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Comparison of Fluorescence Labelling Techniques for the Selection of Affinity Ligands from Solid-Phase Combinatorial Libraries

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This study reports the comparison of fluorimetric techniques (fluorescence microscopy and spectrofluorimetry on a 96-well format) for the on-bead screening of combinatorial libraries of affinity ligands for chromatographic separations. Two solid-phase libraries of synthetic ligands based on distinct scaffolds were synthesized by combinatorial chemistry. The libraries comprising ligands representing different hydrophobic/hydrophilic properties and sizes were tested for binding to randomly selected biomolecules (labelled with a fluorophore). Fluorescence microscopy was revealed to be a reliable and reproducible technique for the detection of lead ligands which strongly bound the target biomolecule. Results obtained by fluorescence intensity measurements in a 96-well format were less consistent, mainly due to challenges related with the accurate dispensing of the solid support.

Keywords affinity ligands; combinatorial chemistry; HTS

INTRODUCTION

The concept of affinity chromatography emerged in the 1970s with biospecific adsorbents, comprising of ligands originated from biological sources or from *in vitro* techniques. These are natural receptors which target molecules with high selectivity and affinity, such as peptides, antibodies, antigens, and binding or receptor proteins (1–3), and are associated with high costs of production and purification, poor stabilization under sterilization and cleaning-in-place conditions, as well as potential leakage and end-product contamination. Synthetic affinity ligands have been developed in an attempt to overcome these disadvantages of natural ligands. They tend to combine molecular recognition features with high resistance to chemical and biological degradation, high scalability, as well as low costs and low toxicity (1,3,4). A number of synthetic affinity ligands have been developed including the biomimetic or *de novo* designed ligands targeted at specific proteins and based on the triazine scaffold (5–12). Recently, affinity ligands for immunoglobulins and their fragments based on the Ugi reaction scaffold have also been developed (13). Independent of the scaffold selected, the research strategy followed for the development of these ligands involves ligand design using *in silico* molecular modelling tools as a first step (2,14), followed by the on-bead combinatorial synthesis and high-throughput screening (HTS) of ligand libraries (Fig. 1). The screening process is crucial for the correct selection of lead structures for further optimization. This stage assesses molecular binding interactions between the ligand and the target biomolecule (15). Although it would be possible to screen *in silico* a large number of ligands against a target and to identify the main candidates (16), several important factors which are introduced upon ligand immobilization on the solid support would not be taken into account (17,18). Recent studies have confirmed that the strength of binding between immobilized ligands and proteins can be indirectly affected by interactions with the support material (18). This reinforces the need to carry out synthesis and screening of combinatorial libraries on the same support (17). Fluorimetric assays are quite commonly used for the on-bead HTS of combinatorial libraries (14,17,19). In general, fluorescence-based techniques are simple to perform, easily automated, relatively inexpensive, highly sensitive, and require small amounts of ligand and target biomolecule (14). However, these techniques are fluorophore dependent and, due to this, some effects such as fluorophore interference and quenching or bleaching can be observed. Furthermore, fluorimetric techniques usually require expensive

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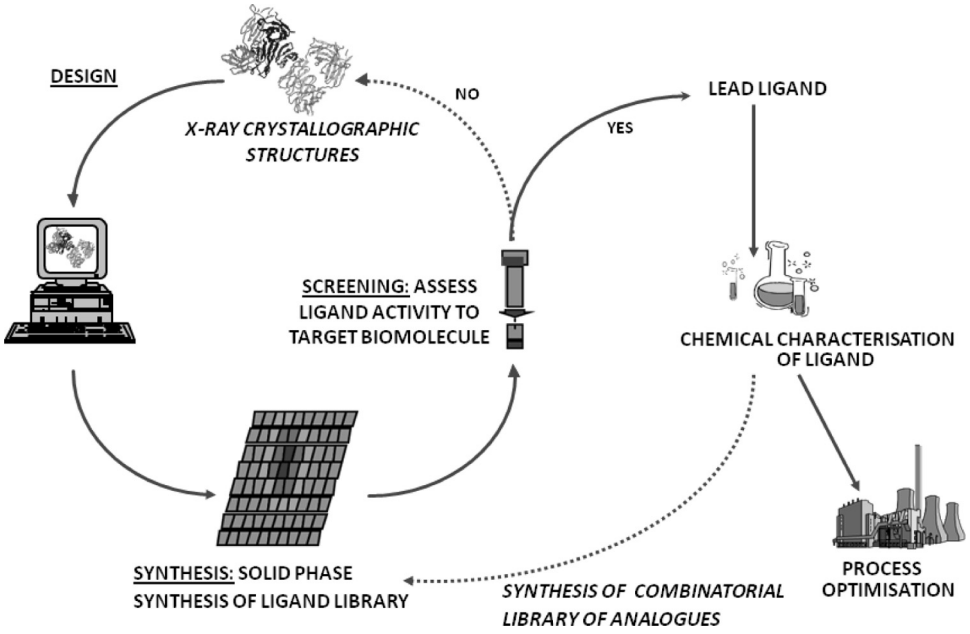

FIG. 1. Research strategy for the development of *de novo* designed synthetic affinity ligands for biomolecule separation by affinity chromatography.

instrumentation. Fluorescence microscopy has been described as a reliable qualitative technique for the detection of strongly binding ligands (based on the triazine skeleton) against human IgG conjugated to FITC (fluorescein isothiocyanate) (17) and FITC-labelled cutinase (10). This rapid screening of libraries helped to reduce the number of potential lead ligands for further evaluation and optimization (10,17,20). The second stage of screening involves a more detailed assessment of binding between the lead ligand and the target biomolecule, and the determination of affinity and kinetic constants by label-free techniques, such as liquid chromatography, Surface Plasmon Resonance, Mass Spectroscopy, X-Ray Crystallography, or Nuclear Magnetic Resonance spectroscopy (15,19).

The aim of this work was to compare two fluorescence-based techniques for the screening of solid-phase combinatorial libraries of ligands against random biomolecules. Combinatorial libraries of affinity ligands were synthesized using two different methodologies, a modified "mix-and-split" procedure (8,21) and a one-pot reaction (13). The screening of the on-bead libraries was conducted by fluorescence microscopy (Method A) (17,20) and fluorescence intensity measurement on a 96 well plate format (Method B). All biomolecules (except green fluorescent protein – GFP) were conjugated to FITC. The two methods were compared both in terms of reliability in the detection of strongly binding and non-binding ligands, independent of the target biomolecule and the ligand scaffold, and reproducibility.

MATERIALS AND METHODS

Materials

All chemicals used were at least 98% pure and the solvents were pro-analysis. BSA protein (Albumin from bovine serum) min. 98% and recombinant green fluorescent protein rTurboGFP (Evrogen) were purchased from Sigma and Biocat GmbH respectively. The FITC labelled peptides were obtained from Antagen Inc (USA). Cross-linked agarose (Sepharose^{TM} CL-6B) and PD-10 Columns – Sephadex^{TM} G – 25 M (17-0521-01) were obtained from GE Healthcare. Captiva 96-well 20 µl Polypropylene columns (0.8 × 6.0 cm) were purchased from Varian. BRAND microplates were supplied from VWR International.

Instrumentation

The synthesis of the ligands was performed on a Big SHOT III^{TM} Hybridization oven from Boekel Scientific. The combinatorial synthesis was performed in a Captiva^{TM} 96 well-block from Varian. The fluorescence studies were carried out by using a Sartorius Centrifuge 1–15 K from Alfagene, as well as a Fluorescence Microscope Olympus BX 51 with an objective U-RFL-T (40× amplification), U-MWB ($\lambda_{exc} = 460$–490 nm; $\lambda_{em} = 515$–570 nm), an Olympus U-RFL-T lamp, an objective Uplam FLN, and Cell F software for monitoring. The images were acquired by using a soft imaging system of Olympus F-View (U-CMAD3). Image J was utilized for analysis of data obtained from fluorescence microscopy. The measurement of the fluorescence intensity (top reading) on a black 96

well plate (flat bottom) was conducted on a Microplate Titre Infinite F200 with the respective Tecan filters (λ_{exc} = 485–505 nm; λ_{em} = 535–560 nm).

Methods

Combinatorial Synthesis of the Ligand Libraries

Two different solid-phase libraries were synthesized. Library I possessed 1,3,5-tricholoro-*sym*-triazine as the main scaffold molecule, and was obtained by nucleophilic substitution of triazine chlorine atoms by different amines (17,21). Library II was based on the Ugi reaction scaffold as recently described by Lowe and co-workers (13).

Conjugation of BSA with FITC

The labelling of the BSA protein was performed with the FluoroTag™ FITC conjugation kit, according to the supplier instructions (Sigma) and previous work (17). The fluorescein/protein molar ratio (F/P) of the BSA-FITC conjugate was estimated at approximately 1.

Screening of the Combinatorial Libraries

The screening assays were performed at room temperature and divided into three main steps: Resin regeneration, biomolecule loading and incubation, and resin washing (17), after which, bead samples were analyzed by fluorescence microscopy (Method A) or spectrofluorimetry on 96-well plates (Method B). The biomolecules tested included BSA-FITC, GFP, and five randomly selected peptides conjugated to FITC. The screening by Method A involved the observation of samples in the fluorescence microscope (17). For the affinity ligands based on the ugi reaction scaffold, the exposure time was 10 ms, and for the ligands based on the triazine scaffold, the exposure time was 100 ms. The images were saved as jpeg with the dimensions 1376×1032 pixels without further downsizing or image compression. The fluorescence intensity of the images obtained was then analyzed using

(i) the software Image J and
(ii) by calculation of a percentage of fluorescence intensity (Fig. 2).

The scoring of the relative fluorescent intensities was made taking into account typical images as shown in Fig. 2b. In Method B, the resins (80 µl) were transferred into wells of a 96 well black microplate. PBS buffer (10 mM sodium phosphate, 150 mM NaCl, pH 7.4) was added to each well up to a final volume of 100 µl. The fluorescence intensity on each well was measured in the microplate reader. Controls of plain agarose were subjected to the same treatment and evaluation, and no fluorescence was observed.

Reproducibility of the Screening Systems

Resins with different binding assessments obtained by fluorescence microscopy (Method A) were randomly chosen to perform reproducibility tests. For Method A, three samples of the same resin (3×1.5 µl) were observed under the fluorescence microscope. Photographs of ten random fields along each sample were recorded, first under phase contrast (to count the total number of agarose beads), and then with the fluorescence filter (to count the number of fluorescent beads). The images were analysed according to Fig. 2. For Method B, defined volumes of each resin were pipetted in triplicate (10, 30, 50, 80, and 100 µl) in different wells, and the PBS buffer was added up to a final volume of 100 µl. The fluorescence intensity on each well was measured in the microplate reader as described above.

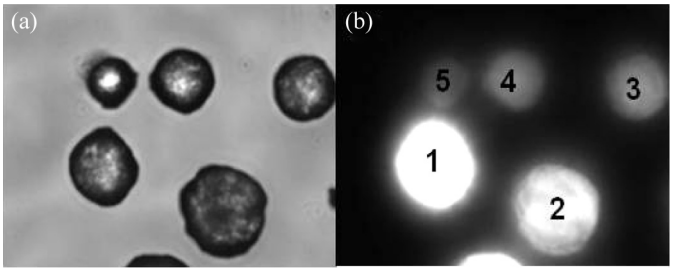

FIG. 2. Example of the assignment of the fluorescent intensity percentage for each agarose bead. (a) Image taken under phase contrast and (b) image taken with the fluorescence filter. The corresponding fluorescence intensities are represented in Fig. 2b, being (1) 100%, (2) 50%, (3,4) 25%, and (5) 0%. The percentage of the fluorescence intensity is given by the formula % fluorescence intensity = $(100 + 50 + 25 \times 2 + 0)/5 = 40\%$.

RESULTS AND DISCUSSION

This work aimed at comparing two on-bead fluorescence screening methods as a generic approach for the selection of lead affinity ligands from combinatorial libraries. The techniques studied were fluorescence microscopy (Method A) and spectrofluorimetry in a 96 well plate format (Method B). Two solid-phase libraries comprising ligands bearing a diversity of hydrophobic and hydrophilic characters and different ligand scaffolds (Libraries I and II with triazine and Ugi-based skeletons, respectively) were screened for binding to model biomolecules, including two proteins and five randomly selected peptides. The results obtained were translated into percentages of fluorescence intensity (related with the amount of FITC-labelled biomolecule bound to the agarose beads), and then correlated with a classification of the ligands as non-binding, intermediate binding and strongly-binding.

According to previous reports, the screening of triazine ligand-libraries by fluorescence microscopy was shown to be reliable for the selection of non-binding and strongly binding ligands for proteins, with no false negatives observed (17). The Fluorescein/Protein (F/P) ratio was shown to influence the results for fluorescence microscopy

screening, as for lower F/P values ($F/P \leq 2$) the existence of non-specific interactions and the number of false positives observed decreased (17). In this study, all biomolecules labelled with FITC presented an F/P ratio of 1.

Screening of Libraries by Fluorescence-Based Techniques

Libraries I and II were first assessed for binding to the model biomolecules by Method A. Typical images obtained are shown in Fig. 3. A non-binding ligand (or low binder) interacts very weakly with the target biomolecule and little or no fluorescence is observed, which results in a black image (Fig. 3a). A ligand considered an intermediate binder reveals moderate fluorescence on the beads (Fig. 3b), whereas a strongly binding interaction is characterized by a strong fluorescence on all agarose beads (Fig. 3c). The microscope images obtained (all with the same dimension and quality) were analyzed with the software Image J using the mean grey value parameter. This is related to the brightness of pixels in an image and the values are given within the range 0–255, where 0 corresponds to a black image and 255 to a white image.

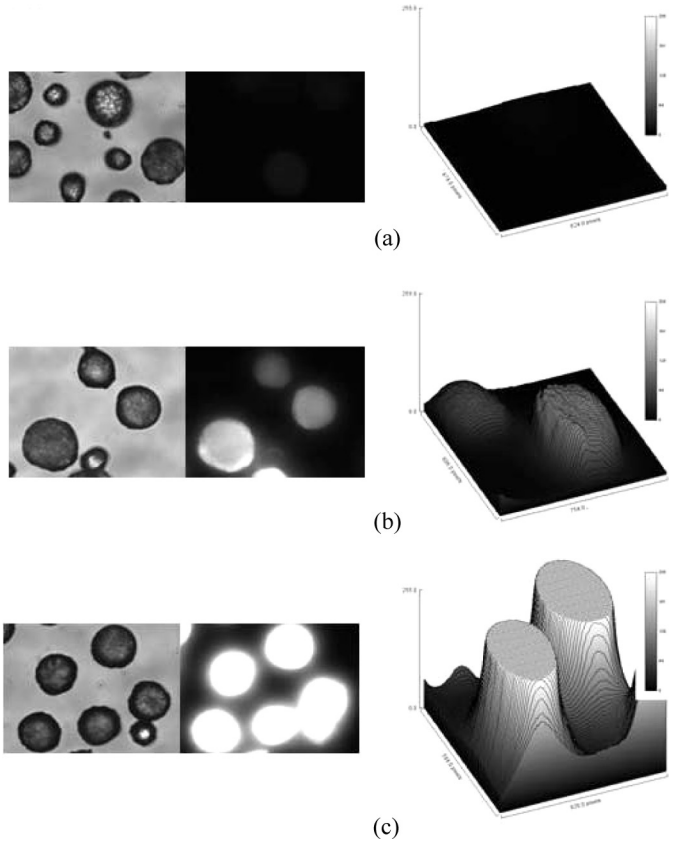

FIG. 3. Images showing typical on-bead fluorescence for (a) non-binding ligand, (b) intermediate binding ligand, and (c) strongly binding ligand.

Although easy to perform, this evaluation does not take into account the number of agarose beads in the field as well as the black background area between beads.

In parallel, microscope images were also analyzed by counting the total number of agarose beads in the field and the number of agarose beads presenting fluorescence (17). A value of the percentage of fluorescence intensity was assigned to each agarose bead and the total fluorescence of the sample calculated (Fig. 2). The maximum fluorescence corresponds to 100% (Fig. 3c) and the minimum to 0% (Fig. 3a). This method is more laborious as both bright field and microscope images need to be analyzed for each sample, and is also dependent on the criteria used for the assignment of percentage of fluorescence per bead. In order to understand if the two methods for analysis of fluorescence microscopy images lead to similar conclusions regarding the binding character of each ligand, the two sets of results were plotted (Fig. 4). The two methods showed a linear correlation indicating that the classification of ligands in non-binding, binding, and strongly binding can be equally performed by both methods. However, the results obtained by Image J analysis can be misleading as the number of beads per image is not taken into account. This fact could justify the absence of higher values of mean grey value ($\geq$200), even for those ligands presenting very high fluorescence on the agarose beads. Therefore, some strongly binding ligands were classified as intermediate binding ligands. For the reasons stated, the remaining assessment of fluorescence microscope

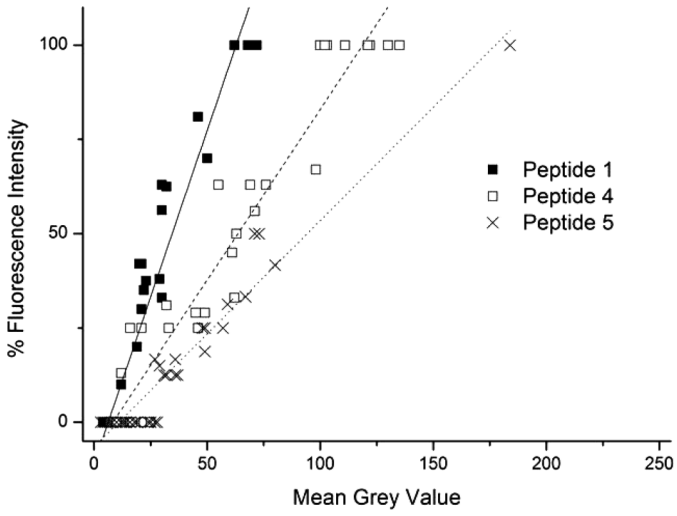

FIG. 4. Correlation between the results obtained from software Image J (mean grey value) and the assignment of the percentage of fluorescence intensity for three selected biomolecules used in the screening of the combinatorial libraries. The equations expressing the linear correlation (calculated from OriginPro 8 software) are: peptide 1 $y = 1.77(\pm 0.09)x - 11.06(\pm 2.73)$ with $r^2 = 0.84$, peptide 4 $y = 0.91(\pm 0.04)x - 7.60(\pm 2.94)$ with $r^2 = 0.86$ and peptide 5 $y = 0.60(\pm 0.02)x - 6.60(\pm 1.03)$ with $r^2 = 0.87$.

images was performed by calculating the percentage of fluorescence intensity on each sample.

When the libraries were screened by Method B, the fluorescence intensity results were first translated into percentages. The maximum fluorescence attained in each system was taken as 100% and the remaining fluorescence values calculated accordingly. The results obtained from the screening of the two libraries against the six random biomolecules by Methods A and B were plotted against each other (Fig. 5). We observed that most of the data points showed no direct correlation between the two methodologies. In order to analyse the results better, each graph was divided in four quadrants (Fig. 5a). Data points in quadrants 1 and 4 are those giving discrepant ligand classification results by the two methods. On the other hand, data points in quadrants 2 and 3 show correlation between the results obtained by Methods A and B. In quadrant 3, we can find data points correspondent to ligands binding less than 50% for the biomolecules. This is where most of the results fit. In quadrant 2 we can find the most promising lead ligands as high percentages of fluorescence intensity ($\geq$50%) were given by both methods.

Reproducibility Studies

The discrepancies found in the results obtained by Methods A and B (Fig. 5) lead us to perform reproducibility assays in order to select the most reliable technique to undertake the screening of combinatorial libraries of adsorbents for binding to target biomolecules. Random ligands with different binding classifications (strong binders, intermediate binders, and low binders) for a target biomolecule were selected to perform these studies.

For Method A, a total of 30 fluorescence microscope images were taken per ligand. As already shown in the past (10,17), this screening methodology is reliable for the detection of non-binding and strongly-binding ligands. Our results also confirmed this observation. As an example, Fig. 6 shows microscope images obtained for two ligands with extreme binding characters. Figure 6a represents several fields of the same sample of a non-binding ligand. Figure 6b shows an example of a strongly binding ligand that displays a very high fluorescence in all beads. The graph plotting the percentage of fluorescence intensity taken from one single microscope image against the average of 30 microscope images, shows a linear correlation (Fig. 7). This indicates that, in general, a single microscope field is sufficient for correct ligand classification. Error bars are more significant for intermediate binding ligands.

For the studies with Method B, different volumes of the same ligand were pipetted into 96-well microplates and the fluorescence intensities were measured (Fig. 8). For each resin, an increase in the number of beads per well results in an increase in fluorescence intensity. In Fig. 8, ligands in the x-axis present increasing percentages of fluorescence

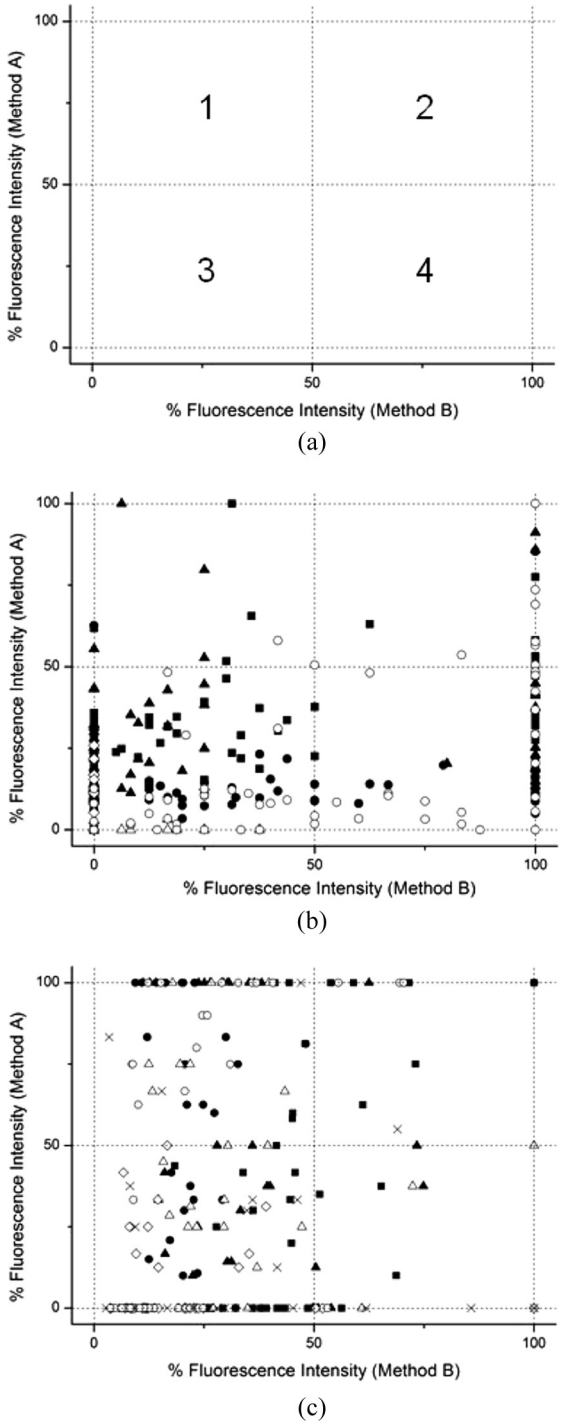

FIG. 5. Relationship between the results obatined from the screening of the combinatorial libraries by Methods A and B (Fig. 5a), where quadrant (1) and (4) corresponds to a non correlation of the methods, quadrant (2) represents the potential lead ligands and quadrant (3) present the ligands with a percentage of fluorescence intensity less than 50% by both methods. The Fig 5B represents the correlation of results obtained from library I screened against biomolecules (■) Peptide 1, (●) Peptide 2, (▲) Peptide 3, (Δ) Peptide 4, (◇) Peptide 5, (x) GFP, and (○) BSA by the two methods. Figure 5c corresponds to the correlation of the screening results of library II against biomolecules.

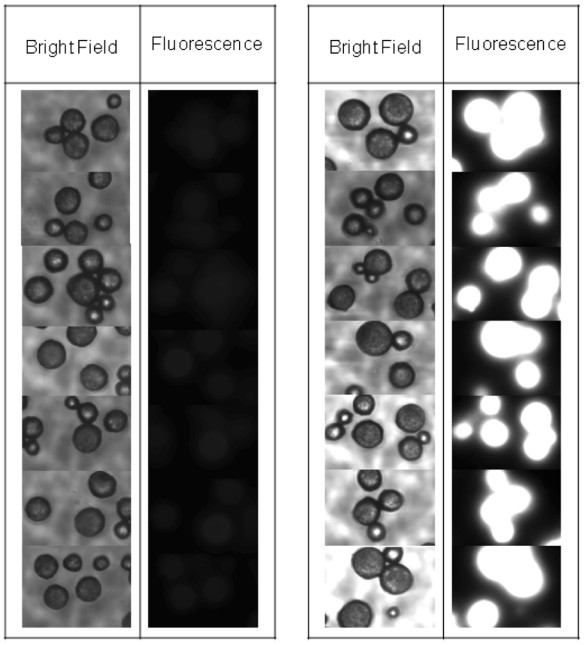

FIG. 6. Microscope images of agarose beads bearing ligands with a (a) non-binding character and (b) a strongly binding character for a target biomolecule (400× magnification).

obtained by Method A, where L1 presents 0% binding and L8 100%. The fluorescence intensity does not increase linearly from L1 to L8, as ligands L1 to L5 (0–50% fluorescence intensity by Method A) yield similar results.

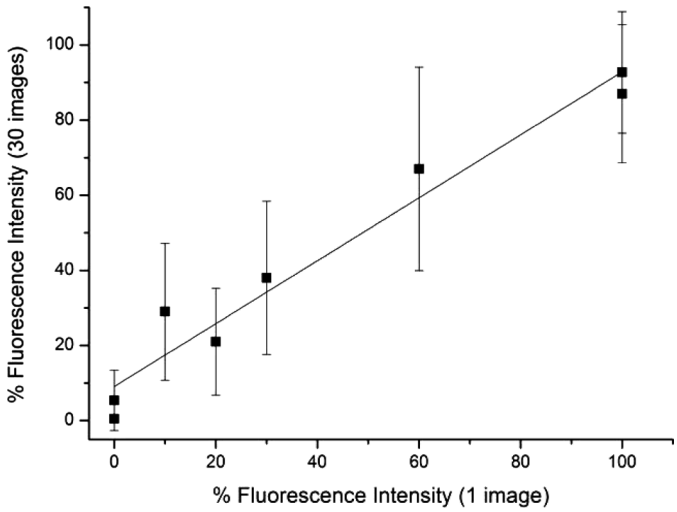

FIG. 7. Relationship of the percentage of binding between 1 image and 30 images taken from the fluorescence microscopy for several ligands with different binding assessments. Equation expressing the linear correlation (calculated from OriginPro 8 software): $y = 0.84(\pm 0.07)x + 9.08(\pm 3.87)$ with $r^2 = 0.91$. The error bars correspond to the standard deviation of the average of 30 images.

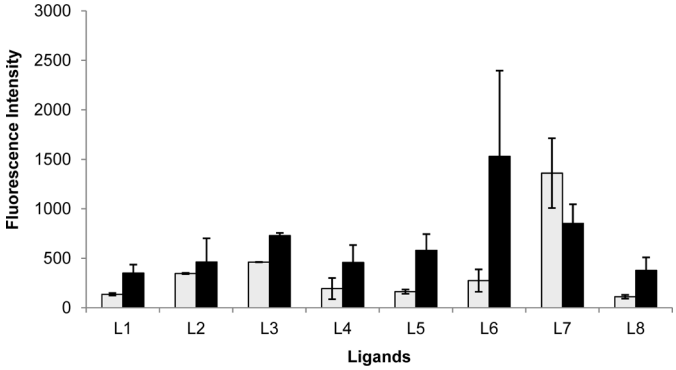

FIG. 8. Fluorescence intensity measurements by using 96-well format fluorimetry technique of several ligands with different binding assessment by fluorescence microscopy, (□) 30 µl of resin and (■) 80 µl of resin.

Ligands L6 to L8 (75–100%) present higher fluorescent intensities (except for L8), but the increase in the resin volume does not always give more fluorescence intensity in the well. Ligands L1 and L8, each possessing extreme binding characters from Method A, present the same fluorescence intensity in Method B. Coffmann and co-workers performed extensive studies on the reproducibility of HTS techniques for chromatography adsorbents (22). One of the challenges encountered was the accurate pipetting and dispensing of agarose suspensions (22). The authors found that the use of automatic liquid-handling systems tend to minimize these limitations, as well as the reduction of pipetting steps during the screening assays. We believe that these are the main reasons explaining the discrepancies observed between Methods A and B and also the low reproducibility of Method B.

CONCLUSIONS

Affinity chromatography on agarose beads is still the workhorse for the purification of many biomolecules. For the discovery of new *de novo* designed synthetic affinity ligands, solid-phase libraries are synthesized and screened for binding against target molecules. As the support and the chemical functionalities interposed between ligand and support can interfere with the interaction with the target molecule, on-bead screening has shown to be an ideal approach in the search of ligands for affinity chromatography. In this work, we report the comparison between two fluorescence-based methodologies for the screening of solid-phase combinatorial libraries of ligands against several biomolecules. Fluorescence microscopy (Method A) has been previously described as a reliable technique for the selection of non-binding and strongly binding affinity ligands for target proteins (17). In this work, we have confirmed this observation, as well as the higher error in the determination of percentage of binding for intermediate binders. Although shown to give false positive results,

this method can be used as a first generation screening assay. On the other hand, Method B appears to be strongly dependent on the number of beads per well and therefore both false positive and negative results were observed. False negative results are undesirable as potential lead ligands might not proceed for ligand optimization. Resin-pipetting is the major factor accounting for the unreliability of Method B, including resin retention in the tip after dispensing and resin settling during the aliquotting procedure, and variability in the aliquotted slurry volume (22). In future screening assays, these factors must be avoided for a more accurate assay, so that the 96-well format fluorimetry can be used as a qualitative and quantitative screening of combinatorial libraries.

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REFERENCES

1. Clonis, Y.D.; Labrou, N.E.; Kotsira, V.P.; Mazitsos, C.; Melissis, S.; Gogolas, G. (2000) Biomimetic dyes as affinity chromatography tools in enzyme purification. *J. Chromatogr. A*, 891: 33.
2. Roque, A.C.A.; Lowe, C.R. (2006) Advances and applications of the novo designed affinity ligands in proteomics. *Biotechnol. Adv.*, 24: 17.
3. Lowe, C.R. (2001) Combinatorial approaches to affinity chromatography. *Curr. Opin. Chem. Biol.*, 5: 248.
4. Lowe, C.R.; Lowe, A.R.; Gupta, G. (2001) New developments in affinity chromatography with potential application in the production of biopharmaceuticals. *J. Biochem. Bioph. Meth.*, 49: 561.
5. Soto Renou, E.N.; Gupta, G.; Young, D.S.; Dear, D.V.; Lowe, C.R. (2004) The design, synthesis, and evaluation of affinity ligands for prion proteins. *J. Mol. Recognit.*, 17: 248.
6. Teng, S.F.; Sproule, K.; Hussain, A.; Lowe, C.R. (2000) Affinity chromatography on immobilized ''biomimetic'' ligands: Synthesis, immobilization, and chromatographic assessment of an immunoglobulin G-binding ligand. *J. Chromatogr. B*, 740: 1.
7. Roque, A.C.A.; Taipa, M.A.; Lowe, C.R. (2005) Synthesis and screening of a rationally designed combinatorial library of affinity ligands mimicking protein L from Peptostreptococcus magnus. *J. Mol. Recognit.*, 18: 213.
8. Roque, A.C.A.; Taipa, M.A.; Lowe, C.R. (2005) An artificial protein L for the purification of immunoglobulins and Fab fragments by affinity chromatography. *J. Chromatogr. A*, 1064: 157.
9. Gupta, G.; Lowe, C.R. (2004) An artificial receptor for glycoproteins. *J. Mol. Recognit.*, 17: 218.
10. Ruiu, L.; Roque, A.C.A.; Taipa, M.A.; Lowe, C.R. (2006) De novo design, synthesis and screening of a combinatorial library of complementary ligands directed towards the surface of cutinase from Fusarium solani pisi. *J. Mol. Recognit.*, 19: 372.
11. Melissis, S.; Labrou, N.E.; Clonis, Y.D. (2006) Nucleotide-mimetic synthetic ligands for DNA-recognizing enzymes: One-step purification of Pfu DNA polymerase. *J. Chromatogr. A*, 1122: 63.
12. Wu, F.; Yu, J. (2007) Novel biomimetic affinity ligands for human tissue plasminogen activator. *Biochem. Bioph. Res. Co.*, 355: 673.
13. Haigh, J.M.; Hussain, A.; Mimmack, M.L.; Lowe, C.R. (2009) Affinity ligands for immunoglobulins based on the multicomponent Ugi reaction. *J. Chromatogr. B*, 877: 1440.
14. Entzeroth, M. (2003) Emerging trends in high-throughput screening. *Curr. Opin. Pharmacol.*, 3: 522.
15. Shiau, A.K.; Massari, M.E.; Ozbal, C.C. (2008) Back to basics: label-free technologies for small molecule screening. *Comb. Chem. High T. Scr.*, 11: 231.
16. Langer, T.; Wolber, G. (2004) Virtual combinatorial chemistry an in silico screening: Effect tools for lead structure discovery? *Pure Appl. Chem.*, 76: 991.
17. Roque, A.C.A.; Taipa, M.A.; Lowe, C.R. (2004) A new method for the screening of solid-phase combinatorial libraries for affinity chromatography. *J. Mol. Recognit.*, 17: 262.
18. Zamolo, L.V.B.; Moiani, D.; Moscatelli, D.; Cavallotti, C. (2008) Molecular dynamic investigation of the interaction of supported affinity ligands with monoclonal antibodies. *Biotechnol. Progr.*, 24: 527.
19. Renaud, J.P.; Delsuc, M.A. (2009) Biophysical techniques for ligand screening and drug design. *Curr. Opin. Pharmacol.*, 9: 1.
20. Sousa, I.T.; Ruiu, L.; Lowe, C.R.; Taipa, M.A. (2008) Synthetic affinity ligands as a novel tool to improve protein stability. *J. Mol. Recognit.*, 22: 83.
21. Teng, S.F.; Sproule, K.; Hussain, A.; Lowe, C.R. (1999) A strategy for the generation of biomimetic ligands for affinity chromatography. Combinatorial synthesis and biological evaluation of an IgG binding ligand. *J. Mol. Recognit.*, 12: 67.
22. Coffman, J.L.; Kramarczyk, J.F.; Kelley, B.D. (2008) High-throughput screening of chromatographic separations: I. method development and column modeling. *Biotechnol. Bioeng.*, 100: 605.