Published in final edited form as:

Biotechnol Bioeng. 2017 April ; 114(4): 934–938. doi:10.1002/bit.26207.

Cholestyramine as a Promising, Strong Anion Exchange Resin for Direct Capture of Genetic Biomarkers from Raw Pancreatic Fluids

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Abstract

The ability to capture cell-free DNA from the gastrointestinal tract, in a minimally invasive manner, could enhance our ability to diagnose gastrointestinal disease, or gain a better understanding of the spatial mapping of the intestinal microbiota. We therefore sought to identify a class of capture agents that could directly and efficiently sequester genetic material from intestinal fluids. As a particular case study, we examined the ability to capture DNA from pancreatic secretions, for potential application in enabling the sequestration of early, genetic biomarkers of pancreatic disease. We hypothesized that the cholestyramine series of strong cation exchange resins, which are FDA approved for the treatment of high cholesterol, may be capable of capturing DNA from pancreatic secretions. We identified a particular cholestyramine resin, DOWEX 1×2 100–200 mesh, which is able to efficiently capture and purify DNA from pancreatic fluid. Using only 200 μ l of pancreatic secretions, we are able to recover 247 ± 182 ng of amplifiable human DNA, giving an estimated pancreatic fluid DNA content of 1.23 ± 0.91 ng/ μ l. To our knowledge, this is the first demonstration of a material that can effectively capture and purify DNA directly from untreated pancreatic fluids. Thus, our approach could hold high utility for the *in vivo* capture of DNA and disease biomarkers if incorporated into an appropriate sampling device.

Graphical TOC

A FDA-approved anion exchange resin is re-purposed for direct DNA capture from raw pancreatic fluids. Such resins hold promise for incorporation into smart capsules for *in vivo* DNA biomarker sequestration to aid in enhanced diagnosis of gastrointestinal diseases.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

The ability to isolate and sequester human or bacterial genomic material from the gastrointestinal tract, in a minimally invasive manner, could have a substantial impact in advancing the diagnosis of digestive diseases, or in enhancing our understanding of microbial diversity in the small and large intestine. One example of a disease that could benefit from such a technology is pancreatic cancer (PC), for which there are currently no effective screening tools for early detection. PC is a lethal, recalcitrant cancer with a median survival of 6 months and 5-year survival rate of 5% (Hezel et al, 2006). Despite significant progress in the detection and treatment of other cancers, the grim prognosis of PC has remained unchanged over the past 40 years (American Cancer Society, 2016), and PC is predicted to become the 2nd leading cause of cancer-related deaths by 2030 (Rahib et al, 2014). This is partly due to the late diagnosis of pancreatic cancer, with only 20% of patients presenting with surgically resectable tumors (Tamburrino et al, 2014), and the majority of patients presenting with metastatic disease (Hidalgo, 2010). Despite its grim prognosis, genetic analysis shows that PC progresses slowly, with at least a decade of time between the initiating mutation and cancer development (Yachida et al, 2010). This provides a significant window of opportunity for early detection.

Recently, analysis of genetic and epigenetic changes in pancreatic cancer has begun to show promise for the detection and treatment of this disease. Whole-genome bisulfite sequencing efforts have identified >500 differentially methylated regions in PC tissues, and follow-up analysis on 6 of these regions using methylation-specific PCR (MSP) on DNA isolated from pancreatic fluid demonstrated AUCs (area-under-the-curve) ranging from 0.83–0.92 (Kisiel et al, 2015). Beyond epigenetic changes, genetic mutations are also observed in the pancreatic fluid of individuals with pancreatic cancer (Kanda et al, 2013; Sadakari et al, 2014), which could lead to the development of combined genetic and epigenetic diagnostic panels. Recently, whole-genome sequencing efforts have elucidated recurrently-mutated pathways that can lead to therapeutic targets (Waddell et al, 2015; Witkiewicz et al, 2015), and one study showed that 4 out of 5 PC patients with defective DNA maintenance pathways

(BRCA1/2 or PALB2 mutations) responded well to platinum therapy (Waddell et al, 2015). Therefore, analyzing pancreatic fluid DNA using a combination of MSP and next-generation sequencing (NGS) could potentially enable both early detection and personalized treatment of the disease.

Pancreatic secretions represent an ideal biomarker source for the early detection of pancreatic cancer. Because approximately 90% (Pellegata et al, 1994) of pancreatic cancers are exocrine in nature, pancreatic fluid is a rich source of protein and genetic biomarkers that are shed into the pancreatic duct, and secreted into the upper intestine (Aleksandra, 2013). As an example, in patients with malignant pancreatic tumors, concentrations of protein biomarkers, such as CA19-9 and CEA, are much higher – 30 and 1000 fold respectively – in pancreatic juice compared with blood (Kawai et al, 2004).

An existing challenge for using pancreatic secretions for biomarker diagnostics is that this fluid is difficult to collect. Currently, the only available method for sample collection is upper endoscopy, which is highly invasive, expensive, and time consuming, thereby making it unsuitable for population level sampling and triage. One alternative to endoscopy would be to identify a material that is capable of *in vivo* genetic biomarker capture from pancreatic fluids. With recent advances in smart-capsule technologies (Slawinski et al, 2015), such a material could be incorporated into a capsule-based device, thereby allowing for the binding and storage of genetic markers from the GI tract, which could then be analyzed post-passage.

Here, we sought to identify a DNA sequestration agent that could be used under *in vivo*-compatible conditions. Such a material should be capable of binding DNA from raw gastrointestinal fluids at their native pH. This immediately rules out silica-based adsorption technologies, which are the foundation for many commercially available DNA and RNA purification kits, since these technologies require high concentrations of chaotropic agents, acidic pH, and the presence of alcohol (IPA or EtOH) to facilitate nucleic acid adsorption (Boom et al, 1990). In addition, it rules out some polymeric systems, such as chitosan, which rely on the protonation of primary amines for DNA binding, and therefore exhibit pH-dependent binding of nucleic acids (Cao et al, 2006). In contrast, quaternary ammonium salts are able to interact strongly with DNA via coulombic interactions under a range of conditions, and are not influenced by fluctuations in pH. Therefore, we hypothesized that cholestyramine resin, which is presently FDA approved as a bile acid sequestrant for the treatment of high cholesterol, could potentially be repurposed for direct DNA capture from gastrointestinal fluids. We ultimately identified the cholestyramine resin, Dowex 1×2 100–200 mesh, as an effective material for sequestering nucleic acid from both simulated intestinal fluids and raw pancreatic secretions.

Cholestyramine is a divinylbenzene-crosslinked, polystyrene-based polymer that is further functionalized with quaternary ammonium groups to generate a strong anion exchange resin (Fig 1a). These cationic, quaternary ammonium functional groups can facilitate binding and capture of nucleic acids through coulombic interactions with the phosphate groups of the nucleic acid backbone. In the present study, we evaluated a number of cholestyramine resins to determine their efficacy of DNA capture using 100 mg of capture resin in a spin column

format with centrifugally-driven flow. The general workflow is depicted schematically in Figure 1b–d. Briefly, the DNA-containing sample is passed through the column by centrifugal flow, during which DNA is bound by the resin. The bound DNA is then washed several times with intermediate ionic strength buffer in order to remove low affinity contaminants. Finally, DNA is eluted under high ionic strength conditions (7.0 M GuHCl, 50 mM MES, pH 6.0).

Using this spin column format, we were able to identify an optimal resin, DOWEX 1×2 100–200 mesh, for DNA capture and purification. For determining an optimal resin candidate, DNA capture was evaluated using fasted-state simulated intestinal fluid (FaSSIF, biorelevant.com), supplemented with 10 mg/ml bovine serum albumin (BSA), in a series of spike-and-recovery experiments. The BSA was added in order to simulate the high protein content of pancreatic secretions. Spiked nucleic acid consisted of cell-line DNA that had been isolated using a DNeasy Blood and Tissue Kit (Qiagen). For the purposes of this experiment, spiked and eluted nucleic acid concentrations were evaluated using a NanoDrop Spectrometer. The experimental results are shown Figure 2(b), in which the amount of recovered DNA is plotted against the quantity that was spiked. As can be seen from the data, the DOWEX 1×2 100–200 mesh resin consistently outperformed the other resin candidates, recovering greater than 80% of the spiked DNA under all conditions. Analysis of absorbance spectra also confirmed the selective enrichment of DNA using these resins. Figure 2a shows absorbance spectra from the sample flow through and eluted DNA fractions. As can be seen in the figure, the sample flow through exhibits a dominant protein peak at 280 nm, which can be attributed to the high BSA concentration in the simulated intestinal fluid sample. In contrast, the eluted DNA shows a peak absorbance near 260 nm, which is consistent with nucleic acid absorbance.

After identifying a promising resin candidate using simulated intestinal fluids, we sought to validate the capture resin using clinical pancreatic fluid samples. For the processing of patient samples, we re-optimized the wash and elution conditions for removal of the vast majority of protein, while selectively enriching for nucleic acid. In particular, we found that a larger number of wash steps – five total, with two washes at 0.25 M GuHCl and three washes at 0.50 M GuHCl – was required in order to maximize the removal of protein from the biological samples, as evaluated by measuring the absorbance at 280 nm in the flow throughs from the wash steps (Fig 3a). Specifically, we found the five wash steps was sufficient for reducing the concentrations of protein in the wash flow throughs to baseline levels. While protein is removed during the wash steps, DNA remains bound to the capture resin. This was confirmed using the PicoGreen dsDNA Assay to analyze the washes for the presence of dsDNA (Fig 3b). These results indicated that DNA remains bound during all wash steps, and is selectively eluted with 7M elution buffer. DNA recovery could be enhanced by using multiple consecutive elution steps (Fig 3c–d), with 85–90% of bound DNA being recovered in the first elution step. In order to maintain as high of a concentration of DNA as possible in our eluted samples, all clinical samples were eluted in a single elution step. However, it should be noted that a second (or third) elution step could be used if maximal recovery is necessary.

Using these optimized wash conditions, along with a single elution step, we then evaluated the performance of our resin across a larger number of patient-derived samples from individuals with suspected chronic pancreatitis. Samples were obtained from six individuals undergoing secretin-stimulated endoscopic Pancreatic Function Testing (ePFT) for suspected chronic pancreatitis, with fluid being collected at 15, 30, and 45 minutes post-secretin administration. For each sample, 200 μ l of pancreatic fluid was applied to a 100 mg resin bed, and the sample was processed as above. Recovered quantities of DNA varied widely across both individuals and collection times (Fig 4a), and we recovered an average of 247 ± 182 ng of DNA from 200 μ l of pancreatic secretions, which would give an estimated fluid DNA content of 1.23 ± 0.91 ng/ μ l. Given the widely varying concentrations of DNA within pancreatic secretions (present data and ref (Sadakari et al, 2014)), this value is in reasonable agreement with the value of 2.39 ± 2.70 ng/ μ l that has been previously reported for duodenal aspirates of secretin-stimulated pancreatic secretions (Sadakari et al, 2014).

In addition to testing the DNA capture performance of our resin, we also wanted to compare the performance of cholestyramine to the DNeasy Blood and Tissue Kit (Qiagen), which is frequently used for the *in vitro* isolation of DNA from biological fluids, including pancreatic juice (Matsubayashi et al, 2006; Sadakari et al, 2014). While the DNeasy Kit is useful for *in vitro* DNA isolation, it is unsuitable for *in vivo* applications due to the fact that it requires pH buffering, chaotropic salts, and alcohol in order to facilitate DNA binding. Despite this limitation, the method provides a gold-standard to which we can compare our resin capture performance. A parity plot depicting the amount of DNA recovered for the two different approaches is shown in Figure 4b. We found that there was a very good correlation between the amount of DNA recovered using the DNeasy kit and the amount recovered using resin-based capture. Using the optimal DOWEX 1 $\times$ 2 100–200 mesh resin, with no pre-treatment of the pancreatic fluid, we find that we are able to successfully recover approximately 70% of the DNA that is obtained using the DNeasy Blood and Tissue Kit. This is quite striking given the fact that the silica-based kit requires substantial pre-treatment of the sample in order to facilitate DNA capture. In addition, we note that these resin-based DNA recoveries were achieved using a single elution step, and that it should be possible to further increase this value by maximizing DNA recovery with second or third elutions.

In this study, we evaluated the ability of cholestyramine, a strong cation exchange resin, to sequester DNA from unprocessed, gastrointestinal fluids, using pancreatic fluid as a model system. We identified a particular cholestyramine resin, DOWEX 1 $\times$ 2 100–200 mesh, that is able to efficiently capture and purify DNA from pancreatic fluid. Using only a 200 μ l sample volume, we were able to recover 247 ± 182 ng of amplifiable human DNA from clinically collected, pancreatic fluid samples. To our knowledge, this is the first demonstration of a material that can effectively capture and purify DNA directly from untreated pancreatic fluids, making it a promising candidate for *in vivo* biomarker sequestration.

Materials and Methods

Resin-based DNA Isolation

Resin performance was evaluated in a spin column format. Briefly, 100 mg of capture resin was added to a fritted centrifugal column (Pierce, Cat# 69705). The resin was then washed

with three volumes (300 μ l) of PBS buffer prior to the application of a 200 μ l sample volume. After sample application, the bound DNA was washed 2–5 times with 300 μ l of 0.25–0.50 M GuHCl (50 mM MES, pH 6.0) before eluting with 200 μ l of 7 M GuHCl (50 mM MES, pH 6.0). Two washes were used for simulated intestinal fluid (2×0.50 M GuHCl), while 5 washes were used for biological fluids (2×0.25 M GuHCl + 3×0.50 M GuHCl).

DNA-Spiked, Simulated Intestinal Fluid

Human genomic DNA was isolated from cultured WI-38 cells using a DNeasy Blood and Tissue Kit (Qiagen), and was stored at -20°C until use. Prior to spike-and-recovery experiments, DNA was buffer exchanged to water using 7 kDa MWCO Zeba Spin Desalting columns (Thermo Scientific, Cat# 89882). Nucleic acid concentration was evaluated using a NanoDrop spectrometer, using the absorbance at 260 nm. For resin testing, varying amounts of nucleic acid were spiked into 1x fasting-state simulated intestinal fluid (FaSSIF, Biorelevant.com), which was further supplemented with 10 mg/ml BSA in order to simulate the high protein content of pancreatic secretions.

Collection of Patient-derived Pancreatic Fluid Samples

Pancreatic juice samples were obtained from 6 patients who were undergoing screening for suspected chronic pancreatitis (SCP). Fluid was collected using the standard protocol for endoscopic Pancreatic Function Testing (ePFT) (PMID: 19806083). Briefly, pancreatic secretion was induced by intravenous injection of 0.2 mcg/kg of human synthetic secretin (ChiRhoClin, Inc., Burtonsville, MD) over the course of 1 minute. Approximately 10 ml of fluid was then collected at 15, 30, and 45 minutes post-secretin administration by suctioning fluid through the accessory channel of the endoscope. The pancreatic juice samples were aliquoted without further processing, and stored at -80°C within 1 hour.

DNA Buffer Exchange and Sample Storage

For patient derived samples, it was necessary to buffer exchange the eluted DNA samples prior to measuring DNA concentration, since high concentrations of guanidine hydrochloride interferes with PCR or other analytical methods. Buffer exchange was accomplished by dialysis against 1x TE at room temperature using a Pierce, 96-well microdialysis plate (10 kDa MWCO, Cat. 88260). Dialysis buffer was changed six times during buffer exchange, allowing at least 4 hours for equilibration at each step. Purified DNA samples were then stored at -20°C prior to analysis.

Acknowledgments

Research reported in this publication was supported by the National Institute of Diabetes, Digestive, and Kidney Diseases (NIDDK) of the National Institutes of Health under fellowship award number F32 DK101211 to A.J.H. and R01 DK063158 to C.K. We also acknowledge support from the Canary Center at Stanford University for Early Cancer Detection.

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Figure 1.

(a) General structure of the cholestyramine resins that were utilized in this work. (b)–(d) Schematic of sample processing protocol for isolating DNA from biological fluid samples. (b) DNA is bound to the capture resin by passing the fluid sample through the resin bed by centrifugation. (c) The bound DNA is then washed 3–5 times using an intermediate ionic strength buffer before (d) eluting the bound DNA with a high ionic strength buffer.

Figure 2.

(a) Demonstration of DNA purification using Dowex 1×2 100–200 mesh resin. The flow through during sample application has a dominant protein peak at 280 nm, which is consistent with predominantly protein absorbance, whereas the spectrum of the eluted DNA is shifted toward an absorbance maximum at 260 nm, which is consistent with nucleic acid absorbance. (b) Results of spike-and-recovery experiments using different amounts of WI-38 cell line DNA spiked into simulated intestinal fluid. DNA concentrations for the spike-and-recovery experiment were evaluated using a NanoDrop spectrometer. DOWEX 1 × 2 100–200 mesh resin consistently gave the greatest recoveries across all conditions. Samples were processed in triplicate, and error bars represent ± 1 SD.

Figure 3.

Demonstration of protein elimination and DNA elution using patient-derived pancreatic fluid samples. (a) Analysis of protein content in the original pancreatic fluid sample, as well as the sample flow through and wash steps from four patient samples, evaluated using the absorbance at 280 nm. Five wash steps were used for processing patient-derived samples, since this number of washes reduced the protein absorbance to baseline in the wash flow throughs. (b) Eluted DNA, as evaluated using the PicoGreen dsDNA Assay. DNA remains bound during all wash steps, as demonstrated by the lack of measureable DNA content in the wash step flow throughs, but is eluted upon switching the buffer conditions to 7 M GuHCl. (c)–(d) Evaluation of recovered DNA if multiple elution steps are employed. Over 85% of the bound DNA can be successfully recovered in a single elution step. If desired, additional elutions can improve DNA yields.

Figure 4.

(a) Recovered DNA quantities using resin-based capture from endoscopically collected pancreatic fluid samples from 6 patients with suspected chronic pancreatitis at three different time points. (b) Parity plot comparing the amount of DNA recovered using the resin capture approach and DNeasy spin columns. Cholestyramine resin demonstrates a similar performance to the DNeasy spin columns, which represent the state-of-the-art technology for in vitro DNA capture from biological fluids. Samples were processed in duplicate, and error bars represent ± 1 SD. DNA concentrations were evaluated using the Quantifiler Human DNA Quantification Kit.