Mechanisms of biliary stent clogging: confocal laser scanning and scanning electron microscopy

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Submitted for publication
ABSTRACT
Introduction: In order to compare early events in biliary stent clogging and identify distribution of bacteria in unblocked stents, we performed Confocal Laser Scanning (CLS) and Scanning Electron Microscopy (SEM) on two different stent materials, polyethylene (PE) and hydrophilic polymer coated polyurethane (HCPC).

Methods: Ten consecutive patients with postoperative benign biliary strictures were included in the study. Two 10 Fr 9 cm stents, one PE and one HCPC, were inserted. Stents were electively exchanged after 3 months and examined by CLS and SEM.

Results: No differences between the two types of stents were seen. The inner stent surface was covered by a uniform amorphous layer. On top of this layer a biofilm of living and dead bacteria was found which in most cases was unstructured. The lumen was filled with free floating colonies of bacteria and crystals surrounded by movable laminar structures of mucous. In all stents an open network of large dietary fibres was seen.

Conclusion: In both PE and HCPC stents the same early clogging events occurred. The most remarkable observation was the identification of networks of large dietary fibres resulting from duodenal reflux acting as a filter. This seems an uniform mechanism responsible for stent clogging.

INTRODUCTION
Endoscopic insertion of plastic biliary endoprostheses is a well established treatment for obstructive jaundice. The major limitation of this technique is late stent occlusion after a median duration of 3-6 months which necessitates stent exchange (1,2). Stent obstruction is caused by biliary sludge which consists of crystals of calcium bilirubinate and calcium palmitate, as well as proteins, mucopolysaccharides, cholesterol crystals and bacteria (3,4).

It is generally assumed that the initial event in stent blockage is adherence of proteins and bacteria to the inner wall of the stent forming a biofilm. Subsequently, biliary components such as calciumbilinearubinate and calcium fatty acid soaps precipitate because bacteria secrete β-glucuronidase and phospholipases (5-7). Probably the bacteria are already introduced during transpapillary placement of the stent. Additionally, after the stent is in place, bacteria can colonize the biliary tract due to reflux from the duodenum.

The attachment of bacteria to the inner wall of the stent may depend on polymeric surface properties (8). Different materials have been used for stent construction: polyethylene, polyurethane and teflon. In vitro studies have found a direct relation between the friction coefficient and the amount of encrusted material (5,9). Teflon has the lowest friction coefficient and therefore the maximum potential for preventing stent clogging. A hydrophilic polymer coating was demonstrated to be effective in reducing bacterial adherence in in vitro studies (10). The hydromer stent not only has a smooth texture but also a coating that absorbs water and provides a hydrophilic sheath. Because bac-
teria initially attach by hydrophobic interactions, this coating might decrease bacterial adhesion and therefore increase stent patency. However, the positive results of different in vitro studies could not be confirmed in prospective clinical trials (11-14).

In this study we performed Confocal Laser Scanning (CLS) and Scanning Electron Microscopy (SEM) in two different stent materials, polyethylene and hydrophilic polymer coated polyurethane, in order to compare early events in stent clogging and identify distribution of (living and dead) bacteria in unblocked biliary stents.

**MATERIALS AND METHODS**

From February 2002 to December 2002, 10 consecutive patients with postoperative benign biliary strictures were included in the study. There were 5 men and 5 women with a mean age of 50 years (range 30-64 years). Two 10 Fr 9 cm stents were inserted in patients with a postoperative bile duct stenosis: one standard polyethylene stent (PE) (Wilson Cook, Winston-Salem, N.C) and one hydrophilic polymer coated polyurethane stent (HCPC) (Biosearch, Somerville, N.J). The HCPC stent was soaked in water for 5 minutes before use. Elective stent exchange was performed, as a standard treatment, after 3 months to avoid cholangitis by clogging. None of the patients received prophylactic antibiotics. The stents were then examined by CLS within 30 minutes after stent removal and by SEM.

**Confocal laser scanning microscopy**

Small rings were cut from the stents: at the distal end, in the centre and at the proximal end of the stent. Specimens were stained for 15 min with SYTO 9 and propidium iodide as live/dead stain, rinsed in buffer and imaged. The live/dead viability test we used relies on the fact that membranes of dead cells are permeable to many dyes that cannot cross them in the living state. SYTO 9 is a very effective stain with minimum non-specific binding during staining of complex communities (15). For imaging a Leica SP2 confocal microscope was used. Excitation was done with the 488 nm line of the Argon-Ion laser. SYTO 9 was detected between 500 nm and 535 nm and propidium iodide was detected between 650 nm and 700 nm. For additional structural information the reflected image of the 488 nm line was acquired.

Images were acquired in a 512*512 format (8 bit). A HCL PL APO 20.0 x 0.70 Imm/Corr UV objective was used and a zoom factor between 1 and 5. The pinhole was set at a diameter of 1 airy disc corresponding with a z-resolution of approximately 3 μm. Data stacks were generated over a depth of up to 120 μm with a step size of 2 μm. Using the Leica software, images of SYTO 9 and propidium iodide were merged and stereo pairs were generated from the 3D image stacks.

**Scanning electron microscopy**

The specimen were fixed in McDowell's fixative for at least 48 hours, dehydrated and finally dried with hexamethyldisilazane. The dried specimen were mounted on stubs
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and coated with approximately 10 nm gold. Specimens were imaged with a Philips SEM 525 operated at either 5 or 10 kV and equipped with an Orion frame grabber.

RESULTS

The endoprostheses of each group were analyzed using both Confocal Laser Scanning and Scanning Electron Microscopy. No patient presented with symptoms of stent clogging and no patient received prophylactic antibiotics. All analyzed stents were patent as judged by eye. There was no difference in the amount and distribution of sludge and bacteria between polyethylene and hydrophilic coated polyurethane stents on both CLS and SEM.

Confocal laser scanning microscopy

Both polyethylene and hydrophilic polymer coated polyurethane stents showed a similar phenomenon. The layer attached to the internal wall of the stent was a highly reflective amorphous layer (thickness approximately 15-30 mm) which for each stent was extremely uniform in thickness and distribution. This layer formed the substratum for the attached bacteria.

In some cases a structured biofilm was observed, with dead bacteria at the top layer which are in direct contact with the bile while covering the underlying living cells (Figure 1). However, in most cases this layer was without any structure: dead and living bacterial cells were interspersed or cloudy areas with living bacteria covered by dead bacteria were seen (Figure 2). Crystals embedded in these biofilms were found occasionally. Especially in and around these unstructured biofilms mucoid like sheets were found which, in many cases, extended into the lumen of the stent. The thickness and density of unstructured biofilms were more irregular when compared with the structured biofilms. As far as could be observed, the biofilm attached to the stent wall never extended more than 100 μm into the lumen of the stent. Taken into account the inner diameter of the stent, the decline of the luminal radius and effect on bile flow is negligible.

The lumen of all stents examined was filled with free floating mixed colonies of living and dead bacteria. The size of these colonies varied considerably, from approximately 50 μm cross-section to a major part of the stent diameter (2.5 mm). These colonies were aggregated in a substance with a slightly higher reflectance than the surrounding bile. Small crystals (5 μm or smaller) were often found dispersed in these loosely attached floating colonies. If large free floating crystals were found (up to 150 μm), in many cases they or at least their fringes were covered with a mix of living and dead bacteria.

In a number of cases these free floating colonies were surrounded by laminar structures (probably mucous), part of a highly movable complex network of sometimes anastomosing structures with highly reflective surfaces. These structures sometimes were attached to the wall of the stent. In cross section the size of these empty spaces...
varies from 50 μm to over 500 μm. This is best seen in the 3 dimensional picture shown in Figure 3.
In all stents refluxes of dietary fibres were found. These fibres formed a more rigid tangled network which formed a kind of filter (Figure 4).

Scanning electron microscopy
Directly on the inner surface of both stent types, the same amorphous layer of uniform thickness, as observed with CLS was seen (Figure 5A). This layer is loosely attached to the wall of the stent. Consequently, after dehydration and drying it becomes easily detached from the stent. This layer was covered by bacteria and yeasts starting to form a biofilm (Figure 6). The shape and structure of this layer was variable. Sometimes a clear structure could be identified with the bacteria oriented perpendicular to the stent wall and spaces in the biofilm empty of bacteria (Figure 5C). More often no distinct orientation was found with mucous and clusters of microorganisms dispersed.
We also discerned a network of amorphous material, probably mucus, in which colonies of bacteria were embedded (Figure 7 with inset).
In some of the stents crystals were present, whereas in other stents they were absent. These crystals were found both organised in biofilms and dispersed throughout mucous and biomaterial (Figure 5B).
Reflux material from the gut was found over the whole length of the stent, often covered with microorganisms.
The HCPC stent also showed a very porous wall of the endoprosthesis in which contrast additives and little holes were seen (Figure 5A), whereas the polyethylene stent showed a solid plastic surface.
Contrary to the results from the confocal microscopy which show a volume filled with a highly flexible and transparent structures (in fluorescence and reflected mode), scanning electron microscopy gives the impression that this biomaterial in whatever shape always forms a solid obstruction.

DISCUSSION
The first finding in our study, found on both CLS and SEM, was the presence of an amorphous layer covering the stent wall which has not been described before. In earlier studies mostly blocked stents were studied without the use of CLS. Investigation of patent stents has the advantage that, initial stages of clogging can be studied. Particularly in the final stage of occlusion when biliary flow is almost absent, significant transformations in the clogged material may take place. Different studies reported about amorphous material into the stent lumen but did not report an organized layer (6,15).
In this study we used both CLS and SEM to collect information from the same specimen using two microscopic techniques in order to integrate these findings.
The most important difference between CLS and SEM is that CLS allows fluorescently labelled *in vivo* images and non invasive structural images with the possibility of three dimensional reconstructions. This enables to examine complex organized structures like biofilms without disruption or fixation. The live/dead viability test we used relies on the fact that membranes of dead cells are permeable to many dyes that cannot cross them in the living state. This staining system is intended for the use with pelagic bacteria and its role in mixed species biofilm has not been established. However, some impression of the distribution of live and dead cells can be obtained. SYTO 9 has been shown to be a very effective stain with minimal non-specific binding during staining of complex biofilm communities (15).

An interesting finding in our study was that in a number of stents, living bacteria were attached to the stent wall covered by a layer of dead bacteria. This distribution however, was not uniform in all stents, neither in one stent nor between the stents. This finding implies that living bacteria may be protected by dead bacteria which form a physical barrier to e.g. bacterial penetration. This may also explain the fact that antibiotics do not prolong stent patency in clinical trials (16).

Another finding was a movable cloudy network of mucus, bacteria and crystals including large empty spaces which was best seen by three dimensional CLS pictures. The occurrence of crystals seems to be a patient dependent factor. After an amorphous layer on the stent surface, growth of the biofilm connects bacteria to other bacteria, mucus and crystals forming microcolonies which also are connected to each other in a highly flexible three dimensional networks.

The most remarkable observation was the presence of large numbers of plant fibres that have refluxed from the duodenum. In our three dimensional pictures, as well as in the SEM pictures, we found tangled networks of fibres acting as a filter. Large fibres may suddenly obstruct the stent lumen independent of accumulation from the amount of biliary sludge. This view is in accordance with a recent study which also reported about the causal role of duodenobiliary reflux and suggested change in stent design to prevent reflux (17).

Because in all patients two types of stents were placed (and removed) simultaneously, we were able to study the effect of different stent materials on the formation of a biofilm. In this study we used the standard biliary stent made of polyethylene and a hydrophilic polymer coated polyurethane stent. In theory, the coated stent might prevent bacterial adhesion because bacteria initially attach by hydrophobic interactions. However, in this study no difference in bacterial growth between the two types of unblocked stents could be observed. This is in accordance with clinical studies in which no difference in stent patency is observed (13,14).

In the past, many investigators have used SEM techniques for biofilm studies where fixation may induce morphological changes. CLS can demonstrate these complex networks in aqueous biofilms by non disruptive techniques. Both microscopic techniques gave an explicit additive impression of the clogging mechanism. SEM gave a
very solid impression of the biofilm development process suggesting that bacterial growth and biofilm formation significantly contributes to stent obstruction. However, CLS proved that these structures are open, transparent and extremely flexible, forming at most a minor obstruction to the bile flow. In conclusion, in both PE and HCPC type of stents the same early clogging events occurred. The thickness of the biofilm is so small compared to the inner diameter of the stent that it has negligible effects on bile flow. The most remarkable observation was the identification of networks of large dietary fibres resulting from duodenal reflux acting as some sort of filter. This seems to be the uniform mechanism responsible for stent clogging. It also explains why different stent surface materials and administration of antibiotics or bile salts do not have any effect on stent patency.
**Figure 1.** CLS showing a merged colour image of live cells (green) and dead cells (red) in a stent. Dead and live bacteria are in a mixed colony semi attached to the amorphous layer of the stent wall. The stent material shows a strong fluorescence. Scanned area $750\mu m \times 750\mu m$.

**Figure 2.** CLS showing an organised biofilm attached to the stent wall. Dead bacteria (red) in direct contact with bile, covering the living cells (green). Scanned area $800\mu m \times 800\mu m$.

**Figure 3.** CLS showing a three dimensional stereograph of mucous sheets (MS) mixed with a few colonies of bacteria (C) and crystals (arrows). Image dimensions: $750\mu m \times 750\mu m \times 100\mu m$. Please use a red-green 3-D viewer to get three-dimensional impression.

**Figure 4.** CLS showing a three dimensional stereograph of vegetable fibres (reflux from the duodenum) covered with bacteria. The fibres are surrounded by small floating colonies and some crystals. Image dimensions: $500\mu m \times 500\mu m \times 100\mu m$. Please use a red-green 3-D viewer to get three-dimensional impression.
Figure 5A. SEM of an amorphous layer of uniform thickness directly deposited on the stent wall. The stent is porous and contains contrast additives (Bar=20 μm).
Figure 5B. SEM showing cholesterol crystals in various size deposited on the stent wall (Bar=20 μm).
Figure 5C. SEM showing a biofilm growing on the stent wall (Bar=100 μm).
Figure 6. SEM showing the first growth of bacteria and (in this case) yeasts on the amorphous layer (*) on the stent wall (Bar=20 μm).

Figure 7. SEM of mucus sheets with embedded bacterial colonies (inset) (Bar=0.1 mm).
REFERENCES