Self-Assembling Ligands for Multivalent Nanoscale Heparin Binding**

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Heparin is a highly negatively charged sulfated polysaccharide with a heterogeneous mixture of diverse chains with different lengths that consist of repeating copolymers of 1–4 linked iduronic acid and glucosamine residues in a semirandom order (Scheme 1).^[1] This polysaccharide regulates a



Scheme 1. Structures of heparin (typical repeat unit) and self-assembling ligand G1.

variety of cellular processes, plays important roles in biological systems, and interacts with a wide range of protein targets.^[2] Heparin is widely used as an anti-coagulant during surgery—however, after surgical intervention, it is necessary to remove heparin in order to allow clotting to take place.^[3] Protamine is currently the only clinically approved heparin binder, but its use can cause adverse clinical outcomes.^[4] Protamines are highly positively charged proteins with multiple arginine groups on their surfaces, and as a consequence they bind heparin through electrostatic ion–ion interactions.^[5]

Heparin is a fascinating target for supramolecular chemistry, and as a consequence, interest has recently developed in creating synthetic heparin receptors and sensors. There have been a number of reports in which fluorescent/colored dyes were functionalized with cationic units capable of binding

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heparin, and were demonstrated to exhibit a spectroscopic response to heparin.^[6] Cationic units have also been attached to a foldamer in order to bind heparin;^[7] also relatively small cationic molecules, such as surfen, can bind heparin, with the mode of binding being predominantly electrostatic.^[8] In elegant work, Anslyn and co-workers reported a heparin receptor based on a trivalent scaffold that displayed protonated amino acids to bind to the anionic charges of heparin in combination with boronic acids to interact with the diols of heparin.^[9] A similar combination of noncovalent interactions was employed by Schrader and co-workers to develop fluorescent polymeric heparin sensors.^[10]

Nanoscale structures, which to some extent mimic the structure of protamine, have also been reported as heparin binders. For example, spherical cationic dendrimers, such as polyamidoamines (PAMAMs) have been of particular interest, because they have similar sizes as protamine and bind heparin by a similar mechanism, displaying multiple cationic ligands on their polyvalent scaffolds.^[11] In some cases, specific biomimetic units such as arginine-rich peptides have been attached to dendritic scaffolds and have been shown to bind heparin.^[12] In recent work mimicking this approach, viruslike particles have been used for the display of multivalent cationic ligand arrays for heparin binding.^[13] In addition, calix[8]arene has been used as a scaffold for the organization of multivalent amines, and it was demonstrated that the conformational flexibility of the calixarene scaffold plays an important role in heparin binding-in contrast to protamine, which is conformationally rigid.^[14]

We have an active research programme for the development of dendritic molecules capable of binding polyanionic DNA^[15] and have recently reported amphiphilic systems that self-assemble into micellar spherical "pseudo-dendrimers" prior to DNA binding.^[16] We therefore became interested in the development of protamine mimics that, instead of being large covalent structures, were held together by noncovalent interactions between branched ligands. Although such systems are well known for binding to DNA, there has only been one previous report about their exploitation for heparin formulation.^[17] Such systems are synthetically straightforward, with programmed self-assembly of simple building blocks being used as the key nanofabrication step (Scheme 1), and can potentially show high-affinity heparin binding-in analogy to proteins such as protamine. Herein we report our initial studies of self-assembling heparin binders that act as protamine mimetics, and uncover their ability to form organized nanostructured assemblies in the presence of heparin.

The ligand designed for this project (G1) contains peripheral amines that are protonated at physiological pH and are hence capable of electrostatic binding to polyanionic heparin (Scheme 1). These amines are supported on the Communications



Scheme 2. Synthesis of self-assembling dendron G1. *p*-TsOH: *p*-toluenesulfonic acid, DCC: *N*,*N*'-dicyclohexylcarbodiimide, DMAP: 4-dimethylaminopyridine, Boc₂O: di-*tert*-butyl dicarbonate, DIPEA: *N*,*N*-diisopropylethylamine, MsCI: methanesulfonyl chloride.

degradable, biocompatible scaffold first introduced by Hult, Fréchet, and co-workers.^[18] A hydrophobic unit located at the focal point of the structure drives the self-assembly of the ligands into a larger nanoscale architecture in aqueous media as a consequence of the hydrophobic effect. The synthesis of G1 was achieved using a modular approach (Scheme 2). The Fréchet dendron scaffold was synthesized using their previously described methodology;^[18d] in an early step, though, modification of the focal point with propargyl alcohol led to the incorporation of an alkyne group in this position.^[19] The use of *p*-nitrophenylchloroformate methodology enabled the incorporation of the N.N-di-(3-aminopropyl)-N-methylamine surface ligands, with N-tert-butoxycarbonyl (Boc) protecting groups being used to control the reaction. In the key step, Cu(I)-catalyzed "click" chemistry was used to attach 1azidodocosane 9, synthesized by previously reported methodologies from docosan-1-ol.^[20] The click chemistry product 10 was purified by gel permeation chromatography. Finally, deprotection of the surface ligands by using HCl gas in methanol gave rise to target ligand G1. Full synthetic methods and characterization data are given in detail in the Supporting Information.

To probe self-assembly, we performed a Nile Red assay, in which the solubilization of the hydrophobic dye, as monitored by fluorescence spectroscopy, acts as a probe for the minimum concentration at which self-assembly can take place.^[21] The studies were performed in phosphate buffered saline (PBS) at pH 7.5. As illustrated in Figure 1, Nile Red solubilization occurred at concentrations above $(3.88 \pm 0.25) \,\mu\text{M}$, which therefore can be regarded as the critical aggregation concentration (CAC). We are confident that self-assembly occurs



Figure 1. Fluorescence intensity of Nile Red in the presence of increasing amounts of G1; Nile Red is used to detect the self-assembly of G1 and determine the critical aggregation concentration (CAC).

even at relatively low concentrations, and that during the heparin-binding assay (see below), compound **G1** will be present in self-assembled form. Further evidence for self-assembly was provided by TEM, with spherical aggregates being observed when an aqueous sample of compound **G1** was allowed to settle on the TEM grid, was negatively stained with uranyl acetate while wet, and was then allowed to settle for further 10 min before imaging (Figure 2). The diameters of the spherical aggregates observed were approximately (8.5 ± 1.5) nm.

In order to monitor the heparin-binding ability of compound G1, we chose to employ a previously reported



Figure 2. TEM image of compound G1 dried from aqueous solution, indicative of spherical self-assembled nanostructures.

methylene blue (MB) displacement assay.^[22] As a cationic dye, MB forms moderate strength electrostatic interactions with heparin, and importantly, when bound to heparin, the UV/Vis spectrum of the dye changes significantly—unbound MB has a λ_{max} at 664 nm, whereas MB bound to heparin has a λ_{max} at 568 nm. The addition of a more strongly binding ligand to a mixture of heparin and MB displaces the MB from heparin, and consequently leads to changes in the UV/Vis spectrum of the dye. Hence, in this competition assay, UV/Vis spectroscopy is capable of indirectly reporting on the interactions between synthetic receptors and heparin.

The experiment was performed using the optimized conditions (MB (10 µm) and heparin (72.5 µm) in tris(hydroxymethyl)aminomethane hydrochloride (Tris HCl; 1 mM)). Heparin concentrations were calculated assuming the structure shown in Scheme 1, with an apparent "molecular" mass $(M_{\rm r})$ of 605.04 per repeat unit. For comparison, the novel compound G1 and protamine were both assayed for their abilities to bind heparin. Protamines are not available in monodisperse form, because they are mixtures of polyarginylated peptides; however, the following typical protamine structure was assumed for purposes of calculation: NH-Pro-Arg₄-Ser-Arg-Pro-Val-Arg₅-Pro-Arg₂-Pro-Arg₂-Val-Ser-Arg₆-Gly-Arg₄-COOH. Compound **G1** (or protamine) was added in increasing aliquots to the heparin-MB complex and normalization of the UV/Vis data against unbound MB allowed us to calculate the concentration at which 50 $\%\,$ of the MB was displaced from its complex with heparin.

Table 1 shows the effective concentration at which 50% displacement was achieved (EC₅₀ value). It becomes apparent that the lower the concentration, the more effective the binding. In addition, we have converted the data into +/-

Table 1: Data from methylene blue (MB) displacement assay.^[a]

ratios that indicate the charge ratio between the receptor and the heparin required for 50 % MB displacement.^[23] This ratio indicates how efficiently the receptor uses each of its positive charges to bind each negative charge of heparin—the smaller the value, the more effectively the cationic charge of the receptor is being used. To calculate +/- ratios, we assumed four positive charges on **G1**, four negative charges per repeat unit of heparin, and twenty four positive charges for the typical protamine sulfate structure. Finally, we also converted the data into the form of an "effective dose"; expressed in terms of the mass of the receptor (drug) required to bind a certain number (100 international units (IU)) of active units of heparin, this is the way in which protamine activity would normally be expressed for clinical administration.^[24]

As indicated in Table 1, G1 shows effective binding of heparin with an EC₅₀ value of $(102 \pm 3) \mu M$. This value corresponds to a +/- ratio of (1.41 ± 0.05) . This result indicates that more than one positive charge on the receptor is required for each negative charge of heparin and hence it is likely that not all positive charges are directly involved in the binding of heparin. The effective dose of G1 was calculated to be (0.48 ± 0.01) mg/100 IU heparin. Under the same assay conditions, protamine had an EC_{50} value of $(20 \pm 1) \mu M$. This value is lower than that observed for G1, but it must be remembered that protamine has a significantly higher mass than **G1** and thus the apparent EC_{50} is artificially improved. The +/- ratio leads to a better comparison of the receptors, and for protamine, the obtained value is (1.64 ± 0.11) , thus indicating that protamine uses its positive charge a little less efficiently than compound G1. Furthermore, the dose of protamine required for 50% MB displacement is $(0.46 \pm$ 0.02) mg/100 IU heparin, which is very similar to the effective dose of G1 under these conditions.

The binding assays were then repeated in the presence of 5 mM aqueous NaCl in order to determine the effect of electrolytes on heparin binding. Given that receptor-heparin interactions are predominantly based on electrostatics, it might be expected that increasing the ionic strength would decrease the binding affinity. However, Table 2 indicates that in this assay, both **G1** and protamine appear to bind heparin more efficiently. It must be remembered that this assay is a competitive one, and we can therefore conclude that the binding between MB and heparin is more adversely affected by salt than the interaction between the receptors and heparin. Evidence for this hypothesis is provided by the observation that with NaCl concentrations above 5 mm, binding of the probe dye molecule to heparin was no longer observed. Comparison of the data for **G1** and protamine,

Table 2: Data from methylene blue (M	ИВ) displacement assay. ^{[a}
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Tuble 1. Data non methylene blue (Mb) displacement assay.						
Receptor	EC ₅₀ [µм] ^[b]	+/- Ratio ^[c]	Effective Dose ^[d]			
G1	(102±3)	(1.41±0.05)	(0.48±0.01)			
Protamine	(20±1)	(1.64±0.11)	(0.46±0.02)			

[[]a] Performed in 1 mm Tris-HCl. [b] EC_{50} is the concentration required for 50% displacement of MB from its complex with heparin. [c] The +/- ratio represents the receptor/heparin charge ratio required for 50% displacement. [d] The effective dose is reported in mg of receptor per 100 IU heparin.

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Receptor	EC ₅₀ [µм] ^[b]	+/- Ratio ^[c]	Effective Dose ^[d]
G1 Protamine	(47±8) (14±2)	(0.65±0.10) (1.16±0.15)	(0.23±0.03) (0.34±0.05)

[a] Performed in 1 mm Tris-HCl in the presence of 5 mm NaCl. [b] EC_{s0} is the concentration required for 50% displacement of MB from its complex with heparin. [c] The +/- ratio represents the receptor/heparin charge ratio required for 50% displacement. [d] The effective dose is reported in mg of receptor per 100 IU heparin.

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however, would indicate that **G1** becomes a significantly more effective heparin binder than protamine in the presence of electrolyte. Indeed, **G1** operates at an effective dose of (0.23 ± 0.03) mg/100 IU heparin, which is significantly lower than protamine, for which the dose is (0.34 ± 0.05) mg/100 IU. Thus, the ability of protamine to compete against MB increases by a factor of 1.5, while the ability of **G1** to compete with MB more than doubles. This result indicates that salt is less capable of screening the effective charge of the **G1** assemblies, which therefore exhibit better multivalent binding to heparin.^[15g]

In a final experiment, we used TEM to probe the interaction between G1 and heparin in water (Figure 3). We observed micellar aggregates on the TEM grid, which



Figure 3. TEM image of self-assembled spherical **G1** nanostructures in the presence of heparin, leading to linear organization of the nanostructures.

correspond to aggregates of G1. Indeed, the size of these aggregates was approximately (6.5 ± 1.0) nm, which is reasonably consistent with the observations in the absence of heparin (Figure 2). However, remarkably, these micellar aggregates, unlike those observed in the absence of heparin, were aligned in an organized manner within domains on the TEM grid. The micelles appear to be arranged in closely packed linear assemblies. This heparin-induced nanoscale organization of micelles was further confirmed by cryo-TEM methods (see the Supporting Information). We propose that heparin, as a relatively inflexible polymer, organizes the micellar aggregates along its backbone as a consequence of electrostatic interactions (Figure 4). This mode of binding has often been observed for the binding of spherical cationic systems to DNA, and is commonly referred to as "beads on a string".^[25] This model helps explain, why more than one positive charge on the G1 aggregate is required to effectively neutralize each anionic charge on the heparin (Table 1), since some of the positive charges on the micellar structures will be unable to make effective contact with the linear heparin polymer and as such will not be useful for the neutralization of the overall heparin charge. This mode of binding has also previously been suggested for spherical covalently bound dendritic macromolecules that bind to heparin,^[11c] and the experimental evidence presented herein therefore clearly illustrates that our self-assembled nanoscale architectures



Figure 4. Schematic showing the organization of self-assembled micelles formed by **G1** in the presence of heparin.

effectively behave like pseudo-dendrimers when binding to heparin, with the individual ligands being combined noncovalently to give a spherical aggregate. This architecture is an intriguing form of nano-orientated soft matter, in which the interactions between a biological polymer and a selfassembled soft nanostructure are able to control the eventual nanoscale morphology (Figure 4).

In summary, we can conclude that **G1** self-assembles into a polycationic spherical form that is capable of binding polyanionic heparin in a multivalent manner. These selfassembled architectures mimic the behavior of covalently constructed proteins such as protamine and covalent spherical macromolecules such as dendrimers in terms of binding affinity and mode of binding. TEM indicates that binding to heparin induces nanoscale organization; such simple materials may have potential biomedical applications. In future work, the ability of these self-assemblies to intervene in biological processes will be studied, as will higher-generation dendritic systems and the potential of these systems to degrade and disassemble, thus leading to controlled heparin binding and release.

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- [1] D. L. Rabenstein, Nat. Prod. Rep. 2002, 19, 312-331.
- [2] a) I. Capila, R. J. Linhardt, Angew. Chem. 2002, 114, 426-450;
 Angew. Chem. Int. Ed. 2002, 41, 390-412; b) T. M. Laremore, F. Zhang, J. S. Dordick, J. Liu, R. J. Linhardt, Curr. Opin. Chem. Biol. 2009, 13, 633-640.
- [3] a) L. Roden, D. S. Feingold, *Trends Biochem. Sci.* 1985, 10, 407–409; b) R. J. Linhardt, N. S. Gunay, *Semin. Thromb. Hemostasis* 1999, 25, 5–16.
- [4] M. Nybo, J. S. Madsen, Basic Clin. Pharmacol. Toxicol. 2008, 103, 192-196.
- [5] R. Balhorn, Genome Biol. 2007, 8, 227.
- [6] a) T. Briza, Z. Kejik, I. Cisarova, J. Kralova, P. Martasek, V. Kral, *Chem. Commun.* **2008**, 1901–1903; b) K.-Y. Pu, B. Liu, *Macromolecules* **2008**, 41, 6636–6640; c) S. Wang, Y.-T. Chang, *Chem.*

Angew. Chem. Int. Ed. 2011, 50, 4675-4679

Commun. 2008, 1173–1175; d) M. Wang, D. Zhang, G. Zhang, D. Zhu, Chem. Commun. 2008, 4469–4471; e) K.-Y. Pu, B. Liu, Adv. Funct. Mater. 2009, 19, 277–284; f) L. Zeng, P. Wang, H. Zhang, X. Zhuang, Q. Dai, W. Liu, Org. Lett. 2009, 11, 4294–4297; g) H. Szelke, S. Schübel, J. Harenberg, R. Kramer, Chem. Commun. 2010, 46, 1667–1669; h) R. Zhan, Z. Fang, B. Liu, Anal. Chem. 2010, 82, 1326–1333; i) H. Szelke, S. Schubel, J. Harenberg, R. Kramer, Bioorg. Med. Chem. Lett. 2010, 20, 1445–1447.

- [7] a) S. Choi, D. J. Clements, V. Pophristic, I. Ivanov, S. Vemparala, J. S. Bennett, J. L. Klein, J. E. Winkler, W. E. De Grado, *Angew. Chem.* 2005, *117*, 6843–6847; *Angew. Chem. Int. Ed.* 2005, *44*, 6685–6689; b) C. Kelly, S. Khaja, A. Vena, A. Yu, J. M. Esson, *Anal. Chim. Acta* 2010, *681*, 1–7.
- [8] M. Schuksz, M. M. Fuster, J. R. Brown, B. E. Crawford, D. P. Ditto, R. Lawrence, C. A. Glass, L. Wang, Y. Tor, J. D. Esko, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 13075–13080.
- [9] a) Z. L. Zhong, E. V. Anslyn, J. Am. Chem. Soc. 2002, 124, 9014–9015; b) A. T. Wright, Z. L. Zhong, E. V. Anslyn, Angew. Chem. 2005, 117, 5825–5828; Angew. Chem. Int. Ed. 2005, 44, 5679–5682.
- [10] W. Sun, H. Bandmann, T. Schrader, Chem. Eur. J. 2007, 13, 7701–7707.
- [11] a) S. Bai, C. Thomas, F. Ahsan, J. Pharm. Sci. 2007, 96, 2090–2106; b) S. Bai, F. Ahsan, Pharm. Res. 2009, 26, 539–548; c) X. Feng, Y. Cheng, K. Yang, J. Zhang, Q. Wu, T. Xu, J. Phys. Chem. B 2010, 114, 11017–11026; d) Q. Zhang, S. Chen, E.-X. Zhuo, X.-Z. Zhang, S.-X. Cheng, Bioconjugate Chem. 2010, 21, 2086–2092; e) M. F. Ottaviani, R. Mazzeo, M. Cangiotti, L. Fiorani, J. P. Majoral, A. M. Caminade, E. Pedziwiatr, M. Bryszewska, B. Klajnert, Biomacromolecules 2010, 11, 3014–3021.
- [12] a) S. Kasai, H. Nagasawa, M. Shimamura, Y. Uto, H. Hori, Bioorg. Med. Chem. Lett. 2002, 12, 951–954; b) M. Donalisio, M. Rusnati, A. Bugatti, D. Allemand, G. Pirri, A. Giuliani, S. Landolfo, D. Lembo, Antimicrob. Agents Chemother. 2010, 54, 4290–4299; c) A. Luganini, A. Giuliani, G. Pirri, L. Pizzuto, S. Landolfo, G. Gribaudo, Antiviral Res. 2010, 85, 532–540.
- [13] A. K. Udit, C. Everett, A. J. Gale, J. R. Kyle, M. Azkan, M. G. Finn, *ChemBioChem* 2009, 10, 503-510.
- [14] a) T. Mecca, G. M. L. Consoli, C. Geraci, R. La Spina, F. Cunsolo, Org. Biomol. Chem. 2006, 4, 3763–3768; b) T. Mecca, F. Cunsolo, Polym. Adv. Technol. 2010, 21, 752–757.
- [15] a) M. A. Kostiainen, J. G. Hardy, D. K. Smith, Angew. Chem.
 2005, 117, 2612-2615; Angew. Chem. Int. Ed. 2005, 44, 2556-2559; b) J. G. Hardy, M. A. Kostiainen, D. K. Smith, N. P. Gabrielson, D. W. Pack, Bioconjugate Chem. 2006, 17, 172-178; c) M. A. Kostiainen, D. K. Smith, O. Ikkala, Angew.

Chem. 2007, 119, 7744–7748; Angew. Chem. Int. Ed. 2007, 46, 7600–7604; d) D. J. Welsh, S. P. Jones, D. K. Smith, Angew. Chem. 2009, 121, 4107–4111; Angew. Chem. Int. Ed. 2009, 48, 4047–4051; e) M. A. Kostiainen, G. R. Szilvay, D. K. Smith, M. B. Linder, O. Ikkala, Angew. Chem. 2006, 118, 3618–3622; Angew. Chem. Int. Ed. 2006, 45, 3538–3542; f) M. A. Kostiainen, G. R. Szilvay, J. Lehtinen, D. K. Smith, M. B. Linder, A. Urtti, O. Ikkala, ACS Nano 2007, 1, 103–113; g) G. M. Pavan, A. Danani, S. Pricl, D. K. Smith, J. Am. Chem. Soc. 2009, 131, 9686–9694; h) S. P. Jones, G. M. Pavan, A. Danani, S. Pricl, D. K. Smith, J. 4519–4532.

- [16] a) S. P. Jones, N. P. Gabrielson, D. W. Pack, D. K. Smith, *Chem. Commun.* **2008**, 4700–4702; b) P. Posocco, S. Pricl, S. Jones, A. Barnard, D. K. Smith, *Chem. Sci.* **2010**, *1*, 393–404.
- [17] P. Y. Hayes, B. P. Ross, B. G. Thomas, I. Toth, *Bioorg. Med. Chem.* 2006, 14, 143–152.
- [18] a) H. Ihre, A. Hult, E. Soederlind, J. Am. Chem. Soc. 1996, 118, 6388-6395; b) H. Ihre, A. Hult, J. M. J. Fréchet, I. Gitsov, Macromolecules 1998, 31, 4061-4068; c) H. Ihre, O. L. Padilla de Jesus, J. M. J. Fréchet, J. Am. Chem. Soc. 2001, 123, 5908; d) E. R. Gillies, J. M. J. Fréchet, J. Am. Chem. Soc. 2002, 124, 14137-14146; e) C. C. Lee, E. R. Gillies, M. E. Fox, S. J. Guillaudeu, J. M. J. Fréchet, E. E. Dy, F. C. Szoka, Proc. Natl. Acad. Sci. USA 2006, 103, 16649-16654.
- [19] P. Wu, M. Malkoch, J. N. Hunt, R. Vestberg, E. Kaltgrad, M. J. Finn, V. V. Fokin, K. B. Sharpless, C. J. Hawker, *Chem. Commun.* 2005, 5775–5777.
- [20] E. W. Sugandhi, R. V. Macri, A. A. Williams, B. L. Kite, C. Slebodnick, J. O. Falkinham, A. R. Esker, R. D. Gandour, *J. Med. Chem.* 2007, *50*, 1645–1650.
- [21] M. C. A. Stuart, J. C. van de Pas, J. B. F. N. Engberts, J. Phys. Org. Chem. 2005, 18, 929–934.
- [22] a) A. Leone-Bay, D. R. Paton, B. Variano, H. Leipold, T. Rivera, J. Miura-Fraboni, R. A. Baughman, N. Santiago, J. Controlled Release 1998, 50, 41–44; b) Q. C. Jiao, Q. Liu, C. Sun, H. He, Talanta 1999, 48, 1095–1110.
- [23] The +/- ratio is calculated assuming that the M_r of heparin is 605.04 (i.e. one typical repeat unit, which possesses four negative charges). For protamine we assume 24 positive charges on a hypothesized typical structure of NH-Pro-Arg₄-Ser-Arg-Pro-Val-Arg₅-Pro-Arg₂-Pro-Arg₂-Val-Ser-Arg₆-Gly-Arg₄-COOH, while compound **G1** has four associated positive charges.
- [24] The reported standard medical dose of protamine for heparin is 1 mg/100 IU; this is the dose required for full neutralization (binding) of heparin, and is therefore in line with the EC₅₀ value reported for 50% heparin binding presented in Table 1.
- [25] H. Wong, J. M. Victor, J. Mozziconacci, PLos One 2007, 2, e877.