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Laboratory note

Synthesis of versatile chemical tools toward a structure/properties relationships study onto targeting colloids

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Abstract

As part of a drug delivery project, four aldehydes of the type Pam-Lys(Pam)-spacer-CO-CHO were synthesized to be included in targeting colloids. Though amphiphilic, they were obtained within reasonable yields (18–55%) and with high RP-HPLC purity (~90%). Parallely, six complementary targeting peptides of the type H_2N-NH-CH_2-CO-spacer-YGRGDSP-NH_2 were prepared to be anchored onto colloids. Isolated yields are related to the spacer length and nature. To easily and rapidly modulate the distance between the peptide and the vesicle, every partners were elaborated on solid phase and the expected constructions were obtained by hydrazone ligation. One possible application is presented here with multilamellar vesicles targeting HUVEC cells. Preliminary results prove that the fine-tuning of the spacer length permits to optimize the recognition toward the target cells.
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Keywords: Amphiphilic; Chemical ligation; Targeting colloids; Structure/properties relationships; Vectorisation; Multilamellar vesicles

1. Introduction

In drug delivery, the targeting is permitted by a molecular recognition pattern (MRP) specific to the target cells (via a receptor, a specific metabolic route, an enzyme, or a particular glycoprotein for example). This MRP can be directly bound to the drug to be delivered or covalently attached to a 'cargo', usually a vesicle or a micro/nano sphere/particle. Among every MRPs already in use, peptides and proteins are compounds of choice because their chemistry is versatile, well-mastered and high-yielded. To site-specifically immobilize peptides and proteins onto vesicles or micro/nano entities – popular bonds (like amide [1] thioether [2–6] and disulfide [7]) left apart – the hydrazone bond was proposed by some authors as an alternative [8]. Conjugates are classically prepared by mild periodate oxidation of N-terminus Seryl or Threonyl peptides [9] or sugar of antibodies' Fc fragment [10] followed by the incubation of the product with a hydrazide-phosphatidylethanolamine [11]

Abbreviations: AcOEt, ethyl acetate; Boc, tertiobutyloxycarbonyl; Chol, cholesterol; CryoTEM, transmission electronic microscopy; DIEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; Fmoc, 9-fluorenylmethyloxycarbonyl; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; HUVEC, human umbilical vein endothelial cells; IPT, isopropylidene tartrate; MALDI-TOF, matrix assisted laser desorption ionisation-time of flight; MRP, molecular recognition pattern; NMP, *N*-methyl pyrrolidone; OSu, *N*-hydroxysuccinimide ester; Pbf, 2,2,4,6,7-pentamethyl-dihydrobenzofurane-5-sulfonyl; PBS, phosphate buffered saline; PC, phosphatidylcholine; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; SPR, structure/properties relationships; SPS, solid phase synthesis; TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; *t*Bu, tertiobutyl; TFA, trifluoroacetic acid; TIS, triisopropyl silane; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

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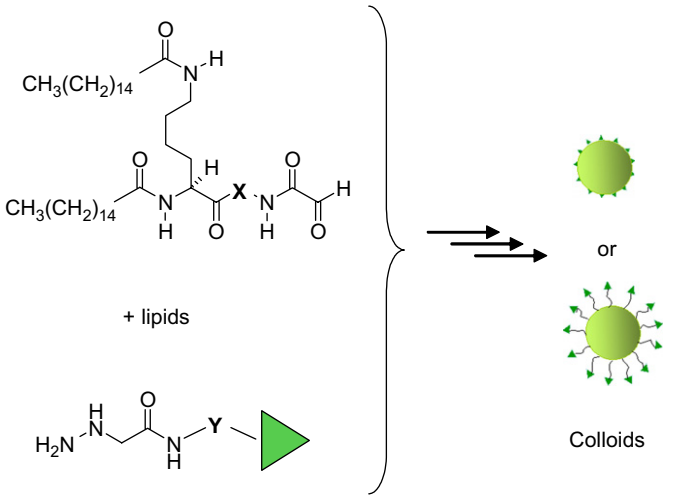
contained in the bilayers, and acting as a chemical 'anchor'. Advantageously, this method neither requires any activating agent nor generates any side-product.

The reverse reaction consisting of the coupling of amphiphilic aldehyde-like anchors with synthetic hydrazino acetyl peptides remained unprecedented until recently. This strategy is now envisaged owing to the recent progress concerning the preparation of the latter compounds. The introduction of a glyoxylyl moiety on amphiphilic compounds is permitted by the preparation of an isopropylidene tartrate (IPT) resin and a periodic oxidation [12]. Moreover, the use of the *N*,*N*′,*N*′-tri(*tert*-butyloxycarbonyl)-hydrazino acetic acid[2] allows the facile introduction of the hydrazino-group on peptides using solid phase peptide synthesis [13,14]. Notably, this approach neither requires extra steps to functionalize the peptides after cleavage from the resin, nor purification compared to the synthesis of N-terminus glyoxylyl-peptides, where a deleterious periodic oxidation and a second purification are necessary. For the same reasons, sulfur-containing peptides are more easily accessible. At any case, this reverse strategy now complements and reinforces the attractiveness of the hydrazone chemoselective ligation. Thus, this methodology was employed successfully in the preparation of some peptidoliposomes [15] and, in another field, of a lipopeptide vaccine mixture [16]. Embarked on a drug delivery project, we worked at obtaining easy, versatile chemical tools to study the structure/properties relationships in targeting peptidyl vesicles. The synthetic advantages, the simplicity and the efficiency of the hydrazone bond formation were good reasons for applying the hydrazone ligation in the present work.



Scheme 1. Stake of the present work. Triangles represent the MRP that can be close to or distant from the colloids acting as 'cargos'. Spacers are written as **X** and **Y**.

2. Chemistry

2.1. Anchors' design and synthesis

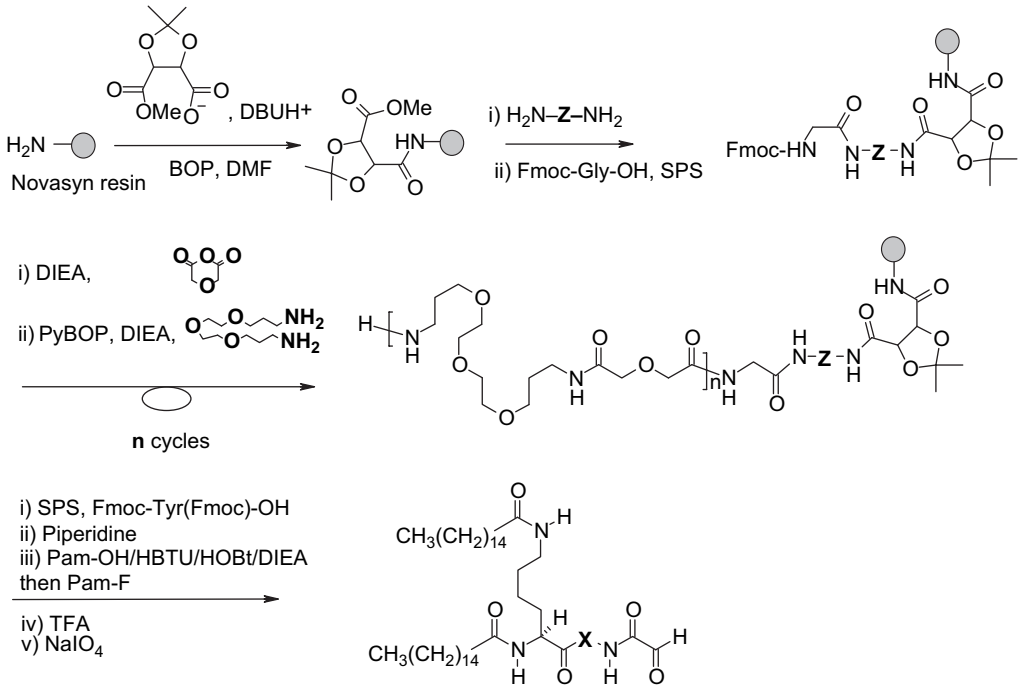
The difficulty in preparing amphiphilic compounds resides in the fine-tuning of the physico-chemical properties to allow their solubilization, purification and characterization. In addition, here, the anchored peptide should be close to or distant from the membrane surface (Scheme 1). Thus, to allow the spacer arm to be easily modulated, anchors were elaborated on solid phase using standard Fmoc/*t*Bu protocols [17,18] and our IPT resin [19,20]. A first anchor **A1/2** of type Pam-L-Lys(Pam)-spacer-CO-CHO was prepared previously [12] and isolated with a 25% overall yield (non-optimized at that time).

In the present work, three analogs of **A1/2** were synthesized. The difference resides in the choice of the initial amine to obtain the starting IPT resin (**Z**, Scheme 2). 4,7,10-Trioxa-1,13-tridecanediamine was chosen for its hydrophilicity. Anchors **A0**, **A1** and **A2** were reached after $n = 0$, 1 and 2 cycles, respectively, of the following: the amino resin was acylated by diglycolic anhydride in the presence of DIEA and the resulting carboxylic resin was amidified by 4,7,10-trioxa-1,13-tridecanediamine under BOP in situ activation in the presence of DIEA (Scheme 2). **A1/2**, whose synthesis was already reported [12], needed one cycle of the above-mentioned cycle. At this stage, the amino derivatives were all acylated by Fmoc-L-Tyr(*t*Bu)-OH in the presence of PyBOP and DIEA. Then after Fmoc removal, the resin was treated by Fmoc-L-Lys(Fmoc)-OH under the same conditions. Following an ultimate deprotection, palmitic acid reacted first under HBTU/HOBt activation with DIEA onto the deprotected diamino resins. To prepare the second acylation, palmitic acid was activated by cyanuric fluoride in the presence of pyridine under argon [21]. The fluoride reacted on the resins in the presence of DIEA. Every coupling and deprotection was checked by a rapid colorimetric test based on the reaction of trinitrobenzenesulfonic acid (TNBS) with the amino resins [22]. The acetonide bridges' cleavage and the periodic oxidation of the diol and the extraction were performed as reported in Ref. [12]. Anchors' identity was controlled by MALDI-TOF mass spectrometer and their purity by RP-HPLC (on a C3 Zorbax® column). Overall yields were between 18 and 56% according to the spacer **X** in a 100–500 mg scale (see Table 1). It is noteworthy that the longer the spacer arm was, the lower the yield was. The spacer length is correlated to the hydrophilic part of the molecule: the most hydrophilic compounds are isolated with the lowest yields. **A1/2** overall' yield was increased to 40% due to better final extraction and purification.

2.2. Hydrazino acetyl peptides' design and synthesis

In parallel, six α-hydrazino acetyl peptides of the type H_2N-NH-CH_2-CO-spacer-YGRGDSP-NH_2 were produced (Scheme 3). Their sequences – GRGDSP – corresponded to a part of the fibronectin 'RGD' domain [23] and the tyrosin is added to obtain traceable peptides by UV monitoring. The Fmoc peptidyl resin Fmoc-YGRGDSP-NH-PAL-PEG-PS® was automatically obtained with a 10 equiv in situ activation

[2] Now commercially available at Novabiochem.



Scheme 2. General route to anchors **A0**, **A1**, **A2** (**Z** = $(CH_2)_3$-[O-(CH_2)]$_2$-O-$(CH_2)_3$) and **A1/2** (**Z** = $(CH_2)_3$), $n = 0$, 1 and 2, respectively. A general formula for the four anchors is presented, for **X**, see Table 1.

of the amino acids in the presence of HBTU, HOBt and DIEA. The resin was then divided into five equal portions.

Peptides **RGD1**, **RGD2** and **RGD3** were reached after 1, 2 and 3 cycles, respectively, of the following: the deprotected amino resin was acylated by the diglycolic anhydride in the presence of DIEA and the resulting carboxylic resin was amidified by 4,7,10-trioxa-1,13-tridecanediamine under BOP in situ activation in the presence of DIEA. In peptide **RGD0**, no anhydride and no diamine were coupled, only the Fmoc was removed manually. Every free N-terminal amino resins were then acylated parallely by *N*,*N*′,*N*′-tri(*tert*-butyloxycarbonyl)-hydrazino acetic acid using an HBTU/HOBt/DIEA activation. The peptidyl resins were deprotected and cleaved by TFA following a classical solid phase synthesis workup. It is noteworthy that hydrazino acetyl peptides are instable in the presence of acetonitrile and should be purified with RP-HPLC eluents containing isopropanol as was exposed in Refs. [13,14]. The peptides' identities were checked on a MALDI-TOF spectrometer and their purities (over 90% in every cases) were controlled by RP-HPLC. Overall yields were between 16 and 37% (Table 2). It is noteworthy, that, once again, the yield decreases with the length (and the hydrophilicity) of the spacer.

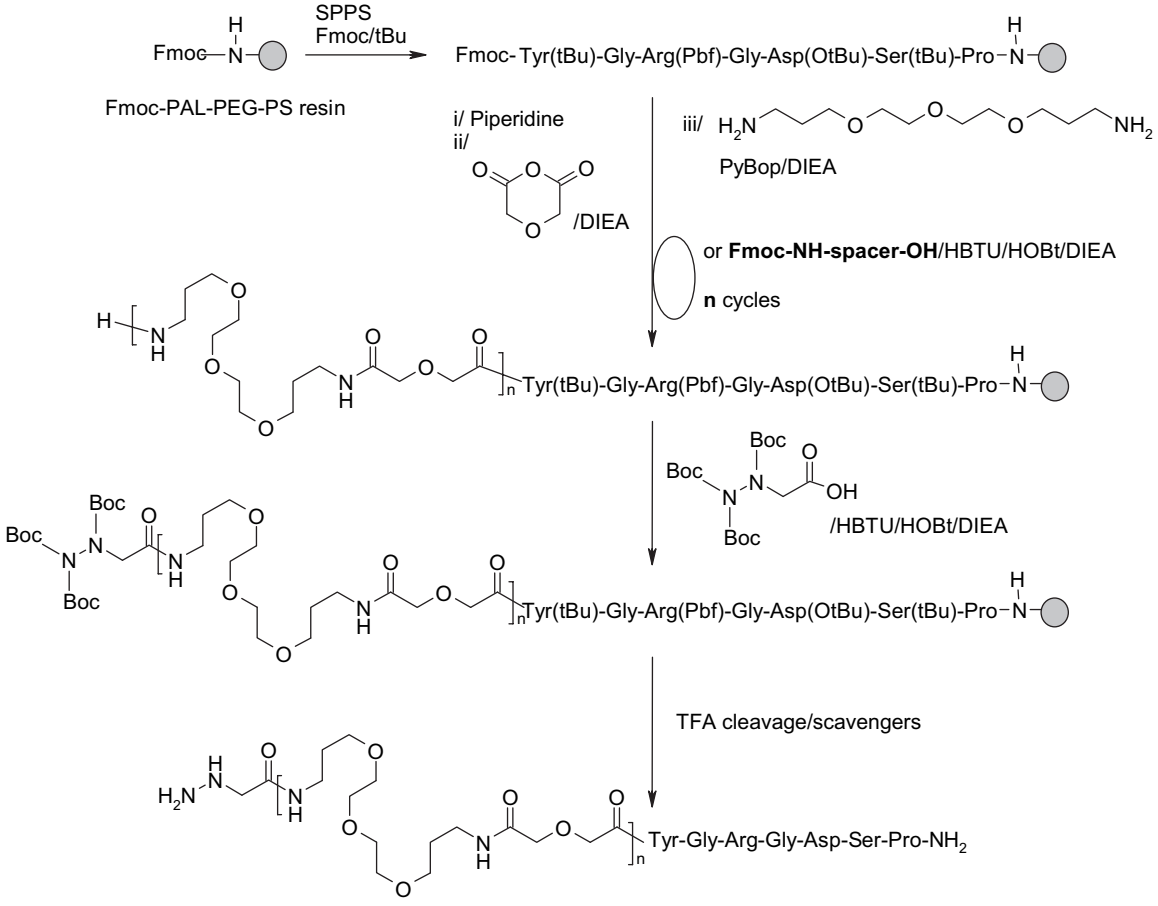
2.3. Conception and synthesis of a versatile spacer for longer MRP

RDG6 was produced starting from an Fmoc-YGRGDSP-NH-PAL-PEG-PS® resin, as for above-mentioned peptides and a new Fmoc-protected amino acid of the type **Fmoc-NH-spacer-OH** designed to be handled in solid phase synthesis. On a Wang resin, a first amount of the expected **Fmoc-NH-spacer-OH** amino acid was obtained in good purity and acceptable yield (30%, data not shown). The synthesis was optimized and transposed to solution phase basing on already reported works [24–26]. Thus, diglycolyl anhydride reacted with 4,7,10-trioxa-1,13-tridecanediamine in such conditions that only one amine reacted to get the monoamide (Scheme 4). The free amine was then protected by Fmoc-OSu to lead to the expected bifunctional monoprotected compound

Table 1
The four anchors synthesized: spacers' formula, yields and lengths

Anchor entry	X =	Yield (%)	Estimated length[a] (Å) (out of the membrane)
A0	Tyr-Gly-NH-$(CH_2)_3$-[O-(CH_2)]$_2$-O-$(CH_2)_3$-	56	22
A1/2	Tyr-NH-$(CH_2)_3$-[O-$(CH_2)_2$]$_2$-O-$(CH_2)_3$-NH-CO-CH_2OCH_2CO-Gly-NH-$(CH_2)_3$-	40	37
A1	Tyr-NH-$(CH_2)_3$-[O-$(CH_2)_2$]$_2$-O-$(CH_2)_3$-NH-CO-CH_2OCH_2CO-Gly-NH-$(CH_2)_3$-[O-(CH_2)]$_2$-O-$(CH_2)_3$-	34	49
A2	Tyr-{NH-$(CH_2)_3$-[O-$(CH_2)_2$]$_2$-O-$(CH_2)_3$-NH-CO-CH_2OCH_2CO}$_2$-Gly-NH-$(CH_2)_3$-[O-(CH_2)]$_2$-O-$(CH_2)_3$-	18	76

[a] Lengths are estimated from the Nα of the tyrosin to the alpha-oxo aldehyde. As an example, -NH-$(CH_2)_3$-[O-$(CH_2)_2$]$_2$-O-$(CH_2)_3$-NH-CO-CH_2OCH_2CO- length is ∼27.5 Å.



Scheme 3. Solid phase synthesis of RGD peptides. $n = 0$, **RGD0**; $n = 1$, **RGD1**; $n = 2$, **RGD2**; $n = 3$, **RGD3**.

(Fmoc-NH-$(CH_2)_3$-[O-$(CH_2)_2$]$_2$-O-$(CH_2)_3$-NH-CO-CH_2OCH_2 COOH) within a good yield (64%) in two steps, which is better than the five steps previously reported [24].

2.4. The difficulty in synthesizing longer MRPs

Fmoc-NH-spacer-OH was used six times manually onto H-YGRGDSP-NH-resins. The solid phase synthesis included a capping step to prevent truncated peptide from side reactions. The coupling yield decreased with the couplings (Fig. 1), despite several coupling agents and solvents were tried (HOBt/HBTU in DMF, or in a mixture of Triton®100 1% in NMP, DMF and ethylene carbonate 4 M in CH_2Cl_2 called 'magic mixture', or PyBOP in DMF, Fig. 1a, estimated by Fmoc removal UV test [27], an attempt with an *O*-succinimide ester was totally disappointing in solution). A small amount of the Fmoc-(spacer)$_6$-YGRGDSP-NH-PAL-PEG-PS® resin was cleaved before introducing the final *N*,*N*′,*N*′-tri(*tert*-butyloxycarbonyl)-hydrazino acetic acid. The RP-HPLC profile showed the successive cappings of some truncated peptides indicating an incomplete coupling of **Fmoc-NH-spacer-OH** at each step (Fig. 1b, admitting that the relative areas were correlated to the relative amounts of truncated peptides since the major absorbance was given by the tyrosin side chain, whereas the expected peptidyl resin contained a Fmoc).

Finally, the Fmoc-(spacer)$_6$-YGRGDSP-NH-resin was deprotected and submitted to *N*,*N*′,*N*′-tri(*tert*-butyloxycarbonyl)-hydrazino acetic acid coupling for the unambiguous introduction of the hydrazino acetyl group upon the selectively deprotected α-amino group of the N-terminal amino acyl resin. This synthesis was not optimized, though, and a 0.3% overall yield was obtained after preparative RP-HPLC, producing enough peptide (1 mg) in sufficient purity for a preliminary study.

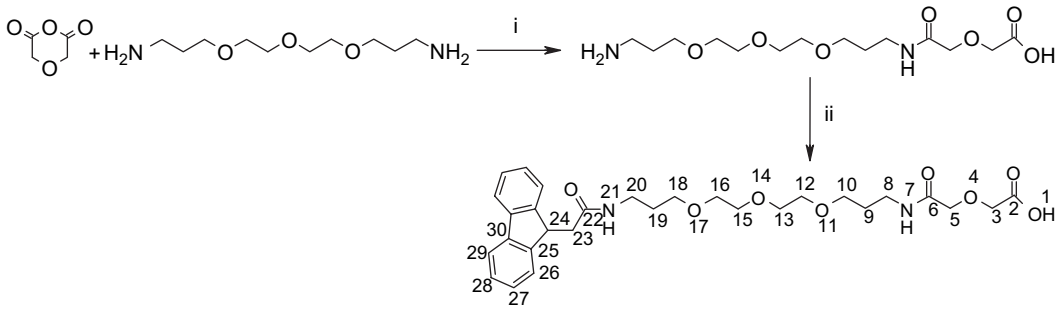
Table 2
Yields and lengths estimated for the six peptides synthesized for this study

Peptide entry	Yield (%)	Estimated length[a] (Å)
RGD0	37.2	29
RGD1	31.6	55
RGD2	22.6	81
RGD3	15.7	108
RGD6	0.3	186
RGD-PEG$_{2000}$	5.4	186

[a] 24 Å for the peptidic sequence Tyr-Gly-Arg-Gly-Asp-Ser-Pro and around 27.5 Å for a spacer -NH-$(CH_2)_3$-[O-$(CH_2)_2$]$_2$-O-$(CH_2)_3$-NH-CO-CH_2OCH_2CO- are estimated.

2.5. Alternative route toward longer peptides

Reconsidering the route to **RGD0**, **RGD1**, **RGD2** and **RGD3**, a **RGD-PEG$_{2000}$** peptide was synthesized by using



Scheme 4. Synthesis of **Fmoc-NH-spacer-OH**. (i) 2 h at 0 °C then 2 h at rt in CH_3CN. (ii) Fmoc-OSu in CH_3CN/H_2O 1:1.

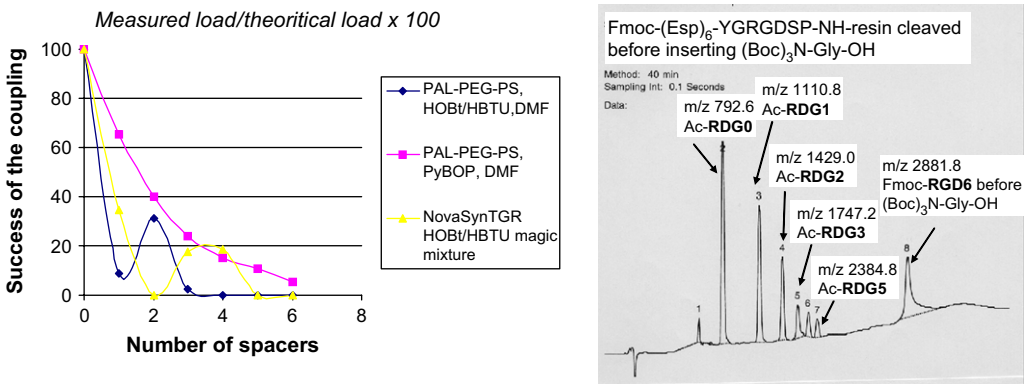


Fig. 1. (a) Successes of the couplings (%) as a function of the number of spacers of the type **Fmoc-spacer-OH** introduced in solid phase with diverse coupling agents and solvents estimated by Fmoc removal UV test. (b) RP-HPLC profile of a crude Fmoc-(spacer)$_6$-YGRGDSP-PAL-PEG-PS® resin cleaved after six couplings of **Fmoc-spacer-OH**. Ac = acetyl (the SPS includes a capping step to prevent truncated peptide from side reactions).

a commercially available PEG_{2000} diamine, '*O*,*O*′-Bis(2-aminoethyl)polyethylene glycol 2000', in place of 4,7,10-trioxa-1,13-tridecanediamine which was previously employed. An overall yield of 5.4% was reached for **RGD-PEG$_{2000}$** (8 mg, not optimized, see Table 2).

3. Preliminary biological results. One possible application for these molecular recognition patterns with spherulites™

3.1. Introduction: spherulites™ as model targeting colloids

Spherulites™ are multilamellar vesicles with no aqueous core obtained by shearing a lipidic lamellar phase [28] and whose diameter is between 200 nm and 1 μm (Fig. 2). Their monodispersed size (usually less than 5% have an other diameter [29]) is related to their formulation and the shearing strength and lasting. Easy to produce (Scheme 5), these vesicles were first designed and studied at the laboratory scale [30] and further developed industrially[3]. Spherulites™ are usually constituted of alternate lipidic bilayers, formed by phospholipids (especially phosphatidylcholine, PC), sometimes co-surfactants, and aqueous layers, and therefore display an onion-like structure. Here, we work on spherulites™ whose diameter is between 200 and 500 nm, containing only PC and water. Precisely, the mean here is 400 nm. Thus, spherulites™ can encapsulate either hydrophilic [31,32] or hydrophobic [33] active compounds, in the aqueous layer or in the lipidic bilayers, respectively. This indicates their potential interest in drug encapsulation and further, in drug targeting [34]. We thus envisaged the use of spherulites™ in that field and especially chose either the dendritic [35] or the endothelial [36] cells targeting as a way to validate these vesicles. Applications can thus be found in the delivery of anticancer drugs [37] or vaccines [38].

3.2. The background of this study

The system using the RGD/integrin couple as a targeting model for spherulites™ was first investigated by some of us, especially Chenevier, Delord and Roux [36]. Spherulites™ are designed neutral in surface to avoid their non-specific adhesion. Their targeting was first envisaged thanks to lipopeptides. Incorporated as lipids in the initial formulation, their lipidic double chain permitted the anchoring of a peptidic head, acting as an MRP. The study demonstrated the specificity of the interaction between a GRGDSP sequence (from fibronectin) presented by the vesicles and integrins of the sub-type $\alpha_v\beta_3$ present, for example, at the surface of

[3] Industrially developed by Capsulis for non-pharmaceutical applications (cosmetology, detergents…) and by Ethypharm for pharmaceutical applications.

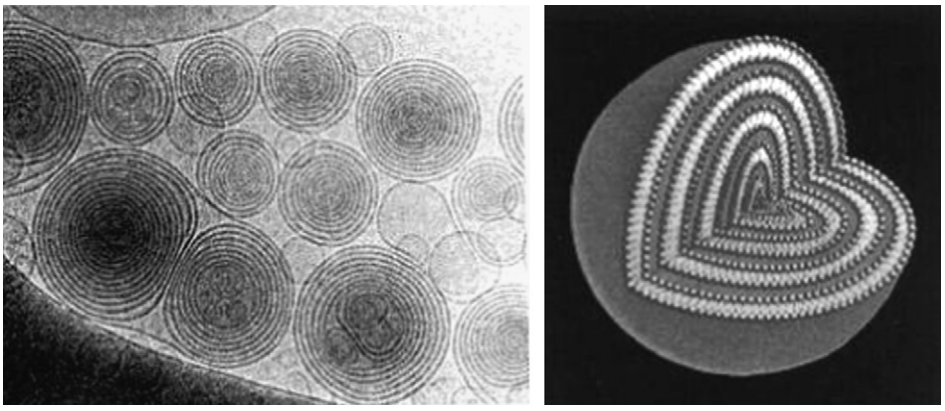


Fig. 2. Spherulites™. Left, by CryoTEM [30] and right, 3D-scheme [31].

HUV endothelial cells. Nevertheless, lipopeptides are amphiphilic compounds difficult to produce, especially when large amounts are needed, essentially for analytical reasons and low purification yields. Moreover, to perform a structure/properties relationship (SPR) study, a versatile strategy and facile synthetic routes are needed. Hence, the great interest to develop the ligation strategy based on the hydrazone bond previously described is obvious.

Another study was performed by Poirier and co-workers [39] and was considered as a second useful basis for the present work. It revealed that (1) a precise quantification of particles' specific binding using flow cytometry is feasible. It focused on a statistical kinetic study on a population of cells with a population of particles and showed how such experiments can give information on the microscopic behaviour; (2) chemical versatile ligation is equivalent to known and validated lipopeptide insertion in terms of binding to the target cells.

3.3. Preparation of the peptido vesicles

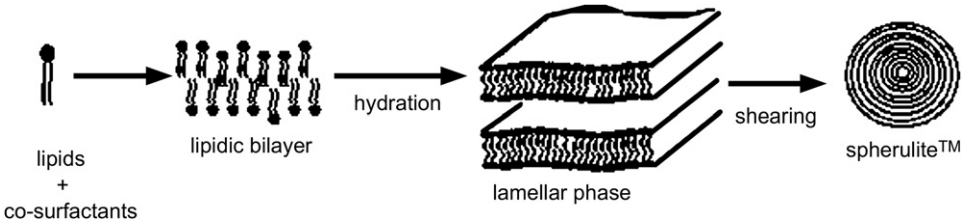
Thermodynamic and kinetical aspects of the chemoselective hydrazone ligation were extensively studied [40] and are not further tackled in the present work. To summarize, the reaction takes 4 h at pH 4.6, the conversion rate yields 80–90%, the non-specific adsorption is inferior to 10% and the alpha-oxo hydrazone bond is stable at least 14 days at pH 4.6. The expected peptido vesicles were obtained according to a protocol reported in Section 5 (ligand surface density $= 2 \times 10^{-2}$ nm^{-2}) to get a final 1/100 w/w concentration. To remove non-grafted peptides from the colloidal suspension, the dispersions were submitted to a gel filtration step. The MRP's length was between 51 Å and 262 Å.

3.4. Structure/properties preliminary relationships on HUVEC endothelial cells

We used RGD ligands with different spacers to functionalize the spherulites™ and compared their binding efficiency to HUV endothelial cells (Fig. 3). Since all RGD vesicles were provided from the same original preparation (lipid mixture, shear and dispersion), only the effect of length spacers is compared here; all other parameters, and particularly particle sizes are considered to be equal.

Colloids and cells were centrifuged together (10 min, 400 g, 4 °C) and incubated for 4 h at 37 °C. Then, the cells were washed and suspended in a fresh phosphate saline buffer. Flow cytometry measurements were carried out as described in Section 5.

Several combinations have been studied and the most relevant are shown here. The comparison between the fluorescence profiles obtained reveals that the adhesion decreases when the length of the spacer increases. So, the most efficient binding is obtained with the shorter spacer (**A0–RGD0** combination) whereas the weaker adhesion is observed for a longer combination **A2–RGD6** (Fig. 4). These results seem to imply that a long spacer between the surface of the colloids and the recognition pattern RGD does not favor the adhesion of the particles on the integrins' receptors. Though surprising, this result is interpretable. The hydrophilic spacer used is flexible and thus, a longer spacer leads to a more important number of conformations for its head RGD. Regarding the chemical nature of the spacer, **RGD6** is as long as **RGD-PEG$_{2000}$** but certainly less flexible. Some 'privileged' structures can even be encountered considering the large potential for intramolecular hydrogen bonds. Such bonds can also be put forward to explain the poor reactivity of



Scheme 5. Preparation of spherulites™.

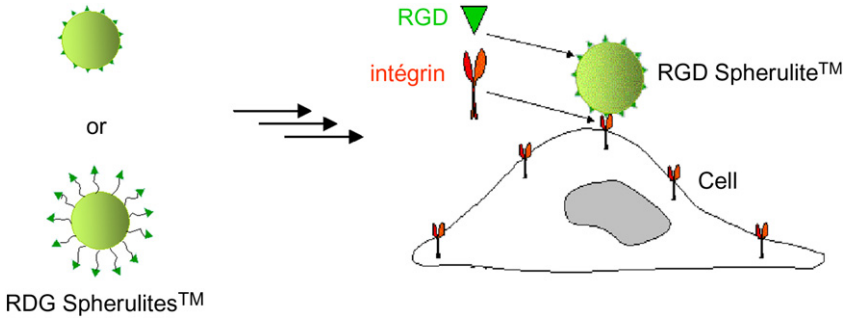


Fig. 3. Stake of the present work. Triangles represent molecular recognition patterns (MRP) close to (left) or distant from (right) the vesicle's membrane.

the **Fmoc-spacer-OH** compound. Here, it seems that the same argument can be claimed to explain the difference between the combinations **A2–RGD6** and **A2–RGD-PEG$_{2000}$**.

Moreover, the recognition between the integrin receptors and their ligands strongly depend on their conformations. A number of papers underline the importance of dynamic changes in integrins' conformations. This complexity allows in vivo a very fine adjustment of the binding affinity with the extracellular matrix. Crystallographic studies showed that cations play an important role, favouring an active conformation (with Mn^{2+}) [41] or on the contrary, inhibiting recognition (with Ca^{2+}) [42]. This strong dependence on geometric conformations of this ligand/receptor couple could explain why a long and rigid spacer becomes so unfavourable in case of RGD integrin recognition whereas it seems to be of an advantage in case of biotin/streptavidin binding.

4. Conclusion

To summarize, we have in hands some easy chemical tools and a versatile ligation bond to prepare targeting vesicles. Considering the spacer between the colloid and an RGD recognition pattern, its length, its flexibility and its capabilities to fold up via hydrogen bonds are to be finely tuned for an optimized recognition. In our case, when a favorable conformation becomes less probable, the adhesion is consequently less efficient. Our results are opposite to those reported with the biotin/streptavidin couple. We think that for each ligand/receptor couple, a preliminary study like the one presented here has to be performed. Even if our results are to be confirmed, a general rule seems to be difficult to establish. Relationships between the structure (length, then flexibility) and the recognition properties are dependent on (1) the nature of the receptor, (2) the strength of the interaction (the biotin/streptavidin is the strongest interaction in biology and the RGD/integrin couple is 4–5 units of logarithm lower) and, least, (3) the chemical nature of the ligand. In our model, the encapsulation of active compounds is currently in progress. The design of even more complex structures is conceivable: PEG-covered ('stealthy™') or multi-ligand constructs are permitted by orthogonal ligation strategies and a fine-tuning of the reaction conditions. Remarkable vesicles fully defined at the molecular level are already obtained [43].

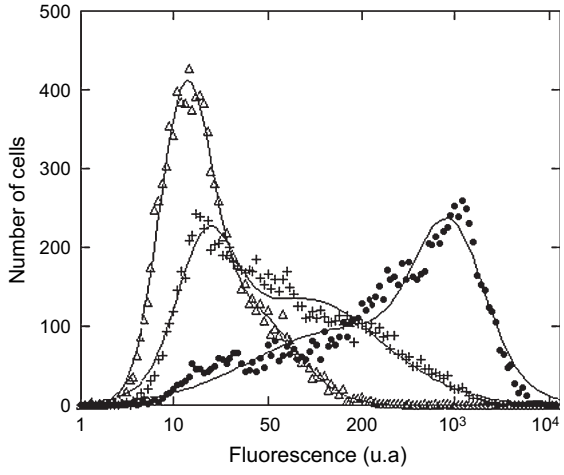


Fig. 4. Fluorescence distribution obtained by flow cytometry after 4 h of incubation at 37 °C between HUVEC cells (10^6 mL^{-1}) and RGD colloids (5×10^{-4}). ●: **A0–RGD0**, △: **A2–RGD6**, +: **A2–RGD-PEG$_{2000}$**.

5. Experimental protocols

5.1. Generalities

5.1.1. Chemicals

All Fmoc-protected amino acids (L-Arg(Pmc), L-Asp(O*t*Bu), L-Glu(O*t*Bu), Gly, L-Lys(Fmoc), L-Pro, L-Ser(*t*Bu), L-Tyr(*t*Bu)), *N*,*N*′,*N*′-tri(*tert*-butyloxycarbonyl)-hydrazino acetic acid, coupling agents (HOBt, HBTU, BOP, PyBOP) and resins (Novasyn®, PAL-PEG-PS®) were purchased from Novabiochem. Chemicals were from Sigma–Aldrich except cyanuric fluoride which was from Lancaster. Solvents were from Acros.

5.1.2. RP-HPLC eluents

A: TFA 0.05% in H_2O, **B**: TFA 0.05% in CH_3CN/H_2O: 80/20 (v/v), **C**: TFA 0.05% in propan-2-ol/H_2O: 40/60 (v/v), **D**: TFA 0.05% in CH_3CN/H_2O: 90/10 (v/v).

5.1.3. The mass spectra

The mass spectra were acquired on a Perspective Biosystems Voyager-DE™ STR, Biospectrometry™ Workstation

MALDI-TOF spectrometer and measures were acquired after deposition on a dihydroxybenzoic acid (DHB) matrix.

5.2. Syntheses of anchors *A0*, *A1*, *A2* and *A1/2*

Two Fmoc-Gly-IPT resins were prepared either with 4,7,10-trioxa-1,13-tridecanediamine (anchors **A0**, **A1** and **A2**) or with diaminopropane (anchor **A1/2**) on commercial Novasyn® resin. The reactions were performed on the basis of 0.2 mmol of resin (0.78 g, loading = 0.256 mmol/g). After deprotection of the Fmoc-Glycyl residue (by a mixture of piperidine/DMF (2/8: v/v) for 2 min then 20 min), anchors **A0**, **A1** and **A2** were reached after 1, 2 and 3 cycles, respectively, of the following: the amine was acylated by diglycolic anhydride (92.85 mg, 4 equiv) in the presence of DIEA (134.4 μL, 4 equiv) in DMF and the resulting carboxylic resin was amidified by 4,7,10-trioxa-1,13-tridecanediamine (438.43 μL, 10 equiv) under BOP (177 mg, 2 equiv) in situ activation in the presence of DIEA (104.56 μL, 3 equiv). **A1/2**, whose synthesis was already reported, needed one cycle of the above-mentioned run. At this stage, the amino derivatives were all acylated by Fmoc-L-Tyr(*t*Bu)-OH (367.68 mg, 4 equiv) in the presence of PyBOP (417.92 mg, 4 equiv) and DIEA (278.81 μL, 4 equiv) in DMF (1 mL) for 40 min. Then after Fmoc removal, the resin was treated by Fmoc-L-Lys(Fmoc)-OH (472.56 mg, 4 equiv) under the same conditions. Following an ultimate deprotection of the lysine by piperidine (see above), palmitic acid (205.14 mg, 4 equiv) reacted first under HBTU (303.44 mg, 4 equiv), HOBt (108.1 mg, 4 equiv) activation with DIEA (557.63 μL, 16 equiv) in DMF (1.5 mL) onto the diamino resins. To prepare the second acylation, palmitic acid (410.33 mg, 8 equiv) was activated by cyanuric fluoride (864.33 μL, 32 equiv) in the presence of pyridine (129.33 μL, 8 equiv) under argon (in a sealed round glass flask) for 2 h in 15 mL of CH_2Cl_2/*t*BuOMe: 4/1 (v/v) mixture. Following a rapid aqueous washing, CH_2Cl_2 was evaporated and dried on $MgSO_4$, the acyl fluoride was solubilized in 2 mL of DMF and reacted on the resins in the presence of DIEA (279 μL, 16 equiv) for 3 h. Every coupling and deprotection was checked by a rapid colorimetric test based on the reaction of TNBS with amino resins. The acetonide bridges were then cleaved by 20 mL of a TFA/H_2O/TIS mixture (95/2.5/2.5: v/v/v) for 2 h. Anchors were cleaved by $NaIO_4$ (254.6 mg, 6 equiv) in 10 mL of a water/AcOH/methyl-2-propanol-2 (2/1/3: v/v/v) mixture for 5 min the excesses of which were quenched by ethanolamine (608.8 μL, 50 equiv) for 5 min more. Anchors were obtained by two extractions by 10 mL of *t*BuOH/H_2O (1/1: v/v) at rt followed by five extractions with 10 mL of *t*BuOH/CH_2Cl_2 (1/1: v/v) at 50 °C. The seven extraction phases were pooled, CH_2Cl_2 was added to obtain a CH_2Cl_2/*t*BuOH/water (1/1/1: v/v/v) ratio. Following drying and evaporating the organic phase, anchors were obtained as very light yellow powders, soluble in $CHCl_3$/MeOH (1/1: v/v) mixture at rt and in propan-2-ol at 37 °C. Anchors' identity was controlled by MALDI-TOF mass spectrometer and their purity by RP-HPLC (C3 Zorbax®, 4.6 × 200 mm, 1 mL min^{-1}, $\lambda = 215$ nm, 50 °C, eluents **A** and **D** (**D** 0–100% in 30 min, 100% **D** for 10 min).

5.2.1. Anchor *A0*

NMR ($CDCl_3$/CD_3OD 1/1: v/v, 300 MHz) ^1H δ ppm 0.79 (t, $J = 6.9$ Hz, 6H), 1.17 (m, 50H), 1.37 (m, 2H), 1.50 (m, 6H), 1.75 (m, 4H), 2.11 (m, 4H), 2.75 (m, 1H), 3.10 (m, 3H), 3.25 (m, 4H), 3.50 (m, 12H), 3.75 (m, 2H), 4.00 (s, 1H), 4.35 (m, 1H), 6.65 (d, $J = 8.5$ Hz, 2H), 6.74 (d, $J = 8.5$ Hz, 2H). MALDI-TOF *m/z* obsd: 1123.91, $[M + Na]^+$ calcd = 1123.57 g/mol. TLC performed on silica with CH_2Cl_2/MeOH/AcOH (8.75/1.25/0.05: v/v/v) ($R_f = 0.3$). RP-HPLC purity > 99%. Isolated yield = 56% (123 mg).

5.2.2. Anchor *A1*

NMR ($CDCl_3$/CD_3OD 1/1: v/v, 300 MHz) ^1H δ ppm 0.89 (t, $J = 6.9$ Hz, 6H), 1.27 (m, 50H), 1.43 (m, 2H), 1.63 (m, 6H), 1.83 (m, 6H), 2.21 (m, 4H), 3.00 (m, 2H), 3.15 (m, 2H), 3.34 (m, 12H), 3.62 (m, 22H), 3.92 (s, 2H), 4.12 (s, 2H), 4.18 (s, 2H), 4.48 (m, 1H), 6.74 (d, $J = 8.5$ Hz, 2H), 7.02 (d, $J = 8.5$ Hz, 2H). MALDI-TOF *m/z* obsd: 1442.25, $[M + Na]^+$ calcd = 1142.16 g/mol. A preparative chromatography performed on silica with CH_2Cl_2/MeOH/AcOH (8.95/1.00/0.05: v/v/v) ($R_f = 0.33$) as eluent gave the expected doubly purified compound. RP-HPLC purity > 99%. Isolated yield = 34.4% (97.6 mg).

5.2.3. Anchor *A2*

NMR ($CDCl_3$/CD_3OD 1/1: v/v, 300 MHz) ^1H δ ppm 0.88 (t, $J = 6.5$ Hz, 6H), 1.27 (m, 50H), 1.45 (m, 2H), 1.68 (m, 6H), 1.83 (m, 10H), 2.21 (m, 4H), 3.01 (m, 2H), 3.15 (m, 2H), 3.36 (m, 14H), 3.62 (m, 34H), 3.75 (t, $J = 5$ Hz, 2H), 3.92 (s, 2H), 4.08 (m, 5H), 4.45 (m, 1H), 6.74 (d, $J = 8.5$ Hz, 2H), 7.01 (d, $J = 8.5$ Hz, 2H). MALDI-TOF *m/z* obsd: 1761.48, $[M + Na]^+$ calcd = 1761.35 g/mol. A preparative chromatography performed on silica with CH_2Cl_2/MeOH/AcOH (8.75/1.20/0.05: v/v/v) ($R_f = 0.3$) as eluent gave the expected doubly purified compound. RP-HPLC purity > 99%. Isolated yield = 18.4% (53.9 mg).

5.2.4. Anchor *A1/2*

Synthetic details are found in Ref. [26]. NMR ($CDCl_3$/CD_3OD 1/1: v/v, 300 MHz) ^1H δ ppm 0.80 (t, $J = 6.7$ Hz, 6H), 1.15 (m, 50H), 1.35 (m, 2H), 1.50 (m, 5H), 1.66 (m, 7H), 1.80 (m, 2H), 2.15 (m, 4H), 2.85 (m, 2H), 3.10 (m, 4H), 3.20–3.50 (m, 16H), 3.50 (m, 16H), 3.80 (s, 2H), 4.00 (s, 2H), 4.05 (s, 2H), 4.15 (t, $J = 5.6$ Hz, 1H), 4.35 (m, 1H), 6.65 (d, $J = 8.3$ Hz, 2H), 6.90 (d, $J = 8.3$ Hz, 2H). ^{13}C δ ppm 13, 28, 36, 42, 48, 53, 60, 69, 95. MALDI-TOF *m/z* obsd: 1295.0, $[M + Na]^+$ calcd: 1295.7. RP-HPLC purity > 99%. Isolated yield = 40% (101.8 mg).

5.3. Syntheses of hydrazino acetyl peptides

The synthesis of an Fmoc peptidyl resin of the type Fmoc-YGRGDSP-NH-resin was performed on 0.25 mmol of PAL-PEG-PS® resin in a Pioneer synthesizer (Applied Biosystems, UK) with a 10 equiv in situ activation of amino acid in the

presence of HBTU, HOBt, DIEA and protected amino acids. The resin was then divided into five equal portions of 0.05 mmol (0.323 g). Peptides **RGD1**, **RGD2** and **RGD3** were reached after 1, 2 and 3 cycles, respectively, of the following: the deprotected amino resin was acylated by diglycolic anhydride (23.21 mg, 4 equiv) in the presence of DIEA (34.85 μL, 4 equiv) in DMF and the resulting carboxylic resin was amidified by 4,7,10-trioxa-1,13-tridecanediamine (109.6 μL, 10 equiv) under PyBOP (52.24 mg, 2 equiv) in situ activation in the presence of DIEA (26.1 μL, 3 equiv). In peptide **RGD-PEG$_{2000}$**, a commercially available PEG_{2000} diamine, *O*,*O*′-Bis(2-aminoethyl)polyethylene glycol 2000 (1 g, 10 equiv) in 4 mL of DMF replaced the above-mentioned diamine. In peptide **RGD0** no anhydride and no diamine were coupled, only the Fmoc was removed manually. Every free N-terminal amino resins were then acylated by *N*,*N*′,*N*′-tri(*tert*-butyloxycarbonyl)-hydrazino acetic acid (23.43 mg, 1.2 equiv) using an HBTU/HOBt/DIEA (22.72 mg/8.11 mg/41.8 μL) activation in 0.5 mL of DMF. The peptidyl resins were deprotected and cleaved by TFA in the presence of H_2O/phenol/ethanedithiol/thioanisole: 0.5 mL/0.75 g/0.25 mL/0.5 mL in 10 mL TFA for 2 h. The crude peptides were then precipitated in cold Et_2O, washed and purified by preparative RP-HPLC (C18 Nucleosil® column, 15 × 500 mm, 4 mL min^{-1}, $\lambda = 225$ nm, 50 °C, eluents **A** and **C** (**C** 0–100% in 30 min, 100% **C** for 10 min). The peptides' identities on a MALDI-TOF spectrometer and their purities were controlled by RP-HPLC. **RGD 0**: *m*/*z* obsd: 822.38, $[M + H]^+$ calcd: 821.85 g/mol, purity > 99%, overall yield: 37.2% (15.3 mg). **RGD 1**: *m*/*z* obsd: 1140.59, $[M + H]^+$ calcd: 1140.23 g/mol, purity = 94%, overall yield: 31.6% (18.0 mg). **RGD 2**: *m*/*z* obsd: 1458.76, $[M + H]^+$ calcd: 1458.64 g/mol, purity = 92%, overall yield: 22.6% (16.5 mg). **RGD 3**: *m*/*z* obsd: 1776.90, $[M + H]^+$ calcd: 1776.97 g/mol, purity = 89%, overall yield: 15.7% (14.0 mg). **RGD-PEG$_{2000}$**, *m*/*z* obsd: a gaussian curve centered on 2962.9, $[M + H]^+$ calcd: 2962.42 g/mol for $n = 45$, purity = >84%, overall yield: 5.40% (8 mg of a sticky powder).

RDG6 was produced starting from an Fmoc peptidyl resin of the type Fmoc-YGRGDSP-NH-resin, as for shorter peptides and a new synthon of the type **Fmoc-NH-spacer-OH**.

Fmoc-NH-spacer-OH was obtained in solution phase. 4.40 g (20 mmol) of 4,7,10-trioxa-1,13-tridecanediamine was solubilized in CH_3CN (100 mL) in a glass flask at 0 °C. Parallely, 2.56 g of diglycolyl anhydride (20 mmol, 1 equiv) was solubilized in CH_3CN (50 mL) and added drop by drop to the cold diamine. Under magnetic stirring, a precipitate was rapidly observed. The medium was left 2 h at room temperature, it was milky and contained a non-soluble phase. 150 mL of distilled water was added, the medium was stirred for 30 min at 0 °C and returned to limpidity. Then, 3.5 mL of DIEA (20 mmol, 1 equiv) and a solution of 8.76 g of Fmoc-OSu (20 mmol, 1 equiv) in CH_3CN (75 mL) were added to the medium kept at 0 °C. The solution's pH was checked and adjusted to 9 with drops of DIEA. The reaction was left over 12 h at room temperature under stirring. A thin layer chromatography was performed to monitor the conversion into the expected compound followed by UV detection and phosphomolybdic acid mineralisation ($HCCl_3$/MeOH = 70/30: v/v, $R_f = 0.3$). Solvents were evaporated under reduced pressure. The white viscous compound obtained was extracted by an aqueous solution of $NaHCO_3$ 5% (200 mL), then washed by AcOEt (100 mL, several times if necessary to remove the unreacted diamine). The aqueous phase was acidified by HCl 1 N to get pH = 2 and the expected compound was extracted three times by 100 mL of AcOEt. Organic phases were pooled and washed three times by 75 mL of distilled water before being dried over $MgSO_4$ and evaporated under reduced pressure. A colorless oil was obtained. MALDI-TOF *m*/*z* obsd: 559.4 $[M + H]^+$, 576.4 $[M + H_2O]^+$ and 581.3 $[M + Na]^+$ calcd: 559.63 ($[M + H]^+$). RP-HPLC purity > 91%. Isolated yield = 64% (7.2 mg). NMR (CD_3OD, 300 MHz) ^1H δ ppm 1.51 (m, 4H, H_9 and H_{19}), 2.94 (t, $J = 6.66$ Hz, 2H, H_8), 3.07 (m, 3H, H_{20}, N*H*), 3.31 (m, 13H, H_{10}, H_{12}, H_{13}, H_{15}, H_{16}, H_{18}, N*H*), 3.79 (s, 2H, H_5), 3.93 (s, 3H, H_3 and H_{25}), 4.12 (d, 2H, $J = 6.71$ Hz, H_{24}), 7.06 (td, 2H, $J = 7.45$, 1.06 Hz, 7.14 (t, 2H, $J = 7.16$ Hz, H_{18}), 7.39 (d, 2H, $J = 7.32$ Hz, H_{26}), 7.54 (d, 2H, $J = 7.45$ Hz, H_{29}). ^{13}C δ ppm 29.30, 29.73, 36.64, 38.07, 48.00, 66.44, 68.16, 68.69, 69.03, 70.20, 70.42, 70.50, 119.94, 125.13, 127.14, 127.76, 141.62, 144.36, 157.80, 170.91, 172.70.

5.3.1. *RDG6 synthesis*

Fmoc-YGRGDSP-NH-resin was submitted to standard Fmoc/*t*Bu solid phase synthesis using **Fmoc-NH-spacer-OH** as an ordinary amino acid. Several attempts were performed, one of them required the preparation of a 'magic mixture' (Triton®100 1% in NMP/DMF/ethylene carbonate 4 M in CH_2Cl_2: 1/1/1: v/v/v) as a solvent before coupling *N*,*N*′,*N*′-tri(-*tert*-butyloxycarbonyl)-hydrazino acetic acid and standard workup. Overall yield = 0.3% (not optimized), purity > 99%, *m*/*z* obsd: 2731.5 $[M + H]^+$, calcd: 2730.0 ($[M + H]^+$).

5.4. Vesicles' formulation and preparation

Phosphatidylcholine purified from soybean lecithin was purchased from Lipoid and 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a, 4a-diaza-*s*-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-*glycero*-3-phosphocholine) (named Bodipy® lipid) from Avanti Polar Lipids. Spherulites™ were prepared by shearing a lamellar phase made of lipids (52% w/w phosphatidylcholine) and water (48% w/w). To begin with, lipids were all solubilized and mixed into a methanol/chloroform (50/50 v/v) solution. After evaporation under reduced pressure, a homogeneous lipidic film was obtained, the water was dropped and the lamellar phase was then formed. After shearing by hand (in an Eppendorf test tube) with a well-adapted piston, dispersion of the paste in 50 times its volume in PBS buffer led to the formation of spherulites™ of ~300 nm in diameter (consistent with previously reported work) at a 2×10^{-2} volume fraction (20 mg mL^{-1}). Fluorescent colloids were obtained by addition of 0.1% (w/w) of the Bodipy® fluorescent lipid to the initial mixture of lipids (before addition of water). To graft the MRPs on the colloids, the reactive anchor, **A0**, **A1/2**, **A1** or **A2**, was added in the formulation allowing the coupling reaction with the RGD peptide. To this purpose, 6% molar of the phospholipids is replaced by the anchor so that the colloids,

after shear and dispersion 50 times in aqueous phase, bear on the surface reactive alpha-oxo aldehyde functions.

5.5. Ligation of a peptide onto functionalized spherulites™

A solution of **RDGn** at 1 mM was prepared in distilled water. 40 μL of this solution was added to 50 μL of spherulites™ suspension (1/50 w/w) with 10 μL of acetate buffer solution (0.1 M), pH = 5. After 3 h of reaction at rt under a soft stirring, RGD grafted colloids were obtained (ligand surface density = 2×10^{-2} nm^{-2}) to get a final 1/100 w/w concentration (10 mg/mL).

5.6. Gel filtration

An NAP-5™ column (Sephadex™ G-25 as stationary phase, Amersham Biosciences) was emptied and stabilized with 3 × 3 mL of PBS. Then 300 μL of a suspension of spherulites™ in water 1% (w/w) was dropped on the column. 300 μL of PBS was added to complete the dead volume of the column. Then 900 μL of PBS eluted the vesicles that were collected at the end of the column. Nevertheless, gel filtration separation induced dilution of the sample; that is why it was necessary to determine the new concentration of the dispersion as described thereafter.

5.7. Determination of unknown concentration dispersion: fluorimetry

Between the coupling reaction and the filtration, 10 μL of the well-known concentration dispersion was taken off. A reference curve 'fluorescence as a function of the particles' concentration' was carried out with a spectrofluorimeter (Fluorolog 3 FL 1008) (excitation at 488 nm − emission at 515 nm). Maximum emission spectra were recorded so as to obtain a reference line. After gel filtration, fluorescence of a little fraction (4 μL) of the dispersion collected at the end of the column was measured. By means of the reference line, the concentration could be precisely determined.

5.8. Cell culture

The primary human umbilical vein endothelial cells (HUVEC) were kindly provided by J. Amédée and cultured as described in Ref. [44]. Endothelial cells were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% fetal calf serum (v/v), 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin, 90 μg mL^{-1} heparin, and 20 μg mL^{-1} endothelial cell growth supplement (ECGS). Adherent cells were detached from their support by 5 min action of trypsin solution (0.25% in PBS buffer). The presence of integrin of the sub-type $\alpha_v\beta_3$ was checked by immunomarking and flow cytometry as described in Ref. [45]. After centrifugation (10 min, 400 g, 4 °C), they were washed in fresh phenol red free Dulbecco's modified Eagle's medium (DMEM). The concentration was adjusted at 10^6 cells mL^{-1}. Colloids were then added at the desired concentration and gently stirred to get homogeneous mixture of colloids and cells. After an appropriate step of centrifugation and 4 h of incubation at 37 °C, cells were washed and analysed by flow cytometry.

5.9. Flow cytometry

Experiments on living cells (no fixation) were carried out using a Facscalibur™ cytometer (Becton Dickinson Immunocytometry Systems) equipped with an argon laser (488 nm). For each sample, at least 10 000 events were counted. The fluorescence of each cell was provided and was directly proportional to the number of fluorescent colloids bound on the cell surface. Then, cell population measurement gave a fluorescence distribution. Using forward and side scatter parameters, the software (Cell Quest Pro® software) was used to determine the events of interest. On the gated area, the mean fluorescence intensity of the sample cell population was finally deduced from the mean geometric value of the Gaussian distribution.

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References

[1] V.T. Kung, C.T. Redemann, Biochim. Biophys. Acta 862 (1986) 435–439.
[2] P. Schelré, C. Boeckler, B. Frisch, F. Schuber, Bioconjug. Chem. 11 (2000) 118–123.
[3] J.R. Silvius, F. l'Heureux, Biochemistry 33 (1994) 13093–13101.
[4] J. Heeremans, J. Kraaijenga, P. Los, C. Kluft, D. Crommelin, Biochim. Biophys. Acta 1117 (1992) 258–264.
[5] M. Fleiner, P. Benzinger, T. Fichert, U. Massing, Bioconjug. Chem. 12 (2001) 470–475.
[6] J. Kamps, P. Swart, H. Morselt, R. Pauwels, M.P. De Béthune, E. De Clerck, D. Meijer, G. Scherphof, Biochim. Biophys. Acta 1278 (1996) 183–190.
[7] J. Barbet, P. Machy, L. Leserman, J. Supramol. Struct. Cell. Biochem. 16 (1981) 243–258.
[8] D. Horak, M. Karpisek, J. Turkova, M. Benes, Biotechnol. Prog. 15 (1999) 208–215.
[9] S. Zalipsky, B. Puntambekar, P. Boulikas, C.M. Engbers, M.C. Woodle, Bioconjug. Chem. 6 (1995) 705–708.
[10] J.A. Harding, C.M. Engbers, M.S. Newman, N.I. Goldstein, S. Zalipsky, Biochim. Biophys. Acta 1327 (1997) 181–192.
[11] S. Zalipsky, Bioconjug. Chem. 4 (1993) 296–299.
[12] L. Bourel-Bonnet, H. Gras-Masse, O. Melnyk, Tetrahedron Lett. 42 (2001) 6851–6853.
[13] D. Bonnet, N. Ollivier, H. Gras-Masse, O. Melnyk, J. Org. Chem. 66 (2001) 443–446.

[14] D. Bonnet, C. Grandjean, P. Rousselot-Pailley, P. Joly, L. Bourel-Bonnet, V. Santraine, H. Gras-Masse, O. Melnyk, J. Org. Chem. 68 (2003) 7033–7040.
[15] L. Bourel-Bonnet, E.I. Pécheur, C. Grandjean, A. Blanpain, O. Melnyk, B. Hoflack, H. Gras-Masse, Bioconjug. Chem. 16 (2005) 450–457.
[16] L. Bourel-Bonnet, D. Bonnet, F. Malingue, H. Gras-Masse, O. Melnyk, Bioconjug. Chem. 12 (2003) 494–499.
[17] G.B. Fields, R.L. Noble, Int. J. Pept. Protein Res. 35 (1990) 161–214.
[18] W.C. Chan, P.D. White, in: W.C. Chan, P.D. White (Eds.), Fmoc Solid Phase Synthesis, A Practical Approach, Practical Approach Series, Oxford University Press, Oxford, 2000, pp. 9–76 and 277–302.
[19] J.S. Fruchart, H. Gras-Masse, O. Melnyk, Tetrahedron Lett. 40 (1999) 6225–6228.
[20] O. Melnyk, J.S. Fruchart, C. Grandjean, H. Gras-Masse, J. Org. Chem. 66 (2001) 4153–4160.
[21] G.A. Olah, M. Nojima, I. Kerekes, Synthesis (1973) 487.
[22] W.S. Hancook, J.E. Battersby, Anal. Biochem. 71 (1976) 260–264.
[23] S. Verrier, Ph.D. Thesis, University of Paris 7, 1998.
[24] A.M.P. Koskinen, T. Valo, S. Vihavainen, J.M.L. Hakala, Bioorg. Med. Chem. Lett. 5 (1995) 573–578.
[25] G.R. Ehteshami, S.D. Sharma, J. Porath, R.Z. Guzman, React. Funct. Polym. 35 (1997) 135–143.
[26] M. Braun, X. Camps, O. Vostrowsky, A. Hirsch, E. Endress, T.M. Bayer, O. Birkert, G. Gauglitz, Eur. J. Org. Chem. 7 (2000) 1173–1181.
[27] Novabiochem Synthesis Notes, Fmoc removal test, catalog 2004/5, p. 3.4.
[28] T. Gulik-Krzywicki, J.C. Dedieu, D. Roux, C. Degert, R. Laversanne, Langmuir 12 (1996) 4668–4671.
[29] A. Richard, Ph.D. Thesis, University of Lille 2, 2005.
[30] O. Diat, D. Roux, J. Phys. II France 3 (1993) 9–14.
[31] T. Pott, D. Roux, FEBS Lett. 511 (2002) 150–154.
[32] N. Mignet, A. Brun, C. Degert, B. Delord, D. Roux, C. Hélène, R. Laversanne, J.C. François, Nucleic Acids Res. 28 (2000) 3134–3142.
[33] O. Freund, Ph.D. Thesis, University of Bordeaux 2, 1998.
[34] O. Freund, J. Amédee, D. Roux, R. Laversanne, Life Sci. 67 (2000) 411–419.
[35] P. Chenevier, C. Grandjean, E. Loing, F. Malingue, G. Angyalosi, H. Gras-Masse, D. Roux, O. Melnyk, L. Bourel-Bonnet, Chem. Commun. (2002) 2446–2447.
[36] P. Chenevier, B. Delord, J. Amedee, R. Bareille, F. Ichas, D. Roux, Biochim. Biophys. Acta 1593 (2002) 17–27.
[37] P. Simard, D. Hoarau, M.N. Khalid, E. Roux, J.C. Leroux, Biochim Biophys Acta 1715 (2005) 37–48.
[38] S. Gaubert, B. Delord, D. Roux, J. Amedee, E. Bayer, C. Auriault, R. Laversanne, Drug Deliv. 12 (2005) 69–74.
[39] C. Poirier, Ph.D. Thesis, University of Bordeaux 1, 2005.
[40] P. Chenevier, L. Bourel-Bonnet, D. Roux, J. Am. Chem. Soc. 125 (2003) 16261–16270.
[41] A.P. Mould, S.J. Barton, J.A. Askari, S.E. Craig, M.J. Humphries, J. Biol. Chem. 278 (2003) 51622–51629.
[42] J.F. Chen, A. Salas, T.A. Springer, Nat. Struct. Biol. 10 (2003) 995–1001.
[43] C. Boeckler, D. Dauter, P. Schelté, B. Frisch, D. Wachsmann, J.-P. Klein, F. Schuber, Eur. J. Immunol. 29 (1999) 2297–2308.
[44] F. Villars, L. Bordenave, R. Bareille, J. Amedee, J. Cell. Biochem. 79 (2000) 672–685.
[45] P. Chenevier, Ph.D. Thesis, University of Bordeaux 1, 2001.