Dendrimers as size selective inhibitors to protein–protein binding†

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This communication describes how the "quantized" size effect of dendrimers can be exploited towards a size selective binding mechanism for the inhibition of protein–protein binding.

Protein–protein interactions play an essential role in many biological processes.[1] The interacting surfaces are large and range from 500 Å^2 up to 5000 Å^2 and usually possess hydrophobic centers surrounded by regions of high charge.[2] The predominant interactions involve simple hydrophobic and polyvalent electrostatic interactions.[3] When protein–protein interactions occur in an undesirable or uncontrolled fashion, disease is often the result.[1] Polymer and/or macromolecular based systems capable of interrupting these unwanted interactions represent viable and realistic therapeutic targets (due to the size of the interacting surfaces and the number of interacting groups, traditional small molecule approaches are rarely successful).[4] Pioneering work by Hamilton and co-workers has shown that success can be achieved through the use of hydrophobic scaffold molecules possessing charged groups at their periphery, which bind to the "hot spot" or interfacial area of their target proteins.[5] Notwithstanding the considerable success of these initial systems, most of the effort has been directed at the binding and inhibition of proteins that have relatively small interfacial areas.[6] Ideally a series of macromolecular scaffold molecules that are capable of interacting across a range of interfacial areas, and therefore a range of differing proteins is desirable.

This communication describes our initial studies into protein binding using a series of dendrimers as size selective ligands.[7] Dendrimers are synthesized using a controlled procedure that generates a series of macromolecules of particular and discrete size.[8] This synthesis is in effect a "quantized" process and its this quantization effect we wish to exploit in this study. The dendrimers selected were the G1.5 to G4.5 acid terminated PAMAM dendrimers possessing 8 to 64 terminal groups, respectively. It has previously been shown that these dendrimers have relatively flexible structures, which are below the densely packed and rigid structures associated with higher generation dendrimers.[9] As such these carboxylate dendrimers are capable of interacting with a positive surface across their full diameter. The surface areas that each dendrimer can address (referred to as the addressable area) can be calculated from their diameters and these values are shown below each dendrimer schematically represented in Fig. 1. It should be appreciated that although the larger G4.5 dendrimer may not be able to utilize its entire compliment of terminal groups when interacting with a surface, it remains capable of interacting with surfaces utilizing its full diameter (and therefore its full addressable area).

The proteins selected were chymotrypsin and cytochrome-c. Both these proteins have been well studied and their interfacial areas are well known. In addition, chymotrypsin has an interfacial area more than twice that of cytochrome-c, (interfacial areas of around 2400 and 1100 Å^2, respectively).[10] When comparing these interfacial areas to the maximum addressable areas of the dendrimers, we were interested to discover whether or not a simple size based mechanism for selective protein binding would occur. Utilizing such a mechanism led us to predict that the smaller protein cytochrome-c should bind best to the relatively small G2.5 dendrimer (cytochrome-c has an interfacial area of 1100 Å^2, which matches up best to the G2.5 dendrimer with a maximum addressable area 1200 Å^2). Extending this idea further, we should be able to predict/show that the larger protein chymotrypsin should bind best to the larger G3.5 dendrimer (chymotrypsin has an interfacial area of 2400 Å^2, whilst the maximum addressable area of the G3.5 dendrimer is 2250 Å^2). To test these predictions all dendrimers were individually screened against both proteins.

Initially, we tested cytochrome-c, whose active site has a porphyrin moiety capable of quenching suitable chromophores bound to the interfacial area. This feature was exploited by Hamilton and co-workers as a method for

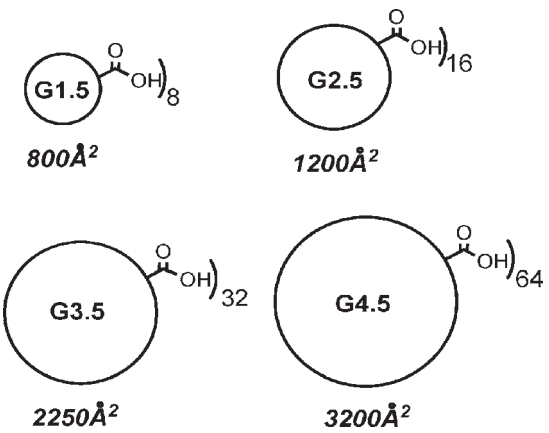

Fig. 1 Schematic showing the series of PAMAM dendrimers investigated. The maximum area that each dendrimer can address is shown below.

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directly assessing the binding of tetracarboxyporphyrins (TCP) to cytochrome-c.[5] In a modification of this procedure we proposed to study dendrimer binding using a competitive/displacement assay. In these experiments solutions of dendrimers would be titrated into a solution containing a preformed protein–TCP complex. When the dendrimers bind, the porphyrins are displaced and return to bulk solvent. When this occurs the porphyrins are freed from their quencher (*i.e.* the protein) and fluorescence returns. The association constant of TCP[5] is around 10^5 M^{-1}. Solutions of cytochrome-c and TCP were made up such that the concentration was 5×10^{-4} M (sodium phosphate buffer, 0.1 M ionic strength). At this concentration some unbound TCP remained free in solution and could be detected using fluorescence spectroscopy (emission at 650 nm).

The intensity of this free peak was observed to increase as solutions of dendrimer were gradually titrated in. Plots of (log) dendrimer concentration *vs.* change in emission intensity produced the characteristic sigmoidal shaped graphs indicative of a competitive binding process.[11] The experiment was repeated for each dendrimer and the plots obtained fitted to a 1 : 1 competitive binding equation. From this analysis binding constants in the 10^2–10^3 M^{-1} range were obtained (±15%). The results for each dendrimer are shown graphically in the top graph of Fig. 2. The G2.5 dendrimer with 16 terminal acid groups and a maximum addressable area of around 1200 Å^2 was found to bind best (interfacial area of cytochrome-c around 1100 Å^2). This fits in well with the prediction based on a sized based binding/inhibition mechanism. A control experiment using a neutral dendrimer that cannot bind to the charged hot spot area of the protein and displace the porphyrin probe was also undertaken (using a PAMAM G2.0 dendrimer with 16 terminal OH groups). When this experiment was carried out, no change in the emission intensity was observed. This control experiment confirms that the increase in porphyrin emission from the original experiment was not due any undesirable porphyrin dendrimer interactions.

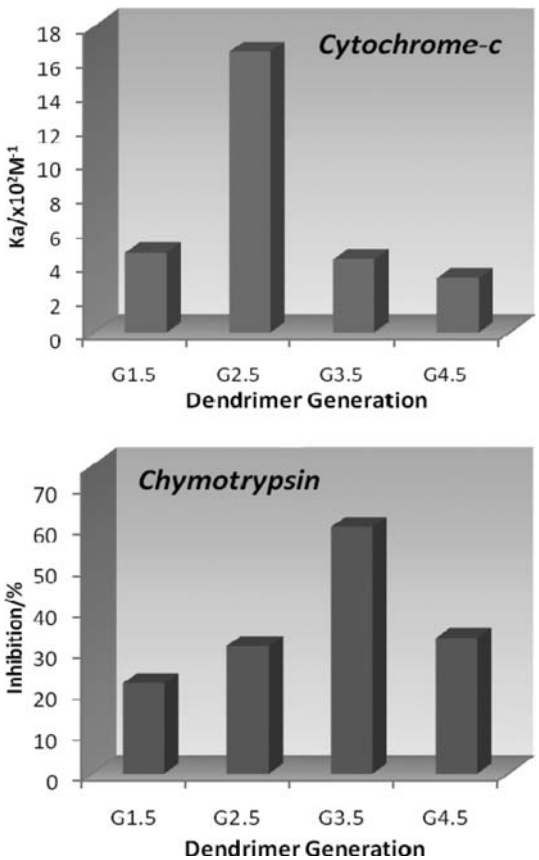
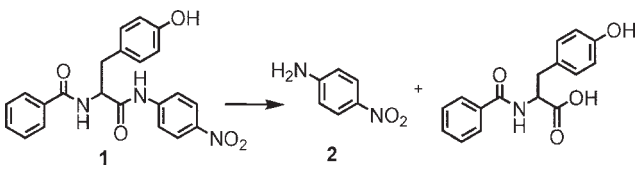


Fig. 2 Binding and inhibition data show that a dendrimer with a maximum addressable area of 1200 Å^2 binds best to the smaller protein cytochrome-c (interfacial area ~1000 Å^2), whilst chymotrypsin, (interfacial area ~2400 Å^2), binds best to a larger dendrimer with a maximum addressable area of 2250 Å^2.

In a further effort to demonstrate the proposed size based mechanism we then turned our attention to the larger protein chymotrypsin. This protein is a member of the serine protease family of enzymes and catalyzes the hydrolysis of peptide bonds. Although not selective, chymotrypsin has a preference for the large hydrophobic side chains of aromatic amino acids, cleaving them at the carboxyl end of the aromatic residue. The active site entrance of chymotrypsin is at the centre of its interfacial/binding area, allowing for the assessment of binding *via* simple inhibition studies.[12] The dendrimer that binds best will also inhibit the best—as such relative binding can be directly related to inhibition efficiency (*i.e.* 60% inhibition equals 60% binding). As the amide substrate N-(1-(4-nitrophenylcarbamoyl)-2-(4-hydroxyphenyl)ethyl)benzamide BTNA **1** is hydrolyzed, the UV active by-product 4-nitrobenzenamine **2** is generated, Scheme 1. The catalyzed reaction can therefore be followed by measuring the rate of 4-nitrobenzenamine production using UV spectrophotometry (λ_{max} 418 nm). A background rate for the uninhibited reaction was measured by adding BTNA **1** to an aqueous solution containing just chymotrypsin (final concentrations 1.0×10^{-4} and 5.0×10^{-7} M, respectively). For this uninhibited reaction (*i.e.* no dendrimer present), a typical reaction profile was observed and an initial rate of 7.78×10^{-8} M^{-1} s^{-1} was obtained. The reaction was then repeated in the presence of equimolar amounts of each dendrimer and chymotrypsin (5.0×10^{-7} M in both dendrimer and chymotrypsin) and initial rates calculated for all dendrimers. In all cases the reaction profiles and initial rates were reduced when compared to the uninhibited reaction. By comparing the initial rates to that obtained from the uninhibited reaction, we were able to calculate a level of inhibition (and therefore binding). As a control reaction, the BTNA amide substrate was added to a solution of G2.5 dendrimer and any reaction monitored *via* UV. Over the time scales studied (1200 s) no reaction could be detected, confirming that the dendrimers do not play a direct part in the hydrolysis reaction. Graphs showing the inhibition data for each dendrimer generation are shown in the bottom graph of Fig. 2 (results



Scheme 1 The chymotrypsin catalyzed hydrolysis used for the inhibition and binding assay.

obtained are an average of three runs and errors are within ±10%). On this occasion it is the larger G3.5 dendrimer with 32 terminal groups that binds best, with a 60% inhibition/binding being observed. Satisfyingly, this is again the result predicted using the size based binding analysis. The interfacial area of chymotrypsin matches up best with the maximum addressable area of the G3.5 dendrimer (2400 and 2250 Å^2, respectively), which therefore binds best.

The proteins selected for our initial study possessed well studied and well known interfacial areas. Specifically, proteins were selected such that their interfacial areas differed by 100% with respect to each other (*i.e.* interfacial areas of around 2400 and 1100 Å^2 for cytochrome-c and chymotrypsin, respectively). As such they provided useful and reliable systems by which to study size selective protein binding. However, our sized based argument, which simply states that the best interaction will occur between a dendrimer and a protein of similar interfacial/addressable areas, is probably over simplistic. In reality the dendrimer best able to interact with a protein surface will be the one that maximizes the thermodynamic situation *via* specific interactions and specific properties.[13] With respect to the thermodynamics of binding, a number of factors are important. These include enthalpic factors, such as charge to charge and site to site interactions, as well as entropic forces including solvation/desolvation and hydrophobic binding. The size and number of interacting groups are all important in this respect. Another important factor includes flexibility, *i.e.* the capacity of the dendrimers to "stretch" or squeeze" onto or into a protein surface (accompanied by a thermodynamic cost), whilst maximizing enthalpic/entropic factors. This is one reason why larger dendrimers may bind to their protein targets more weakly than the optimum sized dendrimers. That is, as the dendrimers become larger, there is an increase in surface crowding and rigidity.[14] So although there are more terminal groups capable of interacting over the same protein area (a potentially favourable process), this is accompanied by a significant thermodynamic cost as the dendrimers restructure themselves to stretches or squeeze into/onto the protein surface. A similar argument could of course be applied to the protein. The real situation is therefore a balance between structure (*i.e.* the size, shape and functionality of the dendrimer and/or protein) and thermodynamics (*i.e.* enthalpic and entropic factors). All of these areas are currently being explored towards the development of a general strategy for the construction and isolation of functionalized dendrimers capable of binding specifically to a range of target proteins. Nevertheless, the initial data presented in this paper allows us to conclude that the "quantized" size effect of dendrimers can be exploited towards a size selective binding mechanism for the inhibition of protein–protein binding.

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