fully extended structure (49 Å). In our case, about 30% of cross-linking was obtained from constructs **6** and **9** where the biotin units are connected by slightly shorter non-PEG spacers (40 Å). Constructs **11** and **14**, containing two PEG spacers of higher length (73.4 and 76.6 Å, respectively) gave a minor amount of Av oligomers. In addition, a comparison between these latter compounds, having almost the same distance between the biotin heads, showed that the introduction of a rigid, aromatic, spacer into the PEG chain significantly lowers the propensity to give avidin oligomers (Fig. 6 – panel D).

Compounds **16** and **18**, containing shorter spacer arms respect to those previously prepared, showed a spacer length of 27.6 and 19.5 Å, respectively, instead of the 40.6 Å of **6**, 40.0 Å of **9**, 73.4 Å of **11** and 76.6 Å of **14**. These compounds were left to interact with avidin as above reported. The SEC-HPLC chromatograms of the resulting mixtures were recorded with the same modality already described for the other bis-biotins and are displayed in Fig.7. The chromatograms clearly showed that the two latter constructs possess a higher cross-linking ability respect to the others, compound **18** giving even 100% of a unique Av polymer. These results were in good agreement with predictions of the molecular modelling, confirming the close relationship between the spacer's length and the percentage of avidin oligomers formed.

Multi-angle laser light scattering analysis of the oligomers formed by 16 and 18

The determination of the molecular weight of the avidin oligomers formed by the action of the bis-biotins has been limited only to the two cases that gave the best results. For this reason, were taken into consideration only the avidin oligomers formed by compounds **16** and **18**.

Determination of proteins molar mass is a key parameter for their characterization[22] and an efficient method is represented by the combination of SEC-HPLC analysis in apparatus endowed of MALLS detector. MALLS technique is very usefully in the determination of organic and biological polymers like proteins. In particular, protein characteristics[23] such as molar mass and hydrodynamic radius can be evaluated.[24,25,26,27] A *dn/dc* value of 0.180 mg/mL is generally accepted for apo-proteins[28,29] but in the case of glycoproteins this value is unknown because the *dn/dc* value of the sugar residues often changes for different fragments.[30] Therefore, we calculated the *dn/dc* value with software Astra 6 related to avidin directly from refractive index analysis of the native avidin. The calculated value was 0.168 mL/g and we assumed that this value is the same for avidin and its aggregates.

As reference, we analysed the native avidin and the biotinylated-avidin. Native avidin has a molecular weight of

65.9 kDa and a hydrodynamic radius (Rh) of 3.42 nm. The Rh value was obtained from QELS (Quasi Elastic Light Scattering) experiment, assuming a spherical structure for the native avidin[31,32] (see the correlation function in Fig. 9S). MALLS analysis of Av/**16** oligomers show that the avidin (monotetramer) is the most abundant species (Table 1). At lower ^{t}R avidin oligomers are present in significant concentrations. We can assign the peak at ^{t}R 9.44 min (referred to the Fig. 7) to a biotinylated avidin dimer (calculated molecular weight: 140.6 kDa) and that at ^{t}R 8.54 min to a biotinylated avidin trimer (calculated: 209.5 kDa). A shoulder of the last peak at ^{t}R 8.11 min, which we estimate at a concentration of 1.14% and molecular weight of 414 kDa, can be tentatively attributed to an hexameric avidin polymer. All measurements are affected by errors due to the incomplete resolution of the chromatographic peaks and to the assumptions made about the value of *dn/dc*.

However, the data collected are in good agreement with the theoretical values, and coherent to give a correct interpretation about the number of avidin involved in each oligomer formed.

Rh values for the different species were calculated from the correlation functions, assuming a spherical structure for peak at ^{t}R 10.76 min and a stick structure for peaks at ^{t}R 9.44, 8.54 and 8.11 min (referred to the Fig. 7 – panel A).

From MALLS analysis we cannot discriminate native avidin from perbiotinylated-avidin because their molecular weight variation falls into the experimental error. However, the models and the assumptions on the shapes of the species in solution, used for fitting the experimental data points, turned out quite realistic as it appeared from the good agreement with the theoretical values.

Analysis of the Av aggregation induced by the bis-biotin **18** shows, instead, the presence of only one polymeric species with very high molecular weight (1465 kDa, Rh of 418.0 nm) and low polydispersity index (PDI) of 1.1. A low value of PDI highlights the presence of a polymer composed of a well-defined number of structural units. The data are in good agreement with an aggregate of perbiotinylated 21 tetramers, corresponding to a calculated molecular weight of 1461 kDa. The good agreement with the theoretical assumption was due to the presence of a single well-resolved peak (Fig. 7 – panel B).

Table 1. Molecular weight and hydrodinamic radius obtained by SEC-HPLC-MALLS analysis of the reaction mixtures of Av/**16**.

Retention time[a] [min]	Molecular Weight[b] [kDa]	Rh[b] [nm]
10.76	67.7	3.7
9.44	145.4	4.5
8.54	233.4	6.3
8.11	414.9	233.7

[a] Retention times are referred to the chromatogram depicted in Fig. 7 – panel A. [b] The maximum error in molecular weight values is ±2 %, and ±6 % is the highest error associate to the calculated Rh values.

Table 2. Molecular weight and hydrodinamic radius obtained by SEC-HPLC-MALLS analysis of the reaction mixtures of Av/**18** at different incubation times.

Incubation time [min]	Molecular weight[a] [kDa]	PDI[b]	Rh[c] [nm]
0	965.6	1.4	266.9
45	1482	1.2	270.1
150	1465	1.2	245.3
360	1541	1.3	392.8

[a] Maximum error: ±2 %. [b] PDI was calculated in accordance with literature.[33] [c] Maximum error: ±6 %.

To study the rate aggregation of Av by the action of compound **18** we analyzed the sample also at different incubation times (Table 2).

Rh values were calculated from a correlation function of the aggregates of avidin induced by **18**, assuming that their structure have a random coil shape.

We can observe that polymerization occurred rapidly and that after 150 min of incubation time the polymer was essentially the same with little increase of molecular weight and Rh after 360 min of incubation time. We hypothesize that this difference may be due to the adsorption of modified biotin and fragment of avidin from the solution media.

The hydrodynamic radius of the complex Av/**18** is very high if compared with the native avidin or other biotinylated-avidin samples and it is another evidence of the formation of a complex with high molecular weight.

All the correlation functions calculated for native avidin, Av/**16** and Av/**18** oligomers are reported in the Supplementary Informations.

Radiolabeling test and stability studies on bis-biotins

All the synthetic bis-biotin, **6**, **9**, **11**, **14**, **16** and **18** were radiolabeled with ^{111}In using the same procedure and specific activity (2.6 MBq/nmol) as previously described for ^{90}Y-DOTA-biotin in clinical trials.[10,14] Radiochemical purity (RCP) above 98% was obtained for all the six bis-biotins analyzed.

Moreover, for compounds **16** and **18**, stability studies were performed by incubating at 37 °C the radiolabeled samples in human serum pooled from ten different individuals diluted 1:1 with saline. Biotinidase degradation was assessed by ITLC-SG method[6] up to 48h. During this time the RCP remained unchanged (>98%) for both derivatives, proving the effectiveness of the L-Abu residue in preventing the enzymatic degradation in human serum and the effective stability of all the synthetized bis-biotins.

Experimental

General Procedures

Materials and Instrumentation. DOTA-tris(*t*Bu)ester was purchased from Chematech (Dijon, France) while standard (+)-biotin (B4501), *N*,*N*-Bis[3-(Fmoc-

amino)propyl]glycin hydrogen sulphate potassium salt, HABA, avidin from egg white and HPLC-grade CH_3CN were purchased from Sigma-Aldrich. Fmoc-L-Lys(Mtt)-Wang resin (100-200 mesh), Fmoc-NH-PEG-COOH, Fmoc-NH-$(PEG)_5$-COOH and Fmoc-PABA-OH were from Novabiochem AG (Laufelfingen, Switzerland). Fmoc-L-2Abu-OH, Fmoc-Gly-OH, Fmoc-EDA*HCl, HATU, HBTU were from Iris Biotech (Marktredwitz, Deutschland). Peptide-synthesis grade DMF was from Scharlau (Barcelona, Spain). Dry solvents were distilled immediately before use. All other chemicals were commercial compounds and were used as received. ^1H NMR spectra were recorded on a Varian Gemini 200 MHz or Varian Inova 400 MHz spectrometer in DMSO-d_6. Chemical shifts are reported in ppm (δ) downfield from tetramethylsilane. Electrospray ionization mass spectra (ESI-MS) were acquired on LCQ-Advantage ESI ion trap spectrometer (Thermo Finnigan) for positive and negative ions detection. The purity of the final compounds (>98%) was checked by HPLC and, in some cases, also by combustion analysis, performed on a Flash E1112 Thermo Finnigan elemental analyser. LC was performed in automatic with a Biotage Isolera One. The solid phase syntheses were performed using a semi-automatic synthesizer from MultiSyn Tech (German) and the microwave-assisted steps were carried out with a CEM Explorer microwave reactor (Matthews, NC, USA).

Chromatography. TLC was carried out on SiO_2 (Merck, 60 Å F254), and spots were located with UV light (254 and 366 nm), methanolic ninhydrin, Fluram (Fluka; fluorescamine, 4-phenyl-spyro[furan-2(3*H*),10-isobenzofuran]-3,30-dione) in acetone. SPE was performed on RP-LiChroprep silica. Semipreparative RP-HPLC was carried out by a Phenomenex Jupiter C18 180 Å (250 mm × 10 mm, 5 μm, metals <10 ppm) column using a Beckman Gold Noveau instrument. Analytical RP-HPLC was run on a Waters Alliance 2695 equipped with a Phenomenex Kinetex C18 column (100 mm × 3 mm, 2.6 μm). The solvent systems used for gradients were A (0.1% TFA in H_2O) and B (0.1% TFA in CH_3CN). The flow rates were 0.6 mL/min for analytical HPLC and 4 mL/min for semipreparative HPLC, with the linear gradients indicated when necessary. LC-ESI-MS analyses were performed by a Phenomenex aqua C18 column 300 Å 5 μm (250 mm × 4.6 mm) (flow rate, 1000 μL/min) on a Thermo Finnigan Surveyor HPLC system coupled to the ESI-MS, using the same solvents system as previously described. Silica gel ITLC paper strips for the labeling procedure were from ITLC-SG, Gelman Science (Ann Arbor, MI). The radiochromatographic profile was determined by Cyclone (Packard BioScience, Meriden, CT).

Solid Phase Synthesis

General procedures. The products **6**, **9**, **11**, **14**, **16**, **18** were synthesized using classical SPPS method. The products were synthesized on Fmoc-Lys(MTT)-Wang resin (400 mg, 0.216 mmol) using the semiautomatic synthesizer. Before starting the synthesis the resin was swelled for 20 min in DMF.

Step 1: Fmoc deprotection was performed by 20% piperidine in DMF (2×10 min). The resin was washed twice with DMF, one time with DCM and twice with DMF.

Step 2: coupling reactions.

Method 1: HBTU/NMM method with formation of active esters. The coupling reaction was performed by using the reagent (2.5 eq.), HBTU (2.5 eq.) and NMM (5 eq.) in DMF, under vortex mixing for 45 min (in case of coupling concerning the two spacer arms the eq. were doubled). The resin was then washed with DMF (×3), DCM (×2) and DMF (×1). Coupling reactions were checked by the Kaiser test.

Method 2: HATU/NMM method; the coupling reaction was performed by using the reagent (2.5 eq.), HATU (2.5 eq.), and NMM (5 eq.) in DMF, under vortex mixing for 45 min. The resin was then washed with DMF (×3), DCM (×2) and DMF (×1). Coupling reactions were checked by the Kaiser test.

Step 3: removal of Mtt protecting group was performed by a solution containing 2% TFA, 2.5% TIS and 95.5% DCM (about 35 washing for 1.5 min). Initially the solution became fluorescent yellow, indicating the presence of Mtt. Washing was continued until the solution became colorless again.

Step 4: cleavage from the resin and removal of the *tert*-butyl protection was performed with 2.5% TIS, 2.5% H_2O and 95% TFA for 3 h. The resin was filtered off and the solution was concentrated by flushing with N_2. The peptide was precipitated from cold diethyl ether, centrifuged, dissolved in water and lyophilized. The crude products were pre-purified by solid phase extraction and, subsequently, by semi preparative reverse-phase HPLC. Eluents: A (0.1% TFA in H_2O) and B (0.1% TFA in CH_3CN). Characterization of the products was performed by ESI-MS and LC-ESI-MS.

The volume of solvents used in washes and couplings was calculated with the general principle of 1 mL for 100 mg of resin.

Synthesis of 16-(2-((1-carboxy-5-(2-(4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)acetamido)pentyl)amino)-2-oxoethyl)-8,11,21,24-tetraoxo-2,30-bis(5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)-7,12,16,20,25-pentaazahentriacontane-1,31-dioic acid (6). After Fmoc deprotection from Fmoc-Lys(Mtt)-Wang resin, (**5**) (400 mg, 0.216 mmol) (step 1), the spacer-C_3 (**2**) conjugation to the lysine side chain was performed using step 2, method 2: 2 (2.5 eq, 415 mg), HATU (2.5 eq, 205 mg) and NMM (5 eq, 119 μL). in DMF. Fmoc removal was performed by treating the resin **3** following the step 1. The succinic anhydride (20 eq, 432 mg) and NMM (40 eq, 894 μL) was coupled to the on resin compound **3** by a microwave reactor using anhydrous THF as solvent. Microwave program: 20 cycles (60 Watt for 1 min and

cooling for 30 sec), Tmax = 65 °C. The resin **4** was washed with DMF, 0.25M NaOH in THF and DMF. Fmoc-Lys-O*t*Bu was introduced by activation of the on-resin carboxyl function **4** following step 2, method 2 with HATU (3 eq, 246 mg), NMM (6 eq, 133 µL) in DMF and vortexed for 1 min. Fmoc-Lys-O*t*Bu (3 eq, 275 mg) in DMF was then added and vortexed for 1 h. Biotin (5 eq, 263 mg) was activated with HATU (5 eq, 410 mg), NMM (10 eq, 223 µL) in DMF and reacted for 50 min (step 2, method 2). The MTT protecting group was then removed in accordance with step 3. DOTAtris-*t*Bu ester (2.5 eq; 309 mg) was activated with HBTU (2.5 eq, 205 mg), NMM (5 eq, 112 µL) in DMF and reacted for 50 min. Cleavage from the resin and removal of the tert-butyl protection was performed using step 4. The crude product **6** was purified by semipreparative RP-HPLC to give pure **6** (90 mg, 26%). RP-HPLC (15 to 50% of B in 5 min): ^{t}R = 3.12 min; ESI-MS: *m/z* calculated $[M+H]^{+}$, 1612.91; $[M+2H]^{2+}$ 806.95; $[M+3H]^{3+}$ 538.30, found 1613.34 $[M+H]^{+}$; 807.44 $[M+2H]^{2+}$; 538.53 $[M+3H]^{3+}$. Elemental analysis. Found: C, 44.14; H, 6.11; N, 11.37%. Calculated $C_{70}H_{117}N_{17}O_{22}S_2\bullet 3H_2O\bullet 4TFA$; C, 44.13; H, 6.03; N, 11.22%.

Synthesis of 2,2',2''-(10-(8-carboxy-26-ethyl-12-(14-ethyl-5,8,13,16-tetraoxo-20-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-4,9,12,15-tetraazaicosyl)-2,10,17,20,25,28-hexaoxo-32-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-3,9,12,16,21,24,27-heptaazadotriacontyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (9). The on-resin carboxyl function of **4** were activated with HBTU (5 eq, 410 mg), NMM (10 eq, 224 µL) in DMF and vortexed for 1 min (step 2, method 1). Fmoc-ethylendiamine (5 eq, 344 mg) dissolved in DMF was then added and vortexed for 45 min. Fmoc removal from **7** was performed following the step 1 and Fmoc-L-α-aminobutyric acid (5 eq; 352 mg) was coupled by activation with HBTU (5 eq; 410 mg) and NMM (10 eq; 224 µL) in DMF for 45 min (step 2, method 1). The synthesis was completed as already described for **6**, with the coupling of (+)-biotin (5 eq; 264 mg) with HATU (5 eq; 410 mg) and NMM (10 eq; 224 µL) in 50 min and followed by the step 3 and the coupling with DOTAtris-*t*Bu ester (2.5 eq; 309 mg) activated with HBTU (2.5 eq, 205 mg), NMM (5 eq, 112 µL) in DMF and reacted for 50 min. Cleavage from the resin and removal of the *tert*-butyl protection was performed using step 4. The crude product **9** was purified by semi preparative RP-HPLC to give pure **9** (70 mg, 20%). RP-HPLC (15 to 50% of B in 5 min): ^{t}R = 3.46 min; ESI-MS: *m/z* calculated $[M+H]^{+}$, 1611.94; $[M+2H]^{2+}$ 806.47; $[M+3H]^{3+}$ 537.98, found 1611.07 $[M+H]^{+}$; 806.33 $[M+2H]^{2+}$; 537.89 $[M+3H]^{3+}$. Elemental analysis. Found: C, 44.07; H, 6.23; N, 12.68%. Calculated $C_{70}H_{119}N_{19}O_{20}S_2\bullet 3H_2O\bullet 4TFA$; C, 44.17; H, 6.13; N, 12.55%.

Synthesis of 2,2',2''-(10-(8-carboxy-40-ethyl-12-(28-ethyl-5,27,30-trioxo-34-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-8,11,14,17,20,23-hexaoxa-4,26,29-triazatetratriacontyl)-2,10,17,39,42-pentaoxo-46-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-20,23,26,29,32,35-hexaoxa-3,9,12,16,38,41-hexaazahexatetracontyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (11). 1-Fmoc-amino-3,6,9,12,15,18-esaossaenicosan-21-oic acid (4 eq; 497 mg) was coupled to the on-resin product, **3**, previously deprotected by the Fmoc group (step 1), and activated with HBTU (4 eq, 328 mg), NMM (8 eq, 179 µL) in DMF and vortexed for 45 min (step 2, method 1). Fmoc-L-α-aminobutyric acid (5 eq, 352 mg), HBTU (5 eq; 410 mg) and NMM (10 eq; 224 µL) was coupled in DMF for 45 min (step 1 and step 2, method 1). The final steps require the insertion of (+)-biotin (5 eq; 264 mg) with HATU (5 eq; 410 mg) and NMM (10 eq; 224 µL) in 50 min and followed by DOTAtris-*t*Bu ester (2.5 eq; 309 mg) activated with HBTU (2.5 eq, 205 mg), NMM (5 eq, 112 µL) in DMF and reacted for 50 min. Cleavage from the resin and removal of the *tert*-butyl protection was performed using step 4. The crude product **11** was purified by semi preparative RP-HPLC to give pure **11** (94 mg, 22%). RP-HPLC (20 to 60% of B in 5 min): ^{t}R = 3.54 min; ESI-MS: *m/z* calculated $[M+H]^{+}$, 1998.41; $[M+2H]^{2+}$ 999.70; $[M+3H]^{3+}$ 668.80, found 1998.24 $[M+H]^{+}$; 999.82 $[M+2H]^{2+}$; 666.74 $[M+3H]^{3+}$. Elemental analysis. Found: C, 44.88; H, 7.02; N, 9.28%. Calculated $C_{88}H_{155}N_{17}O_{30}S_2\bullet 6H_2O\bullet 4TFA$; C, 45.01; H, 6.81; N, 9.30%.

Synthesis of 2,2',2''-(10-(19-carboxy-1-(4-(11-ethyl-10,13-dioxo-17-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-3,6-dioxa-9,12-diazaheptadecanamido)phenyl)-15-(1-(4-(11-ethyl-10,13-dioxo-17-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-3,6-dioxa-9,12-diazaheptadecanamido)phenyl)-1,10-dioxo-5,8-dioxa-2,11-diazatetradecan-14-yl)-1,10,17,25-tetraoxo-5,8-dioxa-2,11,15,18,24-pentaazahexacosan-26-yl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (14). Fmoc-8-amino-3,6-dioxaoctanoic acid (4 eq; 333 mg) was coupled to the on-resin product, **3**, previously deprotected by the Fmoc group (step 1), and activated with HBTU (4 eq, 328 mg), NMM (8 eq, 179 µL) in DMF and vortexed for 45 min, in accordance with step 2, method 1. Fmoc-*p*-amino benzoic acid (5 eq, 388 mg), HBTU (5 eq; 410 mg) and NMM (10 eq; 224 µL) was coupled in DMF for 45 min. At this point, the coupling with Fmoc-8-amino-3,6-dioxaoctanoic acid was repeated once again in the same conditions. The intermediate, after the Fmoc removal (step 1), was completed with Fmoc-L-α-aminobutyric acid (5 eq, 352 mg), HBTU (5 eq; 410 mg) and NMM (10 eq; 224 µL), coupled in DMF for 45 min (step 1 and step 2, method 1) and (+)-biotin (5 eq; 264 mg) with HATU (5 eq; 410 mg) and NMM (10 eq; 224 µL) in 50 min. Finally, after step 3, DOTAtris-*t*Bu ester was introduced in the same conditions and quantities reported above in accordance with step 2, method 1. The cleavage from the resin and removal of the

tert-butyl protection was performed using step 4. The crude product was purified by semi preparative RP-HPLC to give pure **14** (83 mg, 18%). RP-HPLC (20 to 60% of B in 5 min): ^{t}R = 3.61 min; ESI-MS: *m/z* calculated $[M+2H]^{2+}$ 1073.74; $[M+3H]^{3+}$ 716.16, found 1073.88 $[M+2H]^{2+}$; 716.09 $[M+3H]^{3+}$. Elemental analysis. Found: C, 45.00; H, 6.03; N, 10.24%. Calculated $C_{96}H_{153}N_{21}O_{30}S_2 \bullet 6H_2O \bullet 5TFA$; C, 45.09; H, 6.07; N, 10.42%.

Synthesis of 2,2',2''-(10-(8-carboxy-21-ethyl-2,10,17,20,23-pentaoxo-27-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-12-(3-(2-(2-(5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)butanamido)acetamido)propyl)-3,9,12,16,19,22-hexaazaheptacosyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (16). To the intermediate **3**, properly deprotected from Fmoc group (step 1), was coupled the Fmoc-Gly-OH (5 eq; 321 mg) activated with HBTU (5 eq; 410 mg) and NMM (10 eq; 224 μL) and reacted for 45 min in DMF (step 2, method 1). The on-resin compound **15** was completed after step 1, with Fmoc-L-α-aminobutyric acid (5 eq, 352 mg), HBTU (5 eq; 410 mg) and NMM (10 eq; 224 μL), coupled in DMF for 45 min (step 1 and step 2, method 1); (+)-biotin (5 eq; 264 mg) with HATU (5 eq; 410 mg) and NMM (10 eq; 224 μL) in 50 min (+)-Biotin and finally, after step 3, DOTAtris-*t*Bu ester (2.5 eq; 309 mg) activated with HBTU (2.5 eq, 205 mg), NMM (5 eq, 112 μL) in DMF and reacted for 50 min. The cleavage from the resin and removal of the *tert*-butyl protection was performed using step 4. The crude product was purified by semi preparative RP-HPLC to give pure **16** (93 mg, 30%). RP-HPLC (20 to 50% of B in 5 min): ^{t}R = 3.36 min; ESI-MS: *m/z* calculated $[M+H]^{+}$, 1441.73; $[M+2H]^{2+}$ 721.36; $[M+3H]^{3+}$ 481.24, found 1441.25 $[M+H]^{+}$; 721.00 $[M+2H]^{2+}$; 480.83 $[M+3H]^{3+}$. Elemental analysis. Found: C, 44.13; H, 6.21; N, 12.72%. Calculated $662H_{105}N_{17}O_{18}S_2 \bullet 3H_2O \bullet 3TFA$; C, 44.46; H, 6.26; N, 12.96%.

Synthesis of 2,2',2''-(10-(8-carboxy-18-ethyl-2,10,17,20-tetraoxo-24-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-12-(3-(2-(5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)butanamido)propyl)-3,9,12,16,19-pentaazatetracosyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (18). To the intermediate **3**, deprotected from Fmoc group (step 1), was coupled the Fmoc-L-α-aminobutyric acid (5 eq; 352 mg) with HBTU (5 eq; 410 mg) and NMM (10 eq; 224 μL) in DMF for 45 min (step 2, method 1). The synthesis required the coupling of (+)-biotin (5 eq; 264 mg) with HATU (5 eq; 409 mg) and NMM (10 eq; 224 μL) in 45 min and followed, after step 3, by the insertion of DOTAtris-*t*Bu ester (2.5 eq; 309 mg) activated with HBTU (2.5 eq, 205 mg), NMM (5 eq, 112 μL) in DMF and reacted for 50 min. After the cleavage from the resin and removal of the *tert*-butyl protections as reported in step 4, the crude product was purified by semi preparative RP-HPLC to give pure **18** (115 mg, 40%). RP-HPLC (20 to 50% of B in 5 min): ^{t}R = 3.42 min; ESI-MS: *m/z* calculated $[M+H]^{+}$, 1327.63; $[M+2H]^{2+}$ 664.31, found 1327.28 $[M+H]^{+}$; 664.01 $[M+2H]^{2+}$; 537.89. Elemental analysis. Found: C, 45.33; H, 6.51; N, 12.69%. Calculated $C_{58}H_{99}N_{15}O_{16}S_2 \bullet 4H_2O \bullet 2TFA$; C, 45.78; H, 6.75; N, 12.92%.

Size-exclusion chromatography and MALLS analysis

HPLC separations of Av and mixtures of Av and biotin derivatives were conducted on Thermo Finnigan Surveyor HPLC system equipped with a Waters Protein Pak Glass 300SW size exclusion column (10μm, 8.0×300 mm; WatersCorp., Milford, MA). The detector was an Uv-vis diode array working in single channel mode @ 285 nm. The mobile phase consisted of 50 mM potassium phosphate (pH 6.8), 300 mM NaCl, 1 mM EDTA, and 1 mM NaN_3 in water milli-Q. A flow rate of 0.75 mL/min was used. The retention time of Av under these conditions was 10.75 min. It should be noted that the total peak area for each injection was monitored, and when the area dropped significantly, a 200 μL bolus of 6N guanidine hydrochloride was injected to clear polymerized Av from the column.

The SEC-HPLC apparatus was also interfaced with a refractive index detector Wyatt T-REX Optilab and a MALLS (Multi Angles Laser Light Scattering) detector Wyatt-DAWN HELEOS II (laser source λ = 618 nm). Data collected were elaborated with software Astra 6 from Wyatt Technologies.

The samples analysed with SEC-HPLC were prepared by slow addition of the selected bis-biotin solubilized in PBS, in 100 μL of Av 1 mg/ml in phosphate buffer solution (pH 6.8). For the SEC-HPLC-MALLS analysis the preparation of the sample was the same, but the Av solution was 4 mg/mL.

Molecular modelling

Energy minimizations and molecular dynamic simulations were carried out by using the SANDER module of the AMBER 9[34] with the GAFF and FF03[35,36] force field.

For the dynamic simulation, initially the complexes were subject to energy minimization in two stages. Firstly, the bis-biotin molecules were minimized by holding the avidin monomers fixed with an harmonic constraint strength of 2.0 kcal/(mol Å^2). Secondly, the entire system was minimized without restriction. The first and second stage were performed by using the steepest descent minimization of 100 steps followed by a conjugate gradient minimization of 700 steps.

Molecular dynamic simulations in implicit solvent were performed with GBSA. At this purpose, the dynamic simulations, on the entire complex, were performed with the modified GB model developed by A. Onufriev, D. Bashford and D.A. Case (*GB^{OBC}*) (igb = 2), the surface area was computed and included in the solvation term, and a cut off of 30 Å for non-bonded interactions was used. The simulations were carried out at three different temperatures (150, 300

and 450 K). The increases of temperature of 150 K were obtained in 12 ps by holding the peptide chains fixed with a harmonic constraint of a strength of 0.01 kcal/(mol Å^2).
After heating, 10 ps dynamics simulations, with the avidin constraint (strength of 0.01 kcal/mol Å^2), were performed at a constant temperature with SHAKE turned on for bonds involving hydrogens, allowing a time-step of 2.0 fs.

Conclusions

In this paper we report a new class of bis-biotins able to generate oligomeric architectures of avidin tetramers targeting the tumor cells surface. The role of these oligomers is planned to avoid the rapid washout of the labelled avidin and to increase the amount of radiation delivered on the cancer cell, minimizing at the same time the amount of the radiopharmaceutical dispersed in the healthy tissue.
In silico calculations based on crystallographic data of Av tetramers, allowed us to develop a collection of new synthetic DOTA-structures bearing two (+)-biotin molecules obtained by efficient solid-phase strategies. In particular, the arms of the structures **6** and **9** contained various amide groups connecting two biotin moieties while the bis-biotins **11** and **14** were built using PEG spacers in order to improve the hydrophilicity of the final molecules. In the bis-biotins **6**, **9**, **11**, and **14** the distances of the two biotin CO groups are in the range of 40-80 Å. Investigation on cross-linking of avidin in solution was carried out for all the synthetic bis-biotins. A small amount of avidin dimers and trimers was detected by SEC-HPLC analysis of 1:1 mixtures of avidin and compounds **6**, **9**, **11**, and **14**, respectively. On the other hand, compounds **16** and **18**, in which the spacers containing a residue of L-Abu were considerably shorter (27.6 and 19.5 Å, respectively) afforded rapidly polymeric avidin architectures. In particular, the structure **16** led up to 53% of avidin dimers and trimers, while the mixture Av/**18** revealed the presence of a unique polymer. MALLS analysis of the cross-link Av/**18** suggested the presence of 21 Av tetramers encompassing the DOTA-biotin **18**. Both **16** and **18** were stable in human serum, confirming the role of L-Abu in blocking biotinidase activity. Moreover, all the synthetic DOTA-conjugated bis-biotins were radiolabelled with ^{111}In, showing in all cases a radiochemical purity above 98%.
From *in silico* calculations it emerged quite clearly that the chemical nature of the spacer arms is not determinant for the formation of avidin oligomers. The experimental data highlighted that only the length of the spacer arms is involved in forming the oligomers amount, provided a flexible, tri-functional spacer connecting the DOTA moiety to the arms is present. In conclusion, we described herein a new and useful class of bis-biotins conjugated to DOTA with appropriate structural features to create molecular weight-tuned avidin oligomers through modifications of the length of the spacers connecting the (+)-biotin heads.

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Abbreviations

DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; HATU, 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate, *N*-[(Dimethylamino)-1*H*-1,2,3-triazolo-[4,5-b]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HBTU, *N,N,N',N'*-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate, *O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate; ITLC-SG, instant thin layer chromatography-silica gel; MTT, 4-methyltrityl; NMM, 4-methylmorpholine; RP-HPLC, reverse phase HPLC; SEC, size exclusion chromatography; TIS, triisopropyl silane.

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1 G. Paganelli, C. Grana, M. Chinol, M. Cremonesi, C. De Cicco, F. De Braud, G. Robertson, A. Siccardi, and U. Veronesi, *Eur. J. Nucl. Med.,* 1999, **26**, 348-357.

2 H. P. Lesch, M. U. Kaikkonen, J. T. Pikkarainen, and H. S. Yla, *Expert opinion on drug delivery,* 2010, **7**(5), 551-564.

3 C. Rousseau, F. Kraeber-Bodere, J. Barbet, and J. F. Chatal, *Eur. J. Nucl. Med. Mol. Imaging,* 2013, **40**, 1373-1376.

4 S. Papi, C. Grana, M. Bartolomei, L. Ravasi, M. Cremonesi, M. Ferrari, L. Martano, L. Garaboldi, M. Chinol, and G. Paganelli, *Methods of cancer diagnosis, therapy and prognosis.* M. A. Hayat, Ed.; Springer: New York, 2010; Vol. 7, Chapter 7, pp 81-98.

5 D. B. Axworthy, J. M. Reno, M. D. Hylarides, L. W. Mallet, L. J. Theodore, L. M. Gustavson, F. M. Su, L. J. Hobson, P. L. Beaumier, and A. R. Fritzberg, *Proc. Natl. Acad. Sci.,* 2000, **97**, 1802-1807.

6 G. Sabatino, M. Chinol, G. Paganelli, S. Papi, M. Chelli, G. Leone, A. M. Papini, A. De Luca, and M. Ginanneschi, *J. Med. Chem*., 2003, **46**, 3170–3173.

7 N. Urbano, S. Papi, M. Ginanneschi, R. De Santis, S. Pace, R. Lindstedt, L. S. Ferrari, J. Coi, G. Paganelli, and M. Chinol, *Eur. J. Nucl. Med. Mol. Imaging*. 2007, **34**(1), 68-77.

8 A. Pratesi, F. Bucelli, I. Mori, M. Chinol, A. Verdoliva, G. Paganelli, V. Riveccio, L. Gariboldi, and M. Ginanneschi, *J. Med. Chem*., 2010, **53**, 432–440.

9 G. Paganelli, M. Ferrari, L. Ravasi, M. Cremonesi, C. De Cicco, V. Galimberti, G. Sivolapenko, A. Luini, R. De Santis, L. Travaini, M. Fiorenza, M. Chinol, S. Papi, C. Zanna, P. Carminati, and U. Veronesi, *Clin. Cancer Res.,* 2007, **13**, 5646s-5651s.

10 G. Paganelli, C. De Cicco, M. Ferrari, G. Carbone, G. Pagani, M. C. Leonardi, M. Cremonesi, A. Ferrari, M. Pacifici, A. Di Dia, R. De Santis, V. Galimberti, A. Luini, R. Orecchia, S. Zurrida, and U. Veronesi, *Eur. J. Nucl. Med. Mol. Imaging,* 2010, **37**, 203-211.

11 Z. Yao, M. Zhang, H. Sakahara, Y. Nakamoto, T. Higashi, S. Zhao, N. Sato, Y. Arano, J. Konishi, *J. Nucl. Med.,* 1999, **4***,* 479-483.

12 M. Chinol, O. De Cobelli, G. Trifirò, E. Scardino, M. Bartolomei, F. Verweij, S. Papi, D. V. Matei, and G. Paganelli, *Eur. Urol.,* 2003, **44**, 556-559.

13 F. Botta, M. Cremonesi, M. Ferrari, E. Amato, F. Guerriero, A. Vavassori, A. Sarnelli, S. Severi, G. Pedroli, and G. Paganelli, *Eur. J. Nucl. Med. Mol. Imaging,* 2013, **40**, 1047-1056.

14 M. Chinol, O. De Cobelli, and G. Paganelli, *Am. J. Urol. Rev.,* 2004, **2***,* 190-194.

15 D. S. Wilbur, P. M. Pathare, D. K. Hamlin, and S. A. Weerawarna, S. A. *Bioconj. Chem.,* 1997, **8**, 819-832.

16 E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. I. Cook, P. I. *Anal. Biochem*., 1970, **34**, 595-598.

17 J. C. Kappel, and G. Barany, *Lett. Pept. Sci.,* 2003, **10**, 119-125.

18 D. C. Jagesar, F. Hartl, W. J. Buma, and A. M. Brouwer, A. M. *Chem. Eur. J*., 2008, **14**, 1935-1946.

19 F. Stieber, and H. Waldmann, H. *Chem. Comm.,* 2002, **16**, 1748-1749.

20 D. S. Wilbur, D. K. Hamlin, M. K. Chyan, B. B. Kegley, J. Quinn, and R. L. Vessella, *Bioconj. Chem.,* 2004, **15**, 601-616, and references therein.

21 D. S. Wilbur, D. K. Hamlin, and M. K. Chyan, *Bioconj. Chem*., 2006, **17**, 1514-1522.

22 M. Sakuma, M. Nakamura, H. Koike, and M. Suzuki, M. *Proc. Japan Acad. Ser. A,* 2005, **81**, 110-116.

23 M. Rocco, M. Molteni, M. Ponassi, G. Giachi, M. Frediani, A. Koutsibas, A. Profumo, D. Trevarin, B. Cardinali, P. Vachette, F. Ferri, and J. Perez, *J. Am. Chem. Soc.,* 2014, **136**, 5376-5384.

24 H. Ye, *Anal. Biochem.,* 2006, **356**, 76-85.

25 D. Magatti, M. Molteni, B. Cardinali, M. Rocco, and F. Ferri, *Biophys. J.,* 2013, **104**, 1151-1159.

26 I. Printz, D. S. Kalonia, and W. Friess, *J. Pharm. Sci.,* 2012, **101**, 363-372.

27 C. Rosano, P. Arosio, and M. Bolognesi, *Biomol. Eng.,* 1999, **16**, 5-12.

28 A. K. Tomar, B. S. Sooch, B. S. Singh, and S. Yadav, *Int. J. Biol. Macromol.,* 2013, **53**, 133-137.

29 H. Zhao, R. S. Berry, and P. Schuck, *Biophys. J.,* 2011, **100**, 2309-3217.

30 J. Wen, T. Arakawa, and J. S. Philo, *Anal. Biochem.,* 1996, **240**, 155-166.

31 S. Alexander, E. Courtens, and R. Vacher, *Phys. A.,* 1993, **195**, 286-318.

32 T. Nose, and B. Chu, Polymer Science: A Comprehensive Reference; K. Matyjaszewski, M. Möller Eds.; Elsevier: Amsterdam, 2012, p 301.

33 M. Rogošić, H. J. Mencer, and Z. Gomzi, *Eur. Polym. J.,* 1996, **32**, 1337-1344.

34 D. A. Case, T. A. Darden, T. E. Cheatham III, C. L. Simmerling, J. Wang, R. E. Duke, R. Luo, K. M. Merz, D. A. Pearlman, M. Crowley, R. C. Walker, W. Zhang, B. Wang, S. Hayik, A. Roitberg, G. Seabra, K. F. Wong, F. Paesani, X. Wu, S. Brozell, V. Tsui, H. Gohlke, L. Yang, C. Tan, J. Mongan, V. Hornak, G. Cui, P. Beroza, D. H. Mathews, C. Schafmeister, W. S. Ross, and P. A. Kollman, AMBER 9, 2006, University of California, San Francisco, CA.

35 J. Wang, R. M. Wolf, J. W. Caldwell, P. A. Kollamn, and D. A. Case, *J. Comput. Chem.,* 2004, **25**, 1157-1174.

36 Y. Duan, C. Wu, S. Chowdhury, M. C. Lee, G. Xiong, W. Zhang, R. Yang, P. Cieplak, R. Luo, and T. Lee, *J. Comput. Chem.,* 2003, **24**, 1999-2012.