A direct technique for preparation of magnetically functionalised living yeast cells†

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Received 14th July 2009, Accepted 7th October 2009
First published as an Advance Article on the web 6th November 2009
DOI: 10.1039/b914065d

A direct technique for preparation of magnetically functionalised yeast cells by using polyelectrolyte mediated deposition of magnetite nanoparticles is reported. We demonstrate that the cells preserve their viability after the magnetite deposition and show that the magnetic nanoparticles form a multilayered coating on the outer side of the yeast cell's wall. We applied our technique to produce magnetically functionalised yeast cells expressing green fluorescent protein (GFP) under the control of RAD54-GFP reporter and demonstrated that their fluorescence emission is not influenced by the presence of magnetite-polyelectrolyte composite coating. We show that the individual cells can be successfully manipulated by an external magnetic field which can be used for their deposition, holding and subsequent removal from microfluidic devices for genotoxicity and cytotoxicity biosensor applications. Our technique for direct magnetization of cells can find many other biotech applications including biosensors, bioreactors and bioseparation.

1. Introduction

The budding yeast (*Saccharomyces cerevisiae*) is a model organism routinely used in practical applications in biotechnology, as well as in basic biochemistry and molecular biology research. Modification and directed assembly of yeast cells in 2D and 3D structures, including yeast strains with gene-reporters have amplified the applications of yeast strains in novel biotechnology research. Yeast cells have been used in micro-reactors[1] and biosensors.[2] Many different yeast strains incorporating green or red fluorescent protein (GFP or RFP)-reporters have been employed for screening studies for toxicity, in drug discovery and in basic protein analysis[3,4] and recently adapted into micro-fluidics devices.[5] The main advantage of using cells with GFP-reporters is that they fluoresce when GFP is synthesised, without the addition of cofactors or enzyme substrates. Immobilization of yeast expressing fluorescent proteins would allow the development of novel micro-screening systems for a wide range of applications. It is technologically important to be able to manipulate viable yeast cells and to deliver and secure them in a desired position, or to release and remove them after the assay. In this article we describe the achievement of this by using magnetically functionalised yeast cells whose surface has been coated with superparamagnetic iron oxide nanoparticles.[6]

Magnetic modification of the yeast cells with magnetite nanoparticles is regarded as a well-established approach for the fabrication of novel biosorbents or biocatalysts,[7,8] but the non-mediated deposition of "as-prepared" ferrofluids onto the yeast cells[8] may be expected to reduce the viability of the cells, therefore limiting their use in biosensing. There is a challenge to develop a technique of "magnetisation" of living yeast cells which preserves their viability. Recently, fabrication of yeast cells coated with gold and silver nanoparticles and alternating cationic and anionic polyelectrolytes has been reported.[9] Several methods for polyelectrolyte-mediated functionalisation of yeast cells by using magnetic nanoparticles are reported here. This paper describes how GFP-reporter[10] yeast strains are affected by their functionalisation with magnetic nanoparticles.

2. Experimental

2.1. Materials

Poly(allylamine hydrochloride) (PAH, MW = 1.5×10^4 Da), poly(sodium polystyrene sulfonate) (PSS, MW = 7.0×10^4 Da) were purchased from Sigma-Aldrich. Iron(II) and iron(III) chlorides (99%) were purchased from Sigma. 1.0 M aqueous solution of tetramethylammonium hydroxide (TMA) was purchased from Aldrich (A.C.S. reagent). Methyl methanesulfonate (MMS, 99%) used as a genotoxic standard and dimethyl sulfoxide (DMSO, 99.9%) as diluent were purchased from Sigma-Aldrich. All other reagents were of analytical grade and used without further purification. MilliQ water purified by reverse osmosis was used in all our experiments.

2.2. Methods

2.2.1. Synthesis of magnetic nanoparticles. In this study three different types of magnetic nanoparticles were used. (i) Magnetic nanorods were produced following a procedure similar to that

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† Electronic supplementary information (ESI) available: Movies of the response of the magnetized yeast in external magnetic field. See DOI: 10.1039/b914065d

described in ref. 6. Briefly, 2.0 mL of 1 M $FeCl_3$ and 0.5 mL of 2M $FeCl_2$ aqueous solutions were mixed and stirred vigorously. 25 mL of 1.0 M aqueous ammonia solution was then added while stirring and the appearance of a dark magnetite precipitate was immediately observed. The precipitate was separated with a permanent magnet and washed with water until the supernatant reached pH 7. 2 mL of 25% aqueous TMA was then added into the solution resulting in the formation of a stable aqueous suspension of Fe_3O_4 magnetic nanorods (pH $\approx$ 13). (ii) Following the synthesis described in (i) the excess of TMA was removed, by drying up the nanoparticle suspension at 80 °C and resuspending the precipitate in milliQ water. The application of ultrasonic treatment for 20 min at 30% power (Branson Digital Sonifier Model 450, 5 mm tip, 400 W maximal power) yielded a stable aqueous suspension of magnetite nanorods without free TMA (pH $\approx$ 7). (iii) Spherical magnetic nanoparticles were also produced by using PAH as a stabiliser. 100 μL of suspension of magnetite nanoparticles obtained as described above in (i) was diluted in 10 mL of 10 mg mL^{-1} PAH aqueous solution and sonicated for 20 min at 30% power of the sonifier, which produced a stable aqueous suspension of nanoparticles.

2.2.2. Yeast cells and culture media. In this study two different strains of budding yeast were used. Native *S. cerevisiae* was purchased from a local supermarket and used as received after re-hydration in water at approximate weight concentration of 20 mg mL^{-1}. Genetically modified GreenScreen™ yeast strains, specialist resuscitation and assay media were supplied by Gentronix Ltd (Manchester, UK). The yeast reporter consisted of a fusion of the DNA damage-inducible promoter from an endogenous DNA repair gene, RAD54, with a gene encoding a yeast enhanced green fluorescent protein (yEGFP). The yeast cells express GFP when responding to and repairing DNA damage caused by exposure to genotoxic agents and the cells become increasingly fluorescent as the GFP accumulates.

2.2.3. Polyelectrolyte-aided magnetization of yeast. The procedure for coating living yeast cells was adapted from that using gold nanoparticles reported in ref. 9 in this case using magnetite nanoparticles. Briefly, *S. cerevisiae* yeast cells were coated with polyelectrolytes using alternating deposition of PAH and PSS (from 10 mg mL^{-1} solutions in 0.5 M NaCl) adhering to the surface of the cells. Since the hydrated yeast cells in water are negatively charged, they were coated first with PAH. 300 μL of the cell suspension was combined with 1 mL of PAH (aq) solution during constant shaking, incubated during 10 min and then centrifuged. The remaining excess polyelectrolyte was removed and the cells were redispersed and washed three times with milliQ water. The cells were then combined with the PSS solution, and finally the procedure was repeated to obtain PAH/PSS/PAH coatings on the cells. The coated cells were introduced into the suspension of non-coated magnetic nanoparticles, incubated for 15 min and washed with water followed by the deposition of two additional polyelectrolyte layers. The resulting architecture of polyelectrolyte/nanoparticle coating was as follows: PAH/PSS/PAH/MNPs/PAH/PSS. In an alternative procedure, we used PAH-stabilised MNPs by simple mixing of the native yeast cells (300 μL of cells at wet weight concentration of 20 mg mL^{-1}) with the suspension of PAH-stabilised MNPs at various dilutions at magnetite concentrations of 50, 25, 12, 6 and 3 mg mL^{-1} with incubation for 15 min under constant shaking. After the deposition of MNPs cells were separated from the polyelectrolyte solutions by using a permanent magnet.

2.2.4. Viability test. The viability of the magnetized yeast cells was tested using fluorescein diacetate (FDA, Fluka)[10] FDA was dissolved in acetone at 10 mg mL^{-1} and an aliquot (10 μL) of this solution was mixed with 0.49 mL of water and added into the suspension of the magnetized yeast cells. After incubation for 20 min the cells were extracted by magnetic separation, washed with water and examined with a fluorescence microscopy.

2.2.6. Green fluorescence protein (GFP) expression in magnetized yeast. The GreenScreen™ GC assay is used to simultaneously detect genotoxicity, cytotoxicity and test compounds.[3] Prior to the assay, yeast cells were grown in the resuscitation media for 72 h in an orbital incubator (200 rpm) at 30 °C to reach a stationary phase with a concentration approximately 1×10^7 cells per mL determined by optical density (OD) at 620 nm, in a spectrophotometer. 1 mL of yeast culture was collected in 1.5 mL centrifuge tube, centrifuged at 3000 rpm during 1 min and the supernatant removed. The pellets were re-suspended in 200 μL of milliQ water pre-filtered through 0.22 μm Millipore filter and 1 mL of different concentrations of

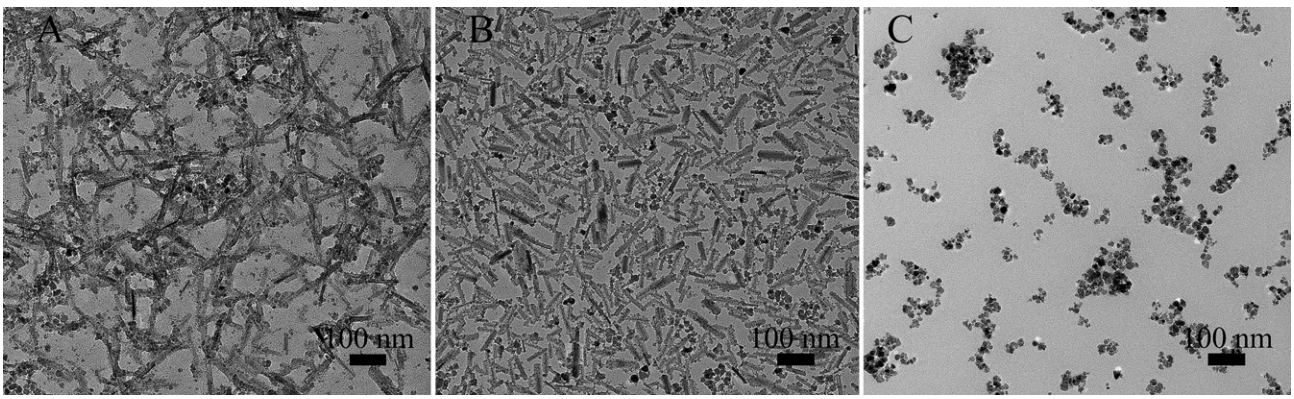

Fig. 1 Typical transmission electron microscopy images of (A) as-made magnetic nanoparticles stabilised with TMA; (B) TMA-stabilised magnetic nanoparticles after drying and re-suspension in water; (C) as-made PAH-stabilised magnetic nanoparticles.

PAH-stabilised MNPs (50, 25, 12, 6 and 3 mg mL^{-1}) was added. Samples were vigorously vortexed for 5 min at room temperature, centrifuged at 3000 rpm for 1 min and separated from the supernatant. Then the yeast cells coated with MNPs, were washed three times with water and finally re-suspended in the culture (assay) medium. 75 μL of the suspension of magnetized yeast cells was mixed with 75 μL of 0.0025% v/v of MMS (genotoxic compound and strong inductor of cellular fluorescence in GreenScreen GC yeast cells) or the vehicle alone (4% DMSO in water) in a 96 well microplate (ScreenMates™, Matrix Technologies Corp.). The plate was sealed with a breathable membrane and incubated for 18 h at 27 °C. The fluorescence emission (excitation at 485 nm, emission at 510 nm) was monitored in a fluorescence microplate reader POLARstar OPTIMA (BMG LABTECH, UK). We compared the fluorescence of the magnetized and the non-magnetized yeast cells to obtain the optimum nanoparticle-to-yeast ratio, in terms of magnetization and fluorescence emission. Comparisons of the fluorescence intensity was performed by analysis of variance (ANOVA) followed by Dunnett's T3 post hoc test, using SPSS 15.0 software.

2.2.7. Characterisation techniques. Optical and fluorescence images were taken with an upright Olympus BX51 microscope equipped with 50× objective and an inverted Olympus IX71 microscope equipped with a 4× objective. Scanning electron microscopy (SEM) images were obtained using an Evo 60 field emission scanning electron microscope (Carl Zeiss). Typically, 5 μL of a sample was placed onto the acetone-wiped glass stubs, dried overnight and sputter-coated with a thin carbon film. Transmission electron microscopy (TEM) images of magnetic nanoparticles and ultrathin sections of resin-embedded yeast cells were obtained using a JEM 2011 (JEOL) transmission electron microscope (Oxford Instruments). ζ-potential measurements were performed using a Zetasizer 3000 HS (Malvern) instrument.

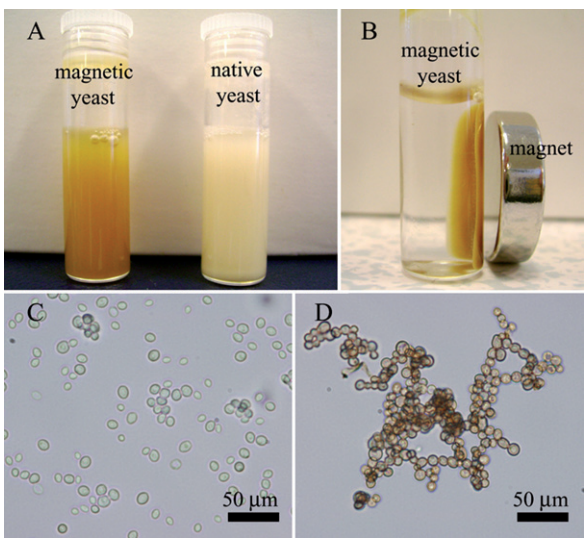

Fig. 2 (A) A photograph of the aqueous suspensions of the native and the magnetic yeast cells; (B) the magnetic behaviour of the magnetized yeast cells illustrated using a permanent magnet; (C) optical microscopy image of the native yeast cells; (D) optical microscopy image of the magnetized yeast cells.

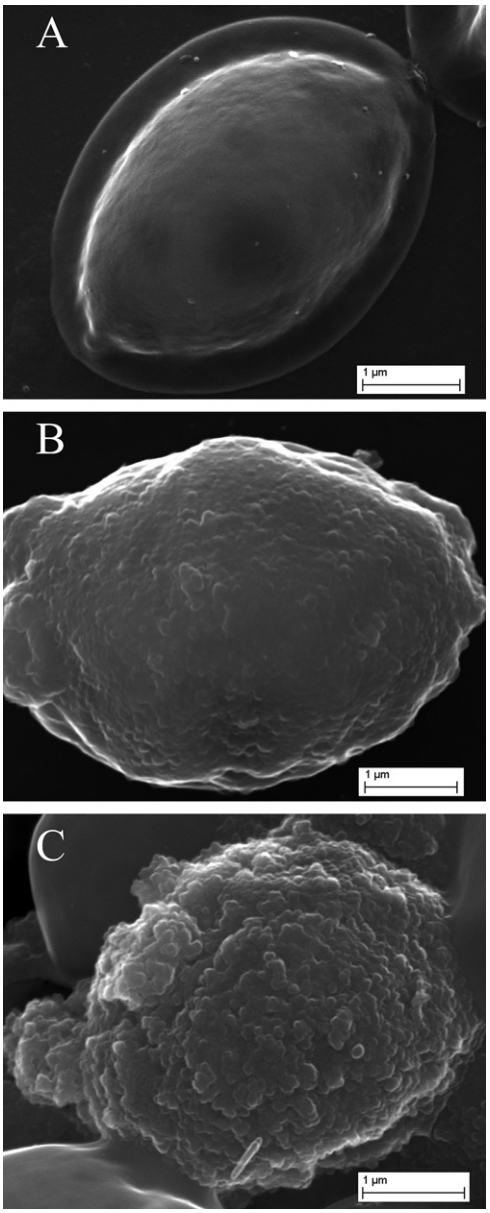

Fig. 3 SEM images of: (A) a native yeast cell; (B) a yeast cell coated with polyelectrolytes and TMA-stabilised MNPs; (C) a yeast cell directly coated with PAH-stabilised MNPs.

3. Results and discussion

3.1. Fabrication of magnetic yeast cells

The major objective of this work was to design a technique for functionalisation of yeast cells with magnetic particles without significantly influencing their viability. We examined the use of three different types of modified magnetite nanoparticles, the synthesis is of which is described in section 2.2.1. In several previous reports TMA-stabilised and boric acid-stabilised nanoparticles were used for the direct assembly of the MNPs on the cellular wall of yeast cells non-mediated by polyelectrolytes,[7,8] but no viability tests were performed. In the present work the following approaches to produce Fe_3O_4 magnetic nanoparticles for application in magnetic functionalisation of the living cells were used. The first approach was based on using TMA as

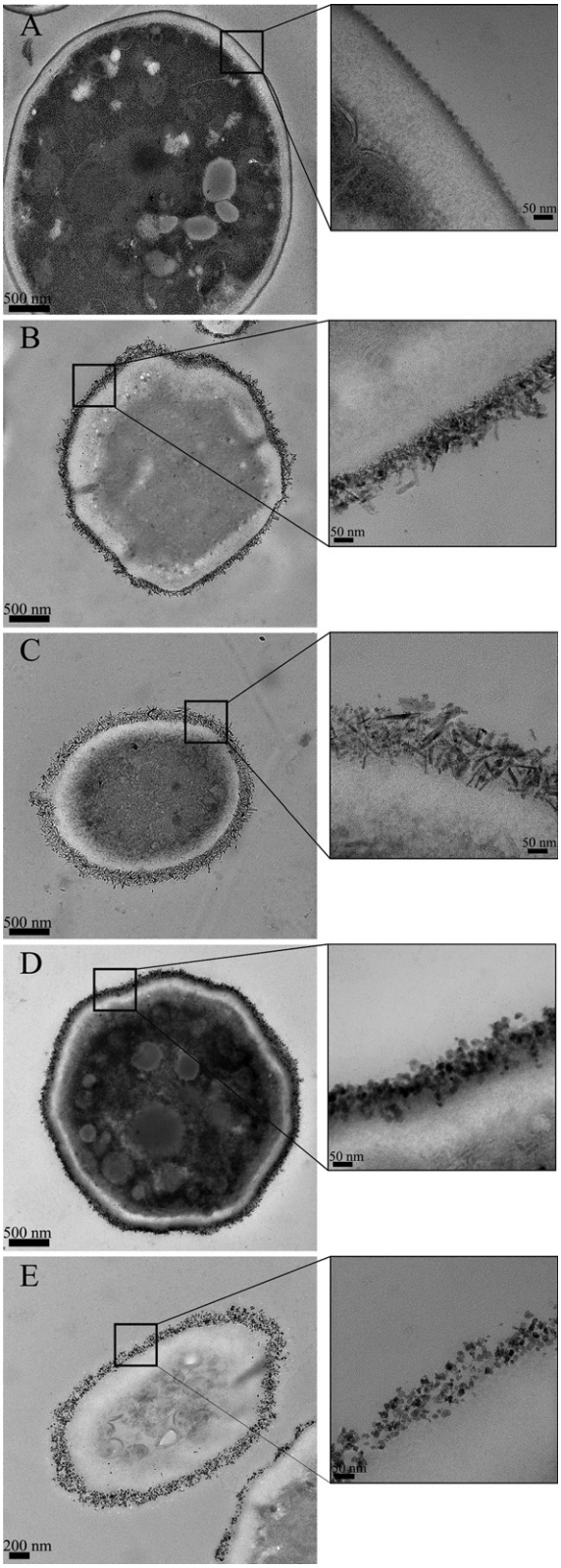

Fig. 4 Transmission electron microscopy images of thin sections of: (A) native yeast cells; (B) yeast cells coated with PAH/PSS/PAH/TMA-stabilised MNPs/PAH/PSS (in presence of residual TMA); (C) yeast cells coated with PAH/PSS/PAH/TMA-stabilised MNPs/PAH/PSS (without residual TMA); (D) yeast cells coated with PAH/PSS/PAH/PSS/PAH-stabilised MNPs/PSS; (E) yeast cells directly coated with PAH-stabilised MNPs.

a capping agent and stabiliser of the magnetite nanoparticles, which resulted in the development of rod-like magnetic nanoparticles. Typical TEM images of samples of these nanoparticles are presented in Fig. 1A and 1B. Two batches of TMA-stabilised MNPs were produced: (i) MNPs capped with TMA with residual amounts of TMA in the aqueous dispersion and (ii) residual TMA-free MNPs obtained by drying and re-dispersing of the MNP suspensions in water. Both batches exhibited negative ζ-potentials (around −60 mV) if measured in milliQ water and were stable for at least 3 months. The magnetite nanorods average length and diameters were 60 ± 15 nm and 13 ± 3 nm, respectively determined by TEM. In addition, PAH-stabilised magnetic nanoparticles were produced by adding an excess of PAH into the prepared magnetite nanoparticle suspension and applying ultrasonic treatment which resulted in the formation of a stable aqueous suspension of the clustered roughly spherical magnetic nanoparticles (as shown in Fig. 1C). These nanoparticles exhibited positive ζ-potential (+140 mV) in MilliQ water and their diameter is 15 ± 2 nm. Yeast cells were magnetized as described in the Experimental section by using the layer-by-layer assembly of polyelectrolyte coating prior to the deposition of MNPs. We used two strategies for coating of the cells with polyelectrolytes and MNPs. In the first approach, the slightly negatively charged native yeast cells (ζ-potential around −10 mV) were coated with the following consecutive polyelectrolyte layers: PAH/PSS/PAH and then with a single layer of negatively charged TMA-stabilised MNPs. If positively-charged PAH-stabilised nanoparticles were used, the cells were coated with PAH/PSS/PAH/PSS prior to coating with MNPs. In order to "seal" the MNPs on the cellular walls, another bilayer (PAH/PSS) or a single PSS layer respectively was deposited on the surface of the cells. The second approach, a single-step technique of the magnetization, utilizes the use of PAH-stabilised MNPs only, which are directly deposited onto the cellular walls of the cells. Both approaches resulted in the fabrication of effectively magnetized yeast cells. As a result, the suspension of the native yeast cells changed its original yellowish colour to brown (as shown in Fig. 2A) due to the deposition of the MNPs on the cellular walls of the cells. The magnetic behaviour of the magnetized cells is illustrated in Fig. 2B and in Movies S1 and S2 (ESI†). As one can see, the cells are magnetically susceptible and can be spatially manipulated, positioned, and immobilised in the required position using an external magnetic field. As long as the magnetite nanoparticles used in this study are superparamagnetic,[6] the magnetized cells do not form aggregates and disassemble after the removal of the external magnetic field, which enabled us to use this approach to manipulate yeast cells in microfluidic devices. Optical microscopy of the native and the polyelectrolyte-MNPs coated yeast cells reveals that in contrast with the native yeast cells the coated cells are brown (as shown in Fig. 2C and 2D). This confirms the previous observations that the layers of nanoparticles can be visualised by optical microscopy, making this technique an important instrument in the characterisation of the coated cells. The image in Fig. 2D shows the yeast cells magnetized using TMA-stabilised magnetic nanorods deposited onto the PAH/PSS/PAH-coated yeast cells. Similar results were obtained when the other two types of nanoparticles were used. Furthermore, SEM images presented in Fig. 3 illustrate the formation of magnetite layers on the surfaces of yeast cells.

We also studied the structure of the MNP/polyelectrolyte-composite layers deposited on the surfaces of yeast cells by slicing thin sections of MNP-coated yeast cells and examining them with TEM. The typical images of the native and magnetized yeast cells are shown in Fig. 4. It can be readily observed that the yeast cell walls are coated with a relatively thick (80–130 nm) layer of magnetic nanoparticles. The coatings are fairly uniform and apparently no nanoparticles were found inside the cells. This data is in good agreement with the previous reports describing the polyelectrolyte mediated coating of fungi cells with gold and silver nanoparticles.[9]

3.2. Viability of magnetic yeast cells

The aim of this work was to produce viable "magnetic" yeast cells which can be used in toxicity and genotoxicity screening applications. The viability of yeast cells was tested using FDA, which is routinely used to differentiate between dead and living eukaryote cells.[11] The method is based on the enzymatic activity of the cells as the non-fluorescent FDA molecules pass through the cellular walls and membranes and undergo hydrolysis inside the cytoplasm by intercellular esterases, resulting in the formation of a fluorescent by-product, thus indicating that the intracellular enzymes and cellular membranes are intact. The results of the viability test are shown in Fig. 5, where the bright field and corresponding fluorescent images of magnetized cells incubated in aqueous FDA solution and then washed in water are compared.

Here the green FDA-mediated fluorescence indicates that the magnetized yeast cells preserve their viability in all cases except if they are coated with TMA-stabilised MNPs in presence of residual amounts of TMA. We suppose that TMA itself causes cellular death by damaging the cellular membranes at the high pH values (typically above 13) of the alkaline TMA solution in spite of the presence of polyelectrolyte shells. Similar results were obtained if the cells are treated with TMA only, where again no viable cells were found. Therefore, the use of TMA-stabilised MNPs in presence of free TMA leads to complete loss of viability of the cells. The previous reports describe the use of TMA-stabilised MNPs for coating of yeast cells in order to produce magnetic sorbents[8] and utilize the cellular enzymes, although no detailed viability tests were reported. Therefore we suppose that the approach described in the present paper is more applicable if the living yeast cells are required. The polyelectrolyte-mediated techniques of magnetization of yeast cells are efficient enough to produce magnetically responsive yeast cells for use in biosensing applications. We found that the most promising route is the direct magnetization of yeast cells using PAH-stabilised MNPs. Due to their positive surface charge they readily assemble on the slightly negatively charged yeast cell walls. This approach considerably reduces the time needed for coating and facilitates the fabrication of magnetized cells in the sterile conditions (*i.e.* by filtration of PAH-stabilised MNPs through 0.22 μm filter). Furthermore, we used this approach to produce "magnetic" GreenScreen GC yeast strains which incorporate the RAD54-GFP reporter.

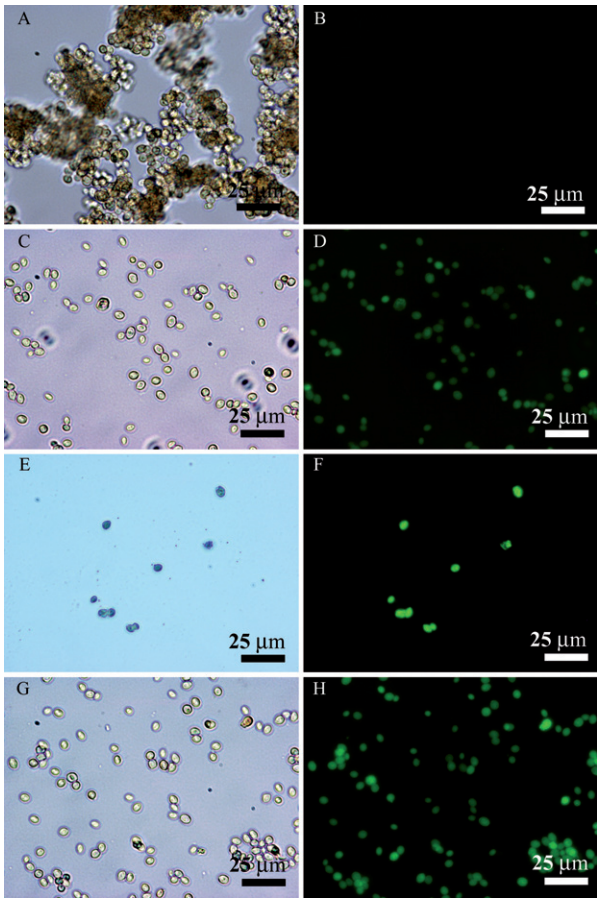

Fig. 5 Results of the viability test of yeast cells coated with magnetic nanoparticles using FDA (bright field and fluorescent optical microscopy images, respectively). (A and B) yeast cells coated with PAH/PSS/PAH/TMA-stabilised MNPs/PAH/PSS (in presence of residual TMA); (C and D) yeast cells coated with PAH/PSS/PAH/TMA-stabilised MNPs/PAH/PSS (without residual TMA); (E and F) yeast cells coated with PAH/PSS/PAH/PSS/PAH-stabilised MNPs/PSS; (G–H) yeast cells directly coated with PAH-stabilised MNPs.

3.3. Bioassay with magnetic RAD54-GFP-reporter yeast

Quantitative analysis of the fluorescence emission showed that PAH-mediated deposition of MNPs on the GFP-reporter yeast does not interfere with the GFP-expression and fluorescence emission. Fig. 6 illustrates the results of the bioassay, confirming the viability of the GFP-reporter yeast strains with MNPs. Surprisingly, the fluorescence emission from the "magnetized" yeast was the same or slightly higher than that in the controls (milliQ water or PAH aqueous solution alone). As expected, the fluorescence intensity was significantly increased when the yeast were exposed to a genotoxic compound MMS ($p < 0.001$, ANOVA) compared with the control samples. GreenScreen™ has been employed for screening both industrial products[12] and environmental samples[13] and has been recently incorporated into microfluidic devices[5] where immobilising the yeast cells was found to be problematic. These results indicated that magnetically modified GFP-reporter yeast could be positioned and immobilised with small permanent magnets in specified compartments of micro-fluidic devices, bioreactors or biosensors

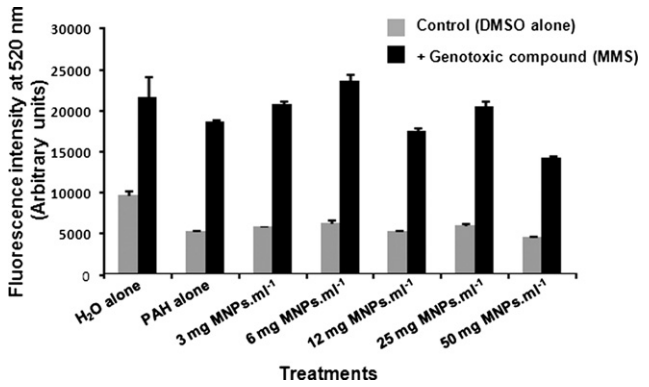

Fig. 6 Fluorescence intensity at 520 nm due to induced green fluorescent protein (GFP) production in magnetically modified yeast cells ("magnetized" GreenScreen™). The yeast samples were treated with different concentrations of MNPs, with milliQ water alone and the PAH solution alone as control treatment. The grey columns represent the fluorescence intensity of the control samples (treated with DMSO only) and the black columns correspond to the samples exposed to the genotoxic compound (MMS).

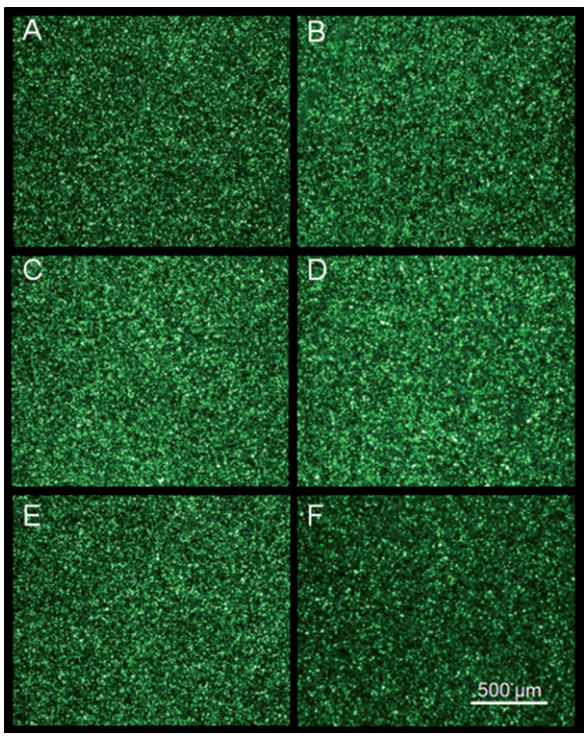

Fig. 7 Fluorescence microscopy images of GFP expressed in magnetically modified GreenScreen™ yeast at 520 nm exposed to a genotoxic compound (MMS): (A) water alone; (B) 3 mg mL^{-1} MNPs; (C) 6 mg mL^{-1} MNPs; (D) 12 mg mL^{-1} MNPs; (E) 25 mg mL^{-1} MNPs; (F) 50 mg mL^{-1} MNPs.

which could greatly improve the versatility, robustness and reliability of these devices and could facilitate their miniaturization. The direct magnetization of the GFP-reporter yeast cells with PAH-stabilised MNPs did not show any interference with the fluorescence signal through the cell wall due to the presence of immobilised magnetic nanoparticles for a wide range of MNP concentrations (see Fig. 7).

Since yeast cells used in this study are eukaryotic they mirror many of the characteristics of mammalian cells providing similar targets sites for toxicity and thus produce relevant data for human risk assessment. However, critically they are far more robust than mammalian cells which gives budding yeast several advantages for the development of viable-cell-based biosensors. They can tolerate a wide pH range, can survive temperatures from freezing to over 40 °C, and they have a wide tolerance of osmotic and electrolyte conditions. As demonstrated in this study, yeast can be magnetised without a loss of viability which shows the potential that this technique for direct magnetization of living cells can also be applied to other cells (including bacteria, algae, plant cells) since it preserves the cells' viability. This will be a subject of additional study which is under way and will be reported in a follow up article.

4. Conclusions

Several approaches have been investigated for the fabrication of magnetic yeast by functionalisation of the surface of living yeast cells with magnetite nanoparticles mediated by polyelectrolytes. We used three different modifications of iron oxide magnetic nanoparticles stabilised by tetramethyl ammonium hydroxide in combination with the layer by layer technique with PAH and PSS polyelectrolytes to produce stable coatings on the surface of the yeast cells. We also developed a direct single step method for "magnetization" of yeast cells by treating them with PAH-stabilised magnetite nanoparticles. We found that all three methods perform well in magnetizing the cell which enabled us to manipulate and position individual cells with an external magnetic field. The deposited magnetic particles were localised on the outer part of the yeast cell wall. It was also demonstrated that after the deposition of the magnetic coating by our method the yeast cells remain viable. Using this method, magnetised GreenScreen™ yeast cells could be incorporated into biosensors for genotoxicity and cytotoxicity and this is the aim of future work. The current study has showed that the fluorescence intensity of the expressed GFP is not influenced by the magnetic coating and demonstrates the cells are viable and functional. The method developed in this article has potential application beyond the fabrication of magnetic yeast and could be used with a range other cells and bacteria as it appears to preserve the cell's vitality which would allow their transportation and positioning with external magnetic field in microfluidic devices and biosensors. Studies to demonstrate this are currently under way. The method could also find application in bioreactors where it may facilitate bioseparation processes.

Acknowledgements

Authors thank Mrs A. Lowry for TEM images; Mr T. Sinclair for the SEM images and Dr Andrew Knight (Gentronix Ltd, Manchester) for kindly supplying the GreenScreen™ yeast cells and his comments on this manuscript. The financial support from the University of Hull, the Government of Tatarstan and the European Commission, TESS COLL-CT-2006 project (6th Framework Programme) is gratefully acknowledged.

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